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Invited Review

Aging, methylation and cancer

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Summary. Alterations in methylation are widespread in cancers. DNA methylation of promoter-associated CpG islands is an alternate mechanism to mutation in silencing gene function, and affects tumor-suppressor genes such as p16 and *RB1*, growth and differentiation controlling genes such as ER and many others. Evidence is now accumulating that some of these methylation changes may initiate in subpopulations of normal cells as a function of age and progressively increase during carcinogenesis. Age-related methylation appears to be widespread and is one of the earliest changes marking the risk for neoplasia. In colon cancer, we have shown a pattern of age-related methylation for several genes, including ER, *IGF2*, *N33* and *MyoD*, which progresses to full methylation in adenomas and neoplasms. Hypermethylation of these genes is associated with gene silencing. Age-related methylation involves at least 50% of the genes which are hypermethylated in colon cancer, and we propose that such age-related methylation may partly account for the fact that most cancers occur as a function of old age. Age-related methylation, then, may be a fundamental mark of the field defect in patients with neoplasia. The causes of age-related methylation are still unknown at this point, but evidence points to an interplay between local predisposing factors in DNA (methylation centers), levels of gene expression and environmental exposure. The concept that age-related methylation is a predisposing factor for neoplasia implies that it may serve as a diagnostic risk marker in cancer, and as a novel target for chemoprevention. Studies in animal models support this hypothesis and should lead to novel approaches to risk-assessment and chemoprevention in humans.

Key words: DNA methylation, CpG islands, Age-related methylation, Tumor-suppressor genes, Colorectal cancer

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Introduction

In recent years, DNA methylation has garnered a lot of attention because of its importance in normal embryonic development and its involvement in carcinogenesis. Widespread changes in methylation patterns have been noted to occur during carcinogenesis as well as during normal development for the past twenty years (Jones, 1996; Baylin et al., 1998). However, the exact function of the process of DNA methylation in the human genome has been much debated during this time, and only recently have some of these functions been elucidated. DNA methylation has been shown to be essential in normal embryogenesis and development, as well as critical in silencing the expression of some tumor-suppressor genes during tumorigenesis.

This review will provide a basic overview of DNA methylation and its role in cancer, with a particular focus on DNA methylation in normal aging and how this may contribute to tumorigenesis.

DNA methylation and CpG Islands

DNA methylation refers to a post-replicative modification by which DNA residues acquire a covalently bound methyl group. DNA methylation is present in most organisms, including bacteria, plants and humans, but is absent in organisms such as Drosophila and Caenorhabditis elegans. In mammals, DNA methylation only occurs at cytosine residues found within CpG dinucleotides and involves methylation of the 5 position of cytosine (C) leading to the formation of 5-methylcytosine (5-mC). DNA methylation is mediated by the enzyme DNA methyltransferase (Mtase) and uses S-Adenosylmethionine as a methyl donor (Bestor and Verdine, 1994). Cytosine methylation, in fact, is the only known naturally occurring modification of DNA in higher eukaryotes and 5-mC has been referred to as the fifth base. This modification is referred to as an epigenetic change since it does not alter the primary DNA sequence.

The CpG dinucleotide is suppressed in higher order

eukaryotes and is present at only 5-10% of its expected frequency (Bird, 1986). This has been attributed to the relative hypermutability of the 5-mCpG dinucleotide and loss of the 5-mC base during evolution, via spontaneous deamination of 5-methylcytosine. In contrast to the genome-wide CpG suppression, there are localized areas of the genome that maintain the expected or higher CpG frequency, called CpG islands. CpG islands are thought to have escaped the phenomenon of CG suppression because they are typically not methylated and hence not subject to the same mutational pressure. CpG islands range in size from 0.5 to about 4 to 5 kb and occur about every 100 kb. CpG islands are defined as having a G+C content greater than 60% and a ratio of CpG to GpC of at least 0.6. Many CpG islands are found associated with the 5' region of expressed genes, and about 60% of human gene promoters are located within CpG islands (Bird, 1986).

