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Chapter 2

DNA Methylation

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Additional information is available at the end of the chapter

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1. Introduction

DNA methylation is a major epigenetic modification that is strongly involved in the physiological control of genome expression. Developmental processes and proper biological functions are tightly dependent on hierarchical and regulated gene expression patterns. Numerous molecular processes control gene expression. DNA methylation is a physiological epigenetic process that leads to long term-repression of gene expression. DNA methylation is a common epigenetic modification involving the methylation of 5'-cytosine residues and is often detected in the dinucleotides of CpG sequences. Methylation is often localized in promoter regions and occasionally in transcriptional regulatory regions in mammals, plants and even prokaryotes. DNA methylation may be classified as hyper-and hypomethylation, according to increased and decreased levels of genomic modification, respectively. Hypermethylation is an epigenetic alteration often leading to gene-inactivating deletions and translocations. Hypermethylated cells may exhibit a phenotype of drug-resistance or malignant proliferation. Aberrant methylation in eukaryotic cells may lead to silencing of important genes, such as tumour suppressor genes, affecting their related transcriptional pathways and ultimately leading to the development of disease such as cancer. Therefore, it is considered to be a hallmark of cancer, it is detected in several types of cancer cells, including colon, breast, ovarian and cervical cancer cells and is associated with alterations in specific gene expression.

Hypermethylation of tumour suppressor gene promoters and global disruption of many histone modifications are characteristic features of cancer. Deregulation of the epigenetic profile alters the transcription profile of many genes. In the case of tumour suppressors DNA methylation reduces gene expression and subsequently removes regulatory proteins required for normal cell growth and development. Therefore, DNA methylation in cancer would be predicted to influence multiple gene networks rather than single genes. Because of heterogeneity of breast cancer at both histological and molecular levels staging breast cancer fails to predict prognosis or therapeutic response of the disease, therefore, DNA methylation targeted

therapies, in recent years, play an increased role in the treatment of breast cancer. DNA methylation targeted therapies, in recent years, play an increased role in the treatment of breast cancer. Two groups of agents targeting epigenetic modifications have been studied previously, namely histone deacetylase inhibitors and DNA methyl transferase inhibitors. The associations between DNA methylation mechanism and breast cancer classification and prognosis will be reviewed in this chapter in detail by describing the DNA methylation mechanism and gene expression in breast cancer, as well as functional genomics and genome wide DNA methylation in breast cancer.

2. What is epigenetics?

The term epigenetic was introduced by Conard Waddington in 1942 as a concept of environmental influence in inducing phenotype modification. His work on developmental plasticity states that the environmental influences during development could induce alternative phenotypes from one genotype, one of the clearest examples is polyphenisms in insects. He showed that exposing the pupae of wild type Drosophila melanogaster to heat shock treatment, results in altered wing vein patterns [1,2]. Breeding individuals who have been exposed to these environmentally induced changes led to a stable population exhibiting the phenotype without the environmental stimulus. The concept of epigenetics was not clarified until the late 1990s when Wolffe and Matzkeset the modern definition, which was 'the study of heritable changes in gene expression that occur without a change in DNA sequence'[4]. Bird came with a wider definition of epigenetic which is 'the structural adaptation of chromosomal regions so as to register, signal or perpetuate activity states' [5]. The term epigenome has emerged to describe the epigenetic modifications all over the epigenome, thus, the epigenome controls the genome in both normal and abnormal cellular processes and events [6]. Epigenetic mechanisms include; DNA methylation, histone modification and non-coding RNAs, which work cooperatively to control gene expression.

3. DNA methylation

DNA methylation is a well conserved process that occurs in eukaryotes and prokaryotes [7]. DNA methylation refers to the covalent addition of a methyl group to carbon number five in the nitrogenous base cytosine at the DNA strand. Only cytosine residues where adjacent to guanine are targets for the methylation by the methyltransferases enzymes and the distribution of methylated and unmethylated CpGs is tissue-specific which leads to cell-specific pattern of DNA methylation [8]. The CpG may occur in multiple repeats which are known as CpG islands [9]. These regions are often associated with the promoter regions of genes. Almost half of the genes in our genome have CpG rich promoter regions. In the whole genome, about 80% of the CpG dinucleotides not associated with CpG islands are heavily methylated [10]. In contrast, the CpG islands associated with gene promoters are usually unmethylated [11].

There are a number of factors that may maintain the undermethylated state of CpG islands, such as sequence feature, SP1 binding sites, specific acting enhancer elements, as well as specific histone methylation mark H3K4me3, which prevents the binding of de novo methylation complexes [12]. Methylation of the CpG islands in the promoter region silences gene expression, and the absence of methylation is associated with active transcription. Thus unmethylated CpG islands are associated with the promoters of transcriptionally active genes, such as housekeeping genes and many regulated genes, such as genes showing tissue specific expression [13]. DNA methylation information at every cytosine can be determined, but it was targeted at few candidate genes using methylation-sensitive restriction enzymes or gene-specific DNA methylation mapping by sequencing bisulfite-converted DNA. In contrast, development of advance technology in DNA methylation mapping, including high-density oligonucleotide arrays, illumina bead arrays and next-generation high-throughput sequencing, together with advances in bioinformatics, have enable examination of broad regions of the genome and provide high-content profiles of DNA methylation.

3.1. DNA Methyltransferases (DNMTs)

The methylation process is catalysed by the DNA methyltransferases enzymes (DNMTs) which are known as DNMTs; DNMT1, DNMT3A, DNMT3B, and DNMT3L [14]. DNMT3A and DNMT3B are the de novo methyltransferases while DNMT1 maintains the methylation patterns during DNA replication (mitosis) [15]. However, the actual function of DNMT2 is not clear, bur several forms of DNMT1 have been detected which differ in their translation start sites and prefer hemimethylated DNA. Overexpression of DNMT1 has been reported in human tumours and may contribute to the global methylation abnormalities seen in cancer cells although increased expression of the DNMTs is likely to be only partially responsible for the observed methylation abnormalities since not all tumours overexpress these enzymes [10]. Cytosine (C^5)-DNA methyltransferases catalyze the transfer of a methyl group from S-adenosyl-methionine onto cytosine residues in specific sequences of duplex DNA, with production of 5-methyl cytosine and S-adenosyl-homocystein (SAMe) (Figure1). For most proteins, cytosine (C^5)-DNA methyltransferases have up to 10 conservative regions arranged in a strictly defined sequence [16]. Comparison of the primary structures of cytosine (C^5)-DNA methyltransferases reveals the association of their major functions with their conservative motifs, whereas the site-specific recognition belongs to a variable region of the target-recognizing domain (TRD) [17]. Among ten conservative blocks of amino acids in cytosine (C^5)-DNA methyltransferases, the N-terminal domain of DNMT1 contains varied specific functional sequences, such as the nuclear localization signal (NLS), the cysteine–enriched zinc-binding motif, and a special sequence directing the methylase into the area of DNA replication. In addition, DNMT1 interact with the proliferating cell nuclear antigen (PCNA) which is required for DNA replication, and the DNMT1-PCNA interaction allow rapid remethylation of the newly synthesised daughter strands before packed into chromatin [18]. A null mutation of the mouse methylase DNMT1 gene resulted in a significant (up to 70%) decrease in the genome methylation and death of developing embryos [19]. The remaining 30% level of DNA methylation and the ability of embryonic stem cells deprived of the DNMT1 methylase for de novo methylation of DNA suggest that these functions were performed by other DNA methylases

[19]. Such methylases were searched for in animals, and new enzymes of the DNMT2 and DNMT3 families were found [20]. Cell-cycle regulators p21 and retinoblastoma gene product Rb can bind to DNMT1 and inhibit its methyltransferase activity during DNA replication in the cell cycle [18]. This observations show complex interaction between DNMT1 and cellular proteins involved in gene regulation and epigenetic signalling during cell replication [21].

