REVIEW

Roles and Causes of Abnormal DNA Methylation in Gastrointestinal Cancers

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Abstract

Evidence now suggests that epigenetic abnormalities, particularly altered DNA methylation, play a crucial role in the development and progression of human gastrointestinal malignancies. Two distinct DNA methylation abnormalities are observed together in cancer. One is an overall genome-wide reduction in DNA methylation (global hypomethylation) and the other is regional hypermethylation within the CpG islands of specific gene promoters. Global hypomethylation is believed to induce proto-oncogene activation and chromosomal instability, whereas regional hypermethylation is strongly associated with transcriptional silencing of tumor suppressor genes. To date, genes involved in regulation of the cell cycle, DNA repair, growth signaling, angiogenesis, and apoptosis, are all known to be inactivated by hypermethylation. Recently developed techniques for detecting changes in DNA methylation have dramatically enhanced our understanding of the patterns of methylation that occur as cancers progress. One of the key contributors to aberrant methylation is aging, but other patterns of methylation are cancer-specific and detected only in a subset of tumors exhibiting the CpG island methylator phenotype (CIMP). Although the cause of altered patterns of DNA methylation in cancer remains unknown, it is believed that epidemiological factors, notably dietary folate intake, might strongly influence DNA methylation patterns. Recent studies further suggest that polymorphisms of genes involved in folate metabolism are causally related to the development of cancer. Identifying epidemiological factors responsible for epigenetic changes should provide clues for cancer prevention in the future..

Key Words: Gastrointestinal cancer, DNA methylation, tumor suppressor, CIMP, aging, inflammation, folate, MTHFR, polymorphism

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Introduction

Gastrointestinal cancer arises through the accumulation of multiple genetic alterations, leading to activation of oncogenes and loss of function of tumor suppressor genes (Kinzler and Vogelstein, 1996). In addition to genetic alterations, a growing body of evidence now suggests that epigenetic changes also play a crucial role in the development and progression of human gastrointestinal malignancies (Jones and Baylin, 2002; Rashid and Issa, 2004). Epigenetics are inherited factors that influence gene activity but do not alter primary DNA sequences, and DNA methylation status is a key epigenetic factor. Within the mammalian genome, methylation takes place only at cytosine bases located 5' to a guanosine in a CpG dinucleotide (Herman and Baylin, 2003) (Figure 1a, b). This dinucleotide is actually under-represented in much of the genome, however, short regions of 0.5_4 kb in length, known as CpG islands, are rich in CpG dinucleotides (Takai and Jones, 2002). The majority of CpG islands are found in the 5' end regions of approximately 50% of genes. While most CpG sites in the human genome are methylated, those within CpG islands generally remain unmethylated in normal cells. Methylation of cytosine is observed in various physiological states, such as X-chromosome inactivation and genomic imprinting (Jones and Baylin, 2002).

The first identified change in DNA methylation in cancer was genome-wide hypomethylation (Feinberg and Tycko, 2004). Genomic hypomethylation is an early and consistent event in colorectal carcinogenesis, and is associated with proto-oncogene activation, genomic instability and an increased number of mutational events (Eden et al., 2003; Chen et al., 1998) (Figure 1). In the past decade, however, it has become apparent that hypermethylation of 5' CpG islands is crucial for silencing tumor suppressor genes, and epidemiological factors seem to influence this epigenetic modification (Figure 2). According to the classical two-hit theory, tumor suppressor genes are inactivated by either gene

