Chapter 3

DNA Methylation in Mammalian Cells

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Abstract

Epigenetic regulation was first studied in the 1970s and has quickly gained global interest. It is involved at many different stages of mammalian cell development. The broad nature of epigenetic regulation has led to it being referred to as an 'all stage' mechanism of regulation as it is implicated in many developmental stages including embryonic development, ageing and in cancer progression. The term 'epigenetic' refers to the alteration of gene expression without changing the genomic sequence. Epigenetic regulation involves the main subtypes of DNA methylation and histone modification, and microRNA expression. Epigenetic alteration is used by mammalian cells to 'turn-on and -off' the gene expression. During embryonic development this process is used to induce cell apoptosis of genes that are no longer useful. During cancer development, epigenetics works to repress tumour suppressor gene expression and activate oncogene expression. The stability and sustainability for the detection of epigenetic markers make it an attractive area for biomarker and drug discovery. In this book chapter we will discuss the key epigenetic processes involved in mammalian cell development and disease progression, specifically in cancer.

Keywords: epigenetic, DNA methylation, histone modification, microRNA, tumour suppressors, cell development

1. Introduction

1.1. Types of epigenetic regulation

The term 'epigenetics' describes the stable modifications of gene expression that occur at different stages of cell development and proliferation [1]. Epigenetic modifications are an essential component for human development and cell differentiation, but can also come about

through random changes and external environmental influences. Different genetic makeups produce different individuals, while the differentiation of organs and various diseases is controlled by epigenetic signatures (**Figure 1**). Epigenetic modification can also be a mechanisms used to protect bacterial or viral modification in the host cell that affect cellular function. There are a number of epigenetic alterations that are required for cellular functions including: DNA methylation and histone modification, and microRNA expression. Epigenetic alterations influence cellular function by altering the protein levels without changing the genomic DNA sequences. These post translational modifications are recognised by specific proteins that are closely associated with DNA as a mediator that causes modifications [2]. The DNA methylation process was first discovered in the 1970s and was later recognised as a major contributor to the stabilisation of gene expression [3].

1.1.1. DNA methylation and histone modification

DNA methylation is an essential process for the normal development of biological systems [4] and its dysregulation has been associated with various pathologies including cancer [5]. Although DNA methylation is known to be integral to the development and is strongly associated with disease, there is limited knowledge on the specifics of its changes during cellular differentiation and its relation to histone methylation and chromatin modifications.

DNA methylation can alter the genome to supress gene expression in mammalian cells. It occurs by the methylation of deocycytosine (dC) bases at the 5′prime position of the cytosine to create deoxymethylcytosine (d^mC). The majority of d^mC are located in CG dinucleotides with approximately 80% of CG pairs being methylated. The majority of these CG pairs are localised to CG-rich DNA regions termed CpG islands. An abundance of transcription factor binding sites exist at CpG islands, which exist in the promoter regions for some genes. CpG islands are present in the promoter regions of approximately half of all mammalian genes [6]. DNA methylation occurs at CG dinucleotides termed CpGs that are distributed globally in 80% of genomes in non-embryonic cells. CpGs sites located at short CpG regions generally remain unmethylated at all times [7, 8]. The majority of CpG islands reside in the promoter

Figure 1. Top panel depicts various species, each with a unique genomic signature. Bottom, within the same individual the differentiation of cell function is controlled by a cell specific epigenetic signature.

region of a gene [9] and are also known to be associated with the transcriptional activity of Sp1 [10]. When CpG islands become methylated during mammalian cell development this can lead to long-term silencing of the associated gene [11].

DNA methyltransferases (DNMT) are a family of enzymes consisting of members DNMT1, DNMT3a and DNMT3b that mediate DNA methylation. These members have different and integral roles to execute DNA methylation; DNMT1 is involved in the maintenance of methylation patterns while DNMT3a and DNMT3b are responsible for *de novo* methylation, that is, methylating DNA that is previously unmethylated [12]. DNA methylation signatures are developed during cell differentiation for the purpose of supressing genes with functions that are unnecessary to the mature cell. This *de novo* methylation is carried out by DNMT3a and DNMT3b [13]. After differentiation, DNMT1 replicates these methylation patterns in the process of mitosis [12]. Unstimulated cells express small amounts of DNMT1, but following mitogenic stimulation the enzyme is upregulated by a sequence of pathways including MAPK/ERK and JNK [14]. DNMT1 is then able to maintain methylation patterns in the DNA by producing symmetrically methylated sites in parent and daughter DNA strands. It accomplishes this by recognising hemi-methylated CG dinucleotides in parent DNA strands and transferring methyl groups from S-adenosylmethione (SAM) to cytosine regions of the unmethylated daughter strand [12]. Alternatively, *de novo* methylation rarely occurs during this normal post-gastrulation development stage and is instead prevalent during processes such as establishment of cell lines *in vitro* [15] and in cancer cells.

