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Review article

DNA methylation in states of cell physiology and pathology

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Abstract: DNA methylation is one of epigenetic mechanisms regulating gene expression. The methylation pattern is determined during embryogenesis and passed over to differentiating cells and tissues. In a normal cell, a significant degree of methylation is characteristic for extragenic DNA (cytosine within the CG dinucleotide) while CpG islands located in gene promoters are unmethylated, except for inactive genes of the X chromosome and the genes subjected to genomic imprinting. The changes in the methylation pattern, which may appear as the organism age and in early stages of cancerogenesis, may lead to the silencing of over ninety endogenic genes. It has been found, that these disorders consist not only of the methylation of CpG islands, which are normally unmethylated, but also of the methylation of other dinucleotides, *e.g.* CpA. Such methylation has been observed in non-small cell lung cancer, in three regions of the exon 5 of the *p53* gene (so-called "non-CpG" methylation). The knowledge of a normal methylation process and its aberrations appeared to be useful while searching for new markers enabling an early detection of cancer. With the application of the Real-Time PCR technique (using primers for methylated and unmethylated sequences) five new genes which are potential biomarkers of lung cancer have been presented.

Key words: DNA methyltransferases, DNA hypomethylation, DNA hypermethylation, "non-CpG" methylation

Introduction

DNA methylation is one of the most frequent chemical modifications in the eukaryotic genome. It occurs in the cells of fungi, plants, non-vertebrates and vertebrates [1-4]. In mammals, this process is important for the normal embryonic development, plays an important role in regulation of gene expression, X chromosome inactivation, genomic imprinting, chromatin modification and the silencing of endogenic genes. Aberrations in the genome methylation pattern may appear while the organism is ageing and in the early stages of cancerogenesis, even 3 years before a clinical diagnosis of cancer is possible [5-14].

The knowledge of a normal methylation process and its aberrations appeared to be useful while searching for new markers enabling an early detection of different types of human cancer.

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Biochemical process of DNA methylation

DNA methylation involves a covalent addition of a methyl group (-CH_3) to the 5'carbon of the pyrimidine ring of deoxycytidine (dC), which leads to the creation of 5- deoxymethylcytidine (dmC). The reaction is catalysed by DNA methyltransferases, whose substrates are palindromic DNA sequences 5'-CG-3', which are also referred to as CpG dinucleotides (Fig. 1) [10,11,15-17].

In genomes of most mammals (70% - 80%), CpG sites with 5-methylcytidine are methylated on both DNA strands, only a few CpG sequences have asymmetrical methylation [10]. The frequency of 5-methylcytidine in mammalian DNA is less than 1% of all nucleotides, and the amount of all CpG dinucleotides is four times smaller than predicted [10,15]. This low frequency of CpG dinucleotides in DNA particles may be related to cytosine methylation. 5-methylcytosines more often undergo spontaneous deamination comparing to unmethylated cytosines. This process leads to the creation of thymine - a natural DNA component, which is rarely recognized by intracellular repair sys-