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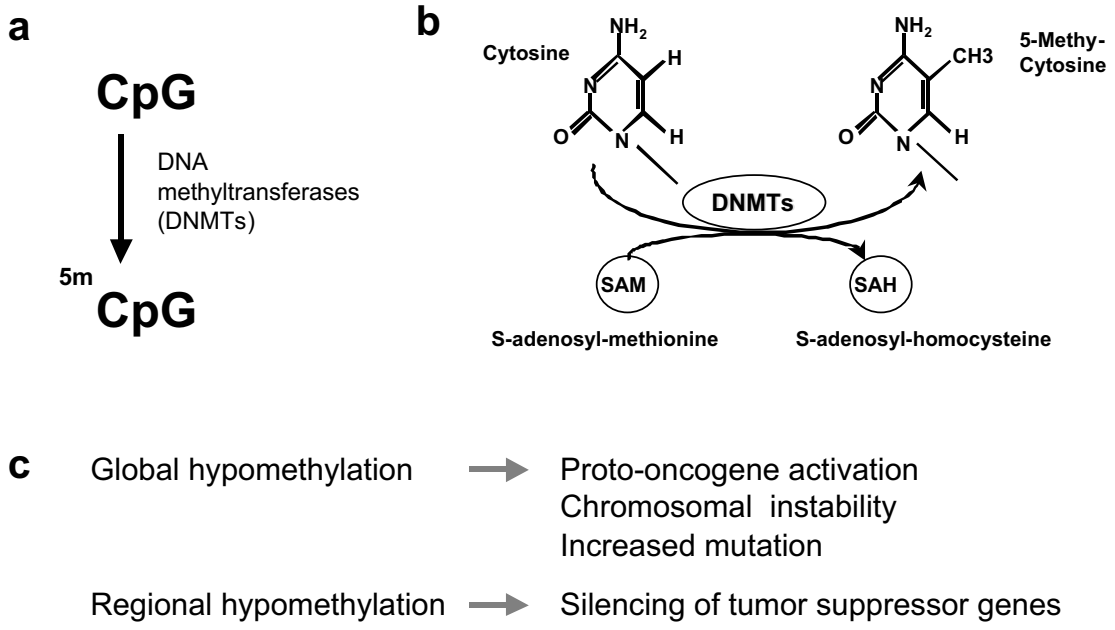

Figure 1. Role of DNA Methylation in Cancer. (a) In mammalian genome, methylation takes place only at cytosine bases located 5' to a guanosine in a CpG dinucleotide. (b) DNA methyltransferases (DNMTs) catalyzes the cytosine methylation using S -adenosyl-methionine as the donor molecule for the methyl group. (c) Two distinct DNA methylation abnormalities and their roles in cancer

mutation or deletion. DNA hypermethylation is now recognized as a third mechanism by which inactivation of tumor suppressor genes occur. Although accumulating evidence suggests that DNA methylation plays a significant role in gastrointestinal tumorigenesis, the specific mechanisms that induce DNA methylation alteration in cancer remain unclear.

Roles of DNA Methylation in Gastrointestinal Tumorigenesis

To date, approximately half of the classical tumor suppressor genes that mutate in familial cancer syndromes, are also known to be inactivated by promoter hypermethylation. These include the retinoblastoma gene

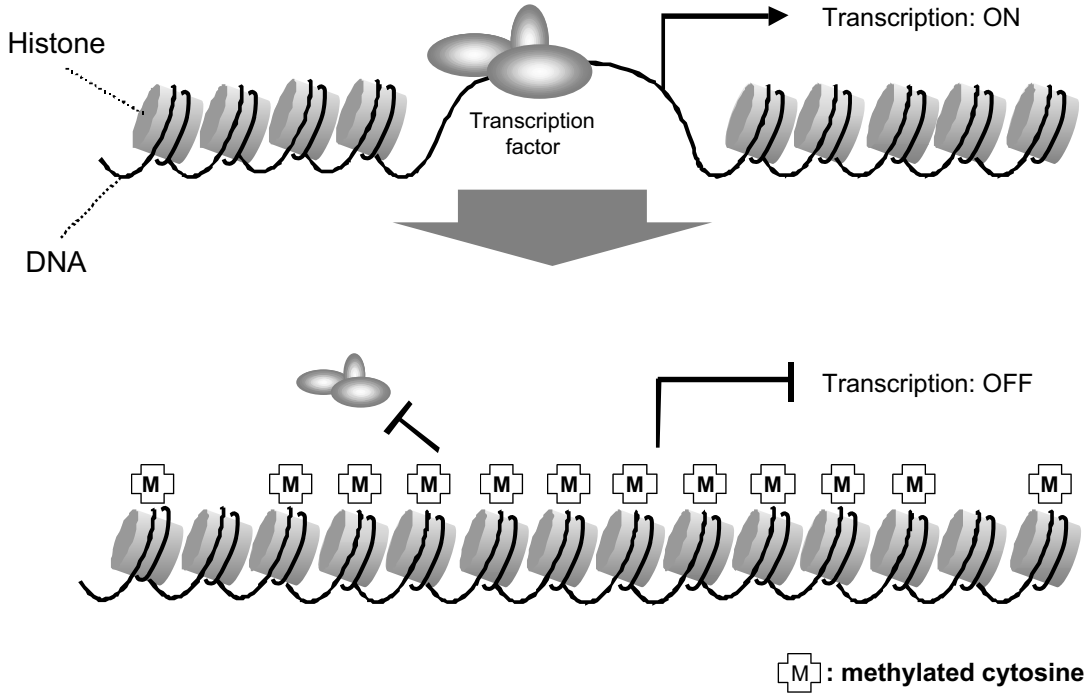

Figure 2. DNA Methylation and Chromatic Structure. Methylation of cytosines in a CpG island leads to chromatin condensation, which inhibits binding of transcription factors, and gene transcription is silenced