The majority of CpG sites (70-80%) in human DNA are methylated. These CpG sites include individual CpGs scattered within the coding region of genes, areas of low CpG density as well as repetitive sequences, such as LINE and SINE (Alus) elements (Turker and Bestor, 1997; Baylin et al., 1998). By contrast, CpG islands with a high CpG density are normally protected from methylation. The protective factors that maintain the methylation-free status of CpG islands are unknown. However, in the mouse *Aprt* gene promoter, the CpG island has been shown to be protected from methylation by a cluster of binding sites for the Sp1 transcription factor (Turker and Bestor, 1997).

Function of DNA methylation

Although, the majority of CpG sites in the human genome are methylated, the exact function of this DNA methylation is still unclear. In prokaryotes, DNA methylation (including both adenine and cytosine) functions as an "immune" system to eliminate foreign DNA. It is now thought that DNA methylation may have a similar function in humans and evolved as a defense mechanism against the harmful effects of invading parasitic sequences such as transposable elements and proviral DNA (Bestor and Tycko, 1996). Regardless of its evolutionary role, DNA methylation is essential in development since homozygous deletion of *Dnmt1*, a Mtase enzyme is embryonically lethal in mice (Li et al., 1992). This is consistent with the fact that, during embryogenesis and development, there is a wave of demethylation followed by "de-novo" methylation that is presumed to be essential for cellular function (Turker and Bestor, 1997). These patterns established during development are fixed and maintained by the activity of Mtase enzymes, which recognize newly synthesized DNA as hemi-methylated and convert it to a fully methylated state (Bestor and Verdine, 1994).

By contrast to the methylated status of CpG sites in most of the genome, CpG islands are regions that are normally unmethylated regardless of the expression of the associated genes (Bird, 1986). When it occurs, DNA methylation of promoter-related CpG islands is known to be associated with silencing of the transcription of the gene. This has been illustrated by two examples that deviate from the rule that CpG islands are devoid of methylation, including (i) genes on the X chromosome, and (ii) imprinted genes (Bestor and Tycko, 1996). In females, one of the X chromosomes is normally silenced by DNA methylation. Similarly, in imprinted genes which demonstrate parental-specific expression, such as *Igf2r* and *H19*, the non-expressing allele is silenced by promoter DNA methylation (Bestor and Tycko, 1996). This silencing via DNA methylation is thought to be normally irreversible, and is maintained through cell divisions. The role of DNA methylation in gene silencing has been supported by several lines of evidence, including: (a) the homozygous mouse knockout of the Mtase gene *Dnmt1* showed reexpression of several imprinted genes which were previously silenced and, (b) pharmacologic demethylation using Mtase-inhibitor drugs such as 5' deoxyazacytidine results in reexpression of such silenced genes.

The mechanism whereby CpG island methylation induces gene silencing are just beginning to be understood. The current model proposes that methylated DNA binding proteins (such as MeCp2) first bind to areas on the CpG island, followed by recruitment of a protein complex that includes histones, deacetylases and other proteins. This protein complex then induces a closed chromatin conformation excluding access to transcription factors and resulting in gene silencing (Kass et al., 1997). The methylated chromatin conformation is one that is hypoacetylated, late-replicating, heterochromatin DNA (Baylin et al., 1998). In recent studies in Neurospora and Xenopus, the methylation-induced gene silencing could be prevented simply by treating the cells with a histone deacetylase inhibitor, such as trichostatin A (Kass et al., 1997). This suggested that hypoacteylation rather than methylation may be crucial for gene silencing in that setting. However, in cancer cells, treatment with trichostatin A appears to be insufficient in causing reexpression of genes such as *p16*, *hMLH1*, and *TIMP-3* (Cameron et al., 1999). Gene silencing could only be reversed by treatment with a demethylating agent such as 5' deoxyazacytidine. On the other hand, a recent paper showed that for *IGF2* (Insulin-like Growth Factor 2), an imprinted gene, the maternal allele silenced by methylation could be reexpressed by exposure to trichostatin A, such that there is biallelic expression (Hu et al., 1998). Further studies should clarify the mechanistic role of acetylation and methylation in gene silencing.

