MDS, Methylation and the Epigenetic Paradigm

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MDS is a pre-cancerous condition of the blood generating cells which often results in AML. It is caused by hypermethylation of the DNA and thus it is driven by the suppressing of the genes which modulate proliferation. Various hematopoietic cell lines produce immature blasts and this process progresses. There are now therapeutics which suppress the hypermethylation but are not curative. When combined with bone marrow transplants and use of Cytokine Induced Killer cells one may have developed a tiered process that could produce curative results. We explore the progress obtained herein. It may represent a powerful paradigm for other cancer treatments.

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Contents

1 INTRODUCTION

Epigenetic factors are appearing to be more prevalent in our understanding of the causes of many cancers. These factors include such elements as methylation, long non-coding RNAs (lncRNA), micro RNAs and acetylation. None of these reflect a fundamental change in the DNA of the underlying genes, but they do reflect a complex process whereby the way the DNA is processed and presented functions. Unlike translocations and gene changes which are difficult to unravel, many of these epigenetic changes may be found to be reversible in part or in whole. We focus on methylation and methylation related disorders herein.

1.1 THE MDS THERAPEUTIC PARADIGM

MDS, the myelodysplastic syndrome, is a multifaceted disease of the bone marrow cells which leads to the over-production of immature blood cells; erythrocytes, lymphocytes, platelets and others. It is often indolent in its early stages but then turns quite virulent and is often fatal, frequently due to the development of AML, acute myelogenous leukemia. However, recent understanding of a key driver of MDS, namely hypermethylation, has resulted in complex therapies which may have proven not only efficacious but curative.

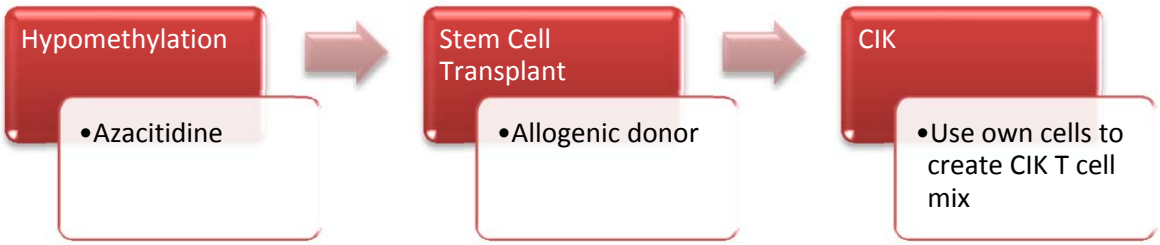
We use this disorder as an example of how methylation causes potential cancers and further how it can be targeted and treated.

The therapeutic responses to MDS are representative to the multi-prong attack on various cancers. The fact that MDS is not per se a cancer but an artifact of a hypermethylation state, and that hypermethylation can be reversed, as compared to a genetic change such as found in CML, the Philadelphia chromosome translocation, and that we know how to deal with hypermethylation, lends MDS to some form of initial treatment. However demethylation does not always work.

Thus the second attack is more aggressive which is a modified hematologic stem cell transplant.

That further reduces the aberrant cell load to an almost miniscule amount. The final hit is using modified T cells called cytokine induced killer cells specifically targeted for the remaining

hypermethylated cells. We depict this below:



This paradigm has been applied to other malignancies with substantial success. The classic cases are the childhood leukemias and Hodgkin's lymphoma. One would suspect that MDS being substantially of the same class would fit this paradigm. Our intent here is to examine the literature across the above spectrum and attempt to make an assessment of progress in this disease.

1.2 HISTORICAL CONTEXT

Methylation has been know of for decades but it has only been in the last fifteen years or so that the connection between methylation and cancers has been somewhat understood. In a 1997 paper by Jones and Gonzalgo the authors state:

DNA methylation is a mechanism for changing the base sequence of DNA without altering its coding function. As a heritable, yet reversible, epigenetic change, it has the potential of altering gene expression and has profound developmental and genetic consequences. The methylation reaction itself is mechanistically complex and involves the flipping of the target cytosine out of the intact double helix, so that the transfer of the methyl group from S-adenosylmethionine can occur in a cleft in the enzyme.

Cytosine methylation is inherently mutagenic, which presumably has led to the 80% suppression of the CpG methyl acceptor site in eukaryotic organisms, which methylate their genomes. It contributes strongly to the generation of polymorphisms and germ-line mutations, and to transition mutations that inactivate tumor-suppressor genes. Despite a 10- to 40-fold increases in the rate of transitions.

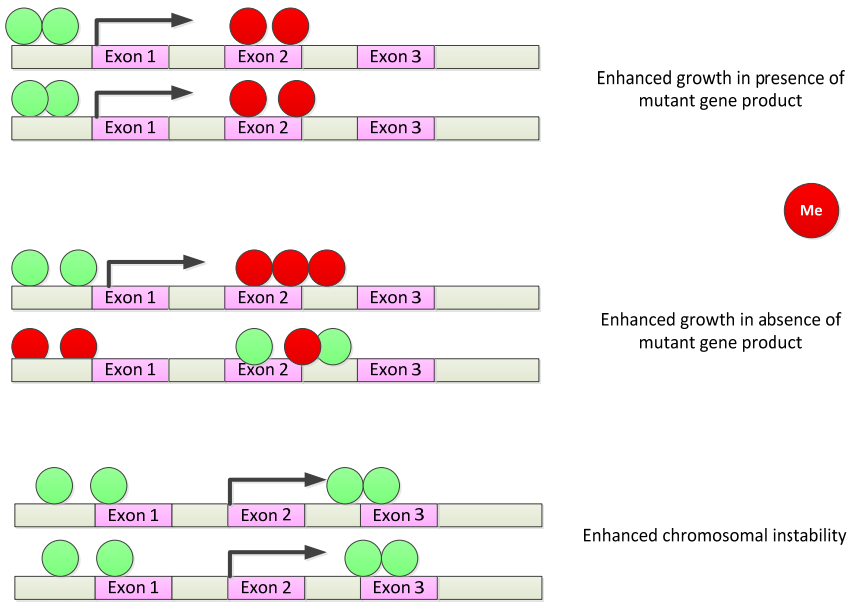
This was somewhat of an opening salvo regarding methylation and cancers. One should remember that this was almost five years before the complete reading of human DNA and also at a time when actually reading the methylated states was complex at best.

The authors hypothesized a mechanism for uncontrolled growth using the methylation construct. They posited three ways in which methylation functioned.

First, it caused a gene change. This was the C to T mutation change.

Second they posited the promoter suppression via methylation of the promoter. This method is seen quite frequently in the process.

Third, there may be a chromosome instability resulting from methylation.



At the same time Robertson and Jones wrote a paper on DNA methylation and its affects and they also suggested a strong link between that and cancer. They stated:

As with the demethylation and de novo methylation observed during development, changes in methylation patterns during neoplasia have been recognized for some time. Initially it was shown that malignant cells have lower levels of methylation than do normal cells. This global hypomethylation accompanies a hypermethylation of CpG islands, DNA regions often associated with promoters of human genes that are normally protected from methylation.

The above statement is a clear description of what we now know to be correct; namely hypomethylation globally but hypermethylation of the CpG islands. The hypomethylation allows expression of a wide variety of proliferation genes while the CpG Island silencing via hypermethylation deactivates control genes. They continue:

The mechanism by which these regions remain unmethylated in the normal cell is not known, but it may be mediated by the binding of certain transcription factors. In malignant cells, these CpG-island regions become methylated and expression of the associated gene is silenced. In the case of a tumor-suppressor gene, this may result in a growth advantage for the cell.

DNA methylation– mediated transcriptional inhibition has thus been proposed as a mechanism that is alternative to mutation and deletion, in the removal of tumor suppressor– gene function. Examples of such genes include the two cell-cycle regulators p16 Ink4a and p15 Ink4b, the von Hippel–Lindau gene VHL in some renal carcinomas, the retinoblastoma gene product Rb, BRCA1, the angiogenesis inhibitor thrombospondin, and the metastasis-suppressor gene E-cadherin.

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PCNA is the polymerase-processivity factor for the d and e DNA polymerases, is homologous to the E. coli b subunit, and is required for DNA replication becomes altered in cancer. It was shown that the DNA methyltransferase is targeted to newly replicated DNA by the replication associated protein PCNA (proliferating cell nuclear antigen). PCNA is the polymerase-processivity factor for the d and e DNA polymerases, is homologous to the E. coli b subunit, and is required for DNA replication

1.3 WHY STUDY MDS?

There are slightly more than 10,000 new cases of MDS each year. There may be a little difficulty in determine them because they can often go un-noticed until they convert to AML at which point the diagnosis would be clear. There may be a slight anemic, thrombocytopenia, and the presence of blasts, immature hematopoietic cells. A true diagnosis requires a bone marrow biopsy. The MDS patient may have one of many variants which we shall discuss latter.

However what seems common is the presence of hypermethylation resulting in the suppression of cell growth and proliferation control genes on the lineage of hematopoietic cells first affected. Thus the thrombocytes may be the initial ones affected and we see a drop in platelets and a presence of blasts. But in all cases it is the hypermethylation. There is as of yet in the process no

genetic change, the excess immature growth is due solely to hypermethylation. Thus the control is simply control of hypermethylation via drugs which block the process. It is a somewhat simple model for developing a therapeutic.

Thus why study MDS? The answers are:

1. MDS is not a full blown cancer. It lacks the genetic breakdown.

2. MDS is a hypermethylation disease. Hypermethylation can be reversed. Thus there is an opportunity to seek a "cure".

3. MDS does lead to cancer, most likely AML. The process that results in that change is worth of study as a means to seek both prevention and cure.

4. MDS can be monitored both genetically as well as via hypermethylation measurements.

1.4 OVERVIEW

In this report we examine several factors in depth. Specifically:

MDS: We present an overview of MDS and its various forms. This is a complex disease and it is almost as if no one patient is identical to any other patient. We consider the cause of methylation at the DNA level but we can at best speculate on the ultimate initiator. We know that many MDS patient had pre-existing malignancies for which the received both chemotherapy and radiation therapy. The nexus there seems to somewhat clear. However, many, if not most, MDS patients have no clearly defined initiating event.

Methylation: We explore methylation and examine how it occurs, and what it does to the functioning of the DNA expression. In many of our cancer models we often just look at gene, RNA and protein flow. As we have indicated before we often look at the epigenetic factors as noise. However it has become clear that the epigenetic elements are integral parts of a cells expression of its genetic capabilities and thus should be included in any model.

Demethylating Therapies: We examine the various demethylating therapies. The specifics are discussed in some detail as well as the efficacy of the therapeutics.

Acetylation: The histones around which the DNA is wound also exhibit acetylation. We examine this phenomenon and relate it to methylation.

Immunotherapy: We discuss immunotherapy focusing on the use of CIKs, cytokine induced killer cells, primed T cells directed at the remaining methylated hematopoietic cells.

We conclude with observations relevant to combined therapies.

2 MDS

We will now examine MDS from a clinical perspective. MDS is a complex set of disorders. There is no single measurement and almost every patient a physician may see presents a somewhat unique set of abnormalities. At the heart of all is a cytopenias of the blood, anemia, thrombocytopenia, leukopenia, and the like. In some cases, as is often the case, it is found as an incidental finding on a blood test leading to more detailed bone marrow studies.

2.1 DEFINITION

To define MDS we use the recent work of Tefferi and Vardiman who state:

The main feature of myeloid neoplasms is stem-cell–derived clonal myelopoiesis with altered proliferation and differentiation. The phenotypic diversity of these neoplasms has been ascribed to different patterns of dysregulated signal transduction caused by transforming mutations that affect the hematopoietic stem cell. There is increasing evidence that haploinsufficiency, epigenetic changes, and abnormalities in cytokines, the immune system, and bone marrow stroma all contribute to the development of the myelodysplastic syndromes.

