DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy

Abstract
The unique properties of a cancer cell are acquired through a stepwise accumulation of heritable changes in the information content of proto-oncogenes and tumor suppressor genes. While gain, loss, and mutation of genetic information have long been known to contribute to tumorigenesis, it has been increasingly recognized over the past 5 years that 'epigenetic' mechanisms may play an equally important role. The main epigenetic modification of the human genome is methylation of cytosine residues within the context of the CpG dinucleotide. De novo methylation of ‘CpG islands’ in the promoter regions of tumor suppressor genes may lead to transcriptional silencing through a complex process involving histone deacetylation and chromatin condensation, and thus represents a tumorigenic event that is functionally equivalent to genetic changes like mutation and deletion. DNA methylation is interesting from a diagnostic viewpoint because it may be easily detected in DNA released from neoplastic and preneoplastic lesions into serum, urine or spu- tum, and from a therapeutic viewpoint because epigenetically silenced genes may be reactivated by inhibitors of DNA methylation and/or histone deacetylase. A better understanding of epigenetic mechanisms leading to tumor formation and chemoresistance may eventually improve current cancer treatment regimens and be instructive for a more rational use of anticancer agents.

Key words: apoptosis; cancer; chemotherapy; DNA methylation; drug resistance; 5-methylcytosine; gene inactivation; tumorigen- esis


Tumorigenesis may best be viewed as a process of somatic evolution in which a cell acquires selective advantages through a stepwise accumulation of changes in gene function. Each advantageous 'hit' can be stably transmitted to the daughter cells and is usually accompanied by a wave of clonal expansion. It has been known for decades that many of the cancer-associated gene changes stem from the gain, loss, or mutation of genetic information. However, it is becoming increasingly clear that 'epigenetic' events, i.e. heritable changes in gene function that cannot be explained by changes in DNA sequence, may also play an important role in many cancers. This review concerns DNA methylation as an epigenetic determinant of tumorigenesis,
focusing on (i) the distribution and maintenance of DNA methylation in normal cells (ii) DNA methylation-mediated silencing of tumor suppressor genes in cancer (iii) the use of DNA methylation events as tumor biomarkers, and (iv) the use of DNA methylation events as targets for anticancer therapy. We also briefly discuss current methods to detect changes in DNA methylation in clinical specimens.

DNA methylation, CpG islands, and DNA methyltransferases

To appreciate how changes in DNA methylation may impinge on the properties of a cell during tumorigenesis, it is necessary to first consider the distribution and function of DNA methylation in normal cells. DNA methylation in the human genome occurs nearly exclusively at cytosine residues within the symmetric dinucleotide, CpG (Fig. 1A). Methylated cytosine accounts for 0.75–1% of the total DNA bases, and ~70% of all CpG dinucleotides are methylated (2). Methylated cytosines are widely spread throughout the genome, with particularly high densities in the promoters of retroviruses and transposons that have accumulated in the genome (2). Unmethylated CpG sites are primarily confined to DNA regions with high relative densities of CpG, so-called ‘CpG islands’. The human genome contains roughly 29,000 CpG islands (3, 4) that are distributed in a non-random pattern, with a preference for the promoter and first exon regions of protein coding genes. While most CpG islands remain free of methylation and are associated with transcriptionally active genes, predominantly so-called ‘housekeeping’ genes, certain CpG islands are normally methylated, including those associated with imprinted genes and genes on the inactive X chromosome (5).

The DNA methylation pattern of a cell is accurately reproduced after DNA synthesis and is stably transmitted to the daughter cells. The covalent addition of methyl groups to CpG sites in the newly synthesized DNA strand is mediated by an enzyme known as DNA methyltransferase 1 (DNMT1) (Fig. 1B) (2). This enzyme is targeted to the replication fork where it efficiently methylates DNA containing hemi-methylated CpGs (postreplicative maintenance methylation). Two additional active DNA methyltransferases have been cloned, DNMT3A and DNMT3B, both of which catalyze the transfer of methyl groups to ‘naked’ DNA (de novo methylation) (2).

