Annu. Rev. Pharmacol. Toxicol. 2005. 45:629–56
doi: 10.1146/annurev.pharmtox.45.120403.095832

ABERRANT DNA METHYLATION AS A CANCER-INDUCING MECHANISM

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Key Words tumor suppressor genes, CpG island, 5-methylcytosine, hypomethylation, DNA demethylating agents

■ **Abstract** Aberrant DNA methylation is the most common molecular lesion of the cancer cell. Neither gene mutations (nucleotide changes, deletions, recombinations) nor cytogenetic abnormalities are as common in human tumors as DNA methylation alterations. The most studied change of DNA methylation in neoplasms is the silencing of tumor suppressor genes by CpG island promoter hypermethylation, which targets genes such as $p16^{INK4a}$, BRCA1, and hMLH1. There is a profile of CpG island hypermethylation according to the tumor type, and genes silent by methylation represent all cellular pathways. The introduction of bisulfite-PCR methodologies combined with new genomic approaches provides a comprehensive spectrum of the genes undergoing this epigenetic change across all malignancies. However, we still know very little about how this aberrant DNA methylation "invades" the previously unmethylated CpG island and how it is maintained through cell divisions. Furthermore, we should remember that this methylation occurs in the context of a global genomic loss of 5-methylcytosine (5mC). Initial clues to understand this paradox should be revealed from the current studies of DNA methyltransferases and methyl CpG binding proteins. From the translational standpoint, we should make an effort to validate the use of some hypermethylated genes as biomarkers of the disease; for example, it may occur with MGMT and GSTP1 in brain and prostate tumors, respectively. Finally, we must expect the development of new and more specific DNA demethylating agents that awake these methyl-dormant tumor suppressor genes and prove their therapeutic values. The expectations are high.

HISTORICAL INTRODUCTION

The field of DNA methylation is attracting the interest of many researchers and clinicians around the world. Some of the best laboratories are gradually changing their old interests and are moving into the emerging fields of epigenetics and, particularly, DNA methylation. Biotechnological and pharmaceutical companies are developing research programs specifically designed to develop new DNA

demethylating drugs and to produce diagnostic kits based on DNA methylation. This exciting new area of research combines questions about basic processes (How are DNA methylation patterns established? What key molecules are involved in the mechanism?) and extremely important clinical questions (Is the hypermethylation of this tumor suppressor gene a good marker of poor prognosis or good response to chemotherapy? Can we use DNA demethylating drugs in chemotherapy regimens?).

The first observation of DNA methylation aberrations in human cancer cells was the finding that tumors were globally hypomethylated (1) only one year after the first oncogene mutation was discovered in the H-ras in a human primary tumor. The idea that the genome of the cancer cell undergoes a reduction of its 5-methylcytosine (5mC) content in comparison with the normal tissue has been firmly corroborated (2, 3). However, genomic hypomethylation does not associate with overexpression of oncogenes as originally thought, and it may be related to the generation of chromosomal instability. Then, as a paradox, gene hypermethylation was also observed in human tumors. To the best of my knowledge, the first discovery of methylation in a CpG island of a tumor suppressor gene in a human cancer was that of the Retinoblastoma (Rb) gene in 1989 (4). Not until 1994 was the idea that CpG island promoter hypermethylation could be a mechanism to inactivate genes in cancer fully restored as a result of the discovery that the Von Hippel-Lindau (VHL) gene also undergoes methylation-associated inactivation (5). However, the true origin of the current period of research in cancer epigenetic silencing was perhaps the discovery that CpG island hypermethylation was a common mechanism of inactivation of the tumor suppressor gene $p16^{INK4a}$ in human cancer (6–8). The introduction of powerful and user-friendly techniques, such as sodium bisulfite modification (9) and methylation-specific polymerase chain reaction (PCR) (10), were also of extreme relevance. From that time forward, the list of candidate genes with putative aberrant methylation of their CpG islands has grown exponentially (11) and it is time to prove the contribution of each gene to tumorigenesis.

THE METHODOLOGY REVOLUTION IN DNA METHYLATION

The emergence of a new technology for studying DNA methylation based on bisulfite modification coupled with PCR has been decisive in the expansion of the field of DNA methylation. Until a few years ago, the study of DNA methylation was almost entirely based on the use of enzymes that distinguished unmethylated and methylated recognition sites. This approach had many drawbacks, from incomplete restriction cutting to limitation of the regions of study. Furthermore, it usually involved Southern blot technologies, which required relatively substantial amounts of DNA of high molecular weight. The popularization of the bisulfite treatment of DNA (which changes unmethylated C to T, but maintains the methylated C as

a C), associated with amplification by specific PCR primers (methylation-specific PCR), taqman, restriction analysis, and genomic sequencing (12), has made it possible for every laboratory and hospital in the world to have a fair opportunity to study DNA methylation, even using pathological material from old archives. We call this change the "universalization of DNA methylation." The techniques described, which are ideal for studying biological fluids and the detailed DNA methylation patterns of particular tumor suppressor genes, can also be coupled with global genomic approaches for establishing molecular signatures of tumors based on DNA methylation markers, such as CpG island microarrays, restriction landmark genomic scanning, and amplification of intermethylated sites (12) (Figures 1 and 2).

Moreover, we now have serious cause to believe that we can study the content and distribution of 5mC in the cellular nuclei and the whole genome thanks to two new tools: the improved immunohistochemical staining of 5mC (13, 14), which allows localization of the latter in the chromatin structure, and high performance capillary electrophoresis (HPCE), a reliable and affordable technique for measuring total levels of 5mC (15) (Figures 1 and 2).

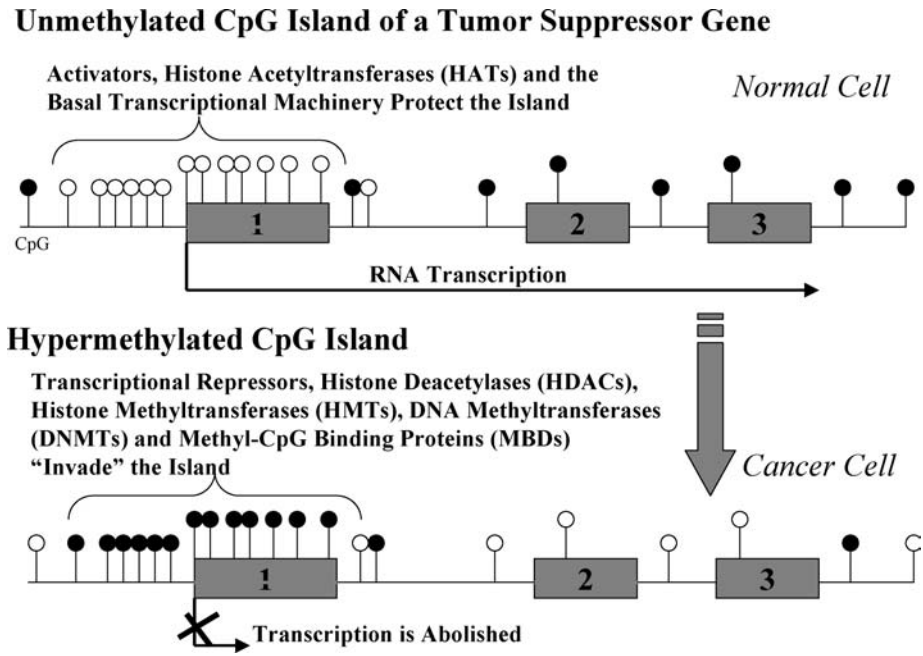

Figure 1 Protein occupancy and methylation status of a promoter CpG island of a tumor suppressor gene in normal and cancer cells. Gray boxes, exons; white circles, unmethylated CpGs; black circles, methylated CpGs.