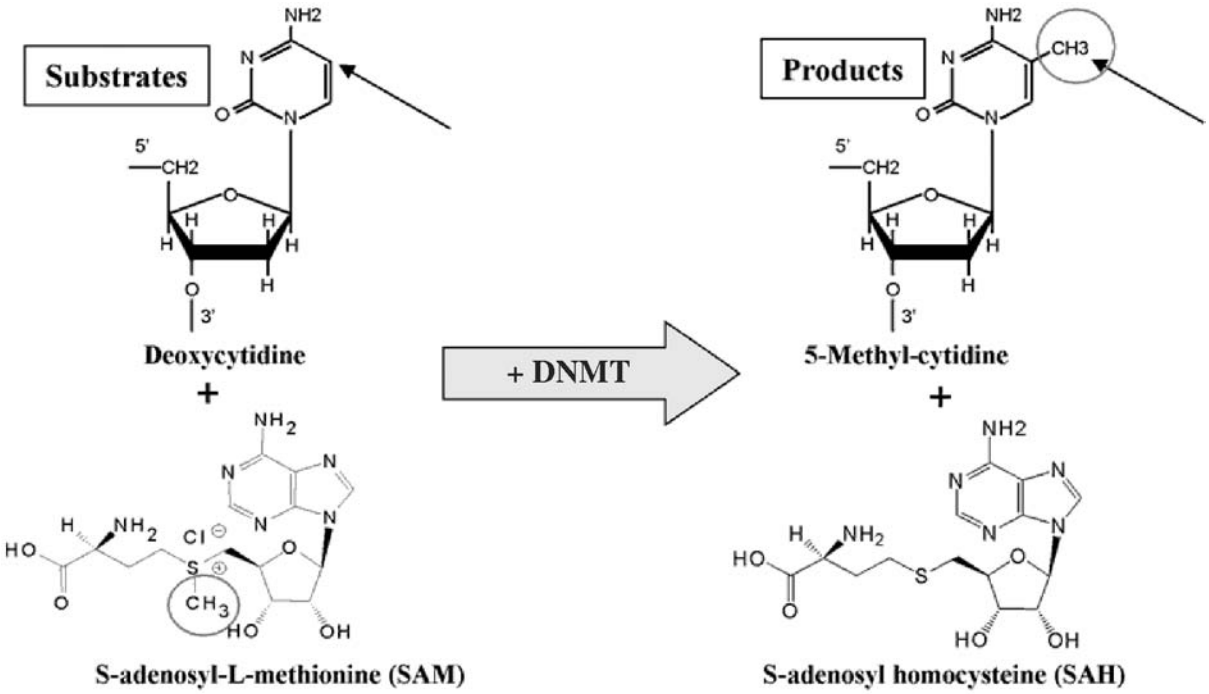

Fig. 1. DNA methylation reaction catalyzed by DNA Methyltransferase (DNMT) [56].

tems while the uracil, which appears after the deamination of unmethylated cytosine, is easily removed from DNA particles [10,15,18,19].

During evolution, the dinucleotides CpG have been progressively eliminated from the genome of higher eukaryotes and now they are present at only 5% to 10% of their predicted frequency [10,19].

Distribution of methylated and unmethylated sequences in the genome

Eukaryotic genomes contain methylated regions interspersed with unmethylated domains. However, the location of CpG dinucleotides and 5-methylocytosines is not random [7,10,15].

Most of the genome shows significant methylation and a low concentration of CpG dinucleotides (extragenic DNA). Such regions are typical of the condensed chromatin that is formed during the late stage of DNA replication as a result of reactions between histones and conformational changes in nucleosomes. Heterochromatin is inaccessible to transcription factors [6,10,18,20].

The rest of the genome (l% - 2%) contains unmethylated DNA. Pairs of CpG (length: 0.5 kb - 2kb) occur in the area from 0.5 to 5 kb every 100 kb (located within genes and gene related sequences) [10,15]. These sequences are rich in CpG dinucleotides, which constitute about 60% - 70%, and, for this reason, are called CpG islands (C+G≥55%; the relation of CpG to predicted CpG≥0.65). Chromatin containing CpG islands is heavily acetylated (histone H_3 lysine 9 - H_3Lys9 is often acetylated), lacks histone H_1 and includes a nucleosome-free region. This, so called, open chromatin configuration may be a consequence of the interaction of transcription factors with gene promoters [15,18,20,21].

CpG islands occur mainly in the 5'end of genes, in the region of promoter, and many of them contain transcription factors binding sites. We differentiate two types of promoters: rich and poor in CpG islands. Approximately half of all human genes contain CpG islands. These are housekeeping genes and some tissue-specific genes (40% of tissue-specific genes) [10,22].

Promoter region CpG islands are usually unmethylated in all normal tissues, regardless of the transcriptional activity of the gene. The genes situated on the inactive X-chromosome and the genes undergoing genomic imprinting are exceptions because one of the parental alleles of these genes may be methylated. It has also been found that CpG islands which are unmethylated in young organisms can become methylated as the organism ages (the, so-called, age-dependent methylation), which may constitute a preliminary stage of cancer-related cell transformation [7,9,10, 18,20,23].

DNA methyltransferases

The enzymes that transfer the methyl group to the phenyl cytosine ring belong to the family of DNA methyltransferases, which includes DNMTl,