The WHO Classification of MDS is as in the following:

1. Acute myeloid leukemia and related neoplasms*
2. Myelodysplastic syndromes
    a. Refractory cytopenia with unilineage dysplasia†
        i. Refractory anemia (ring sideroblasts <15% of erythroid precursors)
        ii. Refractory neutropenia
        iii. Refractory thrombocytopenia
    b. Refractory anemia with ring sideroblasts (dysplasia limited to erythroid lineage and ring sideroblasts ≥15% of bone marrow erythroid precursors)
    c. Refractory cytopenia with multilineage dysplasia (regardless of ring sideroblast count)
    d. Refractory anemia with excess of blasts (RAEB)
        i. RAEB-1 (2–4% circulating blasts or 5–9% marrow blasts)
        ii. RAEB-2 (5–19% circulating blasts or 10–19% marrow blasts or Auer rods present)
    e. Myelodysplastic syndrome with isolated del(5q)
    f. Myelodysplastic syndrome (unclassifiable)
3. Myeloproliferative neoplasms
4. Myelodysplastic–myeloproliferative neoplasms
5. Molecularly characterized myeloid or lymphoid neoplasms associated with eosinophilia

The presence of excess blasts is generally the telling factor. Yet as the above authors state:

The minimal morphologic criterion for the diagnosis of a myelodysplastic syndrome is dysplasia in at least 10% of cells of any one of the myeloid lineages. However, such changes can also be seen in other myeloid neoplasms, which must be excluded before a diagnosis is made. These include AML, which is defined by at least 20% myeloblasts in bone marrow or peripheral blood; MDS– MPN, in which dyserythropoiesis or dysgranulopoiesis is associated with leukocytosis or monocytosis (>1.0×109 cells per liter), as in CMML; and MPN, in which both dyserythropoiesis and dysgranulopoiesis are absent.

From Greenberg et al we have the following IPSS characterization. First the cytogenetic abnormalities must be evaluated. They fall into 5 categories as shown below. One can have from 1 to over 3 abnormalities and the greater the number the higher the risk of low survival.

Prognostic subgroups (% patients)	Cytogenetic abnormalities	Survival* Years, median	AML evolution, 25%* Years, median	Hazard ratios OS/AML*	Hazard ratios OS/AML^
Very good (4%*/3%^)	-Y, del(11q)	5.4	NR	0.7/0.4	0.5/0.5
Good (72%*/66%^)	Normal, del(5q), del(12p), del(20q), double including del(5q)	4.8	9.4	1/1	1/1
Intermediate (13%*/19%^)	del(7q), +8, +19, i(17q), any other single or double independent clones	2.7	2.5	1.5/1.8	1.6/2.2
Poor (4%*/5%^)	-7, inv(3)/t(3q)/del(3q), double including -7/del(7q), complex: 3 abnormalities	1.5	1.7	2.3/2.3	2.6/3.4
Very poor (7%*/7%^)	Complex: >3 abnormalities	0.7	0.7	3.8/3.6	4.2/4.9

Then we take the complete set of five measurements and assign them to the following Table and create a score based upon each one, the result being the cumulative score.

Prognostic variable	*0*	*0.5*	*1*	*1.5*	*2*	*3*	*4*
Cytogenetics	Very Good		Good		Inter-mediate	Poor	Very Poor
BM Blast %	2		2%-5%		5-10%	>10%	
Hemoglobin	10		8-<10	<8			
Platelets	>100,000	50,000-100,000	<50,000				
ANC[1]	0.8	<0.8					

As above the cytogenetics can be 0, or 0.5 to 4.0. The blast score is 0, 1 or 1.5. The platelets can be 0, 0.5 or 1.0. Finally the ANC, absolute neutrophil count, may be high or low, and thus 0 or 0.5.

Category	*Score*
Very Low	≤1.5
Low	1.5-3.0
Intermediate	3.0-4.5
High	4.5-6.0
Very High	>6.0

Thus, we have a patient with the following profile:

1. 1 cytogenetic abnormality; they score Good and have a value 1.0
2. blasts in excess of 15% that yields a 3.0
3. Hemoglobin of 12: that is 0.0
4. Platelets at 80,000 that is a 0.5.
5. ANC in excess of 0.8 that is 0.0

The total score is 4.5. This patient is borderline on Intermediate and High Risk.

2.2 EPIDEMIOLOGY AND ETIOLOGY

[1] See: http://www.mdanderson.org/patient-and-cancer-information/cancer-information/cancer-types/myelodysplastic-syndrome/index.html Absolute Neutrophil Count (ANC) is a measure of the number of WBCs you have to fight infections. You can figure out your ANC by multiplying the total number of WBCs by the percentage of neutrophils ("neuts"). The K in the report means thousands. For example:

WBC = 1000 = 1.0K
Neuts = 50% (0.5)
1000 X 0.5 = 500 neutrophils

Let us examine the overall epidemiology of MDS. From DeVita et al Chapter 135 (8th Ed) we have:

The incidence of MDS in the United States is reported to be 3.4 per 100,000 persons.4 MDS is rare in patients younger than 50 years, but can reach as high as 20 to 50 per 100,000 in individuals older than 70 years.

With around 15,000 new patients diagnosed every year in the United States, MDS has become one of the most common disorders in the section of leukemias. The increase in incidence that is currently observed may relate to increased reporting by clinicians and pathologists. Reasons for not diagnosing MDS in the past may have included little interest in pursuing this diagnosis, especially in older patients (perceived lack of effective therapy other than supportive, comorbidities), and overlap of MDS with other disorders (aplastic anemia, myeloproliferative diseases, and AML).

Thus the incidence is quite small, as say compared to prostate or breast cancer and the complexity of the cellular presentation is also quite complex. After examining the putative predisposing causes we can find that given further the condition of the patient that each case is almost unique, unlike some of the more common cancers.

Let us now continue with etiology.

No etiologic factor is identified in most patients with MDS. MDS is more frequent in men than women by a factor of 1.8.4 It has been associated with smoking and hair dyes, exposure to agricultural and industrial toxins, drugs (e.g., chloramphenicol), and occupational exposures to stone and cereal dusts. MDS has been associated with exposure to ionizing radiation (atomic bomb survivors in Japan, decontamination workers following the Chernobyl nuclear plant accident) and chronic exposure to low-dose radiation (radiopharmaceuticals).5,6 Some inherited hematologic disorders (Fanconi anemia, dy-skeratosis congenita, Shwachman-Diamond syndrome, Diamond- Blackfan syndrome) are also associated with a higher risk of MDS.

There is the putative cause due to such chemicals as benzene and others but this is really limited evidence.

About 20% to 30% of patients with MDS have therapy-related MDS (t-MDS).7 Distinct clinical features have been described based on the nature of the triggering event. t-MDS following exposure to alkylating agents has a longer latency period (3 to 8 years) and is often associated with abnormalities of chromosomes 5 and 7; the latency period following topoisomerase II inhibitors is shorter (2 to 3 years), and cytogenetic- molecular abnormalities tend to involve rearrangements of the MLL gene on chromosome 11q23. Risk factors associated with t-MDS include the cumulative dose of alkylating agents (e.g., cyclophosphamide, melphalan, procarbazine, chlorambucil) or topoisomerase II inhibitors (e.g., etoposide), previous radiation exposure, older age, and use of radiotherapy prior to transplantation.

Indeed a significant number of patients have a clear path backward with aggressive radiation treatment. This is not just casual radiation but extensive radiation.

The number of patients with t-MDS is increasing because of better outcome for tumors that formerly lacked effective therapy. The incidence of t-MDS following therapy for other hematologic (e.g., Hodgkin's disease, non–Hodgkin's lymphoma, chronic lymphocytic leukemia) or non-hematologic malignancies (e.g., breast or testicular cancers) is between 1% and 15% according to which particular study and malignancy is concerned.8–12 Secondary and t-MDS are distinguishable from primary MDS by an earlier age of onset, more prominent dysplasia, more severe cytopenias, more rapid progression to AML, and worse outcome. The worse prognosis may be related to a higher frequency of poor-prognosis cytogenetic abnormalities in these cases.

The causes are often uncertain. As Stanford Medical Center states[2]:

People who have received radiation therapy, chemotherapy with alkylating agents (such as chlorambucil, cyclophosphamide, and melphalan), or who have been exposed to industrial solvents (such as benzene) have a higher risk of developing MDS than people who have not had these exposures. Rarely, genetic disorders are responsible for the disease. Nevertheless, in 60% to 70% of MDS patients, no specific cause can be identified.

Thus women who have been treated for breast cancer may very well have been pre-conditioned or those in the chemical field who may have dealt with benzene.

[2] http://cancer.stanford.edu/blood/mds.html

3 METHYLATION

Methylation is a recently understood process and is a part of the overall set of epigenetic factors which control the classic Watson-Crick paradigm. In classic Watson-Crick structures we have genes (DNA) to RNA to proteins. The next step is the feedback mechanisms amongst proteins and genes. The next step is the collection of dogs and cats we call epigenetic factors. In this section we present an overview of methylation, one of the epigenetic factors and one which dominates in MDS as well as many other cancers.

As Issa and Katarjian state:

We all start life thanks to inhibition of DNA methylation. As soon as embryogenesis begins, a massive decrease in DNA methylation reprograms the epigenome and creates a nearly blank slate on which development and differentiation can be written. Thus, a decrease in DNA methylation is compatible with life, at least in embryogenesis. Nuclear transplantation-induced reprogramming can also erase (if incompletely) DNA methylation in adult cells and, when applied to cancer, seems to reverse the malignant phenotype, even in the face of genetic alterations.

Outside of epigenetic reprogramming, inhibition of DNA methylation can only be achieved by genetic or pharmacologic targeting of DNA methyltransferase enzymes. Given that DNA methylation is a post-DNA synthesis event that needs to be sustained by the presence of methylating enzymes, cellular replication in the face of reduced levels of these enzymes results in significant demethylation in daughter cells, accompanied by gene reactivation.

When applied to cancer cells, this approach does have a therapeutic ratio; normal cells tend to survive hypomethylation whereas cancer cells tend to be killed (or at least stop proliferating) when this happens, perhaps because cancer cells are dependent on critical gene silencing for survival (whereas normal cells are not).

There is a possible restatement of the last paragraph. Some cancer cells if hypomethylated proliferated because the proliferation genes are not deactivated. Thus hyper methylation is not necessarily related to cancer and hypomethylation not so.

3.1 BASIC PRINCIPLES

DNA methylation is a process whereby the cytosine is changed by the insertion of a methyl group on the 5 carbon of the ring. It is a process which is epigenetic and can dramatically modify gene expression. In fact many of the methylation issue in humans are also common to plants, see the work by Zilberman. There has been a great deal of work demonstrating the impact of methylation on cancer progression; specifically the recent summary by Herman and Baylin, that of Palii and Robertson, that of Robertson and Wolffe, Strathdee and Brown, Calin and Croce, are all worth reviewing.

In this report we examine methylation and its impact on several cancers. We will also examine briefly the causes of methylation as well as the therapeutics in use to modulate cancers that cause or persistence is supported by methylation related products, either directly or indirectly.

In the paper by Das and Singal, the authors define epigenetics in a quite clear manner:

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence.

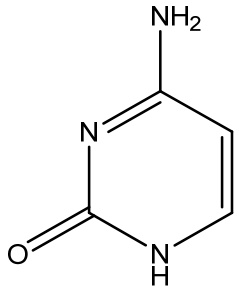
DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target.

Epigenetics has evolved as a rapidly developing area of research.

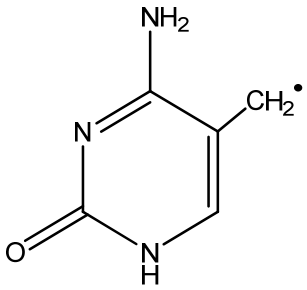
Recent studies have shown that epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation

This is one of the clearest definitions of epigenetics and especially the linking of methylation to epigenetics. The classic Watson and Crick model, now some 60 years old, we had the paradigm of DNA, RNA and protein. It was the proteins which did the work. In the 1953 world the proteins stood one by one and the clarity of gene to protein was unquestioned. Yet as we have come to better understand the details, and the details always count, there are many interfering epigenetic factors that all too often get in the way. Methylation is but one of those factors.

Basic cytosine is shown below. It has two NH groups at opposite poles and single oxygen.



Now when the 5 carbon is replaced by a methyl group we obtain the form below. This is methylated cytosine.



Thus this small change in C, by adding the methyl group, can make for a dramatic difference in the expression of genes. For example a well-controlled gene for proliferation, such as PTEN, may have its control over-ridden by the methylation of Introns of CpG islands, namely collections of C, cytosine nucleotides, and G, guanine nucleotides. The introns may be down from the gene, they may even be on a promoter section. The impact could aberrant cell proliferation and growth.

