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Review

Folate and carcinogenesis: Evidence, mechanisms, and implications

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Introduction

Folate, a water-soluble B vitamin, has recently garnered much attention because of its purported role in the pathogenesis of cardiovascular disease,[1–4] neural tube defects,[5–7] and cancer of certain organs (reviewed in references 8–12). Although the appreciation of the role of folate in carcinogenesis is a recent development, several previous observations have suggested a possible relationship between folate deficiency and the development of cancer. Cytopathologists in the 1950s and 1960s noticed cytologic similarities between exfoliated macrocytic gastric and cervical epithelial cells in individuals with folate and/or vitamin B_{12} deficiency and dysplastic cells.[13,14] Some of these macrocytic epithelial cells persisted even after the correction of folate and/or vitamin B_{12} deficiency.[13] Because the cytologic appearance of these cells had some features of malignancy, it was postulated that these abnormal cells might be a transitional cell type between normal and neoplastic epithelium.[13,15] However, a functional similarity between megaloblastic and neoplastic epithelial cells was not fully appreciated until recently.

The concept that folate deficiency would predispose epithelial cells to a neoplastic transformation appears somewhat contradictory at first glance. Folate is an important factor for a number of metabolic pathways in the cell that involve the transfer of one-carbon groups.[16] Among such pathways are the biosyntheses of purines and thymidylates.[16] It is in this manner that folate plays a key role in DNA replication and cell division.[16] Consequently, a deficiency of folate in tissues with rapidly replicating cells results in ineffective DNA synthesis, resulting in reduced cell proliferation, impaired cellular physiology, and abnormal cytologic morphology. This biochemical function of folate has been utilized in the area of how folate modulates cell proliferation in the process of carcinogenesis. Intuitively, interruption of folate metabolism in neoplastic cells would be expected to lead to ineffective DNA synthesis, resulting in the inhibition of tumor growth. Indeed, this has been the basis for antitumor therapy utilizing a number of antifolate agents, including methotrexate and 5-fluorouracil.[17,18] Clinically, it has been observed that folate treatment of children with acute leukemia results in an "acceleration phenomenon" of the cancer.[19] Experimentally, it has been shown that growth of a transplanted cancer is inhibited in folate-deficient rats,[20] that folate deprivation reduces virally induced cancers,[21] and that the time required for developing neoplasms in transgenic mice, which are predisposed to developing nerve sheath tumors similar to human neurofibromatosis, is significantly delayed by restricting the level of folate in the diet.[22]

Although it is well established that folic acid deficiency will retard the growth of, and occasionally kill, established cancers, it does not necessarily follow that nonneoplastic folate-deficient tissues are less likely to undergo neoplastic transformation. Conversely, a growing body of epidemiologic and clinical evidence suggests that, regardless of the systemic folate status, folate deficiency in certain epithelial tissues may be a factor that predisposes to the development of neoplasms arising from these tissues.[23]

Establishing a cause-and-effect relationship between a dietary factor and cancer is a difficult task. In some cases, correlation studies, which examine the relationship between

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the per capita consumption of a dietary factor and the prevalence or incidence of disease in a population, have provided provocative evidence that a particular factor plays a role in the development of cancer. Case-control studies compare prior use of a putative risk factor between subjects with cancer and matched controls without cancer. Although inherent problems associated with retrospective analyses often limit the interpretation of results from case-control studies, valuable information can be gathered from well-designed case-control studies in a time- and cost-effective manner. The most serious limitation in retrospective studies regarding dietary factors is the accuracy with which intakes of dietary factors or supplementation can be established. A stronger epidemiologic design is the cohort study, in which subjects who have been exposed to a particular agent are followed over time and their cancer incidence or mortality is compared with that of those who have not been exposed. This approach is costly and time consuming, and in the case of a less common disease, requires large numbers of study subjects. The association observed for folate in many of the epidemiologic studies is potentially confounded by other micronutrients in the same foods. Studies utilizing laboratory animals provide greater control of variables, enable interventions to be used that would not be feasible in humans, and are often less expensive than human trials. However, they suffer from their inherent differences from human cancer, which precludes any direct extrapolation of observations from these studies to humans. In theory, randomized intervention studies in humans should provide definitive support for the purported cause-and-effect relationship between a dietary factor and cancer. However, intervention studies are often exceedingly difficult to carry out because of the slowly progressive nature of neoplastic transformation and the large number of subjects necessary to achieve an adequate statistical power. However, several strategies have been developed to circumvent these problems. One is to study those individuals at high risk of developing cancer to determine whether a certain nutritional factor can suppress or prevent the development of cancer. The second strategy is to use so-called intermediate biomarkers as the end point instead of using occurrence or recurrence of cancer. Intermediate biomarkers include alterations in mucosal proliferation indices, aberrant histopathologic features, abnormal immunologic and biochemical markers, and more recently, alterations of several molecular biologic markers. Employing biomarkers in intervention trials of cancer can significantly reduce the number of subjects needed for recruitment as well as the duration of the study, thereby offering cost- and time-effective advantages over using cancer as the end point. However, all intermediate biomarkers have limitations and most have not been validated in clinical studies; therefore, the interpretation of studies utilizing such markers must be done in an intelligent and insightful manner. Furthermore, modulating these intermediate biomarkers have not yet lead to a reduction in cancer incidence and mortality.