DNA methylation and cancer

Changes in methylation patterns are one of the most consistent hallmarks of carcinogenesis. These changes are widespread and include: overall decreases in the

level of 5-methylcytosine, increased levels of Mtase, and regional areas of de-novo methylation of CpG islands. There are several good reviews summarizing these methylation changes in cancer (Jones, 1996; Baylin et al., 1998), and the consensus that has emerged is that methylation and carcinogenesis are linked. The evidence for this relationship has been provided by studies which showed that decreasing Mtase enzyme activity led to decreased colonic polyp formation in mice predisposed to cancer secondary to a germline *APC* mutation (Laird et al., 1995). Simultaneously, a number of laboratories have shown that several well-known tumor-suppressor genes (such as *p16*, *VHL*, etc.) are silenced by *de-novo* methylation of their 5' CpG islands and can be reactivated by demethylation (Jones, 1996; Baylin et al., 1998). These studies have pointed to methylation as an important new pathway in cancer.

One of the first molecular changes noted in cancer was global hypomethylation (Baylin et al., 1998). It has been postulated that this hypomethylation may lead to activation of oncogenes such as *K-ras*. Furthermore, mice fed a methyl-deficient diet develop liver cancer (Poirier, 1994). However, hypomethylation in cancers is usually noted in areas outside the promoter region and how this may affect expression of a gene is uncertain. Recently, it has been proposed that DNA methylation may contribute to overall genetic stability and maintenance of chromosomal integrity, and it has been suggested that hypomethylation may contribute to chromosomal instability in cancer (Chen et al., 1998).

Coexistent with global hypomethylation, there is a several-fold increase in Mtase activity in tumor cell lines and primary cancers. For example, Mtase activity increases progressively during colon cancer progression (Issa et al., 1993). Furthermore, over-expression of Mtase can transform the non-tumorigenic, immortalized cell line NIH-3T3, while forced expression of an Mtase anti-sense construct has been shown to revert the tumorigenic phenotype in a malignant cell line (Baylin et al., 1998). Thus, the current evidence points to a possible etiologic role for increased Mtase levels in neoplasia.

Despite global hypomethylation, some genes also display de-novo methylation of their promoters in cancer and this is associated with transcriptional repression (Jones, 1996; Baylin et al., 1998). This finding has triggered intense interest in the field of DNA methylation in recent years. Promoter hypermethylation has been linked to inactivation of several tumor-suppressor genes in cancer such as *Rb1*, *VHL*, *p16*, p14/ARF and *BRCA1*; growth and differentiation regulatory genes such as *ER* and *AR*; DNA repair genes such as *hMLH1* or *GSTpi* and others (see Table 1) (Jones, 1996; Baylin et al., 1998). It is important to remember that these methylation changes are clonally inherited, and that DNA methylation appears to function as an alternate to coding region mutations in silencing the function of affected genes. For example, in pancreatic cancer there is a high incidence of *p16* mutations/deletions (80%) while the majority of the remaining cases (15-20%) silence *p16* function by DNA methylation (Schutte et al., 1997).

Overall, there is strong data suggesting that methylation changes are important in carcinogenesis. In fact, methylation changes are one of the earliest events in cancer formation. For example, in colon cancer, *p16* promoter methylation is detected very early even in preneoplastic polyps (Toyota et al., 1999a), and in the inflamed mucosa of patients with inflammatory bowel disease (Chih-Jen et al., 1998). Similarly, in a mouse model of lung cancer, exposure to various carcinogens leads to *p16* methylation as a very early event (Belinsky et al., 1998). In the past few years, we and others have found that, for some of the genes involved in cancer, methylation events initiate even earlier in several normal tissues, as a function of aging, and may lead to some of the changes seen in cancer.

Table 1. Partial list of genes affected by DNA methylation in cancer.

