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Chapter 8

Cancer Epigenetics: Role of Epigenetic Events in the Onset and Progression of Cancer

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Abstract

The word "Epigenetics" describes inheritable changes in gene expression that are independent of alterations in DNA sequences. Epigenetics is one of the most rapidly expanding fields in biology and over the past 16 years, the epigenetic regulation of DNA-based processes has been intensely studied. Epigenome is essential for the regulation and in unraveling the stages of normal and abnormal cellular development, including the phases of growth, differentiation, senescence, aging and immortalization during carcinogenesis. The recent characterization of DNA methylome at single nucleotide resolution has allowed the mapping of epigenetic machinery: DNA methylation, post-translational histone and other protein modifications, nucleosome positioning and noncoding RNAs (specifically microRNA [miR] expression) which act in concert to exert their cellular effects. Recent advancements in cancer epigenetics has highlighted the extensive reprogramming of every component of the epigenetic machinery in cancer. Disruption of the epigenome can contribute to cancer via altered gene function and malignant cellular transformation. The reversible nature of gene silencing by epigenetic modifications has facilitated the emergence of the promising field of epigenetic therapy. In contrast to conventional chemotherapy; several epigenetic drugs have been proven to prolong survival and to be less toxic. DNA methylation and histone modifications may serve as a potential targets for the development and implementation of new therapeutic approaches in the clinical settings. Many clinical trials are ongoing with novel classes of agents that target various components of the epigenetic machinery and have already made progress with the recent FDA approval of three epigenetic drugs for cancer treatment. In this book

chapter, we discuss the roles of epigenetic modifications in tumorigenesis; their clinical utility in cancer management as biomarker for detection, diagnosis and prognosis as well as highlight emerging epigenetic therapies being developed for cancer treatment.

Abbreviations

APC: Adenomatosis polyposis coli; CDH13: Cadherin 13; ER-α: Estrogen receptor-α; MLH1: mutL homolog 1; VHL: von Hippel-Lindau tumor suppressor; RAR-b2: Retinoic acid receptor b2; GSTP1:Glutathione S-Transferase Pi 1; MBD: Methyl-binding domain; HDAC: Histone deacetylase; LOI: Loss of imprinting; CDH1: Cadherin-1; ES: Embryonic stem cells; MeCP: Methyl cytosine binding protein; MAGE: Melanoma-associated gene; DPP6: Dipeptidyl peptidase 6; VIM: Vimentin; HOXA2: Homeobox protein Hox-A2; IAP : Inhibitor of Apoptosis (IAP); DNMT: DNA methyltransferase; Rb: Retinoblastoma; HATs: Histone acetyltransferases; NID2: Nidogen 2; CRBP1: cellular retinol binding protein 1; TP73: Tumor Protein P73; RUNX3: Runt-related transcription factor 3; RAR: Retinoic acid receptor; THBS1: Thrombospondin 1; ER-β: Estrogen receptor-β; HDACI: Histone deacetylase inhibitors; SirT1: Sirtuin (silent mating type information regulation 2 homolog) 1; BRCA1: Breast Cancer Type 1 Susceptibility Protein; CDKN2B: Cyclin-dependent kinase inhibitor 2B (p15); PRMT5: Protein Arginine Methyltransferase 5; SUV39H1: Suppressor Of Variegation 3-9 Homolog 1); RASSF1A: Ras association domain family 1 A; MGMT: O6-Methylguanine-DNA-Methyltransferase; ERβ: Estrogen receptor beta; MECP2: Methyl-CpG-binding 2 protein; HP1α: Heterochromatin protein1α; PRC2: Polycomb repressive complex 2; ZBTB 33: Zinc finger and BTB domain containing protein 33; MSP: Methylation specific PCR; SEPT9: Septin 9; DAPK1: Death-Associated Protein Kinase 1; IGF2: Insulin-like growth factor 2; S100P: S100 calcium binding protein P; GATA2: GATA-Binding Protein 2; CDKN1A: Cyclin Dependent Kinase Inhibitor 1A; G9a: Histone-lysine N-methyltransferase 2 (known asEHMT2); SFRP1: Secreted Frizzled Related Protein 1; TMS1: Target Of Methylation-Induced Silencing 1; MBD1: Methyl-CpG binding domain protein 1; PCDH10: Protocadherin 10; ERα: Estrogen receptor alpha; 5-FC: 5-fluoro-2-deoxycytidine; SHOX2: Short stature homeobox 2; TWIST1: Twist Family BHLH Transcription Factor 1; SAHA: Suberoylanilide hydroxamic acid; CDKN2A: Cyclin dependent kinase inhibitor 2A (p16); SAT2: Spermidine/spermine N1-acetyltransferase family member 2.

1. Introduction

Carcinogenesis is driven by the accumulation and interplay of genetic and epigenetic abnormalities that affect the structure and function of the genome [1-3] and result in dysregulated gene expression and function. The term "Epigenetics" coined by C.H.Waddington refers to the study of heritable changes that are independent of alterations in the primary DNA sequence. The epigenetic alternations implicated in the initiation and progression of cancer are DNA methylation, post-translational histone and other protein modifications, nucleosome positioning and noncoding RNAs (specifically microRNA [miR] expression) which act in concert to exert their cellular effects (Fig. 1). These modifications jointly constitute the "epigenome" to modulate the regulation of many cellular processes, including gene and microRNA expression, DNA-protein interactions, suppression of transposable element mobility, cellular differentiation, embryogenesis, X-chromosome inactivation and genomic imprinting [4]. Epigenome is essential for the regulation and in unraveling the stages of normal and abnormal cellular development, including the phases of growth, differentiation, senescence, aging and immortalization during carcinogenesis [5].
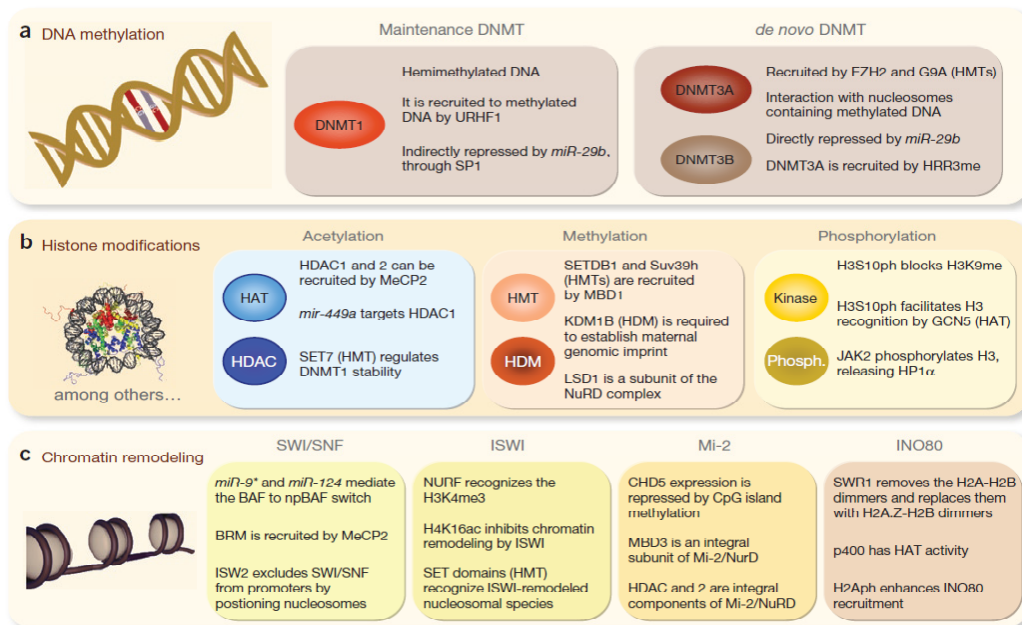
Figure 2 Epigenetic machinery and interplay among epigenetic factors. Epigenetic marks are catalyzed by different epigenetic complexes, whose principal families are illustrated here. (a–c) Epigenetic regulation depends on the interplay among the different players: DNA methylation (a), histone marks (b) and nucleosome positioning (c). The interaction among the different factors brings about the final outcome. This figure illustrates selected examples of the possible interrelations among the various epigenetic players.