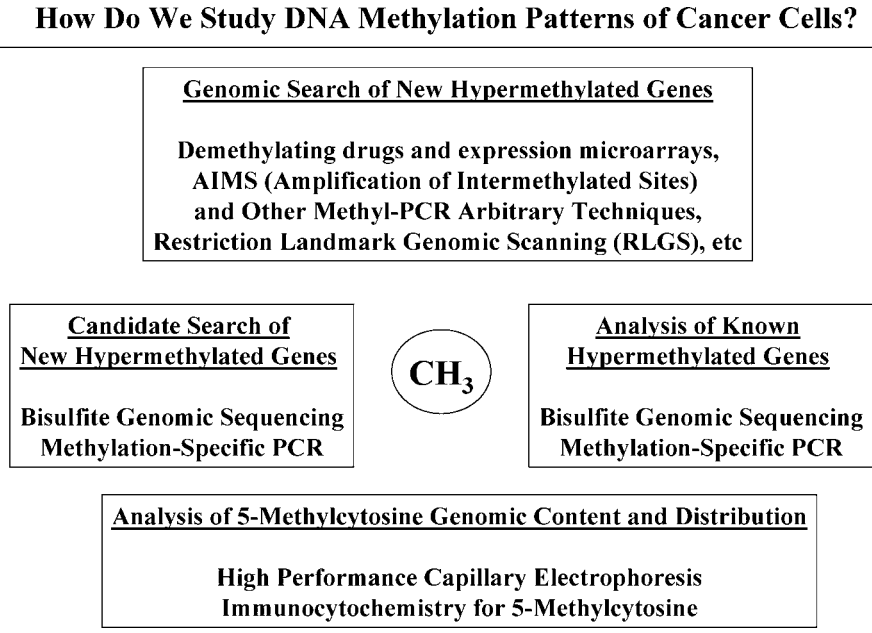

Figure 2 Techniques available for the study of DNA methylation according to the researcher interests.

THE MOLECULAR PLAYERS IN DNA METHYLATION ESTABLISHMENT AND SIGNALING

Methylation occurs at the 5′ carbon of cytosine, a relatively unreactive position. The catalytic mechanism of DNA (cytosine-5)-methyltransferases has been proposed as being similar to that of thymidylate synthetase, in which an enzyme cysteine thiolate binds covalently to the 6-position. This pushes electrons to the 5-position to make the carbanion, which can then attack the methyl group of N5,N10-methylenetetrahydrofolate. After methyl transfer, abstraction of a proton from the 5-position may allow reformation of the 5–6 double bond and release of the enzyme.

The first DNA cytosine-methyltransferase identified was revealed by purification and cloning. It remains the sole mammalian DNA methyltransferase to have been identified by biochemical assay (16). This enzyme, now termed DNMT1, is a protein that contains 1620 amino acids and exhibits a 5- to 30-fold preference for hemimethylated substrates. This property led to the assignment of DNMT1 as the enzyme responsible for maintaining the methylation patterns following DNA replication (16). However, there is no direct evidence that DNMT1 is not also involved in certain types of de novo methylation, and, in fact, DNMT1 is involved in most of the de novo methylation activity in embryo lysates (16).

The remaining DNA methyltransferases were identified by searches of expressed sequence tag (EST) databases. The first of these was DNMT2 (17). This lacks the large N-terminal regulatory domain common to other eukaryotic methyltransferases and does not exhibit comparable DNA methyltransferase activity, although it does seem to have some residual activity in vitro (18). DNMT3a and DNMT3b were soon identified by searching EST databases (19) and were proposed to be the enzymes responsible for de novo methylation (20). Mutations in the human DNMT3B gene are responsible for ICF syndrome. Figure 1*A* shows a schematic representation of the DNMT family.

Although DNMTs were originally classified as maintenance or de novo DNA methyltransferases, there are several strands of evidence that indicate that all three DNMTs not only cooperate but also may possess both de novo and maintenance functions in vivo (21–25).

The information stored by methylation of CpGs has functional significance only in the context of chromatin. Since its discovery, DNA methylation has been associated with a transcriptionally inactive state of chromatin; however, the mechanisms by which DNA methylation is translated into transcriptionally silent chromatin have only recently started to be unveiled.

Historically, several hypotheses have been proposed to explain the way by which DNA methylation is interpreted by nuclear factors. The first possibility is that DNA methylation inhibits the binding of sequence-specific transcription factors to their binding sites that contain CpG (26). In this context, a protein with an affinity for unmethylated CpGs has also been identified that is associated with actively transcribed regions of the genome (27). In this case, methylation of CpGs would result in release of this protein. An alternate model proposed that methylation may have direct consequences for nucleosome positioning, for instance, by leading to the assembly of specialized nucleosomal structures on methylated DNA that silence transcription more effectively than conventional chromatin (28). The third possibility is that methylation leads to the recruitment of specialized factors that selectively recognize methylated DNA and either impede binding of other nuclear factors or have a direct effect on repressing transcription (29).

Although there are examples that support all three possibilities, the active recruitment of methyl-CpG binding activities appears to be the most widespread mechanism of methylation-dependent repression.

MeCP1 and MeCP2 were the first two methyl-CpG binding activities described (29). Although MeCP1 was originally identified as a large multiprotein complex, MeCP2 is a single polypeptide with an affinity for a single methylated CpG. Characterization of MeCP2 in subsequent years led to the identification of the minimum portion with affinity for methylated DNA, i.e., its methyl-CpG binding domain (MBD) (30) and its transcriptional repression domain (TRD).

Database searches led to the identification of additional proteins harboring the MBD, namely MBD1, MBD2, MBD3, and MBD4 (31). Whereas mammalian MBD1 and MBD2 are bona fide methylated DNA binding proteins, MBD3 is able to bind methylated DNA only in certain species (31, 32). In the case of MBD4, this protein binds preferentially to m5CpG x TpG mismatches. The primary product

of deamination at methyl-CpGs and the combined specificities of binding and catalysis indicate that this enzyme functions to minimize mutation at methyl-CpGs.

In 1997, the laboratories of Drs. Adrian Bird and Alan Wolffe reported that MeCP2 represses the transcription of methylated DNA through the recruitment of a histone deacetylase-containing complex (33, 34). This finding established for the first time a mechanistic connection between DNA methylation and transcriptional repression by the modification of chromatin. Additional reports have established the mechanism by which the remaining MBDs connect DNA methylation and gene silencing (32, 35, 36). Ng et al. (35) reported that MBD2 is, in fact, a component of the formerly identified MeCP1 complex, which exhibits histone deacetylase activity. On the other hand, Wolffe's laboratory identified MBD3 as a component of the Mi-2/NURD complex, which exhibits both histone deacetylase and ATPase-dependent nucleosome remodeling activities (32).