DNA methylation is an important process during development and researchers have shown the deletion of *de novo* methylation enzyme DNMT1 in mouse embryonic stem cells led to dramatic DNA hypomethylation [16]. DNMT3a and DNMT3b predominantly exert enzyme activity during the oocyte stage and in early pre-implantation embryos [12]. DNMT3b is also transcribed during zygotic gene activation (ZGA) and highly expressed by blastocysts that acquire epiblast lineage. The absence (by deletion) of DNMT3b led to embryonic lethality and the deletion of DNMT3a was partially viable during development [13]. There is a lower requirement for DNMT3L in DNA methylation which is used predominantly during imprinting control region (ICR) methylation in gametes [17]. However, it is a crucial activating cofactor for DNMT3a [18]. DNMT2 differs structurally from other DNMT and does not present with phenotype modification in knockout mice models which are referred to as a misnomer and depicts methylation activity on RNA [19].

DNA methylation is predominantly found in cytosines of the CG dinucleotide in mammalian cells; this modification is post-replicable. The extent of DNA methylation changes in an orchestrated way during mammalian development, starting with a wave of demethylation during cleavage, followed by genome-wide *de novo* methylation after implantation [20]. Demethylation is an active process that strips the male genome off methylation within hours of fertilisation [21]; by contrast, the maternal genome is only passively demethylated during subsequent cleavage divisions [22]. The extent of methylation in the genome of the gastrulating embryo is high owing to *de novo* methylation, but it tends to decrease in specific tissues during differentiation [17]. *De novo* methylation occurs rarely during normal post-gastrulation development but is seen frequently during the establishment of cell lines *in vitro* [15] and in cancer.

A variety of chromatin modifications can halt the initiation of transposable element (TE) transcription in mammalian cells; modifications of histone tails, chromatin packaging alterations, DNA methylation and condensation are all examples of this. Histone amino (N)-terminal tails modification causes changes to protein factor binding and in turn relays information to transcription factors. DNA methylation of histone H3 at lysine 9 (H3K9) occurs in nucleosomes, that are associated with TE's, leading to transcriptional repression and inactivation of chromatin [23]. Sometimes, mutations can occur in genes that are required for the repression of histone tail modifications, subsequently leading to TE reactivation. A specific example occurred in mouse embryonic stem (ES) cells where mutations of the histone H3K9 DNMT gene suppressor of variegation 3–9 (Su(var)3–9) drove the upregulation of TE transcripts [23].

It is widely understood that histones, specifically H3 and H4, are methylated at lysine (Lys) and arginine (Arg) sites. The predominant regions for Lys-specific methylation on histones that have been catalogued in literature are: Lys9, Lys4, Lys36, Lys27, Lys79 on H3 and Ls20 located on H4 [24]. Additionally, Lys site methylation can occur by mono-, di-, or tri-methylation. The differential manners of Lys residue methylation dictate the variety of functional consequences of Lys methylation.

The pioneering study that uncovered the functions of H3 Lys-methylation determined that one of the well-understood Su(var) genes encodes a histone methyltransferase (HMT). The *Drosophlia* SU(Var)3–9 gene was discovered to have roles in transcriptional silencing associated with heterochromatin [25]. The human homologue of this gene, Suv39H1, underwent biochemical analysis and its protein was found to methylate histone H3 at Lys9 using its enzymatic functions [26]. Expanding on this, specific antibodies for methylation of H3 at different sites revealed a pathway for heterochromatin formation [27]. An example of this occurs in *S. pombe*, where heterochromatin formation is initiated by the deacetylation of histone H3 at Lys9 by a histone deacetylase (HDAC) complex that allows the methylation of this site by histone-lysine N-methyltransferase (Clr4). The chromodomain of heterochromatin protein (HP1) can then recognise and bind to this methylated Lys9 motif. This *in vivo* study demonstrated that disruption to the Clr4 gene caused delocalisation of the HP1 homologue Swi6, depicting the requirement of H3 methylation for HP1 and heterochromatin assembly [27]. Genetics studies in *S. pombe* and *Tetrahymena* have illustrated that heterochromatin formation is dependent on genes that code for elements of the RNA interference (RNAi) machinery [28]. Additionally, small RNAs are thought to target histone-modifying activities at silenced regions [29].

Although the association of Lys4-methylated H3 with euchromatic regions is well established, its role in transcriptional activation is not completely understood. Lys4-methylated H3 was found to be directly bound to the yeast chromatin remodelling enzyme Isw1p [30]. Further supporting their association with transcriptional sites, Lys4 methylated H3 was shown to inhibit the binding of the HDAC complex NuRD (nucleosome remodelling and HDAC) to chromatin in a mammalian system [31]. Together, these findings suggest that Lys4-methylated H3 regions indirectly regulate transcription by maintaining promoter genomic regions in a state that favours transcriptional activation.