(RB), the von Hippel-Lindau gene (VHL), the adenomatous polyposis gene (APC), E-cadherin, and the breast cancer susceptibility gene 1 (BRCA1) (Jones and Baylin, 2002; Feinberg and Tycko, 2004). In addition to these classical tumor suppressor genes, an increasing number of genes related to cell-cycle control, repair of DNA damage, tumor invasiveness, and response to growth factors, are now also known to be inactivated by hypermethylation in gastrointestinal malignancies (Table 1).

Among numerous cell-cycle regulating genes, p16INK4A, a cyclin-dependent kinase inhibitor, is one of the most frequently silenced genes via DNA hypermethylation in gastrointestinal cancer (Toyota et al., 1999a; Suzuki et al., 1999). We found that 14-3-3 sigma, which regulates the G2/M checkpoint, is frequently methylated in stomach and liver cancer (Suzuki et al., 2000; Iwata et al. 2000). In addition, a number of researchers have observed that a mitotic checkpoint gene, CHFR, is silenced by DNA methylation in a wide variety of tumors (Toyota et al., 2004; Corn et al., 2003). Other reports indicate that DNA methylation silences genes associated with apoptosis. These genes include DAP-kinase (Satoh et al., 2002), HRK (Obata et al., 2003), and TMS1/ASC (Yokoyama et al., 2003). DNA methylation also affects genes associated with hypoxia-mediated cell death through silencing of BNIP3 (Murai et al., 2005).

DNA methylation also affects several important signaling pathways that are frequently activated in cancer cells. Using a microarray-based screening technique, we identified SFRP1 as a hypermethylation target in colorectal and gastric cancer (Suzuki et al., 2002). SFRP family members contain an N-terminal domain homologous to the cysteine-rich domain (CRD) of the Wnt receptor Fz, and inhibit signaling by binding directly to Wnt or Fz. SFRP1, SFRP2, and SFRP5 are frequently silenced by hypermethylation in colorectal cancers and adenomas, suggesting that epigenetic inactivation of SFRP genes may be an early event in colorectal tumorigenesis. Introduction of SFRP to silenced cells results in down-regulation of the transcriptional activity of TCF4. These results suggest that SFRP may be a therapeutic target for cancer.

Ras signaling is also affected by DNA methylation. RASSF1 is a negative regulator of Ras mapped to chromosome 3p21, a region in which frequent gene deletions have been observed in human cancers (Dammann et al., 2000). Although RASSF1 is frequently silenced in various types of cancer, including lung, ovarian, bladder, and cervical cancer, alterations in gastrointestinal cancer are not

Table 1. Genes Silenced by DNA Hypermethylation in Gastrointestinal Cancers

Genes	Function	Tumor types
Cell cycle		
p16	Cyclin dependent kinase inhibitor	Colon, stomach, esophagus, pancreas
p57	Cyclin dependent kinase inhibitor	Stomach
14-3-3sigma	G2/M checkpoint	Stomach
CHFR	Mitotic checkpoint	Colon, stomach
DNA repair		
hMLH1	Mismatch repair	Colon, stomach
MGMT	Methylguanine repair	Colon, stomach, esophagus
Signal transduction		
SFRP1	Wnt signaling	Colon, stomach, pancreas
SFRP2	Wnt signaling	Colon, stomach
RASSF1A	Ras signaling	Colon, stomach, esophagus, pancreas
RASSF2	Ras signaling	Colon, stomach
Transcriptional factors		
GATA4	Transcription factor	Colon, stomach
GATA5	Transcription factor	Colon, stomach
Immune system		
CIITA	MHC class II transactivator	Colon, stomach
Invasion and metastasis		
E-cadherin	Cell adhesion	Colon, stomach, esophagus
TIMP3	Matrix metalloproteinase inhibitor	Colon, stomach
Apoptosis		
DAPK1	Serine/threonine kinase	Colon, stomach
HRK	BH3-family	Colon, stomach
BNIP3	Hypoxia	Colon, stomach, pancreas
Others		
p14	MDM2 inhibitor	Colon, stomach, esophagus
RARβ	Retionoic acid receptor	Colon, stomach, esophagus, pancreas
CACNA1G	Calcium ion channel	Colon, stomach