Translating the information content of DNA methylation

The methylation state of promoter CpG islands confers information about the transcriptional activity at these loci. It has been known for many years that, in general terms, there is an inverse relationship between the density of promoter methylation and the transcriptional activity of a gene (Fig. 2). However, the actual mechanisms by which DNA methylation modulates gene expression have remained elusive. Early experiments showed that methylation of specific DNA sequences can prevent the binding of some ubiquitous transcription factors. While this model is certainly an attractive one, it can explain only a minority of cases where methylation causes stable transcriptional silencing of genes. An alternative model implicates changes in the architecture of the nucleosomal core as the repressive element (Fig. 3). This model was reinforced by the recent identification of a family of proteins that preferentially bind to methyl-CpG
Some of these proteins have been directly implicated in methylation-dependent gene silencing by recruiting histone deacetylases to the sites of methylation. Histone deacetylases catalyze the removal of acetyl groups from the core histones, converting the open, transcriptionally competent chromatin structure into a closed structure that can no longer be accessed by the basal transcriptional machinery (Fig. 3). The linkage between methyl-CpG-binding proteins, histone deacetylases and the chromatin remodeling machinery has provided a basis for understanding how DNA methylation may mediate a transcriptionally incompetent chromatin state. Recently, it was shown that DNA methyltransferases can also recruit histone deacetylases to methylated sites (reviewed in (7)). The relative roles of DNA methyltransferases and methyl-CpG-binding proteins in conferring stable transcriptional repression remain to be determined.

Changes in promoter methylation during tumor progression

Compared with their normal counterparts, cancer cells exhibit significant changes in DNA methylation patterns, which can generally be summarized as global hypomethylation of the genome accompanied by focal hypermethylation events (8). The origin of these changes is largely unknown; searches for genetic

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**Fig. 2.** Unmethylated cytosines are found at high densities in CpG islands, which usually map to the promoter and first exon regions of housekeeping genes. (A) The fully unmethylated state of a promoter CpG island (open lollipops) is associated with transcriptional activity, whereas (B) dense methylation (closed lollipops) causes transcriptional silencing.

**Fig. 3.** Model for methylation-dependent gene silencing. The structural element of chromatin is the nucleosomal core, which consists of a 146-bp DNA sequence wrapped around core histones. Acetylation of the histones causes an open chromatin configuration that is associated with transcriptional activity. Methylated cytosines are recognized by methyl-CpG-binding proteins (MBDs), which in turn recruit histone deacetylases (HDACs) to the site of methylation, converting the chromatin into a closed structure that can no longer be accessed by the transcriptional machinery.
defects in components of the DNA methylation machinery have so far been unsuccessful. Much more is known about how changes in DNA methylation may lead to changes in gene expression that are important in tumorigenesis. The most emphasized implication of aberrant DNA methylation is inactivation of tumor suppressor genes. The clear association between promoter methylation and transcriptional inactivation has led to a revision of Knudson’s two-hit hypothesis for tumor suppressor inactivation by adding a new pathway to gene inactivation (9). In this respect, DNA methylation is functionally equivalent to genetic events like mutation and deletion, and the two alleles of a tumor suppressor gene may be inactivated by any combination of genetic and epigenetic events. There are now numerous examples of tumor suppressor genes inactivated by aberrant CpG island promoter methylation in human cancers (See (8) for a recent update). De novo methylation of these genes may occur early in tumor progression and lead to abnormal function of important cellular pathways, including those controlling cell cycle, apoptosis, and cell-to-cell growth signaling.