HDACs drive the deacetylation of acetyl-L-lysine side chains in histones to repress transcription by altering the conformation of chromatin [32]. To date, there are 18 known HDACs including: class I HDACs [33]; class IIa HDACs [34]; class IIb HDACs [35]; class III HDAC enzymes sirtuins [35]; and finally the only class IV enzyme HDAC11 [33]. To catalyse the

deacetylation of histone groups, HDACs together with HDAC related deacetylases, must switch a single metal ion at the metal ion binding site $Mn^{2+}B$ in arginase [36].

1.1.2. microRNA expression

Epigenetic mechanisms play a pivotal role in the regulation of gene expression that forms part of the large complex network that regulates the functioning of eukaryotic cells. MicroRNAs (miRNAs) are a class of small RNA molecules that post-transcriptionally repress gene expression through interaction with the three prime untranslated region/s (3′-UTR) of target messenger RNAs (mRNA)s [37]. miRNAs have a wide variety of functional roles in biological systems that have been extensively studied but the mechanisms controlling their expression are not well understood. In most cases, miRNA expression is initiated by transcription of the miRNA gene by RNA polymerase II. Genes transcribed by RNA polymerase II are frequently regulated by epigenetic mechanisms, so it is likely that DNA methylation regulates the expression of miRNAs. The notion of DNA methylation-based regulation of miRNAs is further supported by the tissue-specific or developmental-stage specific pattern of miRNA expression [38].

A specific study carried out to determine if DNA methylation can alter miRNA expression was carried out using HCT116 colon cancer cells with knockout of DNMT. This model illustrated that approximately 10% of miRNAs studied were regulated by DNA methylation. Furthermore, these miRNAs were shown to be tightly regulated by methylation as shown by a high level of CpG site demethylation required to induce their re-expression. Treatment with 5-aza-2′-deoxycytidine (AZA) or induction of partial demethylation was unsuccessful in upregulating these miRNAs. Collectively, these findings could be directly due to the demethylation of CpG islands in the miRNA promoter sites or be explained instead by the indirect epigenetic regulation of transcription factors acting on these miRNAs [39].

Many miRNAs with tumour suppressor functions have been shown to be silenced by hypermethylation in cancer [40]. miR-148a, miR-34b/c, miR-9-1, miR-9-2 and miR-9-3 were observed to have specific CpG island hyper-methylation associated silencing *in vitro* and *in vivo* in metastatic cancer cells. The metastatic carcinoma cell line SIHN-011B was hypermethylated leading to the repression of miR-148a and miR-34b/c. Transfection of SIHN-011B with expression vectors containing the flanking regions of mature miR-148 and miR-34b/c induced a reduction in migration ability compared to controls in wound-healing assays. Tumour and metastasis formation assays in nude mice depicted a reduction in tumour growth over time following miRNA transfection. These findings illustrate the tumour suppressor activity of these miRNAs, which were found to be explicitly downregulated by CpG island hypermethylation in miRNA promoter regions in these cell lines [41].

The epigenetic regulation of miRNAs is not solely limited to DNA methylation. Two separate studies showed no change in miRNA expression following AZA treatment in lung or bladder cancer cell lines. Combination treatments with a histone deacetylase inhibitor induced miRNA upregulation [42]. To study the mechanisms leading to miRNA regulation more closely a separate group compared the miRNA gene expression profile of a DNMT1 and DNMT3b double knockout cell line model to its associated parental cell line HCT116. Their results depicted notable alterations in miRNA expression in the double knockout model, strongly suggesting that DNA methylation significantly regulates gene expression [39].

2. Epigenetic regulations involvement mammalian cell function gene silencing during development

DNA methylation is the most prevalent form of epigenetic alterations, the most studied and therefore the remainder of this chapter will focus on this area. During mammalian cell development, DNA methylation is an essential component to turn genes 'on and off' [13], however this exact mechanism is still undefined. DNMTs cloning techniques [12] has led to the improved understanding of how DNA methylation proteins and methylation signals influence mammalian cells. The DNA binding protein located on cytosines on the 5′ position of the DNA sequence contributes to the major 'on and off' gene mechanism of mammalian cells. DNA methylation is a heritable trait through mammalian cell development, and these inherited changes of methylation status prompted researchers to develop techniques to identify different stages of cell development. DNA methylation has a multitude of roles in development, specifically methylation of CpG-rich promoter regions and is responsible for the inactivation of the X chromosome and to maintain its silencing. The roles of methylation do not stop with cellular development, the normal functioning of DNA can be affected by methylation and is responsible for the development of human diseases including carcinogenesis. Epigenetic alterations can occur in a mammalian system during different stages of development and can also be effected by external stimuli as shown in **Figure 2** that summarises the regulation of mammalian cells by various epigenetic networks.