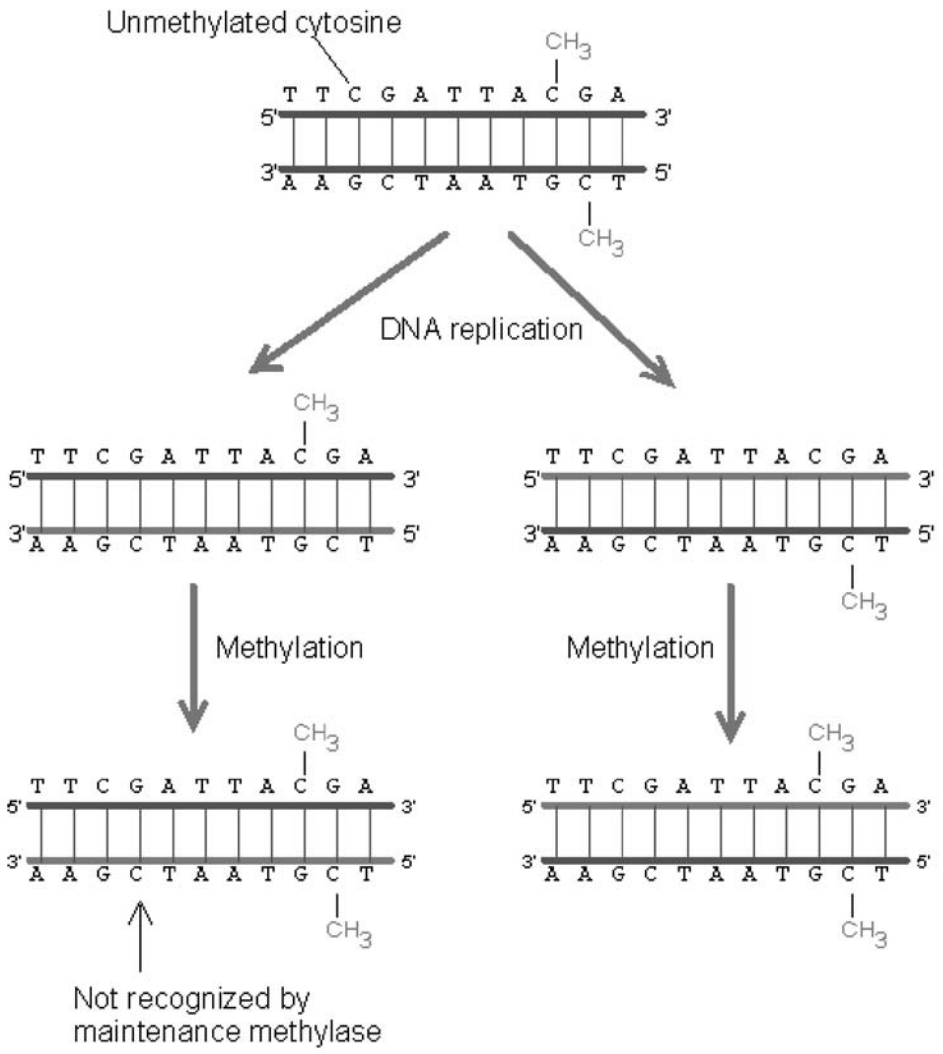

Fig. 2. Inheritance of the DNA methylation pattern. The maintenance methylase can methylate only the CG sequence paired with methylated CG. The CG sequence not paired with methylated CG will not be methylated. Hence, the original pattern can be maintained after DNA replication [57].

DNMT3A, DNMT3B and DNMT3L [11]. These proteins have been found in the nucleolus and the nucleoplasm area. DNMT1 is responsible for maintaining the methylation pattern (it catalyses 97%-99.9% methylation reactions during mitosis) while DNMT3A and DNMT3B conduct from 3% to 5% of methylation in the newly-synthesized DNA (*de novo* methylation) [22,24].

The mechanism of DNMTl activity is well known. In the S phase of cell cycle, this protein reaches its maximum concentration and creates a complex with nuclear antigen of proliferating cells (PCNA). PCNA surrounds the DNA like a ring and moves along making it possible for methyltransferase to fulfill a catalytic function. DNMTl recognizes hemimethylated CpG dinucleotides in maternal and daughter DNA strand and moves the methyl group from the S-adenosylmethionine (SAM) to the cytidine located in the unmethylated daughter strand. As a result of this process, there appear symmetrically methylated sites corresponding to the template DNA particle (Fig. 1,2)[10,15,23].

DNMT3A and DNMT3B are of particular importance in the early stages of embryonic development. They establish the methylation pattern which is transmitted to the differentiating cells and tissues. The role of *de novo* methyltransferases in mature cells has not been fully explained. It is suspected that DNMT3B maintains pericentric heterochromatin in the state of methylation. It is because DNMT3A and -3B have a conservative PWWP domain (binding to DNA) responsible for the localisation of these proteins within the pericentric heterochromatin [25-27].

Moreover, DNMT1 and DNMT3B are involved in the suppression of human rDNA. DNMT3B protein participates in this process probably via the PWWP domain and the ATRX-homology domain (cysteine rich zinc finger DNA binding motif), which takes part in the protein-protein interactions [25,26].

It has also been found that the alternative splicing of the *DNMT3B* primary transcript leads to the formation of one of the five mRNA izoforms (from *DNMTB1* to *DNMTB5*). These particles differ in single exons. *DNMT3B1*, which is the only one to undergo

