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Epigenetic Changes in the Myelodysplastic Syndrome

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Abstract

Epigenetic mechanisms such as DNA methylation and histone modifications drive stable, clonally propagated changes in genes expression and can therefore serve as molecular mediators of pathway dysfunction in neoplasia. MDS is characterized by frequent epigenetic abnormalities, including the hypermethylation of genes that control proliferation, adhesion, and other characteristic features of this leukemia. Aberrant DNA hypermethylation is associated with a poor prognosis in MDS that can be accounted for by more rapid progression to AML. In turn, treatment with drugs that modify epigenetic pathways (DNA methylation and histone deacetylation inhibitors) induce durable remissions and prolong life in MDS, offering some hope and direction in the future management of this deadly disease.

Introduction

The myelodysplastic syndromes are a group of diverse and heterogeneous syndromes characterized by clonal proliferation, bone marrow failure and an increased risk of development of AML⁹⁵. The natural course of the disease ranges from slow progression to rapid evolution into AML. There are several classification systems for MDS such as the International Prognostic Scoring System (IPSS)³⁰, the WHO classification¹⁰, or more recent clinical schemes⁵³ but none captures the full heterogeneity of the disease. Heterogeneity and progression are likely related to molecular changes that drive the disease phenotype and biology, but these remain incompletely understood. Insights into the pathogenesis of cancer has come from studying genetic changes in neoplastic cells^{38;76}. In MDS, several cytogenetic abnormalities have been identified that characterize subgroups of patients such as deletions of chromosome 5 and/or 7 in patients with poor prognosis, isolated deletion of 5q or trisomy 8. Mutations of several genes also characterize subsets of cases, including RAS, TET2, RUNX1 and others. Despite these advances, the molecular causes of MDS and its peculiar clinical features remain poorly understood. Epigenetic changes have been recognized in the past decade as major drivers of the malignant phenotype⁴⁸. Epigenetics refers to the study of clonally inherited changes in gene expression without accompanying genetic changes. There are three general molecular mechanisms carrying epigenetic information – DNA methylation, histone modifications and RNA interference^{15;43}. In cancer, there has been particular recent interest in the involvement of aberrant DNA methylation and histone modifications in gene silencing that then mediate altered physiology^{9;49}. They may be particularly relevant to MDS

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pathogenesis given that the disease responds quite well to drugs that affect DNA methylation⁴⁰, a major epigenetic modulator. In this article, recent progress on MDS epigenetics and epigenetic-based therapies will be reviewed.

DNA methylation in epigenetic regulation

DNA methylation is a covalent modification of cytosines resulting in the formation of 5-methyl-cytosine (5mC), a base that changes the interactions between protein and DNA. CpG methylation can occur anywhere in the genome, but is particularly relevant when it involves CpG rich regions called CpG islands. In turn, these can be present in about half of human gene promoters. CpG island methylation is associated with absent transcription from the involved promoter, and this silencing is stably transmitted through mitosis, thus insuring clonal inheritance¹². This association between CpG island methylation and absent transcription is most striking when one considers the two physiological conditions where this process was described, X-inactivation³⁴ and imprinting⁷. In both cases, one of the two copies of the involved genes is transcriptionally silent in association with promoter methylation, despite continued normal expression of the unmethylated allele. Evidence for a direct role of methylation in maintaining the silenced state came from studies where methylation was relieved via pharmacologic⁴⁷ or genetic⁶⁶ reduction in DNA methyltransferase (DNMT) activity. In most such studies, bi-allelic expression could be restored, in association with decreased methylation of the affected promoters.

