Trans-HHS Workshop:
Diet, DNA Methylation Processes and Health

Folate Status: Effects on Pathways of Colorectal Carcinogenesis[1,2]

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ABSTRACT Many epidemiologic, animal and human studies suggest that folate status modulates carcinogenesis. Although these observations have been made in a number of tissues, the data are clearly most compelling for the colorectum. The mechanism(s) by which this modulation is mediated remains ill defined. Alterations in either genome-wide or gene-specific DNA methylation and/or alterations in DNA stability, resulting from DNA strand breaks or uracil misincorporation, are leading candidates in this regard. Folate has a central role in biological methylation and nucleotide synthesis, and therefore it is not surprising that folate depletion has been observed to alter DNA methylation and diminish DNA stability. The hypothesis that these two pathways are the means by which folate modulates cancer risk is also supported by the epidemiological observation that a common polymorphism in the methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) gene differentially affects the relative risk of colon cancer depending on folate status, because MTHFR catalyzes the reaction that determines whether cellular folate is diverted into biological methylation or nucleotide synthesis. This phenomenon suggests that it is an imbalance between biological methylation and nucleotide synthesis that is responsible for folate-related carcinogenesis. The control of cell proliferation, which also is related to DNA methylation, is another candidate mechanism by which folate status modulates carcinogenesis. In cell culture studies, folate supplementation has been observed to suppress excessive cell proliferation. Understanding the mechanisms by which folate status modulates carcinogenesis is important for advancing insight into cancer biology and for facilitating those efforts to translate research in folate and carcinogenesis into effective and safe public health initiatives. J. Nutr. 132: 2413S–2418S, 2002.

KEY WORDS: • *folate* • *colon carcinogenesis* • *methylenetetrahydrofolate reductase* • *DNA methylation*

Over the past two decades, evidence from several different types of research—epidemiologic, animal models and clinical intervention studies—has increasingly supported the concept that diminished folate status predisposes to the development of several common cancers. Conversely, this body of literature also suggests that habitual ingestion of folate at concentrations that are somewhat above present recommendations is preventive. Such associations have been described with cancers of the cervix, colorectum, lung, esophagus, brain, pancreas, bone marrow and breast. Among these, support for such a relationship is most compelling for colorectal cancer (reviewed in reference 1). Folate deficiency also has been considered an important factor in alcohol-related carcinogenesis, because alcohol alters normal folate metabolism in a number of ways (2).

Folate has a central role in one-carbon metabolism. As such, it is a critical coenzyme for both biological methylation and nucleotide synthesis (**Fig. 1**). These are important functions, because aberrations in the methylation of macromolecules, particularly DNA, as well as disruption in DNA synthesis and repair, are thought to play major roles in carcinogenesis (reviewed in reference 3). Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20),[4] a key enzyme in one-carbon

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[4] Abbreviations used: 5-MC, 5-methyldeoxycytidine; CpG, cytosine guanine dinucleotide; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate; TT, thymine/thymine.

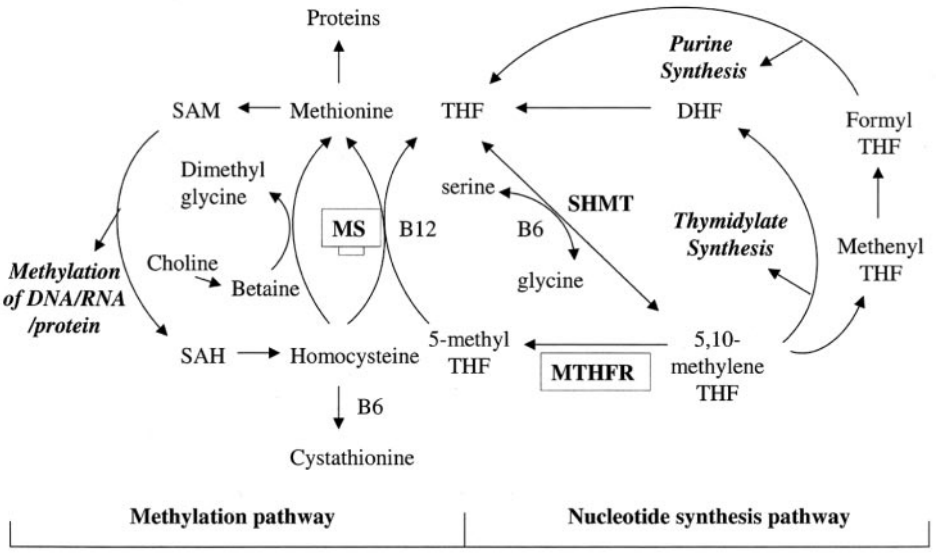

FIGURE 1 Folate in one-carbon metabolism. MTHFR sits as a fulcrum between DNA methylation and DNA synthesis, balancing the two pathways to maintain normal homeostasis. DHF, dihydrofolate; THF, tetrahydrofolate; MS, methionine synthase; MTHFR, methylene tetrahydrofolate reductase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SHMT, serine hydroxymethyl transferase.

