

## Folate and Carcinogenesis: An Integrated Scheme<sup>1-3</sup>

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**ABSTRACT** Collectively, the evidence from epidemiologic, animal and human studies strongly suggests that folate status modulates the risk of developing cancers in selected tissues, the most notable of which is the colorectum. Folate depletion appears to enhance carcinogenesis whereas folate supplementation above what is presently considered to be the basal requirement appears to convey a protective effect. The means by which this modulation of cancer risk is mediated is not known with certainty, but there are several plausible mechanisms which have been described. Folate plays a major role in the formation of S-adenosylmethionine, the universal methyl donor, as well as in the formation of purine and thymidine synthesis for DNA and RNA. Therefore, most mechanistic studies performed to date have focused on alterations in DNA methylation, disruption of DNA integrity and disruption of DNA repair, all of which have been observed with folate depletion. These aberrations in DNA are believed to enhance carcinogenesis by altering the expression of critical tumor suppressor genes and proto-oncogenes. Recently, the role of a common polymorphism of the methylenetetrahydrofolate reductase gene has been highlighted as well. This review presents those mechanisms which are the most likely candidates to explain folate's effects and it proposes an integrated scheme to explain how these mechanisms might interact. *J. Nutr.* 130: 129-132, 2000.

**KEY WORDS:** • folate • carcinogenesis • DNA methylation  
• DNA integrity • MTHFR gene

Low consumption of fruits and vegetables has been consistently related to an increased incidence of cancer. Many components of fruits and vegetables may be responsible for the reduced risk of cancer. Among the candidate components is folate. On a very simplistic level, cancer is thought to arise because of an excess of DNA damage and/or the inappropriate expression of critical genes. Folate has consequently been of particular interest as a potential cancer protective agent because of the important roles it plays in nucleotide synthesis, as well as in the biological methylation of molecules such as DNA, RNA, proteins, and the phospholipids.

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Epidemiologic studies have observed that diminished folate status is associated with cancer of the cervix, colorectum, lung, esophagus, brain, pancreas, and breast. Among these, epidemiologic support for such a relationship is clearly most compelling for colorectal cancer (1). Folate deficiency also has been considered as an important factor in alcohol-related enhancement of rectal carcinogenesis because alcohol alters normal folate metabolism in a variety of ways.

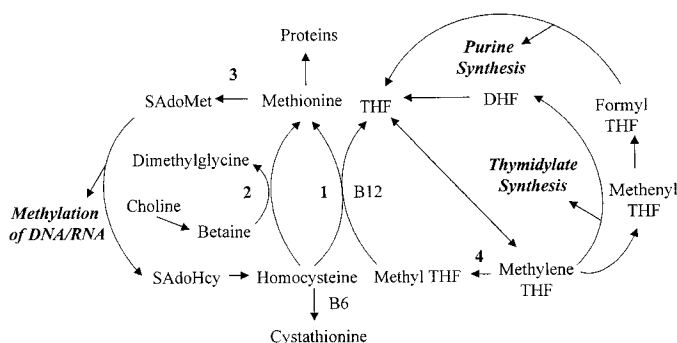
Although some animal studies support the epidemiologic concept that dietary folate is protective against selected cancers (2, 3), the studies are not entirely consistent (4-6). The reasons for these conflicting results are due to several factors: these include the species, the tumor type and model, the timing, dose, and length of application of carcinogen, the stage of carcinogenesis, and the dietary level, and form of folate administered as well as its chronologic relationship to carcinogen administration.

Human intervention trials, designed to determine whether individuals at increased risk of cancer have that risk reduced by supraphysiologic doses of folate, have been performed almost exclusively in regard to cancer of the uterine cervix and colorectum. The studies in the cervix have been inconsistent (7, 8) and, although the eight randomized, intervention trials conducted on colorectal neoplasia have been very promising (reviewed in 1), the small size of the populations studied and the nature of the endpoints has precluded any definite statements regarding efficacy of supplementation.