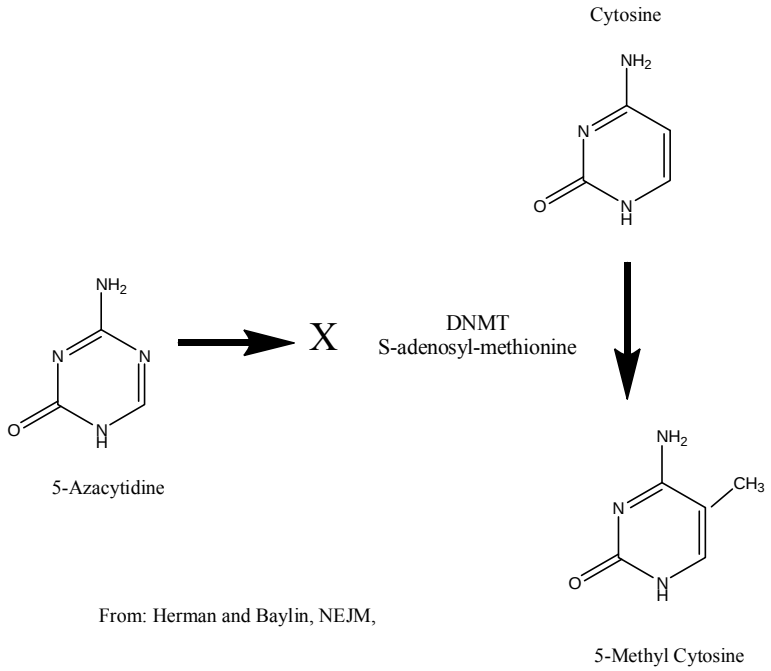
We examine the process; we then look at three types of cancers, a glandular, an epidermal, and a hematopoietic form and then examine some means used to control those cancers through the understanding or methylation and the control of it by therapeutics designed just for that purpose.

What is important about understanding methylation and especially all epigenetic changes is that it may perhaps be simpler to control them rather than a gene mutation. As Brower states:

The move from a purely genetic to an epigenetic model is crucial for prevention strategies. As numerous gene therapy trials have shown, it is very difficult to treat a genetic disease by re-activating the dormant, mutated gene or by replacing it with a non-mutated one. "Epigenetic changes are reversible, and therefore have an edge over genetics," says Mukesh Verma, an epigeneticist at the National Cancer Institute's division of cancer control and population sciences in Bethesda, Maryland. Furthermore, epigenetic changes in cancer occur before genetic mutations. "If you can prevent methylation of those tumour suppressor genes, you might have a valuable prevention strategy," says Baylin.

Thus if we see cancers when they are driven by methylation, then can we actually anticipate reversing the process by reversing the methylation changes. Thus with prostate cancer can we anticipate a preventative measure as one increasing certain methylation preventative therapeutics, can we do the same with say MDS, and can we attempt to do the same with say a melanoma. This is what we examine herein.

What is methylation? Simply, the attachment of a methyl group to the cytosine molecule creates a methylated C. This is not a complicated process but one which happens frequently and may have significant effects. Cytosine gets methylated and is converted to 5-methyl cytosine. This is accomplished by means of two enzymes as depicted below. This occurs when we have a C and G adjacent. It occurs to the C in that pair. We depict that transition below. Note also that by using 5-Azacytadine we can block that transition.



From: Herman and Baylin, NEJM,

5-Methyl Cytosine

Now there are the CpG islands. These are C, cytosine, and G, guanine, adjacent nucleotides which are connected via a phosphodiester bone between the two, and multiple collections of these paired nucleotides. The CpG island is then an area dense in these CG pairs connected by the phosphodiester bond, but the "island" may contain nucleotides other than the CG pairs, but generally are high in CG pair concentration, usually more than 50%.

One should note that the statistical probability of such large CG pairings would normally be quite low. One would anticipate equal probability for any nucleotide and any nucleotide pairing. Furthermore such a high concentration is statistically extremely rare but if often existentially quite common.

The CpG islands may be from 300 to over 3,000 base pairs in total length, and are frequently found in gene promoter regions. Thus when the CpG islands are methylated, namely the C is methylated, then the island gets silenced as does the corresponding gene. Namely methylation of CpG islands can result in gene silencing. This then becomes a critical issue if the gene is a control gene such as PTEN, p53, or many of the critical pathway control genes. The CpG islands are also propagated to cell progeny during mitosis, thus a methylated island remains so in the cells progeny.

However understanding methylation of islands, and having a means to demethylate the islands may present a reasonable way to develop therapeutics for cancers resulting from methylated regions. We shall examine that shortly.

As Laird and Jaenisch state:

The normal pattern of 5-methylcytosine distribution DNA methylation in mammals is found as a covalent modification at the fifth carbon position of cytosine residues within CpG dinucleotides. Most of the CpG dinucleotides in the human genome are methylated.

However, 5-methylcytosine makes up less than 1% of all nucleotides, since CpG dinucleotides are under-represented about five-fold in the mammalian genome. The paucity of CpG dinucleotides in the mammalian genome is attributed to a higher mutation rate of methylated versus unmethylated cytosine residues.

CpG dinucleotides and 5-methylcytosine are unevenly distributed in the genome. Most of the genome is heavily methylated with a corresponding deficit in CpG dinucleotides. About 1 to 2% of the genome consists of islands of non-methylated DNA and these sequences show the expected frequency of CpG dinucleotides.

CpG islands are about 1 kb long and are not only CpG-rich, but generally G/C-rich as well and are found at the 5' end of genes. All known housekeeping genes and some tissue-specific genes have associated CpG islands.

3.2 METHYLATION AND GENE EXPRESSION

We now want to discuss methylation and gene expression. Reference will be made to the work of Herman and Baylin, Jones and Takai, McCabe et al, Allis et al, and Issa and Kantarjian.

We begin with Herman and Baylin and their description of the diagram below:

In most of the mammalian genome, which is depicted here as exons 1, 2, and 3 of a sample gene (boxes 1, 2, and 3), introns of the gene (line between the exons), and regions outside the gene, the CpG dinucleotide has been depleted during evolution, as shown by the small number of such sites (circles).

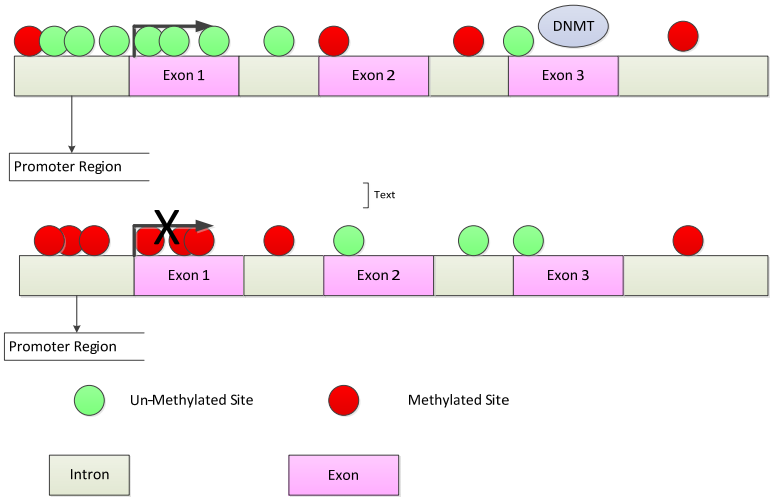
Small regions of DNA, approximately 0.5 to 4.0 kb in size, harbor the expected number of CpG sites and are termed CpG islands. Most of these are associated with promoter regions of approximately half the genes in the genome (numerous circles surrounding and within exon 1 of the sample gene). In normal cells, most CpG sites outside of CpG islands are methylated (black circles), whereas most CpG-island sites in gene promoters are unmethylated (white circles).

This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription (arrow in upper panel). In cancer cells, the DNA-methylation and chromatin patterns are shifted.

Many CpG sites in the bulk of the genome and in coding regions of genes, which should be methylated, become unmethylated, and a growing list of genes have been identified as having abnormal methylation of promoters containing CpG islands, with associated transcriptional silencing (red X at the transcription start site).

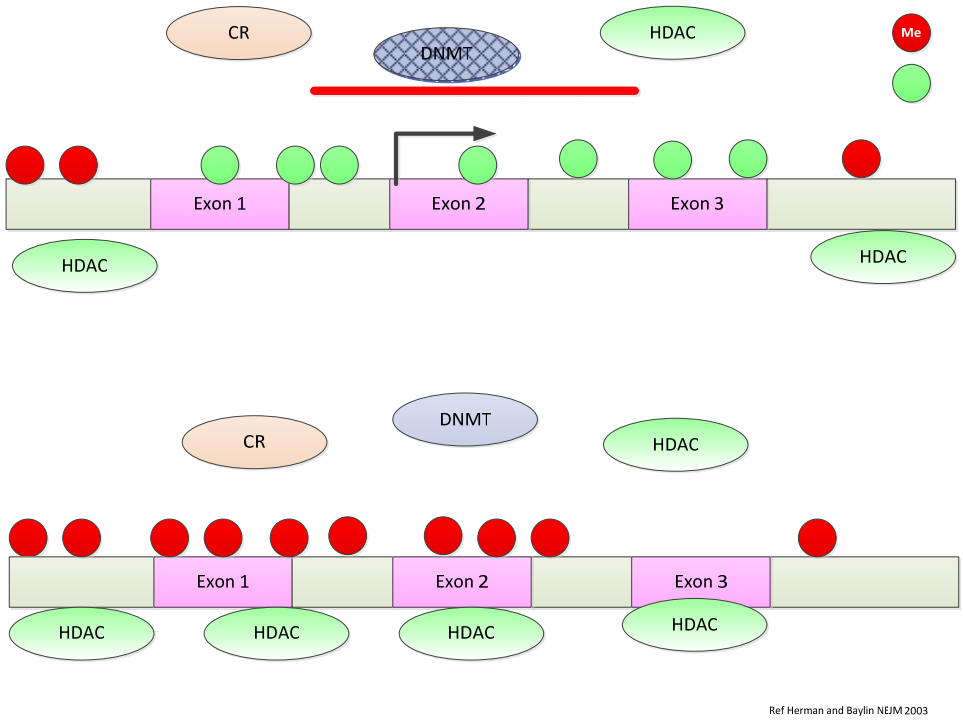
Although there are possible explanations and findings from ongoing investigations, it is not known why the DNA-methylating enzymes fail to methylate where they normally would and which of these enzymes are mediating the abnormal methylation of CpG islands in promoters.

We depict a modified version of their Figure below:



Thus methylation in this case blocks the expression of the targeted gene.

Herman and Baylin also use the following Figure to describe more regarding methylation:



As to the above they state:

The chromatin around the transcriptionally active (green arrow), unmethylated promoter is occupied by widely spaced nucleosomes composed of histone complexes in which key residues in the tails of histone H3 are in the acetylated state (green ovals), and those in the tails of histone H3 are methylated at lysine 4 (yellow asterisks).

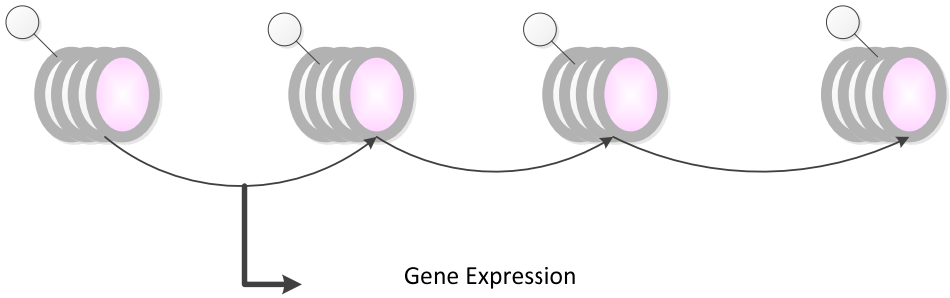
The region is accessible to key components of the gene-transcription apparatus, including primary transcription factors (TF); proteins with histone acetyltransferase activity (HAT), which maintain the histones in an acetylated state; and transcriptional coactivators (CA), which may also have histone acetyltransferase activities.