metabolism (Fig. 1), catalyzes a unidirectional reaction that determines the balance between cellular availability of 5,10-methylenetetrahydrofolate (5,10-methylene THF), which is used for thymidylate and purine synthesis, and methyl-THF, which is used for biological methylation. Recently, a highly prevalent polymorphism of the *MTHFR* gene has been described and reported to have a strong relationship with cancer risk. Not surprisingly, the relationship with cancer risk is modified by folate status (4).

This review will focus on the mechanisms that seem to constitute the means by which folate depletion enhances carcinogenesis. Although these mechanisms are described separately, they operate via interrelated pathways that are synergistic in nature. A prior hypothesis paper describes a unified scheme by which these mechanisms might act together (3). The evolving hypothesis, supported by observations from many laboratories, is that folate depletion enhances carcinogenesis by impairing normal methylation and nucleotide synthesis by creating an imbalance between the partitioning of cellular folates down these two pathways.

DISCUSSION

Folate in one-carbon metabolism. One-carbon metabolism is a network of interrelated biochemical reactions in which a one-carbon unit from a donor compound is transferred to tetrahydrofolate (THF) for subsequent reduction or oxidation and/or transfer to other compounds (Fig. 1). Folate coenzymes in mammalian tissues thereby act as acceptors or donors of one-carbon units in a variety of reactions involved in amino acid and nucleotide metabolism (5). Three of the one-carbon substituted derivatives of THF are each associated with metabolic pathways that are particularly important to that particular form of the vitamin: methyl-THF is needed for methionine synthesis (which is necessary for DNA methylation), 5,10-methyleneTHF for thymidylate synthesis, and 10-formyltetrahydrofolate for purine synthesis (6).

Methionine synthesis plays a major role in one-carbon metabolism (Fig. 1). Methionine is regenerated from homocysteine in a reaction catalyzed by methionine synthase (5-methyltetrahydrofolate—homocysteine S-methyltransferase; EC 2.1.1.13): this is the only known reaction in which the methyl group of methyl-THF is used in mammalian tissues. The enzyme contains a cobalamin (B-12) cofactor, and the reaction proceeds via a methylcobalamin intermediate (7). Regenerated methionine is then adenylated to form *S*-adenosylmethionine (SAM). SAM then donates its labile methyl group to more than 80 biological methylation reactions, including the methylation of DNA, RNA and protein. Although the alternative betaine pathway for methionine synthesis may partially compensate, it is nevertheless well known that dietary folate depletion alone is a sufficient perturbing force to diminish SAM pools (8). As described in the next section, experimental folate depletion in both animals and humans produces demonstrable impairments in biological methylation (9,10).

Folate-derived one-carbon metabolism also is essential for the synthesis of nucleotides. Thymidylate synthase catalyzes the transfer of formaldehyde from folate to the 5-position of deoxyuridylate, producing deoxythymidylate. In addition, one-carbon moieties at the oxidation level of formate are used in de novo purine biosynthesis. Because deoxynucleotides are the immediate substrates for the polymerases involved in DNA replication and repair, the fidelity of DNA synthesis is critically dependent on the correct balance and availability of deoxynucleotides (3). In mammalian cells, the de novo synthesis of deoxythymidylate from deoxyuridylate is a rate-limiting step for DNA synthesis. In cells, inhibition of folate metabolism results in uracil misincorporation in DNA (11). Recently, it has been shown that folate deficiency in the human results in excess uracil incorporation into DNA (12).

Folate and DNA methylation. Approximately 4% of all cytosines in mammalian DNA are methylated. Most of these 5-methyldeoxycytidine (5-MC) residues are found in the palindromic sequence, cytosine guanine dinucleotide (CpG) (13). Maintenance of normal patterns of DNA methylation is important for cellular homeostasis because DNA methylation is thought to play critical roles in the regulation of gene expression and gene integrity (14).