Figure1: Epigenetic machinery and interplay among epigenetic factors (Adapted from A. Portela and M. Esteller, 2010 [97].)

The emergence of epigenetic machinery as key regulators of gene regulation and expression has provided significant insights into oncogenesis. Driven by aberrant DNA methylation and histone modifications, epigenetic aberrations are critically responsible for the disruption of cellular machinery and homeostasis. Failure of the proper maintenance of the epigenetic machinery results in altered gene function and malignant cellular transformation. Aberrant epigenetic modifications occur at an early stage of neoplastic development and serve as an essential player in cancer progression [6].

DNA methylation is characterized by the chemical modification of cytosine with the transfer of a methyl moiety at the 5- carbon of the cytosine base in CG dinucleotides by DNA methyltransferases (DNMTs). DNA methylation play vital role in the regulation of gene transcription and chromatin status. In contrast to normal cell, cancer cell show global hypermethylation mainly of repetitive elements and localized hypermethylation leading to silencing of genes (e.g., tumor suppressor) with associated loss of expression [7]. Nucleosomes the basic unit of chromatin, basically consist of 146bps of DNA wrapped around an octomer of Histone complex (two subunits each of H2A, H2B, H3 and H4 histones). The H1 linker histone binds to the outside of nucleosome and seals two turns of DNA. The less structured N-terminal domains of all core histones protrude from the core histone and are subjected to modifications [8-10]. The epigenetic cross-talk between histone modifications and DNA methylation influences chromatin condensation, stability and nuclear architecture, primarily regulating its accessibility and compactness.

The most common epigenetic modifications observed during malignancies are increased

methylation of CpG islands within gene promoter regions and deacetylation and or methylation of histone proteins which results in aberrant gene expression and altered epigenomic pattern [7,9]. In recent years, tremendous pace of research on epigenetics provides insights into the significant role altered epigenetic alterations plays in mediating tumor onset and progression, their utility as candidate targets being explored for risk assessment, early detection, prognosis, prediction of response to therapy and on the development of compounds that target enzymes which regulate the epigenome as anticancer agents, thereby outlining the great promise this field holds to advance our understanding of oncogenesis and help in the development of strategies for cancer management [11-14].

In the present book chapter, we discuss the current understanding of epigenetic modifications associated with tumorigenesis with focus on histone modification and DNA methylation and provide an overview of the potential utility of methylation markers for cancer detection, diagnosis and prognosis. We also highlight the prospect of epigenetic therapies in designing effective strategies for cancer treatment and prevention.

2. DNA methylation in gene regulation

One of the best characterized epigenetic modifications is DNA methylation which is involved in various biological processes such as the silencing of transposable elements, regulation of gene expression, genomic imprinting, and X-chromosome inactivation [15-17]. (Table 1) Various reports implicate the significant role of DNA methylation in carcinogenesis, right from the silencing of tumor suppressors to the activation of oncogenes and the promoting metastasis [18]. DNA methylation serves as a key element in tissue differentiation during early embryonic development.

Aberrant DNA methylation being recognized as the most common molecular abnormalities during tumorigenesis, are frequently associated with drug resistance [19]. Most CpG sites which are outside the CpG islands are methylated, thereby suggesting its role in the global maintenance of the genome. However, most CpG islands in gene promoters are generally unmethylated, allowing active gene transcription. When a given stretch of cytosine of CG dinucleotide in the CpG island located in the promoter of a given gene is not methylated, the gene is not silenced through methylation. Such CpG island is termed as "hypomethylated'. Contrary, methylation of cytosine of CG dinucleotide in the CpG island located in the promoter of a given gene results in methylation induced gene silencing and such CpG island is termed as "hypermethylated" [20]. Furthermore, methylated cytosines preferentially bind to a protein known as methyl cytosine binding protein, or MeCP, which inhibits the recognition of methylated promoter by transcription factors and RNA polymerase [21].

In normal cells, CpG islands in active promoters are not methylated in order to maintain euchromatin structure, thus allowing active gene expression. However, the CpG islands within

coding regions are often methylated. Reverse patterns are observed in cancer cells, where hypermethylation at CpG island containing gene promoter results in their transcriptional inactivation by changing the open euchromatin structure to compact heterochromatic structure [22].

3. Interrelation between DNA methylation and histone modifications

As mentioned before, all the epigenetic players act in concert to exert their cellular effect. Apart from performing their individual roles, histone modification and DNA methylation machinery interact with each other to determine gene transcription status, chromatin organization and cellular identity. The relationship between DNMT3L and H3K4 is a striking example which reflects the interplay between histone modifications and DNA methylation. The specific interaction of DNMT3L with histone H3 tails induces *de novo* DNA methylation by recruiting DNMT3A. Conversely, this interaction is strongly inhibited by H3K4me [23].

Several histone methyltransferases including G9a, SUV39H1 and PRMT5 have been reported to direct DNA methylation to specific genomic targets by directly recruiting DNA methyltransferases (DNMTs) which in conjugation with repressive histone marks further enhances the suppression of gene expression [24,25]. In addition to direct recruitment of DNMTs, histone methyltransferases and demethylases influence DNA methylation level by modulating the stability of DNMT proteins [26,27]. Early studies have shown that histone H3K9 methyltransferase controls DNA methylation in fungi (*Neurospora crassa*). Mutation of histone H3K9 methyltransferase resulted in reduced methylation thereby signifying H3K9 methylation acts as an upstream epigenetic mark which controls DNA methylation [28].

For the repression of gene expression and chromatin condensation, DNMTs can recruit HDACs and methyl binding protein. DNA methylation can also direct histone modifications. The strongest link between DNA methylation and histone modification is served by Methyl binding proteins which includes methyl CpG binding protein 2 (MeCP2), Methyl-CpG binding domain protein 1 (MBD1), and Kaiso [also known as ZBTB 33 (Zinc finger and BTB domain containing protein 33)]. However, their confinement to methylated promoter mediates the recruitment of histone deacetylases (HDACs) and histone methyltransferases, which suggests that DNA methylation, induces chromatin structural changes via alternation of histone modification. For instance, methylated DNA mediates H3K9methylation through recruitment of effector protein MeCP2, thereby maintaining a repressive chromatin state [29]. (Fig. 2)
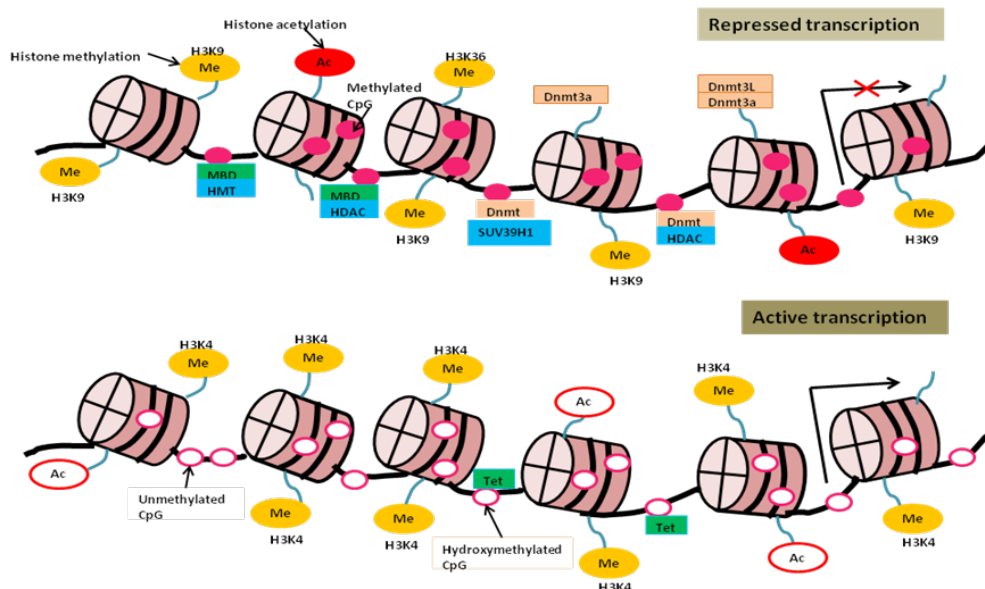
Figure 2: Epigenetic cross talk between DNA methylation and histone modification (Adapted from:K. GrøNbaek et.al. 2007 [12].)