The objective of this review is to provide a comprehensive review of epidemiologic, experimental, and clinical evidence that implicates diminished folate status as a factor in the development of cancer. This review also will highlight some of the proposed mechanisms by which folate deficiency can enhance, and supplementation can protect against, the development of cancer. More speculative, although more provocative, are data indicating that supplementation with supraphysiologic quantities of folate may have a chemopreventive role. The main focus of this review will be on the role of folate in the development of colorectal cancer because the most compelling epidemiologic and clinical evidence exists for this cancer.

Evidence

Lung

It has been observed that smokers have lower plasma/serum and red blood cell (RBC) folate concentrations than nonsmokers.[24–31] Furthermore, smokers who have bronchial metaplasia, a change that frequently precedes the development of bronchiogenic carcinoma, have been found to have lower plasma and RBC folate concentrations than smokers who do not have metaplasia.[28] Smokers also have been observed to have significantly lower folate concentrations in the buccal mucosa than nonsmokers.[29–31] This observation suggests that repeated exposure to cigarette smoke might result in folate depletion in the respiratory epithelium, making the cells more susceptible to the carcinogens in tobacco smoke.[29–31] Some of these studies have demonstrated no significant difference in dietary and/or supplemental folate intake between smokers and nonsmokers,[29–31] thereby ruling out poor dietary intake or habits that are often observed in smokers[32–34] as the cause of apparent differences in systemic and buccal mucosal folate concentrations between smokers and nonsmokers. Chemical components of cigarette smoke, primarily organic nitrites, cyanates, and isocyanates, appear to convert some forms of folate into biologically inactive compounds.[35,36] Although smokers have been shown to have a greater prevalence of chromosomal fragility and breakage in whole-blood cultures[26] and buccal mucosal cells[31] than nonsmokers, it is not clear whether the effect of smoking on chromosomal aberrations is mediated through altered folate status.

Two epidemiologic studies that examined the relationship between dietary folate intake and the risk of lung cancer have produced conflicting results.[37,38] In a case-control study from Hawaii involving 332 cases of lung cancer and 865 controls, no significant association was found between dietary folate intake and the risk of developing lung cancer.[37] However, a recently published prospective study involving 48,000 participants of the New York State Cohort (525 cases of lung cancer), who were followed for 7 years, showed an inverse relationship between dietary folate intake and lung cancer risk in men, but not in women, after adjusting for age, education, cigarettes per day, years smoking, and total energy intake.[38] The apparent protective effect of folate was the strongest in heavy smokers and appeared to be limited to squamous cell carcinoma.[38]

Two randomized folate intervention studies for chemoprevention of lung cancer have been published (*Table 1*). In a double-blind randomized trial using folate and vitamin B_{12} supplements in a group of 73 smokers with bronchial metaplasia detected in sputum samples, the supplemented

Table 1 Intervention trials of folate and lung cancer

Study	Study design	No. subjects	Diagnosis	Folate dose	Duration	Primary end point	End point modification
Heimburger et al.[39]	Randomized, double-blind, placebo-controlled	73 smokers	Bronchial metaplasia	10 mg folate/d + 500 μg B_{12}/d vs. placebo	4 months	Bronchial metaplasia	Significant improvement
Saito et al.[40]	Randomized, double-blind placebo-controlled	38 smokers	Bronchial metaplasia	10–20 mg folate/d + 750 μg B_{12}/d vs. placebo	1 year	Bronchial metaplasia	Significant improvement

group (folate 10 mg/d and vitamin B_{12} 500 μg/d) showed significantly greater reversal of metaplasia than the placebo group after 4 months of intervention.[39] In another randomized study from Japan, 38 subjects with bronchial squamous metaplasia diagnosed on bronchoscopy and sputum samples were randomized to receive either folate (10–20 mg/d) and vitamin B_{12} (750 μg/d) or placebo for 1 year.[40] Folate and vitamin B_{12} supplementation significantly enhanced the regression of bronchial metaplasia compared with controls at follow-up bronchoscopy.[40]

In summary, although a small case-control study showed no significant association between dietary folate intake and the risk of lung cancer,[37] a more recent prospective study with a large number of cohorts demonstrated a significant protective effect of dietary folate on the development of squamous cell carcinoma of the lungs in male heavy smokers.[38] Furthermore, two small randomized folate chemoprevention studies have shown that folate supplementation in conjunction with vitamin B_{12} can reverse bronchial metaplasia, a purported precursor of bronchial squamous cell carcinoma, in smokers.[39,40] More epidemiologic studies and intervention trials using large numbers of subjects are warranted to confirm the modulatory effect of folate in the development of lung cancer.

