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Gene silencing in DNA damage repair

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Abstract. Silencing of DNA repair genes plays a critical role in the development of the cancer because these genes, functioning normally, would prevent the accumulation of mutations leading to carcinogenesis. Epigenetic gene silencing is an alternative mechanism to genetic gene aberration, inactivating those genes in cancer. DNA methylation and histone modification are the major factors for epigenetic regulation of gene expression. Here, we describe recent advances in understanding of epigenetic silencing of DNA repair genes and their epigenetic mechanisms involving DNA methylation and histone modification.

Key words. DNA repair; cancer; DNA methylation; histone modification; heterochromatin.

Introduction

Epigenetics is defined as a heritable change in gene expression that does not involve changes in DNA sequences. Epigenetic regulation of gene expression plays a critical role in development and differentiation, X inactivation, genomic imprinting and several human diseases, including cancer [1]. Recent studies have focused on two molecular mechanisms for epigenetic gene expression, DNA methylation and histone modification. Methylation of cytosine in CpG dinucleotides is the major modification in mammals. The CpG dinucleotides are mainly localized in the CpG islands, which are short stretches of GC-rich sequences associated with promoter regions in approximately half of human genes. These islands are normally unmethylated; however, some of them are aberrantly hypermethylated in cancer cells, leading to gene silencing by transcriptional repression. On the other hand, the DNA of all eukaryotes is packed into chromatin consisting of a repetitive fundamental unit, the nucleosome, which contains a highly conserved histone octamer wrapped twice with 147 bp of DNA. Chromatin structures are divided into two types of domains, euchromatin and heterochromatin, which are generally characterized by the modification state of the histone amino termini. Euchromatin is accessible to DNA-binding factors and is transcriptionally active. In contrast, heterochromatin is inaccessible and transcriptionally inactive. The dynamic change of histone modifications is associated with alteration of chromatin structure and epigenetic gene expression [2]. Here, we review recent studies of epigenetic control of gene expression, especially focusing on DNA repair genes, their role in carcinogenesis and the silencing mechanism involving DNA methylation and histone modification in cancer.

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Epigenetic gene silencing in cancer

It is clear that gene mutations, deletions or genomic rearrangements can cause cancer. These genetic aberrations lose the function of tumor suppressor genes. Recent studies have revealed that a number of those genes are inactivated by aberrant promoter hypermethylation in human cancer and that these genetic aberrations and epigenetic promoter hypermethylation are intricately connected in cancer development from early to late stages. Promoter hypermethylation can have an effect similar to genetic aberration of the gene, leading to a loss of gene function [3–5]. For example, both mutations and promoter hypermethylation of the *VHL* gene occur in renal cancer. Several tumor suppressors, often hypermethylated in cancer, are mapped to chromosomal regions characterized by frequent loss of heterozygosity (LOH). Furthermore, in breast cancer, both genetic and epigenetic aberrations of *BRCA1* produce a similar microarray pattern of gene ex-

pression [4, 5]. Thus, epigenetic gene silencing associated with aberrant promoter hypermethylation is an alternative mechanism to genetic aberration to inactivate tumor suppressor genes. Knudson's two-hit hypothesis may account for the full inactivation of a tumor suppressor gene [6], and this is usually achieved by mutation in one allele and LOH in another allele or homozygous deletion. Recent studies have shown that aberrant promoter hypermethylation of one allele with coordination of mutation or LOH in another allele, or methylation of both alleles, would cause the same effect of two hits, leading to functional inactivation of the gene in cancer [3, 5].

Silencing of DNA repair genes

There are several DNA mismatch repair (MMR) genes – such as *MLH1, MSH2, MSH3, MSH6, PMS1* and *PMS2* – and they form complexes to recognize mismatched pairs and to repair them. Mutations in *MLH1, MSH2, PMS1* and *PMS2* have been found in human hereditary nonpolyposis colorectal cancer (HNPCC) [7]. Of these genes, *MLH1* and another DNA repair gene, *MGMT*, draw special attention with regard to aberrant promoter hypermethylation in cancer. *MLH1* is frequently methylated in sporadic colorectal, gastric and endometrial cancers with microsatellite instability [8, 9]. As a consequence of loss of MLH1 function, mutation would accumulate in the genome. *MGMT*, encoding O^6-methylguanine (O^6-mG)-DNA methyltransferase, prevents G:C to A:T transition mutation by repairing the alkylated base, O^6-mG, and is frequently methylated in a wide spectrum of cancers [9]. Loss of MGMT function also causes mutations leading to tumor induction. In fact, a correlation was found between *MGMT* methylation and transition mutations of *K-ras* and *p53* in various cancers [10, 11]. Strikingly, 71% of non-CpG transition mutations were associated with *MGMT* aberrant methylation [11]. *MLH1* and *MGMT* also frequently exhibit promoter hypermethylation in precancerous tissues [12–14], indicating that inactivation of DNA repair genes seems to be the initial event in tumor development. In other words, DNA repair systems are critically important anti-carcinogenic mechanisms, preventing genetic changes leading to malignant transformation of cells. However, in the situation where the system is overloaded and genetic abnormalities are accumulated in a cell, apoptosis can function as another anti-carcinogenic mechanism to remove precancerous cells (fig. 1) [15]. This model is supported by the results of mice deficient in *Mgmt* and *Mlh1* [16]. $Mgmt^{-/-}$ $Mlh1^{+/+}$ mice were killed by apoptotic response when even low doses of alkylating agents were administrated. In contrast, $Mgmt^{-/-}$, $Mlh1^{-/-}$ (double knockout) mice were resistant to high doses of the chemicals, although a high incidence of cancers was observed.