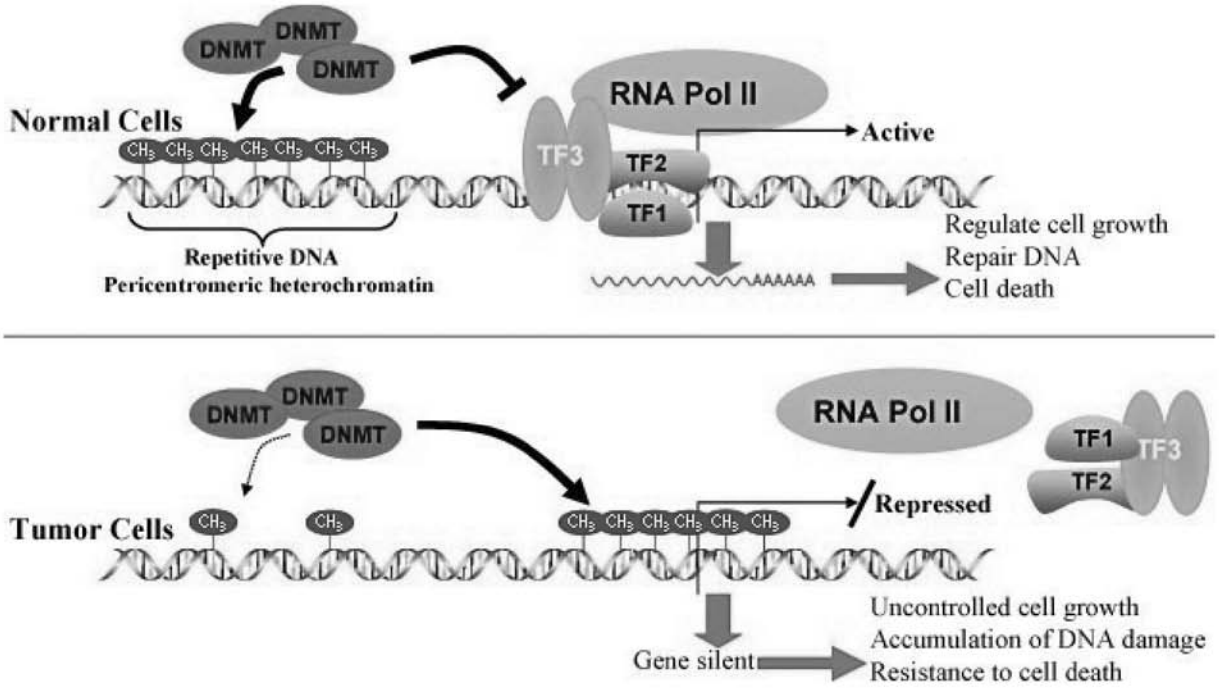

Fig. 3. Methylation DNA in normal and tumor cells [58].

expression in embroynic stem cells, contains a full set of exons. The remaining *DNMT3B* do not have from two to four exons and are active in some cancer cell lines, *e.g. DNMT3B2* in breast cancer, *DNMT3B3* in bladder cancer and *DNMT3B4* in hepatocellular carcinomas (HCC) [28].

The DNMT3L protein may indirectly participate in the process of DNA methylation. While it does not have catalytic activity by iteself, when it accompanies *de novo* methyltransferases, it stimulates their activity and increases their DNA affinity [29].

DNA demethylation

A passive DNA demethylation happens when the activity of methyltransferases is blocked or their concentration is low. Methylation loss usually follows two rounds of replication, between which there is no methylation within the previously methylated DNA sequence. During replication DNMTl may be unable to methylate newly-synthesized DNA, *e.g.* due to the reaction with 5-azacytidine (5-azaC) - an irreversible inhibitor of methylotransferases, or as a result of cytidine methylation blocking by some proteins binding to DNA during the S phase of cell cycle [23,30].

The synthesis of DNMTl and DNMT3B enzymes may be limited already at the transcription stage due to interactions between antisense oligonucleotides - small interfering RNA (siRNA) and 3'untranslated region of genes. The studies perform on CP70 ovarian cancer cell lines has shown that the transfection of cancer cells by adequately designed siRNA molecules induces *DNMT1* and *DNMT3B* mRNA degradation, which inhibits the sythesis of protein products of both genes [31].

The process of demethylation may also have an active character and involve DNA demethylases. One of the proteins showing such activity is 5-methylcytosine DNA glycosylase (5-MCDG). It creates a complex with an RNA molecule (necessary for its activity) and RNA helicase. A similar activity is characteristic for MBD4 - a protein binding methylcytosine and repairing mismatched base pairs (*mismatch repair*) [23].

The influence of methylation on gene expression

A role for DNA methylation in the regulation of gene expression was suggested many years ago. Early research indicated a correlation between the site-specific cytidine methylation within or adjacent to genes and transcriptional repression. It has been proved that the methylation of CpG islands of promoter region is often related to the loss of gene expression while there is a weak relationship between gene expression and the methylation of CpG dinucleotides located in the gene coding sequence situated outside the first exon [28]. The inverse relationship between promoter region methylation and transcription has been observed in many genes, but not in all of them. In the genes without CpG islands, a strong promoter with several CG

pairs may be resistant to silencing by methylation while a weak promoter usually remains sensitive [22].