Changes in DNA methylation may have many cellular consequences other than those affecting the transcriptional activity of tumor suppressor genes. Indeed, DNA hypermethylation events may also cause inactivation of genes involved in the cellular response to chemotherapy (below), or inactivation of ‘caretaker’ genes, i.e. genes that are involved in DNA repair and protect cells from carcinogenic agents. Global hypomethylation, on the other hand, has been implicated in chromosome instability, loss of imprinting, and reactivation of transposons and retroviruses, all of which may contribute to tumorigenesis (8).

Methods to detect DNA methylation events in clinical specimens

Detection of methylated cytosine at specific sites in DNA from clinical specimens is notoriously difficult because the methylation signature is erased during conventional procedures used to amplify DNA, including cloning and polymerase chain reaction (PCR). Southern blot analysis of DNA digested with methylation-sensitive restriction endonucleases has previously been an indispensable tool in the study of DNA methylation, but has now been replaced by PCR methods that are based on initial modification of DNA with bisulfite (Fig. 4). Bisulfite selectively deaminates cytosine residues to uracil, leaving methylated cytosines intact (10), and the modified DNA can be used as a template in a standard PCR using primers specific for the gene of interest (11). Sequence analysis of the resulting PCR product provides an accurate display of methylated cytosines (11), but may be technically difficult and labor intensive. A variety of PCR methods have been developed, including methylation-specific PCR (12), methylation-sensitive single nucleotide primer extension (Ms-SNuPE) (13), and methods based on the use of restriction endonucleases (14, 15), which are simple to use but all suffer from the drawback that only a limited number of CpG sites can be analyzed in each assay. Recently, three PCR methods have been described that resolve differentially methylated DNA sequences on the basis of differences in melting temperature. The overall methylation status of a CpG island may be determined directly after PCR by melting analysis in the reaction tube, provided that the thermal cycler is coupled to a fluorometer (LightCycler or equivalent) (16). For more refined resolution of differentially methylated alleles, the
PCR product may be subjected to melting analysis in a polyacrylamide gel containing a gradient of chemical denaturants (DGGE) (17) or by denaturing high performance liquid chromatography (DHPLC) (18).

The most widely used of the above methods is methylation-specific PCR, which uses primers that are specific for bisulfite-modified DNA and discriminate between methylated and unmethylated alleles of a given gene. A potential pitfall inherent in the use of this method is the very high sensitivity (<0.1%), implying that a positive signal may be obtained from ‘background’ methylation of unknown biological significance (17, 19). Data obtained by methylation-specific PCR should, if not generated in a real-time quantitative format (20), be confirmed by one or more alternative methods to obtain information about the fraction of methylated alleles.

**Changes in DNA methylation as a tumor biomarker**

There is an urgent need of novel approaches for early diagnosis of cancer and detection of recurrence. In this respect, an important discovery was that DNA is released from tumor cells and may be detected at distal sites. Early experiments showed that tumor-derived DNA may be reliably identified by analysis of tumor-specific DNA markers, e.g. mutations in the TP53 tumor suppressor gene (21). However, the large number of different mutations identified in the TP53 gene and the requirement for initial analysis of tumor DNA limit the widespread use of such screenings. A methylation-based approach would have certain useful features compared with other DNA alterations in cancer because there is usually no interindividual variation in the methylation pattern of a particular gene. This allows the establishment of highly sensitive and universally applicable assays based on methylation-specific PCR.

Tumor-specific DNA may be easily obtained in a non-invasive way from the site where the cancer occurs, for example in the sputum of lung cancer patients or the urine from patients with prostate cancer. It has even been documented that significant amounts of circulating tumor DNA may be isolated from the serum of cancer patients (22). A particularly exciting study showed that one of two methylation markers was always positive in tumor and sputum DNA from patients with lung cancer, and that tumor-specific methylation changes could be detected in sputum samples up to 3 years prior to clinical diagnosis (23).

Analysis of the methylation status of certain genes in tumor DNA may also serve as a direct means to gain independent prognostic and predictive information. For example, promoter hypermethylation of the gene encoding the DNA repair enzyme O(6)-methylguanine DNA methyltransferase is a strong favorable prognostic marker in patients with brain tumors treated with alkylating agents (24).

