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Review article

The role of DNA methylation in cancer development

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Abstract: Epigenetic modifications include DNA methylation and covalent modification of histones. These alterations are reversible but very stable and exert a significant impact on the regulation of gene expression. Changes in methylation of promoter or first exon may mimic the effect of mutations of various tumor suppressor genes (TSGs) or protooncogenes. Carcinogenesis can also result from aberrations in genomic DNA methylation that include hypermethylation and hypomethylation of promoter or first exon of cancer-related genes. Hypermethylation of promoter of various TSGs causes their transcriptional silencing. However, hypomethylation of regulatory DNA sequences activates transcription of protooncogenes, retrotransposons, as well as genes encoding proteins involved in genomic instability and malignant cell metastasis. The methylation of genomic DNA in malignant cells is catalyzed by DNA methyltransferases DNMT1 and DNMT3B, revealing significantly elevated expression in different types of cancers. The reversibility of hypermethylation can be used as target of therapeutic treatment in cancer. DNMT1 and DNMT3B inhibitors including 5-Aza-2'-deoxycytidine and antisense oligonucleotides have been applied in clinical trials of such treatment. Identification of aberrations of DNA methylation in cancer cells is a new field of investigation in carcinogenesis. We believe that epigenetic cancer diagnostic and therapy will be achieved in the next decades.

Key words: Tumor suppressor genes - Epigenetic modification - DNA methylation - Histone modifications

Introduction

Development of cancer may result from inherited mutations in the germ line or from changes in DNA sequences arising in somatic tissues during life. These mutations may abnormally enhance the function of protooncogenes, or erase effects of the tumor suppressor gene (TSG) products [47, 86]. Carcinogenesis can also result from aberrations of genomic DNA methylation that include hypermethylation and hypomethylation of promoter or first exon of cancer-related genes. Changes in methylation of promoter or first exon may mimic the effect of mutations of TSGs or protooncogenes. Hypermethylation of promoter and first exon of various TSGs causes their transcriptional silencing. However, hypomethylation of regulatory DNA sequences activates transcription of protooncogenes, retrotransposons, as well as genes encoding proteins involved in genomic instability and malignant cell metastasis. TSG products are normally involved in holding cellular growth at the checkpoint and inhibit expression of the tumorigenic phenotype [79, 82]. Inactivation or loss of TSG products removes a barrier of normal proliferation, which may result in malignant transformation. Oncogenes are derived from normal protooncogenes, that play key roles in cellular processes such as regulation of gene expression or cell growth and cytoplasmic signal transduction. Mutation of protooncogene results in formation of oncogene and its protein product exhibits sustained activity responsible for malignant transformation of cells [71].

5-Methylcytosine (m^5C) was first found in DNA of higher eukaryotes by Hotchkiss in 1948. Methylation of cytosine within cytosine-guanine dinucleotide (CpG) sequences in the 5'-position of the pyrimidine ring is used for epigenetic modulation of chromatin structure and regulation of gene expression in vertebrates. The presence of m^5CpG in genomic DNA is associated with condensation of chromatin, stabilization of chromosomes, transcriptional silencing of X chromosome, genomic imprinting and tissue-specific silencing of gene expression. This epigenetic regulation also coordinates gene expression during cell differentiation in mammalian embryogenesis [8, 88].

The methylation of mammalian genomic DNA is catalyzed by DNA methyltransferases (DNMTs) that can be divided into maintenance and de novo DNMTs. Expression of these DNMTs is significantly elevated in cancers of breast, colon, endometrium, prostate, stomach and in uterine leiomyomata [40, 70, 100, 103, 121, 124].

Two types of DNMT inhibitors are now in clinical trials of cancer treatment: nucleoside analog 5-Aza-2'-deoxycytidine (5-aza-CdR) which is incorporated into

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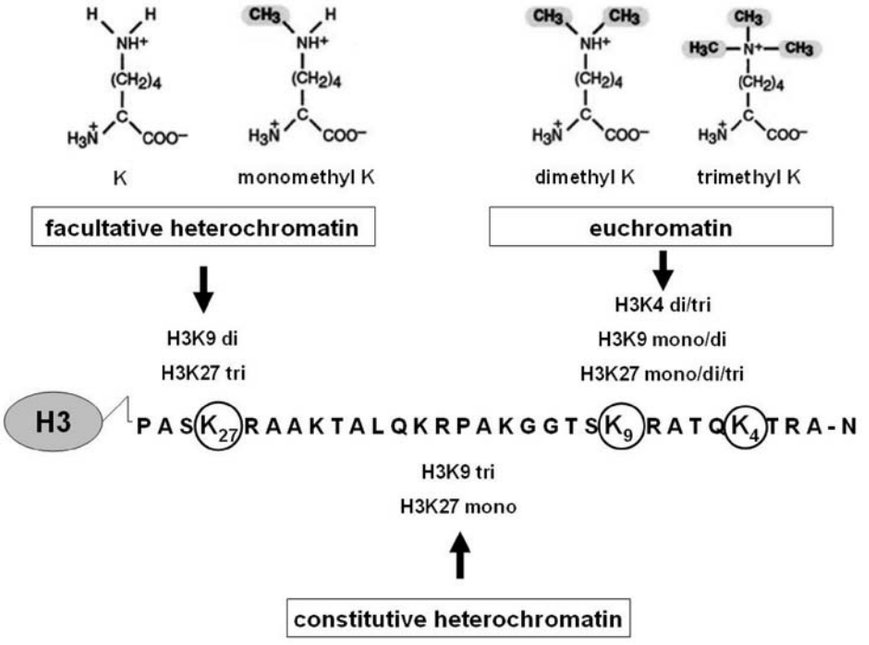

Fig. 1. Methylation level of K4, K9 and K27 amino acid residues of H3 histone corresponds to euchromatin, facultative and constitutive heterochromatin structures, respectively [38, 126].

