



DNA methylation: gene expression regulation

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ABSTRACT

Epigenetic modifications are responsible for the modulation of gene expression without affecting the nucleotide sequence. The observed changes in transcriptional activity of genes in tumor tissue compared to normal tissue, are often the result of DNA methylation within the promoter sequences of these genes. This modification by attaching methyl groups to cytosines within CpG islands results in silencing of transcriptional activity of the gene, which in the case of tumor suppressor genes is manifested by abnormal cell cycle, proliferation and excessive destabilization of the repair processes. Further studies of epigenetic modifications will allow a better understanding of mechanisms of their action, including the interdependence between DNA methylation and activity of proteins crucial to the structure of chromatin and gene activity. Wider knowledge of epigenetic mechanisms involved in the process of malignant transformation and pharmacological regulation of the degree of DNA methylation provides an opportunity to improve the therapeutic actions in the fight against cancer.

KEY WORDS: transcriptional activity, epigenetics, carcinogenesis

Introduction

The human genome is composed of approximately three billion base pairs and contains large amounts of genetic information. Although different types of cells share the same DNA, they display different phenotypes. It indicates that regulated access to the genetic information plays an important role in

understanding cell identity and, thus, human development (Sharma *et al.* 2010, Jurkowski *et al.* 2015). The term "epigenetics" was coined by Conrad Waddington and defined as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into

being” (Goldberg *et al.* 2007, Kunwor *et al.* 2015). Initially, this definition referred to epigenetics in context of embryonic development, however it has evolved over time and nowadays, epigenetics is described as "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence" (Sharma *et al.* 2010, Brait & Sidransky 2011). Most of these changes occur during differentiation and are maintained through multiple cell divisions, allowing cells to develop distinct identities despite having the same genetic information. Epigenetic modifications such as cytosine methylation, histone post-translational modifications as well as the nucleosome positioning along the DNA, mediate heritability of gene expression patterns (Goldberg *et al.* 2007, Carone *et al.* 2010, Greer *et al.* 2011). The set of these modifications, known as the epigenome, regulates the accessibility of the genetic information to the cellular machinery, providing a mechanism for cell diversity (Lee & Lee 2012). Failure to properly maintain epigenetic marks can result in disruption of different signaling pathways by their inappropriate activation or inhibition, and therefore, lead to disease such as cancer. Recent studies show that both genetic and epigenetic alterations are equally important and can contribute to all stages of human cancer development (Kresse *et al.* 2012, You & Jones 2012, Marquardt *et al.* 2013). In contrast to genetic mutations, epigenetic modifications are reversible, which makes them an attractive and promising target for cancer therapy (Esteller 2008, Khan *et al.* 2008, Sadikovic *et al.* 2008, Riggins 2014, Yang *et al.* 2014, Kunwor *et al.* 2015, Nakamura *et al.* 2015).

Methylation patterns in normal cells

Chromatin is composed of repeated structural units, known as nucleosomes,

which consist of approximately 146 base pairs of DNA wrapped around a histone protein octamer made up of two copies of each of the four histone proteins such as H2A, H2B, H3 and H4 (Flis *et al.* 2007, Sharma *et al.* 2010, Lee & Lee 2012). DNA methylation, covalent and non-covalent histone modifications, non-coding RNAs including miRNAs are epigenetic modifications associated with alteration of the dynamics of chromatin structure, its accessibility and compactness. The distinct patterns of these modifications regulate the functioning of the genome and the way it manifests itself in different types of cells, stages of development and various diseases, including cancer, and thus protect the identity of the cell (Sharma *et al.* 2010).

DNA methylation is a reversible addition of a methyl group (-CH₃) to either adenine or cytosine bases. In mammalian cells, methylation occurs at the fifth carbon of the cytosine pyrimidine ring within CpG dinucleotides that can be concentrated in short CpG-rich DNA regions known as CpG islands or regions of large repetitive sequences, such as retrotransposon elements and centromeres (Saxonov *et al.* 2006, Flis *et al.* 2007, Łukasik *et al.* 2009, Guz *et al.* 2010, Sharma *et al.* 2010). CpG islands are frequently located at the 5' regulatory regions of a gene and are associated with approximately 60–70% of human gene promoters. Methylation of the CpG island promoter, catalyzed by DNA methyltransferases (DNMTs) that use S-adenosyl-L-methionine as the donor of methyl groups, prevents binding of transcription factors which results in gene silencing (Saxonov *et al.* 2006, Łukasik *et al.* 2009, Guz *et al.* 2010). DNMT1, often referred to as the "maintenance" methyltransferase, is one of the three active DNA methyltransferases identified in mammals. It recognizes and binds to

