Methylation and Cancer

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We examine methylation and several cancers. There has been statements that all cancers are epigenetic and we have discussed several of these previously. One of the epigenetic factors is methylation, a somewhat understood phenomenon often seen in cancers, and often indicate as causative rather than a consequence. Recent work in anti-methylation therapeutics has raised interest here as well as preventative measures as ways to reduce methylation.

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1 INTRODUCTION

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.

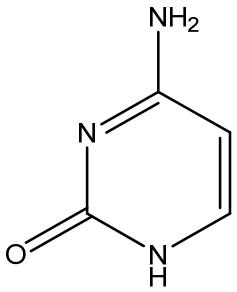
DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target.

Epigenetics has evolved as a rapidly developing area of research.

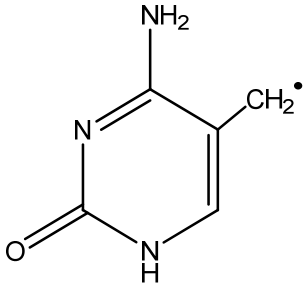
Recent studies have shown that epigenetics plays an important role in cancer biology, viral infections, activity of mobile elements, somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation

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

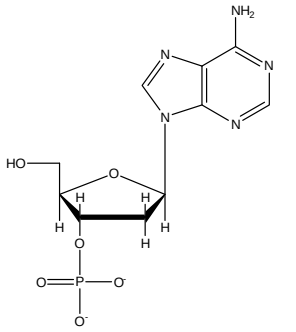
anticipate a preventative measure as one increasing certain methylation preventative therapeutics, can we do the same with say MDS, and can we attempt to do the same with say a melanoma. This is what we examine herein.

2 SOME DNA BASICS

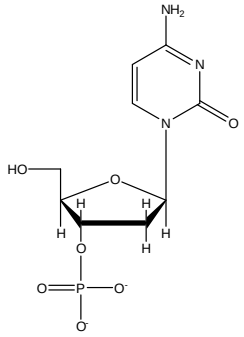
We begin with some simple facts about DNA and then we lead to the methylation of cytosine. But first, the basics of DNA.

DNA is composed of just five basis elements; a ribose backbone with phosphates, and four different nucleotides (C, G, A and T). They align in a double stranded classic DNA pattern.

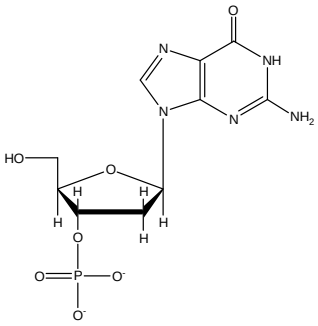
The base pairs and their ribose/phosphate backbone parts are shown below.



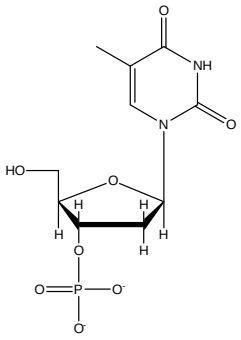
Adenine



Cytosine

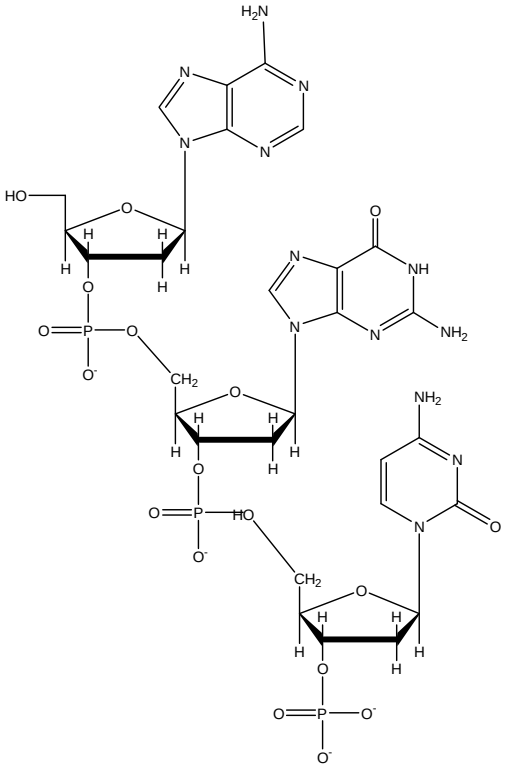


Guanine



Thymine

Now we connect these in the one side of the double helix as is shown below:

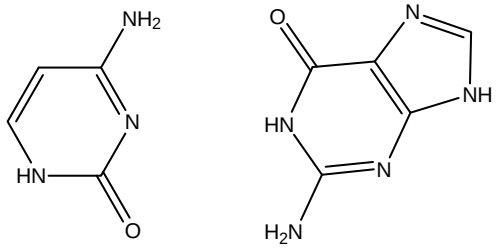


Then from here we can connect the A-T and G-C pairs which make up the DNA as we know it.

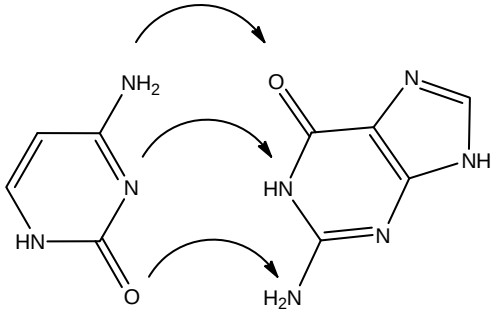
The key observation of Watson and Crick was the hydrogen bonding between base pairs. As Watson and Crick stated in 1953:

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of the bases are perpendicular to the fibre axis. They are joined together in pairs, a. single base from ***one chain being hydrogen-bonded to a single base from the other chain****, so that the two lie side by side with identical z-co-ordinates. One of the pair must be a purine and the other a pyrimidine for bonding to occur. The hydrogen bonds are made as follows: purine position 1 to pyrimidine position 1; purine position 6 to pyrimidine position 6.*

The CG bonding is shown below:



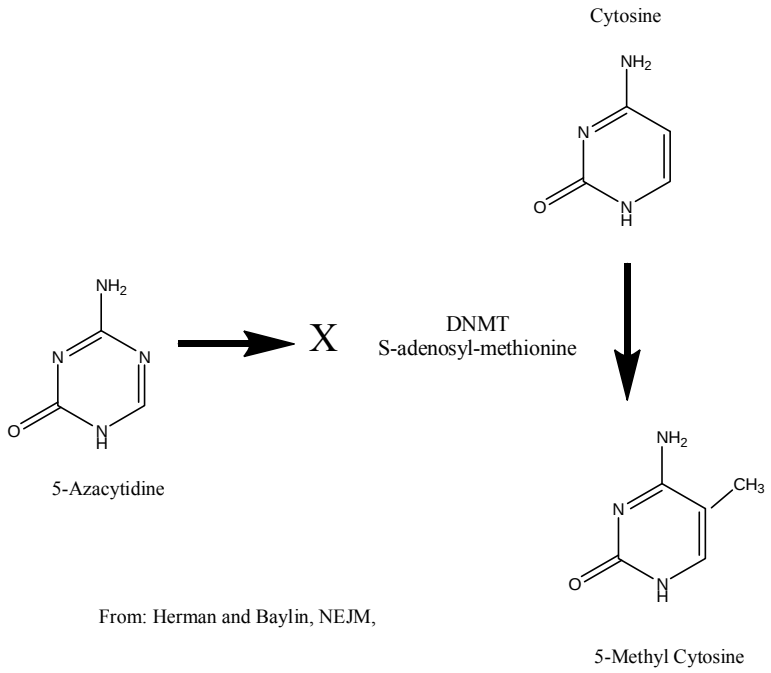
Hydrogen Bonding between C and G



This model is the basis of what we now know as our DNA. The DNA is divided into chromosomes, different strands, and then in the chromosomes we have Introns, non-expressing parts of DNA, and Exons, the expressing parts. The current view is that although the non-expressing parts do not yield proteins they strongly affect that process. That is what methylation does.

3 METHYLATION AT THE MOLECULE

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.

3.1 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).