**Folate in Nucleic Acid Metabolism.** The sole biochemical function of all of the coenzymatic forms of folate in mammalian systems appears to be mediating the transfer of one-carbon units. Within the scope of this function is the synthesis of S-adenosylmethionine (SAAdoMet), a methyl donor used widely for biological methylation reactions, and de novo deoxynucleoside triphosphate synthesis. Each of these two biosynthetic pathways is a means by which folate plays a major role in DNA metabolism. It is through disturbances in normal DNA, and possibly RNA, metabolism that folate depletion appears to produce its pro-carcinogenic effects.

Methionine is regenerated from homocysteine in a reaction catalyzed by 5-methyltetrahydrofolate(methylTHF):homocysteine methyltransferase: this is a reaction for which 5-methylTHF serves as both a cofactor and substrate (Fig. 1). An alternative mechanism for the regeneration of methionine which does not require folate also exists-the methylation of homocysteine by betaine-although the latter reaction seems to only be operative in the liver and kidney. Methionine, in turn, is converted to SAAdoMet in a reaction catalyzed by methionine adenosyl transferase. SAAdoMet then donates the labile methyl group it derived from 5-methylTHF for over 80 biological methylation reactions, including an array of reactions whereby specific sites within DNA and RNA become methylated. Although the alternative betaine pathway may partially compensate, dietary folate depletion alone is a sufficient perturbing force to diminish SAAdoMet pools (9).

The synthesis and turnover of deoxynucleoside triphosphate (dNTP) pools are tightly coupled to DNA synthesis.



**FIGURE 1** Folate in nucleic acid metabolism. THF, tetrahydrofolate; DHF, dihydrofolate; SAdoMet, S-adenosylmethionine; SAdoHcy, S-adenosylhomocysteine; 1, methyltetrahydrofolate:homocysteine methyltransferase; 2, betaine:homocysteine methyltransferase; 3, methionine adenosyl transferase; 4, methylenetetrahydrofolate reductase.

Since dNTPs 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. Folate-derived one-carbon groups are essential for the de novo synthesis of the pyrimidine, thymidylate, as well as the purines. In mammalian cells the de novo synthesis of thymidylate from deoxyuridylate is a rate-limiting step for DNA synthesis and requires 5,10-methylene tetrahydrofolate as a coenzyme.

When the dietary methyl supply is inadequate, such as in folate depletion, the use of folate coenzymes for biological methylation and nucleotide synthesis appear to compete. As SAdoMet concentrations decrease, compensatory mechanisms increase the conversion of 5,10-methyleneTHF to 5-methyl-THF, an irreversible reaction, and thereby compromise folate availability for de novo nucleotide synthesis (9).

**Candidate Mechanisms for Folate Associated Carcinogenesis.** **Altered DNA methylation.** In vertebrate genomes, approximately 4% of cytosine residues are modified postsynthetically to 5-methylcytosine (5mC). Most of these 5mC residues are found in the palindromic sequence, CpG. The cell strictly maintains its particular patterns of methylated residues, although transient changes in methylation occur within promoter sites for certain genes in conjunction with altered expression of the genes. Similarly, the pattern is precisely inherited when mitosis occurs. There is considerable evidence that aberrant DNA methylation plays an integral role in oncogenesis. First, 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 (1). This decrease in genomic methylation appears early in carcinogenesis, and appears to precede the more well described mutation and deletion events that occur later in the evolution of cancer. Genomic hypomethylation has also been observed in some animal models of carcinogenesis (10). Gene-specific hypomethylation may occur even in the absence of genomic hypomethylation and is probably a more important event in carcinogenesis since the prevailing theories of carcinogenesis emphasize damage which occurs at critical loci within DNA. Site-specific aberrancies in DNA methylation within critical genes are also observed in neoplastic tissues, and include both foci of hypomethylation and hypermethylation (11).