hemimethylated CpG sites generated during DNA replication in which the parental strand remains methylated, unlike the newly synthesized one. In order to maintain existing CpG methylation patterns, DNMT1 attaches a methyl group to the cytosines on the daughter strand (Hirasawa *et al.* 2008). Two other methyltransferases, DNMT3A and DNMT3B, target previously unmethylated cytosines and establish DNA methylation patterns early in development, and therefore are called *de novo* methyltransferases (Flis *et al.* 2007, Heinz *et al.* 2007, Łukasik *et al.* 2009, Guz *et al.* 2010, Sharma *et al.* 2010, Ficz & Gribben 2014, Kunwor *et al.* 2015).

The pattern of DNA methylation is not only a consequence of attachment of methyl groups to cytosine but also DNA demethylation (Guz *et al.* 2010, Tan *et al.* 2012, Hill *et al.* 2014). Demethylation is a reaction of removal of the methyl group and can be considered as DNA replication-dependent and independent (Guz *et al.* 2010, Hill *et al.* 2014). This process requires several steps and the first one is oxidation of 5-methylcytosine (5mC) to generate 5-hydroxymethylcytosine (5hmC) with the participation of Tet proteins. It is assumed that the more diverse and stable the cell is, the less 5hmC can be expected. Hydroxylation of 5mC occurs most actively in the zygote and embryo stage, when parental methylation pattern is erased by Tet3 protein. Tet1 Tet2 proteins are active during embryogenesis, making it possible to maintain an adequate level of housekeeping gene expression and sufficient number of stem cells, inhibiting their differentiation (Tahiliani *et al.* 2009, Globisch *et al.* 2010, Tan *et al.* 2012, Hill *et al.* 2014). During the development of the embryo, in which cells divide intensively, 5-hydroxymethylcytosine is transcribed as unmodified cytosine, and therefore is not

recognized by DNMT1. This process is called passive DNA demethylation, however can be also described as DNA replication-dependent, because it occurs when DNMT1 does not methylate newly synthesized DNA strand. In consequence, the second round of replication, which is not accompanied by maintenance methylation, results in a completely unmethylated DNA (Ficz & Gribben 2014, Arand *et al.* 2015). Active DNA demethylation plays an important role in cells that divide less often and can take place in several ways. One of them is further oxidation using Tet proteins, first to the 5-formylcytosine (5fC) and next to 5-carboxylcytosine (5caC), which must be subjected to decarboxylation. There is also the possibility of 5hmC glycosylation or deamination to 5-hydroxymethyluracil. In both cases, the modified nucleotide is considered to be invalid by the base excision repair system (BER) and replaced by cytosine. This is the way of CpG islands demethylation, usually located near the transcription initiation site, to which Tet1-3 proteins preferentially bind, preventing their secondary methylation (Wu & Zhang 2011, Tan *et al.* 2012, Hill *et al.* 2014).

In normal cells (Fig. 1), methylation usually occurs in repetitive regions associated with chromosomal stability, non-coding regions as well as in gene bodies. Although, the majority of CpG islands located in the promoter regions of genes are protected from this epigenetic mechanism and remain unmodified during the development and in differentiated tissues, some of them become methylated. The most classic examples of CpG island methylation during the development, resulting in long-term transcriptional silencing, are X-chromosome inactivation and gene imprinting (Flis *et al.* 2007, Kiefer 2007, Esteller 2008, Illingworth *et al.* 2008, Łukasik *et al.* 2009, Guz *et al.* 2010).

NORMAL CELLS

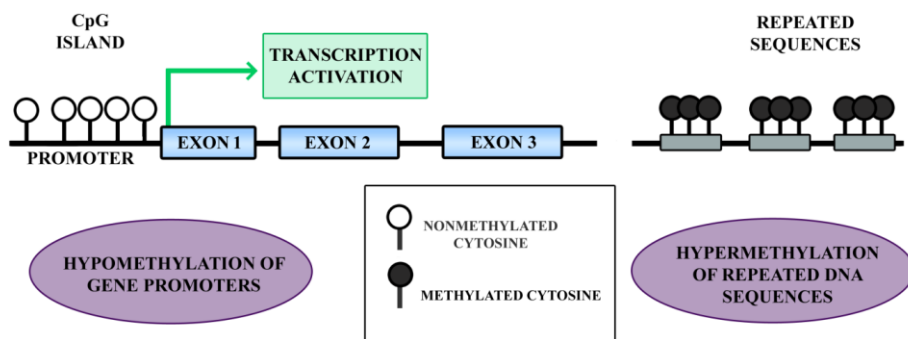


Figure 1. DNA methylation in normal cells.