frequently observed, indicating that other members of RASSF family may play a role. There are six members of the RASSF gene family, and all genes have CpG islands in their 5' regions. Recently, a number of researchers have observed that one gene of the RASSF family, RASSF2, is frequently silenced by methylation in colorectal cancer (Akino et al., 2005; Hesson et al., 2005). Importantly, RASSF2 is frequently silenced in tumors with activating mutations involving K-ras or BRAF, indicating that concurrent activation of oncogenic signaling and inactivation of tumor suppression may be necessary for malignant transformation (Akino et al., 2005). Aberrant methylation of RASSF2 is also observed in gastric cancers (Endoh et al., 2005). Restoration of RASSF2 expression using a demethylating agent or an adenoviral vector leads to suppression of tumor growth, confirming the gene's role in tumor suppression (Akino et al., 2005).

Histone Modification in DNA Methylation-mediated Gene Silencing

Researchers also intensively study the molecular mechanisms between DNA methylation and gene silencing. Recent studies suggest that histone modification might be involved in DNA methylation-mediated gene silencing (Bird and Wolffe, 1999; Magdinier and Wolffe, 2001). Methylated DNA binds to the transcriptional repressor, MeCP2, after which recruitment of a histone deacetylase complex is observed. Thus, histone deacetylation may play a role in DNA methylation-dependent gene silencing. Indeed, inhibition of histone deacetylation and inhibition of DNA methylation have a synergistic effect on induction of gene expression (Cameron et al., 1999). More recently, histone methylation has shown to be linked to epigenetic silencing of gene expression. Using a CpG island microarray coupled with a chromatin immunoprecipitation assay, Kondo et al. found several genes silenced by H3-K9 methylation in association with DNA methylation (Kondo et al., 2004). Further study will clarify the role of complex histone modification in DNA methylation-dependent and -independent gene silencing in gastrointestinal cancer in the future.

DNA Methylation in Association with Aging and Inflammation

Although the mechanism by which CpG island hypermethylation leads to gene silencing has been established, the mechanism that initiates DNA methylation changes in cancer remains to be determined. Studies regarding methylation of cancer-related genes show reduced levels of methylation within normal colorectal mucosa from aged patients (Figure 3). Issa and colleagues found that methylation of a subset of genes, including the estrogen receptor (ER), myogenic differentiation antigen (MYOD), N33 and VERSICAN, also occurs in normal colonic mucosa with aging (Ahuja et al., 1998; Toyota et al., 1999).

Several studies suggest a correlation between chronic inflammation and accelerated DNA hypermethylation. Ulcerative colitis (UC) is a condition associated with a markedly increased risk of colon cancer. Issa et al. found markedly increased methylation of four genes (ER, MYOD, p16 and CSPG2) in dysplastic epithelium from subjects with high-grade dysplasia, compared to non-UC controls (Issa et al. 2001). This suggests that chronic inflammation is associated with high levels of methylation, perhaps as a result of increased cell turnover, and that inflammatory bowel disease can be viewed as akin to premature aging of colorectal epithelial cells. Associations between chronic inflammation and gene methylation have also been identified in cancers involving other organs. For example, hypermethylation of p16INK4A is detected in liver cancer,