Somewhat surprisingly, the patterns of DNA methylation that are so religiously guarded by the cell seem to be susceptible in certain settings to perturbations created merely by altering dietary folate in both animals and man. The induction of genomic hypomethylation in human lymphocytic DNA has been demon-

strated in healthy human volunteers who were placed on a long-term folate deficient diet (12) and this effect was reversible when the deficiency was corrected. Supportive evidence is available from a recent observational study (13) where serum folate levels as well as folate concentrations in the uterine cervix were significantly correlated with genomic DNA methylation in a study of cervical intraepithelial neoplasia. Studies performed in rodents fed diets deficient in folate generally do not show any changes in genomic DNA methylation, although it does appear to be feasible with a severe deficiency state or one deficient in multiple lipotropes such as choline, methionine, vitamin B-12, and folate (10). The resistance to the induction of genomic methylation in rats may be due to the fact that they have a more active betaine pathway than humans.

The induction of site-specific hypomethylation may be more critical to the process of carcinogenesis than genomic effects. In this regard, folate depletion has been shown to induce hypomethylation of the coding region of the p53 tumor suppressor gene even in the absence of genomic hypomethylation (14). Conversely, supplemental folate has been shown to revert the hypomethylation of this region which occurs in association with chemical carcinogenesis (15). Of particular interest is the fact that this region within the p53 gene that is particularly susceptible to hypomethylation by folate depletion or chemical carcinogens (exons 5–8) is precisely that region that is most frequently mutated in human cancer.

Recent studies reveal why changes in site-specific methylation may be related to subsequent mutations at that site. 5mC is more unstable than its unmethylated counterpart. Hydrolytic deamination of 5mC leads to a G/T mismatch and subsequently, if unrepaired, to a C→T transition mutation (16). This probably explains why sites of DNA methylation are mutational hotspots in many human tumors. Paradoxically, unmethylated cytosine can also undergo deamination to yield uracil, particularly under conditions where intracellular SAdoMet is low (such as in folate deficiency) (17). We have found (14, 15) that dietary folate depletion in rodents produces diminished methylation in the so-called 'permutable region' of the p53 gene (exons 5–8); a region where 24% of reported mutations occur at C→T transitions at CpG dinucleotides. This suggests that the phenomenon of DNA methylation may contribute to these mutations.

Transcriptional repression by hypermethylation of promoter sequences has been widely discussed as an alternative means for the inactivation of tumor-suppressor genes in cancer: methylation-induced alterations in the local conformation of the gene can render it inaccessible and transcriptionally inactive and is the presumptive mechanism involved. Hypermethylation of the promoters of p16, calcitonin, and estrogen receptor genes have all been observed in neoplastic tissue (18). Early reports suggested this was due to increased DNA-methyltransferase activity in cancers, although recent reports suggest that methyltransferase activity is not truly elevated in neoplastic tissue when the data are corrected for the proliferation rate of the tissue (19). Paradoxically a lipotrope-deficient diet can induce hypermethylation at selected sites in the genome. Progressive exon-specific hypomethylation of the hepatic p53 gene was seen in animals fed a diet deficient in folate, B12, methionine and choline followed by a rebound hypermethylation at a later time when neoplastic foci became histologically evident in the liver (20). Direct evidence that isolated folate deficiency can similarly produce hypermethylation of critical tumor suppressor gene promoter regions is lacking.

**Altered RNA methylation.** Like DNA, a wide variety of RNA species are methylated at specific sites by SAdoMet-mediated reactions. In some instances, the 5'-methyl cap of RNA is methylated and in other instances, internal nucleotide

residues are methylated. Although the precise functions of RNA methylation sites are only now becoming apparent, it appears that these patterns of methylation in RNA are also judiciously guarded by the cell and serve important purposes in maintaining stability of the RNA species and facilitating transport across the nuclear membrane (21). De-methylation of tRNA was shown some years ago with a severe, methyl deficient diet (22). Only recently, however, has it been shown that folate depletion alone (at least in cell culture) is sufficient to demethylate some RNA species such as small nuclear RNA (snRNA) (23), a species which is a critical component of the machinery necessary for maturation of messenger RNA.