The DNMT3 family consists of two genes, DNMT3a and DNMT3b, which are highly expressed in undifferentiated ES cells but downregulated after differentiation and expressed at low levels in adult somatic tissues and are overexpressed in tumour cells [22]. Both DNMT3a and DNMT3b are required for genome-wide de novo methylation and are essential for mammalian development [22]. Both DNMT3a and DNMT3b had been mapped by the unigene consortium via polymorphisms in 3' –untranslated region sequences. DNMT3b mapped to the region of chromosome 20q that contains the trait for ICFNS (immunodeficiency centromeric instability, facial ubnormalities) syndrome. This syndrome presents with variable combined immunodeficiency, mild facial anomalies and extravagant cytogenetic abnormalities which largely affect the pericentric region of chromosomes 1, 9 and 16. These pericentric regions contain a type of satellite DNA termed classical satellite, or satellites 2 and 3. It is normally heavily methylated, but is nearly completely unmethylated in the DNA of ICF patients. It was found that immunodeficiency centromeric instability (ICF) patients had mutations in the C-terminal DNA methyltransferase domain of DNMT3b. DNMT3b remains the only DNA methyltransferase shown to be mutated in a human disease [15]. DNMT3b has been shown to play a crucial role in hypermethylation of promoter CpG-rich regions of tumour suppressor genes and thus its inactivation within human cancer cells [22].

3.2. How does demethylation occur?

The key question is how the enzymes know where to methylate? Two theories have been suggested. Firstly, it has been suggested that all genes are methylated by default except for active genes [23]. Actively transcribed genes have a preponderance of attached transcriptional factors, giving no physical access to the methyltransferses to reach their targets. On the other hand, inactive DNA is susceptible to the methyltransferases and subsequently become methylated. This model was confirmed by the study of the transcription factor SP1. It has been shown that as long as SP1 is attached to its site, no methylation could occur in the adjacent CpG sites, and removal of the SP1 leads to de novo methylation at this site [24]. The second theory is that methylation is directed by sequence specific binding proteins so the methyltransferases bind with certain proteins such as a histone deacetylases (HDACs) and other transcription repressors, and form a complex would bind to specific sequence on the DNA [23].

Methylated genes may need to be activated in response to environmental signals and thus demethylation is an important dynamic epigenetic mechanism and it was originally thought that demethylation only occured through passive demethylation (Figure 2). However, the rapid demethylation of the paternal genomes upon fertilization and examples of rapid demethylation of genes in post-mitotic neurons suggest that an active demethylase must exist [23,25]. A number of enzymes have been suggested to have demethylase activity these include MBD2b, MBD4, the DNA repair endonucleases XPG (Gadd45a) and a G/T mismatch repair

DNA glycosylase which is glycosidase dependent. In this mechanism, the methylated cytosine is recognized by glycosidase which cleaves the bond between the DNA back bone and base. The base is subsequently removed and replaced with unmethylated cytosine by the DNA repair system.

4. Histone Deacetylases (HDACs)

Histones are five basic nuclear proteins that form the core of the nucleosome and the histone octamer contains two molecules each of histones H2A, H2B, H3 and H4. Histone H1 the linker histone is located outside the core and involve in the packing of DNA [26]. Histone modifications play a major role in regulating gene expression and extend the information potential of the DNA which explains the growing interest of the 'Histone Code' [27]. Modifications to amino acids on the N-terminal tails of histones protruding from the nucleosome core can induce both an open or closed chromatin structure and these affect the ability of transcription factors to access promoter regions to activate transcription. The covalent modification can be acetylation, methylation, phosphorylation and ubiquitination. Methylation of some residues is associated with both transcriptional repression, such as methylation of histone 3 lysine 9 (H3 K9) and others with transcriptional activation, such as methylation of histone 3 lysine 4 (H3 K4) [28,29].

Histone methylation is performed by histone methltransferase (HMTs) which can transfer up to three methyl groups to lysine residues within the tails of the histones with different effects on gene activity. Acetylation which occurs at lysine residue is associated with transcriptional activation [30]. This modification is performed by histone acetylases (HATs) and removed by the HDACs [31]. The HDACs are critical in the regulation of expression of genes important for cell survival, proliferation, differentiation, and apoptosis [32]. HDACs also act as members of a protein complex responsible for recruitment of transcription factors to the promoter region of genes, including those of tumour suppressors, and regulation of acetylation status of specific cell cycle regulatory proteins [33]. High HDAC expression and histone hypoacetylation have been observed in cancer with associated transcriptional repression of genes, providing a rationale for the investigation of HDAC inhibitors in cancer therapeutics [34].

Additionally, acetylation of histones has been extensively studied as one of the key regulatory mechanisms of gene expression [35]. Histone acetylation was found to affect RNA transcription as early as the 1960s [36]. The highly conserved lysine residue at the N-terminal of H3 at position 9, 14, 18 and 23, and H4 lysine 5,8,12 and 16, are frequently targeted for modification [37]. Acetylations of the lysine residues neutralize the positive charge of the histone tails. Therefore, decrease their affinity for DNA which results in open chromatin conformation allowing the transcriptional machinery to reach its target [38]. The acetyltransferases added the acetyl groups from acetyl coenzyme A (acetyl-CoA) to the epsilon-amino group of specific lysine residues [39]. There are eighteen HDAC enzymes in mammalian cells which are divided into two families: a) zinc metalloenzymes that catalyses the hydrolysis of acetylated specific residues on histone tails and include class I, II and 1V HDACs, and b) NAD-dependent Sir2 deactylases which are considered as class III HDACs [40,41].