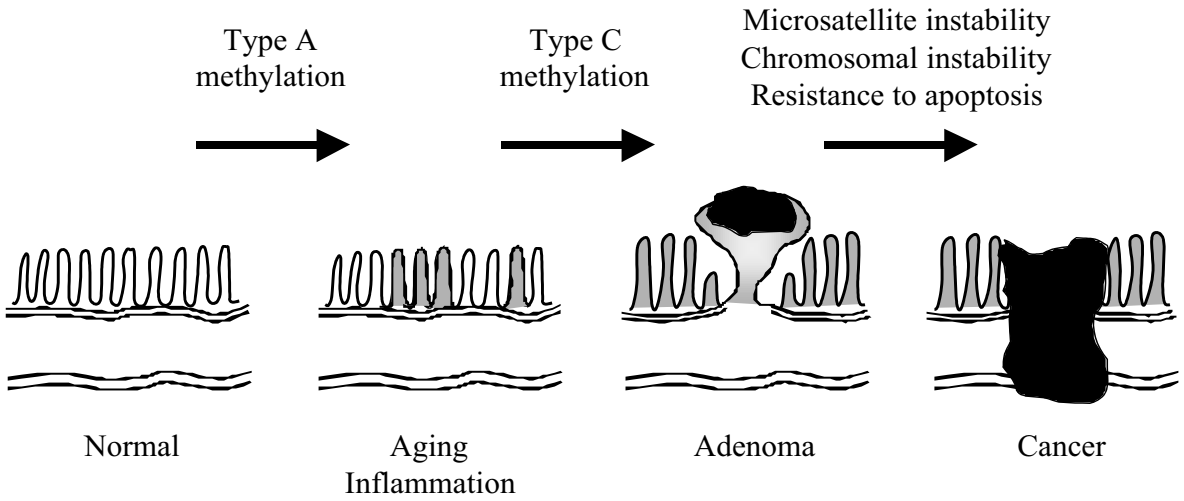

Figure 3. A Model Integrating Aging, Inflammation and DNA Methylation in Colorectal Carcinogenesis. Methylation increases significantly during the course of aging and inflammation, and can be used as a marker for monitoring the patients (Type A methylation). During the course of transformation, cancer specific methylation occurs (Type C methylation), and is involved in silencing of tumor suppressive genes

as well as chronic hepatitis and cirrhosis, in subjects with hepatitis B or C virus (Kaneto et al., 2001).

CpG Island Methylator Phenotype in Gastrointestinal Cancer

The specific cause of changes in DNA methylation in cancer remains unknown. However, recently developed techniques for detecting changes in DNA methylation have dramatically increased the amount of information available regarding the patterns of methylation that occur as cancers progresses. As discussed above, aberrant methylation is known to occur with aging and inflammation. Because aging affects a large number of CpG islands, age-related methylation might explain the increased incidence of cancer seen among older individuals. Other patterns of methylation are cancer-specific and detected only in a subset of tumors exhibiting the CpG island methylator phenotype (CIMP) (Figure 3).

By examining multiple loci identified by genome screening technology, we found a subset of colorectal cancers showing high rates of aberrant promoter methylation events, involving several tumor-suppressor genes (Toyota et al., 1999a; Toyota et al., 1999b). These tumors appear to have a "hypermethylator" phenotype (CIMP), and a strong association with microsatellite instability caused by human MutL homologue (hMLH1) methylation is observed in tumors exhibiting CIMP. Along this line, the majority of sporadic colorectal cancers exhibiting microsatellite instability (MSI) are associated with CIMP. Colorectal cancers with CIMP also show distinct clinicopathological and molecular characteristics. Firstly, tumors with CIMP tend to occur more proximally, and are more commonly observed in women and older patients. Secondly, these tumors demonstrate a higher frequency of K-ras and BRAF mutations and a lower frequency of p53 mutations (Toyota et al., 2000). Recently, Samowitz et al. performed a relatively large, population-based study of DNA methylation in colorectal cancer, confirming most previously published observations regarding CIMP (Samowitz et al., 2005). CIMP is also found cancers involving other organs, including the stomach, liver and pancreas. We recently reported that, in gastric cancer, CIMP is positively correlated with Epstein-Bar virus, and inversely correlated with p53 and K-ras mutations (Kusano et al., 2006). Although the specific mechanism that induce CIMP is not identified yet, it appears that CIMP results in both increased rates of de novo methylation and the spreading of methylation from methylation centers, such as repetitive sequences. Further study will be necessary to clarify the molecular mechanism by which CIMP results in tumorigenesis.