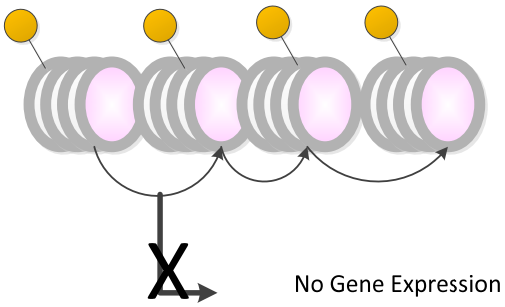
The flanking regions to either side of the unmethylated CpG island contain methylated cytosines. These regions are embedded in chromatin characteristic of transcriptionally silenced regions that is characterized by the binding of methylcytosine–binding proteins (MBPs) to the DNA methylated sites, and by nucleosomes that are more tightly compacted, with deacetylated histones (purple ovals) and methylated lysine 9 residues on the tails of histone H3 (black asterisks). The MBPs are part of complexes containing histone deacetylases (HDAC) that facilitate the deactivated state of the histones.

The blue vertical bars on either side of the unmethylated CpG island depict the molecular events, still to be determined, that prevent the spread of DNA methylation and of transcriptionally repressive chromatin across the CpG island in the promoter region of normal cells. The apparatus for DNA methylation, consisting of the DNA methyltransferases (DNMTs) and their complexes with transcriptional corepressors (CR) and histone deacetylases (HDAC), have access to the flanking areas but not to the CpG island in the promoter region within the barriers.

The lower panel depicts the breakdown of the barriers in a cancer cell, in which the transcriptionally repressive chromatin and DNA methylation have spread into the CpG island in the promoter region and correlate with transcriptional repression (red arrow with X) of the gene. The DNA-methylating complex now has access to the region, and the transcriptional machinery (transcriptional coactivators, histone acetyltransferase, and transcription factors) is excluded.

Now the histones may also be acetylated and drawn together. When histones are drawn closer the genes in between cannot be read and thus they are not expressed. We show that below:





Now we can summarize this as follows:

	Hypermethylated	Hypomethylated
Benign	Suppresses Proliferation Gene	Activates Suppressor Gene
Cancer	Suppresses Control Gene	Activates Proliferation Gene

What this shows is that methylation is good and bad. It is good if it suppresses the bad gene and bad if it suppresses a good gene, and vice versa.

3.3 METHYLATION AND DEAMINATION (C TO T)

Methylation may also progress to more dramatic changes. We discuss here the change of C to T, a serious change in a DNA base pair which can result in dramatic changes in gene expression.

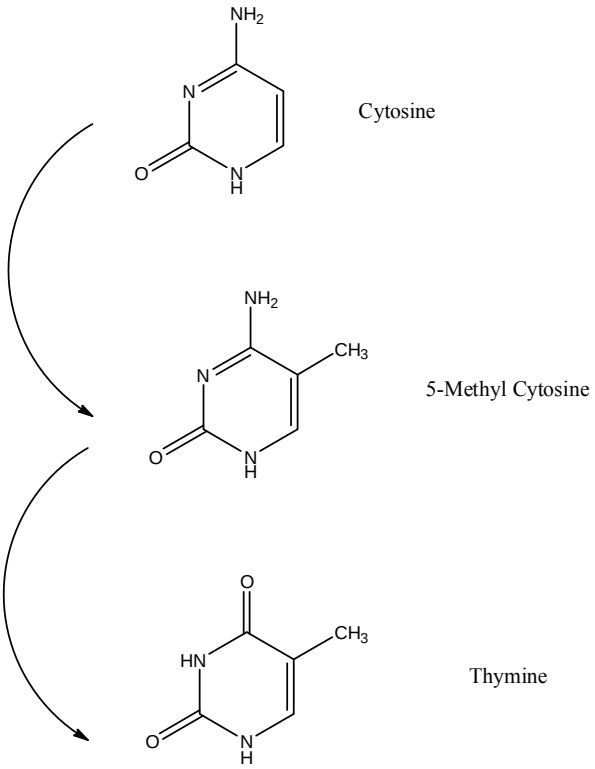
As Herman and Baylin state:

Although only four bases — adenine, guanine, cytosine, and thymine — spell out the primary sequence of DNA, there is a covalent modification of postreplicative DNA (i.e., DNA that has replicated itself in a dividing cell) that produces a "fifth base." Reactions using S -adenosyl-methionine as a methyl donor and catalyzed by enzymes called DNA methyltransferases (DNMTs) add a methyl group to the cytosine ring to form methyl cytosine.

In humans and other mammals, this modification is imposed only on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide). The overall frequency of CpGs in the genome is substantially less than what would be mathematically predicted , probably because DNA methylation has progressively depleted the genome of CpG dinucleotides over the course of time.

The mechanism of the depletion is related to the propensity of methylated cytosine to deaminate, thereby forming thymidine. If this mutation is not repaired, a cytosine-to-thymidine change remains.

The depletion of CpG dinucleotides in the genome corresponds directly to sites of such nucleotide transitions, and this change is the most common type of genetic polymorphism (variation) in human populations.



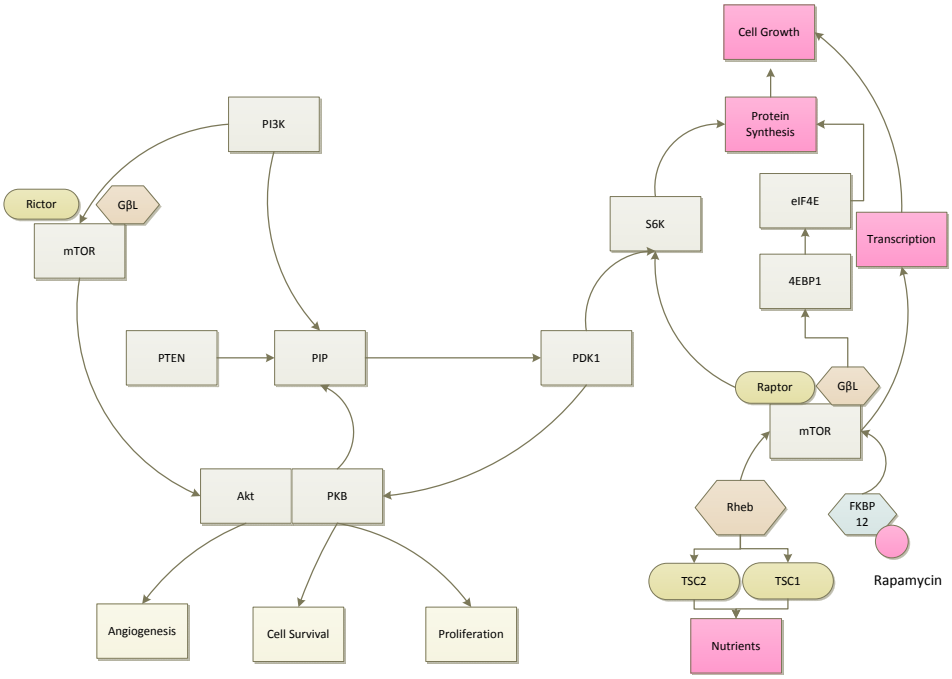
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

CpG-island-associated genes involved in cell growth control or metastasis that can become hypermethylated and silenced in tumors.

We depict the Table below from Robertson on some of the genes impacted by this type of methylation. Most of these are significant regulatory genes.

Gene	*Function*
pRb	Regulator of G1/S phase transition
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds β-catenin, Regulation of actin cytoskeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell-cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTPI	Protects DNA from oxygen radical damage
0^6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metalloproteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by γ interferon
p73	Apoptosis structurally similar to p53

For example we show below some typical pathways and the above genes are seen targeted by methylation.



Methylation may then interfere with many of the genes in the above pathways.

3.4 CAUSES OF METHYLATION

The major question which is often asked is what causes methylation. In Allis et al on p 460 the authors discuss some of the putative cause of methylation and methylation related cancers. Although not confirmative it is consistent with clinical correlations as well.

As Issa and Kartarjian state:

Much remains to be learned about the causes of DNA methylation abnormalities in cancer; for the most part, methylation seems to be gene specific. In some cases, a rare methylation event appears in cancer because of selection , while in others methylation anomalies are downstream of an oncogenic event ...

As McCabe et al state:

DNA methylation patterns in human cancer cells are considerably distorted. Typically, cancer cells exhibit hypomethylation of intergenic regions that normally comprise the majority of a cell's methyl-cytosine content . Consequently, transposable elements may become active and contribute to the genomic instability observed in cancer cells.

Simultaneously, cancer cells exhibit hypermethylation within the promoter regions of many CpG island-associated tumor suppressor genes, such as the retinoblastoma gene (RB1), glutatione S-transferase pi (GSTP1), and E-cadherin (CDH1). As a result, these regulatory genes are transcriptionally silenced resulting in a loss-of-function. Thus, through the effects of both hypo- and hyper-methylation, DNA methylation significantly affects the genomic landscape of cancer cells, potentially to an even greater extent than coding region mutations, which are relatively rare

McCabe et al continue:

Although the precise molecular mechanisms underlying the establishment of aberrant DNA hypermethylation remain elusive, recent studies have identified some contributing etiologic factors.

For example, chronic exposure of human bronchial epithelial cells to ***tobacco-derived carcinogens drives hypermethylation*** *of several tumor suppressor genes including CDH1 and RASSF2A.*

Stable knockdown of DNMT1 prior to carcinogen exposure prevented methylation of several of these genes indicating a necessary role for this enzyme in the molecular mechanism underlying hypermethylation.

The reactive oxygen species (ROS) associated with chronic inflammation is another source of DNA damage with the potential to affect DNA methylation as halogenated pyrimidines, one form

of ROS-induced damage, mimic 5-methylcytosine and stimulate DNMT1-mediated CpG methylation in vitro and in vivo.

Indeed, study of the glutatione peroxidase 1 and 2 double knockout model of inflammatory bowel disease found that 60% of genes that are hypermethylated in colon cancers also exhibit aberrant methylation in the inflamed noncancerous precursor tissues. Although the mechanisms by which DNA damage mediates DNA methylation are not fully understood, O'Hagan and colleagues have examined the process with an engineered cell culture model in which a unique restriction site was incorporated into the CpG island of the E-cadherin promoter.

Thus the actual molecular mechanics leading to methylation are not fully understood but like most cancers inflammation appears to be a driving factor. What the cause of that inflammation may be is not yet clear.

3.5 METHYLATION EFFECTS ON DNA

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5o position of cytosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor.

In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DMNT3a, and DNMT3b.DNAmethylation plays a role in the long-term silencing of transcription and in heterochromatin formation.

As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular divisions.

We continue with the discussion in Mirand and Jones as follows:

Silencing of genetic elements can be successfully initiated and retained by histone modifications and chromatin structure. However, these modifications are easily reversible making them make poor gatekeepers for long-term silencing. Therefore, mammalian cells must possess an additional mechanism for prolong silencing of these sequences. An important component of this process is DNA methylation. DNA methylation is a stable modification that is inherited throughout cellular divisions.

When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development.

We now present a key Figure from Miranda and Joner regarding the methylated reading of DNA. They state regarding the Figure below:

Chromatin structure of CpG islands and CpG poor regions in healthy cells and during cancer. In healthy cells, CpG islands are generally hypomethylated. This allows for an open chromatin structure. However, the CpG poor regions found in repetitive elements within the intergenic and intronic regions of the genome are methylated and thereby maintain a closed chromatin structure. In cancer and on the inactive X chromosome many CpG islands become methylated, forcing these regions into a closed chromatin structure.

When CpG islands located within promoters are methylated, the corresponding genes are persistently silenced. In contrast, the CpG poor regions become hypomethylated allowing for an open chromatin structure.

As Robertson states:

It is now clear that the genome contains information in two forms, genetic and epigenetic. The genetic information provides the blueprint for the manufacture of all the proteins necessary to create a living thing while the epigenetic information provides instructions on how, where, and when the genetic information should be used.

Ensuring that genes are turned on at the proper time is as important as ensuring that they are turned off when not needed.

The major form of epigenetic information in mammalian cells is DNA methylation, or the covalent addition of a methyl group to the 5-position of cytosine predominantly within the CpG dinucleotide. DNA methylation has profound effects on the mammalian genome.

Some of these effects include transcriptional repression, chromatin structure modulation, X chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity.