Different DNMTs are essential during different stages of vertebrate development and will contribute to cell apoptosis in embryos and fibroblasts [43], but not in ES cells or cancer cells [44].

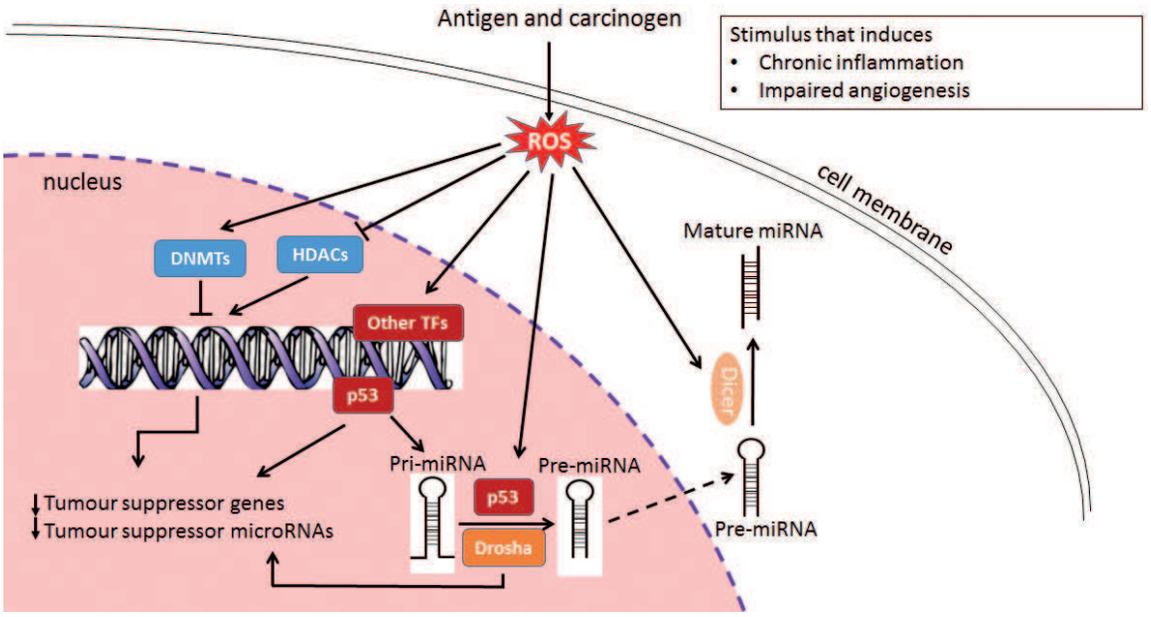

Figure 2. Mammalian cells react to external stimulus such as antigens and carcinogens. The generation of reactive oxygen species (ROS) leading to the alteration of DNA methyltransferase (DNMTs), histone deacetylase (HDACs) and microRNA (miRNA) expression/processing at different stages, will lead to cell apoptosis or epigenetic alterations which allow cells to progress to disease types. TF: transcription factor.

The dysregulated activity of DNMTs results in the repression or activation of gene expression [43] and transcriptional activation elements in diseased mammalian cells [45]. Additionally, aberrant deletion of DNMT1 during brain development has been known to lead to perinatal respiratory distress and malfunction of embryonic development [46]. DNA hypermethylation causes gene silencing via binding of DNMTs to the genome which interferes with the activity of transcription binding proteins that activate gene transcription [47].

2.1. DNA methylation and evaluation of mammalian cells

Genetic variation in individuals can influence phenotypic representation of age and lifespan [48]. However epigenetic and environmental factors also have significant roles in determining physiological changes [49]. This is particularly exemplified by the increasing inherent epigenetic variation present in monozygotic twins with age [50]. DNA methylation status is altered in multiple tissues and specific cell types with age, with the most frequent changes being age-dependent while others remain unchanged with age [51]. Different tissues have specific DNA methylation patterns that results in different cellular functions.

Genome-wide assays have provided a platform for discovering the effect of ageing during the phenotypic alteration in liver cells and a study found that the level of visceral fat is involved in cytosine methylation. This was the first investigation to show that ageing contributes to changes in DNA methylation which is locus-specific in both liver and adipose tissues. These changes appear to cause global hypermethylation in liver specific tissues in genetically identical rat models when exposed to the same environmental conditions throughout their life. This hypermethylated pattern is usually accompanied by hypomethylation at the paired locus. Epigenomic dysregulation has a stable but reversible effect on the genomic sequence. The stability and tissue specific potential of epigenetic marks highlights their potential as biomarkers for the heterogenetic pathophysiology encountered during ageing. In most cases, these epigenetic changes occur in genes that are involved in metabolism and metabolic dysregulation. Therefore, epi-genomic dysregulation is a primary mediator for the pathogenesis of age-related metabolic disease [52]. DNA methylation plays a major role during the ageing process that contributes vastly to loss of tissue homeostasis, the decline of normal cellular functions and the capacity for replication [53]. The accumulation of fat deposits with increasing age in the liver can reduce the capacity of the liver to regenerate and function [54] which may lead to diseases such as diabetes, dyslipidaemia and cardiovascular disease [55]. Ageing is a result of accumulating genomic damage over time that is accompanied by physiological decline [56].