**DNA methylation as a therapeutic target**

While inactivation of tumor suppressor genes by genetic and epigenetic mechanisms are, in many ways, functionally equivalent in tumorigenesis, there are some fundamental differences that may be potentially significant for anticancer therapy. First of all, while genetic hits confer a fixed, irreversible state of gene inactivation, epigenetic events do not interfere with the information content of the affected genes and are potentially reversible. The suppressing activity of epigenetic defects may be alleviated at two different levels: by inhibition of DNA methylation and inhibition of histone deacetylation (Fig. 5). Potent inhibitors of DNA methylation or histone deacetylase are available that can modulate gene transcription in vitro and in vivo at non-toxic concentrations. A very potent specific inhibitor of DNA methylation, 5-aza-2'-deoxycytidine, has been widely used as a demethylating agent in vitro, and is used clinically in the treatment of acute leukemias and myelodysplasia (25). Likewise, cell culture experiments have shown that histone deacetylase inhibitors (e.g. trichostatin A) can reactivate a range of epigenetically silenced genes, and several of these agents are now in clinical trial (26). However, a major drawback of drugs targeting the DNA methylation or histone acetylation machineries is that they lack specificity. Concomitant activation of genes that are normally silenced, but contribute to cellular immortalization and tumor progression in the activated state, may significantly compromise the beneficial effects of these drugs. Future research will be directed at developing more refined strategies to reactivate specific genes.

**Reversal of drug resistance**

Although chemotherapeutic drugs are widely used in the cure and palliation of human malignancies, intrinsic and acquired drug
resistance remains the most unpredictable factor affecting chemotherapy and a major impediment to successful treatment of many types of cancer. Understanding the cellular and molecular mechanisms leading to chemoresistance may dramatically impact on the way chemotherapeutic drugs are designed and used. The possibility that some genes conferring chemoresistance are reversibly switched off by DNA methylation is particularly exciting and may have important clinical implications.

It has become increasingly clear that many chemotherapeutic agents kill susceptible cells through the induction of the physiological cell death program (apoptosis). Accordingly, deregulation of any gene involved in the activation or execution of the apoptotic process may be a major mechanism of chemoresistance (27). A prominent example of this is the association between loss of the apoptosis-related protein caspase-8 and resistance to cytotoxic drugs like doxorubicin and cisplatin. Treatment of cells harboring caspase-8 promoter hypermethylation with 5-aza-2'-deoxycytidine led to re-expression of caspase-8 and restored sensitivity for chemotherapy (28, 29). Although these data are only preliminary and based on cell culture models, they suggest that targeting the silencing mechanism of apoptosis-related genes may increase the efficacy of various forms of chemotherapy, and that simple genetic tests may lead to the identification of patients who might benefit from treatment regimens combining inhibitors of DNA methylation and/or histone deacetylase with chemotherapeutic agents.

Chemoresistance may also be caused by upstream mechanisms, e.g. by deregulation of genes involved in drug-target interaction, interfering with either the stability or accumulation of the drug. Recently, it was shown that cellular accumulation of methotrexate in breast cancer cells can be impeded by promoter methylation of the gene encoding the reduced folate carrier, the protein that normally mediates transport of methotrexate over the plasma membrane into the cell (30). Treatment of the resistant cells with 5-aza-2'-deoxycytidine restored expression of the reduced folate carrier but did not in itself reverse methotrexate resistance due to concomitant up-regulation of one or more proteins involved in methotrexate efflux. Treatment of the cells with a combination of 5-aza-2'-deoxycytidine and an inhibitor of methotrexate efflux reversed methotrexate resistance in a synergistic manner. While this study highlights the significant potential of demethylating agents as chemosensitizers, it also illustrates that the use of these agents may not be trivial, and underscores the need for agents that reverse epigenetic silencing in a gene-specific manner.

References


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