To understand the implications of the connections between DNA methylation and histones, it is important to define the relevance of these posttranslational histone modifications to the determination of different chromatin states. Most histone modifications occur in their protruding N-terminal tails. This specificity in the pattern of modifications under particular conditions led to the proposal of the histone code hypothesis, in which histone modifications act sequentially or in combination to form a code that may be read by nuclear factors (37). There are several modifications that are compatible with gene silencing. In general, histone deacetylation leads to gene silencing. Furthermore, methylation of lysine 9 of histone H3 has been associated with gene silencing.

Following the finding of the coupling between DNA methylation and histone deacetylation by MBDs, additional connections have been found. On one hand, DNMTs are also known to recruit histone deacetylases (38, 39); on the other hand, both DNMTs and MBDs have been reported to recruit histone methyltransferases that modify lysine 9 of histone H3 (40–42).

Therefore, multiple connections are established between hypermethylation of the CpG islands of tumor-suppressor genes in cancer and their transcriptional silencing. The specificity of these connections and the special circumstances in which these different elements participate for different genes remain to be determined.

In the case of MBD proteins, association with hypermethylated promoters and their involvement in silencing their corresponding genes has now been demonstrated in a number of cases (43–45). In fact, MBD proteins appear to be a common feature of the methylated promoter of these genes and also display a remarkable specificity in vitro (46) and in vivo (45). Thus, a MBD-specific profile for hypermethylated CpG islands is starting to be unveiled.

THE DNA METHYLATION SETTING OF HEALTHY CELLS

The inheritance of information based on gene expression levels is known as epigenetics, as opposed to genetics, which refers to information transmitted on the basis of gene sequence. The main epigenetic modification in humans is the

methylation of the cytosine located within the dinucleotide CpG. 5mC in normal human tissue DNAs constitutes 0.75%–1% of all nucleotide bases, and about 4%–6% of all cytosines are methylated in normal human DNA (2, 3, 47).

CpG dinucleotides are not randomly distributed throughout the vast human genome. CpG-rich regions, known as CpG islands (48), are usually unmethylated in all normal tissues and frequently span the 5′-end region (promoter, untranslated region, and exon 1) of a number of genes; they are excellent markers of the beginning of a gene. If the corresponding transcription factors are available, the histones are in an acetylated and unmethylated state, and if the CpG island remains in an unmethylated state, then that particular gene will be transcribed (Figure 3, see color insert).

Of course, there are exceptions to the general rule. We can find certain normally methylated CpG islands in at least four cases: imprinted genes, X-chromosome genes in women, germline-specific genes, and tissue-specific genes (49). Genomic or parental imprinting is a process involving acquisition of DNA hypermethylation in one allele of a gene early in the male and female germline that leads to monoallelic expression (50). A similar phenomenon of gene-dosage reduction can also be invoked with regard to the methylation of CpG islands in one X-chromosome in women, which renders these genes inactive to avoid redundancy. Finally, although DNA methylation is not a widely occurring system for regulating "normal" gene expression, sometimes it does indeed accomplish this purpose, as with the genes whose expression is restricted to the male or female germline and not expressed later in any adult tissue, such as the MAGE gene family. Finally, methylation has been postulated as a mechanism for silencing tissue-specific genes in cell types in which they should not be expressed. However, it is still not clear whether this type of methylation is secondary to a lack of gene expression owing to the absence of the particular cell type–specific transcription factor or whether it is the main force behind transcriptional tissue-specific silencing.

What is the significance of the presence of DNA methylation outside the CpG islands? One of the most exciting possibilities for the normal function of DNA methylation is its role in repressing parasitic DNA sequences (51, 52). Our genome is plagued with transposons and endogenous retroviruses acquired throughout the history of the human species. We can control these imported sequences with direct transcriptional repression mediated by several host proteins, but our main line of defense against the large burden of parasitic sequence elements (more than 35% of our genome) may be DNA methylation. Methylation of the promoters of our intragenomic parasites inactivates these sequences and, over time, will destroy many transposons.

The perfect epigenetic equilibrium of the previously described normal cell is dramatically transformed in the cancer cell. The epigenetic aberrations observed can be summarized as falling into one of two categories: transcriptional silencing of tumor suppressor genes by CpG island promoter hypermethylation and global genomic hypomethylation.

GENOMIC HYPOMETHYLATION OF TRANSFORMED CELLS

At the same time that certain CpG islands become hypermethylated, as discussed below, the genome of the cancer cell undergoes dramatic global hypomethylation. The malignant cell can have 20%–60% less genomic 5mC than its normal counterpart (2, 3). The loss of methyl groups is accomplished mainly by hypomethylation of the "body" (coding region and introns) of genes and through demethylation of repetitive DNA sequences , which accounts for 20%–30% of the human genome. The degree of genomic DNA hypomethylation increases through all the tumorogenic steps, from the benign proliferations to the invasive cancers (14) (Figure 4, see color insert).

How does global DNA hypomethylation contribute to carcinogenesis? Three mechanisms can be invoked: chromosomal instability, reactivation of transposable elements, and loss of imprinting. Undermethylation of DNA might favor mitotic recombination, leading to loss of heterozygosity as well as promoting karyotypically detectable rearrangements. Additionally, extensive demethylation in centromeric sequences is common in human tumors and may play a role in aneuploidy. It has been reported that patients with germline mutations in DNA methyltransferase 3b (DNMT3b) have numerous chromosome aberrations (53). Hypomethylation of malignant cell DNA can also reactivate intragenomic parasitic DNA, such as L1 (long interspersed nuclear elements, LINES) and Alu (recombinogenic sequence) repeats (51, 52). These, and other previously silent transposons, may now be transcribed and even "jump" to other genomic regions where they can disrupt normal cellular genes. Finally, the loss of methyl groups can affect imprinted genes. The best-studied case concerns the effects of the H19/IGF-2 locus on chromosome 11p15 in certain childhood tumors (54, 55).

However, we still know very little about the real role of DNA hypomethylation in the development of cancer cells. Is it really a "causative" factor? Or just a "modulator of cancer risk?" Or only a "bystander passenger?" The studies in mouse models are extremely interesting but puzzling: When the mouse deficient in DNA methylation owing to a defect in DNMT1 is crossed with the colon adenoma-prone Min mouse (with a genetic defect in APC), the resulting mouse has fewer tumors (56); but another DNMT1 defective mouse may have an increased risk of lymphomas (57). This paradox is an important question that needs to be addressed in the near future.