It is of interest to note the expression level of both *MGMT* and *MLH1* genes in human cancer. We found that 20 and 60% of hepatocellular carcinoma lacked *MLH1* and *MGMT* expression, respectively. Ten percent of tumors lacked both expressions, correlating with an advanced pTNM stage [17, 18]. Lack of MGMT alone is a poor prognostic factor in several tumors – such as lymphoma, hepatocellular, biliary tract, gastric and breast cancers – but not in colorectal cancer [19–21]. Furthermore, lack

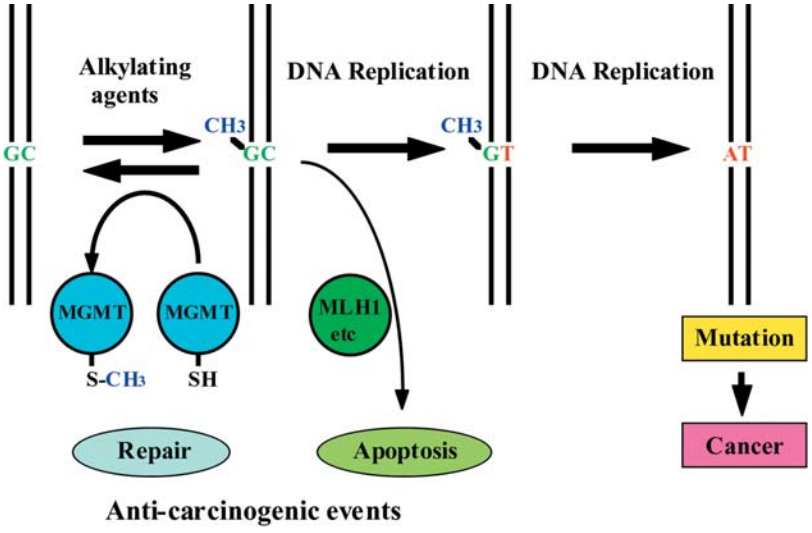

Figure 1. A model for anti-carcinogenic mechanisms. Alkylating agents produce alkylated bases such as O^6-methylguanine in DNA. This methylated guanine pairs with TTP as well as dCTP and leads to GC-to-AT transition mutation through two cycles of DNA replication. Accumulation of such mutations can cause malignant transformation of cells. This process can be prevented by two anti-carcinogenic mechanisms. MGMT protein repairs O^6-methylguanine to prevent the mutation. Cells carrying the mutagenic bases are eliminated through apoptosis, in which a protein complex, composed of MLH1 and some other mismatch repair proteins, is involved.

of both MGMT and MLH1 is much more correlated with a poor prognosis in hepatocellular and biliary tract cancers [18, 21]. On the other hand, glioma with hypermethylated *MGMT* was more sensitive to alkylating agents, suggesting *MGMT* hypermethylation as a good predictive marker for chemotherapy [22].

Epigenetic silencing mechanism: DNA methylation and histone modification

Promoter hypermethylation is associated with gene silencing, as mentioned above. There are three biologically active DNA methyltransferases (DNMTs) in mammals, DNMT1, DNMT3a and DNMT3b. DNMT1 mainly works on maintenance of methylation, whereas DNMT3a and DNMT3b work on de novo methylation. Mice targeted with each DNMT show that each protein is critical for embryonic development. $Apc^{Min/+}$ *Dnmt1*-hypomorphic mice show complete suppression of intestinal polyp formation, accompanied by reduction in frequency of CpG island methylation [23]. However, methylation of CpG islands, including $p16^{INK4a}$, is maintained in human colon cancer cells lacking *DNMT1*, though overall genomic methylation is reduced by 20% [24]. Disruption of both *DNMT1* and *DNMT3b* shows drastic reduction of genomic DNA methylation, resulting in abrogation of silencing of the $p16^{INK4a}$ gene and growth suppression, in human colon cancer cells [25].