Uterine cervix

Folate deficiency as a potential risk factor for cervical cancer was first suggested in 1960s by cytopathologists who observed several cytologic similarities between epithelial cells of the uterine cervix from folate-deficient women and dysplastic cervical cells.[14,41] Subsequently, decreased blood folate concentrations and megaloblastic anemia in women receiving oral contraceptives, a purported risk factor for cervical carcinogenesis,[42,43] were demonstrated in most,[44–46] but not all,[47] studies. Oral contraceptives decrease folate absorption by interfering with intestinal conjugase, which is necessary to deconjugate folylpolyglutamates to folylmonoglutamates, the only form of folate that is transported across the cell membrane.[46] In addition, oral contraceptives increase activities of several folate-metabolizing enzymes.[48] In 1973, Whitehead and colleagues[48] presented evidence that megaloblastic changes can occur in cervical smears of oral contraceptive users even in the absence of anemia and without clinically evident folate deficiency. These investigators, therefore, introduced the concept of localized folate deficiency occurring in target tissue as a result of steroid hormone stimulation and demonstrated that megaloblastic cervical cytology became normal after a few weeks of oral folate supplementation.[48]

Table 2 summarizes eight case-control studies that have examined the relationship between dietary folate intake and the risk of cervical dysplasia [or cervical intraepithelial neoplasia (CIN)],[49–51] carcinoma in situ,[52,53] and invasive cancer.[54–56] The risk of developing cervical dysplasia is reduced by 60 to 70% in individuals with the highest intake of dietary folate compared with those with the lowest

Table 2 Dietary folate intake and cervical neoplasia

Study (year)	Study design	No. cases/controls	Case diagnosis	Odds ratio*	95% CI	*P*-trend for inverse association
McPherson[49] (1989)	Case-control	75/84	Dysplasia	0.34	NA	NA
Buckley et al.[50] (1992)	Case-control	42/58	CIN I, II, III	0.30	0.17–0.53	NA
Van Eenwyk et al.[51] (1992)	Case-control	100/100	CIN I, II, III	0.40	0.10–1.10	NA
Brock et al.[52] (1988)	Case-control	117/196	Cancer in situ	1.30	0.30–5.80	NS
Ziegler et al.[53] (1991)	Case-Control	228/498	Cancer in situ	Dietary source: 0.73	0.37–1.43	NS
				Supplemental: NA		0.03
Verreault et al.[54] (1989)	Case-control	189/227	Invasive cancer	Dietary source: 0.80	0.30–1.70	NS
				Supplemental: NA		NS
Ziegler et al.[55] (1990)	Case-control	271/502	Invasive cancer	Dietary source: 1.18	0.67–2.00	NS
				Supplemental: 0.74	NA	NS
Herrero et al.[56] (1991)	Case-control	748/1,411	Invasive cancer	0.95	0.70–1.30	NA

*Odds ratio for the highest dietary folate intake compared with the lowest intake. Adjusted for confounding factors.
CI–confidence interval. NA–not available. CIN–cervial intraepithelial neoplasia. NS–not significant.

Table 3 Blood measurements of folate status and cervical neoplasia

Study (year)	Study design	No. cases/controls	Case diagnosis	Folate measurements	Odds ratio*	95% CI
Van Eenwyk et al.[51] (1992)	Case-control	98/98	CIN I, II, III	Serum	0.30	0.10–1.10
				RBC	0.10	0.00–0.40
Butterworth et al.[57] (1992)	Case-control	294/170	CIN I, II, III	RBC	0.71	0.40–1.30
Orr et al.[58] (1985)	Case-control	78/240	Invasive cancer	Serum	Inverse association with stages of cancer	
Potischman et al.[59] (1991)	Case-control	330/565	Invasive cancer	Serum	1.05	0.70–1.60

*Odds ratios for the highest folate level compared with the lowest level. Adjusted for confounding factors.
CI–confidence interval. CIN–cervical intraepithelial neoplasia. RBC–red blood cell.

intake.[49–51] However, dietary folate intake appears to have little effect on the development of more advanced cervical neoplasia (i.e., carcinoma in situ and invasive cancer).[52–56]

Four case-control studies have also been conducted to investigate the relationship between blood levels of folate and the risk of cervical neoplasia.[51,57–59] As summarized in *Table 3*, two studies have found that RBC, but not serum, folate concentrations are inversely correlated with the risk of developing cervical dysplasia.[51,57] With respect to invasive cervical cancer, one study has found that serum folate levels were inversely associated with advanced stages of cancer,[58] whereas the other study showed no association between serum folate levels and the risk of invasive cancer.[59] A large, recently published case-control study demonstrated a positive interaction between a modest diminution in RBC folate concentrations and human papilloma virus-16 (HPV-16) infection, a well established risk factor for cervical carcinogenesis,[60,61] in the development of cervical dysplasia.[57] Despite the lack of a statistically significant association between RBC folate and cervical dysplasia, there was a greatly increased risk of cervical dysplasia in women with HPV-16 and an RBC folate of 660 nmol/L or less compared with women without HPV-16 whose RBC folate was greater than 660 nmol/L [odds ratio (OR) = 5.1, 95% confidence interval (CI) 2.3–11.1].[57] This study underscores two important issues regarding diminished folate status and cervical carcinogenesis. First, folate deficiency alone is not associated with an increased risk of cervical dysplasia but rather, it appears to potentiate the effect of other risk factors (e.g., genital HPV-16 infection) for cervical dysplasia. Second, the threshold below which folate status in this study was defined as being diminished was an RBC folate concentration of less than 660 nmol/L (291 ng/mL). This value is well within the range conventionally accepted as normal, suggesting that a modest reduction in folate status is all that is necessary for the enhancement of cervical carcinogenesis.