Tumor Suppressor Genes	*Metastasis/Angiogenesis related Genes*
p16 (CDKN2A)	THBS1
P14/ARF	TIMP3
VHL	E-cadherin
RB1	H-cadherin
BRCA1	*DNA-repair/Detoxification Genes*
APC	HMLH1
WT1	GST-pi
Candidate Tumor Suppressor Genes	MGMT
P15 (CDKN2B)	MDR1
FHIT	*Other Genes*
HIC1	N33
MDGI	H19
Growth/Differentiation Genes	MyoD
ER	ABL1
AR	*IGF2*
PgR	PAX6
RAR-Beta2	CALCA
Endothelin Receptor B	Versican

Age-related methylation

Aging has long been considered one of the most important risk factors for the development of cancers. The incidence of most cancers rises exponentially after age 60 and the median age of patients with cancer in the Unites States is 70 years. This risk factor has generally been attributed to the cumulative exposure to carcinogens over time, as well as the time required to have the multiple hits needed for the onset of neoplasia. However, physiologic aging is also accompanied by widespread functional changes, including gradual loss of immune function, and these findings cannot be all explained solely on the basis of mutations. Unlike classical genetic changes such as mutations, epigenetic changes such as methylation can take effect over several cell generations and cause gradual changes in function which are characteristic of older cells. For this reason, DNA methylation has long been postulated to play a role

in aging and the associated increased risk of cancer because it functions as an epigenetic modification (Holliday, 1987).

As in the field of cancer, the earliest focus in the field of aging and methylation was on overall genomic hypomethylation. 5-methylcytosine was noted to be lost as a function of age, and this was proposed to function as a "counting mechanism" used by cells to monitor the number of divisions allotted over their lifetime (Holliday, 1987). Furthermore, it was also thought that loss of methylation might activate specialized genes in cells where such genes are normally silent. However, most of the changes in 5-methylcytosine content occur at areas outside CpG islands and the consequences of this loss of methylcytosine content with aging is unknown at this point. Several groups have also found areas of regional increased methylation as a function of aging, such as in c-MYC or c-FOS (Ono et al., 1993). However, these hypermethylated areas were also outside the promoter regions, and the functional significance of this change is still unclear.

The most convincing evidence for a functional relationship between aging and DNA methylation was revealed by studying patterns of methylation of promoter-associated CpG islands. The first gene that revealed a pattern of CpG-island methylation as a function of age was the Estrogen Receptor (*ER*) gene. The *ER* gene, on 6q25, gets hypermethylated at its promoter-associated CpG island in all colonic neoplasms and this is associated with absent transcription of the gene. Introduction of an exogenous *ER* expression construct into a colon cancer cell line resulted in marked growth suppression (Issa et al., 1994). These findings suggested that *ER* may function as a candidate tumor-suppressor gene in colorectal cancers and methylation of the *ER* gene may be an important step in the pathophysiology of this disease. However, the surprising finding was that partial *ER* methylation was also seen in normal colonic epithelium as a function of age. In young individuals, *ER* methylation is undetectable, but partial methylation appears in older individuals and the *ER* gene gets hypermethylated in all colonic adenomas and cancers (Fig. 1). This finding led to the concept that aging or age-dependent events may be a significant risk factor for hypermethylation of the *ER* gene.

Subsequently, we have observed that the *IGF2* gene also shows age-related methylation (Issa et al., 1996a). The *IGF2* gene is imprinted and is normally expressed exclusively from the paternal allele. *IGF2* is also highly expressed in several cancers, including colon, and this growth factor may have a putative role in carcinogenesis. *IGF2* is known to have four promoters: the P2-4 promoters are located in close proximity to each other within a CpG island while the P1 promoter is located about 20kb upstream, lacks a CpG island and escapes imprinting. In the normal colon of young individuals, the *IGF2* P2-4 CpG island is silenced by DNA methylation in the maternal allele, but with age, this methylation spreads to the opposite allele. Furthermore, in colon cancers, this methylation continues to spread, and is associated with low or absent expression from the P2-4 promoters, but unaffected expression from the P1 promoter. Thus, the *IGF2* gene also shows age-related methylation in the normal colon, progressing to hypermethylation in cancers.