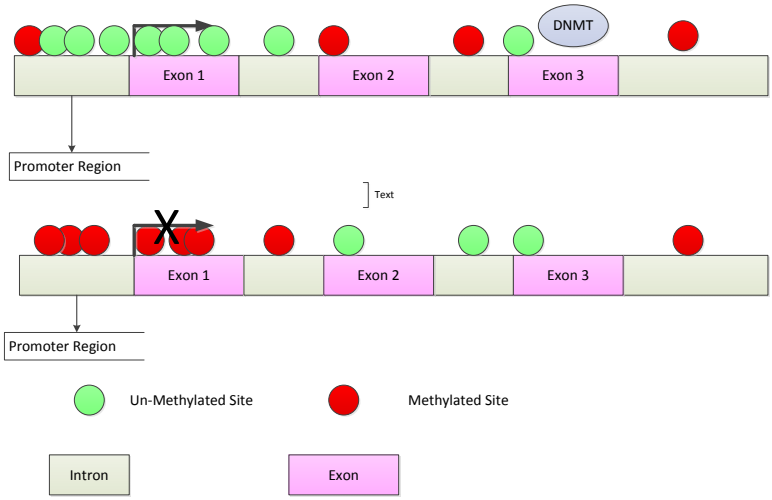
This methylated state in the bulk of the genome may help suppress unwanted transcription, whereas the unmethylated state of the CpG islands in gene promoters permits active gene

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.

3.2 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.

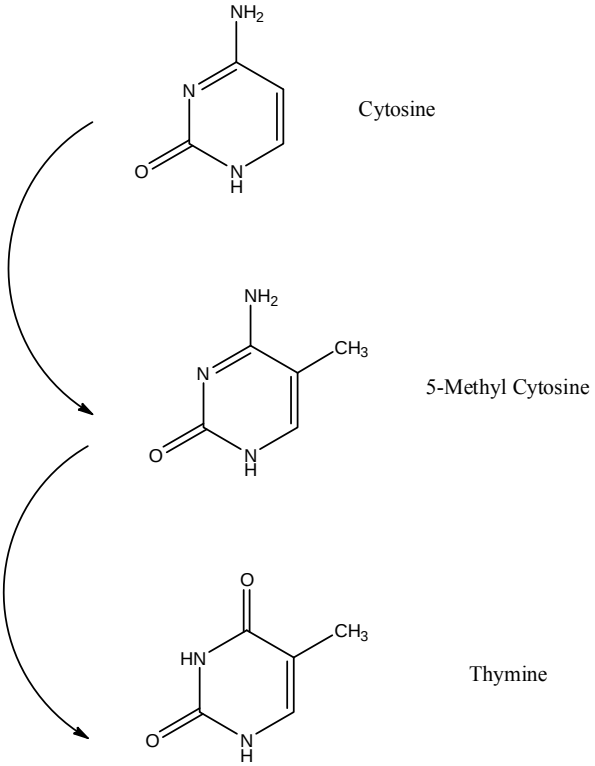
As Herman and Baylin state:

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.