Clinical intervention trials (one uncontrolled and three randomized, double-blind, placebo-controlled), utilizing folate supplementation in women with cervical dysplasia, have produced equivocal findings (*Table 4*). Among 13 oral contraceptive users with megaloblastic and dysplastic features in cervical smear in Shanxi, China, a province with a high prevalence of cervical dysplasia and low folate intakes, 1 to 3 months of oral folate supplementation (5 mg/d) reduced dysplastic change, average nuclear diameter, and hyperploidy.[62] One study from the University of Alabama randomized 47 women with cervical dysplasia (CIN I or II) to receive either folate 10 mg/d or placebo (ascorbic acid 10 mg/d) for 3 months.[63] Folate supplementation was associated with a significant degree of attenuation or regression in cytologic and histologic evidence of dysplasia.[63] However, a more recent study by the same group[64] did not reproduce the attenuation of dysplasia with folate supplementation noted in the earlier study.[63] The latter study involved a larger number of subjects (N = 235; 71% with CIN I and 26% with CIN II; 80% of the participants were current oral contraceptive users; 30% were positive for HPV-16) than the earlier study, followed the subjects for a longer period of time (6 months), and was better controlled. The same dose

Table 4 Randomized, double-blind, placebo-controlled intervention trials of folate and cervical neoplasia

Study	No. subjects	Diagnosis	Folate dose	Duration	Primary end point	Outcome
Butterworth et al.[63]	47	Mild to moderate dysplasia (CIN I, II)	10 mg/d vs. placebo*	3 months	Cytology and biopsy scores	Significant reduction in cytology and biopsy scores with folate
Butterworth et al.[64]	235	CIN I 71% CIN II 26% CIN III 3%	10 mg/d vs. placebo*	6 months	Cytology and biopsy scores	No significant reduction
Childers et al.[65]	331	CIN I 89% CIN II 11%	5 mg/d vs. placebo	6 months	Cytology and biopsy scores	No significant reduction

*Ascorbic acid 10 mg/d.
CIN–cervical intraepithelial neoplasia.

of folate (10 mg/d) was used and ascorbic acid (10 mg/d) was used as placebo. Although RBC folate significantly increased in the intervention group, no significant differences in rates of either CIN regression or progression were observed.[64] After 6 months of observation, approximately half of the participants had normal Pap smear results and two thirds had normal cervical biopsy results, regardless of treatment category.[64] Approximately 66% of the placebo subjects and 63% of the treatment subjects regressed to normal on the final visit.[64] Another large randomized study ($N = 331$; 89% with CIN I and 11% with CIN II; 60% were current oral contraceptive users; HPV-16 status not tested) also failed to demonstrate any beneficial effect of folate supplementation (5 mg/d for 6 months) in the regression of cytologic and colposcopic evidence of dysplasia.[65] After 3 months of treatment, 2.8% of the placebo group and 8.1% of the folate intervention group had lesions that improved based on both cytology and colposcopy ($P = 0.08$). However, at 6 months, 6% of the placebo group and 7% of the folate intervention group had lesions that improved ($P = 0.75$). Upon entry, approximately 70% and 90% of the subjects enrolled in the latter two studies,[64,65] respectively, had the lowest grade of dysplasia (CIN I), a stage of disease that is known to have a spontaneous rate of reversion to normal of more than 50% and a low rate of progression.[66] This may have interfered with the ability to observe an effect of folate supplementation. Furthermore, because both studies included predominantly subjects with CIN I, any definitive conclusion regarding the effect of folate supplementation on regression of CIN II and III cannot be made. Other shortcomings of these studies include a relatively small number of subjects, a short duration of treatment (6 months), and the lack of a true placebo arm in one study[64] where ascorbic acid, which is considered to be protective against the development of cervical neoplasia,[51,54,56] was used as placebo.

In summary, the results from epidemiologic studies that examined the relationship between folate status and the risk of cervical neoplasia are inconclusive. It appears that dietary folate intake and RBC folate concentrations are inversely associated with the risk of developing cervical dysplasia but not more advanced stages of cervical neoplasia (i.e., carcinoma in situ and invasive cancer).[49–59] Furthermore, it appears that folate deficiency is not carcinogenic per se but it enhances an underlying predisposition to cervical carcinogenesis (e.g., HPV-16).[57] Because of the retrospective nature of these epidemiologic studies with inherent limitations, no firm conclusion regarding the association between folate status and the risk of cervical neoplasia can be made. Although two small intervention studies[62,63] demonstrated that folate supplementation can regress cervical dysplasia, more recent randomized, double-blind, placebo-controlled trials with a larger number of subjects failed to confirm the earlier findings.[64,65] Again, because of the limitations associated with the latter two studies, as described earlier, no definitive conclusion can be made with respect to the potential chemopreventive role of folate supplementation in cervical carcinogenesis. Therefore, the role of folate in uterine cervical carcinogenesis is uncertain at present.