DNA and inactivates DNMTs [125] and antisense oligonucleotide which induces degradation of DNMT1 mRNA and inhibits enzyme biosynthesis [69].

Epigenetic modification

Epigenetic modification is defined as a heritable, reversible change in gene expression that does not result from DNA sequence alterations [11, 27, 77, 114, 125].

Epigenetic modifications include DNA methylation and covalent modification of histones. These alterations are reversible but very stable and exert a significant impact on the regulation of gene expression and the development of vertebrates [99]. In mammals, m^5C is primarily located in CpG islands of promoter and first exon sequences, which exhibit highly conservative DNA methylation pattern. CpG islands are 0.5 to several kb DNA sequences that contain 60-70% of CpG dinucleotides [12, 25, 77, 92]. Human genome contains 29000 CpG islands [24] and approximately half of genes possess these islands [5, 27]. Completely methylated CpG islands are found only in promoters of untranscribed autosomal genes and transcriptionally silenced genes of inactive female X-chromosomes [12].

The N-terminal tails of histones are epigenetically modified by histone acetyltransferases (HATs), histone deacetylases (HDACs) and histone methyltransferases (HMTs). These enzymes acetylate, deacetylate or methylate ε and guanidine amine groups of histone Lys (K) or Arg (R) amino acid residues, respectively [126]. The multiple covalent modifications on the same histone tail create specific epigenetic patterns that switch genes between their active and transcriptionally inactive stages and correlate with distinct biological events [76, 126]. HDACs, HATs, HMTs and DNMTs play crucial roles in the epigenetic regulation of gene expression involved in carcinogenesis [36, 110]. Alteration of transcriptionally active euchromatin to transcriptionally inactive heterochromatin requires histone remodeling enzymes HDACs, HATs, HMTs (Suv39h1/2, G9a, EZH2) and ATP-dependent chromatin remodeling enzymes (*e.g.* hSNF2H) [36]. Methylation level of K4, K9 and K27 amino acid residues of H3 histone corresponds to euchromatin, facultative heterochromatin and constitutive heterochromatin structures, respectively (Fig. 1) [36]. HMT (Suv39h1) attaches methyl group to K9 amino acid residues of H3 histone that further recruits heterochromatin protein 1 (HP1). Interactions of HP1 with methylated K9 of H3 histone and transcriptional complex components are essential for formation and maintenance of heterochromatin structure [10, 17, 87, 105, 118].

Mammalian DNMTs interact with HP1, HDAC1, HDAC2, and HMTs, moreover DNMT3B additionally recruits the ATP-dependent chromatin remodeling enzyme hSHF2H involved in heterochromatin structure formation [36]. The covalent modification of histones associated with transcriptional silencing of TSGs and other genes is initiated by DNA methyltransferases.

DNA methyltransferases (DNMTs)

The mammalian DNMTs family encompasses DNMT1, DNMT2, DNMT3A and DNMT3B. This family is divided into maintenance and *de novo* methyltransfer-

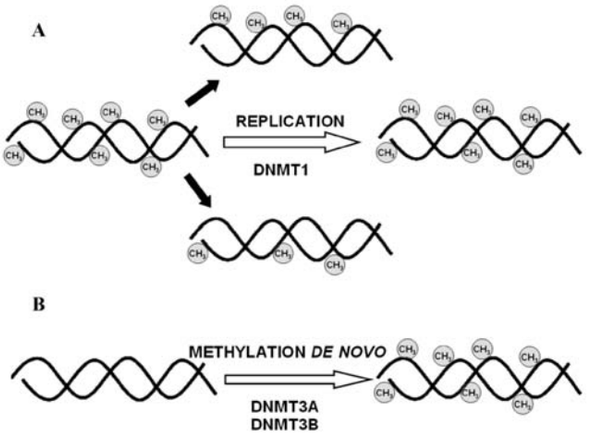

Fig. 2. Maintenance (A) and *de novo* DNMTs (B) methylate DNA. DNMT1 binds methyl groups to the hemimethylated DNA during replication, whereas DNMT3A and DNMT3B can add methyl groups to CpG dinucleotides of unmethylated DNA.