METHYLATION-ASSOCIATED SILENCING OF TUMOR SUPPRESSOR GENES

CpG islands located in the promoter region of tumor suppressor genes, normally unmethylated at these regions like in all the other genes, undergo a dense hypermethylation in cancer cells leading to gene silencing (Figure 3). Not every gene is

methylated in every tumor type, but strong specificity is apparent with respect to the tissue of origin (11, 58). We have recently described the exquisite profile of hypermethylation that occurs in primary human tumors (11). Furthermore, the number of hypermethylated genes increases with the malignant potential (14) (Figure 4).

We do not currently know why some genes became hypermethylated in certain tumors, whereas others with similar properties (a typical CpG island, a history of loss of expression in certain tumors, and the absence of mutations) remain methylation-free. We can hypothesize, as researchers have done before with genetic mutations, that a particular gene is preferentially methylated with respect to others in certain tumor types because inactivation confers a selective advantage, in the Darwinian sense, on the former. Another option is that aberrant DNA methylation is directly targeted. It has been proposed that fusion proteins, such as PML-RAR, can contribute to aberrant CpG-island methylation by recruiting DNMTs and HDACs to aberrant sites (59). This latter activity is somewhat controversial but, in any case, does not seem to be a general mechanism, at least in leukemia patients (60). Selection and targeting are not exclusive events, and they are most probably happening together in the generation and maintenance of hypermethylated CpG islands of tumor suppressor genes.

The tumor suppressor genes, bona fide and "look-alike," that undergo aberrant CpG island methylation in human cancer affect all the cellular pathways and have relevant consequences (49). A brief list of the most significant genes inactivated by DNA hypermethylation is represented in Table 1 and includes the following:

(*a*) Cell cycle. The cell cycle inhibitor $p16^{INK4a}$ is hypermethylated in a wide variety of human primary tumors and cell lines (6–8), allowing the cancer cell to escape senescence and start proliferating. The Rb gene and the cell cycle inhibitor $p15^{INK4b}$ can also suffer occasionally aberrant methylation (4, 61).

(*b*) p53 network. p53 is the most frequently mutated tumor suppressor gene in human cancer; nevertheless, half of human primary tumors are wild-type p53. Another way to inactivate p53 is through the methylation-mediated silencing of the tumor suppressor gene $p14^{ARF}$ (62–64) because in this way the MDM2 oncogenic protein is not inhibited by $p14^{ARF}$ and is free to induce p53 degradation (64). p73, a gene that is a p53-homolog, is also hypermethylated in leukemias (65).

(*c*) APC/β-catenin/E-cadherin pathways. APC is commonly mutated in sporadic colon tumors, but little was known about the relevance of this particular pathway in noncolorectal tumorogenesis until recently. Now it is recognized that aberrant methylation of APC is a common lesion in other neoplasms of the aerodigestive tract (66). E-cadherin, H-cadherin, and FAT tumor-suppressor cadherin promoter hypermethylation is also important in the cancer biology of breast, colon, and other tumor types (25, 67, 68). Finally, methylation-associated silencing of the genes encoding secreted frizzled-related proteins (SFRPs), which possess a domain similar to one in the WNT-receptor frizzled

Table 1 A selected list of genes that undergo CpG island hypermethylation in human cancer

Gene	Function	Location	Tumor profile	Consequences
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor	9p21	Multiple types	Entrance in cell cycle
$p14^{ARF}$	MDM2 inhibitor	9p21	Colon, stomach, kidney	Degradation of p53
$p15^{INK4b}$	Cyclin-dependent kinase inhibitor	9p21	Leukemia	Entrance in cell cycle
hMLH1	DNA mismatch repair	3p21.3	Colon, endometrium, stomach	Frameshift mutations
MGMT	DNA repair of 06-alkyl-guanine	10q26	Multiple types	Mutations, chemosentivity
GSTP1	Conjugation to glutathione	11q13	Prostate, breast, kidney	Adduct accumulation?
BRCA1	DNA repair, transcription	17q21	Breast, ovary	Double-strand breaks?
p73	p53 homolog	1p36	Lymphoma	Unknown
LKB1/STK11	Serine/threonine kinase	19p13.3	Colon, breast, lung	Unknown
ER	Estrogen receptor	6q25.1	Breast	Hormone insensitivity
PR	Progesterone receptor	11q22	Breast	Hormone insensitivity
AR	Androgen receptor	Xq11	Prostate	Hormone insensitivity
PRLR	Prolactin receptor	5p13-p12	Breast	Hormone insensitivity
RARβ2	Retinoic acid receptor β2	3p24	Colon, lung, head, and neck	Vitamin insensitivity?
RASSF1A	Ras effector homolog	3p21.3	Multiple types	Unknown
NORE1A	Ras effector homolog	1q32	Lung	Unknown
VHL	Ubiquitin ligase component	3p25	Kidney, hemangioblastoma	Loss of hypoxic response?
Rb	Cell cycle inhibitor	13q14	Retinoblastoma	Entrance in cell cycle
THBS-1	Thrombospondin-1, antiangiogenic	15q15	Glioma	Neovascularization

(*Continued*)

Table 1 (*Continued*)

Gene	Function	Location	Tumor profile	Consequences
CDH1	E-cadherin, cell adhesion	16q22.1	Breast, stomach, leukemia	Dissemination
CDH13	H-cadherin, cell adhesion	16q24	Breast, lung	Dissemination?
FAT	Cadherin, tumor suppressor	4q34-35	Colon	Dissemination?
HIC-1	Transcription factor	17p13.3	Multiple types	Unknown
APC	Inhibitor of β-catenin	5q21	Aerodigestive tract	Activation β-catenin route
SFRP1	Secreted Frizzled-related Protein 1	8p12-p11	Colon	Activation WNT signaling
COX-2	Cyclooxygenase-2	1q25	Colon, stomach	Antiinflammatory resistance?
SOCS-1	Inhibitor of JAK/STAT pathway	16p13.13	Liver, myeloma	JAK2 activation
SOCS-3	Inhibitor of JAK/STAT pathway	17q25	Lung	JAK2 activation
GATA-4	Transcription factor	8p23-p22	Colon, stomach	Silencing of target genes
GATA-5	Transcription factor	20q13	Colon, stomach	Silencing of target genes
SRBC	BRCA1-binding protein	1p15	Breast, lung	Unknown
SYK	Tyrosine kinase	9q22	Breast	Unknown
RIZ1	Histone/protein methyltransferase	1p36	Breast, liver	Aberrant gene expression?
DAPK	Pro-apoptotic	9q34.1	Lymphoma, lung, colon	Resistance to apoptosis
TMS1	Pro-apoptotic	16p11	Breast	Resistance to apoptosis
TPEF/HPP1	Transmembrane protein	2q33	Colon, bladder	Unknown

proteins and can inhibit WNT receptor binding to downregulate pathway signaling during development, has also been found in colorectal cancer (69).

(*d*) DNA repair. DNA methylation is one of the major players at this crossroads of all cell pathways. Selected examples are the methylation-mediated silencing of the mismatch DNA repair gene hMLH1 in sporadic cases of colorectal (70, 71), endometrial (72, 73), and gastric tumors (74) that cause the unusual

phenotype known as microsatellite instability; the promoter hypermethylation of MGMT (75) that prevents the removal of groups at the O^6 position of the guanine and leads to the appearance of K-ras and p53 mutations (76–78); the hypermethylation of the mitotic checkpoint gene CHFR (79); and the somatic inactivation of BRCA1 by aberrant methylation in breast and ovarian tumors (80), which alters its role in the repair of double-strand breaks in the DNA and leads to the same global expression changes that occur in the carriers of BRCA1 germ line mutations (81).