Robertson then proceeds to detail the genes impacted by hypermethylation. We summarize them below:

Gene	*Function*
pRb	Regulator of G1/S phase transition
p16 INK4a	Cyclin-dependent kinase inhibitor
p15 INK4b	Cyclin-dependent kinase inhibitor
ARF	Regulator of p53 levels
hMLH1	DNA mismatch repair
APC	Binds b-catenin, Regulation of actin cyto-skeleton?
VHL	Stimulates angiogenesis
BRCA1	DNA repair
LKB1	Serine/threonine protein kinase
E-cadherin	Cell ± cell adhesion
ER	Transcriptional activation of estrogen-responsive genes
GSTP1	Protects DNA from oxygen radical damage
O6-MGMT	Repair/removal of bulky adducts from guanine
TIMP3	Matrix metallo proteinase inhibitor
DAPK1	Kinase required for induction of apoptosis by g interferon
p73	Apoptosis?, structurally similar to p53

Regarding PIN, the one which is most concern is the GSTP1 gene and its suppression allowing for DNA damage from inflammation and oxygenation damage.

In the context of cancer generation and progression, the epigenetic effect of hyper and hypo methylation is best described by Esteller:

The low level of DNA methylation in tumors as compared with the level of DNA methylation in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer.

The loss of methylation is mainly due to hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns −regions of DNA that allow alternative versions of the messenger RNA (mRNA) that are transcribed from a gene. A recent large-scale study of DNA methylation with the use of genomic microarrays has detected extensive hypo-methylated genomic regions in gene-poor areas.

During the development of a neoplasm, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer.

Three mechanisms have been proposed to ex-plain the contribution of DNA hypomethylation to the development of a cancer cell:

(i) generation of chromosomal instability,

(ii) reactivation of transposable elements, and

(iii) loss of imprinting.

Under methylation of DNA can favor mitotic recombination, leading to deletions and translocations, and it can also promote chromosomal rearrangements. This mechanism was seen in experiments in which the depletion of DNA methylation by the disruption of DNMTs caused

aneuploidy. Hypomethylation of DNA in malignant cells can reactivate intra-genomic endo-parasitic DNA.

3.5.1 Hypomethylation

We now consider the other extreme, hypomethylation. As Laird and Jaenisch state:

Hypomethylation: Reduced levels of global DNA methylation have been reported for a variety of malignancies in the past decade. Gama Sosa and coworkers found that in a wide variety of tumors, hypomethylation not only correlated with transformation, but also with tumor progression . In their analysis, only 7% of 43 normal tissues had 5-methylcytosine content below 0.8 mol%, whereas 10% of 21 benign tumors, 27% of 62 primary malignancies and 60% of 20 secondary malignancies had 5-methylcytosine content below 0.8 mol%. On the other hand, Feinberg and coworkers did not find a further reduction in DNA methylation levels in the progression from benign to malignant colonic neoplasia, suggesting an early role for DNA hypomethylation in colorectal cancer

3.5.2 Hypermethylation

As again with Laird and Jaenisch we have:

Hypermethylation: There have also been many reports of regional increases in DNA methylation levels. Baylin and coworkers have found regional hotspots for hypermethylation on chromosomes 3p, 11p and 17p in a variety of human tumors. These include CpG island areas that are normally never methylated in vivo, but are found to be methylated in tumor tissues. This is reminiscent of the changes that occur at CpG islands at non-essential genes in tissue culture.

Baylin's group has dissected the sequential order of hypermethylation events in an in vitro model for lung tumor progression. There is evidence for inactivation of tumor-suppressor gene function through hypermethylation of the Rb gene in sporadic retinoblastoma. Transient transfection experiments showed that specific hypermethylation in the promoter region of Rb could reduce expression to 8% of an unmethylated control. It is possible, therefore, that hypermethylation of tumor-suppressor genes leading to gene inactivation results in a selective growth advantage of the transformed cells.

From Issa we have:

The mechanism whereby CpG island methylation suppresses gene transcription has been partially elucidated recently (Fig. 1), at least in vitro . 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) methylation . 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 (Fig. 1). Separately, DNMT1 can directly suppress transcription (without DNA methylation) through interactions with histone deacetylases . H3K9 methylation itself appears to set-up a silencing loop by attracting more DNA methylation , and may sometimes precede hypermethylation

3.6 HYPERMETHYLATION INDUCTION

What starts the process of hypermethylation? What are its dynamics? Issa states:

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-methy-cytosine (5mC) content in tumors, and appears to involve primarily satellite DNA, repetitive sequences, and CpG sites located in introns . The cause of this demethylation remains unclear, although it could be related to alterations in proliferation or cell-cycle control .

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 phenotype . An increased mutation rate was demonstrated in cells in which severe hypomethylation (>75%) was achieved by homozygous deletion of DNMT1 , but it is not clear whether this degree of hypomethylation is ever achieved in neoplasms

Thus we still have a great deal of work to fully understand these effects.

3.7 HYPERMETHYLATION AND MDS

We now combine our understanding of methylation and that of MDS to provide insight on the relationship. There has been a great deal of recent literature on the impact of hypermethylation in MDS and we review some of the key contribution here. Issa presents a collection of aberrant CpG islands of hypermethylation found in MDS and we present his Table below:

Gene	*Methylation frequency (%)*	*Function*	*Note*
Calcitonin	50	Differentiation	
CDKN2B	23-80	Cyclin dependent kinase inhibitor; cell cycle/proliferation	Tumor-suppressor; methylation correlates with poor prognosis and progression to AML81
DAPK	50	Proapoptotic serine/threonine kinase	
RASS F1	9	Negative regulator of RAS signaling	Tumor-suppressor
FHIT	50	Purine metabolism	Putative tumor- suppressor; methylation correlates with poor prognosis and progression to AML68
HIC	32	Transcriptional repressor	Tumor-suppressor
CDH	15-27	Adhesion and motility	Methylation correlates with poor prognosis and progression to AML31
CTNNA	10	Alpha catenin	
ERα	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
RIL	36-70	Proapoptotic, tumor- suppressor	
CDH13	21	Adhesion and motility	
NOR1	15	Oxidored-nitro domain- containing protein	
NPM2	20	Nucleophosmin/nucleoplasmin 2, involved in development	
OLIG2	41	Basic helix-loop-helix transcription factor	
PGRA	45	Progesterone receptor	
PGRB	45	Progesterone receptor	

With regards to the Table above Issa comments as follows:

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. 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 simultaneously . 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 . CDKN2B methylation in MDS has also been associated with older age, deletions of 5q and 7q, and a poor prognosis . Interestingly, when

present, CDKN2B methylation in MDS has been shown to affect multiple lineages from clonogenic cells to circulating mononuclear cells .

In a mouse model, loss of CDKN2B was associated with enhanced myeloid progenitor and reduced erythroid progenitor formation , suggesting that its inactivation plays a functional role in MDS.

The interplay between hypermethylation and gene suppression is complex but as we have shown above from Issa it is quite prevalent. As Jiang et al state:

Myelodysplastic syndromes (MDSs) are clonal hematologic disorders that frequently represent an intermediate disease stage before progression to acute myeloid leukemia (AML). As such, study of MDS/AML can provide insight into the mechanisms of neoplastic evolution. In 184 patients with MDS and AML, DNA methylation microarray and high-density single nucleotide polymorphism array (SNP-A) karyotyping were used to assess the relative contributions of aberrant DNA methylation and chromosomal deletions to tumor-suppressor gene (TSG) silencing during disease progression.

Aberrant methylation was seen in every sample, on average affecting 91 of 1505 CpG loci in early MDS and 179 of 1505 loci after blast transformation (refractory anemia with excess blasts [RAEB]/AML). In contrast, chromosome aberrations were seen in 79% of early MDS samples and 90% of RAEB/AML samples, and were not as widely distributed over the genome. Analysis of the most frequently aberrantly methylated genes identified FZD9 as a candidate TSG on chromosome 7. In patients with chromosome deletion at the FZD9 locus, aberrant methylation of the remaining allele was associated with the poorest clinical outcome.

These results indicate that aberrant methylation can cooperate with chromosome deletions to silence TSG. However, the ubiquity, extent, and correlation with disease progression suggest that aberrant DNA methylation is the dominant mechanism for TSG silencing and clonal variation in MDS evolution to AML.

Tumor Suppressor Gene silencing is a significant if not the dominant factor. As has been discussed elsewhere, the cell has a complex control mechanism to ensure that uncontrolled proliferation.

4 DEMETHYLATING THERAPEUTICS

Myelodysplastic Syndrome is an uncommon hematological cancer mostly caused by excess exposure to radiation, chemicals such as benzene, and insecticides. The specific genetic causes are still a work in progress. However, there is certain therapeutics which addresses some of the pathway aberrancies which characterize the disease, specifically hypermethylation.

4.1 EPIGENETIC CONTROL PARADIGMS

As Taferri and Vardiman state:

According to the 2008 World Health Organization (WHO) classification system for hematologic cancers, the primary myelodysplastic syndromes are one of five major categories of myeloid neoplasms. The main feature of myeloid neoplasms is stem-cell–derived clonal myelopoiesis with altered proliferation and differentiation. The phenotypic diversity of these neoplasms has been ascribed to different patterns of dysregulated signal transduction caused by transforming mutations that affect the hematopoietic stem cell.

There is increasing evidence that haploinsufficiency, ***epigenetic changes****, and abnormalities in cytokines, the immune system, and bone marrow stroma all contribute to the development of the myelodysplastic syndromes.*

Thus MDS is both complex in presentation and complex in development. Melanoma and prostate cancer are more clearly characterized morphologically and generally in genetic development. The presentation may involve the white cells, red cells or platelets, or any combination thereof. It is often discovered as an incidental finding on a blood test with lowered amounts of one or several of the constituents. If it has progressed more it may also present in the bone biopsy with more than normal blasts, immature cells.

As DeVita et al state:

Myelodysplastic syndromes (MDSs) are a group of complex and heterogeneous clonal hematopoietic stem cell disorders whose defining characteristics are dysplasia of one or several hematopoietic cell lineages, hypercellular marrows, and blood cytopenias.

1 Although historically considered as a preleukemic state, most patients with MDS do not transform into an acute myeloid leukemia (AML), but will instead succumb to complications of persistent cytopenias. Indeed, the pathophysiology of MDS extends from immune-mediated mechanisms and excessive apoptosis resulting in marrow failure to arrest of maturation and proliferation resembling the mechanisms at play in AML.

2 The diverse pathophysiology of factors that contribute to the development of MDS is reflected in vast differences of patients' prognosis, which is increasingly recognized and reflected in the design of more elaborate systems of diagnosis, classification, and prognostication.

Let us begin with a simple set of statements regarding the micro RNA elements which are often seen at the heart of the disease. As Croce states:

Several of the miRNAs that have been described as suppressors have been found to be deleted or mutated in various human malignancies. For example, loss of miR-15a and miR-16-1 has also been observed in prostate cancer and multiple myeloma (TABLE 1). Members of the miR-29 family have been found to be deleted in a fraction of myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) patients.

As Croce further states:

MicroRNAs as targets of epigenetic changes. The most studied epigenetic changes in cancer cells are the methylation of cytosines in the dinucleotide CpG in DNA62. Such 'methylable' sites, known as CpG islands, are preferentially located in the 5' region (which consists of the promoter, 5' uTR and exon 1) of many genes, are non-methylated in normal cells and are transcribed in the presence of the appropriate transcription factors. Methylation of the CpG islands of tumour suppressors results in their silencing and contributes to malignant transformation.

As mentioned above, the expression of miRNAs can be affected by genetic changes, such as deletion, gene amplification and mutation, and by transcription factors. In addition, the expression of miRNAs can be affected by epigenetic changes, such as methylation of the CpG islands of their promoters. Saito et al. reported that miR-127 is silenced by promoter methylation in bladder tumours and that its expression could be restored by using hypomethylating agents such as azacitidine.

This miRNA targets BCL6, an oncogene that is involved in the development of diffuse large b cell lymphoma. Therefore, the silencing of miR-127 may lead to the overexpression of bCL6. Other investigators have described additional miRNAs that are silenced by methylation in various cancers and that can be reactivated by hypomethylating agents.

