

CHEMOPREVENTIVE ROLE OF FOLIC ACID IN COLORECTAL CANCER

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1. ABSTRACT

Mortality from colorectal cancer, a leading cause of death in the U.S.A. and other western countries, has remained unchanged over the past 45 years. Therefore, the search for strategies to prevent the development and progression of colorectal cancer has markedly intensified. Chemoprevention is one such strategy. Accumulating evidence suggests that folic acid, a water soluble vitamin, could be an effective chemopreventive agent for colorectal cancer. Results from several studies have demonstrated that a diet deficient in folic acid may be associated with an increased risk of colonic neoplasia, whereas dietary supplementation of this nutrient may be chemopreventive. Although the mechanisms by which folic acid exerts its chemopreventive role in colorectal carcinogenesis remain to be fully elucidated, supplemental folic acid has been shown to arrest the loss of heterozygosity (LOH) of the tumor suppressor gene DCC (deleted in colorectal cancer) and to stabilize its protein in normal appearing rectal mucosa of patients with colorectal adenomas. Data from *in vitro* studies utilizing colon cancer cell lines suggest that supplemental folic acid or its metabolite 5-methyltetrahydrofolate (5-MTF) attenuates the expression and activation of EGF-receptor (EGFR) as well as proliferation of cells. The folic acid mediated reduction of EGFR function could partly be the result of suppression of EGFR gene through increased methylation of CpG sequences within its promoter.

2. CHEMICAL STRUCTURE AND SOURCES

Folic acid is water soluble B vitamin, composed of a pterin ring connected to p-aminobenzoic acid (PABA) and conjugated with one or more glutamate residues (Figure 1). Humans do not generate folate endogenously because they cannot synthesize PABA, nor can they conjugate the first glutamate. It is distributed widely in green leafy vegetables, beans, whole grains, citrus fruits, and animal products.

3. DISTRIBUTION AND METABOLISM

Folates are present in natural foods and tissues as polyglutamates. In plasma and urine, they are found as

monoglutamates, so that they can be transported across membranes. Enzymes in the lumen of the small intestine convert the polyglutamate to monoglutamate that is absorbed in the proximal jejunum via both active and passive transport. Within the plasma, folate is present, mostly in the 5-methyltetrahydrofolate (5-methyl THFA) form, and is loosely associated with plasma albumin in circulation. The 5-methyl THFA enters the cell via a diverse range of folate transporters. Once inside, 5-methyl THFA is demethylated to THFA, which is derived by the 2-step reduction of folate involving dihydrofolate reductase. THFA is the biologically active form participating in folate-dependent enzymatic reactions.

THFA plays a key role in the transfer of 1-carbon units (such as methyl, methylene, and formyl groups) to the essential substrates involved in the synthesis of DNA, RNA, and proteins. THFA is involved in the enzymatic reactions necessary for the synthesis of purine, thymidine, and amino acids (Figure 2). The most important carrier of methyl groups is S-adenosylmethionine (SAM). Methylation of DNA and RNA requires SAM as a source of methyl groups. The degree of methylation correlates with transcriptional activity. Cobalamin and folate metabolism are intricately related, and abnormalities in these pathways are believed to lead to the attenuated production of DNA. Manifestations of folate deficiency thus involve impairment of cell division, accumulation of possibly toxic metabolites such as homocysteine, and impairment of methylation reactions involved in the regulation of gene expression, thus increasing neoplastic risks, as discussed later.