The mechanism whereby CpG island methylation suppresses gene transcription has been partially elucidated recently (Fig. 1), at least in vitro⁵¹. Methylated CpG islands form excellent binding sites for methylated-DNA binding proteins (often with transcriptional repression properties), such as MeCP2. MeCP2 binding is followed by the recruitment of a protein complex that includes histone deacetylases (HDAC), and eventually leads to a closed chromatin configuration. This closed chromatin configuration results in exclusion of transcription factors, thus insuring allele-specific inactivation. Methylation-related epigenetic silencing has also been found to be associated with histone H3 lysine 9 (H3K9) methylation⁴³. Evidence suggests that H3K9 methylation is a critical modification that is associated with closed chromatin at DNA methylation sites, and it was proposed that a cascade of events follows DNA methylation (MeCP2 binding, H3K9 deacetylation, H3K9 methylation) and ensures transcriptional suppression^{22;59;74} (Fig. 1). Separately, DNMT1 can directly suppress transcription (without DNA methylation) through interactions with histone deacetylases^{25;89}. H3K9 methylation itself appears to set-up a silencing loop by attracting more DNA methylation⁹⁷, and may sometimes precede hypermethylation⁵.

DNA methylation changes in cancer

There are complex changes in DNA methylation in cancer. For the most part, these changes involve simultaneous global demethylation, increased expression of DNMTs and de-novo methylation at previously unmethylated CpG islands. Demethylation was first discovered by studying overall 5-methyl-cytosine (5mC) content in tumors, and appears to involve primarily satellite DNA, repetitive sequences, and CpG sites located in introns^{23;44}. The cause of this demethylation remains unclear, although it could be related to alterations in proliferation or cell-cycle control²⁹. The functional consequences of hypomethylation are not entirely clear, but there is mounting evidence that gene-specific hypomethylation can cause increased expression of various genes that could contribute to the neoplastic phenotype²¹. An increased mutation rate was demonstrated in cells in which severe hypomethylation (>75%) was achieved by homozygous deletion of DNMT1¹⁶, but it is not clear whether this degree of hypomethylation is ever achieved in neoplasms¹⁰⁷.

Increased enzymatic DNMT activity is a property of nearly all transformed cells⁵⁵. Increased mRNA levels for DNMT1, DNMT3a and DNMT3b have also been described in some neoplasms including leukemias^{19;41;85}, and these three DNA-methyltransferase genes probably account for the observed increase in activity. The causes and functional significance of these increases remain unclear. DNMTs appear to be cell-cycle regulated⁹⁶, and it has been argued that DNMT levels do in fact reflect the physiologic state of increased proliferation in neoplasia⁶³. On the other hand, DNMT1 has been reported to increase following oncogene activation^{6;88}, and it is possible that its levels in neoplasia reflect the various molecular defects seen in tumors. The functional significance of increased DNMT activity is also poorly defined. In several systems, increased DNMT activity has been found to be transforming^{6;106}, but it is not clear whether this is due to increased CpG island methylation and tumor-suppressor gene silencing, or due to direct effects on the cell-cycle. The facts that, in primary tumors and cell lines, no correlation was found between DNMT activity and gene silencing^{67;100}, and that DNMT-related transformation is reversible when the oncogenic stimulus is removed⁶ support the latter possibility. Nevertheless, simultaneous inhibition of multiple DNMTs does inhibit cancer cell growth⁸², making them potential therapeutic targets.

In parallel to global hypomethylation and increased DNMT activity, there also are distinct and frequent localized increases in methylation, often involving CpG islands^{9;49}. Because CpG island methylation is associated with repressed transcription that is stably inherited through mitosis, this de-novo methylation in transformed cells has been proposed to serve as an alternate mechanism for inactivating tumor-suppressor genes⁴⁸. Indeed, several genes have now been shown to be transcriptionally silent in neoplasia, in association with CpG island methylation, including in leukemias. The most convincing evidence for CpG island methylation as a true alternative to mutations in neoplasia came from studies of *RBI*⁹⁰, *p16*⁷³, *VHL*³⁶ and *MLH1*⁵². For each of these genes, the tumor-spectrum of methylation events is virtually the same as that for mutations, and there are described cases where one allele of the gene is inactivated by methylation while the other is mutated, suggesting an equivalent growth advantage for each event in neoplasia. For example, in colorectal cancer, the HCT116 cell line carries one mutated unmethylated *p16* allele, while the second allele is unmutated but densely hypermethylated and transcriptionally silent⁷³. Moreover, the *MLH1* mismatch repair gene is unmutated but densely methylated in the mismatch repair deficient cell line RKO, and nearly normal levels of mismatch repair can be restored by inhibiting DNMT activity³⁷.