These early findings in the *ER* and *IGF2* genes led us to investigate the incidence of age-related methylation in colorectal cancer where methylation events are frequent and have been well studied. The methylation status of six additional genes, known to be frequently methylated in colon cancer, was studied in the normal colon. These genes included the following: (a) the *N33*

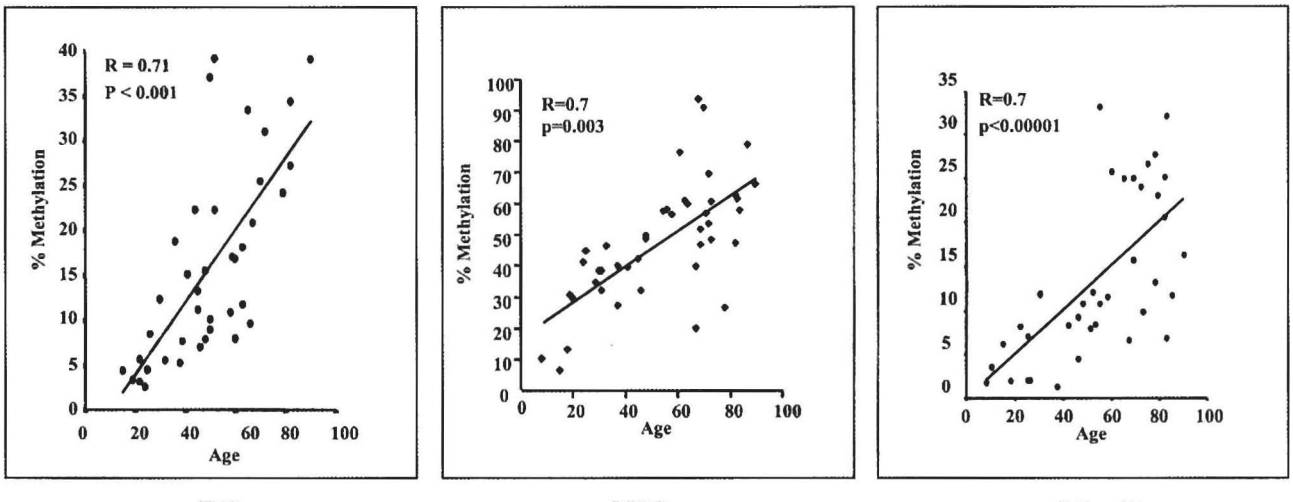

Fig. 1. Age-related methylation of several genes in normal colorectal mucosa. The percentage of methylation of DNA from normal colorectal mucosa is plotted against the age of the patients. Each of the graphs shows that the percentage of methylation increases with age in a linear manner.

gene, a candidate tumor-suppressor gene, on 8p22, which is highly methylated and silenced in several colon cancer cell lines; (b) the *MyoD* gene, on 11p, which is hypermethylated in several cancers including bladder, breast and colon cancers; (c) the *p16* gene, on 9p21, which functions as a cyclin-dependent kinase inhibitor and is hypermethylated in most common human cancers, including colon cancers; (d) the *THBS1* (Thrombospondin-1) gene, an angiogenesis inhibitor on 15q, which is predominantly methylated in a subset of colorectal cancers with microsatellite instability; (e) the *HIC-1* (Hypermethylated In Cancer 1) gene, a zinc-finger transcription factor on 17p13.3, which was cloned on the basis of the presence of a large CpG island in this area that becomes frequently methylated in various cancers, including colon, and (f) the CALCA (Calcitonin) gene, on 11p15, which is methylated in lung and colon cancers.

Our study showed that both *N33* and *MyoD* genes also have a pattern of partial methylation in the normal colorectal mucosa that increases as a function of age (Ahuja et al., 1998) (Fig. 1). Most polyps and cancers showed progressive increases in methylation of these two genes eventually leading to complete methylation. However, the remaining four genes, including *p16*, *THBS1*, *HIC-1* and CALCA showed no evidence of methylation in any normal colonic mucosa, but were *de-novo* methylated in colon cancers. Thus, age-related methylation is gene-specific and only certain promoters are susceptible to this process. Overall, out of the eight genes known to be hypermethylated in colorectal cancers, four or 50% (*N33*, *MyoD*, *ER* and *IGF2*) showed a pattern of age-related methylation. These data suggested that many hypermethylation events observed in cancer are related to the fact that neoplasia begins in aging cell populations.