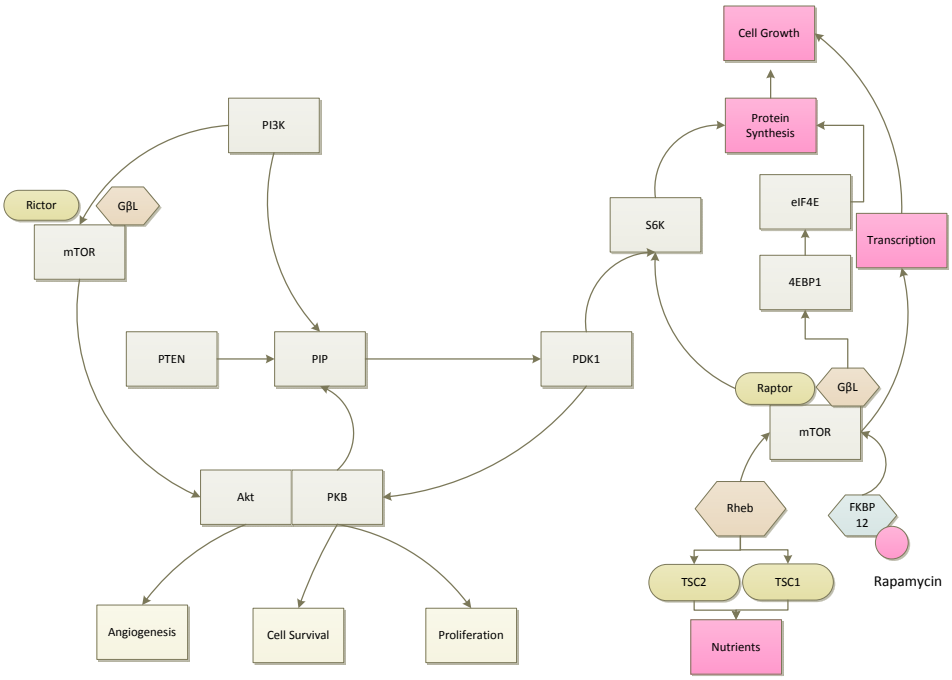
From Robertson (2001) we have some of the genes influenced by methylation or as he states:

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.

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.

5 METHYLATION EFFECTS ON DNA

As is stated in the paper by Miranda and Jones:

DNA methylation is a covalent modification in which the 5₀position 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
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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.

5.1 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 a 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 a 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

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 reduced 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.

6 METHYLATION AND CANCER

We now examine the impact of methylation on several cancers. We have selected three different types:

1. Glandular: This is prostate cancer. Many adenocarcinomas are typical of this type. Glands seem often to be the source of cancers and one could surmise it is because they are continually active cell sites with high mitotic activity.

2. Epidermal: We select melanoma as an example. This is especially interesting because it is a cancer which is often attributed to UV radiation, since the melanocytes are so close to the skin surface, a few dozen keratinocytes deep.

3. Hematopoietic: The majority of blood/bone generated cancers result from a variety of changes. CML is a classic model with a Philadelphia chromosome abnormality, a translocation. MDS, myelodysplastic syndrome, on the other hand, is a pre-cancerous state where hypermethylation is the driving factor. This is interesting in that unlike CML which has a clear genetic change, MDS has a clear hypermethylated state. It may result in a genetic change and thus AML but the progression may be mitigated by drugs which mitigate methylation.

We examine the literature on each as regards to methylation impact.

6.1 PROSTATE

Prostate cancer is a complex malignancy of a glandular element. It may be indolent or highly aggressive, and at this time it is quite difficult to determine the difference based solely on pathology examination. One of the themes we shall see in methylation and cancers will be exogenous effects such as sunlight in melanoma, such as chemicals and radiation in MDS and such as free radicals and infections in prostate cancer. These factors all seem to impact methylation.

In a recent (2013) paper by Vasiljevic et al they state:

Our data indicate CpG methylation of the first HSPB1 intron to be an important biomarker that identifies aggressive PCas otherwise regarded as low risk by current clinical criteria but that, biologically, require immediate active management.

This is a very powerful conclusion. It is a step to identifying indolent from aggressive. They continue:

Heat shock protein 27 (Hsp-27), encoded by the gene HSPB1 located on chromosome 7q11.23 has been shown in several independent studies to be a reliable biomarker of poor clinical outcome in human prostate cancer (PCa) as well as in human breast cancer, colorectal cancer and malignant melanoma.

Biologically, Hsp-27 is an anti-apoptotic protein that induces intracellular homeostasis and allows cellular repair and recovery after physical and chemical insults. Although Hsp-27 is constitutively expressed in most human cells, induced overexpression during carcinogenesis can lead to increased survival of the malignant cells.

Therefore, it is not surprising that studies link high expression of Hsp-27 to unfavorable prognosis in many cancer types. The prognostic potential has been confirmed in prostate cell lines14 as well as in prostate tissues where overexpression has been linked with hormone resistance and poor clinical outcome.

During early prostate carcinogenesis, expression of Hsp-27 protein becomes universally abrogated but may be re-expressed subsequently, in which case the malignancy develops an aggressive phenotype.