The histone amino termini are susceptible to a variety of covalent modifications, such as acetylation, methylation, phosphorylation and ubiquitination. The most prominent among these are acetylation and methylation of lysine residues, which are modified by histone acetyltransferase (HAT) and histone methyltransferase (HMT), respectively. These play a pivotal role in chromatin assembly and epigenetic gene regulation. Acetylation of histone H3 and H4 is associated with euchromatin with increased accessibility to transcriptional factors. Methylation of histone H3 lysine 4 (H3K4) is also associated with euchromatin and correlated with transcriptional activity. In contrast, histone H3 lysine 9 (H3K9) methylation is associated with the formation of stably silenced heterochromatin [2, 26, 27]. It has been found that heterochromatin protein-1 α (HP1α) is important in establishing heterochromatin. It binds to methylated H3K9 residue and interacts with histone methyltransferases to recruit them to sites of methylated H3K9, leading to propagation of heterochromatin [2, 27].

The silencing process via DNA methylation was initially thought to be simply due to physical interference between transcription factors and their binding sequences [28]. However, it seems that DNA methylation does not lead to gene-silencing by itself. Recruitment of proteins to methylated DNA is required for the formation of heterochromatin to silence genes. Recent studies of methyl-CpG binding proteins (MBDs) have reported a gene silencing mechanism based on CpG methylation. MBDs also function as transcriptional repressors in vitro. MBDs bind to CpG nucleotides in a methylation-dependent manner and interact with a corepressor complex containing histone deacetylases (HDACs), resulting in highly deacetylated nucleosomes. A recent report showed that MBD1 interacts with histone methyltransferase, Suv39h1, and binds to HDACs via Suv39h1 [29]. MeCP2 is also associated with methyltransferase activity on H3K9 in vivo, though the identity of the H3K9 methyltransferase is unknown [30]. Thus, interaction of those proteins results in heterochromatin containing tightly compacted, highly deacetylated and highly H3K9-methylated nucleosomes (fig. 2). On the other hand, when genes with a CpG island promoter are transcriptionally active, chromatin configuration changes to a euchromatic state, in which histones are acetylated and methylated at H3K4 and the nucleosomes are sparsely and irregularly spaced, leading to high accessibility of transcriptional factors [2, 4, 5, 31].

In cancer cell lines, in which either *MGMT* or *MLH1* is silenced, the promoter region of each gene shows hypermethylation of CpG and H3K9, hypomethylation of H3K4, and hypoacetylation of H3 and H4, resulting in inactive heterochromatin [32–34]. Recently, we actually revealed the precise methylation status at each of 97 CpG sites in the *MGMT* promoter CpG island and the histone modification status in both *MGMT*-negative and -positive cancer cell lines. In addition, we also showed that MeCP2 rather than MBD1 tends to bind to methylated *MGMT* promoter [32]. The result suggests a silencing mechanism of *MGMT*, in which MeCP2 binds to CpG-methylated promoter followed by recruitment of HDAC(s) and H3K9 methyltransferase, resulting in heterochromatin. For both *MGMT* and *MLH1* genes, HDAC inhibitor, TSA, increases histone acetylation in highly DNA-methylated promoters, with little effect on DNA methylation, gene expression and H3K9 methylation. However, a DNA demethylation agent, 5Aza-dC, substantially reactivates gene expression with the same degree of increased histone acetylation and considerably decreased H3K9 methylation [33, 34, Zhao W. et al., unpublished observations]. Thus, derepression of the gene correlates with DNA demethylation and decreased H3K9 methylation rather than with increased acetylations. 5Aza-dC treatment of colorectal cancer RKO cells, in which *MLH1* is silenced with hypermethylation of DNA and H3K9, hypomethylation of H3K4 and hypoacetylation of H3 at the promoter region, leads first to promoter demethylation, second to gene derepression and finally to complete reversal of histone modifications [33]. This result suggests that DNA hypermethylation is the dominant epigenetic mechanism, rather than the repressive histone modifications, in main-