Age-related CpG island methylation is, however, not limited to the colon. Several studies suggest that it is a universal phenomenon and one of the most frequent events contributing to hypermethylation in cancers. For example, in a recent study of hypermethylation of multiple genes in glioblastoma multiforme, we found a striking concordance in the methylation status of the *ER* and *N33* genes, which were both much more frequently methylated in the tumors of older patients. By contrast, just as in the colon, *p16* and *THBS1* methylation was not related to the age of the patients studied (Li et al., 1998). Similarly, the *HIC-1* gene which is frequently methylated in prostate cancer, is also partially methylated in normal prostate, and it appears that this methylation is also age-related (Morton et al., 1996) (Table 2). We have also observed this finding in the cultured human fibroblasts IMR90 which undergo senescence with repeated passaging. This in-vitro model of aging is accompanied by methylation of the *ER* and *IGF2* CpG islands in the aging fibroblasts (Issa et al., 1996a; Baylin et al., 1998). Recently, Shiraishi et al. have used a combination of methylated DNA binding column and denaturing gradient gel electrophoresis to isolate methylated CpG islands from human lung adenocarcinomas. Out of the 1067 clones analyzed, 9 were CpG islands and 8 of the 9 clones had methylation that started in the normal lung, and it appears that this methylation may be age-related (Shiraishi et al., 1999). Similarly, a strategy to clone hypermethylated CpG islands from breast cancer cell lines found that 44% (13/30) of the clones that showed methylation in cancer initiated the process in normal breast (Huang et al., 1999). Although, the pattern of methylation in normal breast tissues was not studied in detail, it appears likely that these clones show age-related methylation with increasing methylation in cancer.

An important point in studying the phenomenon of age-related methylation is that the "normal" tissue adjacent to cancer may not always be representative of the cell populations that are predisposed to neoplasia. For example, "normal" breast is largely composed of supportive stromal cells and is unmethylated at the *HIC-1* locus while purified breast epithelium is highly methylated at this locus (Fujii et al., 1998). This factor may lead to underestimation of the contribution of age-related methylation in normal tissues to the ultimate frequency of methylation in neoplasia.

The evidence, so far, seems to indicate that most tissues show a pattern of age-related methylation, but that the patterns are both gene and tissue-specific. For example, the *ER* gene shows very high levels of methylation in the normal liver as compared to the normal colon in age-matched tissues. In contrast, the *N33* gene shows much higher levels of methylation in the normal colon compared to normal liver (Ahuja et al., 1998). Further studies of the methylation patterns of different genes in various normal human tissues may shed insight into the factors modulating tissue-specific patterns of age-related methylation.

Given the widespread prevalence of age-related methylation, it may be reasonable to hypothesize that all hypermethylation events in cancer are simply related to initial methylation in normal tissues. This hypothesis needs to be explored in detail. At the sensitivity level of Southern blot analysis and bisulfite-PCR, however, there appears to be two distinct types of CpG island methylation events in cancer: (a) methylation which can be detected in both normal aging cells and neoplastic cells, and (b) methylation which can only be detected in neoplastic cells.

Table 2. List of genes demonstrating age-related methylation.

GENES INVOLVED	TISSUES AFFECTED
ER	colon, liver and heart muscle
N33	colon, liver
HIC1	prostate and brain
IGF2	Colon
MyoD	Colon
PAX6	Colon
RAR-Beta1	Colon
Versican	Colon

The paradigm of the two types of methylation in cancer has been confirmed recently. Our laboratory has used a technique called Methylated CpG island Amplification to identify novel genes methylated in colon cancer. Using this strategy, we have isolated 33 differentially methylated clones (Toyota et al., 1999b). Twenty nine of the 33 recovered clones met the criteria of CpG islands and, of these, 6 matched to already known genes (Versican, alpha-tubulin, CSX, PAX6, OPT homologue and ribosomal RNA gene). The methylation patterns of these 29 CpG islands was analyzed in a large number of normal colon and primary cancer tissues. We found that a majority of the recovered clones (70%) showed age-related methylation in the normal colon tissue with progressive hypermethylation in adenomas and cancers (Toyota et al., 1999a). The remaining clones were de-novo methylated in cancer. This phenomenon has also been observed by Huang et al. (1999) using a strategy called differential methylation hybridization to isolate methylated CpG islands from breast cancer. About half the clones showed methylation in the normal breast and frequent methylation in cancer, whereas the remaining clones showed de-novo methylation in cancer (Huang et al., 1999).