**Disruption of DNA integrity.** Folate deficiency induces breaks in chromosomes and such breaks are associated with an increased risk of cancer in humans. More recently, folate-deficient conditions in both cell culture and animal experiments have been shown to create an excess of breaks in the phosphodiester backbone of DNA, which is presumed to be the molecular basis for chromosomal breaks (24). There are several mechanisms by which folate deficiency might create such breaks: these include the incorporation of uracil from the cellular nucleotide pool into DNA and by *in situ* deamination of cytosine. Folate deficiency reduces thymidylate synthesis from deoxyuridylate and the ensuing nucleotide imbalance increases the misincorporation of uracil bases into DNA as most DNA polymerases do not effectively distinguish between deoxyuridylate and thymidylate. Uracil in DNA is excised by a repair glycosylase, and in the process a transient single-strand break develops in the DNA. Simultaneous removal and repair of two adjacent uracil residues on opposite strands can result in a double-strand DNA break, further exacerbating genetic instability. Unrepaired double-strand DNA breaks enhance cellular transformation in culture and increase cancer risk. 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 (25). Folate supplementation above the RDA was also observed to lessen chromosome breakage below levels observed in normal, folate-replete individuals (26).

In cell culture, folate deficient media enhanced DNA strand breaks induced by an alkylating agent and  $\gamma$ -irradiation (24) and in a recent rodent study, a folate deficient diet increased gene specific DNA strand breaks in the hypermutable region of p53 (14). Site-specific hypomethylation was also noted at this site, thereby supporting the speculation that deamination of nonmethylated C turns to uracil and removal of uracil induces strand breaks.

In those instances where cancers are enhanced by particular viruses, the phenomena of hypomethylation and strand breaks may have an additional significance. For instance, human papilloma virus 18 is widely accepted as a risk factor for human cervical neoplasia. It is incorporated into the human genome at four loci, three of which are in or near a constitutive 'fragile site' that is created by folate depletion. More recently, integrated Human Polyomavirus JCV DNA sequences have been identified in human colon cancer DNA, raising the question as to whether the virus plays an etiologic role (27). Methylation of specific sites is known to block the integration of certain viruses into the genome and strand breaks are thought to perhaps enhance integration. Whether the hypomethylation or strand break sites produced by folate deficiency might enhance the incorporation of tumorigenic viruses remains a provocative concept.

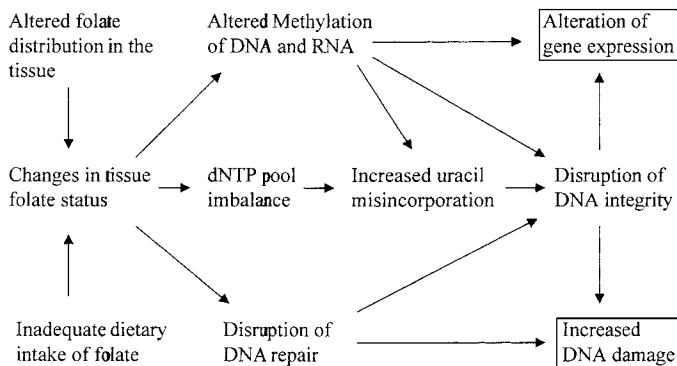
**Disruption of DNA repair.** DNA is constantly damaged by a host of endogenous and exogenous factors, and therefore sophisticated repair mechanisms are available in all cells to