(*e*) Hormonal response. Aberrant methylation of the estrogen, progesterone, androgen, and prolactin receptors occurs in breast and uterine tumors and may render these cancer cells unresponsive to steroid hormones (45, 82–84). The differentiating action of the retinoids may also be abolished in tumors that show promoter hypermethylation of the retinoic acid receptor-β2 (60, 85–87) and the cellular retinol-binding protein I (87).

(*f*) Cytokine signaling. The suppressor of cytokine signaling (SOCS) family of proteins has been implicated in the negative regulation of several cytokine pathways, particularly the receptor-associated tyrosine kinase/signal transducer and activator of transcription (Jak/STAT) pathways of transcriptional activation. SOCS-1 and SOCS-3 undergo methylation-associated silencing in human cancer (88–90).

(*g*) The remaining pathways. This is not an exhaustive list, but I would like to emphasize that every imaginable molecular route can be affected by a candidate gene: the proapoptotic death-associated protein kinase (DAPK) (91) and TMS1 (92); the kidney tumor and hemangioblastoma-related VHL gene (5); the serine-threonine kinase LKB1/STK11 in hamartomatous neoplasms (93); the ras-effector genes RASSF1A (94, 95) and NORE1A (96); the antiangiogenic factor thrombospondin-1 (THBS-1) (97); the prostaglandin generator cyclooxygenase 2 (98); the TPEF gene that contains epidermal growth factor domains (99); the electrophilic detoxifier glutathione S-transferase P1 (GSTP1) in prostate, breast, and kidney tumors (100, 101); the transcription factors GATA-4 and GATA-5 (102); and many more.

Finally, it is important to mention that as a consequence of the increasing number of hypermethylated genes in human cancer, we need to demonstrate a role for the methylation-associated silencing of the studied gene in tumor biology. For example, we can check if the reintroduction of the gene in a deficient cancer cell line reduces colony formation (25, 45, 103) or inhibits xenograft growth in nude mice (95); if the hypermethylation of that gene correlates with a particular molecular or clinical phenotype, as is the case with the MGMT methylation that is associated with the appearance of transition mutations and chemosensitivity to alkylating agents (78); if the methylation-mediated silencing has the same effects as a frameshift mutation, as it has been shown for BRCA1 (81); or if mutations for that gene are not described, generating a knockout mouse, as has been done for HIC-1 (104).

HOW TO FIND NEW HYPERMETHYLATED GENES IN CANCER

Classical DNA methylation research concentrates on investigating the methylation status of cytosines occurring in known (or partially known) DNA sequences. However, alternate ways of investigating genome-wide methylation by searching for unidentified spots have been developed. They all rely on the distinctive properties of the CpG islands to find new methylated sequences in the genome.

The restriction landmark genomic scanning (RLGS) technique is one of the earliest ways reported for genome-wide methylation-specific searching (105). DNA is radioactively labeled at methylation-specific cleavage sites and then size-fractionated in one dimension. The digestion products are then digested with any restriction endonuclease that is specific for high-frequency targets. Fragments are separated in the second dimension, yielding a number of scattered methylation-related hot spots. The location and strength of a spot reveal its locus and the copy number of the corresponding restriction site. This approach led to the discovery of a number of CpG islands, for which there was no previous sequence knowledge (58).

Another suitable tool for screening the genome for regions displaying altered patterns of DNA is methylation-sensitive arbitrary primed PCR (AP-PCR), a simple DNA fingerprinting technique that relies on AP-PCR amplification followed by digestion with restriction isoschizomers (106). Strain-specific arrays of DNA fragments are generated by PCR amplification using arbitrary oligonucleotides to prime DNA synthesis from genomic sites that accidentally or roughly match. Usually, two cycles of PCR are performed under low stringency conditions, followed by PCR at high stringency with specific primers. DNA amplified in this manner is digested with a couple of methylation-sensitive isoschizomers, and fragments displaying differential methylation patterns are cloned and used as probes for Southern analysis to corroborate differential methylation of such DNA regions. Another approach is CpG island amplification (MCA) (107). DNA is digested with restriction isoschizomers and restriction products are PCR-amplified after end-adaptor ligation. Even though methylated CpG islands are preferably amplified, cloning of truly CpG-rich DNA regions is frequently a laborious task. A new technique based on DNA arbitrary PCR enriched in methyl-sequences, amplification of intermethylated sites (AIMS) (108) has enormous potential to "catch" new hypermethylated genes in human cancer (25).

Another original approach to isolated methylated CpG-rich regions has recently been described (109). This method employs affinity chromatography of a fragment of the methyl-CpG binding domain of MeCP2 to purify methylated CpG-rich fragments from mixtures obtained by digestion with methylation-specific restriction endonucleases. Chosen fragments are then cloned into a lamda Zap II vector, and fragments that are mostly rich in CpG dinucleotides are isolated by segregation of partially melted molecules (SMP) in polyacrylamide gels containing a linear gradient of chemical denaturant. Despite the advantages of this approach, the

specificity of the methylated DNA binding column needs to be improved for it to be a first-class method.

Undoubtedly, one of the most effective means of genome-wide searching for CpG islands is the use of the novel CpG island arrays technology. The best proposed array-based method, termed differential methylation hybridization (DMH), allows the simultaneous determination of the methylation rate of >276 CpG island loci (110). The CpG island library DNA fragments are gridded on high-density arrays. Genomic DNA from the tissues of interest is digested with MseI, which yields a large number of fragments containing intact CpG islands. Then, half of the subtracted DNA is digested with methylation-sensitive endonuclease BstU I, whose sequence target occurs frequently within CpG islands. Digestion products are used as templates for linker PCR. Unmethylated targets are differentiated from methylated ones because the former are cut and no PCR product is obtained, whereas the latter can be amplified by linker PCR. Resulting oligonucleotides, termed pretreated amplicons, are used as probes for screening hypermethylated sequences within the CpG island library. DMH has been applied to the screening of CpG methylation in both cancer cell lines and breast cancer using CpG arrays of 300 and 1104 targets, respectively. Therefore, DMH can be used as a sophisticated screening tool for selecting putative DNA fragments to be analyzed in greater depth by other more specific methods. DMH technology has unveiled new hypermethylated genes in colon cancer (25) and breast cancer, the latter using MBD-immunoprecipitated DNA, the ChIP on CHIP approach (chromatin immunoprecipitation + microarray) (45).

A modification of this method for the study of DNA methylation in cancer is the methylation-specific oligonucleotide (MSO) microarray (111). After bisulfite treatment and PCR amplification, products are array hybridized. MSO microarray is designed to detect methylation at specific nucleotide positions. Quantitative differences can be obtained by fluorescence detection.