As Das and Singal state:

Hypermethylation is associated with many leukemias and other hematologic diseases. *Many genes, such as the calcitonin gene, p15INK4B, p21Cip1/Waf1, the ER gene, SDC4, MDR, and so on, were seen to be hypermethylated in a variety of hematologic cancers.*

The calcitonin gene and p15 were hypermethylated in 65% of myelodysplastic syndromes*, and it was found that p15 methylation at diagnosis was associated with lower survival and transformation to acute myeloid leukemia.*

Also acquisition of p15 methylation at a later date signaled disease progression. These may suggest the role of p15 as a marker of leukemic transformation. Acute myeloid leukemia demonstrated frequent hypermethylation of ER, MYOD1, PITX2, GPR37, and SDC4

Thus MDS is closely related to methylation, and in effect is caused by methylation. In addition as we show below its management is also performed through an understanding of methylation and managing that process.

From DeVita et al (pp 479-480)we have:

Originally synthesized as cytotoxic antimetabolite drugs in the1960s, 21azacytosine nucleosides were recognized as inhibitors of DNA methylation in the early 1980s. 5-Azacitidine (5AC) and 2-deoxy-5-azacitidine induced muscle, fat, and chondrocyte differentiation in mouse embryo cells, in association with reversal of DNA methylation. Incorporation of azacytosine nucleosides into DNA in lieu of cytosine residues was shown to be associated with inhibition of DNMT activity. DNMT inhibition requires incorporation of DAC triphosphate into DNA in lieu of cytosine residues. The incorporated azacytosine nucleoside forms an irreversible inactive adduct with DNMT.

Sequential reversal of DNA methylation results when DNA replication proceeds in absence of active DNMT. 5AC must be dephosphorylated and converted to DAC diphosphate by ribo-nucleotide reductase before it can be activated through triphosphorylation; DAC does not require the ribo-nucleotidereductase. 5AC can also be incorporated into RNA; this inhibits tRNA cytosine methyltransferase.

This may contribute to an inhibition of protein synthesis. The azacytosine nucleosides exhibit complex dose-response characteristics. At low concentrations (0.2 to 1 mcM), the "epigenetic" activities of these drugs predominate, with reversal of DNA methylation and induction of terminal differentiation in some systems. As concentrations are increased, apoptosis be-comes more prominent.

Cell lines with 30-fold resistance to the cytotoxic effects of DAC continue to reverse methylation in response to this nucleoside, suggesting that the methylation re-versing and cytotoxic activities of this compound can be sepa-rated.27The ability of these drugs to inhibit cell cycle, at least in part through induction of p21WAF1/CIP1expression, complicates the goal of reversing DNA methylation because the latter re-quires DNA replication with the azacytosine nucleoside incorporated into the DNA.

The two azacytosine nucleosides in clinical use are highly unstable in aqueous solution. In aqueous solutions, the drugs readily hydrolyze and inactivate.28In clinical practice, the drugs must be administered shortly after reconstitution. The drugs are also metabolized by cytidine deaminase, leading to a short half-life in plasma.

Thus there have been significant developments in methylation control. Recent papers by Blum and by Lubbert et al discuss some of the therapeutic issues as well.

4.2 AZACITIDINE AND DECITABINE AND MDS

Understanding the impact of methylation in MDS recent efforts have led to certain therapeutics which have been of help.

As Issa and Kantarjian state:

Two nucleoside inhibitors of DNA methylation, azacitidine and decitabine, are now standard of care for the treatment of the myelodysplastic syndrome, a deadly form of leukemia. These old drugs, developed as cytotoxic agents and nearly abandoned decades ago were resurrected by the renewed interest in DNA methylation.

They have now provided proof of principle for epigenetic therapy, the final chapter in the long saga to provide legitimacy to the field of epigenetics in cancer. But challenges remain; we don't understand precisely how or why the drugs work or stop working after an initial response. Extending these promising findings to solid tumors faces substantial hurdles from drug uptake to clinical trial design.

We do not know yet how to select patients for this therapy and how to move it from life extension to cure. The epigenetic potential of DNA methylation inhibitors may be limited by other epigenetic mechanisms that are also worth exploring as therapeutic targets. But the idea of stably changing gene expression in vivo has transformative potential in cancer therapy and beyond.

They continue:

Drugs that inhibit DNA methylation were discovered by pure serendipity . Cytosine analogs developed as cytotoxic anticancer agents in the 1960s and tested in the clinic in the 1970s were found to induce peculiar differentiation phenotypes in vitro (16). This DNA hypomethylating property is limited to cytosine analogs with modifications of the ring. Other cytosine or nucleoside analogs do not affect DNA methylation directly. Eventually, this property of the two main analogs, 5-azacytidine (AZA) and 5-aza-deoxycytidine (DAC), was traced to their ability to incorporate into DNA, trap DNA methyltransferases (DNMTs), and target these enzymes for degradation. DNA synthesis in the absence of these enzymes then results in hypomethylation in the daughter cells and eventually to reactivation of silenced gene expression. Several other modified nucleoside analogs have been described either in preclinical studies or in early stage clinical trials.

As Li has stated:

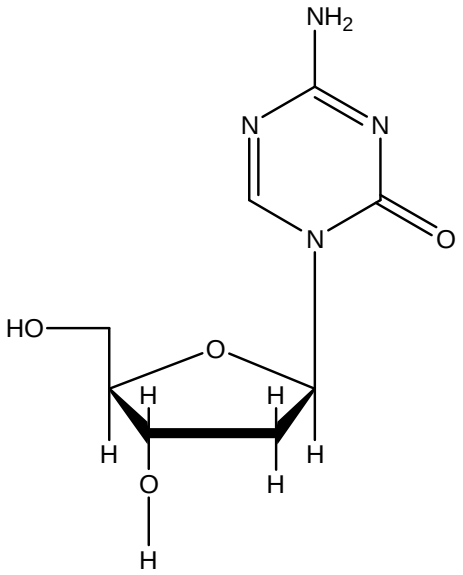
The strategies targeting DNA methylation. Epigenetic control of gene expression by DNA methylation has a great impact on cell proliferation and differentiation. Hypermethylation of promoter regions results in specific suppression of gene expression, including the expression of tumor suppressors, which could promote cancer development.

Conversely, demethylation of DNA may enhance cell apoptosis or reduce cell growth. This concept has been proven by a recently approved anticancer drug decitabine for the treatment of

myelodysplastic syndrome. Decitabine (Dacogen; MGI Pharma) is a nucleoside analogue that inhibits DNA methylation.

It demethylates the p73 promoter and induces reexpression of p73, thus activating the caspase cascade and leading to leukemic myeloid cell death.26 DNA hypermethylation in tumor cells may be involved in resistance to interferon (INF)-induced apoptosis, and inhibition of DNA methylation may also enhance the therapeutic effect of INF. Treatment of cancer cells with specific DNA demethylating nucleoside analogue was shown to augment the effect of INF.

Now decitabine is shown below in detail. It is a cytosine derivative with several modifications. It functions in a manner similar to azacitidine. We have discussed that previously.



From Boumber et al we have the following regarding therapeutics for epigenetic drugs:

What Is Epigenetic Therapy? The understanding that epigenetic changes are prevalent in cancer and play a causative role in its biology has led to the development of new therapeutic approaches that target the epigenetic machinery. The first successful drugs developed as epigenetic agents were DNA methyltransferase inhibitors; these were followed by histone deacetylase inhibitors (HDIs).

Both classes of drugs aim at reversing gene silencing and demonstrate antitumor activity in vitro and in vivo. Several other classes of drugs have been developed that target various other components of the epigenetic machinery; one such class is the histone methyltransferases, with new drugs in this class currently in early preclinical development

The authors continue:

What Has Been Done? The inhibitors of DNA methylation used clinically are nucleoside analogues that get converted into deoxy-nucleotide-triphosphates (dNTPs) and become

incorporated into DNA in place of cytosine during DNA replication. They trap all DNA methyltransferases and target them for degradation. At low doses these drugs do not inhibit proliferation; they reactivate gene expression and have shown clinical activity as anticancer agents.

Azacitidine was the first hypomethylating agent approved by the FDA; its approval, in 2004, for the treatment of myelodysplastic disorders and leukemia, was followed by the approval, in 2006, of decitabine. Both drugs produce remissions or clinical improvements in more than 30% of patients treated. Features of responses have included the requirement for multiple cycles of therapy, slow response, and relatively few side effects.

On the molecular level, demethylation, gene reactivation, and clonal elimination were observed in treated patients. The data in myelodysplastic syndrome (MDS) represent a proof-of-principle for epigenetic therapy for cancer, in particular in myeloid disorders.

From Boumber et al we have the following Table of many of the recent therapeutics:

Drug Class	Compound
DNMT Inhibitor	Azacitidine
	Decitabine
	S110
	CP-400
	Nanaomycin
HDAC Inhibitor	Vorinostat
	Romidepsin
	Panobinostat
	Valproic Acid
	Belinostat
HMT Inhibitor	Deazaneoplanocin
	Quinazoline
	Ellagic Acid
Histone demethylase inhibitor	Polyamine analogues
	Hydroxamate analogs
GAT inhibitor	Spermidinyl
	Hydrazinocurcumin
	Pyrazolone

As Stressman et al state:

Aberrant DNA methylation patterns play an important role in the pathogenesis of hematologic malignancies.

The DNA methyltransferase inhibitors azacytidine and decitabine have shown significant clinical benefits in the treatment of myelodysplastic syndrome (MDS), but their precise mode of action

remains to be established. Both drugs have been shown the ability to deplete DNA methyltransferase enzymes and to induce DNA demethylation and epigenetic reprogramming in vitro. However, drug-induced methylation changes have remained poorly characterized in patients and therapy-related models.

We have now analyzed azacytidine-induced demethylation responses in myeloid leukemia cell lines. These cells showed remarkable differences in the drug-induced depletion of DNA methyltransferases that coincided with their demethylation responses. In agreement with these data, DNA methylation analysis of blood and bone marrow samples from MDS patients undergoing azacytidine therapy also revealed substantial differences in the epigenetic responses of individual patients.

Significant, transient demethylation could be observed in 3 of 6 patients and affected many hypermethylated loci in a complex pattern. Our results provide important proof-of-mechanism data for the demethylating activity of azacytidine in MDS patients and provide detailed insight into drug-induced demethylation responses.

4.3 ENVIRONMENTAL AND GENETIC CAUSES AND FACTORS

The main problem with MDS is that there is not clear genetic pathway and causal relationship. As DeVita et al state:

No etiologic factor is identified in most patients with MDS. MDS is more frequent in men than women by a factor of 1.8. It has been associated with smoking and hair dyes, exposure to agricultural and industrial toxins, drugs (e.g., chloramphenicol), and occupational exposures to stone and cereal dusts. MDS has been associated with exposure to ionizing radiation (atomic bomb survivors in Japan, decontamination workers following the Chernobyl nuclear plant accident) and chronic exposure to low-dose radiation (radiopharmaceuticals). Some inherited hematologic disorders (Fanconi anemia, dy-skeratosis congenita, Shwachman-Diamond syndrome, Diamond- Blackfan syndrome) are also associated with a higher risk of MDS.

Thus there is no clear causal factor or factors recognized at this time.

In a recent paper by Suzuki et al the authors discuss some of the causes of methylation and in turn cancers. They state:

Evidence now suggests that epigenetic abnormalities, particularly altered DNA methylation, play a crucial role in the development and progression of human gastrointestinal malignancies. Two distinct DNA methylation abnormalities are observed together in cancer.

One is an overall genome-wide reduction in DNA methylation (global hypomethylation) and the other is regional hypermethylation within the CpG islands of specific gene promoters. Global hypomethylation is believed to induce proto-oncogene activation and chromosomal instability, whereas regional hypermethylation is strongly associated with transcriptional silencing of tumor suppressor genes.

To date, genes involved in regulation of the cell cycle, DNA repair, growth signaling, angiogenesis, and apoptosis, are all known to be inactivated by hypermethylation. Recently developed techniques for detecting changes in DNA methylation have dramatically enhanced our understanding of the patterns of methylation that occur as cancers progress. One of the key contributors to aberrant methylation is aging, but other patterns of methylation are cancer-specific and detected only in a subset of tumors exhibiting the CpG island methylator phenotype (CIMP).

Although the cause of altered patterns of DNA methylation in cancer remains unknown, it is believed that epidemiological factors, notably dietary folate intake, might strongly influence DNA methylation patterns.