eliminate such damage. As mentioned above folate deficiency induces dNTP pool imbalance and uracil misincorporation into DNA. Such misincorporation results in abnormal DNA replication and imposes greater dependence on the repair system. Cells grown in folate-deficient media show various types of chromosomal aberrations but cells grown in hypoxanthine-supplemented folate-deficient medium exhibit a significantly lower frequency of damaged mitotic figures (28). Hypoxanthine is a purine precursor which bypasses the need for folate-dependent purine biosynthesis. In another cultured cell study, folate deficiency acted synergistically with alkylating agents to increase somatic mutations and, with  $\gamma$ -irradiation, to promote DNA strand breaks, by limiting DNA repair (24). In folate deficient rodents we found that the DNA excision repair was impaired in folate deficient colonic mucosal cells compared to normal mucosal cells (29). Supplementation of the colonocytes with hypoxanthine as a purine precursor and thymidine as a pyrimidine precursor, which together preclude the need for folate dependent nucleotide synthesis, partially reversed the impaired excision repair. This suggests that folate deficiency disrupts excision repair in part by altering the cellular pool of deoxyribonucleotides. Similarly, diminished DNA repair capability of human lymphocytes was seen in a folate deficient medium (30). Since the p53 gene product is an important regulator of DNA repair and the cell cycle, folate deficiency-induced impairment of DNA repair might feasibly be mediated by its effects on the p53 gene; this is consistent with animal studies mentioned above in which folate depletion led to strand breaks within the p53 gene (14). Folate deficiency has also been observed to impair the other major cellular DNA repair system, mismatch repair, in ulcerative colitis patients (31). It was suggested that increased microsatellite instability in these patients might translate into an increased risk for mutations.

Aberrations in normal patterns of DNA methylation, which can be induced by folate depletion as mentioned above, might adversely impact on the efficacy of DNA repair systems because DNA methylation plays an important role in strand discrimination during postreplication mismatch repair. Therefore, site-selective DNA hypomethylation induced by folate deficiency might affect the methyl-directed mismatch repair. Also, methylation of CpG sites in the hMLH1 gene, one of the major mismatch repair genes, has been associated with microsatellite instability in colon cancer and stomach cancer (32).

**Methylenetetrahydrofolate Reductase Gene and Risk of Colon Cancer.** Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF. A common polymorphism of this gene (C677T) causes thermolability and reduced activity of MTHFR. Men with this homozygous mutation have half the risk of colorectal cancer than do homozygous wild-type or heterozygous genotypes (33). Among men with adequate folate levels, a three-fold decrease in risk was observed, but protection associated with the mutation was largely absent in men with low systemic folate status. It was suggested that the cancer protective effect of the MTHFR mutation was related to increased availability of 5,10-methyleneTHF, and therefore increased ease of nucleotide synthesis. One might speculate that, under low but not high folate conditions, availability of 5-methylTHF for biological methylation constitutes a more critical determinant of whether the cell is pushed down the pathway towards neoplasia.

Recently Bagley and Selhub (34) found that human subjects possessing the homozygous polymorphism have formylated forms of tetrahydrofolate in their red cells; this compares



**FIGURE 2** Molecular effects of folate depletion. 'Alterations of gene expression' and 'Increased DNA damage' are enclosed in boxes to emphasize the concept that these are the two pathways through which carcinogenesis is enhanced.

with wild type individuals, whose cells contain only methyl-THF. In a preliminary study, we have also found that lymphocytic DNA from subjects with the MTHFR polymorphism is significantly less methylated than DNA from wild type subjects (35). These observations suggest that the protective effect of the polymorphism may be conveyed by an alteration in the forms of folate contained within the cell, and it explains how the protective effect might be operable even when total folate levels are normal.

Figure 2 summarizes the molecular effects of folate depletion that are described in this article and provides a framework of how these phenomena are interrelated. Although it is an oversimplification, increased DNA damage without a compensatory increase in DNA repair and alterations in gene expression are generally agreed upon to be major pathways towards cancer. The premise of this unified scheme, therefore, is that all mechanisms described in this paper conspire to enhance carcinogenesis by either increasing net DNA damage and/or altering the expression of critical genes. At this point in time, the schema presented in Figure 2 has not been conclusively proven to constitute the means by which folate depletion enhances cancer; nevertheless, considerable work is presently underway that should give us a more definitive answer in the near future.

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