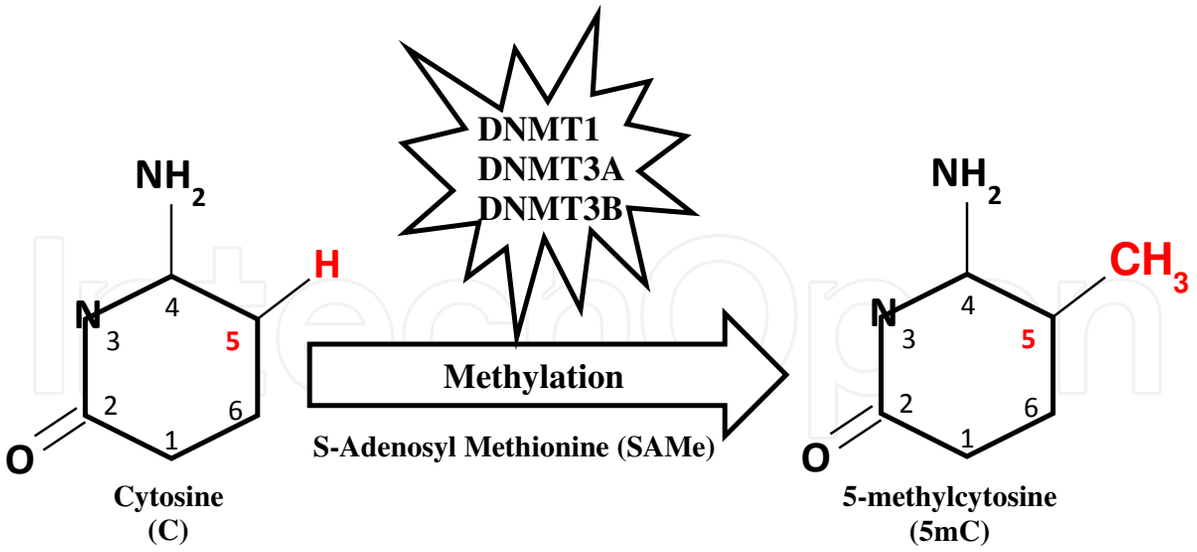

Figure 1. Methylation of DNA by DNA methyltransferases enzymes (DNMTs) DNMT1, DNMT3A, DNMT3B. A methyl group transfer from S-adenosyl-methionine onto cytosine residues leading to production of 5-methyl cytosine and S-adenosyl-homocystein (SAMe).

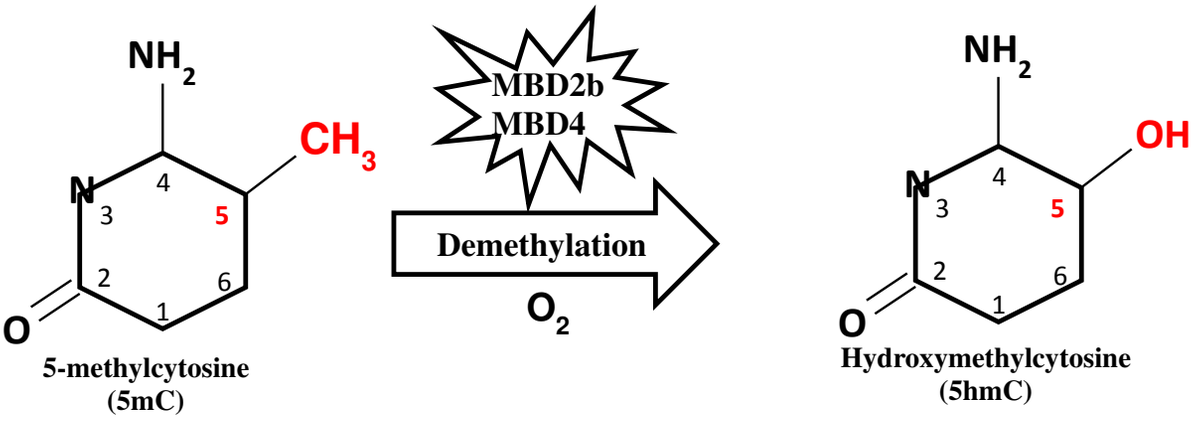

Figure 2. DNA demethylation appears to be a shared attribute of reprogramming events, and understanding DNA methylation dynamics is thus of considerable interest. Some enzymes such as MBD2b and MBD4 convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC).

Class I is a group of four enzymes known as HDAC1, 2, 3 and 8 and this class is associated with gene regulation. They are expressed ubiquitously and they function exclusively in the nucleus [40]. Class II is subdivided into class IIA, which includes HDAC 4, 5, 7 and 9 and class IIB that includes HDAC 6 and 10. Class II enzymes shuttle between cytoplasm and nucleus, and they involve mainly in cell differentiation and are highly expressed in certain tissues [40]. Class III includes the NAD-dependent deacetylases which is a group of seven enzymes that are involved in maintaining the chromatin stability. They can remove the acetyl groups from histones besides other proteins [42]. Class IV contains one member which is HDAC11 which is closely related to class I thus some reviewers consider it as a member of that class. The function of HDAC11 has not been characterized yet [43], however, there is increasing evidence

showing that changes in chromatin structure would alter DNA methylation patterns. The targeting of DNA methylation enzymes to gene promoters is guided by chromatin modifying enzymes. The fact is that chromatin configuration is dynamic and that chromatin modifying enzymes are activated by cellular signalling pathways. This provides a link between the extracellular environment and the state of DNA methylation [44]. Evidence of the link between chromatin modelling and DNA methylation in humans and mice arises from mutations of the SWI-SNF proteins which are involved in chromatin remodelling. These mutations result in defects in DNA methylation [44]. A number of histone methyltransferases, such as G9a, SUV39H1 and EZH2, a member of the multi-protein polycomb complex PRC2 can regulate DNA methylation by either recruiting or regulating the stability of DNMTs. DNMTs in turn can recruit HDACs and MBPs to achieve chromatin condensation and gene silencing [45]. This relationship between the epigenetic machinery makes the epigenetic mechanisms of genome expression a tightly regulated process.

5. DNA methylation and breast cancer

During the last decade, the study of epigenetic mechanisms in cancer, such as DNA methylation, histone modification, nucleosome positioning, and micro RNA expression, has provided extensive information about the mechanisms that contribute to the neoplastic phenotype through the regulation of expression of genes critical to transformation pathways. Regarding DNA methylation, the low level of CpG methylation in tumours compared with that in their normal-tissue counterparts was one of the first epigenetic alterations to be found in human cancer this let us to think that the cancer cells have a specific epigenome [46]. Hypomethylation in cancer cells is associated with a number of adverse products, including chromosome instability, activation of transposable elements, and loss of genomic imprinting [47].

Breast cancer has traditionally been staged by histopathological standards that are based on size, level of invasiveness and lymph node infiltration, and by immunochemical characterization of cell surface receptors, including oestrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). However, in many instances staging breast cancer fails to predict prognosis or therapeutic response because of the heterogeneity of the disease. Changes in gene expression that reset a cell program from a normal to a diseased state involve multiple genetic circuitries, creating a characteristic signature of gene expression that defines the cell's unique identity and to classify subtypes of breast cancers [48]. Detailed knowledge of the DNA methylation status of all cytosines (the methylome) is paramount for understanding the mechanisms and functions underlying DNA methylation and led to extend our ability to classify breast cancer and the outcome prediction. DNA methylation is a forceful biomarker, greatly more stable than proteins or RNA, and is therefore a promising target for the development of new approaches for diagnosis and prognosis of breast cancer and other diseases. Because DNA methylation is critical in gene expression programming, a change in methylation from a normal to diseased state should be similarly reflected in a signature of DNA methylation that involves multiple gene pathways. Whole-genome approaches have been used with different levels of success to distinguish breast-

cancer-specific DNA methylation signatures, and to test whether they can classify breast cancer and whether they could be associated with specific clinical outcomes [48].