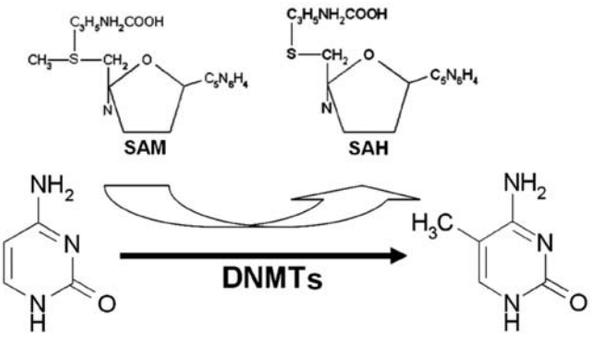

Fig. 3. Methylation of cytosine within CpG dinucleotides is catalyzed by DNMTs. S-adenosylmethionine (SAM) donates methyl groups and is converted to S- adenosylhomocysteine (SAH).

ases. Maintenance DNMT1 binds methyl groups to the hemimethylated DNA during replication, whereas *de novo* DNMT3A and DNMT3B add methyl groups to CpG dinucleotides of unmethylated DNA (Fig. 2). Homozygous loss of DNMT1, DNMT3A and DNMT3B is lethal in mice [15], that suggests the crucial role of these enzymes in the regulation of embryogenesis. DNMT1, DNMT3A and DNMT3B are required for formation of the established pattern of methylation in promoters and first exons of human genomic DNA [27]. In somatic cells, the pattern of DNA methylation is highly conservative and during cell division is kept by maintenance DNA methyltransferase (DNMT1) [27, 77, 92]. This enzyme is a component of DNA replication complex [111] and maintains the DNA methylation via addition of a methyl group to the 5-position of the cytosine ring within the CpG dinucleotides of newly synthesized DNA strand (Figures 2A and 3). DNMT1 forms three isoforms, which were found in somatic cells, pachytene spermatocytes, oocytes and preimplantation embryos. These transcript isoforms are produced by alternative usage of multiple first exon of DNMT1 primary transcript [73]. DNMT3A and DNMT3B enzymes are responsible for establishment of new methylation pattern in genomic DNA (Fig. 2B) [15, 27, 121].

Mammalian DNMT1, DNMT3A and DNMT3B are composed of the N-terminal regulatory and the C-terminal catalytic domains that are linked by a short fragment of repeated GK dipeptides (Fig. 4). The N-terminal domains of DNMT1 and DNMT3B do not exhibit extensive homology of primary structure. These differences are responsible for distinct functions of N-regions in these enzymes. The DNMT1 requires interaction between the N- and C-terminal domains for catalytic activity. Separated C-terminal domain of DNMT1 is catalytically inactive despite the presence of the highly conserved sequence motifs

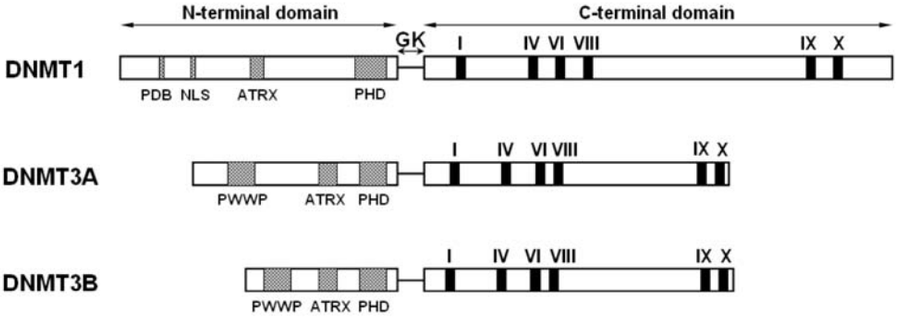

Fig. 4. Members of mammalian DNMTs family. DNMT1, DNMT3A and DNMT3B consist of N-terminal regulatory and C-terminal catalytic domains that are linked by repeated GK dipeptides. The N-terminal domain possesses nuclear localization signal sequence (NLS) responsible for localization of DNMTs in the nucleus. The N-fragment of DNMTs also contains proliferating cell nuclear antigen binding domain (PDB), a cysteine rich zinc finger DNA binding motif (ATRX), and polybromo homology domain (PHD) targeting DNMTs to the replication foci. However, PWWP tetrapeptide is only present in N-terminal domains of DNMT3A and DNMT3B and interact with histones [48]. The C-terminal domain contains six conservative motifs I, IV, VI, VIII, IX and X. Motifs I and X form S-adenosylomethionine binding site, motif IV binds cytosine at the active site, motif VI possesses glutamyl residue donating protons, and motif IX maintains the structure of the target recognition domain (TRD) usually located between motifs VIII and IX, that makes base-specific contacts in the major groove of DNA [14, 91, 122, 123].

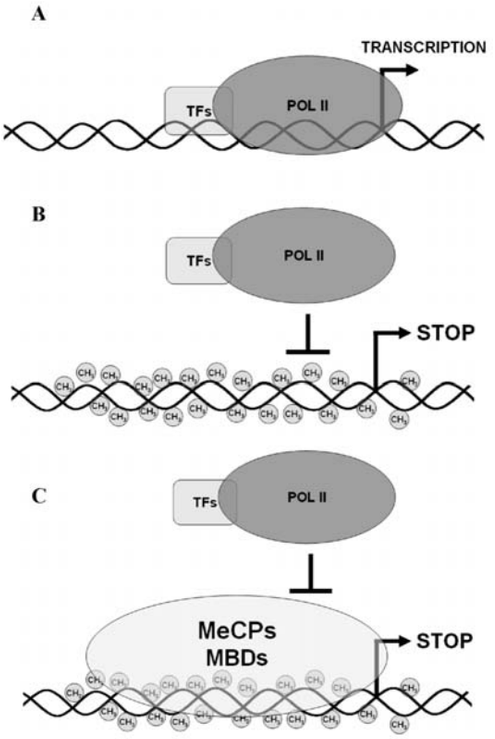

Fig. 5. Repression of transcription via CpG dinucleotide methylation. Promoter sequence binds transctiption factors (TFs) and RNA polymerase II (POL II) that initiates transcription (A). Methylation of CpG within promoter binding site directly inhibits requirement of TFs and represses transcription (B). Methylated DNA binds m^5CpG binding (MeCPs) and m5CpG-binding domain (MBDs) proteins forming spatial obstacle that prevents binding of TFs to promoter sequence.

typical of active DNMTs. In contrast to DNMT1, C-terminal domain of DNMT3A and DNMT3B is active without interaction with their N-regulatory regions. These differences between DNMT1 and *de novo* DNMTs indicate significantly disparate mechanism that regulate methylation activity of these enzymes.