DNMT1 is responsible for the maintenance of DNA methylation levels and also acts as a catalyst for methylation [57]. DNA methylation becomes more frequent when there is existing methylation which ensures the self-perpetuation of methylation status. This process results in *de novo* methylation extending from the already methylated regions to surrounding sequences during ageing and cancer progression, leading to an overall reduction in global methylation as methylation-specific enzymes are concentrated in these areas [58]. The direction and strength of the correlation between age and methylation is dependent on the CpG island status of loci. During ageing CpG island loci gain methylation and non-islanded CpGs become less methylated. Researchers have frequently shown that methylation status is increased within CpG

islands with age [59, 60]. Tra et al. and Bjornsson et al. showed bi-modal age-related methylation in aged tissues vs. normal tissues using microarray. A direct comparison of the top 50 most age-related and normal methylation genomes show an overall decreased methylation status in non-CpG island regions of normal samples [51]. They also indicated an inverse correlation trend in other tissue types, where they found that although methylation occurs at non-CpG island regions, there is a strong correlation between age and methylation at the CpG island regions [61].

Studies have indicated that age-related methylation is a common mechanism of dysregulation regardless of tissue specificity [62]. There is a reduction in the fidelity to maintain DNMTs activity with ageing and this potentially results in the age-related reduction of methylation [63]. On the other hand, age-related hypermethylation is a reflection of accumulating stochastic methylation events over time [64]. However, these methylated CpGs in mammalian cells may not have dramatic functional consequences due to the absence of pathologic phenotype differences [65]. The accumulation of alterations without functional consequences should not be considered biologically insignificant, because age-related alteration of the normal epigenome without changes in gene expression may confer a significantly increased risk to pathologic phenotypes and altered gene expression or genomic instability [64]. For example, the methylation of 'non-functional' CpG islands in promoter regions of an aged individual can continue to accumulate methylation events (methylation spreading) and increase the chance of methylation induced gene silencing in the future [64]. The increasing spread of methylation can also affect gene expression of distant loci via silencing of important genomic regions, such as enhancers resulting in progression to a diseased phenotype [66]. Fortunately, the aberrant CpG methylation causing gene silencing on a single allele can be compensated by the complementary allele that is not methylated. Thus, the clusters of mono-allelic gene expression will increase the risk of pathologic phenotype alteration, for example the loss of the 2nd functional allele. As a result, it is necessary to study the potential of quantification of age and/or environmental exposure that is associated with DNA methylation which can act as an indication of disease risk [67].

Other DNA methylation mechanisms are involved in cellular ageing and provide a large area of discovery, these include: endogenous hypomethylation and exogenous hypermethylation [68]. Age-dependent decreases in DNA methylation, including possible endogenous changes that alter gene expression or the function of DNMT and demethylases, as well as exogenous factors like diet, drugs and UV may also result in gene expression alteration [69]. Endogenous DNMT alteration can arise at different stages of cellular development from new-born, middle aged, to elderly individuals. All of these factors may lead to gene overexpression and the increase of 'transcriptional noise', however this is not fully understood in the mammalian system. Exogenous agents that affect DNA methylation may affect cellular function in the long-term [47]. Cells treated with demethylation agents can become re-methylated due to the action of their DNA methylation maintenance mechanisms which endure multiple insults with accumulating age [70]. Dietary deficiencies in folate, choline, methionine, zinc and/or selenium can result in alteration of DNA methylation status [71]. Folate and choline deficiency may contribute to DNA hypomethylation in the liver that leads to liver cancer development [72]. Folate deficiency can also lead to an increase of homocysteine levels and promote degeneration of neurons in Alzheimer's and Parkinson's disease [73].

The mechanisms involved in hypermethylation of CpG islands with ageing are not well understood. Wu et al., showed increased levels of DNMT1 leading to CpG island methylation and phenotype changes in fibroblasts, suggesting the increased level of the protein contributed to process of phenotypic modification. Under normal cellular development DNMT1 may increase in response to DNA hypomethylation that is caused by drug intake and dietary deficiencies which lead to increased DNA methylation and contributes to the DNA methylation balance of the cell.