DNA methylation in cancer cells

Hypermethylation of CpG islands and global hypomethylation are characteristic of cancer cells (Fig. 2). The low level of methylation in the rest of the genome can induce the activation of oncogenes located nearby and too frequent methylation within CpG islands - silencing of tumor suppressor genes (Flis *et al.* 2007, Łukasik *et al.* 2009, Guz *et al.* 2010, Sharma *et al.* 2010, Hansen *et al.* 2011, Yang *et al.* 2014, Kunwor *et al.* 2015).

CANCER CELLS

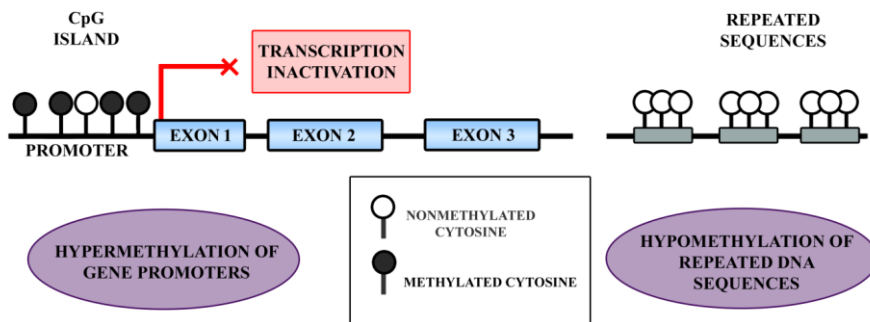


Figure 2. DNA methylation in cancer cells.

In colorectal cancer, the 10-30% reduction was observed in the overall methylation as well as significant reduction in the amount of 5-methylcytosine in premalignant stages of the adenoma (Wilson *et al.* 2007, Ehrlich

2009, King *et al.* 2014). Hypomethylation of over 50% was noted in the tumors of the chest (Wilson *et al.* 2007, Rauch *et al.* 2008). Hypomethylation in tumors of blood occurs in chronic lymphocytic leukemia

(CLL), whereas in the chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) and multiple myeloma there is only a small change in the pattern of DNA methylation (Stach *et al.* 2003, Lyko *et al.* 2004, Wilson *et al.* 2007). The global demethylation occurs in the early stages of tumors of the chest, colon and in chronic lymphocytic leukemia. In addition, in colorectal cancer hypomethylation is present in normal tissues adjacent to the tumor. In other tumors, eg. hepatocellular carcinoma hypomethylation increases with advancing stage and histological tumor stage (Lin *et al.* 2001, Wilson *et al.* 2007). Hypomethylation of specific genes was observed in the tumors of colon, pancreas, chest, stomach, prostate and in leukemia (Sadikovic *et al.* 2008). Usually, these genes regulate growth, encode enzymes important for the organism's development, tissue-specific genes and oncogenes (Flis *et al.* 2007, Guz *et al.* 2010, Kunwor *et al.* 2015).

The most common regions of hypermethylation in different kinds of tumors are chromosome 3p, 11p and 17p (Rush *et al.* 2001, Choi *et al.* 2007, Sulewska *et al.* 2007, Stöcklein *et al.* 2008). This phenomenon occurs within CpG islands which are normally unmethylated in the genome. The most important consequence of this event is silencing the function of tumor suppressor genes, for example promoter hypermethylation of p16 gene (INK4A), which occurs in many tumors. p16 is an inhibitor of cyclin-dependent kinase, which negatively regulates cell cycle progression from G1 to S phase (Flis *et al.* 2007, Li *et al.* 2011). Abnormal expression leads to disruption of the cell cycle and the loss of control, which stimulates proliferation and affect tumor progression. This phenomenon was noted in bladder, nose, throat, pancreas, colon, lung cancers as well as in melanomas, leukemias and glioblastomas. In the