Esophagus, stomach, pancreas, and liver

Several ecological studies have suggested a possible association between diminished blood concentrations of folate and the risk of esophageal dysplasia/cancer.[67–70] In those rural South African areas where extremely high incidence and mortality rates from esophageal cancer are noted, significantly lower RBC and serum levels of folate were observed compared with other areas with lower rates of cancer.[67–69] Esophageal cytology studies in the high incidence areas revealed morphologic features very similar to those associated with folate deficiency.[68,69] However, it was found that levels of vitamins A, E, and B_{12} and of selenium were generally lower in the high cancer incidence areas than in the low cancer incidence areas.[67] These data suggest that esophageal cancer is more common in regions where overall nutritional status is poorer, but neither single out folate nor disclose the nutriture of localized tissues. Another ecological study from China also found that both serum and RBC folate concentrations were significantly lower in areas with high esophageal cancer incidence than in areas with low incidence.[70] One case-control study from India found that individuals with esophageal cancer had significantly lower levels of RBC folate, vitamin A, and zinc than those without cancer.[71]

Recently, a randomized study conducted in China investigated the effect of vitamin and mineral supplementation on esophageal dysplasia and cancer.[72–74] Rates of esophageal cancer in Linxian, located in north central China, are among the highest in the world due to multiple nutritional deficiencies.[75] In this study, 3,318 adults with a cytologic diagnosis of esophageal dysplasia received a daily supplement of 14 vitamins including folic acid 800 μg/d and 12 minerals in doses typically two to three times the U.S. Recommended Daily Allowances (RDA), or placebo, for 6 years. At the end of the study, cumulative esophageal cancer incidence and death rates were 14% [relative risk (RR) = 0.86 (0.54–1.38)] and 8% lower (RR = 0.92; 95% CI 0.67–1.28), respectively, among individuals receiving supplements than among those receiving placebo, a nonsignificant difference.[72,73] However, there was a significant increase in reversion of dysplastic cytology to normal among the group receiving the active treatment compared with the placebo group.[74] It is unclear whether the reversion of dysplastic cytology will translate into a significant drop in esophageal cancer rates with a longer follow-up.

Two case-control studies have examined the relationship between dietary folate intake and the risk of gastric cancer.[76,77] In a study from Italy involving 723 cases of gastric cancer and 2,024 controls, individuals taking the highest amount of dietary folate had a 43% reduction in the risk of developing gastric cancer than those taking the lowest amount of dietary folate.[76] However, the protective effect of folate was no longer evident when the effect of other micronutrients was taken into account.[76] A recent hospital-based case-control study from New York (67 subjects with gastric cancer and 96 subjects without cancer) showed that individuals with the highest dietary folate intake had a 90% reduction in the risk of developing distal gastric adenocarcinoma compared with those with the lowest dietary folate intake (OR = 0.1; 95% CI 0.04–0.6; $P = 0.033$).[77]

One case-control study from Adelaide, South Australia, which involved 104 cases of pancreatic cancer and 253 controls, assessed the association between dietary factors and the risk of pancreatic cancer.[78] It was found that folate intake was inversely correlated with the risk of pancreatic cancer after correcting for total energy intake, alcohol and tobacco use, and other nutrients ($P = 0.002$ and 0.014, respectively, linear trend).[78] The risk of developing pancreatic cancer was reduced by 64% in individuals with the highest dietary intake of folate (OR = 0.36; 95% CI 0.18–0.74) compared with those with the lowest intake.[78]

The effect of dietary folate deficiency on liver cancer was examined in male weanling Holtzman-Rolfsmeyer rats in which liver cancer was induced by gastric infusion of 3′-methyl-4-dimethylaminobenezene.[79] Although most of the folate-deficient animals died before developing tumors (15 of 21 rats), after 28 weeks 80% of the surviving control animals (2 mg folate/kg diet) and 3 of the 6 surviving folate-deficient animals (50%) had liver tumors.[79] This study indicated that the folate deficiency did not enhance the development of liver tumors. However, the very low survival rate (29%) among the folate-deficient animals and the high incidence (80%) of liver tumors among the folate-sufficient controls suggest that either the deficiency was too severe or the amount of carcinogen was so high that the modulatory effects of folate deficiency, if present, would not have been detectable. In another animal study in which liver tumor was induced by diethylnitrosamine in male Wistar rats, dietary folate supplementation at 23.5 times the normal level did not affect tumor incidence or survival time.[80] The lack of the association between dietary folate deficiency and liver cancer is in contrast with that observed with methyl deficiency.

In rodent models of hepatocarcinogenesis, diets deficient in all sources of methyl group donors (choline, folate, methionine, and vitamin B_{12}), in methionine and choline, and in choline alone have been shown to enhance the development of spontaneous and chemically-induced hepatocellular carcinoma.[81,82] No epidemiologic studies that examine an association between folate deficiency and the risk of liver cancer have been published to date.