Causes of age-related methylation

Although the existence of age-related methylation has been well documented in a number of tissues, the etiologic factors causing this phenomenon are still largely unknown. Studies in our laboratory in the normal colon indicate that age-related methylation appears to show gene-specificity. Age-related methylation is not simply a random event with selection of affected cells, for example based on a growth advantage, since hypermethylation is not seen for the *p16* locus in normal colon. Also, age-related methylation appears to be tissue-specific, and involves both genes that are expressed in the colon (*ER* and *N33*) and genes that are not expressed, or expressed at low levels (*IGF2*, *MyoD*) (Ahuja et al., 1998).

Several factors have been shown to be associated with aberrant DNA methylation. Some of these factors include: (a) Changes in local DNA structure, as in the Fragile X syndrome in which the presence of a CGG repeat triggers methylation, leading to the full-blown clinical syndrome (Bestor and Tycko, 1996). (b) Carcinogen exposures-Nickel exposure has been shown to cause gene silencing by inducing DNA methylation and condensation of chromatin to heterochromatin in a Chinese hamster cell line (Lee et al., 1995). Also, in a rodent model of lung carcinogenesis, the amount of *ER* methylation varied as a function of the type of carcinogen exposure (Issa et al., 1996b). (c) Increased Mtase activity- Over-expression of Mtase driven by a CMV promoter into human fibroblasts resulted in *de-novo* methylation in CpG islands of susceptible loci (Vertino et al., 1996). (d) Microsatellite Instability- Sporadic colorectal cancers demonstrating microsatellite instability demonstrate a "hypermethylator phenotype" by silencing several tumor-suppressor genes via DNA methylation (Ahuja et al., 1997). Furthermore, these cancers demonstrating microsatellite instability have an underlying defect in their DNA repair gene, usually hMLH1 or hMSH2. It has recently been shown that the DNA repair gene (hMLH1) is, itself, silenced by DNA methylation in 70-80% of the cases (Kane et al., 1997; Herman et al., 1998).

These above-named factors may also have a putative role in some of the age-related methylation changes. However, since age-related methylation is widespread while de-novo methylation in cancer highly selective for certain genes, it is likely that each is governed by different mechanisms. De-novo methylation in cancer probably arises from a specific defect in the methylation machinery whereas age-related methylation is more likely due to a stochastic event by which methylation originates within a CpG island and then spreads via the action of the Mtase. Age-related methylation may thus initially arise from local triggering factors, such as chromatin structure or proximity of Alu sequences which are highly methylated normally and may act as methylation centers from where methylation spreads into the heart of a CpG island (Bestor and Tycko, 1996; Turker and Bestor, 1997).

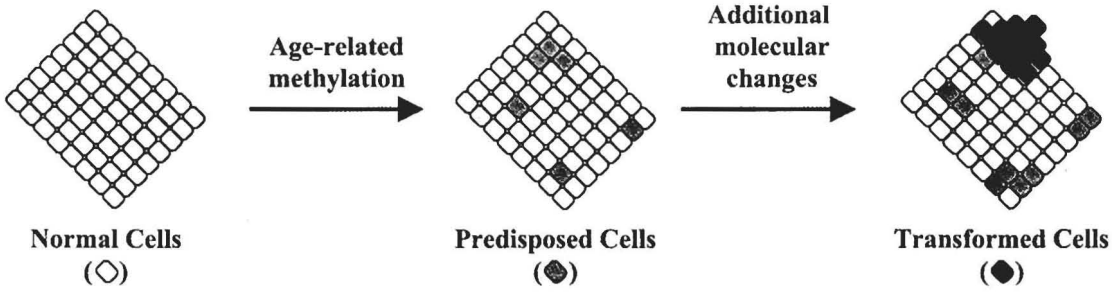

Fig. 2. A proposed model of the impact of age-related methylation on carcinogenesis. Initially, all normal colonic epithelial cells in younger individuals are unmethylated (left). Some of these cells undergo age-related methylation changes at several loci resulting in an altered physiology (middle, shown as light gray cells). Some of these cells may be then be predisposed to gaining further genetic or epigenetic changes and may have a growth advantage. These age-related methylation changes constitute a type of field defect for cancer predisposition. Ultimately, some of these cells with age-related methylation then acquire further defects, such as mutations, deletions and de-novo methylation of tumor-suppressor genes, which results in transformation to malignant cells (right, dark gray cells).