A healthy individual has about 500-20,000 μg of folate in body stores with 50% concentrated in the liver. Humans need to absorb approximately 50-100 μg of folate per day in order to replenish the daily degradation and loss through urine and bile. Signs and symptoms of deficiency can manifest after 4 months. The RBC folate level indicates folate stored in the body, whereas the serum folate level reflects acute changes in folate intake. Data from the Second National Health and Nutrition Examination Survey indicate that 10% of the US population may have low folate stores. Folic acid deficiency is one of the most common

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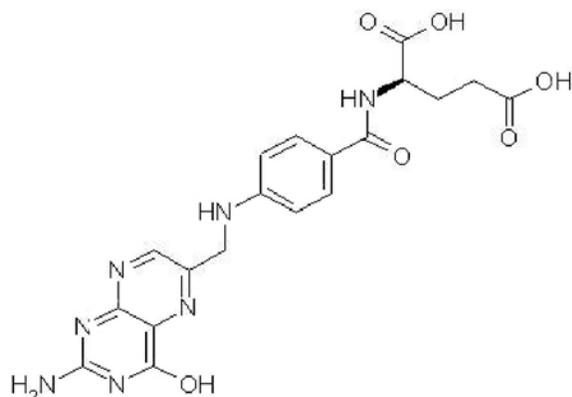


Figure 1. The Chemical Structure of Folic Acid.

vitamin deficiencies in the United States, due to its association with excessive alcohol intake (5% of US population) as well as pregnancy. The significance of folic acid deficiency is compounded by the following attributes:

- An association of folate deficiency with elevated homocysteine, leading to increased risk of arteriosclerosis
- Increased incidence of neural tube defects has been noted with folate deficiency.
- Increased risk of colorectal cancer has been noted with folate deficiency.

Hence, folic acid clearly has a profound impact on public health in the United States, especially since heart disease and cancer constitute the number 1 and number 2 causes of mortality in this country.

4. CHEMOPREVENTION WITH FOLIC ACID

Although the epidemiology of colorectal cancer is related to genetic susceptibility, dietary factors such as vitamins and micronutrients are thought to influence tumorigenic processes (1). Considerable interest has recently been focused on the water-soluble vitamin folic acid. Folic acid is an essential factor in the generation of S-adenosylmethionine, the critical methyl donor for DNA methylation. Although the specific mechanism(s) by which inadequate folate availability might enhance colorectal carcinogenesis have not been fully elucidated, it was hypothesized that aberrations in DNA methylation might certainly contribute to abnormalities in DNA synthesis and genomic instability.

Several large case-controlled studies have noted an inverse relationship between dietary folic acid and the development of colorectal cancer (2- 5). Results from several studies have demonstrated that a diet deficient in folic acid may be associated with an increased risk of colonic neoplasia (1, 6, 7), whereas dietary supplementation of this nutrient may be chemopreventive (4, 8, 9). Initial epidemiological studies in patients with chronic ulcerative colitis (8, 10), known to be associated with an increased risk of colonic dysplasia/carcinoma, demonstrated that folate supplementation was associated with a 62% lower incidence and decreased relative risk for the development

of colorectal neoplasia. Folate deficiency in ulcerative colitis may be induced by reduced intestinal absorption from competitive inhibition by sulfasalazine, intestinal losses related to disease activity and reduced oral intake. Reduced RBC folate or failure to supplement ulcerative colitis patients with folate is associated with the development of dysplasia and cancer (4, 10).

Several studies performed in patients with benign colorectal adenomas, known to be the precursors of colorectal carcinomas, have also suggested a role for folic acid in preventing colorectal cancer. Meenan *et al.* (11) have measured folate levels in adenomas, carcinomas and adjacent normal appearing mucosa noting that levels were lower in adenoma and carcinoma compared to adjacent normal mucosa. Another study (7) noted that the mean RBC folate levels were significantly lower in patients with adenomas compared to healthy controls.

A recent study by Pufulete *et al.* (12) compared differences in DNA methylation and folate status between patients with adenomas or colorectal cancer with healthy controls. Cancer patients were found to have a 26% lower folate status, a score derived from serum and dietary intake levels, compared to controls. High folate status was associated with a decreased risk of cancer. Finally, colonic and leukocyte DNA hypomethylation were associated with an increased risk for adenoma. It was further hypothesized that an inadequate supply of folate may increase the risk of neoplasia by inducing DNA hypomethylation which may alter DNA stability and the subsequent aberrant expression of proto-oncogenes and tumor suppressor genes involved in colon carcinogenesis. In an elegant study by van Engeland *et al.* (13), the effect of folate intake on the promoter methylation of several genes reported to be involved and methylated in colon carcinogenesis were examined. The prevalence of promoter hypermethylation was higher in colorectal cancers from patients with low folate intake, although the difference did not achieve statistical significance. Furthermore, the number of colorectal cancers with at least one gene methylated was higher in the low folate intake group compared to those with high intake. We recently determined that supplemental folic acid prevents loss of heterozygosity (LOH) and stabilization of colonic mucosal protein levels of the deleted in colon cancer (DCC) gene in patients with a history of colorectal adenomas (14).