In summary, no conclusion can be drawn regarding the association between dietary folate deficiency and cancer of the esophagus, stomach, pancreas, and liver because of insufficient data.

Breast

One case-control study from western New York that involved 439 postmenopausal women with breast cancer and 494 controls found that women with breast cancer had a substantially smaller ingestion of dietary folate compared with those without ($P < 0.05$).[83] Furthermore, this study showed that dietary folate intake was inversely associated with the risk of developing breast cancer (P trend = 0.03).[83] Although total caloric intake between the two groups was similar, women with breast cancer were shown to ingest significantly smaller quantities of dietary fiber, α-tocopherol, vitamin C, and carotene compared with those without cancer.[83] This observation suggests that women with breast cancer may have ingested smaller quantities of food items containing the aforementioned macro- and micronutrients compared with those without cancer because of anorexia and/or change in taste sensation associated with the underlying malignancy. Due to the retrospective nature of this study, the observed lower dietary intake of folate in women with breast cancer does not establish folate deficiency as a cause of breast cancer.

Two relevant animal studies published to date have produced conflicting results.[84,85] The effect of fermentation *Lactobacillus casei* factor, pteroyltriglutamate (folate with 3 glutamates), on mice with confirmed spontaneous mammary cancers was studied in 1940s.[84] In mice injected daily with the fermentation factor, new mammary tumor formation and lung metastases were significantly decreased, and tumor regression was significantly increased.[84] In contrast, liver *L. casei* factor, a monoglutamate form, had no significant effects on these parameters compared with controls.[84]

Another animal study investigated the effects of nutritional folate deficiency and supplementation in female Fischer 344 rats on initiation and early promotion of methylnitrosourea (MNU)-induced mammary cancer.[85] Rats were fed diets containing 0, 2 (the basal dietary requirement for the rat), or 40 mg folate/kg diet or 20 mg folinic acid/kg diet for 30 days, injected with MNU, and subsequently fed the control diet containing 2 mg folate/kg diet for 180 days. All four groups of rats gained weight equally well. Although the incidence of mammary cancer was not significantly different among the four groups, the number of mammary cancers per tumor-bearing animal (cancer multiplicity) was significantly lower in rats fed the folate-deficient diet (0 mg folate) than in those fed diets supplemented with 2 mg folate ($P = 0.009$), 40 mg folate ($P = 0.002$), or folinic acid ($P = 0.0001$).[85] Furthermore, the time required for 50% of the rats to develop palpable mammary cancer was significantly longer in the folate-depleted rats than in the rats supplemented with folate (40 mg folate, $P = 0.04$) or folinic acid ($P = 0.01$).[85] Therefore, this study demonstrated that folate supplementation can enhance the initiation or early promotion of mammary cancer and that folate deficiency may retard or protect against the development of mammary tumors in rats.

In summary, the role of folate deficiency in the development of breast cancer is unclear at present, mainly because of insufficient data available in the literature.

Colorectum

The roles of folate deficiency and supplementation in colorectal carcinogenesis have been extensively studied since Rosenberg and Mason suggested this provocative relationship.[85a] Diminished folate status, assessed by dietary intake or by the measurement of blood folate levels, has been found in several, but not all, epidemiologic studies to be associated with an increased risk of colorectal neoplasia (*Tables 5 through 7*).

Chronic ulcerative colitis. The role of folate deficiency and the risk of colorectal cancer was first investigated by Lashner et al.[86] in a case-control study of patients with extensive chronic ulcerative colitis. Chronic ulcerative colitis is associated with a 10- to 40-fold increased risk of

Table 5 Dietary folate intake and colorectal adenoma

Study	Location (year)	Study design	No. cases/controls	Odds ratio*	95% CI	*P*-trend for inverse association
Benito et al.[98]	Majorca, Spain (1993)	Case-control	101/242	0.27	NA	<0.01
Bird et al.[99]	USA (1995)	Case-control	M: 180/189	0.70	0.36–1.34	NS
			F: 152/161	1.47	0.73–2.95	NS
Tseng et al.[100]	USA (1996)	Case-control	M: 105/165	0.84	0.29–2.43	NS
			F: 131/245	0.39	0.15–1.03	0.08
Boutron-Ruault et al.[101]	France (1996)	Case-control	Small adenoma (<1 cm)			
			154/426	0.50	0.30–1.00	0.03
				M: 0.80	M: 0.30–2.30	NS
				F: 0.40	F: 0.20–1.00	0.03
			Large adenoma (≥1 cm)			
			208/426	0.50	0.30–1.00	0.04
Giovannucci et al.[102]	USA (1993)	Propective	M: 331/9159	0.63	0.41–0.98	0.03
			F: 564/15420	0.66	0.46–0.95	0.04
Baron et al.[103]	USA (1998)	Prospective	449/260	0.65	0.41–1.04	0.04

*Odds ratio for the highest dietary folate intake compared with the lowest intake. Adjusted for confounding factors.
CI–confidence interval. NA–not available. NS–not significant. M–male. F–female.