Finally, one approach that it is becoming broadly used is the study of gene expression by microarrays comparing RNA from cancer cell lines before and after treatment with a demethylating drug (103, 112). This methodology has proven to be very useful in identifying newly hypermethylated genes. However, not all the genes that became reexpressed after the use of the demethylating agent are going to be methylated: rigorous bisulfite genomic sequencing, expression, and functional analysis are always required. However, this approach promises to be very successful.

USING DNA METHYLATION FOR TRANSLATIONAL PURPOSES

There is an increased necessity and obligation to translate these new results on DNA methylation aberration in cancer from the basic research laboratory to the clinic. There are several open fronts.

CpG Island Hypermethylation as a Marker of Cancer Cells

In recent years, we and other groups have extensively mapped an increasing number of gene CpG islands aberrantly hypermethylated in cancer (11, 49). These now include examples from most classes of human neoplasia. Such DNA methylation mapping has brought to light the existence of a unique profile of hypermethylated CpG islands that define each neoplasia (11, 25, 49, 58). Following this lead, many groups are currently providing us with the "methylotype" or "DNA methylation signature" of each form of human cancer. Only those methylation markers that are always unmethylated in normal "healthy" cells can be included in this panel. Combining 3–4 methylation markers, we can reach an informativity of 100% because hypermethylation events at different loci are unrelated (11). In some cases, such as prostate cancer, a single hypermethylated marker, GSTP1, is informative in 80%–90% of the cases (100, 113).

If we wish to use these epigenetic markers in a clinical setting, we need to use quick, easy, nonradioactive and sensitive ways of detecting hypermethylation in CpG islands of tumor suppressor genes, such as the methylation-specific PCR technique (MSP) (10). A careful design of the MS PCR primers with strong estringency conditions and the inclusion of positive and negative controls to avoid false positive results are always strongly encouraged. In this spirit, CpG island hypermethylation has been used as a tool to detect cancer cells in all types of biological fluids and biopsies: broncoalveolar lavage, lymph nodes, sputum, urine, semen, ductal lavage, and saliva (114, 115). An exciting new line of research was also initiated in 1999 when we demonstrated that it was possible to screen for hypermethylated promoter loci in serum DNA from lung cancer patients (116). Following our observation, many studies have corroborated the feasibility of detecting CpG island hypermethylation of multiple genes in the serum DNA of a broad spectrum of tumor types (114, 115), some even using semiquantitative and automated methodologies. Thus, DNA hypermethylation has proven its versatility over a wide range of tumor types and environments.

Another aspect worth considering is whether promoter hypermethylation of the CpG island of tumor suppressor genes occurs early on in tumorigenesis. Several examples of this can be mentioned, such as the presence of $p16^{INK4a}$, $p14^{ARF}$, APC, and MGMT hypermethylation in colorectal adenomas and hMLH1 aberrant methylation in atypical endometrial hyperplasia (49). Thus, the presence of aberrant CpG island methylation alone does not signal the presence of an invasive cancer. This may be the case, but premalignant or precursor lesions on their way to full tumorigenesis can also carry this epigenetic culprit. In fact, this finding may be useful in early detection screenings, especially in those people with a high familiar risk of developing cancer because they have patterns of CpG island hypermethylation similar to the sporadic cases (3). For those interested in cancer epidemiology, it should also be emphasized that aberrant DNA methylation has been found up to three years before lung cancer diagnosis in subjects, such as uranium miners and smokers, who are overexposed to carcinogens (117).

CpG Island Hypermethylation as a Marker for Tumor Behavior

All of us would like to look at a tumor and be able to predict its behavior. In recent years, attempts have been made in the field of genetics to establish reliable tumor prognosis, but they have faced a twofold problem: First, only a few genes are somatically mutated in human solid tumors (the oncogenes K-ras and Braf and the tumor suppressor gene p53 are the most reliable) and, second, owing to the heterogeneous cell population of a human primary neoplasm, no single marker can perfectly and completely predict the behavior of the neoplasm. Although this second problem cannot be solved by CpG island hypermethylation-based techniques, we can nevertheless take care of the first: Methylation-associated silencing affects many genes in all existing cellular pathways (11, 49). From apoptosis to cell adherence, from DNA repair to cell cycle, no route can escape from aberrant DNA methylation. As examples of DNA methylation markers of poor prognosis, we can mention that the DAPK and $p16^{INK4a}$ hypermethylation has been linked to tumor virulence in lung and colorectal cancer patients (118, 119). Further candidates awaiting analysis to determine their relation to enhanced metastasizing or angiogenic activity in primary tumors include the aberrant methylation of E-cadherin (CDH1), H-cadherin (CDH13), FAT tumor suppressor-cadherin, and thrombospondin-1 (THBS-1).

CpG Island Hypermethylation as a Predictor of Response to Treatment

The case for this concept needs to be made for each gene separately. The most compelling evidence is provided by the methylation-associated silencing of the DNA repair MGMT in human cancer. The MGMT protein (O^6-methylguanine DNA methyltransferase) is directly responsible for repairing the addition of alkyl groups to the guanine (G) base of the DNA (78). This base is the preferred point of attack in the DNA of several alkylating chemotherapeutic drugs, such as BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea], ACNU [1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea], procarbazine, streptozotocin, or temozolamide. Thus, tumors that had lost MGMT owing to hypermethylation (75) would be more sensitive to the action of these alkylating agents because their DNA lesions could not be repaired in the cancer cell, leading to cell death. We gave proof of principle for this hypothesis, and MGMT promoter hypermethylation effectively predicts a good response to chemotherapy, greater overall survival, and longer time to progression in glioma patients treated with BCN (carmustine) (120). This study has been followed up by other groups, who have obtained similar results (121). It is important to note that MGMT hypermethylation alone, without treatment with an alkylating agent, is not a good prognostic factor per se. In fact, it is a poor prognostic factor, probably owing to the finding that patients with epigenetic silencing of MGMT accumulate more mutations, as demonstrated for p53 and K-ras in colorectal, brain, and lung tumors (75). The potential of MGMT to predict the

chemoresponse of human tumors to alkylating agents is not limited to BCNU-like alkylating agents; it also extends to other drugs such as cyclophosphamide (122). This has been demonstrated in diffuse large cell lymphomas treated with cyclophosphamide, where MGMT hypermethylation was the strongest predictor of overall survival and time to progression and was far superior to classical clinical factors such as the international prognostic index (122). Finally, we have extended in gliomas the use of MGMT methylation as a marker of good clinical response for another drug recently introduced, temozolomide (123).

Similar cases to that described for MGMT can be cited for other DNA repair and detoxifier genes that also undergo aberrant DNA methylation. For example, the response to cisplatin and derivatives may be a direct function of the methylation state of the CpG island of hMLH1 (124), the response to adriamicine may be related to the methylation status of GSTP1 (101), and the response to certain DNA-damaging drugs could be a function of the state of BRCA1 hypermethylation (80, 125).