Although the specific factors controlling these changes are presently unknown, one plausible mechanism is DNA methylation (DNAme) of the HSPB1 gene. The majority of CpG dyads in the human genome are methylated with the exception of CG-rich regions called CpG islands.16 CpG islands mainly cover gene promoters and first exons and their hypermethylation is associated with repressed transcription of many tumor-suppressor genes.

Therefore, we test the hypothesis that the DNAme status of HSPB1, particularly the HSPB1 promoter, exon and intron regions, is an important determinant of PCa behavior.

Thereafter, we assess any potential relationship between DNAme and Hsp-27 protein levels. Our objectives are also to investigate the diagnostic biomarker potential, by comparing the methylation status of BPH vs PCa, and the prognostic potential of DNAme, by analyzing the association between the methylation and PCa-specific death in the well-characterized Transatlantic Prostate Group (TAPG) cohort.

They conclude:

In conclusion, HSPB1 is essentially unmethylated in BPH but with increasing neoplastic changes through to PCa, the gene becomes increasingly methylated, proceeding from the promoter in a 3' direction. In PCas with low Gleason score, higher methylation within the HSPB1 gene independently identifies patients with poor clinical outcome and hence is an objective biomarker identifying the immediate need for active intervention in the clinical management of this cohort of patients.

This is a powerful observation and sets the path for improved prognostics on PCa.

In an older paper by O'Shaughnessey et al they state:

PIN and prostate cancer lesions share a number of somatic genome abnormalities, including loss of DNA sequences at 8p and increased GSTP1 CpG island DNA methylation, among others. Finally, transgenic mouse strains prone to developing prostate cancers typically develop PIN lesions in advance of the appearance of invasive cancer.

We have discussed elsewhere the HGPIN issue regarding PCa and the questions raised by the assumed linear progression from HGPIN top PCa, except in certain cases where we hypothesize the removal of stem calls upon biopsy.

6.2 MELANOMA

Melanoma is a solid tumors which has the tendency to metastasize very rapidly. Melanoma is fundamentally a malignancy of the melanocytes and the melanocytes are often changed into a malignant state due to their proximity to the skin surface, at the basal layer of the epidermis, and the impact of UV light on their progress. We have argued elsewhere that methylation of portions of the DNA due to such factors as backscatter radiation may be a significant factor as well. The skin being so thin absorbs the radiation more strongly that the viscera and thus is millions of times more sensitive.

From Bennett we have:

A primary event in progression would be a cellular change that is clonally inherited, that contributes to the eventual malignancy, and that occurs independently rather than as a secondary result of some other oncogenic change.

These events are either genetic (gene mutation, deletion, amplification or translocation), or epigenetic (a heritable change other than in the DNA sequence, ***generally transcriptional modulation by DNA methylation*** *and/or by chromatin alterations such as histone modification). In clonal evolution of cancer, such a primary event would initiate a new, more progressed, clone with a growth advantage over its neighbors, or an alternative selective advantage such as migration…. The β-catenin pathway can be upregulated by several kinds of primary and secondary changes in melanoma. These include uncommon activating mutations of b-catenin (CTNNB1) itself,* ***methylation or mutation of APC****, overexpression of proto-oncoprotein SKI….*

In a recent paper by Mazar et al they report on melanoma as follows:

Here, we report that cell lines derived from malignant melanomas and melanoma patient samples have hypermethylated CpG islands in the 59-upstream regions of several miRNA coding genes, including that of miR-34b. We engineered two cell lines derived from metastatic melanoma to ectopically express miR-34b, and show that these cells exhibit reduced cell motility, decreased substrate attachment, and reduced invasion.