Application of DNA methylation profiling becomes important for breast cancer diagnosis and prognosis only if it provides additional classification value to other currently used methods like immunohistochemistry and mRNA expression analysis. A recent whole-genome DNA methylation analysis by using the Illumina 27 K arrays suggests that DNA methylation profiling might expand current classifications of breast cancer subtypes [49,50]. The analysis of 248 breast cancer tumour samples, comprising a 'main set' of 123 samples (4 normal and 119 infiltrating ductal carcinomas (IDCs)), and a 'validation set' of 125 samples (8 normal and 117 IDCs), revealed an immune 'signature' in a mixed tumour stromal population, as also reported [51]. Methylome analysis performed on frozen primary tumour samples, led to the identification of six different methylation clusters [52]. It was shown for the first time that DNA methylation profiles can reflect the cell-type composition of the tumour microenvironment, with a T lymphocyte infiltration of these tumours in particular in HER2-enriched and basal-like tumours. High expression of certain immune-related genes were found to be associated with improved relapse-free survival providing further insight into the importance of the immune system and tumour microenvironment in certain breast cancer subtypes [53].

Furthermore, aberrations in DNA methylation patterns of the CpG islands in the promoter regions of tumour-suppressor genes are accepted as being a common feature of human cancer [54]. CpG island promoter hypermethylation affects genes from a wide range of cellular pathways, such as cell cycle, DNA repair, toxic catabolism, cell adherence, apoptosis, and angiogenesis, among others [54], and may occur at various stages in the development of cancer [55]. The CpG-island-containing gene promoters are usually unmethylated in normal cells to maintain euchromatic structure, which is the transcriptionally active conformation allowing gene expression. Yet, during cancer development, many of these genes are hypermethylated at their CpG-island-containing promoters to inactivate their expression by changing open euchromatic structure to compact heterochromatic structure [56,57]. These genes are selectively hypermethylated in tumourigenesis for inactivation owing to their functional involvement in various cellular pathways that prevent cancer formation. Some of the methylated genes identified in human cancers are classic tumour suppressor genes in which one mutationally inactivated allele is inherited. According to Knudson's (2000) two-hit model, complete inactivation of a tumour suppressor gene requires loss-of-function of both gene copies [58]. Epigenetic silencing of the remaining wild-type allele of the tumour suppressor gene, thus, can be considered as the second hit in this model. For example, some well-known tumour suppressor genes, such as the cyclin-dependent kinase inhibitorp16INK4a, APC and BRCA1, are mutationally inactivated in the germline occasionally lose function of the remaining functional allele in breast epithelial cells through DNA hypermethylation [59].These advances in the knowledge of the breast methylome strongly indicate that DNA hypermethylation mechanism plays a crucial role in initiation, promotion and maintenance of breast carcinogenesis, which cooperatively and synergistically interact with other genetic alterations to promote the development of breast cancer. In addition to cell-cycle regulatory genes, DNA methylation-mediated silencing of DNA repair genes, such as BRCA1 and MGMT, could result

in further inactivation of tumour suppressor genes or activation of oncogenes, which further drive breast tumourigenesis [60]. The genes that function as inhibitors of WNT oncogenic pathway such as SFRP1 and WIF1 have been found to be frequently hypermethylated in primary breast tumours [61]. Accordingly, epigenetic gene silencing is another mechanism that fosters malignant transformation of the mammary gland by aberrantly activating oncogenic signalling pathways in addition to the genetic mutation-mediated mechanism [62].

In vitro experiments showed that decreased BRCA1expression in cells led to increased levels of tumour growth, while increased expression of BRCA1 led to growth arrest and apoptosis. The magnitude of the decrease of functional BRCA1 protein correlates with disease prognosis [63]. Phenotypically, BRCA1-methylated tumours are similar to tumours from carriers of germline BRCA1 mutations. BRCA1 promoter hypermethylation was observed in one of two tumours from BRCA1 carriers lacking LOH [64]. In other study of population-based ovarian tumours, two of eight tumours with germline BRCA1mutations showed neither LOH nor promoter methylation [65]. Another study of 47 breast tumours from hereditary breast cancer families identified three BRCA1 carriers of which two showed BRCA1 promoter methylation in their tumours [66]. All these investigated studies suggest that methylation of BRCA1 may be serve as a second hit in tumours from a subset of BRCA1 mutation carriers [67].Tumours with BRCA1 mutations are usually more likely to be higher-grade, poorly differentiated, highly proliferative, ER negative, and PR negative, and p53 mutations. BRCA1 mutated breast cancers are also associated with poor survival in some studies [68]. BRCA1 promoter methylation was more frequent in invasive than in situ carcinoma and there were no correlation between BRCA1 promoter methylation and ER/PR status in a subset population [69]. However, they also found a higher prevalence of BRCA1 promoter methylation in cases with at least one node involved and with tumour size greater than 2cm. Based on their findings higher methylation levels may correlate with more advanced tumour stage at diagnosis. They also observed a 45% increase in mortality of individuals with BRCA1 methylation positive tumours compared those who had unmethylated BRCA1 promoters [69]. Another study conducted a familial breast cancer based study and found contradicting results. They found no overall correlation of ER, PR, or grade with hypermethylation of BRCA1 in the tumours from BRCA1 mutation negative families. However, seven individuals had both promoter hypermethylation and LOH; the majority of these tumours had a basal-like phenotype and were triple negative [70].

In addition, discriminate between tumour and normal or histologically non-malignant breast tissue has been applied widely by genome wide DNA methylation. One of the first genome wide DNA methylation studies in breast cancer developed methylation-specific digital karyotyping (MSDK) to assess epithelial, myoepithelial, and stromal fibroblasts from normal abreast and cancer tissues [71]. Furthermore, genome wide DNA methylation studies in breast cancer identified gene families that were commonly identified as differentially methylated between non-malignant and tumour included transcription factors (FOX, KLF, PRDM, ZBTB, and ZNF) and gene families involved in cell transport of proteins or vesicles(RAB and SLC) or involvement in cell adhesion (CDH and PCDH) [71-74]. The pathways and gene families do not appear to have a strong link to hormone metabolism or signalling, it is likely that these

genes are not drivers of cancer but rather are secondary events that occur as part of the tumourigenic process [75,76].

Genome wide DNA methylation studies have supported correlation between DNA methylation and gene expression, particularly the association between CpG islands DNA hypermethylation and gene repression [49,74,77,78]. Using familial breast cancers and BRCA1/2-mutated tumours combined DNA methylation profiles that alone predicted BRCA status, with gene expression and copy number variation (CNV) and found that genes with reduced expression were more likely to be in genomic regions with loss of heterozygosity and/or high levels of DNA methylation. It has also been shown that the combination of gene dosage in breast cancer cell lines, allelic status, and DNA methylation explains more gene expression changes than either genomic element alone [79]. Combining DNA methylation profiling with CNV and gene expression can be promising tool to facilitate the identification of critical genes involved in tumourigenesis. In genome wide methylation analysis, several platforms have been recently developed to allow genome wide methylation analysis. The Golden Gate methylation array was the first platform which allowed methylation of 1536 CpG loci to be investigated. The Infinium Human Methylation 27 increased CpG investigation with the use of 27,578 probes. Most recently was the Infinium Human Methylation 450K array, designed by Illumina. This array utilises florescence microarray hybridisation technique, often associated with expression studies, to provide a methylation profile of 485,764 CpG loci including CpG associated in CpG islands, shores, shelves and the isolated loci in the open sea regions of the genome and promoter regionshave used Illumina Infinium Human Methylation 27 Bead Chip to analyse normal breast tissues from ten healthy individuals and compared this to 62 breast tumour samples (19 were inflammatory breast cancer) [73].