During development, both DNA methylation and histone modification are involved to establish patterns of gene repression. Certain forms of histone methylation results in generation of local heterochromatin, that is readily reversible. In contrast, a highly stable long term repression is maintained by DNA methylation. Recently several studies provide insight that DNA methylation and histone modification pathways can be dependent on each other and this cross talk can be achieved through biochemical interactions between SET domain histone methyltransferases and DNA methyltransferases [30].

For instance, in embryonic stem cells (ES), the pluripotency genes such as Oct3/4 and Nanog are inactivated after lineage commitment. This silencing process involves the recruitment of repressor complex: the SET domain containing histone methyltransferases G9a together with histone deacetylase. Subsequently methyltransferases DNMT3A and DNMT3B, which mediate *de novo* methylation, are recruited by G9a through its ankyrin (ANK) domain, at the promoter [31,32]. In context to G9a, it seems that the different protein domains are responsible to carry out the histone methyltransferases activity and the link with DNA methyltransferases activity. Therefore, mutation of the SET domain disrupts H3K9 methylation without affecting DNA methylation thereby suggesting that DNA methylation is not dependent on histone modification; instead on the recruitment of G9a (in particular, ankyrin motif) and the interrelation between histone modification and DNA methylation is generated through enzyme interactions [24,33].

Cooperation between histone modifications and DNA methylation in order to achieve silencing is reflected by the Polycomb targeted genes. (Fig. 3) In normal cells, repression involves formation of local heterochromatin – the SET domain histone methytransferase (EZH2), as a part of Polycomb repressive complex 2 (PRC2) mediates the histone H3 lysine 27 trimethylation leading to heterochromatinization through the PRC1 complex, that consist

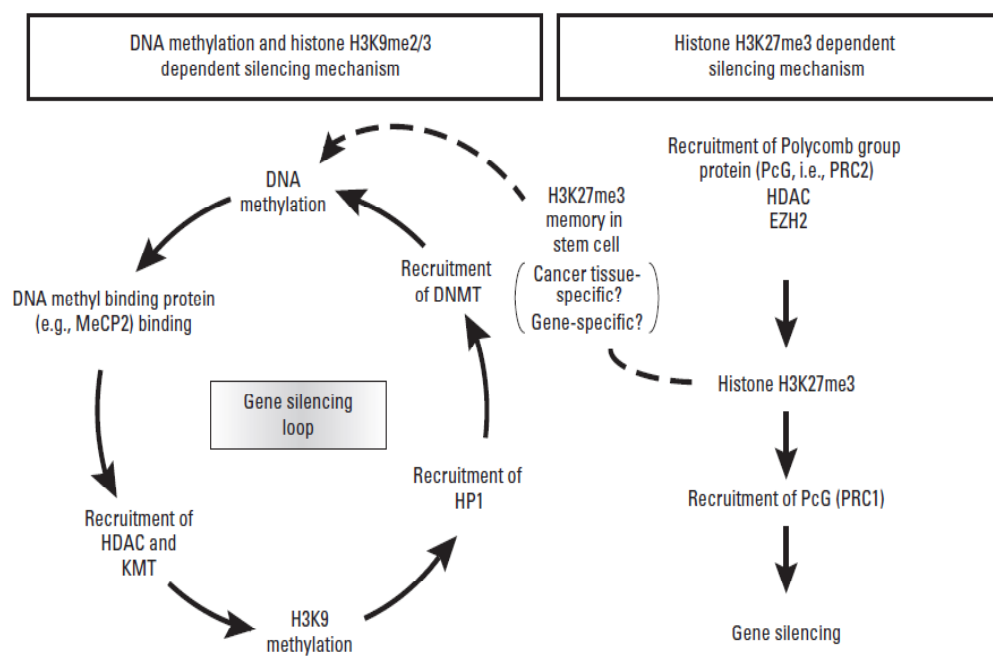of chromodomain protein PC, thereby blocking the recruitment of transcriptional

Figure 3: Two distinct histone modifications for gene silencing in human cancers(Adapted from Y. Kondo, 2009 [40].)

activation factors [34,35]. Interestingly, polycomb induced repression are easily reversible and in ES cells, almost all polycomb targeted genes are marked by both the repressive H3K27me3 modification as well as activating modification H3K4me3. This bivalent modification pattern confers the potential of a gene to be driven either to its active or inactive state. Those genes which were silenced by this mechanism might readily get activated during differentiation. On contrary, genes in their active conformation might revert to the repressed state [36,37].

Most of the genes repressed by polycomb complexes are generally associated with unmethylated CpG islands. However under certain circumstances (such as cancer), a number of these genes might become targets of *de novo* methylation, possible through the interaction between EZH2 and the methyltransferases DNMT3A and DNMT3B [38, 39]. Upon methylation, some of these genes lose their polycomb repressive proteins, but still remain inactive due to the DNA methylation, as an alternate silencing mechanism. This epigenetic switch reduces epigenetic plasticity, locking the silencing of key regulators and contributing to carcinogenesis. However, in some genes H3K27me3 and DNA methylation co-exist on the same promoter, in such cases PcG-mediated H3K27me3 is the dominant silencing machinery [40]. (Fig. 4)

4. Epigenetic modifications in cancer

Recent studies indicate that tumorogenesis cannot be accounted by genetic alternations alone but also involve epigenetic modifications. Thus, tumour cells are activated by both genetic and epigenetic alterations. The interplay among the different players such as DNA methylation, histone modifications and nucleosome positioning is critical for the regulation of gene and noncoding RNA expression. During carcinogenesis, these epigenetic marks play an

important role in tumor development and progression by modulating the chromatin structure, gene and miRNA expression**.** (Fig. 5) Additionally, tumor cells reflect a profoundly distorted epigenetic landscape. The epigenetic alternations, their possible mechanisms and associated biological consequences by which they promote tumorigenesis have been discussed in Table 1.
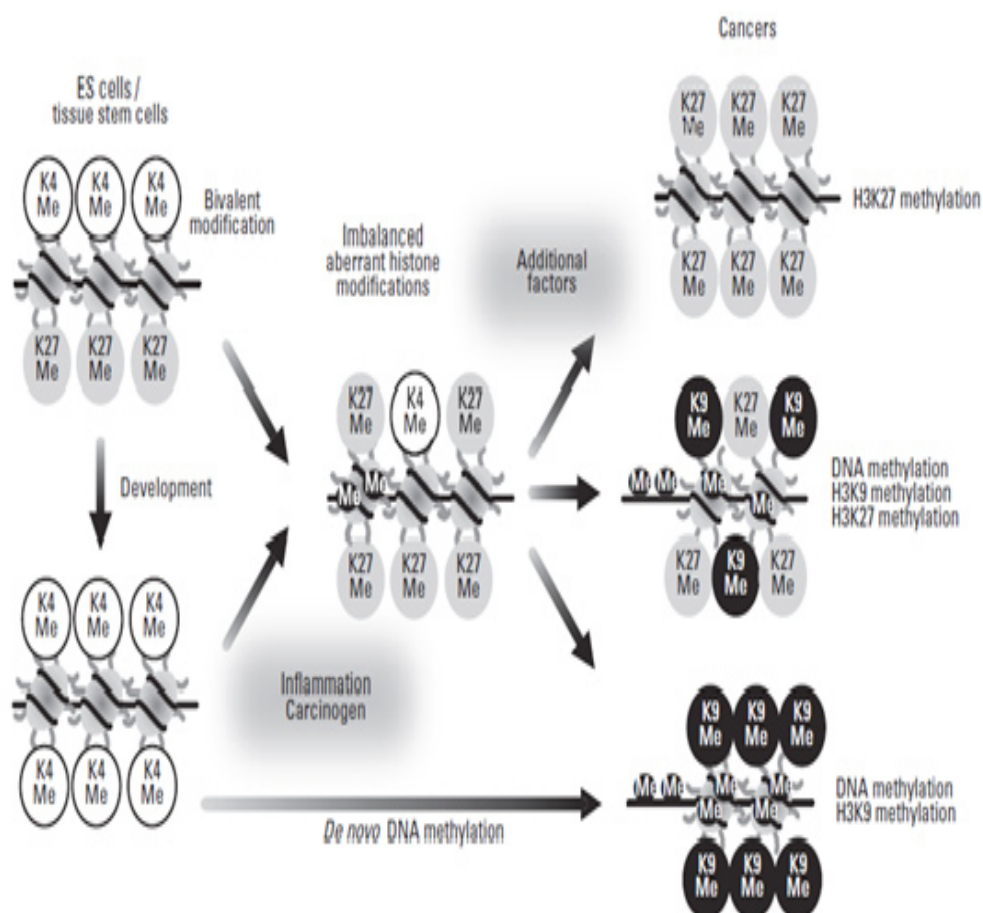