Accumulated data from murine studies have also supported a role for folic acid in the prevention of colon carcinogenesis. Folate deficient rats demonstrate an increased susceptibility to dimethylhydrazine induced colonic neoplasia when compared to folate replete animals (15). In a similar model, folate supplementation protected against the development of macroscopic colonic neoplastic lesions in a dose dependent manner (16). We have previously demonstrated that folic acid supplementation can reduce the age related susceptibility of murine colorectal mucosa to carcinogenic stimulation as reflected by indices of proliferation which are essential to the carcinogenic process (17).

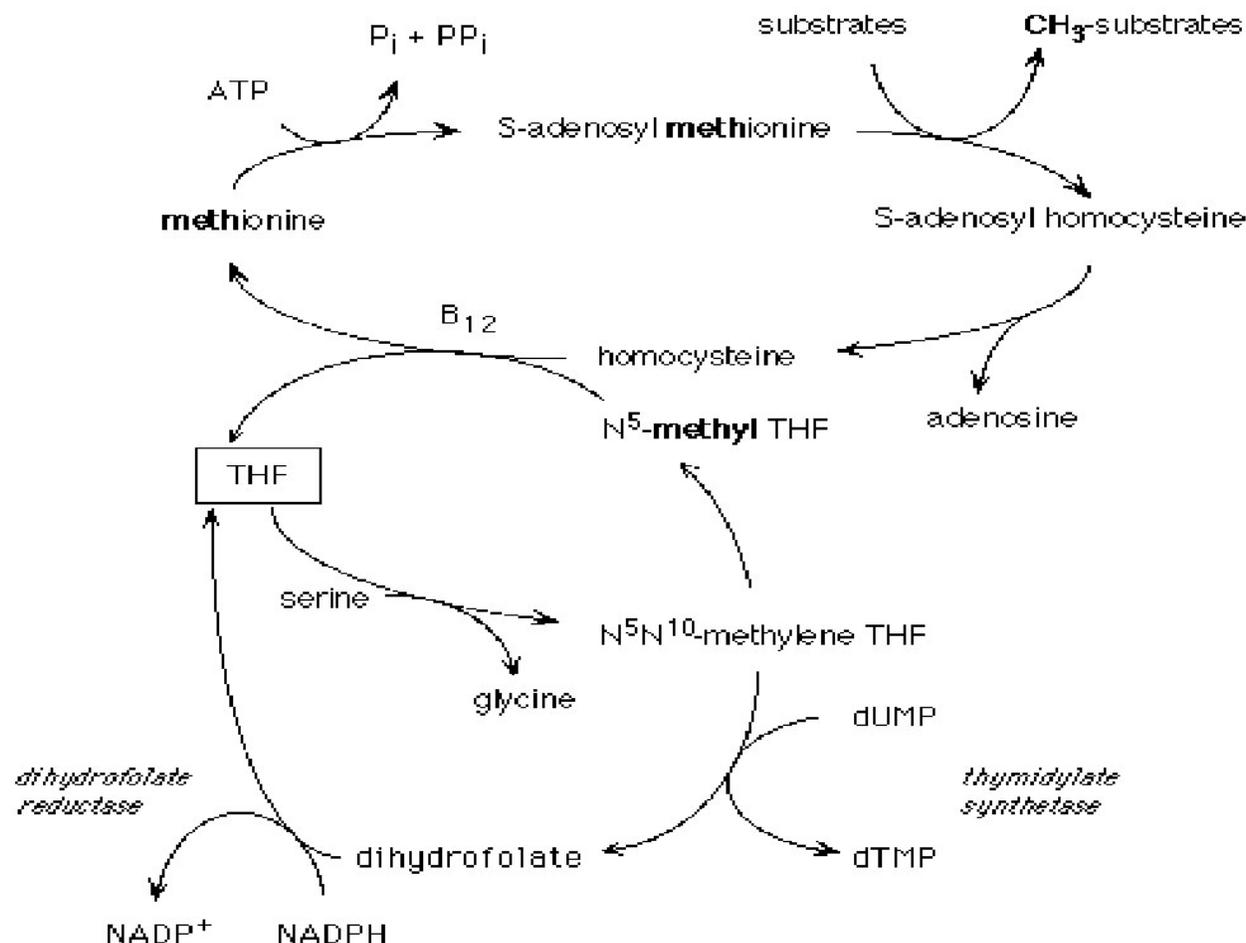


Figure 2. Overview of key reactions involving one-carbon transfer.

Recently developed genetic murine models which spontaneously develop small intestinal and colonic tumors have provided an opportunity to examine the effects of environmental and nutritional factors on colorectal carcinogenesis (18, 19). Kim *et al.* (18, 19) studied the effect of folic acid in the Min mouse model which carries a heterozygous germ-line mutation of the Apc gene which resembles the human syndrome of familial adenomatous polyposis coli. One study (18) demonstrated that folate supplementation significantly decreases the number of small intestinal and colonic adenomas if begun before the establishment of neoplastic foci. A subsequent investigation (19) noted that increasing dietary folate levels significantly reduced the number of ileal polyps in a dose-dependent manner. Furthermore, the number of ileal polyps was inversely correlated with serum folate concentrations. Finally, increasing dietary folate levels significantly decreased the number of colonic aberrant crypt foci (ACF) which are believed to be the precursors of colorectal adenomas (20).