Further studies have also compared tumour to non-malignant tissue and the number of genes identified that discriminates the two depends on the filtering or analyses utilized. For instance, Kim et al. (2012) used several filtering processes to identify six genes [80], whereas, Faryna et al, (2012) identified 214 CpG islands but only one CpG island (TAC1) was methylated in all ten cancer samples [81]. The DNA methylation profiles divided the samples into three groups based on high, intermediate, and low DNA methylation levels, with the normal samples having low DNA methylation levels. When comparing DNA methylation between normal and tumour samples, 1352 CpG loci (1134 genes) were differentially methylated [73]. There was significantly greater methylation in tumours compared with normal and 77% of these are CpG loci. Another study using the same technology found 6309 CpGs differentially methylated between 119 tumours and four normal breast tissue samples identified several hundred differentially methylated loci between 11 adjacent non-malignant breast tissues and 108 tumours [49;74]. Kim et al, (2011) pooled DNA from ten cancers and ten non-malignant matched adjacent tissues and identified 1181 differentially methylated CpGs (corresponding to 1043 genes) with the vast majority (972) hypermethylated [82]. Another study found 291 probes (264 genes) hypermethylated in breast cancer (n=39) compared with non-malignant breast tissue (n=4) after removal of imprinted genes and X chromosome genes [83].

In addition, numbers of studies have investigated whether genome wide DNA methylation profiling can cluster breast cancers into hormone receptor status (ER/PR positive or negative)

or subtype (luminal A or B, basal or HER2). These investigations differentiate hormone receptor-positive breast cancers from hormone receptor-negative cases using DNA methylation profiles [49,77,83-85]. The majority of genome wide DNA methylation studies have found that ER+PR+tumours have higher levels of DNA methylation compared with ER−PR− tumours [77,82,85,86]. Li et al, (2010) found 148 altered CpG sites (93 hypermethylated and 55 hypomethylated) in ER+PR+breast cancers relative to ER−PR− tumours [85]. Other study have identified 40 CpG probes that had an overall specificity of 89% and sensitivity of 90% for classifying ER+from ER− tumours [86].

Moreover, Hill et al, (2011) have used cluster analysis to show that ER+PR+tumours had high methylation, whereas triple-negative breast cancers had low methylation status [83]. Breast cancer cell lines have also shown clustering according to hormone receptor status based on DNA methylation levels [78]. Thus, all these genome wide DNA methylation studies demonstrate that an adequately results of appropriate clinical samples should identify methylation differences based on hormone receptor status. These studies may serve with additional future studies as a basis for the development of an improved clinical test to identify the hormone status of breast cancers.

In addition, in DNA methylation cluster analysis found that one cluster was predominantly luminal A (22/30 samples), the second cluster was highly correlated with basal-like (7/8 samples), and the third cluster contained a mixture of subtypes [74]. Recently, the Cancer Genome Atlas (TCGA) [87] and genome-wide profiling of DNA methylation has been also performed in primary breast tumours and revealed genes whose hypermethylation was significantly correlated with relapse-free survival, including RECK, SFRP2 and ACADL. Tumour specificity of methylation was confirmed for these genes by sequencing of an independent set of normal/breast tumour samples. Other investigation observed that the reduction of RECK methylation has been associated with worst prognosis in other tumours [88]. Genome-wide analysis has also been employed to characterize the DNA methylation profile of primary breast cancer with different metastatic potential. A global breast CpG island methylation phenotype (B-CIMP) was identified as an epigenetic profile associated with low risk of metastasis. Parallel gene expression analyses identified genes with both significant hypermethylation and down-regulation in B-CIMP tumours, including those involved in epithelial-mesenchymal transition (EMT), such as LYN, MMP7, KLK10 and WNT6 and the genes in the B-CIMP repression signature showed genes whose differential expression correlated with prognosis across several BC cohorts [89].

6. HDAC inhibitors and breast cancer

As we mentioned previously, abnormal HDAC activity has been documented in a variety of tumour types and led to the development of HDAC inhibitors as anticancer therapeutics. Currently available HDAC inhibitors target a variety of HDAC isoenzymes with class 1 (HDAC 1, 2, 3 and 8), class 2 (HDAC 4–7 and 9–10), and class 4 (HDAC 11) activity. Modest clinical benefits were previously reported with relatively weak HDAC inhibitors such as

valproic acid and phenylbutyrate in advanced solid tumours or hematologic malignancies [89]. Laboratory research conducted to date supports the investigation of HDAC inhibitors for the treatment of breast cancer. Recently, vorinostat as HDAC inhibitor induces differentiation or arrests growth of a wide variety of human carcinoma cells including breast cancer cells [90].Vorinostat also reduced tumour incidence in NMU-induced rat mammary tumourigenesis by 40 % [91]. In vitro studies demonstrated that vorinostat inhibits clonogenic growth of both ER-positive and ER-negative breast cancer cell lines by inducing G1 and G2/M cell cycle arrest and subsequent apoptosis [92].

The ability of the HDAC inhibitors to relieve transcriptional repression in preclinical breast cancer models has also been investigated. The accumulation of acetylated H3 and H4 histone tails in conjunction with re-expression of a functional ER in ER-negative breast cancer cell lines has been observed with a novel HDAC inhibitor known as scriptaid [93].Treatment of ER-negative breast cancer cell lines with vorinostat is associated with reactivation of silenced ER, as well as down regulation of DNMT1 and EGFR protein expression [94]. The significance of an epigenetically reactivated ER was demonstrated when tamoxifen sensitivity was restored in the ER-negative MDA-MB-231 breast cancer cells following treatment with both HDAC (trichostatin A) and DNMT inhibitors (DAC) [95]. Entinostat has been shown to induce not only re-expression of ERα, but also the androgen receptor and the aromatase enzyme (CYP19) both in vitro and in triple-negative breast cancer xenografts [96]. In addition, the combination ofletrozole and entinostat resulted in a significant and durable reduction in the xenograft tumour volume when compared to treatment with either agent alone. These experiments have provided the strong rationale for combining epigenetic modifiers with hormonal therapy in breast cancer clinical trials [96]. Interestingly, many of these studies also indicate that a strategy which combines HDAC and DNMT inhibitors is more efficacious than either agent alone with respect to both re-expression of silenced genes and restoration of response to tamoxifen and aromatase inhibitors [93.97].

Moreover, pretreatment of various tumour cell lines with HDAC inhibitors increases the cytotoxicity of chemotherapy. Administering the HDAC inhibitor after chemotherapy did not achieve the same results, suggesting that pretreatment with these agents may open the chromatin structure and thus facilitate an enhanced anti-cancer effect of chemotherapy drugs that target DNA [98]. In breast cancer cell lines with amplification and overexpression of HER2, HDAC inhibitor use depleted HER2 by attenuation of its mRNA levels and promotion of proteosomal degradation. HDAC inhibition also had been reported to enhance apoptosis induction by trastuzumab, docetaxel, epothilone B, and gemcitabine [99]. HDAC inhibitors also significantly enhance trastuzumab-induced growth inhibition in trastuzumab-sensitive, HER2-overexpressing breast cancer cells, providing a strong rationale for clinical studies with this combination in patients with HER2-positive disease [100].