Figure 4: A model representing *de novo* methylation and *de novo* histone modifications in human cancer (Adapted from Y. Kondo, 2009 [40].)
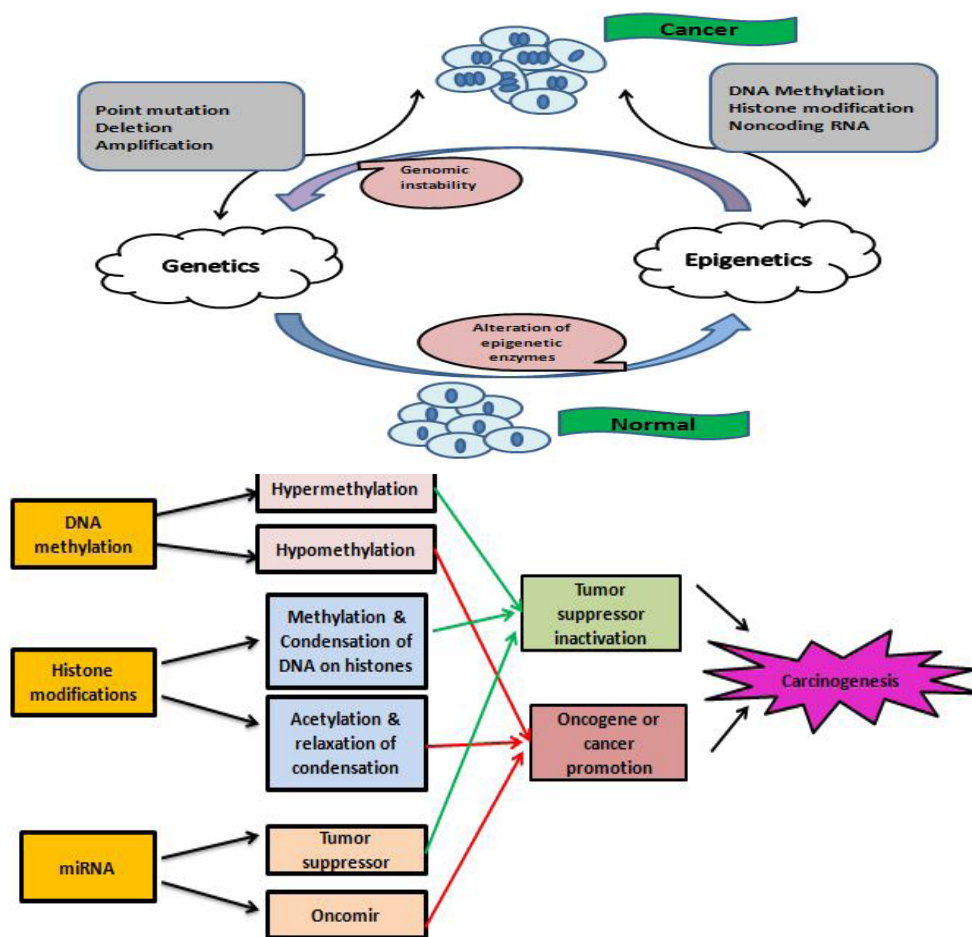
Figure 5: Epigenetic alternations that contributes to carcinogenesis

Table 1: Summarized outline of the epigenetic changes and possible mechanisms by which they promote tumorigenesis

Epigenetic Alteraions	Mechanism	Consequences
DNA hypermethylation	*De novo* hypermethylation at promoter CpG islands leads to silencing of tumor suppressor genes and cancer –associated genes	Genomic and chromosomal instability, growth advantage , increased proliferation
DNA hypomethylation	Activation of cellular oncogenes Activation of transposable element	Increased proliferation, growth advantage, Genomic instability, transcriptional noise
Loss of imprinting (LOI)	Reactivation of silent alleles, biallelic expression of imprinted genes	Expansion of precursor cell population
Relaxation of X –chromosome inactivation	Mechanisms is still unknown, but appears to be age related	Altered gene dosage, growth advantage
Histone acetylation	Gain -of - function Loss-of - function	Activation of tumor promoting genes Defects in DNA repair and checkpoints
Histone deacetylation	Silencing of tumor suppressor genes	Genomic instability, increased proliferation
Histone methylation	Loss of heritable patterns of gene expression (cellular memory)	Genomic instability, growth advantage

4.1 DNA methylation in cancer

Cytosine methylation is the most extensively studied epigenetic modification in humans, which primarily occurs by the covalent modification of cytosine bases in the CpG dinucleotide. These CpG dinucleotides are not evenly distributed across the human genome, but tend to cluster in short stretches called "CpG islands" [7] which is defined as regions of more than 200 bases with a G+ C content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6 as well as regions of large repetitive sequences (e.g. centromeric repeats, retrotransposon elements, rDNA etc.) [41,42].In mammalian genomes, CpG dinucleotides are usually quite rare (~1%). CpG islands occupy about (~60%) at the promoter of human genes, which are normally unmethylated, thereby allowing transcription. However, during early development or in differentiated tissues some of them (~6%) become methylated in a tissue-specific manner [43].

CpG-island methylation is associated with gene silencing and transcription regulation. Aberrant hypermethylation leads to transcriptional inactivation [44]. DNA methylation plays a key role in X chromosome inactivation, imprinting, embryonic development, silencing of repetitive elements and germ cell-specific genes, differentiation, and maintenance of pluripotency [45-47]. DNA methylation is vital for the regulation of non-CpG islands, CpG island promoters, and repetitive sequences to maintain genome stability [44,45]. Repetitive sequences appear to be hypermethylated which prevents chromosomal instability, translocations and gene disruption by the reactivation of endoparasitic sequences [48]. The DNA methylation at CpG island shores, which are located up to 2 kb upstream of the CpG island, is closely associated with transcriptional inactivation. Most of the tissue-specific DNA methylation seems to occur at CpG island shores and are conserved between human and mouse [49,50].

DNA methylation regulates gene silencing by different mechanisms. Methylated DNA can promote the recruitment of methyl-CpG-binding domain (MBD) proteins, such as MeCP2, MBD1, MBD2, and MBD4, which in turn recruit histone modifying and chromatin-remodeling complexes to the methylated sites, leading to transcriptional repression [48,51,52] or by precluding the recruitment of DNA binding proteins from their target site (e.g.,c-myc and MLTF) ,which directly inhibits transcription [53]. Long-term repression of active genes through DNA methylation is performed by DNA methyltransferases (DNMTs). However an active gene with unmethylated CpG islands generates an open chromatin structure favorable for gene expression by the recruitment of Cfp1 and its association with histone methyltransferases Setd1, thereby creating domains enriched with histone marks such as acetylation and H3K4 trimethylation [54].