Role of age-related methylation in cancer

These age-related methylation changes seen in promoter-associated CpG islands could progress to have significant functional consequences for a cell by altering expression of important genes. Based on our current data that methylation may start for some genes as a function of age and become more prominent in cancer, it appears that either cells with preexisting methylation are selected for in the neoplastic process, or that tumors greatly extend methylation at these genes. Some of the genes affected by age-related methylation may have little physiologic relevance to colorectal cancer pathogenesis, such as the *MyoD* gene which is not expressed in the normal colon (Ahuja et al., 1998). However, other genes may be important to the neoplastic process, such as the *ER* gene, which appears to modulate growth and differentiation in normal colon (Issa et al., 1994). Methylation and loss of expression of these latter genes may impart a selective growth advantage to the affected cells which then become more susceptible to acquiring further genetic defects that ultimately lead to neoplasia (Fig. 2-model). Our current model postulates that age-related methylation constitutes a type of "field-defect" in the colon and that this phenomenon may partially explain the dramatic age-related increase in CRC incidence. This model would further predict that patients with high levels of methylation in their colorectal mucosa may be at higher risk for developing colorectal adenomas and cancer, and that normal mucosa from colorectal cancer patients would have higher levels of methylation than mucosa from patients without cancer. Answers to such questions will eventually require large-scale epidemiological studies.

Clinical implications of age-related methylation

These changes in methylation provide new opportunities for cancer treatment and prevention, and may also serve as useful risk markers in cancer. Age-related methylation changes in normal tissues are one of the earliest known alterations in the pathogenesis of cancers. As such, monitoring the levels of age-related methylation in different tissues may serve as a useful biomarker of cancer risk. The development of sensitive PCR techniques has facilitated the rapid methylation analysis of clinical specimens (Herman et al., 1996). One would predict that patients with high levels of age-related methylation changes are at a higher risk for cancer formation and may need closer surveillance. Furthermore, the number of genes affected by age-related methylation may predict relative cancer risk. Doing large, epidemiological surveillance studies should provide reliable answers to such questions.

This research into the interface between aging and cancer may also allow the use of these strategies for chemoprevention. Age-related methylation changes are well suited for preventive strategies since they appear long before any mutations or other genetic events. One would predict that preventing age-related methylation would act at the tumor initiation stage and may efficiently prevent the emergence of tumors. Support for this observation comes from experiments done by Laird et al. where lowering the levels of methylation decreased the number of colonic polyps dramatically in a mouse model of Familial Adenomatous Polyposis (Laird et al., 1995). Other possible chemopreventive strategies include dietary modifications. There is some evidence, for example, to indicate that folate-deficient diet may have a role in carcinogenesis by altering methylation pathways (Poirier, 1994). Thus, it would be interesting to study the impact of folate supplementation on age-dependent methylation in the colon. Finally, because methylation changes act via an epigenetic mode without altering the genetic code, reversal of such changes can be conceived to occur with minimal morbidity to the patient. One of the most exciting prospects in this field then is the potential ability to reverse some of these epigenetic changes using demethylating agents.

In fact, demethylating strategies have already been used clinically in some studies to treat leukemias, and some responses have been observed (Kantarjian, 1997). However, the demethylating agents, 5-azacytidine and 5-deoxyazacytidine, incorporate into DNA and may have had effects other than inducing demethylation. These studies did not specifically address whether the response was accompanied by demethylation of specific genes. Newer strategies specifically targeting methylation, such as antisense strategies to target the Mtase enzyme or newer demethylating agents, may provide useful tools in the treatment of cancer.

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