Additionally, HDAC inhibitors such as entinostat or valproic acid, have been tested in breast cancer cells and efficiently restored both ERα expression and letrozole sensibility in ER$^-$BC in vitro and in vivo [101,102].The association of HDAC inhibitors or 5-azadeoxycytidine with a treatment inducing overexpression of TFAP2C might improve ESR1 expression in ER$^-$patients. A combined HDAC inhibitors and 5-azadeoxycytidine treatment induces the most significant

increase in ERα content. Surprisingly however, addition of tamoxifen does not produce a tumourigenic response in ER-BC cells demonstrated that a better response to tamoxifen in BC cells, correlated with a lower level of the RNA-stabilizing HuR protein [103]. Tamoxifen treatment increased HuR content, and contributed to its own resistance while HDAC inhibitors /5-azadeoxycytidine decreased HuR. Preliminary treatment with HDAC inhibitors /5-azadeoxycytidine was given before delivering tamoxifen to attempt to obtain the best tamoxifen sensitivity. The precise roles of tamoxifen are complex: although it competes with 17β-estradiol to bind to ERα, ERα bound to tamoxifen is still able to target the TFF1 (also called pS2) promoter without constitutive activation of gene transcription. The loss of transcriptional activity of the tamoxifen-ERα complex is mediated by changes in the balance of co-activators/co-repressors and ERα-interacting partners [104].

7. DNMTs inhibitors and breast cancer

The human DNMTs 1, 3A, and 3B coordinate mRNA expression in normal tissues and overexpression in tumours and the expression levels of these DNMTs are reportedly elevated in breast cancer [105,106]. The mean levels of DNMT1, DNMT3a, and DNMT3b overexpression have turned out to be quite similar among different tumour types. The DNMT3b gene has shown the highest range of expression (81.8 for DNMT3a compared with 16.6 and 14 for DNMT1 and DNMT3a, respectively). About 30% of patients revealed overexpression of DNMT3b in the tumour tissue as compared to normal breast tissue. Taking only these overexpressing tumours into account, the DNMT3b expression change was 82-fold, thus being significantly higher [106]. Interestingly, DNMT1 and DNMT3a were overexpressed in only 5 and 3% of breast carcinomas [107]. As a result of these studies, DNMT3b plays the predominant role over DNMT3a and DNMT1 in breast tumourigenesis. This is consistent with a recent study in breast cancer cell lines, which demonstrated a strong correlation between total DNMT activity and overexpression of DNMT3b, but not with the expression of DNMT3a or DNMT1 [107,108].

Cancer was the first group of diseases to be associated with DNA methylation and to be considered for DNA-methylation-targeted therapeutics, and it serves as a prototype for determining the role of DNA methylation and DNA-methylation-targeted therapeutics in other diseases [109]. As we mentioned previously, several types of aberration in DNA methylation and in the proteins involved in DNA methylation occur in cancer: hypermethylation of tumour suppressor genes, aberrant expression of DNMT1 and other DNMTs, and hypomethylation of unique genes and repetitive sequences [110,111]. Silencing of tumour suppressor genes by DNA methylation provides a powerful molecular mechanism by which DNA methylation can trigger cancer, and also provides a rationale for therapeutics aimed at inhibition of DNA methylation and re-expression of silenced tumour suppressor genes. Multiple genes are hypermethylatedin breast cancer compared to non-cancerous tissue [112]. These include genes involved in evasion of apoptosis (RASSF1A, HOXA5, TWIST1), limitless replication potential (CCND2, p16, BRCA1, RARβ), growth (ERα, PGR), and tissue invasion

and metastasis (CDH1) [113]. These genes are not only hypermethylated in tumour cells, but show increased epigenetic silencing in normal epithelium surrounding the tumour site.

Unlike genetic alterations which are almost impossible to revert, DNA methylation is a reversible event. Reactivation of hypermethylated tumour-suppressor genes can be considered as a possible therapeutic target which will lead to develop pharmacological inhibitors of DNA methylation. Moreover, the use of DNMT inhibitors is good tools for cancer treatment because the restoration of expression of tumour-suppressor genes could restore the protective effect of these genes on tumour divisions [114]. The nucleoside analogues, 5-azacytidine (vidaza or AZA,) and 5-aza-2′-deoxycytidine (decitabine or DAC) are two DNMT inhibitors that are effective hypomethylating agent that inhibit cell proliferation [115]. These two drugs represent the two most prominent DNMT inhibitors being under preclinical and clinical investigation for over 30 years [116]. Moreover, these agents are pro-drugs that need to be incorporated into DNA to act as inhibitors of DNMTs [116]. The nucleoside analogues are first phosphorylated to the triphosphate nucleotide and incorporated into DNA during DNA synthesis. DNMT1 forms a covalent bond with the carbon at position 6 of the cytosine as well as 5-aza-cytosine ring. Under normal conditions, as mentioned previously, the enzyme transfers the methyl group from SAMe to the fifth carbon position of the cytosine ring. This enables the release of the enzyme from its covalent bond with cytosine. When a 5′-aza-cytosine ring replaces cytosine in the DNA, the methyl transfer does not take place and the DNMT is trapped on the DNA (Figure 3). The replication fork progresses in the absence of DNMT1 resulting in passive loss of DNA methylation in the nascent strand but not the template [116].

Because they are cytidine analogues, both agents are incorporated into DNA after activation to a triphosphate moiety. After formation of an irreversible complex with DNMT1, degradation of the enzyme occurs [117]. This prevents methylation of daughter DNA in CpG islands during DNA replication. In addition, AZA (but not DAC) is converted into a ribonucleoside moiety and is incorporated into RNA, interfering with protein translation. At low concentrations (e.g. 30nM DAC, 300nM AZA), these inhibitors exhibit potent DNA hypomethylation properties, whereas high concentrations (≈3–10 μM) are cytotoxic [119]. The doses of AZA and DAC that were employed in many of the early clinical trials in solid tumours were cytotoxic, reflecting maximum tolerated doses, which likely accounts for the excessive toxicity, and possibly also to lack of overall efficacy, observed in these studies [120]. Previous study indicated that the DNMT inhibitors were associated with response rates as high as 18% in breast cancer [120]. The doses of AZA that were employed in these studies, however, were far higher than doses used in clinical trials today and likely exerted cytotoxic activity as opposed to relief of transcriptional repression as an anti-cancer strategy [120].

Current clinical studies with administration of DNMT inhibitors at the presumed optimal epigenetic dose aim to elucidate the biological effects of these agents, and to assess clinical efficacy, alone or in combination with other anti-cancer agents. The ability of single agent AZA to induce expression of the ER and PR genes in patients with triple-negative breast cancer who are awaiting definitive breast cancer surgery is under investigation using a 75 mg/m^2/day dosing schedule [121]. Based on the preclinical evidence previously described which suggests that a combination of epigenetic modifiers may be more successful in re-expression of silenced

genes and restoration of hormonal therapy responsiveness, patients with advanced triple-negative and hormone-resistant breast cancer are being enrolled in an ongoing multi-center phase 2 clinical trial and receive the combination of low dose AZA (40 mg/m^2) on days 1–5 and 8–10, and entinostat 7 mg on days 3 and 10 of a 28 day cycle. Tumour biopsies prior to and after therapy are collected to assess modulation of candidate gene methylation and expression, such as the ER gene. Patients may transition to an optional continuation phase at the time of disease progression in which the same epigenetic therapy is administered with the addition of hormonal therapy [122].