Figure 6: Methylation of cytosine

DNA methylation is mediated by enzymes DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group from *S*-adenosyl methionine to DNA. **(Fig. 6)** Though, five members of the DNMT family have been reported in mammals: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L, only DNMT1, DNMT3a and DNMT3b possess methyltransferase activity. The maintenance DNMT, DNMT1 has a 30- to 40-fold preference for hemimethylated DNA and is the most abundant DNMT in the cell, transcribed mostly during the S phase of the cell cycle. DNMT1 also has *de novo* DNMT activity and is responsible for post- replicative methylation i.e., to methylate hemimethylated sites generated during semi-conservative DNA replication. The *de novo* DNMTs (DNMT3A and DNMT3B), highly expressed in embryonic stem (ES) cells and downregulated in differentiated cells are responsible for establishing the pattern of methylation during embryonic development [55-57](Fig. 7)

Figure 7: Establishment and propagation of methylation patterns. Cellular DNA methylation patterns seem to be established by a complex interplay of at least three independent DNA methyltransferases: *de novo* (by DNA methyltransferases DNMT3A and DNMT3B) and maintained (by DNMT1).

Epigenetic dysregulation in malignant cells is characterized by global hypomethylation and focal hypermethylation. During tumor initiation and progression , the epigenome undergoes

massive global loss of DNA methylation (20–60% less overall 5-methyl-cytosine) and acquisition of specific patterns of hypermethylation at the CpG islands of certain promoters resulting in their transcriptional inactivation [5,58]. In normal cell, CpG island-containing gene promoters are usually unmethylated, thereby maintaining euchromatic structure, which is the transcriptional active conformation allowing gene expression. However, during cancer development, DNA hypermethylation of several tumor suppressor genes at their CpG island-containing promoters has been shown to result in their abnormal silencing by changing open euchromatic structure to compact heterochromatic structure. DNA methylation mediated epigenetic silencing results in gene inactivation and promotes carcinogenesis, thus signifying that DNA methylation impinges on carcinogenesis [59].

Thus, DNA methylation plays a vital role in promoting tumorogenesis by local hypermethylation associated with the promoter of tumor suppressor genes resulting in their silencing and in parallel by global hypomethylation triggering the reactivation of several cellular protooncogenes. (Table 1).

4.2 Hypermethylation in cancer

DNA methylation is the first epigenetic alterations which were identified in cancer. Aberrant DNA methylation is deeply associated with cancer initiation and progression. The cancer epigenome typically reflects genome-wide hypomethylation and site-specific CpG island promoter hypermethylation [60,61] The underlying mechanism for these global changes initiation is still under investigation. However, recent studies have shown that some changes occur very early in cancer development.

Hypermethylation, typically observed at specific CpG islands, is a significant mechanism of tumor suppressor genes silencing that contributes to tumor initiation and progression [21], [62]. The transcriptional inactivation which is caused by promoter hypermethylation, typically affect various genes that are involved in the main cellular pathways such as DNA repair (hMLH1 , MGMT, WRN, BRCA1), Ras signaling (RASSFIA, NOREIA), cell cycle control (p16INK4a, p15INK4b, RB), apoptosis (TMS1, DAPK1, WIF-1, SFRP1) vitamin response (RARB2, CRBP1) p53 network (p14ARF, p73 (also known as TP73), HIC-1) metastasis (CDH1, CDH13, PCDH10) detoxification (GSTP1) [63,64] (Table 2). Several other tumor suppressor genes have also been reported to undergo tumor silencing by hypermethylation [48,65].

Furthermore, promoter DNA hypermethylation can indirectly inactivate additional classes of genes by silencing transcription factors and DNA repair genes. For instance, promoter hypermethylation-induced silencing of transcription factors, such as RUNX3 in esophageal cancer [66] and GATA-4 and GATA-5 in colorectal and gastric cancers [67] which further contributes to the inactivation of their downstream targets has been reported. Silencing of

DNA repair genes such as MLH1, BRCA1 facilitates cells to accumulate further genetic lesions resulting in the rapid progression of cancer. Thus promoter hypermethylation provides tumor cell with growth advantage, increase in their genetic instability and aggressiveness. It has been proposed that the hypermethylated promoters are associated with molecular, clinical and pathological features of cancer and can serve as potential biomarkers, holding great diagnostic and prognostic promise for clinicians [60].

Table 2: Epigenetically regulated genes in cancer

Cancer- associated Pathway	Gene
Cell cycle	RB, $p16^{INK4a}$, $p15^{INK4b}$, cyclin D2, cyclin E
Signal transduction	RASSF1, APC, ErbB2
Apoptosis	DAPK, Caspase-8 gene
DNA repair	MGMT, MLH1, BRAC1
Carcinogen metabolism	GSTP1
Hormonal response	Oestrogen receptor gene, retinoic acid receptor b2 (RAR-b2)
Senescence	TERT, TERC
Invasion/ metastasis	E-cadherin gene, VHL, TIMP-3
Transcription	Runx3, Twist, ER α, ER β, RAR, vitamin D receptor
Drug responsiveness	Gluthionefy S-transferase, thymidylate synthase
Angiogenesis	THBS1

Characterization of human cancer has been reported to be associated with an overall miRNA downregulation [68] as a result of hypermethylation at miRNA promoter [69]. Repression of miR-124a by hypermethylation mediates CDK6 activation and Rb phosphorylation [70]. Hypermethylation induced inactivation of miRNA expression is not only associated with cancer but also to metastasis development. For example, promoter hypermethylation induced silencing of miR-148, miR-34b/c and miR-9 facilitates tumor metastasis [71].

4.3 Hypomethylation in cancer

Global DNA hypomethylation which can occur at various genomic sequences including repetitive elements, retrotransposons, CpG poor promoters, introns and gene deserts, plays a significant role in tumorigenesis [72]. Furthermore, the DNA hypomethylation at repetitive sequences promotes chromosomal instability, translocations, gene disruption and reactivation of endoparasitic sequences [73,74]. Genomic instability established as an outcome of DNA hypomethylation in cancer cells are primarily caused by the loss of methylation from repetitive regions and are characterized as a hallmark of tumor cells. For example, the LINE family member L1, has been reported to be hypomethylated in a wide range of cancers, including breast, lung, bladder and liver tumors.

The association of hypomethylation with oncogenes has been reported in cancers. A striking example is served by c-Myc, a transcription factor that acts as an oncogene. In cancers, it has been widely reported as hypomethylated genes [65]. Hypomethylation at specific promoters activates the aberrant expression of oncogenes and induces loss of imprinting (LOI) in some loci. MASPIN (also known as SERPINB5), a tumor suppressor gene hypermethylated in breast and prostate epithelial cells [75], has been reported to be hypomethylated in other tumor types. On account of hypomethylation, the expression of MASPIN increases with the degree of dedifferentiation of certain cancer cell types [76,77].

Other well-studied examples of hypomethylated genes in cancer include S100P (pancreatic cancer), S-100 (colon cancer) SNCG (breast and ovarian cancers) and melanoma-associated gene (MAGE) and dipeptidyl peptidase 6 (DPP6) (melanomas) [50,78]. (Table 3) The most common LOI event induced by hypomethylation is IGF2 (insulin-like growth factor 2) and has been widely reported in various tumor types such as breast, liver, lung and colon cancer [79]. LOI of IGF2 has been also linked with an increase risk of colorectal cancer [80]. Thus, DNA hypomethylation induced aberrant activation of genes and non coding regions contributes to cancer development and progression.