Folate Deficiency and DNA Methylation in Cancer

Although much remains unclear regarding the mechanism of DNA methylation during malignant transformation, environmental factors have long been thought to play a crucial role. Inadequate dietary folate has been implicated in the development of several types of cancers, with the most convincing evidence linking low folate intake with an increased risk of malignancy in colorectal cancer (Duthie et al., 2004). Prospective cohort (Giovannucci et al., 1993; Giovannucci et al., 1995; Giovannucci et al., 1998; Konings et al., 2002) and case control (Benito et al., 1991; Benito et al., 1993; Tseng et al., 1996) studies report an association between low folate intake and increased risk of colorectal adenomas and cancer. Plasma and erythrocyte folate levels are decreased in patients with colorectal cancer, compared to normal subjects (Porcelli et al. 1996). Conversely, supplementation with folic acid protects against the development of colorectal neoplasia in high-risk patients with ulcerative colitis (Lashner et al., 1997). The mechanism by which folate deficiency enhances, and supplementation suppresses, colorectal carcinogenesis has not been clearly elucidated. One proposed mechanism is that folate deficiency might induce DNA hypomethylation, which can affect DNA stability and the expression of proto-oncogenes (Jones and Baylin, 2002; Feinberg and Tycko, 2004).

Folate has a crucial role in DNA metabolism and function through its ability to methylate cytosine and regulate gene expression, as well as its role in nucleotide synthesis and DNA repair. Folate is required to maintain an adequate cellular pool of the methyl donor S-adenosylmethionine (SAM). 5-Methyltetrahydrofolate, the major circulating form of folate, is involved in re-methylation of homocysteine to methionine, which is a precursor of SAM. SAM is the primary methyl group donor for most biological methylations, including cytosine methylation within DNA. After transfer of the methyl group, SAM is converted to S-adenosylhomocysteine (SAH), a potent inhibitor of most SAM-dependent methyltransferases. SAH is immediately hydrolyzed to homocysteine and adenosine. Homocysteine is then either recycled back to methionine and S-adenosylmethionine through the vitamin B12-dependent enzyme methioninesynthase (MS), a reaction in which folate is the methyl donor, or catabolized to pyruvate through cystathionine-b-synthase (CBS). When the supply of folate is limited, plasma and cellular levels of homocysteine increase. This leads to an increase in cellular levels of SAH, which inhibits methyltransferase activity, and leads to DNA hypomethylation (Yi et al. 2000). High alcohol intake may also induce DNA hypomethylation by reducing intracellular levels of SAM. Alcohol has been shown to cleave folate, impair folate absorption, increase folate excretion, and interfere with MS activity (Kenyon et al., 1998). Genome wide hypomethylation is believed to cause inappropriate proto-oncogene activation and transcription, and malignant transformation (Jones and Baylin, 2002; Feinberg and Tycko, 2004).

However, much remains to be clarified about the effect of folate deficiency on genomic DNA methylation in human colon. Evidence showing a role of folate deficiency in modulation of DNA methylation is inconsistent. DNA

hypomethylation in colon tissue and low folate status are both associated with an increased risk of colorectal neoplasia (Pufulete et al., 2003a). Few studies have measured DNA methylation in normal colorectal mucosa, although a recent study suggests that genomic DNA methylation in the colon is inversely associated with folate status (Pufulete et al., 2003b). DNA hypomethylation is induced in lymphocytes isolated from healthy post-menopausal women with subclinical folate deficiency (Jacob et al., 1998). These effects on methylation have been confirmed in a study of elderly women consuming a moderately folate-depleted diet (Rampersaud et al., 2000). In contrast, in subjects with normal folate status, lymphocyte genomic DNA methylation is unaffected by folate supplementation (Fenech et al., 1998).

Although methyl depletion may lead to genomic hypomethylation, it is unclear how regional hypermethylation of gene promoters in the presence of low folate levels occurs in cancer. A recently published cohort study examined the relationship between promoter methylation of 6 genes involved in colorectal cancer (CRC) carcinogenesis (APC, p14ARF, p16INK4A, hMLH1, O6-MGMT, and RASSF1A), folate levels, and alcohol intake (van Engeland et al., 2003). For each gene tested, the prevalence of promoter hypermethylation was greater among patients with CRC and low folate / high alcohol intake (n=61), compared to patients with CRC and high folate / low alcohol intake (n=61), however, none of the results reached statistical significance. The number of cases in which at least one of the six genes was methylated was higher (84%) in the low folate intake / high alcohol intake group, compared to the high folate intake/ low alcohol intake group (70%; P=0.085). Thus, it has been suggested that folate and alcohol intake may be associated with changes in promoter hypermethylation in CRC, although further study with a larger population is necessary to confirm this.