The DNMT inhibitors combination with standard chemotherapy has not been extensively evaluated in the breast cancer setting and preclinical evidence have shown the AZA could overcome platinum resistance through DNA hypomethylation, patients with both platinum resistant and refractory ovarian cancer received the combination of AZA and carboplatin after being enrolled [122,123]. Since DNMT inhibitors like AZA and DAC are known to be effective in the clinic for diseases like myelodys plastic syndromes that may result in part from transcriptional dysregulation due to epigenetic changes, there is interest in developing novel DNMT inhibitors that would be more effective and less toxic. One such putative agent is zebularine, a cytidine which has been reported to prevent early tumour development and also to inhibit growth of mammary gland tumours and breast cancer cells lines [124,125]. Zebularine is a novel DNMT inhibitor, which was developed as a more stable and less toxic drug [126]. Zebularine, similar to AZA-CR and 5-AZA-CdR, incorporates into DNA and forms a covalent irreversible complex with DNMT preventing the enzyme from methylating position 5 of cytosines clustered in regulatory CpG islands [127]. Recent studies showed the ability of zebularine to sustain the demethylation state of the 5′ region of the tumour suppressor gene CDKN2A/p16 and other methylated genes in T24, HCT15, CFPAC-1, SW48, and HT-29 cells [127]. It was also reported that zebularine inhibits growth of cancer cell lines but not normal cells [128].

Zebularine acts as a cytidine analogue containing a 2-(1H)-pyrimidinone ring that was originally developed as a cytidine deaminase inhibitor to prevent deamination of nucleoside analogues [129,130]. Zebularine is also a versatile starting material for the synthesis of complex nucleosides and is a mechanism based DNA cytosine methyltransferase inhibitor [131]. It acts primarily as a trap for DNMT protein by forming tight covalent complexes between DNMT protein and zebularine-substituted DNA [132]. In contrast, to other DNMT inhibitors, it has low toxicity in most tested cell lines and is quite stable with a half-life of 510 h at pH 7.4 [131, 133,134]. Because of its low toxicity, continuous administration of effective doses of zebularine alone or in combination with other DNMT inhibitors is feasible and this can result in the enhanced re-expression of epigenetically silenced genes in cancer cells [128].

Zebularine treatment led to increased p21 protein expression coupled with decreased cyclin B and D protein expression in MCF-7 cells and an increased percentage of cells in S-phase that indicates a zebularine induced S-phase arrest [135].This finding suggests errors in chromatin assembly that contribute to genome instability [136]. S-phase arrest can also be triggered by repression of histone synthesis in human cells [137]. The genomic instability induced by DNMT1 down regulation and repression of histone synthesis triggers the activation of S-phase

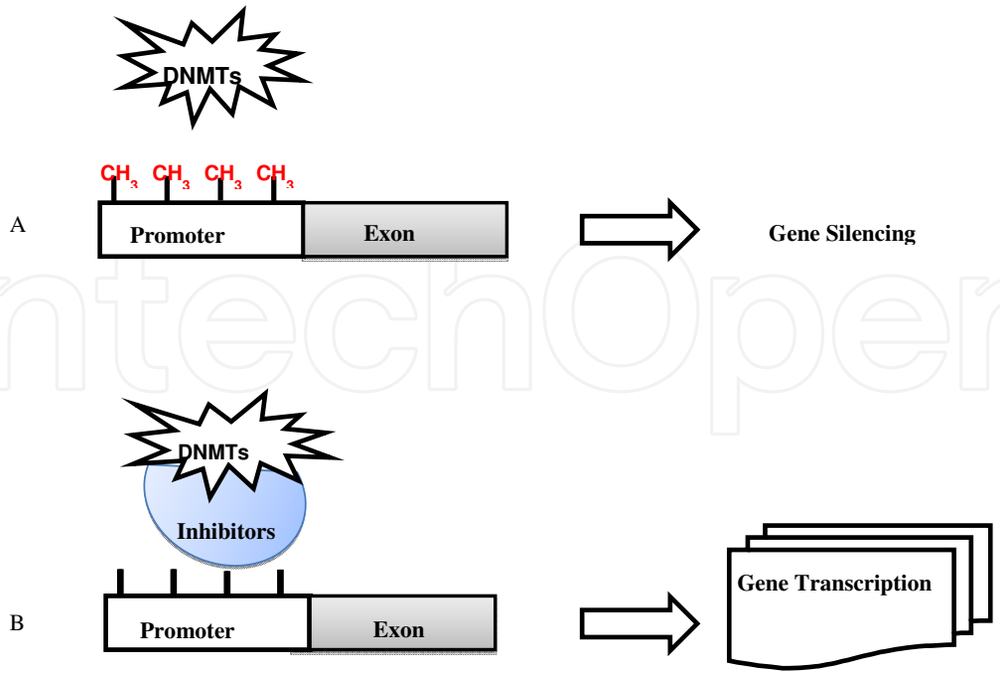

Figure 3. Activation of gene expression by nucleoside analogues, 5-azacytidine (vidaza or AZA,) and 5-aza-2′-deoxycytidine (decitabine or DAC), both are DNMTs inhibitors. (A) In active transcription is characterized by the presence of methylated cytosines within CpG dinucleotides (CH_3) which is sustained by DNMTs. (B) When a 5′-aza-cytosine ring replaces cytosine in the DNA, the methyl transfer does not take place and the DNMT is trapped on the DNA and the gene expression could restored again.

check point proteins like p21 (in MCF-7 cells) and/or down regulates cyclin-D to permit DNA repair before entering G2 phase.

The zebularine-mediated decrease in expression of global acetylated histones observed in our studies further supports our hypothesis. Several preclinical studies have evaluated zebularine as a possible therapeutic in cancer cell lines. Zebularine preferentially incorporates into DNA, leading to cell growth inhibition and increased expression of cell cycle regulatory genes in cancer cell lines compared with normal fibroblasts [135]. Additionally, to determine the ability of zebularine to prevent or treat breast cancer, Min et al, 2012 tested if daily oral treatment with zebularine affects mammary tumour growth in these MMTV-PyMT mice [124]. They observed a significant delay in tumour growth and a reduction of total tumour burden in the zebularine-treated mice. They have reported that the depletion of DNMTs in tumours excised from zebularine-treated mice and identified upregulation of 12 genes previously characterized as silenced by DNA hypermethylation. Zebularine treatment was shown to be associated with a dose-dependent depletion of DNMT1, DNMT3a, and DNMT3b proteins in the breast cancer cell lines MCF-7 and MDA-MB-231 [124]. Zebularine also depletes DNMT1 in T24 bladder carcinoma cells after 24 hours of treatment and partially depletes DNMT3b after 3 days of drug exposure [128]. Recently, Chen et al, (2012) have proofed in in vivo study that DNMT1 was depleted, and DNMT3b was significantly lowered (50% depletion) in the mammary tumours derived from zebularine-treated mice as compared with untreated mice [138]. Regardless of the mechanism of tumour growth inhibition, tumour cells eventually develop resistance to

zebularine treatment. Because it has been shown that zebularine and the HDAC inhibitor depsipeptide have a synergistic effect on the inhibition of breast cancer growth a combinatorial treatment with DNMT inhibitors and a combinatorial treatment with DNMT inhibitors and HDAC inhibitors may be warranted to overcome resistance to single-drug therapy.