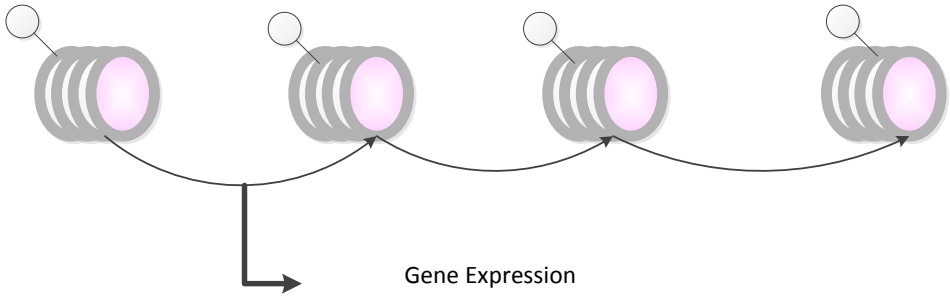
Recent studies further suggest that polymorphisms of genes involved in folate metabolism are causally related to the development of cancer.

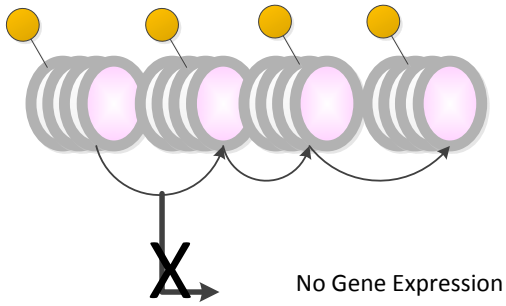
5 ACETYLATION AND HISTONES

Histones also play a role in the control of genes and their expression. Whereas we can have methylated or non-methylated CpG regions we can have acetylated histones which can have another layer of control on gene expression. It is not our intend to discuss this in detail but merely to point out its significance.

5.1 DNA AND HISTONES

Let us consider the DNA as it is wrapped around histones, and how it may wind out or become more closely packed. We demonstrates this below.





5.2 ACETYLATION

5.3

5.4 DEACETYLATION THERAPEUTICS

From p 481 in DeVita et al:

The increasing recognition of the critical importance of his-tone modifications in regulating the transcriptional permissivity of chromatin has led to intense interest in compounds that can inhibit the activity of HDAC proteins, facilitating acetylation of lysines associated with transcriptional activation of genes. The first generation of HDAC inhibitors were small chain

fatty acids, including sodium butyrate, arginine butyrate, sodium phenylbutyrate, and valproic acid.

These agents require sub millimolar to millimolar concentrations to inhibit HDACs. Like the DNMT inhibitors, these compounds have complex pharmacodynamic properties. At the lowest concentrations associated with HDAC inhibitory activity, these com-pounds may increase cellular proliferation. At high concentrations, cell cycle arrest occurs, associated with induction of p21WAF1/CIP1and evidence of differentiation.

At concentrations exceeding 1 mM, apoptosis is induced. Second-generation HDAC inhibitors include hydroxamic acids, cyclic depsipeptides, and benzamides. The hydroxamic acid HDAC inhibitors include vorinostat (suberoylanilide hydroxamic acid, SAHA), which was synthesized as a derivative of the differentiation inducer hexamethylene bisacetamide.

Other hydroxamic acid HDAC inhibitors under clinical investigation include belinostat (PXD1010)48and LBH589.Hydroxamic HDAC inhibitors fit into and interact with the catalytic core of HDACs. Hydroxamic acids inhibit HDACs of class I and II.51Romidepsin (FK228) is a depsipeptide with potent HDAC inhibitory activity. Romidepsin requires reduc-tion for optimal activity and appears to specifically inhibit class I HDACs.52The benzamide HDAC inhibitors include SNDX 275 (formerly known as MS275),CI994, and MGCD0103 are selective for class I HDACs. Many proteins in addition to histones serve as substrates for protein acetylases and can thus be impacted by HDAC inhibitors. These include transcription factors such as p53,E2F1, and GATA 1.DNA binding proteins such as HMG-158and tubulin can also be acetylated by acetyl transferases.

Protein acetylation can result in increased DNA binding, impact protein-protein interactions, and increase protein stability.

Given that a wide variety of proteins can undergo acetylation in the presence of HDAC inhibitors, it is not surprising that administration of HDAC inhibitors has been associated with a panoply of effects on cellular physiology. As predicted, administration of HDAC inhibitors induces alterations in gene expression. This includes both up and down regulation. Expression profiling has suggested that between 2% and 10%of genes studied may have their expression altered by exposure to HDAC inhibitors; however, the number of genes whose expression is reliably altered in a number of different cancer cell lines in response to a variety of HDAC inhibitors is few.

6 BONE MARROW TRANSPLANTS

One of the most successful techniques used in many hematopoietic malignancies is bone marrow transplants ("BMT"). Allogeneic transplants, with HLA matches being high, are often the only techniques which and effectively restart the system and allow for regrowth of a stable hematopoietic system. We briefly discuss BMT as applied to MDS. The intent is not to provide any substantial detail on BMT but to allow for an understanding of its place in the treatment of MDS.

6.1 BASIC PRINCIPLES

BMT is the ultimate approach, despite the ability to reduce methylation with azacytidine, it is not curative. As Sekeres in a summary article states:

Those who treat patients with myelodysplastic syndromes (MDS) have been forced to become comfortable with a rather uncomfortable truth. MDSis a bone marrow failure syndrome that represents the most commonly diagnosed myeloid malignancy and predominantly affects older adults, with a median age at diagnosis of 71 years.

The only cure for MDS is hematopoietic stem-cell transplantation (HSCT). For a variety of reasons, including patient comorbidities, availability of related or matched donors, related donor comorbidities, physician and patient preference, and treatment-related adverse events, transplantation is only considered in approximately 5% of patients with MDS.

Thus, even when we offer disease-modifying therapies such as azacitidine, decitabine, and lenalidomide, we are ultimately palliating 95% of our patients. Despite this, patients often perceive these drugs to have curative potential in this setting, but cure is unfortunately not possible with these agents.

As DeVita et al state (note, they use SCT, stem cell transplants, for our use of BMT, and the difference for this purpose is minimal):

Allogeneic SCT (stem cell transplants) remains the only treatment modality that can lead to long-term disease-free survival. Given the demographics of MDS, only few patients will ultimately benefit from SCT. Treatment-related morbidity and mortality remain substantial impediments to SCT. SCT with reduced intensity conditioning can decrease the toxicity of the procedure, but at the cost of higher relapse likelihood. Matched unrelated donor transplants may overcome some of the shortage of suitable donors. Although effective, they carry a higher risk of toxicities. Judicious selection of patients for SCT is therefore crucial, particularly in the context of therapies such as lenalidomide or DNMT inhibitors.

Outcome is generally most favorable in patients who may need transplant the least, such as younger patients with low-risk MDS. In a study by the International Bone Marrow Transplant Registry, 452 recipients of HLA-identical sibling transplants with a median age of 34 years and

high-risk MDS in two-thirds, overall survival at 3 years was 42%. Survival was more favorable with young age and platelet counts less than 100 × 109/L. Relapse was highest in patients with high percentages of marrow blasts at transplantation, with high IPSS scores, and with T-cell depleted transplants. Diseases-free survival was 60% in the low-risk, 36% in the intermediate-1, and 28% in intermediate-2 risk groups. This compared to 5-year survival rates of 55%, 35%, and 7%, respectively, for unselected patients not receiving SCT, suggesting a benefit of SCT mostly for high-risk MDS patients.

A key issue remains the optimal timing of SCT. Using a Markov decision model, three transplant strategies were compared: (1) SCT at diagnosis, (2) SCT at the time of progression to leukemia; and (3) SCT sometime after diagnosis but prior to leukemic progression.63 Delaying transplant was most beneficial for patients in the low and intermediate-1 IPSS groups, an effect that was more noticeable in patients younger than 40 years. Earlier transplantation, on the other hand, improved survival in the intermediate-2 and high IPSS groups.

6.2 EFFICACY

One of the recent putative studies was by Koreth et al which state:

Erythropoiesis-stimulating agents may offer a survival advantage for anemic patients or those with RBC transfusion-dependent low/ intermediate-1 IPSS MDS.22,23 Hypomethylating agent therapy can reduce rate of AML progression in patients with intermediate-2/high IPSS MDS, and azacytidine has been demonstrated to improve survival.24-26 Unfortunately these treatments seldom induce durable remissions, and none are curative. Allogeneic hematopoietic stem-cell transplantation is potentially curative.

In myeloablative conditioning (MAC) transplantation, IPSS risk is correlated with MDS relapse and disease-free survival.27 Treatment-related mortality (TRM) is 35% to 80%, varying with age and other factors. In a prior analysis, we documented that for patients 18 to 60 years of age with intermediate- 2/high IPSS MDS, early MAC transplantation provides maximal quality-adjusted survival.

However, 75% of patients with MDS are 60 years at diagnosis and are typically not considered MAC transplantation candidates.

In patients 60 years of age, reduced-intensity conditioning (RIC) transplantation is potentially curative but is also associated with mortality risk. Retrospectively, TRM was 26% to 41%, with long-term MDS/AML survival of 27% to 54%.

RIC transplantation in older patients remains uncertain because MDS prognosis differs from that of younger patients, and RIC and MAC transplantation risks and benefits may also differ. A retrospective report suggests that transplantation benefits patients with advanced MDS/AML who are 60 to 70 years old, but head-to-head comparisons of RIC transplantation versus nontransplantation approaches are lacking for MDS.

Koreth et al conclude:

For 223 patients with intermediate-2/high IPSS, early RIC transplantation had an LE of 36 months versus 28 months with nontransplantation therapy. ... a Kaplan- Meier plot derived from the Monte Carlo simulation. QoL inclusion also indicated QALE benefit with early RIC transplantation, and sensitivity analyses supported RIC transplantation as a preferred option across the range of plausible state utilities for patients with intermediate-2/high MDS receiving hypomethylating agent therapy (0.33 to 0.73) versus the range of plausible state utilities after RIC transplantation (0.6 to 0.92). Explicitly modeling a plateau of long-term post-transplantation survival or discounting future survival also did not change the conclusion

....In conclusion, we undertook decision modeling to quantify benefit of RIC transplantation versus non-transplantation therapies in patients with de novo MDS aged 60 to 70 years. We conclude that early RIC transplantation offers survival benefit for intermediate-2/high IPSS MDS, but not for low/intermediate-1 IPSS MDS. These simple but robust findings may help clinical decision making for the older patient with MDS.

Sekeres comments on the above as follows:

In the article that accompanies this editorial, Koreth et al9 report on a Markov decision analysis exploring the role of reduced-intensity allogeneic HSCT in older patients with MDS. This statistical technique relies on assumptions, which themselves are based on best estimates of outcome given in previously published studies, to play out scenarios of what would happen in real life to a given patient if he or she decided to undergo HSCT early, at or near diagnosis, or instead to pursue supportive care, growth factor, or disease-modifying therapy.

Although this approach is not perfect, it does allow for sensitivity analyses in which assumptions can be changed to see if the same conclusion holds, and it is the best substitute available in the absence of prospective, randomized studies….

The analysis by Koreth et al9 addresses these shortcomings. Now, given the non myeloablative preparative regimen, the median age of the 132 patients undergoing transplantation gleaned from the Center for International Blood and Marrow Transplant Research, Dana- Farber Cancer Institute, and Fred Hutchinson Cancer Research Center data sets is 64 years—closer to what we see in clinic. Patients who did not undergo transplantation included 132 with lower-risk disease (IPSS low and intermediate-1) receiving best supportive care; 91 anemic or transfusion-dependent patients receiving erythropoiesis stimulating agents; and 164 higher-risk patients with MDS receiving azacitidine or decitabine.

Patients being treated with lenalidomide, immunosuppressive approaches, or drug combinations were not included. Primary end points of the model were life expectancy (LE) and quality-adjusted life expectancy, an end point adjusted for quality of life, the values of which were derived from studies in which patients may not reflect those included in the current analysis.

The authors tried to keep the assumptions used in an already complicated model to a minimum, and in so doing ignored some real-life scenarios, such as a patient initially in the non-transplantation arm deciding at a later time to undergo transplantation.

7 IMMUNOTHERAPY

The third general step is the use of CIK, or cytokine induced killer cells. These are somewhat akin to NK cells and have been developed specifically for cancers of these type. We briefly discuss how they are prepared. The efficacy is yet to be fully determined but there is a large base of Phase I and II Trials demonstrating efficacy.