5. MECHANISMS OF ACTION

The mechanisms by which folic acid exerts its chemopreventive role in colorectal carcinogenesis are

poorly understood. Carcinogenesis, which is a multi-step process, results from the accumulation of mutations during progression from normal epithelium to carcinoma (21, 22). It is becoming increasingly apparent that tumor suppressor genes play a key role in the development and progression of carcinogenesis since the products of these genes normally function to regulate cell growth and differentiation and their loss of function contributes to the neoplastic phenotype (23). The mechanisms of gene inactivation include allelic deletion (loss of heterozygosity or LOH), chromosomal rearrangement, point mutation and inactivation of suppressor gene products by viral or cellular inactivation agents. Genetic changes that occur at different stages of epithelial cell carcinoma have been extensively studied by Vogelstein and his colleagues in human colon cancer (21, 22). At least for colon cancer, it has been suggested that the loss or inactivation of the tumor-suppressor gene APC initiates genomic instability that may produce the phenotypic appearance of an adenoma. The advanced tumors, however, possess mutations and/or deletion of a number oncogenes and tumor-suppressor genes not seen in the early adenoma (22, 24, 25). For example, mutations of *ras* and *p53* and deletion of *DCC* are thought to be involved in cellular adhesion and metastasis. Multiple allelic mutations may also occur in