Accumulating evidence implicates aberrations in DNA methylation as a cause of carcinogenesis rather than as just an epiphenomenon. A decreased level of genomic methylation is a nearly universal finding in tumorigenesis: this has been observed in cancers of the colon, stomach, uterine cervix, prostate, thyroid and breast (reviewed in reference 1). This decrease in genomic methylation appears early in carcinogenesis and, at least in the case of colorectal cancer, seems to precede the better described mutation and deletion events that

occur later in the evolution of cancer (15). However, DNA methylation does not seem to be confined solely to the neoplasm. It has been observed to be present in normal-appearing rectosigmoid mucosa of patients harboring adenomas or carcinomas and therefore seems to be a characteristic of colonic mucosa that is predisposed to undergo neoplastic transformation. Whether it is responsible for that predisposition remains a matter of speculation. Genomic hypomethylation of DNA also has been observed in the colonic mucosa of animals genetically engineered to develop intestinal neoplasms (16), as well as in rats exposed to the colonic carcinogen dimethylhydrazine (17).

In 1993 Balaghi and Wagner demonstrated that hepatic DNA from folate deficient rats was hypomethylated on a genomic level compared with DNA from pair-fed control animals (9). When this phenomenon has been reexamined in the liver (18) or in other tissues such as the colon (19), the effect has not always been reproduced; therefore, it may be dependent on the severity or duration of the deficiency and/or it may be a tissue-specific effect.

In human studies using lymphocytic DNA, a consistent relationship between folate status and genomic DNA methylation has been observed. Jacob and colleagues reported that marginal folate depletion in volunteers housed in a metabolic unit diminished genomic DNA methylation; repletion of folate status then reversed the hypomethylation (10). Rampersaud and colleagues reported a similar effect in their metabolic unit study where a less restrictive diet (120 μg folate/d) was used (20). The induction of genomic hypomethylation under these circumstances probably is due primarily to the associated rise in homocysteine, which in turn increases tissue concentrations of *S*-adenosylhomocysteine (SAH), an inhibitor of methylation reactions (21). It is for this reason that plasma SAH and not SAM concentrations have been found to be most predictive of DNA methylation (22).

From the perspective of carcinogenesis, hypomethylation at critical loci is probably a more important factor than genomic hypomethylation. Observations in folate-deficient rats have confirmed that dietary deprivation of folate is a sufficient force to produce hypomethylation within the "hypermutable region" of the *p53* gene (18). Subsequent studies have demonstrated that this depletion also decreases steady-state concentrations of p53 mRNA (23). Consistent with the concept that these changes are involved in carcinogenesis are observations which indicate that hypomethylation within exon 8 of the colonic *p53* gene is also observed in an animal model of chemical carcinogenesis. Most recently, Jhaveri and colleagues reported that folate depletion produces gene-specific, rather than genomic, DNA hypomethylation in human nasopharyngeal carcinoma KB cells (24). Cell culture studies indicate that these foci of aberrant methylation within the coding region may serve as initiators of mutation (25) and may induce susceptibility to breakage of the DNA backbone at the site of hypomethylation (26) by increased uracil insertion and strand breaks (12). Hypomethylation of the coding regions of critical genes, which have been observed experimentally in models of folate depletion (18), can lead to instability of the region either because it becomes more susceptible to endogenous nucleases (27) or because the site of hypomethylation is more likely to undergo deamination to uracil (11,12). The latter situation is particularly prone to occur under conditions in which intracellular SAM concentrations are low, such as in folate depletion (28).

Alterations in methylation status within promoter regions is a very important epigenetic mechanism in gene control (29). Because methylation of the promoter generally suppresses transcription, promoter hypomethylation can activate protooncogenes, and promoter hypermethylation can inactivate tumor suppressor genes. In a rat model of hepatocellular carcinoma, a choline-deficient diet induced hypomethylation of 5′ upstream CpG sites of the *c-myc* gene as well as over expression of this gene (30). Interestingly, experimental deficiency of one-carbon nutrients in animals may instead cause a paradoxical (and presumably compensatory) hypermethylation of certain loci (31), which is of interest because the promoter regions of several tumor suppressor genes, such as *p16*, *p53*, and *APC*, are frequently found to be hypermethylated in cancers (32–34). Jhaveri and colleagues also reported that the H-cadherin gene, which showed hypermethylation of 5′ sequences in response to folate depletion, was down-regulated in human nasopharyngeal carcinoma KB cells. This hypermethylation suppresses expression of the gene (35) and is therefore hypothesized to contribute to the evolution of the cancer.