Due to the fact that the methylation pattern influences gene expression and is inherited during the mitotic division without causing changes in DNA sequence, DNA methylation has been classified as an epigenetic gene regulation mechanism. Several ways of transcriptional suppression by methylation have been suggested.

The modification of cytidine (to 5-methylcytidine) located in the site recognized by specific transcription factors may block the binding of these factors and inactivate the gene (Fig. 3). Transcription factors which are sensitive to methylation include, among others: AP-2, cMyc/Myn, CREB, E2F, NFκB. Other transcription factors (*e.g.* SPl, CTF) bind both to methylated and unmethylated sequences [10,28,32]. The SPl transcription factor reacts with *Alu* hypermethylated sequences, which surround the promoters of some housekeeping genes. The binding of such a factor blocks the spreading of methylation to CpG islands and maintains the gene in an active state [15].

Transcription factors binding sites may be occupied by proteins stimulating chromatin condensation. Changes in chromatin structure are caused by proteins binding methylated cytosine: MeCP-2, MBD1, MBD2, MBD3, MBD4, which bind to the methylated sites of CpG regardless of the neighbouring sequences [10,20,33].

MeCP-2 is not necessary to keep embryonic stem cells alive, but it is vital for the embryonic development. The human *MeCP-2*, gene, which consists of 3 exons, is situated in Xq28 and the protein it codes contains 489 amino acids. This gene is alternatively polyadenylated in 3' untranslated regions (UTR) producing several transcripts more than 10.1 kb long. Transcript expression is different in the brain than in other tissues [33].

MeCP-2 contains two domains: MBD (*methyl-CpG binding domain*), which binds unmethylated DNA and TRD (*target recognition domain*), which ihibits transcription and may bind to unmethylated DNA constituting nucleosomes or a more compacted form of chromatin. 85 amino acids (from 78 to 162) situated at the N-end of MBD recognize single methylated CpG. 104 amino acids of TRD (from 207 to 310) react with a large comlex of Sin3 co-repressor, which consists of at least seven subunits and covers histone deacetylases HDAC1 and 2, proteins binding histones RbAP48 and 46 and Sin3A, SAP30 and SAP18. Sin3 complex shares four proteins (HDAC1, 2 and RbAP48, 76) with NuRD complex, which is a part of MBD4. RbAP48 reacts directly with histone deacetylase and strengthens enzymatic activity, probably by means of binding deacetylase to the proper site, which causes deacetylation of the N-end domain of histone H_4 and makes chromatin more compact [33].

The structure of MeCP-2 protein suggests that it takes part in two main gene regulation mechanisms: DNA methylation and histone deacetylation. In some cases, it has been proved, however, that in transcription inhibition DNA methylation dominates histone deacetylation. MeCP-2 participates in the formation of a more stable, inactive chromatin structure, but it can also inhibit the transcription of even “naked” DNA, which means that the formation of chromatin is not necessary for its activity. The silencing of gene expression by proteins binding methylated DNA is probably significant while inheriting X chromosom inactivation [5,10,20].

Methylation during embryonic development

DNA methylation is tissue-specific and may control gene expression during embryonic development. The lack of a functional gene of DNA methyltransferase in mice causes their death within the first eight days of pregnancy [10].

Changes in DNA methylation pattern occur during the normal development and differentiation of mammalian cells. In the zygote, immediately after the fertilization, there are dynamic histone modifications and fluctuations of DNA methylation levels. Using methylation-sensitive restriction enzymes, it was found that a dramatic reduction in methylation levels (the, so-called, global demethylation) occurs in the early embryo. After implantation, this is followed by a wave of *de novo* methylation involving most CpG sites. During gastrulation, tissue-specific genes are demethylated in their tissues of expression, but the majority of the genome remains methylated. The last wave of *de novo* methylation is sex-dependent and takes place during gametogenesis. A slow decrease in the DNA methylation level has been observed during postembryonic life as well as *in vitro* in ageing cells. [19,34-36].

Aberrations in the methylation pattern in cancer

Changes in the overall methylation level and methylation pattern of particular genes are characteristic for different types of cancer cells. Research has shown that both hypomethylation and hypermethylation of DNA are connected with cancer. The influence of DNA methylation on oncogenesis may involve one of the following mechanisms:

1. DNA hypomethylation (a global demethylation inside the genes and a local one in gene promoters)
2. hypermethylation of tumor suppressor genes (within normally unmethylated CpG islands)
3. transition of 5-methylcytosine to thymine and "non-CpG" methylation in tumor cells
4. induction of chromosomal instability.