developing colorectal dysplasia and cancer compared with the general population.[87,88] Although megaloblastic anemia is rare, patients afflicted with this condition often demonstrate depressed serum and RBC folate levels due to inadequate nutritional intake, intestinal losses from inflammation, and the frequent use of sulfasalazine.[89–91] Sulfasalazine impairs folate absorption, principally through competitive inhibition of both folate transport enzymes and enzymes involved with folate metabolism.[92,93] Among 99 patients with extensive chronic ulcerative colitis of longer than 7 years' duration who were undergoing routine surveillance colonoscopy, 35 had dysplasia and 64 did not.[86] A history of chronic folate supplementation was associated with a 62% lower incidence of neoplasia among patients not receiving supplementation (OR = 0.38; 95% CI 0.12–1.20).[86] Furthermore, chronic administration of sulfasalazine was associated with a 50% increase in the risk of dysplasia (OR = 1.50; 95% CI 0.43–5.19).[86] Both of these observations fell short of conventionally accepted levels of statistical significance, but they were not attenuated by adjustment for other known risk factors for colon cancer in this population. In another case-control study involving 67 patients with ulcerative pancolitis (six cases with colonic neoplasia and 61 controls), Lashner[94] found that RBC folate concentration was 13% lower in those with colonic neoplasia (453.7 ± 123.0 ng/mL) compared with controls

Table 6 Dietary folate intake and colorectal cancer

Study	Location (year)	Study design	No. cases/controls	Odds ratio (OR)*	95% CI	*P*-trend for inverse association
Benito et al.[106]	Majorca, Spain (1991)	Case-control	286/295 (population)	0.53	NA	<0.05
			286/203 (hospital)	0.56	NA	<0.05
Freudenheim et al.[107]	USA (1991)	Case-conrol	Colon cancer			
			M: 205/205	1.03	0.56–1.89	NS
			F: 223/223	0.69	0.36–1.30	NS
			Rectal cancer			
			M: 293/273	0.31	0.16–0.59	<0.01
			F: 151/146	0.50	0.24–1.03	NS
Meyer and White[108]	USA (1993)	Case-control	M: 238/224	1.24	NA	NS
			F: 186/190	0.54	NA	OR = 0.81 (0.66–1.00)
Ferraroni et al.[109]	Italy (1994)	Case-controll	1,326/2,024	0.52	0.40–0.68	<0.05
Glynn et al.[110]	Finland (1996)	Nested Case-control	136/249	0.51 0.21†	0.20–1.31 0.06–0.74	0.15
Boutron-Ruault et al.[101]	France (1996)	Case-control	171/309	1.00	0.50–2.0	NS
White et al.[111]	USA (1997)	Case-control	444/427	0.51	0.34–0.77	<0.001
			M: 251/233	0.59	0.34–1.01	0.04
			F: 193/194	0.44	0.24–0.80	0.007
La Vecchia et al.[112]	Italy (1997)	Case-control	1,953/4,154	0.83	0.60–1.10	0.06
Giovannucci et al.[113]	USA (1995)	Prospective	M: 251/47,680	0.86	0.54–1.36	0.72
				0.30‡	0.14–0.63	<0.01

*Odds ratio for the highest dietary folate intake compared with the lowest intake. Adjusted for confounding factors.
†High folate–low alcohol–high protein intake compared with low folate–high alcohol–low protein intake.
‡High folate–low alcohol–high methionie intake compared with low folate–high alcohol–low methionine intake.
CI–confidence interval. NA–not available. NS–not significant. M–male. F–female.

Table 7 Blood and colonic mucosal concentrations of folate and colorectal neoplasia

Study	Location (years)	No. cases/control	Case diagnosis		Folate level Case	Control	*P*-value
Paspatis et al.[114]	Greece (1995)	62/50	Adenoma	Serum (ng/mL)	4.57	5.09	NS
				RBC (ng/mL)	536.0	743.8	<0.01
Bird et al.[99]	USA (1995)	332/350	Adenoma	Plasma (ng/mL)	M: 9.0	10.2	<0.05
					F: 8.8	10.0	NS
				RBC (ng/mL)	M: 206.6	230.3	<0.05
					F: 232.5	238.5	NS
Glyn et al.[110]	Finland (1996)	144/276 smokers	Cancer	Serum (ng/mL)	4.0	3.8	NS
Kim et al.[115]	USA (1994)	20/10	Adenoma*	Serum (ng/mL)	22.9	29.5	NS
				RBC (ng/mL)	307.1	353.2	NS
				Plasma homocysteine (nmol/mL)	9.5	7.1	0.04
				Colon (ng/g tissue)	343.9	520.1	0.04
Meenan et al.[116]	Ireland (1997)	12 cancer 7 adenoma 8 control	Cancer or adenoma	Serum (ng/mL)	6.8	8.6	NS
				RBC (ng/mL)	361	341	NS
				Colonocytes (pg/μg DNA)			
				Adenoma	15.5	18.5	<0.06
				Cancer	15.1	18.5	<0.02

*Hyperplastic polyps were used as controls.
NS–not-significant. RBC–red blood cell.