Lin and Hui provide a definition for CIK cells:

Cytokine-induced killer (CIK) cells are polyclonal T effector cells generated when cultured under cytokine stimulation. CIK cells exhibit potent, non-MHC-restricted cytolytic activities against susceptible tumor cells of both autologous and allogeneic origins. Over the past 20 years, CIK cells have evolved from experimental observations into early clinical studies with encouraging preliminary efficacy towards susceptible autologous and allogeneic tumor cells in both therapeutic and adjuvant settings. ... we anticipate that the continuous therapeutic application of CIK cells will likely be developed along two major directions: overcoming the challenge to organize large prospective randomized clinical trials to define the roles of CIK cells in cancer immunotherapy and expanding its spectrum of cytotoxicity towards resistant tumor cells through experimental manipulations.

Jiang et al add to this description as follows:

The number of immune cells, especially dendritic cells and cytotoxic tumor infiltrating lymphocytes (TIL), particularly Th1 cells, CD8 T cells, and NK cells is associated with increased survival of cancer patients. Such antitumor cellular immune responses can be greatly enhanced by adoptive transfer of activated type 1 lymphocytes.

Recently, adoptive cell therapy based on infusion of ex vivo expanded TILs has achieved substantial clinical success. Cytokine-induced killer (CIK) cells are a heterogeneous population of effector CD8 T cells with diverse TCR specificities, possessing non-MHC-restricted cytolytic activities against tumor cells. Preclinical studies of CIK cells in murine tumor models demonstrate significant antitumor effects against a number of hematopoietic and solid tumors. Clinical studies have confirmed benefit and safety of CIK cell-based therapy for patients with comparable malignancies.

Enhancing the potency and specificity of CIK therapy via immunological and genetic engineering approaches and identifying robust biomarkers of response will significantly improve this therapy.

The preparation and creation of CIK cells is done as described by Jakel et al:

*CIK cells are generated by culturing **peripheral blood lymphocytes (PBL)** with*

1. *interferon-γ (**INF-γ**) monoclonal*
2. ***antibody against CD3 (anti-CD3)** and*

3. ***IL-2*** *in a particular time schedule.*

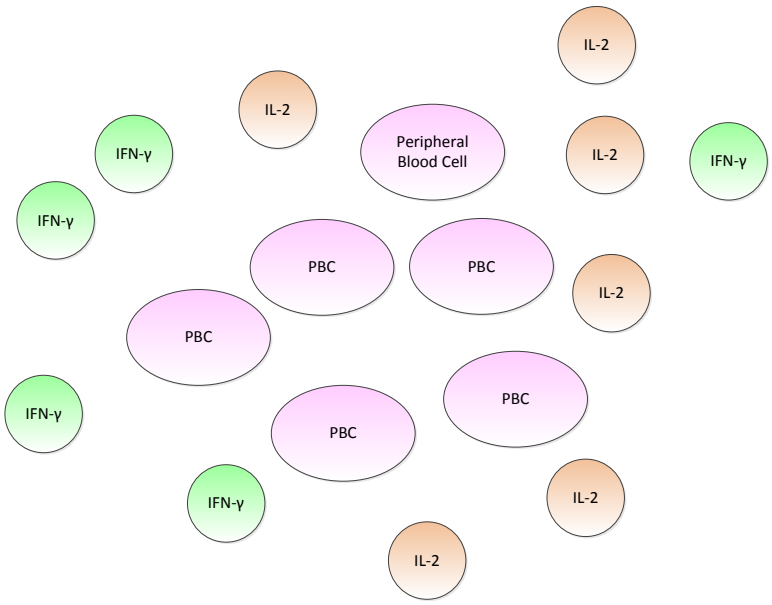
*The **cytokines INF-γ and IL-2 are crucial for the cytotoxicity** of the cells and anti-CD3 provides mitogenic signals to T cells for proliferation. Most of these CIK cells (87%) are positive for CD3 and for one of the T-cell coreceptor molecules CD4 (37.4%) or CD8 (64.2%), respectively.*

IFN-γ, added at day 0, activates monocytes providing crucial signals to T cells via interleukin-12 (IL-12) and CD58 (LFA-3) to expand CD56+ cells.

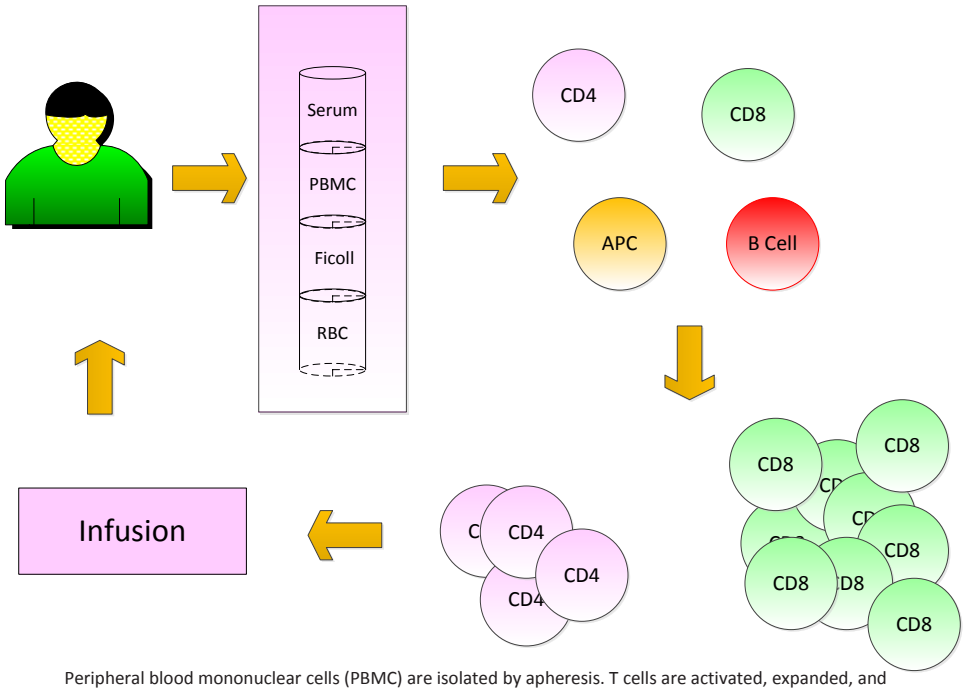
After 14 days of culture, 37.7% of cells are CD3+CD8+CD56+. These cells are referred to as natural killer T (NK-T) cells and represent the cell type with the greatest cytotoxicity in the CIK cell population.

Interestingly, these CD3+CD56+ double positive CD8+ T cells do not derive from the rare CD3+CD56+ cells in the starting culture but from proliferating CD3+CD8+CD56− T cells.

Their cytotoxicity is nonmajor histocompatibility complex (MHC)-restricted and they are able to lyse a variety of solid and hematologic tumors. Cell lysis is not mediated through FasL but through perforin release. CIK cell cytotoxicity depends on NKG2D recognition and signaling.



Jiang et al propose the following:



Peripheral blood mononuclear cells (PBMC) are isolated by apheresis. T cells are activated, expanded, and differentiated by anti-CD3 in the presence of cytokines including IFN-γ, IL-1α, and IL-2 for 14 to 21 days. These T cells, commonly called CIK, are then infused into patients. Jiang et al

Jiang et al prepare their cells as follows:

CIK cells have been evaluated as an adoptive cell immunotherapy for cancer patients in a number of clinical trials.

Peripheral blood mononuclear cells (PBMC) were isolated by apheresis.

T cells were then activated, expanded, and differentiated by

1. ***anti-CD3*** *in the presence of cytokines including*
2. ***IFN-γ,***
3. ***IL-1α, and***
4. ***IL-2***

*for **14 to 21** days to generate CIK, which were subsequently infused into patients.*

There are no significant clinical results for this in MDS but there are many Trials underway. One could suppose that this is a substantial third step after a BMT procedure. Logically it could be curative.

8 OBSERVATIONS

We now want to make some general and specific observations. WE shall discuss each as a separate topic.

8.1 COMPLEXITIES OF EPIGENETICS

Epigenetics has become as significant a factor in cancer as the pathway and immunological approaches. The impact of miRNA, lncRNA, methylation, acetylation, and other epigenetic elements are now understood as causative. However the drivers initiating many of these are not clearly understood. The methylation in MDS for example is understood as a cause but what leads to the methylation is still speculative. For example in melanoma one could speculate that backscatter X-rays in full body airport scans provide just the driver for methylation if it is applied at the right time. However that is also speculative and no studies have been done. It is speculated that excess radiation, excess CAT scans or radiation for cancers can cause the methylation seen in MDS. Proof is lacking however.

8.2 DOWNSIDE OF METHYLATION

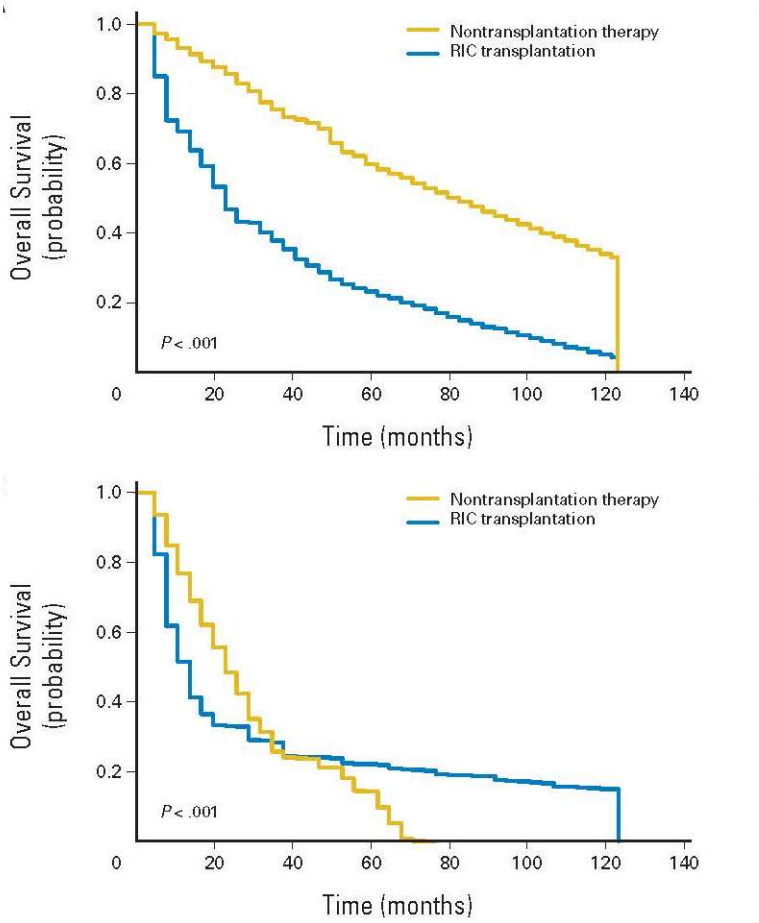
Methylation treatment with DNMT suppressors is known to drive down the blast percentage. However it is a broad based therapeutic and demethylates many other cell. This may also give rise to secondary neoplasia, by activating proliferation genes in other cells in the body. It is not known how significant this is. It might result in sequelae as is found in Hodgkin's lymphoma but the sequelae there are often found 20-30 years later. Thus since MDS occurs at 70 years of age that well exceeds any life expectancy.

8.3 HYPO VS HYPER METHYLATION

The problem with MDS is known to be hypermethylation. But there are many cases of hypomethylation as well. One then wonders if the approach taken herein applies to those cases as well.

8.4 EFFICACY; REMISSION OR CURE

Limited survival data is clinically available using the CIK approach. Koreth et al present data based upon a Markov model but we have considerable concerns about the approach. The results are shown below.



It should be noted that the top graph is for low to low intermediate and the bottom graph is high intermediate to high, using IPSS scoring. These Kaplan Meir curves show that for the high case we have a rapid drop and then a slow decline with about 20% at 10 years. Since the average age is about 70, the average life expectancy is 14 years and so 20% seem to have reached average life expectancy. In contrast the opposite is the case for the more indolent forms. The problem that we see is the initial conditions. Perhaps one would expect most patients have initial health conditions which would bias them against a BMT survival. Perhaps other health conditions are also a concern. The problem is that MDS is so complex and given the patients initial health status conditions it is expected that any case is different and thus any generalized result is problematic.

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