The presence of m^5CpG dinucleotides in DNA sequence directly inhibits transcription or recruits proteins that specifically recognize methylated DNA and initiate the remodeling of euchromatin into heterochromatin structure.

DNA hypermethylation inhibits gene transcription

Two mechanisms have been proposed to account for transcriptional repression via DNA methylation. In the first mechanism, DNA methylation directly inhibits the binding of transcription factors (TFs) such as AP-2, c-Myc/Myn, E2F and NFkB to their binding sites within promoter sequence. In this mechanism, CpG dinucleotides have to be present within the binding site of TFs, which are sensitive to methylation of CpG dinucleotides (Fig. 5).

The second mode of repression includes a binding of proteins specific for m^5CpG dinucleotides to methylated DNA. Methylated DNA recruits m^5CpG-binding (MeCP) and m^5CpG-binding domain (MBD) proteins. MeCP1 and MeCP2 bind specifically to methylated DNA in whole genome and form spatial obstacle that unable binding of TFs to promoter sequences (Fig. 5). MeCP1 represses transcription of specific genes, which are controlled by densely methylated promoters containing more than ten m^5CpG dinucleotides. MeCP2 can bind to a single symmetrically located m^5CpG pair in two DNA strands [44].

MBD protein family includes MBD1, MBD2, MBD3 MBD4, and uncharacterized Kaiso complex, which binds to methylated DNA. MBD1 binds to symmetrically methylated CpG dinucleotides and inhibits gene expression by blocking TFs interaction with the promoter [33]. MBD2 may bind to methylated DNA and actively demethylates DNA *in vivo* and *in vitro* [81, 111]. MBD3 is targeted to methylated DNA through association with the MBD2 and is a component of the chromatin remodeling protein complex [9]. MBD4 is thymine and uracil glycosylase involved in DNA mismatch repair, formed during C and m^5C deamination, respectively [34, 45]. MBD1, MBD2, MeCP2, and Kaiso complex are able to interact with HDAC1 and HDAC2, which deacetylate histones and remodel chromatin structure [12, 15, 27, 67, 92].

Malignant cells also exhibit the hypomethylation of various regions in DNA that is responsible for increase in expression of genes promoting proliferation, invasions and metastases of cancer cells.

Role of DNA hypomethylation in cancer development

Global hypomethylation of genomic DNA is observed in numerous tumor cells and is responsible for overexpression of protooncogenes, growth factors and genes which via their protein products are involved in cancer cell proliferation, invasion, and metastasis [111]. Expression of tumor genes such as urokinase type plasminogen activator (*PLAU*), heparanase and calcium binding protein (S100A4) is induced by DNA hypomethylation. Protein products of these genes promote movement of single malignant cells through the extracellular matrix to lymphatic or blood capillaries. Advanced stage tumors produce proteases that are involved in degradation of the extracellular matrix components and promote metastasis of malignant cells. Heparanases are released from malignant cells and degrade heparan sulfate proteoglycans of the extracellular matrix. S100A4 protein regulates production of matrix-degrading enzymes responsible for remodeling of the extracellular matrix and increase in tumor cell proliferation and motility [102].

The most common protease involved in malignant cell metastasis and invasiveness is the PLAU enzyme. This protease is expressed in cancers of breast, prostate and other organs [83, 85]. The expression of *PLAU* in invasive human breast cancer MDA-231 cells is con-

stitutive, since promoter of this protease gene is unmethylated [84]. Szyf *et al.* [111] treated breast cancer MDA-231 cells with S-adenosylmethionine, a donor of methyl group and activator of DNMTs (Fig. 2). The activated DNMTs increased DNA methylation that inhibited prometastatic gene expression and reduced invasiveness of MDA-231 cells in mouse model. *PLAU* promoter is methylated in breast cancer MCF-7 cells and in noninvasive breast tumor cells. The treatment of breast cancer MCF-7 cells with the DNMT inhibitors resulted in production of PLAU protease and increase in invasiveness of MCF-7 cells in mouse model [111].

Insulin-like growth factor 2 (IGF2) is a growth factor that stimulates malignant cell proliferation [104]. In nonmalignant cells, *IGF2* is transcribed only from one allele, while the second one is imprinted and transcriptionally inactive. Hypomethylation of DNA in tumor genome results in a loss of imprinting of the second allele and increased biallelic expression of *IGF2* that efficiently stimulates proliferation of cancer cells [74, 120].