DNA hypomethylation

It has been found that there are differences between hypomethylation levels in the same type of cancer as well as between different types of cancer. Hypomethylation, in comparison to normal tissues, has been observed in numerous solid tumors and in some haematological malignances. In colorectal cancer, there was a 10%-30% average reduction in the overall methylation and in precancerous forms of adenomas there was a significant decrease of 5-meC. There is a clear hypomethylation (over 50%) in chest tumors. As regards haematological malignances, hypomethylation is observed in chronic lymphocytic leukaemia (CLL) whereas in the case of chronic myelogenous leukaemia (CML), acute myelogenous leukaemia (AML) and in multiple myeloma, only small changes in total DNA methylation have been observed. In the case of haematological and some other malignances, it is difficult to define correctly normal cells or tissues, which would serve as a control [20].

Global demethylation occurs at different moments and its role in the initiation and progress of cancer is different for its particular types. Global demethylation appears in the early stages of chest tumors, colorectal cancer and CLL. In colorectal cancer, hypomethylation is observed in healthy tissues adjacent to the tumor, which suggests its role in disease initiation. In other tumors, *e.g.* hepatocellular carcinoma, hypomethylation increases together with tumor's histological stage and advancement [20].

Total DNA methylation is significantly influenced by the activation of usually inactive transpozones and endogenous retroviruses, which are present in the human genome. LINE (*long interspersed nuclear elements*), which belong to retrotranspozones, show a high degree of methylation in all types of mammalian tissues. Moreover, most mammalian genes contain retrotranspozones situated inside introns. The activation of a strong promoter connected with these elements may change completely the transcription level and modify the expression of genes within which these elements appear. It is also believed that unmethylated transpozones cause mutations in the genome and abnormal chromosomal recombinations in the dispersed form of chromatin [20,28].

Gene-specific methylation occurs in numerous colon, pancreas, chest, stomach, prostate cancers, lukaemia and others. It happens in the genes regulating the growth, coding enzymes, genes significant for the development, tissue-specific genes and oncogenes. In liver cancer, was observed hypomethylation of *C-MYC, C-FOS, H-RAS, K-RAS* protooncogenes. There is a strong correlation between the state of specific CpG dinucleotide methylation in the third exon of *C-MYC* gene and human hepatocellular tumours [37]. This dinucleotide is also methylated in some chronic leukaemia and colorectal cancers. In leukaemia, there is also a decrease in the level of ERB-A1 and BCL-2 gene methylation whereas in lung and colorectal cancer we observe a decrease in the amount of K-RAS protooncogene [20].

DNA hypermethylation

Hypomehtylation of the genome leads to nonrandom changes in the localisation of methyl groups. Some DNA regions show hypermethylation even if the total concentration of 5-methylcytidine is decreasing [10]. It has been found that there are, so-called, hot spots for hypermethylation on chromosomes 3p, 11p and 17p in various human cancers. They include CpG islands, which are normally never methylated *in vivo*, and in tumor cells they undergo methylation, sometimes even in the early stage of cancerogenesis [13].

In addition to point mutations or gene deletions, promoter and first exon hypermethylation constitutes an additional factor which inactivates suppressor genes. This process silences over 90 genes in various types of carcinoma [28,38].

Hypermethylation was first described for the calcitonin gene located on the chromosome 11p, in lung cancer and lymphoma. Later research revealed the methylation of this gene also in colorectal cancer and CML [22]. The retinoblastoma (*RB*) gene appeared to be the first classical tumor suppressor gene where hypermethylation was discovered within 600 bp long CpG islands situated at the 5' end: in retinoblastomas (ca. 10%), gliomas (25%), bladder cancers, hypophyseal adenomas, in many small cell lung cancers (SCLC) and some non-small lung cancers (NSCLC) (30%)) [10,38,39]. This gene is located on the chromosome, 13q, in band 14 and consists of 27 exons. It has also been shown that *in vitro* methylation of *RB* gene promoter region blocks the activation of this promoter and may limit expression to 8% in relation to the unmethylated conrol [40].

One of important cell cycle regulatory proteins is p16 (*INK4A*) coded by the gene located at 9p21, which - using an alternative reading frame - also codes a structurally and functionally different protein - p14 (*ARF*) [8,41,42]. P16 binds cyclin-dependent kinases (cdk4 or cdk6) stopping them from creating an active complex with cyclin D, which halts the pRb/p130 pathway in the early G1 phase of cell cycle. A spontaneous arrest of *p16* expression has been observed in small colonies of normal human breast epithelial cells growing in a standard, serum-free medium, which increased the number of divisions by 40-50 in relation to the normal division. It is believed that the methylation of *p16* CpG islands plays a role in the spontaneous silencing of this gene, and treating cells with 5-aza-2'-deoxycytidine (methylase inhibitor) causes the renewal of *p16*

expression and a normal growth inhibition. In some types of human cells the level of p16 tends to accumulate, which may cause an increase of this gene's expression in ageing cells increase by up to forty times in comparison with freshly passaged cells [42]. Mutations and methylation resulting in *p16* silencing are often observed in human primary tumours: bladder tumor, nose and throat tumour, melanoma, glioma, pancreas cancer, leukaemia, colorectal and lung cancer [43].