4.4 Histone modification in cancer

Nucleosome is the fundamental repeating unit of chromatin, which consists of 147-bp segment of DNA wrapped in 1.65 turns around the histone octomer of following core histone proteins: : H2A, H2B, H3, and H4 and neighboring nucleosomes are separated by, on average, ~50 bp of free DNA. The core histones are predominately globular except for their amino-terminal tails that protrude from the nucleosome, which are less structured [81]. All histones are subject to post- transcriptional modifications. Several posttranscriptional modifications that histone tail domain is subjected to includes: acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADPribosylation [40,82].(Fig. 8)
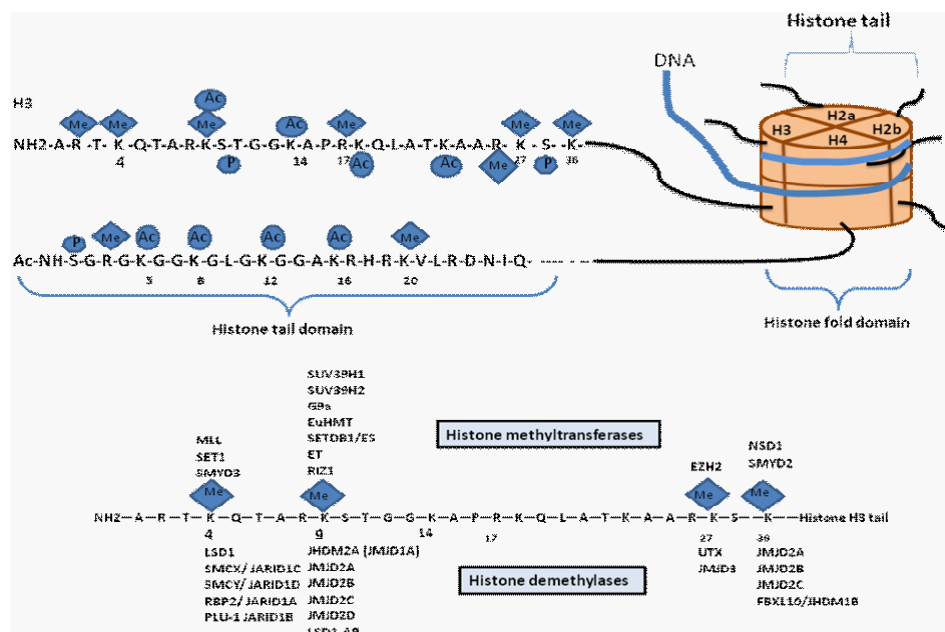
Figure 8: Schematic representation of Histone modifications and modifiers

The complexity of post translation modifications is attributed to histone modifying enzymes which can either activate or repress transcription, on the basis of the type of chemical modification and its location in the histone protein [83]. Recruitment of activating or repressive complexes to DNA can reshape chromatin into relaxed or a tightly packed structure on the basis of the modification pattern of histone and is associated with gene function during development as well as tumorigenesis.(Table1) With respect to its transcriptional state, the human genome can be roughly divided into euchromatin and heterochromatin. Actively transcribed euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79 whereas transcriptionally inactive heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation.(Fig. 9) Recent studies have revealed that histone modification levels are predictive for gene expression [84].
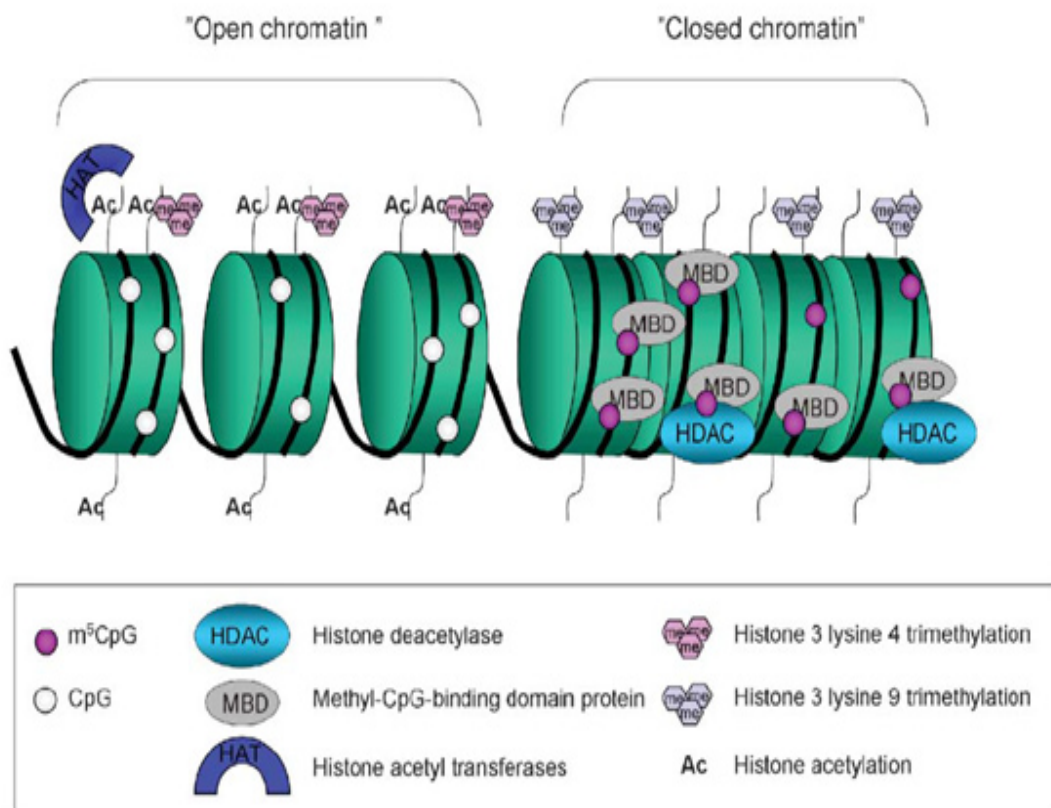
Figure 9: Chromatin structure of active and inactive promoters. (Adapted from: K. GrøNbaek et.al. 2007 [12].)

Post translational modifications patterns dynamically regulated by enzymes which either catalyze or remove the covalent modifications to histone proteins, have been described [85,86]. Histone modifying enzymes such as Methyltransferases, histone demethylases and kinases have been reported to be the most specific to individual histone subunits and residues [8].On contrary, most of the histone acetyltransferases (HATs) and histone deacetylases (HDACs) modify more than one residue, so are not highly specific.

Many transcriptional co-activators (e.g., GCN5, PCAF, CBP, p300, Tip60 and MOF) with intrinsic HAT activity as well as several transcriptional co-repressor complexes (e.g., mSin3a, NCoR/SMRT and Mi-2/NuRD) with HDAC activity have been reported to play important part in chromatin remodeling and gene transcription [87]. It has been reported that phosphorylated RNA polymerase II targets both HDACs and HATs to transcribed regions of active genes, where most HDACs function to reset chromatin by removing acetylation at active genes inhibiting transcription. On other hand HATs are mainly associated with transcriptional activation [27]. It is now evident that the interaction between these histone-modifying enzymes as well as other DNA regulatory mechanisms is essential to tightly link chromatin state and gene transcription.

Histone modifications play important roles in various cellular processes such as transcriptional regulation, DNA repair [88], DNA replication, alternative splicing [89] and chromosome condensation [81]; however their deregulation is implicated in human malignancies [90,91].

In various cancers, the global reduction of monoacetylated H4K16 has been reported as the most prominent alternations in histone modification [92]. HDACs are found to be overexpressed or mutated in different cancer, mediate the loss of acetylation [93]. The Sirtuin family of proteins is the main class of HDACs which are involved in this process. Upregulation in gene expression and deacetylase activity of SirT1 is observed in various cancers. Interaction of SirT1 with DNMT1 affects DNA methylation patterns [94]. The expression of HDAC is also regulated by miRNAs, such as *miR-449a,* induces growth arrest in prostate cancer cells by repressing in the expression of HDAC-1 [95]. Additionally, mutations or deletions as well as translocations in HATs and HAT-related genes has been observed in several cancer such as colon, uterus, lung and leukemia, which contributes to the global imbalance of histone acetylation [96].