Finally, gene inactivation by promoter hypermethylation may be the key to understanding the loss of hormone response in many tumors. The inefficacy of the antisteroids, estrogen-progesterone-androgen-related compounds such as tamoxifen, raloxifene, or flutemide, in certain breast, endometrial, and prostate cancer cases may be a direct consequence of the methylation-mediated silencing of their respective cellular receptors (ER, PR, and AR genes). A similar picture can be painted for the retinoids: Why has chemoprevention with these agents not produced the results that we so desire and expect? A highly convincing explanation is that the tumors and the premalignant lesions become insensitive to these compounds owing to epigenetic silencing of genes that are crucial in the retinoid response, especially the retinoic acid receptor $\beta 2$ (RAR$\beta 2$) (60, 85–87) and the cellular retinol binding protein I (CRBPI) (87). This is a dynamic process, and we have demonstrated that a suitable supply of dietary retinoids prevents the aberrant methylation of RAR$\beta 2$ and CRBPI in colorectal tumorigenesis (87).

DNA DEMETHYLATING DRUGS IN CANCER THERAPY

A patient does not respond to drugs in the same manner as a cancer cell line grown in vitro or a mouse with an implanted tumor. Since the mid-1980s, we have been capable of reactivating hypermethylated genes in vitro. One obstacle to the transfer of this technique to human primary cancers is the lack of specificity of the drugs used (126). Demethylating agents such as 5-azacytidine or 5-aza-2-deoxycytidine (Decitabine) inhibit DNA methyltransferases and cause global hypomethylation, and we cannot reactivate solely the particular gene we would wish to (126). Furthermore, the demethylating effect of 5-aza-2-deoxycytidine seems to be universal, affecting all human cancer cell lines (127), although this may not be the case for its cytotoxic capacity (128). New chemical inhibitors of DNA methylation are being introduced, such as procainamide (129) and procaine

(130), which give us more hope; however, the nonspecificity problem persists. If only tumor suppressor genes were hypermethylated, this would not be a great problem. However, we do not know if we have disrupted some essential methylation at certain sites, and global hypomethylation may be associated with even greater chromosomal instability. Another drawback is the toxicity to normal cells. Indeed, this phenomenon was observed when higher doses were first used.

The first analog tested as a possible inhibitor of DNA methylation was 5-azacytidine (131). This substance causes covalent arresting of DNMTs, resulting in cytotoxicity, and tumors with increased levels of these enzymes are expected to present higher sensitivity toward the drug (131). 5-azacytidine was tested as an antileukemic drug before its demethylating activity was known (132, 133). It is reported that 5-azacytidine has interference at very low concentrations (below 0.1 μM) in RNA processing, tRNA methylation, and protein synthesis owing to its incorporation preferential into RNA in vivo and in cultured cells. Treatment with equimolar amounts of both cytidine and 5-azacytidine inhibits the incorporation of the latter one in nucleic acids, resulting in no alteration of the cell cycle either in vivo or ex vivo. 5-azacytidine is degradated by a nucleoside deaminase, so cells that highly express this enzyme are not sensitive to this compound (132). Therefore, 5-azacytidine is much less employed in studies related to methylation. However, more recently, it has been concluded that irreversible cell cycle arresting at phases G1/G0, G2, and S caused by this compound when used at micromolar concentrations is due to its effects on DNA methylation and not on RNA metabolism (134). It is still used in clinical trials (135, 136).

The analog 5-aza-2′-deoxycitidine (Decitabine) is one of the most used demethylating drugs for assays with cultured cells. It overcomes the major incorporation of 5-azacytidine into RNA and reduces its side effects. Indeed, Decitabine is only incorporated into DNA. However, it has been shown that cytidine deaminase can degradate 5-aza-2′-deoxycytidine to 5-aza-2′-deoxyuridine (137), resulting in the complete loss of DNMT's inhibition. The high level of cytidine deaminase in liver and spleen may reduce the half-life of this compound to 15–20 min when tested in vivo (138). A Phase I clinical trial has suggested that deamination is the major pathway for this compound (133).

In reference to new DNA demethylating agents, in addition to the previously mentioned procaine and procainamide (129, 130), we should discuss zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one]. Zebularine is the first DNA demethylating agent that can be administered orally and exhibits chemical stability and minimal cytotoxicity both in vitro and in vivo (139, 140).

These compounds and their derivatives have been used in the clinic with some therapeutic benefit, especially in hematopoietic malignancies such as myelodysplastic syndrome and acute myeloid leukemia (141–143). Lower doses of 5-azacytidine associated with inhibitors of histone deacetylases (such as trichostatin, depsipeptide, SAHA, or sodium butyrate) may also reactivate tumor suppressor genes (126). This was an encouraging discovery with respect to avoiding toxicity. Hypermethylation of the CpG island is not a solitary phenomenon, but it occurs

in the context of the action of methyl-binding proteins, histone hypoacetylation, and histone methylation, which cooperate with DNA methylation in the cellular mechanisms that lead to a closed chromatin state and transcriptional silencing (144). Several clinical trials to test this and other similar strategies in human cancer patients are underway in the United States and Europe. It is extremely important to define the parameters of response, clinically and molecularly. With respect to the latter, the demethylation of the CpG islands of tumor suppressor genes, such as that demonstrated for $p15^{INK4b}$ (145), and the quantitative measurement of the 5-methylcytosine DNA levels after treatment using high performance capillary electrophoresis (HPCE) (15, 127) could be excellent surrogate markers. One of the most promising models for testing some of these drugs is acute promyelocytic leukemia, where the transcriptional disruption induced by the PML-RARα translocation is the main guilty party. In acute promyelocytic leukemia, treatments that combine inhibitors of histone deacetylases, inhibitors of DNA methylation, and differentiating factors (the rediscovered arsenic trioxide may have all three functions) have met with success in several cases (146). On the other hand, 5-aza-2′-deoxycytidine alone can induce, by mechanisms that are not fully understood, the reexpression of certain silenced tumor suppressor genes that do not have apparent CpG island hypermethylation, such as the proapoptotic gene APAF-1 (147). Furthermore, it is well known that 5-aza-2′-deoxycytidine has cytotoxic effects in cancer cells over and above its DNA demethylation activity. These last two activities expand the killing capabilities of these compounds, thus increasing their power in cancer treatment.

These new findings have proven very attractive to several pharmacological and biotechnology companies that are now studying how to achieve demethylation of cancer cells using novel approaches such as antisense constructs, ribozymes, and RNA interference against the DNA methyltransferases or other elements of the DNA methylation machinery (methyl-CpG binding proteins) (45). Nevertheless, we are left with the obstacle of nonspecificity. Other companies are tackling the problem using gene therapy-like strategies whereby they specifically reactivate a targeted methylated gene. These studies are still in their infancy, and we still have the unsolved problem of achieving efficient delivery to the target tissue. The classical demethylating agents are no strangers in this context: They all have to be administered by injection as no oral compound is yet available. I hope that the Phase II and III studies will answer some of our questions while we await better epigenetic agents.