A different study focused on the hypermethylation profile of 15 types of primary tumours (colon, stomach, pancreas, liver, kidney, lung, head and neck, breast, ovaries, bladder endometrium, brain, leukaemia, lymphoma). The analysis covered 3 groups of genes: tumor suppressor genes (*p16*, *p15*, *p14*, *p73*, *APC* and *BRCA1*), DNA repair or xenobiotic metabolising genes (*hMLH1, GSTP1, MGMT*) and the genes responsible for metastases and invasiveness (*CDH1, TIMP3, DAPK*). Methylation in at least one gene was found in every type of carcinoma. Methylation profiles appeared to be both tumour and gene specific. Some genes, like *p16*, as well as *MGMT* and *DAPK* were methylated in various carcinoma (colon, ovarian, lung, head and neck cancer, leukaemia, bladder, lymphoma). *P14* and *APC* hypermethylation tended to spread in gastrointestinal tumours (colon, stomach) and in *GSTP1* it was characteristic for steroid-dependent tumours (breast, liver, prostate) [77].

The above results have been confirmed by research focusing on single types of tumour. *MGMT* and *APC* have been analysed in colorectal cancer, *p14* in stomach cancer, *DAPK* in bladder cancer, and *GSTP1* in liver cancer. Methylation appeared to depend on tumor type in the following genes: *BRCA1* (breast and ovarian cancer), *hMLH1* (rectal, endometrial and gastric cancer), *p73* and *p15* in leukaemia [44].

Subsequent analyses have also proved the influence of epigenetic inactivation on molecular pathways engaged in cell immortalisation and tumour transformation [8]. Promoter hypermethylation was observed in connection with the silencing of genes involved in cell cycle (*p16* i *p15*), DNA repair (*hMLH1, MGMT* i *BRCA1*), cell adhesion and metastasis formation (*CDH1, TIMP3, DAPK*), related to p53 (*p14* i *p73*) and to metabolic enzymes (GSTP1) and APC/β-cathenin (*APC*) pathway. Aberrations in the methylation patterns of genes belonging to different pathways were diversified in various tumour types. Considering the gene-specific methylation profile, it was noticed that gastrointestinal tumors (colon, stomach) share few of hypermethylated genes (*p16*, *p14, MGMT, APC, hMLH1*) whereas lung, head and neck cancers have different patterns of hypermethylated genes: *DAPK, MGMT, p16* and similar in the case of *hMLH1* and *p14*. Breast and ovarian cancers show a tendency to methylate *BRCA1, GSTP1* and *p16*. Changes in haematological malignances differ from those in solid tumours, which is apparent in the high methylation frequency in the case of *p73* and *p15*, which are not methylated in tumours of epithelial origin. Epigenetic lesions were often found already in the early stages of tumor development. Hypermethylation of *p16*, p*14, MGMT* and *APC* gene promoters occurred in colorectal adenomas, *p16* in basal cell hyperplasia squamous metaplasia and in the carcinoma *in situ* of the lung, and *hMLH1* was epigenetically silenced in endometrial hyperplasia and in ulcerative colitis, both in the uterus and in colorectal cancer [44].

Other studies concentrated on lung tumors. The analysis covered adenocarcinoma and squamous cell carcinoma, the adjacent cancer-free lung parenchyma, peripheral blood mononuclear cells (PBMC) and saliva. The research focused on 11 genes involved in cell pathways such as: signal transduction (*3-OST-2*), apoptosis (*DcR1, DcR2, DAPK*), adhesion (*APC, E-CAD, H-CAD*), cell cycle (*p16*, *RASSF1A*), cytokine singalling (*SOCS1, SOCS3*). Methylation aberrations in at least one gene were found in 98% of primary tumors. Methylation frequency ranged from 90% for *3-OST-2* to 40% for *DcR2*. In the adjacent non-cancer tissues methylation frequency was 58% for *APC* and 2,5% for *DAPK*. No methylation was observed in PBMC or it was statistically insignificant. The analyses of saliva samples (patients with and without tumours) showed that 4 out of 11 genes may be used as potential biomarkers in cancer. Methylation of genes *3-OST-2, RASSF1A*, *p16* and *APC* was 62% in the examined samples and zero in control. Taking into account the whole examined material, it was stated that methylation in 9 genes may be a useful biomarker in non-small cell lung cancer (*3-OST-2, RASSF1A, p16, DcR1, DcR2, SOCS1, APC, DAPK, H-CAD*). Each of the genes could be useful for the differentiation of lung cancer from the adjacent non-cancer tissues, tumors from PBMC and adenocarcinoma from squamous cell carcinoma. The authors were the first to propose the usage of five of these genes as lung cancer markers (*3-OST-2, DcR1, DcR2, SOCS1 i SOCS2*) [45].