Additionally, a global loss of active mark H3K4me3 and repressive mark H4K20me3 as well as a gain in the repressive marks H3K9me and H3K27me3 has been described during carcinogenesis [97]. Aberrant expression of histone methyltransferases and histone demethylases results in altered distribution of histone methyl marks in cancer cells. **(Table 3)** Inactivation of histone modifying genes - histone methyltransferase SETD2 and histone demethylases UTX and JARID1C has been revealed in renal carcinomas [98]. The histone methyltransferase EZH2, overexpressed in various cancers, is a subunit of PRC2/3 complexes which enhances proliferation and malignant transformation [39]. In breast cancer, overexpression of the lincRNA *HOTAIR* reprograms chromatin state to promote cancer metastasis [99]. Histone methyltransferases such as NSD1 undergoes promoter DNA methylation dependent silencing in neuroblastomas [100], while DOT1L, essential for the establishment of euchromatic state allows the expression of tumor suppressor genes [101]. Upregulation of several histone

demethylases such as GASC1, LSD1, JmjC and UTRX have been reported in prostate cancer and squamous cell carcinomas [102]. Histone phosphorylations are key players in DNA damage – repair response, chromosomal stability and apoptosis. JAK2, a nonreceptor tyrosine kinase phosphorylate H3Y41, which in turn prevents the binding of heterochromatin protein1α (HP1α) to this region of H3 resulting in an increase in the expression of the genes located there. In hematological malignancies, chromosomal translocations or point mutations are responsible for JAK2 activation [103].

Table 3: Consequences of DNA methylation and histone modifications in cancer

Aberrant epigenetic modification		Consequences	Genes affected and resulting disease
Cancer			
DNA methylation	CpG island hypermethylation	Transcription repression	MLH1 (colon, endometrium) BRAC1 (breast, ovary), MGMT (several tumor types) p16^{INK4a} (colon) [55]
	CpG island hypomethylation	Transcription activation	MASPIN (pancreas), S100P (pancreas), MAGE (melanomas) [104]
	CpG island shore hypermethylation	Transcription repression	HOXA2 (colon), GATA2 (colon) [49]
	Repetitive sequence hypomethylation	Transposition, recombination genomic instability	L1 [55], IAP[55], SAT2[92]
Histone modification	Loss of H3 and H4 acetylation	Transcription repression	CDKN1A[55]
	Loss of H3K4me3	Transcription repression	Hox genes
	Loss of H4K20me3	Loss of heterochromatic structure	Sat2, D4Z4[92]
	Gain of H3K9me and H3K27me3	Transcription repression	CDKN2A, RASSF1[39], [105]

5. DNA methylation as a marker for tumor diagnosis and prognosis

The most well defined epigenetic change in tumors is the aberrant DNA hypermethylation in the promoter regions of genes which is associated with inappropriate gene silencing. This feature can be utilized to explore tumor- specific DNA methylation biomarkers as well as in examining potential candidate DNA biomarker for clinical use as diagnostic, prognostic, or predictive marker [1,106]. DNA methylation biomarkers are molecular target that undergo DNA methylation changes during carcinogenesis. Such a biomarker is essential for early diagnosis of cancer, detection of recurrence as well as for predicting and monitoring therapeutic responses.

DNA methylation biomarkers offer several advantages over genetic and serum markers [107] such as higher incidences of aberrant DNA methylation of specific CGIs, their selective detection in cancer cells, even when it is embedded in substantial amount of contaminating normal DNA, technically simple detection (for instance., can be detected using MSP) and their occurrence at early stage of tumor development, causing gain or loss of function of key processes implicate its potential as early indicator of existing cancer and for evaluation of risk assessment for future development of cancer [108]. Though DNA methylation biomarker has several advantages over genetic markers, it has been reported that combination of the two might serve better outcome. For instance, combination of both markers in stool DNA facilitated the detection of curable stage colorectal cancer and large adenomas with higher accuracy [109].

Moreover, DNA methylation has been recognized as a potential ideal biomarker (diagnostic/ prognostic) due to its methylation stability, amplification ability, high sensitivity, the possibility of localization to a specific gene region, relatively low cost and potential of development as a high-throughput screening method specific for cancer detection [107,110, 111]. Furthermore, the diagnostic and prognostic use of DNA methylation has been reported in various types of cancer, particularly in glioma [7].

A large number of potential DNA methylation marker genes and their role in carcinogenesis have rapidly increased due to the development of recent genome wide techniques for their identification and functional analyses [7,112]. The detection of methylation signatures in virtually any body fluid such as serum/plasma, smears, nipple fluid aspirate and vaginal fluid, among others has been highlighted in numerous reports [113,114]. As blood samples which can be obtained through minimal invasive procedure, serves as ideal substrate for DNA methylation analysis. On other hand, analysing DNA methylation in body fluids remains challenging because of relatively low mount of cell free DNA (cfDNA) compared with cell- derived DNA and for the fact that cfDNA is highly fragmented. DNA methylation markers which are detected in urine or sputum are site directed; however those markers which are detected in serum, plasma or saliva can originate from anywhere in the body. So the methylation markers identified in these substrate should hold specificity for a particular disease or small group of disease thereby enhancing their diagnostic utility [115].

Regarding the clinical implementation of DNA methylation biomarkers, we briefly discuss the established markers as well as the current methylation marker validation studies. Currently, several ongoing studies have focused on testing the utility and clinical implementations of DNA methylation biomarkers as early diagnostic biomarker and disease progression and predictive biomarkers in various malignancies [116,117].

For the early detection of lung, colon and prostate cancer, DNA methylation marker based kits are already available in market. Methylation of septin 9 (SEPT9) and vimentin

(VIM) is used for early detection of colon cancer by analyzing blood (SEPT9) or stool (VIM) samples of patients [118,119]. Improved sensitivity and specificity was exhibited by both markers upon comparison with the fecal occult blood test. Similarly, methylation of SHOX2 is used as a biomarker for distinguishing malignant and benign lung diseases.A sensitivity of 78% and a specificity of 96% have been reported when SHOX2 methylation was analyzed in bronchial aspirates [120]. The methylation of TWIST-1 and NID-2 along with other biomarkers is used to detect bladder cancer [121]. Methylation of Vimentin and NID-2 is associated with assessment of recurrence of bladder cancer [122]. MGMT gene encodes a DNA repair protein, O6-methylguanine DNA methyltransferases. Its methylation has been reported to be associated with survival benefit of glioblastoma patients after treatment with the temozolomide, which is an alkylating drug thus highlighting its predictive potential in clinical settings [123,124]. **(Table 4)**

Table 4: Commercially available tests based on DNA methylation biomarkers

Biomarker	Application	Disease	Material	Sensitivity/ Specificity (%)	Commercial test	References
SEPT9 + VIM	Early detection	Colorectal Cancer	Blood	80-82/ 89-99	EpiproColon® 2.0 (Epigenomics), ColoVant age™ (Quest Diagnostics), Real-Time mS9 (Abbott)	deVos et al. (2009)
SHOX2	Early detection	Lung Cancer	Sputum	81/95	EpiproLung® BL 1.0 (Epigenomics)	Kneip et al. (2011)
MGMT	Predictive	Brain Cancer	Tumor	-	PredictMDx™ Brain Cancer (MDxHealth)	Hegi et al. (2005)
TWIST2 + NID2	Predictive	Bladder Cancer	Urine	87.9/99.9	CertNDx™ Bladder Cancer Assay Hematuria Assessment (Predictive Biosciences)	Renard et al. (2010)
VIM + NID2	**VIM + NID2**	Bladder cancer	Urine	90.5/95.5	CertNDxTM Bladder Cancer Assay Hematuria Assessment (Predictive Biosciences	Reinert et al. (2012)

6. Prospective on Epigenetic therapy

The reversible nature of gene silencing by epigenetic modifications has facilitated the emergence of the promising field of epigenetic therapy as a treatment option. The aim of epigenetic therapy is to restore gene function which is silenced by epigenetic changes during tumorigenesis. The three critical components of epigenetic regulation which have been targeted for development of epigenetic therapies for cancer prevention and treatment include: DNA methylation, post-translational histone and protein modification (e.g., acetylation, methylation)

and more recently, post-transcriptional gene regulation by miR [125,126].