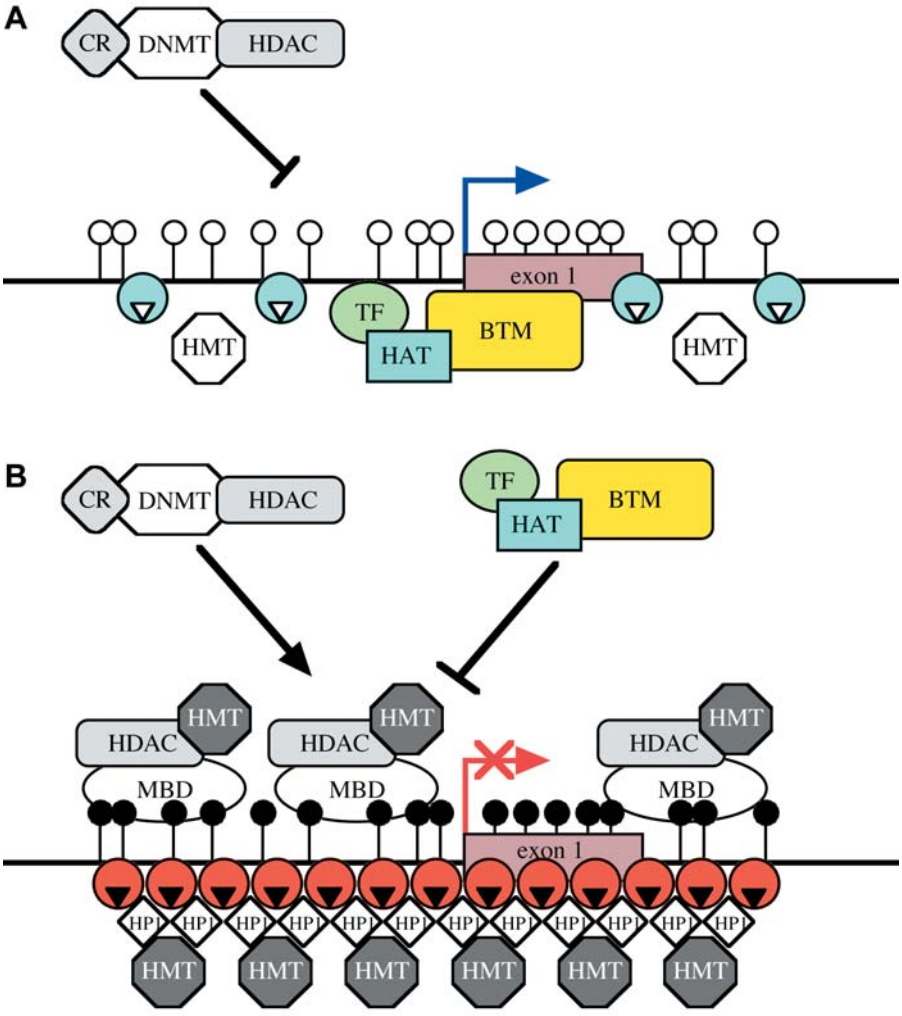

Figure 2. Aspects of the chromatin at promoter with CpG island. (*a*) Transcriptionally active euchromatin. CpGs are not methylated. The nucleosomes are distributed sparsely and irregularly, with histones acetylated and methylated at H3K4. Thus, transcriptional factors are accessible to their target sequences. (*b*) Transcriptionally inactive heterochromatin. CpGs are heavily methylated and bound by MBD protein(s), forming a complex with HDAC(s) and HMT. Histones are highly deacetylated and highly methylated at H3K9, and then the nucleosomes are tightly compacted. Finally, transcriptional factors are inaccessible to the targets, leading to gene silencing. Otherwise, DNMTs directly interact with HDACs and transcriptional corepressor. It is unknown whether all these phenomena simultaneously occur at the same locus. As for *MGMT* silencing, MeCP2 rather than MBD1 binds to methylated DNA. The identity of HMT is still unknown, and binding of HP1 is not confirmed. White lollipop, unmethylated CpG; black lollipop, methylated CpG; blue circle, acetylated histone; red circle, deacetylated histone; white triangle, methylated H3K4; black triangle, methylated H3K9; CR, transcriptional corepressor; TF, transcription factor; BTM, basic transcriptional machinery.

taining silencing of the *MLH1* gene in colorectal cancer. However, recent studies have revealed that DNMTs directly interact with HDACs and transcriptional corepressor, suggesting a contribution to heterochromatin by a mechanism other than DNA methylation [5]. In *Neurospora crassa* and *Arabidopsis thaliana*, the presence of H3K9 methylation is essential for all or a subset of DNA methylation [34, 35], though it is not clear how the H3K9 methylation directs DNA methylation. The mechanism may be more intricate in mammalian cells. Further studies must be conducted to understand the relationship between DNA methylation and histone modifications in establishment and maintenance of gene silencing.

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