2.2. DNA methylation and disease development

Germline and somatic mutations are mainly the result of cytosine methylation during cancer development [74]. Abnormal promoter methylation of the regulatory genes can lead to gene silencing and is an important mechanism of cancer progression [75]. Rare diseases such as immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF) [13] and mental retardation in young girls (Rett Syndrome) are the potential consequence of abnormal methylation alteration [76]. For example, ICF patients are found to be have a mutated DNMT3b gene that leads to the downregulation of satellite DNA methylation and chromosomal de-condensation. Methylation binding domains (MBDs, MeCP2) were found to be aberrantly methylated in Rett Syndrome patients, resulting in the interruption of the methylation signal [76]. Together this suggests methylation is not completed after embryonic development, requires maintenance and is essential in mammalian cells. Alternatively, the increasing methylation of mammalian cells may contribute to the risk of cancer development. Therefore, the balance of methylation is essential in maintaining healthy cellular function.

Many studies have indicated that the imbalance of DNA methylation occurs in the disease mechanism which leads to the discovery of pharmacological agents that reverse epigenetic abnormalities [77]. The interaction of DNA methylation and histone modification machinery were further investigated and proved to be an important contribution that led to disease development. Another group of epigenetic alterations caused by small RNAs also play a major role at different disease stages that could also be exploited to monitor treatment results [78].

2.3. DNA methylation and cancer development

DNA methylation and cancer development was first studied in the 1980s, showing DNA hypomethylation in their normal counterparts of cancer cells [79]. The loss of DNA methylation in the repetitive regions of the genome was referred to as hypomethylation which led to genomic instability and is a hallmark of tumour cells [80]. Hypomethylation can also lead to over-expression of oncogenes that contributes to cancer progression [81]. Transcriptional interference describes the reactivation of transposon promoters via demethylation that contributes to aberrant gene regulation in cancers [82]. Down-regulation of DNA methylation occurs at the early stages of cancer and correlates with disease progression and metastatic potential in many cancer types [81]. The melanoma antigen (MAGE) family of cancer genes are a gene-specific hypomethylation in cancer cells that encodes tumour antigens of unknown function that are frequently demethylated and re-expressed in cancer [83]. Specific genes that are hypomethylated in specific types of cancer include: S100 calcium binding protein A4 (S100A4) upregulated in colon cancer [84], serine protease inhibitor gene SERPINB5 (also known as maspin) in gastric

cancer [85] and the putative oncogene γ-synuclein (SNCG) in breast and ovarian cancers [86]. Global hypomethylation that occurs at early stages of tumorigenesis may be protected by genomic instability and further genetic changes. However, gene-specific hypomethylation could allow tumour cell adaptation to their local environment and promote metastasis. Research on genome-wide demethylation in cancer cells has been largely overshadowed by studies of gene-specific hypermethylation events, which occur concomitantly with the hypomethylation events discussed above. In cancer cells, aberrant hypermethylation usually occurs at CpG islands that are mostly unmethylated in normal somatic cells [87]. Histone deacetylation leads to changes in chromatic structure that effectively silence transcription. In a subset of tumour types that are referred to as CpG island-methylator phenotypes, there was a 3–5 fold increase in aberrant methylation [88]. Most of the involved genes were regulators of cell-cycle, tumour cell invasion, DNA repair, chromatic remodelling, cell signalling, transcription and apoptosis. Additionally these genes are known to be aberrantly hypermethylated and silenced in most cancers, favouring cancer cell growth and increasing their genetic instability causing them to metastasize. In the case of colon cancer, aberrant hypermethylation is detectable in the earliest precursor lesion, indicating DNA hypermethylation is an early, detectable event during colon cancer development and can be used as a biomarker [89].

Cancer can be described as a disease of ageing, Issa et al., reported global (repeat element) hypomethylation and promoter hypermethylation of cancer cells are also found in normal tissues with ageing [58]. Other studies have described the age-related methylation in normal human prostate and colon tissues that contain CpG island bearing genes [90]. The alteration of DNA methylation is age-related but also tissue-dependent. The process of DNA methylation associated with ageing and promoter CpG methylation is complex. CpG islands are known to contribute to gene silencing in cancer cells. Gene silencing of retinoic acid receptor- β (RAR β) 51 is an example of *de novo* methylation of the CpG island that causes leukaemia to develop in humans [91]. During cancer development, interactions between DNMTs and HDACs may facilitate *de novo* methylation to maintain permanent methylation of tumour suppressor genes that are already down-regulated [92].