Many epigenetics drugs which can effectively reverse DNA methylation and histone modification alternations have been discovered in the recent past. Currently, several agents that target DNA methylation (DNMT inhibitors) and protein acetylation (histone deacetylase inhibitors [HDACIs]) are in clinical development (ClinicalTrials.gov; www.clinicaltrials.gov.) So far, three epigenetic drugs have been approved by The US FDA which includes: decitabine and Vidaza® for myelodysplastic syndromes [127] and vorinostat for cutaneous T-cell lymphoma [128,129]**(Table 5).**As such no compound that specifically targets miR activity is in clinical development, however chromatin modifying agents hold the potential to re- activate miR expression thereby resulting in target protein modulation [130].

Table 5: Examples of approved agents in epigenetic therapy for cancer management

Agent	Class	Disease indications	FDA approval data	Main study institution	Number of patients	Basis of approval
5-azacitidine	DNMT inhibitor	Myelodysplastic syndrome	2004	Memorial Sloan-Kettering; Mount Sinai	191	Phase III trial; 23% response rate; significantly improved median survival compared to supportive care (18 months vs 11 months)
Decitabine	DNMT inhibitor	Myelodysplastic syndrome	2006	MD Anderson	170	Phase III trial; 17% response rate; trend toward improved median survival compared to supportive care (12 months vs 8 months)
Vorinostat	HDAC inhibitor	Cutaneous T-cell lymphoma	2006	Duke	74	Phase IIB trial; 30% response rate; median time to progression was 5 months
Romidepsin	HDAC inhibitor	Cutaneous T-cell lymphoma	2009	National Institute of Health; King's College London	167 (96+ 71)	Phase II trial; 34% - 38% response rate; median response duration was 11 -15 months

DNA demethylating compounds are the first epigenetic drugs approved for use as cancer therapeutics, can be categorized into two distinct mechanistic groups: "**nucleoside analogs**" that are incorporated into the DNA of rapidly growing tumor cells during replication, covalently bind and trap the DNA methyltransferases (DNMTs) blocking their activity, followed by their proteosomal degradation(e.g. Vidaza (5-azacytidine)) [131]and the "**non-nucleoside inhibitors**" which effectively inhibit DNA methylation without being incorporated into the DNA (e.g. quinolone-based small molecule, SGI-1027) [132].

These DNMT inhibitors tend to induce the de-repression of hypermethylation-induced gene silencing thereby reactivating tumor suppressors and other cancer related genes[133]. They have been also demonstrated to reverse resistance to chemotherapy in vivo [134].

The most clinically advanced nucleoside DNMT inhibitors are the azanucleoside prodrugs, decitabine (5-aza-2-deoxycytidine) and Vidaza (5-azacytidine). Originally being developed as cytotoxic agents, these compounds were subsequently reported to have demethylating properties at lower concentration [135]. Their mode of action is yet not well defined. In addition, they are chemically unstable [136]. Cytidine deaminase metabolizes Vidaza and decitabine to inactive forms [135]. SGI-110, a novel DNMT inhibitor is protected from enzymatic degradation by Cytidine deaminase is progressing through preclinical trials [137]. 5-fluoro-2-deoxycytidine (5-FC) is the most recent agent of this class to enter clinical trial [138]. However, there are drawbacks such as the chemical instability and S-phase specificity has resulted in poor efficiency against cancer stem cells and tumors with low proliferation index, thereby limiting the clinical application of nucleoside DNMT inhibitors. The formation of bulky DNA adducts results in cytotoxicity, which is dose limiting and is manifested as bone marrow suppression and neutropenia [135], [139].

In contrast non-nucleoside DNMT inhibitors are less toxic and potentially more chemically stable [140]. MG98 is an antisense oligonucleotide to DNMT1, with antitumor activity and has completed phase I trials [141]. Quinolone based small molecules such as SGI-1027 and RG108, are inhibitor of DNMT1 which do not bind to DNA or RNA. Being comparatively less Cytotoxic, they might serve as promising clinical candidate [142].

Treatment with HDAC inhibitors, in order to re-establish normal histone acetylation patterns, has been reported to exhibit antitumorigenic effects which are mediated by their ability to reactivate silenced tumor suppressor genes [143]. HDAC inhibitor, such as Suberoylanilide hydroxamic acid (SAHA) has been clinically approved for T cell cutaneous lymphoma treatment. Furthermore, other HDAC inhibitors for instance, depsipeptide and phenylbutyrate are under clinical trials [144].

Recently various combinatorial cancer treatment strategies that involves both DNA methylation and HDAC inhibitors together has been explored and have proved out to be more effective than the individual treatment approaches. Combined treatment with 5-Aza-CdR and trichostatin A exhibited the de-repression of certain putative tumors suppressor genes [145]. Enhanced antitumorigenic effects of depsipeptide were observed upon simultaneous treatment of leukemic cells with 5-Aza-CdR [146]. Combined treatment with phenylbutyrate and 5-Aza-CdR demonstrated greater reduction of lung tumor formation in mice, thus implicating the synergistic activities of DNA methylation and HDAC inhibitors [147].

Recently, the role of HMT inhibitors has also been explored. DZNep, a HMT inhibitor

has been reported to induce apoptosis in cancer cells, preferentially targeting PRC2 proteins, generally overexpressed during carcinogenesis [148]. However, its specificity still remains contradictory [149]. Further development of specific HMT inhibitors is critically needed.

For epigenetic therapy, miRNAs may also serve as promising targets. It was demonstrated that the treatment with 5-Aza-CdR and 4-phenylbutyric acid downregulates the oncogene BCL6 via the reactivation of miR-127, which strongly highlightsthe potential of a miRNA-based treatment strategy [69]. Synthetic miRNAs that mimic tumor suppressor miRNAs can also be used to selectively repress oncogenes [150]. For the targeted delivery of synthetic miRNAs to tumor cells, development of efficient vehicle molecules is highly essential.

The development of several drugs which can potentially modulate the epigenome to restart transcription of epigenetically silenced genes, thereby augmenting the action of conventional cancer treatment methods, offers an entirely new approach to cancer therapy. On the same note, better understanding of the pharmacokinetics of epigenetic drugs is critically required to identify clinically beneficial properties as well as to develop newer and more efficacious treatments.

7. Key Highlights

- Epigenetic machineries are essential for normal mammalian development and regulation of gene expression.

- Hypermethylation of CpG islands is known to be common event during carcinogenesis.

- Aberrant promoter methylation leads to epigenetic gene silencing leading to loss of gene function in cancer.

- Hypermethylation of tumor suppressor genes is associated with their transcriptional silencing thereby contributing to oncogenesis.

- Methylation analysis of CF-DNA in preferentially any body fluid serves a novel approach for non invasive cancer detection

- Epigenetic drugs targeting the epigenome to induce functional re- expression of aberrantly silenced genes, offers new approach to cancer therapy.

8. Conclusion

An unanticipated progress in unrevealing the molecular mechanisms associated with the epigenetic regulation of normal development and its far implication in treatment of human diseases has been explored over the past 20 years. The deregulation of epigenetic mechanisms

which is responsible for tumorogenesis also augments the effect of oncogenic mutations. Targeting early tumor development and its progression serves as a logical therapeutic approach for the management of aberrant epigenetic alternations. Therefore, epigenetic alternations which are associated with the onset and progression of cancer, serves as potential clinically useful targets. Extensive research testing the utility and clinical implementation of DNA methylation based markers for early detection, diagnosis, prognosis or prediction of cancer cases is in progress. However, DNA methylation marker kits for the early detection of various cancers (such as lung, colon and prostate cancer) are already commercialized. Exploration of the molecular events that initiates and maintains epigenetic gene silencing has facilitated the discovery of epigenetic drugs targeting the epigenome, including DNA methylation and histone modifications. Several epigenetic agents have mapped their way in clinical utility upon approval by US Food and Drug Administration (FDA). The future will see the utility and success of combination of epigenetic drugs along with other therapy for the management of cancer significantly and with efficacy.

9. References

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