Histone modifications and the histone code

Histones are small proteins that form a core around which DNA is wrapped, forming nucleosomes. Nucleosomes are the basic in-vivo structural unit of DNA, and consist of 8 histone molecules (2 each of Histones H2A, H2B, H3 and H4) around which a loop of DNA is wrapped⁵⁶. While H2A and H2B are thought to play primarily a structural role, it has become apparent that Histones H3 and H4 are key integrators of a variety of signals that regulate gene transcription^{43;62;83}. In particular, these two histone proteins have histone “tails” or strings of amino acids that protrude outside of the basic nucleosomal structure and make contact with DNA. Specific post-translational modifications of the amino acids in these histone tails (e.g. methylation, acetylation, phosphorylation, ubiquitination, sumoylation) interact with other proteins to create nucleosomes that are relaxed and promote transcription, or nucleosomes that are closed, exclude transcription factors and result in gene silencing. These histone modifications occur relatively dynamically and are mediated by specific histone modifying proteins. Targeting of these histone modifiers to specific gene promoters in-turn is achieved by transcriptional activator/co-activator complexes in response to physiologic stimuli. Thus, histone modifications form a “code” that integrates gene activation/inactivation/silencing signals, such that the transcriptional activity of a given promoter can be predicted by looking at the specific histone modifications⁴³.

Currently, the best understood histone modifications are acetylation and methylation of specific residues, although it is clear that other modifications also play a role in the process⁴³. Acetylation of specific residues on histone H3 and H4 is typically associated with active gene transcription³². Methylation of specific residues can be associated with either activation of transcription (H3K4) or silencing (H3K9, H3K27)⁶². The different modifications also significantly interact. Thus, H3K4 methylation promotes H3K9 acetylation. Moreover, H3K9 can be either acetylated or methylated (but not both), and the switch from acetylation to methylation at this residue appears to be a key element of silencing across many organisms. H3K9 promotes silencing by recruiting HP1 and a silencing complex that changes chromatin structure locally and results in exclusion of transcription factors. An added complexity to the process was demonstrated when residues were shown to have several possible methylation states^{77;84;92}. For H3K9, mono and dimethylation are associated with euchromatin silencing, and trimethylation is associated with pericentric heterochromatin silencing. The H3K9 methylation/silencing switch appears conserved across evolution and is active in mammalian cells⁶². H3K27 methylation appears distinct from H3K9 methylation; H3K27 trimethylation has been reported to occur early in X-chromosome inactivation⁸⁷, and leads to silencing by recruitment of polycomb group (PCG) proteins⁷⁵. By contrast H3K9 trimethylation is primarily a mark of heterochromatin, and leads to silencing by recruitment of HP1 proteins⁶². Thus, these two processes, which are mediated by different enzymes, are potentially functionally distinct. There is now considerable interest in the involvement of the histone code in stemness and differentiation. A bivalent chromatin domain has been described in embryonic stem cells¹¹, whereby some unexpressed genes have co-existence of activating (H3K4Me) and silencing marks (H3K27Me). Upon differentiation, this bivalency resolves either into the activated or the suppressed state.

While histone modifications are relatively stable, histones are eventually degraded, and the nucleosome structure is substantially altered during DNA synthesis²⁸. Moreover, histone acetylation and methylation can be directly reversed by a large number of histone deacetylases and demethylases. Thus, for histone modifications to retain epigenetic memory, some form of targeting has to be operative. DNA methylation is one such form of targeting; so is persistent expression of transcription factors. It has also been argued that the histone modifications themselves could be a form of epigenetic memory in the absence of DNA methylation or other proteins to direct the process³³.