The importance of promoter methylation of tumor suppressor genes in cancer is underscored (36) by the following observations: *1*) gene silencing associated with promoter hypermethylation is a fully heritable event for individual alleles of a given gene. For examples, in cell culture, a mutated copy of the *p16* gene allele that has an unmethylated promoter is expressed, whereas the wild-type allele is hypermethylated and silenced (37); *2*) hypermethylation of promoters nearly always occurs only in those tumors that lack coding-region mutations in the involved gene (38); and *3*) the selective survival advantage for a clone of cells that has undergone a loss of function associated with altered methylation seems to be identical to the loss of function arising from a coding-region mutation (38).

Folate and DNA synthesis/repair. It has been known for some decades that folate deficiency induces breaks in chromosomes and that such breaks are associated with an increased risk of cancer in humans. DNA strand breaks, whereby breaks occur in the phosphodiesterase backbone of DNA, seem to be the molecular basis for this disruption in chromosomal integrity. Folate-deficient conditions in both cell culture (39) and animal experiments (26) have been shown to create an excess of strand breaks in DNA (39). In a study of rats, a folate-deficient diet increased gene-specific DNA strand breaks in the hypermutable region of *p53* in the rat colon (18) and decreased steady-state *p53* gene expression in that organ (23). Preliminary data from a human study also indicate that genome-wide DNA strand breaks are related to folate status (40).

Two likely mechanisms by which folate deficiency may create such breaks are uracil misincorporation (41) and impaired DNA repair (42). Folate deficiency reduces deoxythymidylate synthesis from deoxyuridylate, and the ensuing nucleotide imbalance increases the incorporation of uracil bases into DNA. Uracil in DNA is excised by a repair glycosylase and, in the process, a transient single-strand break develops in the DNA (12). Simultaneous removal and repair of two adjacent uracil residues on opposite strands can result in a double-strand DNA break, which is difficult to repair and further exacerbates genetic instability. Unrepaired double-strand DNA breaks enhance cellular transformation in culture and increase cancer risk. Similarly, an in vitro study using immortalized human colon epithelial cells demonstrated that folate deficiency induces uracil misincorporation and increases DNA strand breaks (41). Excessive DNA uracil content, as well as increased numbers of chromosomal breaks, are observed in folate deficient humans, and both defects are reversed by folate administration (12). Increased DNA strand breaks induced by

folate depletion also seem to arise as a result of impaired DNA repair. In a folate-deficient rat model, DNA excision repair was impaired in deficient colonic mucosal cells compared with normal mucosal cells (42). Fenech and colleagues (43) also found that folate supplementation above the recommended dietary allowance lessened chromosome breakage below concentrations observed in healthy, folate-replete persons. They suggest that this is an indication that basal requirements of folate are higher than formerly thought.

Folate and cell proliferation. Folate status also affects cell proliferation. Abnormal cell proliferation has essential roles in carcinogenesis, including the processes of initiation and promotion, although its precise role is still not clarified (44). A net increase in cell division is undesirable because it is generally agreed that it leads to hyperplastic and/or neoplastic growth. During clonal expansion, proliferating cells divide by a process defined by the cell cycle, which is strictly controlled at several check points by numerous oncoproteins, tumor suppressor gene proteins, and cyclins.

Folate status, in many settings, modifies proliferation rates. James and colleagues reported increased excess proliferation in the livers of folate/methyl-deficient rats (45). Conversely, folate supplementation has been observed to decrease colorectal mucosal proliferation in both animal and human studies. Nensey and colleagues reported that in an animal model, folate supplementation reduces carcinogen-induced ornithine decarboxylase and tyrosine kinase activity, both of which are indices of cellular proliferation (46). Biasco and colleagues also reported that folate supplementation significantly reduces rectal mucosal proliferation in patients with long-standing ulcerative colitis, a condition that carries an increased risk of colorectal cancer, a predisposition to which is thought to be due in part to reduced availability of folate (47). Most recently, Akoglu and colleagues reported a human colon cancer cell line in which dihydrofolate and methyl-THF served as growth-inhibitory factors (48).

Cell proliferation is affected by DNA methylation. Fournel and colleagues reported that specific reduction of cellular DNA methyltransferase in human cancer cells inhibits cell proliferation (49). DNA hypomethylation at critical loci might enhance expression of proto-oncogenes, such as *ras* and *c-myc*, or other genes related to carcinogenesis, resulting in excess cell proliferation (50,51).