Folate Metabolism and Genetic Susceptibility to Cancer

Recent molecular epidemiological studies indicate that polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene may modulate colorectal cancer risk by influencing DNA stability and cytosine methylation. MTHFR is a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby playing an important role in DNA synthesis, maintenance of the nucleotide pool, and DNA methylation (Kim, 1999; Kim, 2000) (Figure 4).

The MTHFR gene is located at 1p36.3, and several polymorphisms of the MTHFR gene have been identified (Goyette et al., 1994). The most common variant of the MTHFR gene is located at nucleotide 677 (C677T) and results in an alanine to valine conversion in the protein associated with decreased enzyme activity (Frosst et al., 1995). Heterozygotes (CT) or homozygotes (TT) variants have markedly reduced in vitro enzyme activity (Frosst et al., 1995). Compared with homozygotes for the common variant (CC), heterozygotes have 65% of their enzyme activity levels in vitro, homozygous variant (TT) have only 30% of the enzyme activity. MTHFR polymorphism is associated with changes in the blood folate and homocysteine levels. Red blood cells from individuals homozygous for the TT variant have decreased 5-methylfolate levels with a concomitant increase in formylfolate derivatives (Molloy et al., 1997; Bagley and Selhub, 1998). Persons with the TT variant also have lowered plasma folate and vitamin B12 levels and raised homocysteine levels (Ma et al., 1997; Ma et al., 1999; Jacques et al., 1996). The frequency of homozygosity for the C677T variant of the MTHFR gene varies between geographic areas and according to ethnic origin. TT genotype frequency in White populations in Europe, North America, and Australia, ranges from 8–20 percent. In Europe, there appears to be a trend of increasing frequency of this variant from north to south. Twelve percent of the Japanese population are TT homozygotes.

On the basis of the functional effects of the MTHFR polymorphism, and the inverse association between folate status and disease, it might be expected that the C677T variants are associated with an increased risk of disease. However, most studies demonstrate a reduced risk among homozygous variant (TT) subjects, compared with homozygotes for the common allele (Chen et al., 1996; Ma et al., 1997; Park et al., 1999; Slattery ML et al, 1999; Keku et al., 2002; Le Marchand et al., 2002). Individuals homozygous for the variant (TT) generally have a reduced risk of developing colorectal cancer, compared with heterozygotes or wild type individuals. The observed relative risk among individuals homozygous for the variant (TT) ranges from 0.45 to 0.9, although most studies do not show statistical significance (Sharp and Little, 2004). The relationship between MTHFR genotype and risk of colon cancer appears to be heavily influenced by diet and environmental factors, such as reduced folate levels, methyl donor status, and high alcohol intake (Sharp and Little, 2004).

Studies regarding the influence of MTHFR C677T polymorphism on DNA methylation status reveal inconsistent results. Global DNA methylation status in one study of lymphocytes isolated from approximately 200 healthy subjects was unaffected by genotype or folate status (Narayanan et al. 2004). However, in another study, global DNA hypomethylation was observed in leucocytes from individuals with TT, compared to subjects with the CC genotype (Stern et al. 2000). The results of a larger study demonstrated reduced genomic DNA methylation in peripheral blood mononuclear cells in TT, compared to CC individuals, when plasma folate concentrations were low (Friso et al., 2002). CT and TT individuals with colon, breast, and lung cancer, have all demonstrated reduced levels of 5-methylcytosine in their normal counterpart tissues, and tumors in these patients did not achieve severe degrees of global hypomethylation (Paz et al., 2002). However, similar