Hypomethylation of retrotransposons also contributes to carcinogenesis via destabilization of the genome by insertional mutagenesis and recombination between non-allelic repeats. Long interspersed nuclear elements (LINEs) belong to retrotransposons, which are heavily methylated in all cell types in mammals. Hypomethylation of LINEs induces transcriptional activation of these sequences, which contributes to genomic instability and facilitates tumor progression. The methylation of CpG dinucleotides in LINEs and other retrotransposon sequences is host defense against retrotransposon activation [14]. Moreover, most cellular genes contain multiple retrotransposons within introns, where their transcriptional activation may interfere with regulation of host gene expression. Hypomethylation of LINEs has been observed in colon cancer and chronic lymphocytic leukemia and contributed to the development of malignant phenotype of these cells [18].

The exact reasons for global DNA hypomethylation in malignant cells are still unclear. It has been suggested that it can result from complete or partial deficiency of numerous enzymes involved in methyl transport at the cellular level [107]. However, this hypothesis does not explain the simultaneous hypermethylation of TSGs promoter and hypomethylation of other promoter genes. Some findings suggest that overexpression of catalytic inactive variants of DNMT3B specific for DNA sequences may shield CpG dinucleotides from active DNMTs [122].

DNMT3B isoforms contribute to hypermethylation and hypomethylation of genomic DNA promoting development, invasion and metastases of cancer cells

DNMT3B gene is composed of 23 exons, 22 introns and is located on 20q11.2. The enzyme is abundantly expressed in embryonic stem (ES) cells, but DNMT3B expression is decreased upon differentiation of ES cells and remains low in adult somatic tissues. However, overexpression of various DNMT3B splice variants (Fig. 6) has been reported in tumor cells suggesting that this enzyme is responsible for epigenetic modification of DNA [13].

Primary transcript of DNMT3B can be spliced into five different mRNA isoforms DNMT3B1, DNMT3B2, DNMT3B3, DNMT3B4 and DNMT3B5 (Fig. 6). DNMT3B1 and DNMT3B2 contain all the highly conserved motifs I, IV, VI, IX and X as well as target recognition domain (TRD) sequence in the C-terminal domain (Figures 4 and 6). DNMT3B2, DNMT3B3, DNMTB4 and DNMT3B5 isoforms do not contain exons 10 and 11 in the mRNA sequences. Moreover, DNMT3B3, DNMT3B4 and DNMT3B5 lack 21-22, 21 or 22 exons, respectively (Fig. 6). Huntriss *et al.* [50] and Weisenberger *et al.* [122] demonstrated that transcript for DNMT3B1 mRNA was only present in ES cells but was absent from differentiated somatic cells. This suggests that different DNMT3B isoforms are predominantly expressed in human somatic as well as malignant cells. DNMT3B2 variant contains all conserved motifs of the active site and is abundantly expressed in breast cancer cell line MCF-7. However, the effect of depletion of polypeptide fragment corresponding to exons 10 and 11 on function of this DNMT3B variant is not fully understood [122]. DNMT3B3 lacks the conserved motif VIII, TRD sequence and the nine amino acids of motif IX (Figures 4 and 6). However, the expression of DNMT3B3 variant in LD419 fibroblasts and T24 bladder cancer cells suggests that human DNMT3B3 possesses catalytic activity and can methylate DNA. DNMT3B4 and DNMT3B5 lack exons that correspond to several conservative motifs within the catalytic domain and these isoforms probably are not enzymatically functional [122]. Recently, it has been reported that DNMT3B4 functions as a negative regulator of DNA methylation in human hepatocellular carcinoma (HCC) cells [56 , 97, 122]. DNMT3B4 can bind to DNA sequences and compete with the enzymatically active DNMT3B3 variant for targeting to pericentromeric satellite regions of HCC cells. Overexpression of DNMT3B4 in HCC cells induces DNA demethylation in pericentromeric satellite regions and plays a critical role in the chromosomal instability and aberrant expression of cancer-related genes [97].

Depletion of DNMT1 and DNMT3B but not DNMT3A reactivates the expression of methylation-silenced genes and induces apoptosis of human cancer cells but not nonmalignant cells [13, 115]. This may suggest that methylation silencing of TSGs in numerous types of cancer is mainly catalysed by DNMT1 and DNMT3B [7, 90, 93, 97].