They continue:

The reduced expression of genes that are under the control of CpG island methylation is often reversed by treating the cells with the DNA methyl transferase inhibitor 5-Aza-29-deoxycytidine (5-Aza-dC). To assess the range and extent of miRNA expression under direct or indirect control of DNA methylation, we treated the melanoma cell line WM1552C (derived from a stage 3 malignant melanoma) with 5-Aza-dC and measured changes in miRNA gene expression using

miRNA microarrays (see Methods). Several miRNAs, including miR-34b, -489, -375, - 132, -142-3p, -200a, -145, -452, -21, -34c, -496, -let7e, -654, and -519b, were found to be up-regulated

They conclude:

During melanoma formation, the initial genetic or epigenetic changes are thought to precede additional mutations and further epigenetic changes that affect the function of several signaling pathways. Aberrant DNA methylation patterns at the 59 noncoding region of the INK4a gene was discovered in melanoma , which is consistent with the involvement of epigenetic factors in melanoma development or progression.

Similarly, epigenetic silencing of PTEN expression occurs in certain malignant melanomas with no detectable mutation in the PTEN gene.

While the impact on melanoma development of epigenetic changes in several protein-coding genes is appreciated, there have been few reports of the impact of epigenetic regulation of noncoding RNAs, such as miRNAs.

The epigenetic modification of miR-34b may serve as a useful biomarker for early melanoma detection in humans, and therefore, one could propose to develop a novel sensitive miR- 34b epigenetic biomarker assay to screen skin biopsies in melanoma patients. Including a panel of non-coding RNA epigenetic markers in to widely used pathological and genetic markers will be advantageous for both patients and pathologists.

An investigation of miR-34b regulation and associated CpG island methylation in a large group of melanoma patient samples, in comparison with samples of matched normal tissues or melanocytic nevi, is both relevant and timely. Mir-34 group of miRNAs are known to be useful therapeutic target for various cancers...

The PTEN control of cell proliferation is well known. However here it is shown that methylation can suppress PTEN without a genetic modification. Methylation is thus a powerful tool that surpasses genetic changes. Melanoma is an intriguing cancer because the effects of the environment are so well identified. Upon biopsy one can determine the extent of sun damage and ageing. Thus we can determine how much potential methylations effects are present as well.

6.3 MYELODYSPLASTIC SYNDROME

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 are certain therapeutics which address some of the pathway aberrancies which characterize the disease, specifically hypermethylation.

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.

6.3.1 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.

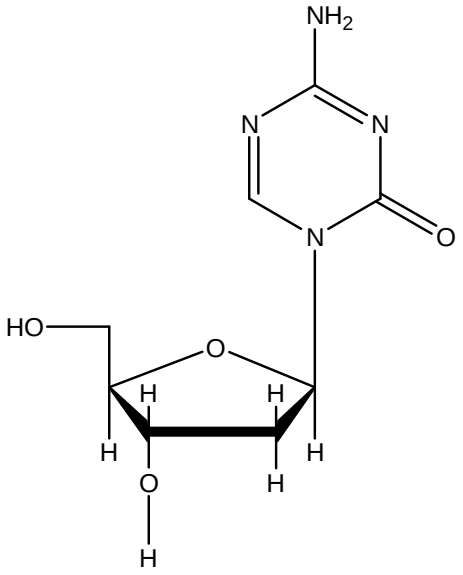
As Li has stated:

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.

6.3.2 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.

7 CONCLUSION

This is a brief overview of methylation. We have attempted to describe it as one of many paths that lead to malignant cells.

7.1 CAUSE AND EFFECT

As we have demonstrated there are no clear causal factors for the methylation we observe in many cancers. Although there are models for the effects of methylation on gene expression there is also not clear understanding of how much methylation is too much. It appears that once methylation begins it continues almost unabated until the expression of the affected gene is suppressed or otherwise modified.

7.2 TARGETING: ACTIVATE OR SUPPRESS

The question is often; hypomethylated or hypermethylated, good or bad? Thus the therapy may also require that answer first. We can examine MDS and see that hypermethylation suppresses the genes controlling cell proliferation. No gene product and thus no control on the cell proliferation. It proliferates but poorly. Now can we just target the hypermethylation, it seems to function with decitabine. Are there other therapeutics such that an appropriate cocktail as used in many other treatments may be applied?

7.3 WHAT CAUSES METHYLATION

The cause of methylation is a critical issue. We have argued that in melanoma it may be the result of UV radiation or even X Ray sources, with exposure as low as what one might see on a backscatter system deployed at airports. However there is still just speculation with limited data. We know that inflammation is a major source of such hypermethylation, and thus any inflammatory state would be a concern, for example as we often see in diabetics and alcoholics.