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other chromosomes. In brief, numerous genetic alterations are necessary for the development of colorectal cancer (22, 24). Inactivation of a number of tumor suppressor genes, including APC (adenomatous polyposis coli), DCC (deleted in colorectal cancer) and p53 has been detected in the development and progression of colorectal cancer (21, 22, 25, 26). Supplemental folic acid has been shown to be protective of mutations involving G→T transversions, which have been related to poor prognosis and a high risk of recurrence in colorectal carcinomas (27). However, it remains to be determined whether mutational status of these and other tumor suppressor genes in the colorectal mucosa might be affected by supplemental folic acid. In a recent study, we examined the changes in mutational status of APC, DCC and p53 genes in macroscopically normal appearing rectal mucosa at the beginning (baseline; i.e. before treatment) and 1 year after treatment with either supplemental folic acid or placebo tablets (14). We have observed that folate supplementation prevented the LOH of DCC gene in 5 out of 5 (100%) patients who demonstrated baseline heterozygosity, whereas 2 out of 4 (50%) placebo-treated patients with baseline heterozygosity demonstrated complete allelic loss (14). Mucosal protein levels of DCC were also reduced in 7 of 10 (70%) placebo treated patients compared to only 2 of 10 (20%) of patients treated with folate. Levels increased, however, in 8 and 3 patients in the folic acid and placebo groups, respectively ($p < 0.02$). Since reduced expression of DCC is thought to play an important role in malignant transformation (28), our observation of a complete loss of one allele in DCC gene in 2 out of 4 placebo-treated subjects together with decreased expression of DCC protein in these and other placebo-treated subjects raises the possibility that they may be vulnerable to malignant transformation.

Lack of folic acid has been shown to cause misincorporation of uracil. This raises the possibility that supplemental folic acid may prevent uracil misincorporation into DNA which is associated with increased chromosome breakage, a risk factor for cancer (29, 30). Another possibility could be due to regional differences in the methylation status of the DCC gene. For example, Sato *et al.* (31) noted discrepancies between methylation status and DCC expression in primary gastric cancer which may have been related to differences in methylation status between the heart of promoter CpG islands and the region which they examined. It has been suggested that a small methylated region of *hMLHI* could itself block expression (32). Although we did not specifically explore the methylation status of *hMLHI* gene, we found no differences in the incidence of mutations in *hMLHI* gene between the placebo and folic acid-treated groups (14).

In contrast to what has been observed for DCC, no LOH was observed for either APC or p53 gene in any of the subjects. This observation suggests that the induction of colorectal adenoma(s) in these subjects was not the result of LOH of either APC or p53 gene (14). However, since LOH of APC and p53 was assessed by studying RFLP in exon 11 and codon 72, respectively, the possibility of other areas of these genes being

affected in the development of colonic adenomas cannot be totally disregarded.

Although in familial cancers genetic instability plays a dominant role, in a vast majority of sporadic cancers hyperproliferation is likely to play a permissive role in initiating the progression of the disease. Proliferative activity in macroscopically normal colonic mucosa also increases in premalignant lesions. In the azoxymethane (AOM) model of colorectal cancer in rats, a single injection of the carcinogen induces a prompt rise in DNA synthesis and ornithine decarboxylase (ODC) activity which is sustained for much of the latent period preceding macroscopic tumor formation (33) [ODC is the rate limiting enzyme in the biosynthesis of polyamines, which are believed to be intracellular mediators of cell proliferation and differentiation (34-37)]. Similar increases in ODC activity and DNA synthesis have been observed in normal appearing colonic mucosa from subjects with colon cancer (38, 39). Increased colonic mucosal proliferative activity has also been observed in numerous premalignant lesions, including familial polyposis (40-42), sporadic adenomas (43-45) and ulcerative colitis (46-48). In addition, animal experiments have demonstrated that aging, which is associated with an increased incidence of colorectal neoplasia, is accompanied by a rise in colonic mucosal proliferative activity and decreased apoptosis (49-52). Recent *in vitro* studies from this and other laboratories have further demonstrated that supplemental folic acid greatly inhibits proliferation of colon cancer cell lines (53, 54). We have further observed that in polypectomized patients, supplemental folic acid for one year decreases colonic mucosal proliferative activity (14). Although the regulatory mechanisms for folic acid mediated inhibition of mucosal proliferation are not fully understood, we hypothesize that tyrosine kinases, which are associated with a number of growth factor receptors and the products of many protooncogenes (55-57), play a role in regulating this process. A positive relationship between hyperproliferative state and tyrosine kinase activity and tyrosine phosphorylation has been demonstrated in various precancerous lesions in the gastrointestinal tract. In humans, this relationship was seen in ulcerative colitis (58, 59) and in the rectal mucosa of patients harboring adenomatous polyps (60, 61). In rats, induction of colonic mucosal proliferative activity by AOM (azoxymethane) or its active metabolite MAOM (methylazoxymethanol) has also been shown to be accompanied by an increase in tyrosine kinase activity and tyrosine phosphorylation of several membrane proteins (62).