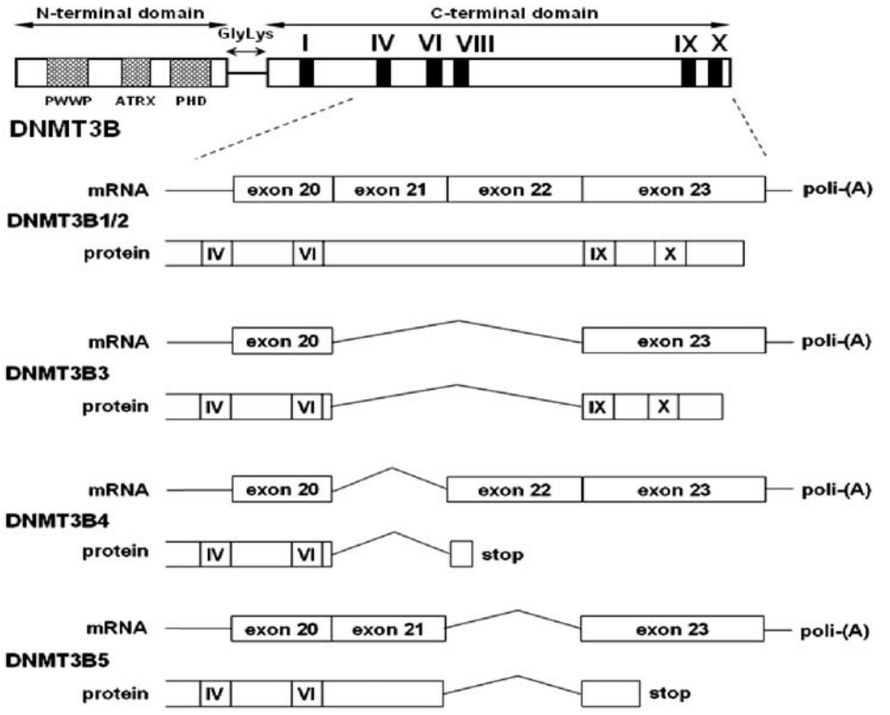

Fig. 6. Human primary DNMT3B transcript is spliced into five different isoforms: DNMT3B1, DNMT3B2, DNMT3B3, DNMT3B4 and DNMT3B5. DNMT3B1 and DNMT3B2 contain all the highly conserved motifs I, IV, VI, IX and X as well as TRD sequence in the C-terminal domain (Fig. 4). DNMT3B2, DNMT3B3 DNMTB4 and DNMT3B5 isoforms do not contain exons 10 and 11 in the mRNA sequences. Moreover, DNMT3B3, DNMT3B4 and DNMT3B5 lack exons 21-22, 21 or 22, respectively.

Methylation of tumor suppressor gene promoters in cancer

DNA hypermethylation is responsible for epigenetic inactivation of TSGs expression in cancer cells. Increase in mRNA and protein biosynthesis of DNMT1 and DNMT3B in various cancer types significantly correlates with hypermethylation of CpG islands located in the promoter regions of cyclin-dependent kinase inhibitor 2A (*CDKN2A)*, cyclin-dependent kinase inhibitor 2B (*CDKN2B*), E-cadherin (*CDH1*), human mismatch repair gene (*MLH1*), retinoblastoma 1 (*RB1*), TIMP metallopeptidase inhibitor 3 (*TIMP3*) and other TSGs (Table 1). Methylation of these TSG promoters is associated with the complete loss of TSG protein products in cancer cells and development of malignant phenotype [75].

Rhee *et al.* [89] reported that DNMT1 and DNMT3B cooperated in order to silence *CDKN2A* and *TIMP3* gene expression in human colon cancer HCT116 cells. Replacement of conservative motifs in the C-terminal domain of DNMT1 and DNMT3B by neomycin or hygromycin resistance genes was used to produce double knockout cells with inactive DNMTs. This resulted in greater than 95% reduction of genomic DNA methylation and reexpression of *TIMP3* and *CDKN2A*, which was associated with growth suppression of HCT116 cells.

Etoh *et al.* [31] showed in gastric cancer cells significantly reduced expression of the *CDKN2A*, *MLH1*, and cell-cell adhesion protein encoded by *CDH1* gene. Loss of expression of mRNAs and proteins of TSGs correlated with intracellular elevation of DNMT1 content and complete methylation of the *CDKN2A*, *MLH1* and *CDH1* promoters in gastric cancer cells. Similar relationship between DNMT1 and DNMT3B expression and *CDH1* promoter methylation was observed by Girlaut *et al.* [40] in breast carcinoma. Increased expression of DNMT1 and DNMT3B were also correlated with the increase in breast cancer aggressiveness [40]. The epigenetic inactivation of *CDKN2A* and *MLH1* genes expression was also associated with increased level of DNMT1 and DNMT3B contents in colon cancer cells [55].

Overexpression of DNMT1 and DNMT3B were also found in ovarian cancer cell lines HeyA8, HeyC2, SK-OV-3 and PA-1 [3]. HeyA8 and HeyC2 cells exhibited higher expression of DNMT1 while SK-OV-3 and PA-1 cells overproduced DNMT3B compared to normal ovarian epithelial cells [3].

Agoston *et al.* [2] revealed increase in DNMT1 protein stability in breast cancer MCF-7 cells compared to normal human mammary epithelial cells (HMECs). It has been observed that protein levels of DNMT1 are elevated in breast cancer MCF-7 cells without increase in DNMT1 mRNA level. Elevation of DNMT1 protein level in MCF-7 cells was dependent on the absence of the N-terminal, 120-amino acid fragment which was responsible for longer half-life of this enzyme. The 120-amino acid sequence functions as a domain for ubiquitination and proteasome degradation of DNMT1 in HMECs. The lack of N-terminal domain in DNMT1 may result from improper post-translational modifications of this enzyme in MCF-7

cells. The longevity of DNMT1 can be responsible for elevation of this enzyme activity and increase in methylation of DNA sequences corresponding to promoter of TSGs [2]. Increase in DNMT1 protein stability was also observed in prostate cancer cell line LNCaP and colon cancer cell line HCT116 compared to normal prostate epithelial cells and normal human dermal fibroblasts [2].

Acute myelogenous leukemia (AML) cells tend to express higher levels of DNMT1 and DNMT3B. Furthermore, AML cells are characterized by intense methylation of *CDKN2A*, *CDKN2B*, estrogen receptor 1 (*ESR1*), and *RB1* promoters [72].