2.4. DNA methylation, biomarkers, treatment and monitoring of disease

Promoter hypermethylation of DNA can be used as biomarkers for the detection of different cancerous cells when compared with methylation status of 'normal-healthy' cells. There is a growing trend to develop DNA methylation based biomarkers for cancer diagnosis. This area of development is attractive to researchers because of the stable and sustainable nature of detection (even in circulation). Aberrant DNA methylation can also be used as a biomarker for malignant transformation. The development of a methylation-specific PCR technique in 1996 by Herman et al., became popular soon after their first publication [93]. It offers a quick, easy, non-radioactive and sensitive way to detect hypermethylated CpG regions of tumour suppressor genes and can detect unmethylated CpG regions in 'normal-healthy' cells. Recently, the new droplet digital PCR technology and next generation sequencing has moved methylation detection in clinical samples a step forward which is sensitive enough to look at the traced methylation status of tumour suppressor genes in

circulation. The advantage of this detection technology is built on the basis of the following: positive PCR signals are not masked by the contamination of normal cells, promoter hypermethylation can occur at an early stage of cancer which allows early diagnosis and all tumours have one or more loci that contain hypermethylated tumour suppressor genes. The development of detection techniques also allowed promoter hypermethylation to be identified using bronchoalveolar lavages [94], lymph nodes [95], stool and sputum [96] to screen methylation of tumour suppressors. The screening of promoter hypermethylation in serum DNA from non-small cell lung cancer also opens the avenue for researchers to further develop this technology for diagnosis [97]. Septin 9 (Sept9) was the first FDA approved DNA methylation marker that utilised non-invasive serum samples from patients [98]; MLH1 is used for colon cancer diagnosis [99] and O6-methylguanine-DNA-methyltransferase (MGMT1) is used for brain cancer [100] diagnosis in the clinic.

The process of methylating d^mC in cancer is complicated as malignancies have different origins; it can either be direct or indirect influence by oncogenes or as part of cancer cell adaptation to external stress response, environmental factors and exposure to therapeutic genes and others [101, 102]. The distribution of d^mC during cancer development will allow cancer cells to abject their phenotypes, adapt to different tissue microenvironments and also become resistant to therapeutic drugs [103]. Cancer associated upregulation of d^mC at promoters or enhancers of a genome can also cause tumour suppressor genes to become silenced [104]. The commonly silenced tumour suppressor genes in most cancers CDKN2A, RB and MLH1 are associated with aggressive cancer types and a poor prognosis [104]. DNA methylation can also be used to detect silenced genes involved in immune recognition or modulate a response to chemotherapy, resulting in disruption of immune surveillance that induced chemotherapy resistance [105]. However, these epigenetic alterations are reversible and it is possible to reverse aberrant methylation with DNMT inhibitor (DNMTi) or a demethylating agent (i.e. decitabine) which allows the restoration of the genomic functions [67]. The advantages of these drugs are their ability to reprogram cancer cells to undergo terminal differentiation, induce chemosensitisation, loss of self-renewal properties or become visible to immune system. DNMTi and decitabine can also be used to induce anti-tumour response by induction of endogenous stimulation of interferon response pathways [106].

The multifaceted and easy detection of d^mC highlights its great potential to be used as a biomarker with utilisation of the improved detection methods of methylated DNA using modified DNA fragments. d^mC biomarkers have now become a convincing predictor of clinical outcomes and are able to predict response to DNMTi in the clinic. They can also be used to classify cancer into biological and clinically distinct disease subtypes, providing guidance for chemotherapeutic drug selection. The use of tumour suppressor gene CDKN2A methylation status has shown to be prognostically significant in many cancer types [107]. In leukaemia patients, clinicians use the panel 16 methylated gene panel as a biomarker for detection using microarray and showed that differentially methylated regions (DMTs) are useful to predict outcomes of patients and their clinical variability. This d^mC detection technology has been developed further as biomarkers and is validated in acute myeloid leukaemia (AML) patients [108]. The DMRs can also be used to predict response of patients with chronic myelomonocytic leukaemia to DNMTi [109]. Hypermethylation and silencing

of SMAD1 was useful as a predictive biomarker for chemotherapy resistance in patients with high-risk diffuse large B-cell lymphoma (DLBCL) [110]. SMAD1 silencing also showed contribution to chemotherapy resistance that can be reversed by DNMTis in patients [110]. Results were favourable for the use of DNMTi in patients diagnosed with high-risk DLBCL, in combination with decitabine before rituximab in combination of cyclophosphamide, doxorubine, vincristine and prednisone chemo-immunotherapy. MGMT which is a DNA repair enzyme has also proved to be useful as a predictive marker for alkylating agent response [111].

3. Epigenetic regulation and drug discovery

Other epigenetic factors are useful for drug discovery, such as acetylation, methylation and phosphorylation, ubiquitination, sumoylation and ADP-ribosylation [112, 113]. A list of these agents is summarised in **Table 1a** and **Table 1b** [114, 115]. Vincent Alfrey et al., suggested modifications of acetylation have functional roles in modulating transcription and it was later established by others that the process of chromatin and post-translational modification of epigenetic regulation may be interrupting DNA [116]. HDAC are a family of enzymes responsible for chromatin modification. Aberrant regulation of this family of genes has been studied in many cancers and used in pharmacological target discovery. The inhibition of chromatin-modification enzymes is a key process to modulate transcription in eukaryotic cells which led to the development of novel pharmacologic agent discovery. HDAC inhibitor (HDACi), vorinostat, was the FDA approved treatment for patients with advanced refractory cutaneous T-cell lymphoma. This provides evidence that HDACi is a useful therapeutic treatment, however the different subtypes of HDAC should be considered to achieve promising therapeutic interventions. The discovery of both HDACi and DNMTi provide options for clinical treatment alone or possible use with other agents in combination therapies for the treatment of various diseases that are related to epigenetic abnormalities [117].