carcinogenesis of esophageal adenocarcinoma promoter methylation of the p16 gene can occur already in the metaplasia (Auerkari 2006, Li *et al.* 2011). In addition, the repression of transcription of another gene, MLH1 encoding DNA mismatch repair protein, increases the frequency of mutations and, therefore, the abnormal expression of other genes (Tsai & Baylin 2011). Hypermethylation profile of 15 cancers such as colon, stomach, pancreas, liver, kidney, lung, head, neck, breast, ovary, bladder, endometrium, brain, lymphoma and leukemia was examined. Analysis consisted of 3 groups of genes: tumor suppressor genes: p16, p15, p14 (cyclin-dependent kinase inhibitors), p73 (p53-related protein), APC (adenomatous polyposis coli protein) and BRCA1 (breast cancer type 1 susceptibility protein); genes responsible for DNA repair or metabolism of xenobiotics: hMLH1, GSTP1 (glutathione S-transferase pi-1), MGMT (O⁶-methylguanine DNA methyltransferase); genes involved in invasion and metastasis: CDH1 (cadherin-1), TIMP3 (metalloproteinase inhibitor 3), DAPK (death-associated protein kinase). Methylation in at least one gene was present in every type of tumor. Methylation profiles were dependent on both the gene and the tumor. Some genes, for example p16, MGMT, DAPK were methylated in various types of cancer (colon, lung, head, neck, ovary, bladder, lymphoma and leukemia) (Esteller *et al.* 2001, Flis *et al.* 2007). Hypermethylation of p14, APC, p16, MGMT, hMLH1 occurred in gastrointestinal tumors (colon, stomach) and GSTP1 in steroid tumors (breast, liver, prostate). Another study confirmed these reports. Methylation depends on the type of cancer for the following genes: BRCA1 - breast and ovarian cancer, hMLH1 - rectal, endometrial, gastric

cancer, p73 and p15 in leukemia (Flis *et al.* 2007, Esteller 2008).

Methods of detection and potential therapies

Detection methods must have a high sensitivity due to the material from which the DNA is isolated, and the specificity to distinguish methylation of tumor cells from methylation present in normal cells. None of the methods is universal and during the selection attention should be paid to the type, quantity and quality of the biological material. The correct choice of method should minimize the risk of contamination of the sample and ensure reproducibility of results (Łukasik *et al.* 2009). The most commonly used methods are: REP (restriction enzyme PCR), MS-PCR (methylation specific PCR), BSSCP (bisulfite single-strand conformation polymorphism), BGS (bisulfite genomic sequencing) (Majchrzak & Baer-Dubowska 2009, Łukasik *et al.* 2009). There are also other methods: MS-nested PCR, QAMA (quantitative analysis of methylated alleles), Heavy Methyl. The main objective of the analysis is the differentiation of methylated and unmethylated sequences. This can be achieved either by using methylation sensitive restriction enzyme or chemical modification of DNA by sodium bisulphite. Sodium bisulfite deaminates cytosine to uracil, also m5C can undergo this reaction, however, very slow formation of the intermediate product significantly limits the speed of the process. Defined DNA fragments are then subjected to allele-specific PCR (MS-PCR), SSCP (BSSCP) or sequencing (BGS) (Łukasik *et al.* 2009).

DNA methylation pattern of adults is tissue specific and relatively stable. It is known that it can be changed in the early stages of embryonic development, during cell differentiation. Significant changes in the profile of DNA methylation are commonly detected in cancer cells

(Ogoshi *et al.* 2011, You & Jones 2012). In many tumors it has been shown that inactivation of tumor suppressor genes is accompanied by hypermethylation of the promoter regions. Hypermethylation within CpG islands which are normally unmethylated in the genome, is a factor that inhibits transcription and expression of genes (Deaton & Bird 2011). Considering that tumor suppressor genes are involved in cell differentiation and regulation of the cell cycle, apoptosis and repair of DNA, the consequences of hypermethylation of the promoter sequences resulting in silencing genes are evident. Therefore, compounds which inhibit DNA methylation can play a role in tumor therapy (Guz *et al.* 2010, Kunwor *et al.* 2015).