As Brower states:

Epigenetics has also provided clues that link environmental factors with cancerous genetic changes. Changes in methylation can be detected in the blood of cancer-free individuals who smoke and eat high-fat diets, and these changes have been shown to precede genetic mutations3. More recently, Karl Kelsey, a molecular epidemiologist at Brown University in Providence, Rhode Island, has uncovered independent associations between epigenetic patterns in breast cancer tumours, the tumour size, alcohol consumption and folate intake.

A prime candidate at the interface of environment and genetics is chronic inflammation, which is known to precede the development of numerous types of precancerous lesions — and indeed certain cancers themselves, including oesophageal, liver and colon cancers. Inflammation has been linked with increased DNA methylation in otherwise healthy looking tissue. Issa calls chronic inflammation "a truly epigenetic phenomenon".

Long-term inflammation may result from infection with Helicobacter pylori or hepatitis C virus, or from autoimmune diseases such as ulcerative colitis (a form of inflammatory bowel disease). People with ulcerative colitis often develop colon cancer at a younger age — for example in their 50s — than the 60 to 70 year average age of onset.

Thus it still is clear that causal factors are speculative.

7.4 WHAT CAN PREVENT METHYLATION

Prevention of methylation will require clear causation. Reducing inflammation, perhaps various well accepted ways to do that may assist, but no clear path is laid out.

7.5 CAN DAMAGING METHYLATION BE REVERSED

Having resulted in a hyper or hypo methylated state can it be reversed? That is a major clinical question. Decitabine is a typical example of a drug which appears to work.

As Brower states:

Drugs and dietary substances that alter epigenetic pathways are currently being tested. During his research on RCC, for example, Baylin and colleagues were able to reverse hypermethylation of the VHL gene with the drug 5-azacytidine. Trials of demethylating drugs as adjuvant treatments to prevent lung cancer recurrence are underway. If successful, prevention trials are the next logical step. "We need five- and ten-year survival data with current drugs to be sure there are no secondary effects before we give them to reasonably healthy people for prevention," says Issa. He sees a different source for the first wave of preventive medications. "I would bank on discovering more 'gentle' approaches to epigenetic manipulation for cancer prevention — be they natural products, existing drugs with a good safety records, or even vitamins or diet."

Thus many trials are underway but few solutions have been presented.

In conclusion, as Palii and Robertson state:

Epigenetic modifications are defined as heritable changes in gene expression occurring without alteration of underlying DNA sequence. A great deal of data has been accumulated showing the connection between neoplasia and dysregulated epigenetic processes.

Furthermore, cancer is now regarded as a multifaceted disease with a complex etiology, involving both mutational (genetic) and epigenetic alterations (such as DNA methylation and histone tail modifications).

DNA methylation status of biomarker genes is beginning to be employed for the assessment of patient samples and for prognostic purposes, and new techniques are being evaluated in the quest for yet unidentified TSGs as potential therapeutic targets DNA methylation represents a defense mechanism against selfish DNA elements, preserves the structural integrity of the

genome by "masking" repetitive sequences, and contributes to transcriptional repression and gene silencing.

Methylation targets CpG dinucleotides, which are generally underrepresented in mammalian genomes, except for promoter associated CpG islands, the only genomic regions in which CpG occurs at the expected frequency.

In normal cells, methylation is mostly present in pericentromeric regions, repetitive DNA, retro elements, and non-island CpGs, whereas methylation events in promoters and the body of genes have regulatory functions. Additionally, methylation is physiologic in the differentially methylated regions of imprinted genes where it ensures selective expression from a single parent of origin allele and in the inactive X chromosome in females.

CpG islands are generally protected against DNA methylation and therefore lack this modification in normal cells, although exceptions to this generalized rule have been found.

Thus methylation has both positive and negative effects. It is the serious and life threatening effects which we are concerned with. Palii and Robertson argue that Cancer is almost always epigenetic; methylation, miRNAs, and the like. However there are clear indications that pure genetic changes play a significant role as well.

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