"Non-CpG" methylation and 5-methylcytosine - thymine transition

In mouse embryonic stem cells there occurs methylation which is unconnected with CpG sequences (*non-CpG methylation*) [46]. It has also been detected in promoter-enhancer sequences in an adenoviral vector containing the *CMV-PE-hFGF-4* gene sequence. The mechanism of different methylation may result from the DNMT3 activity [41]. DNMT3A and -3B are responsible for *de novo* methylation, which, apart from

CpG, can occur in CpA, CpC and CpT nucleotides situated in the DNA of embryonic stem cells and episomal DNA [46].

Minimum CpG methylation has also been discovered during the inactivation of virus sequences in cell lines and in animals. At least four types of viruses involved in human lung cancerogenesis can be inactivated in this way: EBV, HPV, HIV, SV40 [47-50]. This type of cytosine methylation has not been found in human tumours for many years.

In human cells, deamination of methylcytosine to thymine in CpG sequences often leads to mutagenesis. Several such sequences have been described as mutation hot spots (in lung cancer - codons 157, 248 and 273 of gene *p53*) [51,52]. It has been found that in non-small cell lung cancer, in gene *p53* there is a significant percentage of GC to AT transitions in "non-CpG" sites, which, according to the expectations, should not be methylated (44.09% of non-CpG sites) [53]. "Non-CpG" methylation occurred in three regions of exon 5, which contain CpA (codons 156-159, codons 175-179, and the sequence at the 3'end of the gene) [53].

It is now suggested that DNMT3 activation can cause "non-CpG" methylation in the human gene *p53*. In the lung, this type of methylation occurs also in the tissues adjacent to the tumor, which indicates that "non-CpG" methylation may appear in the early stage of canceration and serve as a useful tool for early cancer detection [53].

Methylation and chromosomal instability

A methylation pattern defect, in addition to changing the level of gene expression, may facilitate the gain and loss of some chromosomes. These processes lead to the genomic instability preceding the development and progression of cancer.

Microsatellite instability leading to changes in the number of DNA short tandem repeats has been found in 35% of SCLC and in 22% of NSCLC [54].

The loss of heterozygosity (LOH) is a frequent occurrence in lung cancer. In SCLC we observe the loss of 3p, 4p, 4q, 5q, 8p, 10q, 13q, 17p whereas in NSCLC we often deal with the loss of 3p, 6q, 8p, 9p, 9q, 13q, 17p, 18q, 19p, 21q, 22q [13].

LOH tests on chromosome 11 carried out on mouse cell lines have shown that the occurrence of this phenomenon is significantly higher in hypomethylated cells (77%) than in methylated cells (45%). LOH often occurred near the centromere (45% of analysed cases). It is suggested that the increased loss of heterozygoty in hypomethylated cells might have been caused by the influence of hypomethylation on the stability of centromere and pericentric chromatin [55].

There is often a clear cooperation between epigenetic and genetic phenomena during the generation of oncogenic phenotype. The silencing of *MLH1*, which is a gene coding the protein participating in the repair of mismatched base pairs, is connected with hypermethylation and leads to the creation of MIN+ phenotype in lung, endometrial and gastric cancer (70%-80%), and the epigenetic inactivation of the gene coding glutation S-transferase 1 (*GSTP1*) in prostate, breast and other cancers predisposes DNA to being damaged by active forms of oxygen, which may result in adenine mutations occurring during cancer progression [6].

Epigenetic lesions in tumor cells do not always occur before genetic changes. The relation between these aberrations may also be inverse. Genetic changes in the genes coding transcription factors and transcription co-activators may modify chromatin in such a way that, as a result, the normal cell methylation pattern is broken and there is an epigenetic gene silencing [6].

Summary

The relationship between the occurrence of hypermethylation in tumor suppressor gene promoter sequences and its role in cancerogenesis started to generate interest in the mid-eighties of the last century. Further research showed a high degree of total DNA demethylation in tumour cells. The knowledge about the course and role of DNA methylation has enabled the development of efficient and fast methods of detecting methylated and unmethylated sequences. Current research bases on the PCR technique and analyses methylation patterns both in tumor suppressor genes and oncogenes as well as in extragenic DNA. At the beginning of 2007, Shivapurkar *et al.*, using Real-Time PCR technique, a proposed five genes as new markers useful for an early detection of lung cancer [45]. In the future, the knowledge about epigenetic changes occurring in the human genome during cancerogenesis may facilitate an early diagnosis and enable the introduction of new, more efficient methods of cancer treatment.

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