Histone modifications and cancer

Histone modifications have been implicated in the neoplastic process in a variety of ways. Indirectly, a number of genes that are altered in cancer affect gene expression via recruitment of histone modifying enzymes and resulting changes in gene expression. For example, the PML-RAR gene translocation in acute promyelocytic leukemia recruits HDAC to inhibit the expression of target genes, thus contributing to malignant transformation⁷². More directly, several histone-modifying enzymes are molecularly altered in cancer. For example, the histone acetyltransferase CBP is mutated in some acute leukemias⁶⁴, the MLL gene, an H3K4 methylase is rearranged in a significant portion of acute leukemias³, the RIZ1 gene, a putative histone methylase is silenced in some cancers¹⁸ and EZH2, a H3K27 tri-methylase is over-expressed in various malignancies¹⁰³ which also exhibit de-novo H3K27triM mediated silencing⁵⁸. Another mechanism modifying the histone cancer code in cancer is aberrant promoter methylation³⁵. Such DNA methylation may lead to silencing via recruitment of histone modifying enzymes that ultimately alter the histone code in favor of gene silencing.

Epigenetic changes in MDS

Most studies of epigenetics in MDS have focused on DNA methylation so far. Several genes have been shown to be transcriptionally silenced in association with promoter DNA methylation in this disease (Table 1). These include genes involved in cell-cycle regulation (CDKN2A), apoptosis (DAPK1, RIL), adhesion and motility (CDH1, CDH13) and others. Separately, some of these genes clearly have minimal functional impact on the disease, not being expressed in normal hematopoietic cells. MDS cases often show hypermethylation of several genes simultaneously⁹³. Thus, hypermethylation can be viewed in a similar way as mismatch repair deficiency and microsatellite instability in cancer: many loci are affected simultaneously, a few of which likely have functional consequences.

In MDS, CDKN2B (P15) has been the most extensively studied gene. CDKN2B was reported to be methylated in 30-80% of the cases, with the variability being likely due to different methods of measurement, as well as inclusion of different types of MDS. Thus, CDKN2B methylation has been reported to be very frequent in therapy related MDS, as well as in CMML, in RAEB-T or AML arising from MDS^{4;17;81;99;101}. CDKN2B methylation in MDS has also been associated with older age, deletions of 5q and 7q, and a poor prognosis^{4;17;81;99;101}. Interestingly, when present, CDKN2B methylation in MDS has been shown to affect multiple lineages from clonogenic cells to circulating mononuclear cells². In a mouse model, loss of CDKN2B was associated with enhanced myeloid progenitor and reduced erythroid progenitor formation⁸⁶, suggesting that its inactivation plays a functional role in MDS.

In a recent study focusing on quantitative analysis of the methylation status of 10 separate genes, a hypermethylator phenotype was identified that marks a subset of cases with MDS⁹³. This phenomenon, first described in colon cancer¹⁰⁰, results in the simultaneous inactivation of multiple genes by an unknown mechanism. In MDS, this form of intense hypermethylation is associated with rapid progression to AML and a shortened survival in multivariate analyses⁹³. This explains in part why the methylation of so many genes has been reported to be prognostic in MDS (Table 1): In all likelihood, all these studies of individual genes are pointing to a common subset of cases affected by the hypermethylator phenotype.

Whole genome scans for DNA methylation are beginning to reveal the complexity of the disease at an epigenetic level^{24;45}. Hundreds of genes are frequently hypermethylated in MDS, with evidence for enrichment of WNT pathway genes²⁴. As indicated by studies of individual genes, hypermethylation across the genome is associated with poor prognostic features and transformation to AML. Intriguingly, there was a distinct methylation pattern in AML and related AMLs compared to de-novo AML, pointing to distinct pathogenic mechanisms²⁴. Thus, a consensus has emerged that DNA methylation is abnormal early on in MDS, and that progression of the disease is associated with accumulation of additional epigenetic events.