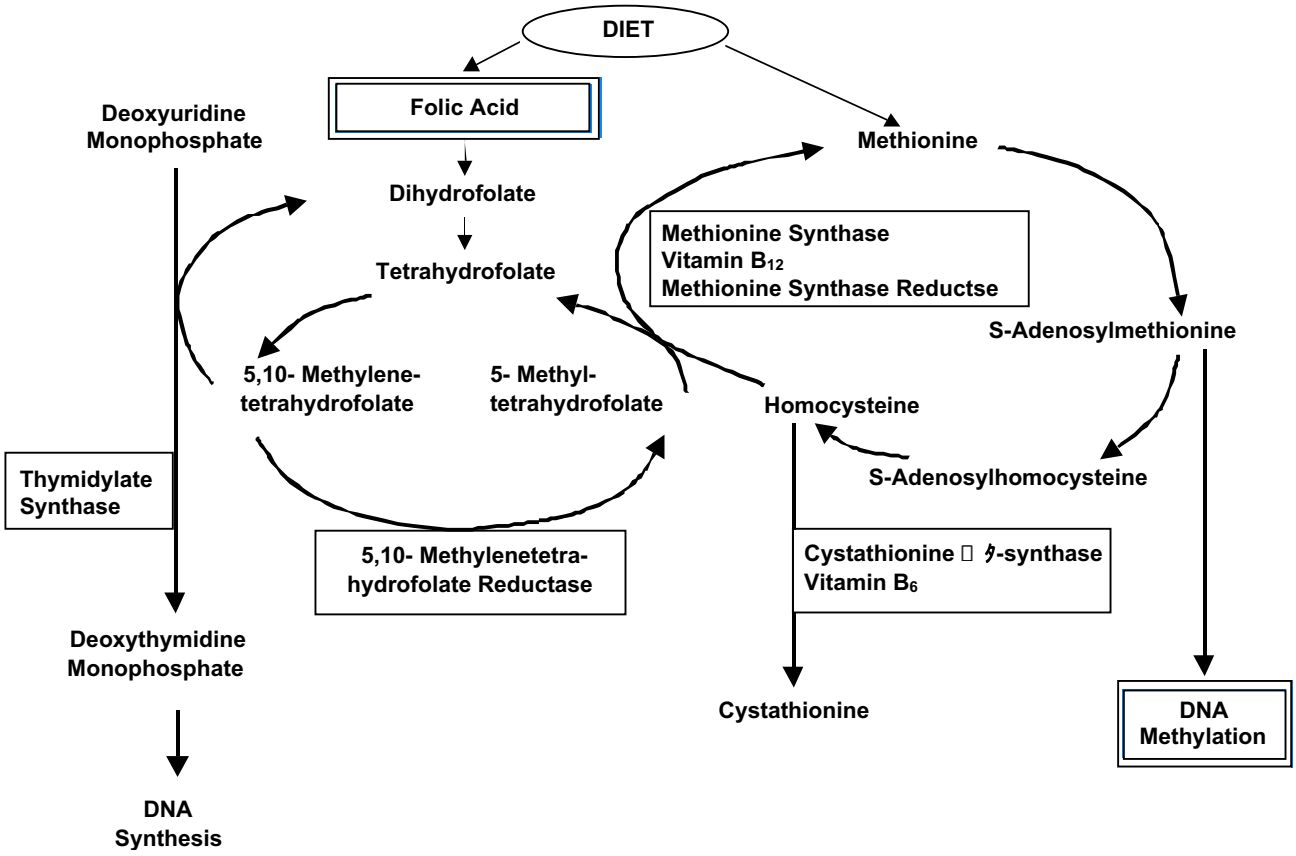

Figure 4. Simplified Scheme of Folate Involvement in DNA Methylation and Synthesis. The roles of folate metabolizing enzymes including methylenetetrahydrofolate reductase, methionine synthase, methionine synthase reductase, cystathionine-b-synthase are shown

levels of CpG island hypermethylation involving specific tumor suppressor genes are observed for all three MTHFR variants (Paz et al. 2002). In contrast, van Rijnsoever et al. found that females who were heterozygous or homozygous for the C677T MTHFR polymorphism were at increased risk of developing colorectal cancer with CIMP (van Rijnsoever et al., 2002). Another group also reported the correlation between MTHFR polymorphism and CIMP in colon cancer (Oyama et al., 2004).

Conclusions and Future Directions

Aberrant DNA methylation in cancer has several characteristic features. It is tumor-specific, gene- and tissue type-specific, and reversible with demethylating drugs. It should therefore be possible to use epigenetic changes as novel molecular to make diagnoses and monitor patients, as well as to assess the benefits of therapy. For instance, detecting aberrant DNA methylation in stool samples might prove useful as a new cancer screening technique. Also, hypermethylation of key genes involved in DNA repair or cell cycle checkpoint regulation, such as O6-MGMT and CHFR, may enable prediction of drug response and prognosis (Herman and Baylin, 2003; Toyota et al. 2003). However, although there is accumulating evidence to suggest that genetic and environmental factors strongly influence the epigenetic changes that occur in cancer, much remains unknown regarding the etiology of these changes. Further molecular and epidemiological studies regarding DNA methylation and other epigenetic changes may provide increased opportunities for cancer prevention.

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