FINAL THOUGHTS

Cancer is a poligenetic disease, but it is also a poliepigenetic disease. We cannot understand the dynamics and plasticity of cancer cells if we do not invoke epigenetic changes. This review has focused on DNA methylation alteration, but the whole epigenetic setting of the transformed cell, including histone

acetylation-methylation and chromatin remodeling factors, is disrupted. We know that the silencing of tumor suppressor genes by CpG island promoter hypermethylation is one of the major epigentic culprits for human tumors. It affects genes important for cell biology, such as $p16^{INK4a}$, BRCA1, or hMLH1. The profile of CpG island hypermethylation is specific to the tumor type, opening the avenue for its use as a biomolecular marker of the disease. An issue strengthened by the development of automatic PCR-based technologies is the easy detection of cancer lesions. But more questions continue to arise: What is the real contribution of DNA hypomethylation to tumorigenesis? Why are some tumor suppressor genes more prone to be hypermethylated than others? How can DNA hypomethylation and hypermethylation coexist in the same cancer cell? Are there any genetic disruptions prompting some of the DNA methylation changes observed or is it the other way around? Will we ever find/create a DNA demethylating agent specific for the hypermethylated tumor suppressor genes?

The *Annual Review of Pharmacology and Toxicology* is online at http://pharmtox.annualreviews.org

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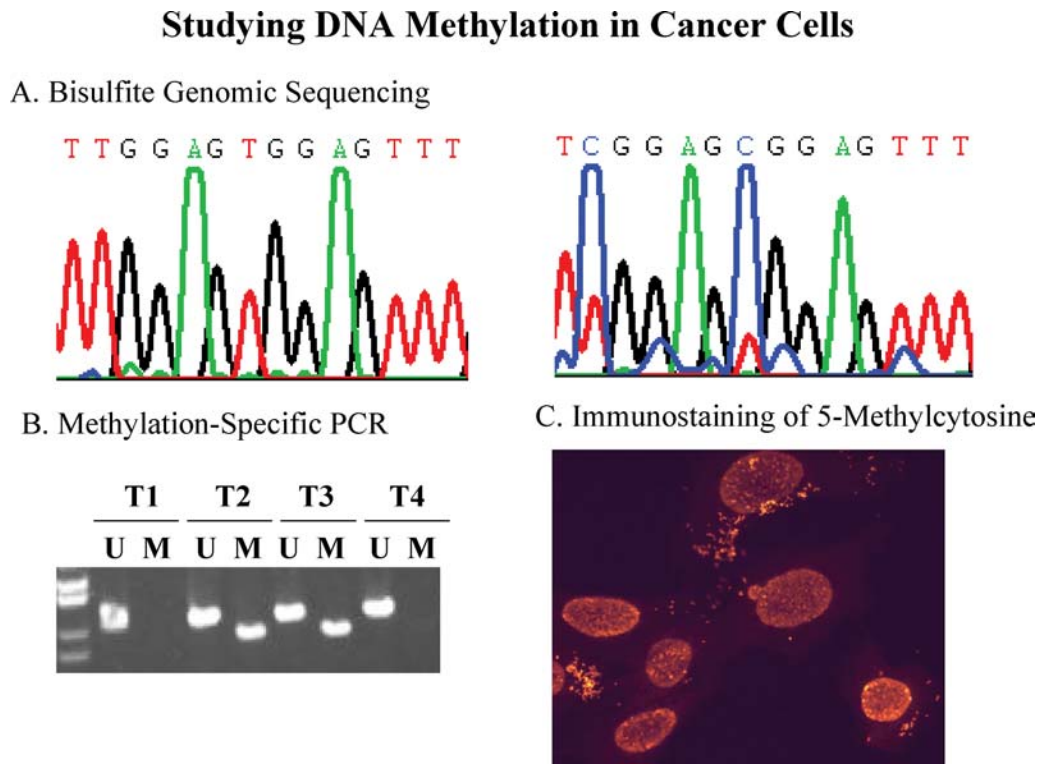

Figure 3 Illustrative examples of DNA methylation analysis. (*A*) Chromatograms of the bisulfite genomic sequencing of a small fragment of a CpG island: left, unmethylated sequence (cytosines changed to thymines); right, methylated sequence (cytosines remined as cytosines). (*B*) Methylation-specific PCR in primary tumors. The presence of a band under the "M" lanes represents hypermethylated neoplasms. (*C*) Staining for the 5-methylcytosine antibody in a cancer cell line.

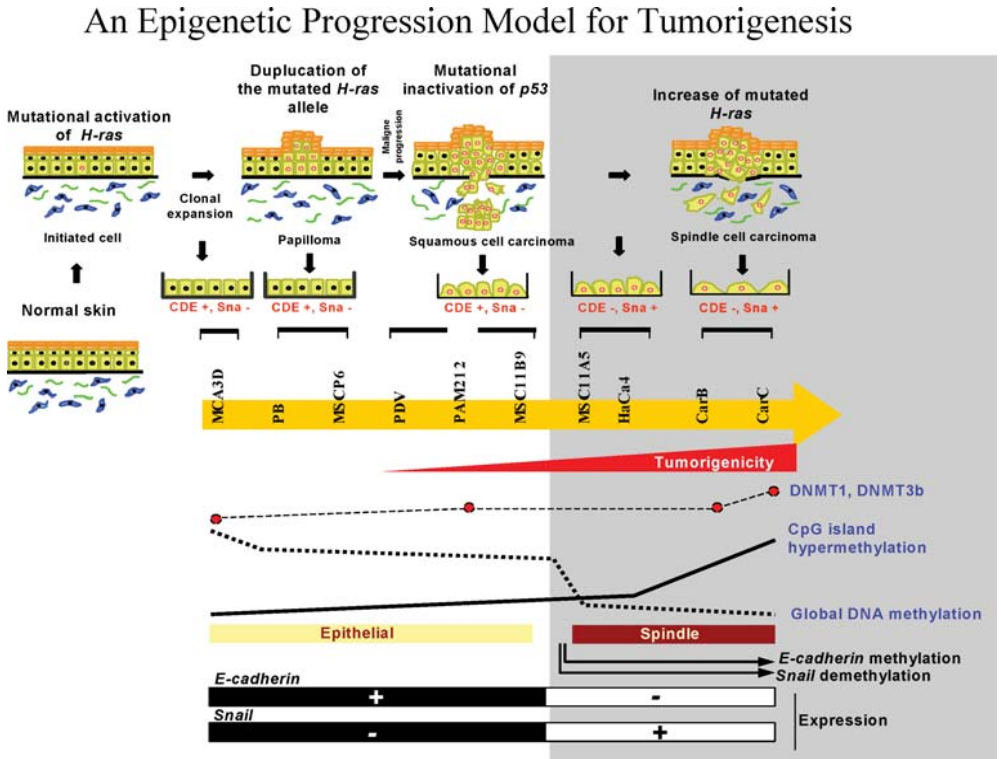

Figure 4 Cancer as a poligenetic and poliepigenetic disease: the model of mouse multistage skin carcinogenesis. Through all the tumoral different stages (from benign lesions to invasive carcinomas) there is an accumulation of gene mutations, but also a double epigenetic lesion: an increase in the number of genes undergoing methylation-associated silencing and in the degree of genomic hypomethylation (14).

Annual Review of Pharmacology and Toxicology
Volume 45, 2005

CONTENTS