By contrast to DNA methylation, detailed studies of histone modifications in MDS remain to be described. A few cases of AML have chromosomal translocations involving MLL, a known epigenetic modifier. Recently, mutations in the polycomb related gene ASXL1 were described in about 10% of cases²⁷, and mutations in the TET2 gene were found in about 25% of cases. TET2 is related to TET1, a gene which converts 5-methylcytosine to 5-hydroxymethylcytosine, and thus may be involved in regulation of DNA methylation. It is not known whether MDS cases with abnormalities in MLL, ASXL1 or TET2 have characteristic epigenetic patterns.

Epigenetic Therapy

Treatment of cancer by targeting epigenetic pathways has been referred to as epigenetic therapy²⁰. The principle of this approach is to reverse pathologic gene expression changes in malignant cells, which is presumed to elicit a therapeutic effect that culminates in tumor

responses. The specific therapeutic effect induced by these agents is still a matter of debate, and there are data favoring many differing pathways as affected by this therapy, including differentiation, senescence, apoptosis, immune recognition etc.⁴⁰ In addition, many epigenetic drugs show dose-dependent cytotoxicity^{8;80}, which could also be a factor in responses. The therapeutic index of this approach lies, in principle, on the fact that cancer cells are more dependent on continued silencing tumor-suppressor genes and like molecules than normal cells. There have been concerns that epigenetic therapy could have dramatic effects on normal cells through reactivating, for example, imprinted genes, and may even lead to cancer formation. In-vivo, however, these fears have not been substantiated in studies so far, with no evidence of unusual side-effects, new chromosomal defects or secondary malignancies¹⁰⁷. It is likely that the doses of drugs that elicit such effects are not achievable in-vivo.

Targeting epigenetics at the present time essentially equates to targeting silencing pathways in cancer and their potential interactions. A central pathway (Fig. 1) involves DNA methylation, methyl-binding proteins, histone deacetylation, histone (H3K9) methylation and eventual binding of a silencing protein complex that, itself, may trigger more methylation⁵⁷. This “silencing loop” is stable, as evidenced by imprinted genes and the inactive X-chromosome, which remain turned off for decades in adult cells. There is also a distinct silencing mechanism in development and cancer that involves the PCG complexes PRC1 and PRC2, resulting in H3K27me3 modification. These complexes also involve histone deacetylases. Each step in these silencing cascades is a potential target for therapeutic intervention. Combining inhibition of multiple epigenetic targets will likely be synergistic, as demonstrated for DNA methylation and HDAC inhibition¹⁴. There are two epigenetic targets for which drugs are available in clinical trials – DNA methylation and histone deacetylation.

5-Azacytidine (azacitidine) and 5-aza-2'-deoxycytidine (DAC, decitabine) are two hypomethylating cytosine analogues with activity in leukemia^{65;91}. Both drugs were synthesized in the 1960's as cytosine analogues, and were shown in the early 1980's to be potent DNA methylation inhibitors and in-vitro differentiation inducers⁵⁰. DNA methylation inhibition is related to the shared modified structure of the cytosine ring with a C to N substitution at the 5 position, resulting in trapping and eventual degradation of DNA methyltransferases. Azacitidine incorporates into RNA and, after intracellular conversion to DAC, incorporates into DNA and inhibits DNA methylation. Unlike azacitidine, DAC does not incorporate into RNA and is directly incorporated into DNA, resulting in 10 fold higher demethylating activity at equimolar concentrations in-vitro⁵⁰. Both drugs have activity in MDS demonstrated in randomized studies^{65;70;94}, and both are now approved by the United States Food and Drug Administration. A feature of both azacitidine and DAC studies in MDS has been delayed clearance of blasts, delayed myelosuppression, slow responses (median number of cycles to best response was >3) and eventual cytogenetics responses. A similar phenomenon has been seen with CML patients treated with DAC⁵⁴. These studies have suggested that the mechanism of action of these drugs is not cytotoxicity, although the exact mechanism of achieving complete remissions is not known.