DNMT3B expression is pronounced in cells of bladder, colon, kidney and pancreas cancers [91]. A significant correlation between DNMT3B expression and cancer grade was observed in endometrial cancers [53]. Saito *et al.* [96] observed high level methylation of CpG dinucleotides in *CDKN2A* promoter and significant overexpression of DNMT3B in hepatocellular carcinomas compared to the corresponding noncancerous liver tissue.

Selective depletion of DNMT1 with DNMT and HDAC inhibitors has been shown to induce demethylation of promoters and reexpression of the silenced TSGs. These observations suggest that hypermethylation of TSGs promoter can be reversed - which may be a target for possible therapy.

DNA methylation and acetylation-targeted drugs in cancer therapy

The reversibility of epigenetic changes can be a target of therapy in cancer [27]. Drugs that epigenetically influence chromatin structure are divided into DNMT and HDAC inhibitors. DNMT inhibitors include 5-Aza-cytidine (5-Aza-CR), 5-Aza-2'-deoxycytidine (5-Aza-CdR) and 1-β-D-ribofuranosyl-2(1H)-pyrimidinone (Zebularine) [22, 67, 125]. 5-Aza-CR and 5-Aza-CdR were first synthesised by Sörm *et al.* in 1964. These drugs *in vitro* profoundly diminish the activity of DNMT1, DNMT3A and DNMT3B at micromolar concentrations and induce demethylation of *CDKN2A, RB1, MLH1* and other TSG promoters in cancer cells [92, 125]. The use of DNMT inhibitors such as 5-Aza-CR and 5-Aza-CdR can correct silent gene expression patterns and revert cells back to more normal functions. 5-Aza-CR is phosphorylated by uridine-cytidine nucleotide kinases to 5-Aza-CR diphosphate which can be reduced by ribonucleotide reductase to 5-Aza-CdR diphosphate and subsequently incorporated into DNA. 5-Aza-CdR is phosphorylated by deoxycytidine, mono- and diphosphate nucleoside kinases and then incorporated into DNA. 5-Aza-CdR nucleotide of DNA forms a covalent bond with the DNMTs and inactivates these enzymes resulting in inhibition of maintenance and *de novo* methylation of genomic DNA (Fig. 7) [74, 92, 111]. Treatment of HCT116 human colon cancer cells with 5-Aza-CdR depletes DNMT1 enzyme activity, induces expression of *MLH1* and causes growth arrest of these cells [90].

Zebularine was first synthesized by Kim *et al.* in 1986 [63] as an inhibitor of cytidine deaminase. This substance is converted to 2'-deoxyZebularine 3-phosphate and then is incorporated into DNA. 2'-deoxyZebularine nucleotide of DNA irreversibly inactivates maintenance and *de novo* DNMTs by covalently binding to these enzymes (Fig. 7) [20, 21, 127]. Cheng *et al.* [22] reported that treatment of bladder cancer T24 cells with Zebularine induced *CDKN2A* gene expression, prevented remethylation of *CDKN2A* promoter and arrested growth of the cancer cells. Zebularine displays two desirable qualities as a chemotherapeutic agent; this drug is highly stable in acid and neutral solutions, and exhibits low toxicity *in vitro* as well as *in vivo* in mouse model [28]. These properties of Zebularine allow continuously treating cancer cells *in vivo* in mouse model and prevent remethylation of TSGs promoter for a long time period [20, 125].

HDAC inhibitors including hydroxamic acid, butyrates, trichostatin A, valproic acid and others (Table 2) block deacetylation of histones and increase gene expression. [15, 125]. Primary structure of the active site of various HDACs is highly conservative and contains Zn^{+2} cation [32]. Hydroxamic acid forms complex with Zn^{2+} that is essential for function of the active and binding sites of HDACs. Nanomolar concentrations of hydroxamic acid inhibit HDACs *in vitro* and induce growth arrest and apoptosis of malignant cells [15].

The novel useful DNA-demethylating agents are antisense RNA and short interference RNA (siRNA). These short RNA fragments are complementary to mRNA and induce degradation of the target transcripts [37]. MG98 is an antisense oligonucleotide, which hybridizes to the 3' untranslated region of the DNMT1 mRNA sequence and causes degradation of this transcript [27, 42]. Antisense oligonucleotides and siRNA directed against DNMT1 mRNA reduced DNMT1 protein levels. Treatment of thoracic malignancies including lung cancer CALU-6, A549 cells and SJGT5, as well as BIC esophageal adenocarcinoma with antisense oligonucleotides for DNMT1 and DNMT3B resulted in depletion of these DNMTs. Decrease in DNMTs activity was associated with *RASSF1A* and *CDKN2A* reexpression and growth arrest of CALU-6, A549, SJGT5 and BIC cells [58]. siRNA directed to DNMT1 mRNA also induced demethylation of *CDKN2A* promoter and expression of this gene; its protein product inhibited growth of human colon cancer HCT116 cells [42, 90]. These pieces of evidence have shown that antisense oligonucleotides and siRNAs are useful as precise anticancer tools activating expression of TSGs and arrest growth of malignant cells.