Moreover, zebularine have been reported to depleted expression of all three DNMT proteins post-transcriptionally in both breast cancer cell lines at most doses tested. It has been reported that human cancer cells lacking DNMT1 or DNMT3b retain significant global methylation and gene silencing, but those lacking both DNMT1 and DNMT3b had >95% reduction in genomic DNA methylation and virtually absent DNMT activity [135]. The zebularine treatment specifically targets DNMT1, and reduced DNMT 3a and 3b protein expression, implying that treated cells may still retain substantial methylation [139]. Another study observed similar results in T24 bladder cancer cells continuously treated with zebularine for 40 days. In these cells zebularine had no effect on the expression of DNMT1, 3a or 3b mRNA but complete loss of DNMT1 and partial depletion of DNMT 3a and 3b protein were observed [128].

Previous findings observed that ER can be epigenetically silenced in some human breast cancer cell lines and HDAC or DNMT inhibitors could reexpress functional ER in ER negative breast cancer cells [140,141]. Further investigation demonstrated that treatment of ER negative MDA-MB-231 breast cancer cells with zebularine results in functional ER reactivation as manifested by expression of ER mRNA and its target gene, PR. This has been reported with a dose as low as 50μM, far lower than doses that induced apoptosis. Chromatin immunoprecipitation analysis of the ER promoter in zebularine-treated cells showed characteristics of an active chromatin as manifested by accumulation of acetylated H3 and H4 and release of DNMT1, 3a and 3b from the ER promoter region. Although reexpression of ER with zebularine was not as robust as with 5-azaDc, the low toxicity could enable continuous administration for sustained re-expression of ER cells [141].

However, several studies have shown that zebularine has some potential limitations such as less potent than the two FDA-approved DNMT inhibitors, azaC and 5-azaDc [133]. It is hypothesized that the reduced inhibitor potency is due to sequestration of the drug by cytidine deaminase, competitive inhibition of zebularine incorporation into DNA by increased cytidine and deoxycytidine that accumulate as a consequence of its cytidine deaminase properties, and preferential incorporation of zebularine into RNA over DNA [142]. For these reasons, the drug is effective only at very high doses, making administration more problematic. Its efficacy combined with a low toxicity profile makes it an attractive agent for combination or sequential therapy with other DNMT or HDAC inhibitors [143].

8. Combination of DNMT inhibitors

Based on the preclinical evidence previously described which suggests that a combination of epigenetic modifiers may be more successful in re-expression of silenced genes and restoration of hormonal therapy responsiveness, we have mentioned previously that the patients with advanced triple-negative and hormone-resistant breast cancer are being enrolled in an ongoing

multi-center phase 2 clinical trial and receive the combination of low dose of AZA [122]. Tumour biopsies prior to and after therapy are collected to assess modulation of candidate gene methylation and expression, such as the ER. Patients may transition to an optional continuation phase at the time of disease progression in which the same epigenetic therapy is administered with the addition of hormonal therapy [123]. Indeed, in a recently published trial exploring the combination of AZA and entinostat in advanced non-small cell lung cancer patients, investigators observed that the regimen was well tolerated and associated with a number of objective responses [144]. These included a complete response as well as a partial response in a patient without progression of disease for 2 years after completing the clinical trial. Interestingly, a number of patients were found to have unexpected major objective responses to subsequent anti-cancer strategies, raising the question as to whether these agents may prime tumour cells to respond to subsequent therapies. A phase 1/2 Canadian trial investigating the combination of decitabine and vorinostat in patients with advanced solid tumours or hematologic malignancies has also indicated clinical activity. Stabilization of disease for 4 or more cycles was observed in 29 % evaluable patients; two of these patients had metastatic breast cancer [145].

Moreover, cytidine deaminase destabilizes DNMT inhibitors like 5-azaDc, resulting in complete loss of their antineoplastic ability [146]. Hence administration of cytidine deaminase inhibitors like zebularine should theoretically potentiate therapeutic effects of 5-azaDc by slowing its degradation and stabilizing activity. Indeed, the combination of 5-aza-Dc and zebularine produced greater inhibition in cell proliferation and clonogenicity than either drug alone in leukemic L1210 and HL-60 cell lines [147]. Similarly, treatment of the AML-193 acute myeloid leukemic cell line, which has a densely methylated p15INK4B CpG island, with zebularine followed by the HDAC inhibitor, trichostatin-A, synergistically enhanced p15INK4B expression [134]. Consistent with these results, the combination of 50μMzebularine and 1μM 5-azaDc in breast cancer cells significantly inhibited cell proliferation compared with either drug alone. Similarly, zebularine significantly inhibited cell proliferation and colony formation in combination with low doses of vorinostat. Cheishvili et al, (2014) have investigated the combination of methylated DNA binding protein 2 (MBD2) depletion and DNMT inhibitor 5-azaCdR in breast cancer cells results in a combined effect in vitro and in vivo, enhancing tumour growth arrest on one hand while inhibiting invasiveness triggered by 5-azaCdR on the other hand. The combined treatment of MBD2 depletion and 5-azaCdR suppresses and augments distinct gene networks that are induced by DNMT inhibition alone. These data point to a potential new approach in targeting the DNA methylation machinery by combination of MBD2 and DNMT inhibitors [148].

The combination of DNMT inhibitors with standard chemotherapy has not been extensively evaluated in the breast cancer setting. Based on strong preclinical evidence that the addition of AZA could overcome platinum resistance through DNA hypomethylation, patients with both platinum resistant and refractory ovarian cancer received the combination of AZA and carboplatin after being enrolled into a phase 1b/2 study. The overall response rate of 22 % was observed in the platinum-resistant patients (disease progression within 6 months of platinum, n=18) suggesting that further evaluation of the combination was warranted [149]. Whether combining DNMT inhibitors with standard therapies or novel agents will result in clinical

benefit for patients with breast cancer remains to be seen. In the meantime, robust preclinical data should support the development of new concepts in order to maximize the chance of success with these agents in the solid tumour arena.

9. Conclusion

Future studies need to include a more detailed investigation of the methylation differences between breast cancer subtypes to determine whether there is a methylation signature that can identify breast cancer subtypes. It is also possible that DNA methylation subtypes are different to the subtypes identified by gene expression and may provide additional information that assists in the clinical setting. Further research is required to delineate these options and determine how subtypes identified by DNA methylation profiling differ to subtypes identified by gene expression. Laboratory studies have shown that AZA and DAC optimally inhibit DNA methylation when used at lower than cytotoxic doses with prolonged exposures. The exact impact of using epigenetic modifiers at an optimally epigenetic dose instead of a cytotoxic dose is yet unknown in solid tumours, despite the supposition that anti-cancer activity will be enhanced. Ongoing clinical trials in breast cancer patients aim to elucidate this question. Optimizing the use of the clinically available epigenetic modifiers is clearly important. An oral form of AZA is currently in development which may be far more convenient for patients than the intravenous and subcutaneous routes employed at this time. A number of new agents are also in development which may circumvent some of the limitations of the currently available drugs such as their in vivo deamination by cytidine deaminase and tendency to be subject to drug resistance.

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