There are a variety of Histone deacetylase inhibitors (HDACi) in clinical trials currently⁷¹. These include drugs discovered through activity in the NCI-60 cell panel (romidepsin), drugs discovered through in-vitro differentiation screens (vorinostat), or drugs discovered to be HDACi serendipitously (Valproic Acid⁷⁸). For all these drugs, in-vitro inhibition of HDAC activity was demonstrated, as well as gene reactivation and induction of apoptosis. Once again, the mechanism of downstream action of these drugs is unclear, with recent data pointing towards DNA damage and effects on reactive oxygen species¹⁰² (in addition to gene expression activation). Clinically, several of these drugs are in phase I/II studies, with activity demonstrated for depsipeptide and SAHA in lymphomas⁷⁹, and both drugs are now approved

for the treatment of cutaneous T-cell lymphoma. Less data is available in MDS and AML, though anecdotal responses have been reported^{26;61}.

Drugs targeting other epigenetic pathways are currently in development or pre-clinical studies. There is particular interest in drugs that can inhibit the activity of various histone methyltransferases^{60;98}, as these could work independently of (and complement) DNA methylation and histone deacetylation inhibitors. It is likely that several drugs targeting epigenetic pathways will enter clinical trials in MDS in the next few years.

Conclusions

Epigenetic pathways mediate cancer-specific gene expression abnormalities in multiple genes involved in determining the neoplastic phenotype. MDS is characterized by multiple epigenetic abnormalities that can help determine prognosis and risk of progression to AML. Drugs that target epigenetic pathways have changed the natural history of MDS and offer some hope that the disease could be treated successfully in the next decade.

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Figure 1.

Two silencing pathways. Left: Model of the DNA methylation associated gene silencing loop. The cascade of events may start with DNA methylation triggering histone modifications or with Histone H3 lysine 9 (K9) methylation triggering silencing, which then promotes DNA methylation. Right: Polycomb group (PcG) based silencing. Targeting PRC2 to a given promoter results in histone deacetylation and H3K27 trimethylation, which triggers PRC1 binding, a closed chromatin structure and gene silencing. In turn, H3K27 trimethylation may also recruit PRC2 binding to sustain silencing through mitosis.

Table 1

Aberrant promoter CpG island hypermethylation in MDS

Gene	Methylation frequency (%)	Function	Note
Calcitonin ³⁹	50	Differentiation	
CDKN2B ¹⁰¹	23-80	Cyclin dependent kinase inhibitor; cell cycle/proliferation	Tumor-suppressor; methylation correlates with poor prognosis and progression to AML ⁸¹
DAPK ¹⁰⁴	50	Proapoptotic serine/threonine kinase	
RASSF1 ⁴⁶	9	Negative regulator of RAS signaling	Tumor-suppressor
FHIT ⁴²	50	Purine metabolism	Putative tumor-suppressor; methylation correlates with poor prognosis and progression to AML ⁶⁸
HIC1 ¹	32	Transcriptional repressor	Tumor-suppressor
CDH1 ¹	15-27	Adhesion and motility	Methylation correlates with poor prognosis and progression to AML ³¹
CTNNA1 ⁶⁹	10	Alpha catenin	
ER α ¹	7-19	Estrogen receptor	Methylation as part of a panel of genes (also including CDH1 and CDKN2A) correlates with poor prognosis and progression to AML ¹⁰⁵
RIL ¹³	36-70	Proapoptotic, tumor-suppressor	
CDH13 ¹⁰⁵	21	Adhesion and motility	
NOR1 ¹⁰⁵	15	Oxidoreductase domain-containing protein	
NPM2 ¹⁰⁵	20	Nucleophosmin/nucleoplasm 2, involved in development	
OLIG2 ¹⁰⁵	41	Basic helix-loop-helix transcription factor	
PGR ¹⁰⁵	45	Progesterone receptor	
PGRB ¹⁰⁵	45	Progesterone receptor	