The best known inhibitors of DNA methyltransferases (DNMTs) are cytidine analogues modified in the 5 position of the pyrimidine: 5-azacytidine, 5-aza-2'-deoxycytidine (decitabine) (Flis *et al.* 2007, Guz *et al.* 2010, Kunwor *et al.* 2015). The mechanism of the pharmacological action of these compounds is their conversion in cells to deoxynucleotide triphosphates and then incorporation into DNA in a place of cytosines during replication (Brait & Sidransky 2011). This modification is recognized by DNMT to which it binds covalently, blocking its activity. Formation of the enzyme-DNA adducts reduces the number of active DNMT molecules in the nucleus, which in subsequent rounds of replication result in passive methylation of DNA, and therefore in the reactivation of epigenetically silenced genes. Covalent binding of DNA methyltransferases may be responsible for the cytotoxicity of the DNMT inhibitors, especially in high doses. Low stability in aqueous solutions and high toxicity of azanucleosides greatly limits their therapeutic potential

(Flis *et al.* 2007, Guz *et al.* 2010). Another cytidine analogue lacking the amino group at C4 of the pyrimidine ring is Zebularine, which has a similar mechanism of action to azanucleosides. Zebularine is a compound less toxic than 5-azacytidine and decitabine, and more stable in aqueous solutions, however, its bioavailability after oral administration is rather low (Cheng *et al.* 2003, Guz *et al.* 2010, Sharma *et al.* 2010, Kunwor *et al.* 2015). Another group of compounds that inhibits DNMTs activity are small molecule inhibitors, including hydralazine (an antihypertensive action), procaine (local anesthetic) or procainamide (antiarrhythmic drug). Procaine and procainamide are derivatives of 4-aminobenzoic acid and are capable of annealing to a sequence rich in CpG, causing the masking target sequences for methyltransferase and thus block the binding of the enzyme with DNA (Guz *et al.* 2010, Kunwor *et al.* 2015). The group of inhibitors, that are not nucleoside analogues, includes the compounds directly blocking the activity of DNA methyltransferase, such as epigallocatechin gallate (EGCG), which is considered to be the most active of green tea polyphenols and L-tryptophan derivative (RG108). The mechanism of action of these compounds consists in blocking the active center of the enzyme. RG108 because of its good fit to the active center of DNMT1 and low toxicity was an attractive candidate for further research on the use of anticancer therapy, however it has been noted that RG108 is

genotoxic (Kunwor *et al.* 2015). An alternative mechanism of DNMT inhibition could be the use of antisense oligonucleotides directed against the DNMT mRNA. Hybridization of an antisense oligonucleotide with the complementary mRNA may block the translation, thus reduce the level of DNA methyltransferases (Flis *et al.* 2007, Guz *et al.* 2010).

Conclusions

DNA methylation plays an important role in the complex and multistep regulation of expression of the genes, whose promoter regions are rich in CpG sequences. The above data indicate that the methylation and gene expression are processes related to each other by several factors, such as the activity of DNA methyltransferases factors transcriptionally, proteins involved in demethylation, protein binding methylated DNA. Further studies of epigenetic processes will allow a better understanding of mechanisms of their action, including the interdependence between DNA methylation and activity of proteins crucial to the structure of chromatin and gene activity. Wider knowledge of epigenetic mechanisms involved in the process of malignant transformation and pharmacological regulation of the degree of DNA methylation provides an opportunity to improve the therapeutic actions in the fight against cancer.

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Streszczenie

Modyfikacje epigenetyczne odpowiedzialne są za modulację ekspresji genów bez ingerencji w sekwencję nukleotydową. Obserwowane zmiany aktywności transkrypcyjnej genów w tkankach nowotworowych w porównaniu do tkanki prawidłowej, bardzo często są wynikiem metylacji DNA w obrębie sekwencji promotorowych tych genów. Modyfikacja ta poprzez przyłączenie grup metylowych do cytozyn wysp CpG skutkuje wyciszeniem aktywności transkrypcyjnej genu, co w przypadku genów supresorowych przejawia się zaburzeniami cyklu komórkowego, nadmierną proliferacją i destabilizacją procesów naprawczych. Dalsze badania nad modyfikacjami epigenetycznymi pozwolą na lepsze zrozumienie mechanizmów ich działania, w tym zależności pomiędzy metylacją DNA, a aktywnością białek decydujących o strukturze chromatyny i aktywności genów. Poszerzenie wiedzy na temat epigenetycznych mechanizmów biorących udział w procesie transformacji nowotworowej i farmakologicznej regulacji stopnia metylacji DNA może stanowić okazję do poprawy działań terapeutycznych w walce z nowotworem.