Aberrations in folate form distribution: the MTHFR polymorphism. Qualitative changes in folate metabolism may be as important as quantitative changes in regard to carcinogenesis. For example, MTHFR catalyzes the irreversible conversion of 5,10-methylene THF to methyl-THF. A common polymorphism of this gene (cytosine-to-thymine transition at position 677 or C677T) causes reduced activity of MTHFR (52). In 1997 Ma and colleagues (4) reported that folate-replete men who are homozygous for the mutation (TT) have half the risk of colorectal cancer compared with the wild-type and heterozygous genotypes. However, protection associated with the mutation was absent in men with low systemic folate status. Subsequent confirmations of this observation suggest that the homozygote may be at even greater risk of cancer than the wild-type subject when folate status is low (53). Several epidemiologic studies indicate that the relationship between this polymorphism and cancer may also exist for other tissues, such as esophagus, lung, bone marrow, breast and ovary (54–56).

It has been suggested that the cancer-protective effect of the MTHFR mutation in folate-replete conditions is related to the increased availability of 5,10-methylene THF and, therefore, increased ease of nucleotide synthesis. On the other hand, the increased risk of colorectal cancer apparent in TT subjects with low folate status may be because of the fact that, below a certain threshold, availability of methyl-THF for biological methylation becomes compromised severely enough to become the critical determinant of whether the cell is pushed down the pathway toward neoplasia (3). Bagley and colleagues reported that persons homozygous for the polymorphism have lower proportions of methylated folates in their red cells (57). This qualitative change, even in the absence of any quantitative differences in cellular folate carcinogenesis, is associated with reductions in genomic DNA methylation, as previously shown in a small population (58) and now confirmed in a large cohort (59).

Thus, the modulation of cancer risk associated with the polymorphism may be conveyed by a shift in proportions of the different coenzymatic forms of folate contained within the cell, thereby changing the balance of biological methylation and DNA synthesis/repair. Qualitative alterations in cellular folate pools related to genotype also may explain how the protective effect associated with the mutant genotype is operable even in the face of plentiful quantities of cellular folate in folate-replete subjects. Therefore, under certain conditions, alterations in folate form distribution (i.e., a qualitative change in folate metabolism) might be more important than total folate concentrations (i.e., a quantitative change) in determining the risk of carcinogenesis (60).

Folate, homocysteine and carcinogenesis. Hyperhomocysteinemia, which is indicative of an impairment of one-carbon metabolism, has been regarded as an important risk factor for cardiovascular disease but as not significant in cancer. However, Kato and colleagues reported in a nested case-control study that the risk of colorectal cancer was significantly elevated among those with a higher total plasma homocysteine concentration. With multivariate analysis, there was a strong trend toward an effect of total plasma homocysteine that was independent of folate concentrations (61). Weinstein and colleagues also reported in a large case-control study that invasive cervical cancer risk is significantly increased for women with high homocysteine concentrations (62). Consistent with earlier studies, Yi and colleagues demonstrated that chronic elevations in plasma homocysteine concentrations, such as those associated with nutritional deficiencies or genetic polymorphisms in the folate pathway, may have an indirect and negative effect on cellular methylation reactions through a concomitant increase in intracellular SAH concentrations (22). Because SAH inhibits biological methylation reactions, increased homocysteine concentrations may directly affect carcinogenesis by diminishing DNA methylation in critical tissues.

In conclusion, compelling evidence indicates that folate status modulates the risk of developing colorectal cancer: folate depletion seems to enhance carcinogenesis, and folate supplementation conveys a protective effect. However, two decades of studies have not yet defined the mechanism(s) responsible for mediating this effect. Nevertheless, such an understanding is being approached.

Because folate has a critical role in both DNA methylation and DNA synthesis, most studies have focused on these effects. Interestingly, alterations in DNA methylation, an epigenetic phenomenon that can affect gene expression and integrity, have been found in folate-depletion studies in both humans and animals. Increased uracil incorporation into DNA, which reflects an imbalance of nucleotide synthesis and can induce DNA strand breaks, also has been observed in human studies that have examined folate-deplete subjects. Both phenomena can be reversed by folate supplementation.

These two mechanisms are highlighted by the common

polymorphism of the *MTHFR* gene and the remarkable decrease in the incidence of colorectal cancer associated with it. This is presumably because the MTHFR reaction sits as a fulcrum between DNA methylation and DNA synthesis, balancing the two pathways to maintain normal homeostasis.

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