4. Conclusion

Epigenetic regulation is an attractive area of research; it provides a broad spectrum of discovery as it is involved in almost all developmental processes of the mammalian cell from

Name	DNMT inhibitor	Clinical status	Treatment
Decitabine	5-AZA-CdR	FDA and the European Medicines Agency	Myelodysplastic syndromes (MDS), acute myeloid leukaemia (AML), and chronic myelomonocytic leukaemia (CMML)
5-AZA	Nucleoside analogues azacitidine	FDA and the European Medicines Agency	Myelodysplastic syndromes (MDS), acute myeloid leukaemia (AML), and chronic myelomonocytic leukaemia (CMML)

Table 1a. DNMT inhibitors.

Class	HDAC inhibitor	Target HDAC Class	Clinical status
Hydroxamic acids	SAHA	pan	Approved for cutaneous T-cell lymphoma
	Belinostat	pan	Approved for peripheral T-cell lymphoma
	Panabiostat	pan	Approved for multiple myeloma
	Givinostat	pan	Phase II clinical trials—relapsed leukaemia and multiple myeloma
	Resminostat	pan	Phase I and II clinical trials—hepatocellular carcinoma
	Abexinostat	pan	Phase II clinical trial—B-cell lymphoma
	Quisinostat	pan	Phase I clinical trial—multiple myeloma
	Rocilinostat	II	Phase I clinical trial—multiple myeloma
	Practinostat	I, II and IV	Phase II clinical trial—prostate cancer
	CHR-3996	I	Phase I clinical trial—advanced/metastatic solid tumours refractory to standard therapy
	Valproic acid	I, IIa	Approved for epilepsia, bipolar disorders and migraine, phase II clinical trials—several studies
Short chain fatty acids	Butyric acid	I, II	Phase II clinical trials—several studies
	Phenylbutyric acid	I, II	Phase I clinical trials—several studies
	Entinostat	I	Phase II clinical trials—breast cancer, Hodgkin's lymphoma, non-small cell lung cancer, phase III clinical trial—hormone receptor positive breast cancer
Benzamides	Tacedinaline	I	Phase III clinical trial—non-small cell lung cancer and pancreatic cancer
	4SC202	I	Phase I clinical trial—advanced haematological malignancies
	Mocetinostat	I, IV	Phase II clinical trials—Hodgkin's lymphoma
	Romidepsin	I	Approved for cutaneous T-cell lymphoma
Cyclic tetrapeptides	Nicotinamide	All class III	Phase III clinical trial—laryngeal cancer
Sirtuins inhibitors	EX-527	SIRT 1 and 2	Cancer preclinical, phase I and II clinical trials—Huntington disease, glaucoma

Table 1b. HDAC inhibitors.

early fertilisation, implantation, embryonic development, ageing and carcinogenesis. As evolutionary stages require different types of epigenetic signature, the various epigenetic patterns have been exploited in biomarker discovery to identify distinctive/unique stages of disease (typically cancer) development. Although DNA methylation is the most studied area of epigenetics, there are not many markers that are currently used as standard clinical diagnostic markers. Due to the stability of DNA in cells and in circulation we believe that with the development of new technologies and methods, DNA methylation biomarkers

have the potential to become a favourable clinical diagnostic marker. Further research is required in this field to ensure the widespread application of DNA methylation markers in the clinical setting.

Abbreviations

3′-UTR	three prime untranslated region
AML	acute myeloid leukaemia
Arg	arginine
AZA	5-aza-2′-deoxycytidine
Clr4	histone-lysine N-methyltransferase
dC	deocycytosin
DLBCL	diffuse large B-cell lymphoma
dmC	deoxymethylcytosine
DMTs	differentially methylated regions
DNMT	DNA methyltransferases
ES	embryonic stem
H3K9	histone H3 at lysine 9
HDAC	histone deacetylase
HMT	histone methyltransferase
HP1	heterochromatin protein
ICF	facial anomalies syndrome
ICR	imprinting control region
Lys	lysine
MBD	Methylation binding domains
MGMT1	O6-methylguanine-DNA-methyltransferase
miRNAs	MicroRNAs
mRNA	messenger RNA
NuRD	nucleosome remodelling

RAR β	retinoic acid receptor- β
RNAi	RNA interference
ROS	reactive oxygen species
S100A4	S100 calcium binding protein A4
SAM	S-adenosylmethione
TE	transposable element
TF	transcription factor
ZGA	zygotic gene activation

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