Since tyrosine kinases are associated with receptors of a number of growth factors and products of many proto-oncogenes (55-57), studies have also been performed to assess the role of different tyrosine kinases in various diseases that represent tissue growth. The type 1 receptor tyrosine kinases constitute a family of transmembrane proteins involved in various aspects of cell growth, survival and differentiation (63). The family includes the EGFR, ErbB-2, ErbB-3 and ErbB-4. EGFR and ErbB-2 have been investigated as potential targets for cancer therapy because of their preponderance in a variety of neoplastic

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tissues (64). Overexpression of EGFR has been implicated in development or progression of many malignancies, including colorectal cancer (65, 66). In particular, EGFR overexpression correlates with high metastatic rate, short survival time (67), and poor prognosis (68) for patients with squamous cell carcinomas of the lung. Increased expression of EGFR or its activating ligands, EGF and TGF- α , correlates with the recurrence or poor prognosis in bladder, pancreatic and colorectal cancer (65). In addition, ErbB-2 expression also correlates with poor prognosis in cancers of the colon, ovary and bladder (69, 70). We have demonstrated that exposure of colon cancer cell lines, HCT-116 and Caco-2 to supra-physiological concentrations of folic acid, that causes a marked reduction in proliferation, is accompanied by a parallel inhibition in EGFR activation (53). Expression of EGFR in both Caco-2 and HCT-116 cells has also been shown to be decreased in response to supplemental folic acid (53).

Although the regulatory mechanisms for folic acid induced inhibition of EGFR expression and activation are not fully understood, the possibility that supplemental folic acid may suppress EGFR expression through hypermethylation cannot be disregarded. Folic acid is the primary methyl donor for DNA methylation, and for producing the purines and pyrimidines required for DNA synthesis. Lack of folate or methyl group nutrients in the diet has been shown to cause DNA hypomethylation in both rats and humans, whereas an opposite phenomenon is noted with excess folic acid (71-73). It is becoming increasingly evident that gene hypermethylation, particularly within the promoter, can result in tissue-specific gene silencing. It has been demonstrated that methylation of CpG sequences within promoters can inhibit binding of transcription factors, which is thought to be one of the mechanisms for gene inactivation in neoplastic cells and tissue-specific gene expression (74-77). In some cancers, CpG islands in the 5' regions of tumor suppressor genes are methylated, and their expression are switched-off. In view of this, we examined whether and to what extent supplemental folic acid or its metabolites 5-methyltetrahydrofolate (5-MTF), dihydrofolate (DF) or tetrahydrofolate (TF) will modulate the basal and serum-induced activation of the EGFR promoter in HCT-116 colon cancer cell line (78). We observed that exposure of the cells to 10% FBS caused a marked stimulation of EGFR promoter activity and its expression; both of which were greatly abrogated by supplemental folic acid and 5-MTF. In contrast, the serum-induced activation of *c-fos* promoter activity was unaffected by 5-MTF. The 5-MTF-induced inhibition of the serum-mediated stimulation of EGFR promoter activity and EGFR expression was reversed when methylation was inhibited by 5-aza-2'-deoxycytidine. Our data suggest that folate and its metabolite 5-MTF inhibit EGFR promoter activity in colon cancer cells by enhancing methylation (78). This could partly be responsible for folic acid mediated inhibition of growth related processes in colorectal neoplasia (78).

6. ACKNOWLEDGEMENTS

The work presented in this communication was

supported by grants to Dr. Majumdar from the National Institutes of Health/National Institute on Aging (AG 14343) and the Department of Veterans Affairs.

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Key Words: Folate, Colon Cancer, Cell Proliferation, EGF Receptor, Hypermethylation, Review

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