Inhibitors of DNMTs and HDACs are currently tested in clinical trials with patients that suffer from

Table 1. TSGs promoter and first exon that are commonly methylated in human cancers

TSGs	Function	Cancer type with identified hyper-methylation	References
AKAP12	signal transduction	gastric	[23]
APC	cell proliferation, migration and adhesion	colorectal	[49]
BRCA1	DNA damage repair	breast, ovarian	[30]
CASP8	apoptosis	neuroblastoma	[19]
CDH1	cell-cell adhesion	breast, prostate, colorectal	[26, 43]
CDKN2A	cyclin-dependent kinase inhibitor	lymphoma	[46]
CDKN2B		leukemia	[72]
DAPK1	interferon-induced apoptosis	lung, B-cell lymphomas	[59]
GSTP1	prevention of oxidative DNA damage	prostate	[68]
ING1	cell growth and apoptosis	lung	[54]
KISS1	chemotaxis and invasion	breast	[106]
LATS2	androgen receptor-mediated transcription		[112]
MLH1	DNA mismatch repair gene	colon	[117]
PTEN	negatively regulating AKT/PKB signaling pathway	colorectal	[41]
RASSF1	cell cycle arrest	kidney	[78]
RB1	represses the transcription of cellular genes	retinoblastoma	[109]
SMAD4	cell proliferation	colorectal	[4]
SOCS1	negative regulator of cytokine signaling	pancreatic	[35]
STK11	signal transduction	lung	[98]
TIMP3	inhibitor of the matrix metalloproteinases	colon, renal, brain	[6]
TP53	cell cycle regulator	leukemia	[1]
WWOX	transcription regulation, protein degradation	lung, breast, bladder	[51]
VHL	promotes angiogenesis	renal carcinoma	[78]

AKT/ PKB - a serine/threonine protein kinase AKT or B

cancer [52, 60, 94, 108]. 5-Aza-CR (Vidaza) is the first DNA hypomethylating agent that was approved by Food and Drug Administration in 2004, for the treatment of all subtypes of myelodysplastic syndrome (MDS). Usage of this drug in cancer therapy exhibits clinical benefits including elimination of transfusion and complete or partial normalization of blood counts and the percentage of bone marrow blasts in responding patients [52]. 5-Aza-CdR (Decitabine) is another DNMT inhibitor used in the phase I clinical trials in patients with hematologic malignancies. Decitabine treatment resulted in myelosuppression in patients that received low doses of this drug [52]. It has been reported that treatment of MDS patients with Decitabine results in partial *CDKN2B* promoter demethylation in peripheral leukocytes or bone marrow cells. Currently, Decitabine

Table 2. DNMT and HDAC inhibitors

Target enzyme	Inhibitors	References
DNMT1 DNMT3A DNMT3B	5-Azacytidine	[29]
	5-Aza-2'-deoxycytidine	[57]
	Hydralazine	[101]
	MG98 (DNMT1 antisense)	[108]
	Procainamide	[101]
	Procaine	[119]
	siRNA	[69]
	Zebularine	[21]
HDACs	Apicidin	[66]
	Butyrates	[115]
	Depudecin	[65]
	Hydroxamic acid	[95]
	Oxamflatin	[63]
	Pyroxamide	[16]
	siRNA	[80]
	Trichostatin A	[116]
	Trapoxin A	[61]
	Valproic acid	[64]

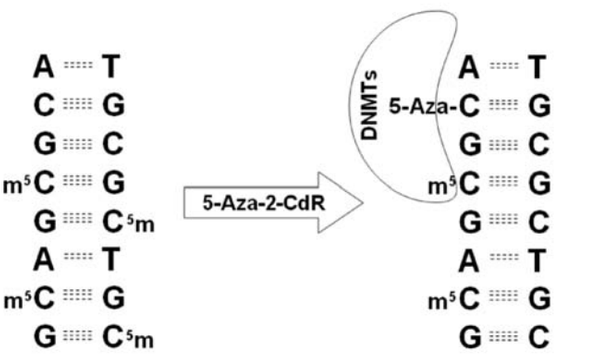

Fig. 7. Mechanism of DNMT inhibition by nucleoside analogs. 5-Aza-CdR or Zebularine nucleotides are incorporated into DNA sequences, forming covalent bonds with DNMTs and irreversibly inactivating these enzymes.

is successfully applied in the phase II clinical trials [52]. The phase I clinical trial study of the MG98 oligonucleotide revealed that this drug was able to inhibit progression of solid tumors in patients for a few months [108]. Currently, MG98 is tested in phase II clinical trials in patients with mesothelioma, cancer of lung, kidney and colon [108].

Hydroxamic acid and phenylbutyrate are two HDAC inhibitors which are tested in phase I and II clinical trials, respectively, in patients with hematological and solid tumors [60]. The phase I clinical trials indicated that these HDAC inhibitors could be administered safely to patients at doses that inhibit HDAC activity *in vivo*. It has been also observed that hydroxamic acid and phenylbutyrate treatment results in regression of tumors and clinical improvement in tumor related symptoms [60].

The best results of transcriptional activation of TSGs are provided by therapy that uses simultaneously inhibitors of DNMTs and HDACs [39]. The pharmacokinetics of 5-Aza-CR and phenylbutyrate were preliminary tested in patients with solid tumors and hematologic malignancies [94]. These results indicate that the combined strategies of anticancer therapy may ultimately offer the most successful approach, leading to reduction in the dose and in side effects of each individual agent [27]. Additionally, the combined use of DNMT and HDAC inhibitors makes these drugs more versatile and results in a synergistic effect on induction of apoptosis, differentiation, and cycle arrest of various solid tumor cells [128].

Epigenetic defects in cancer cells is a new area of carcinogenesis investigation. We presume that epigenetic cancer diagnostics and therapy will open new perspectives in the next decade

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