(519.8 ± 297.7 ng/mL), an observation that also fell short of statistical significance. However, after construction of a logistic regression model that adjusted for confounding effects of age, gender, race, disease duration, and folate supplementation, the risk of dysplasia or cancer was found to be significantly decreased by 18% for each 10 ng/mL increase in RBC folate (OR = 0.82; 95% CI 0.68–0.99).[94] Furthermore, although the subjects with neoplasia had diminished levels of RBC folate compared with the controls, the actual value of RBC folate in those subjects with neoplasia was well within the range conventionally accepted as normal, suggesting that a modest reduction in folate status is all that is necessary for the enhancement of cervical carcinogenesis. This observation is similar to that observed in the cervix.[57] More recently, Lashner and colleagues[95] have corroborated their earlier findings in an historical cohort study. Among 98 patients with ulcerative colitis who had disease proximal to the splenic flexure for at least 8 years, documented folate use of at least 6 months was associated with a 28% reduction in the risk of neoplasia (RR = 0.72; 95% CI, 0.28–1.83), which fell short of statistical significance.[95] However, these investigators were able to demonstrate a weak inverse dose-response relationship between folate intake and the risk of neoplasia and between folate intake and the degree of neoplasia.[95] However, two other studies offered a dissenting view on the role of folate deficiency in ulcerative colitis-associated colorectal carcinogenesis.[96,97] In one case-control study from New York involving 67 ulcerative colitis patients with colonic dysplasia or cancer and 68 without, folate supplementation was not significantly associated with decreased risk of developing colorectal neoplasia.[96] Another case-control study from Sweden involving 102 ulcerative colitis patients with colonic neoplasia and 196 matched controls found that sulfasalazine, a folate antagonist, was associated with a 62% reduction, rather than an increase, in the risk of developing colonic neoplasia (RR = 0.38, 95% CI, 0.20–0.69).[97]

The aforementioned studies suggest a provocative association between folate deficiency and the increased risk of colorectal cancer and dysplasia in chronic ulcerative colitis. However, the strength of this association appears to be tenuous at best, largely due to small sample sizes and retrospective study designs.

Dietary folate intake and the risk of adenomatous polyps. Four case-control[98–101] and two prospective[102,103] studies investigated the relationship between dietary folate intake and the risk of colorectal adenoma, a well established precursor of colorectal cancer[104,105] (*Table 5*). These studies have assessed dietary intake of folate by various food frequency questionnaires. Although these questionnaires are not perfect, they are the best available tools for estimating long-term dietary intake of folate. Four case-control studies indicate, on average, a 35% reduction in the risk of developing colorectal adenoma in subjects with the highest dietary folate intake compared with those with the lowest intake[98–101] (*Table 5*). Most of these studies also showed a statistically significant inverse relationship between dietary folate intake and the risk of developing colorectal adenoma in a dose-dependent fashion.[98,100,101] The protective effect of dietary intake of folate on the development of colorectal adenoma was more pronounced in women than in men in some studies.[100,101]

The most convincing epidemiologic evidence that supports the role of folate deficiency in the development of colorectal adenoma comes from two large prospective studies (the Health Professionals Follow-up Study and the Nurses' Health Study)[102] (*Table 5*). Giovannucci et al.[102] prospectively examined the associations between intake of folate, methionine, and alcohol and the risk of colorectal

adenoma. Their database was derived from two ongoing studies, the Nurses' Health Study (121,700 U.S. female registered nurses, aged 30–55 years) and the Health Professionals Follow-up Study (51,529 U.S. male health professionals, aged 40–75 years). A semiquantitative food frequency questionnaire was used to determine the intake of certain nutrients. To eliminate detection bias, the analysis of the study was restricted to those subjects who had had sigmoidoscopies for routine screening or for unrelated gastrointestinal conditions (9,490 men and 15,984 women). Histopathologically confirmed cases of adenomatous polyps of the distal colon or rectum ($N = 895$) were included in the final analysis. It was found that dietary folate intake was inversely associated with the risk of colorectal adenoma in a dose-dependent manner (P trend = 0.03 for men and 0.04 for women); the RRs of adenoma in subjects in the highest quintile of folate intake compared with those in the lowest quintile of intake were 0.63 for men (95% CI 0.41–0.98) and 0.66 for women (95% CI 0.46–0.95) after adjusting for confounding factors. This association was stronger for adenomas measuring less than 1 cm in diameter than those measuring 1 cm or greater. Folate intake from foods only (i.e., excluding supplement use) had only a weak inverse association with adenoma. The superimposition of moderate to high alcohol intake significantly increased the risk of developing adenoma. Of note is the level of folate intake defined as the lowest quintile. It was less than 166 μg/d for women and less than 241 μg/d for men (mean <200 μg/d), which is the RDA of folate for the U.S. population. This suggests that only a modest decrease in folate intake, slightly below the current RDA, is associated with an increased risk of developing colorectal adenoma. Despite limitations inherent in the assessment of dietary intake of folate and the fact that the investigators included only those individuals with distal colon and rectal polyps in their analysis, this study provides the most convincing epidemiologic evidence to date to establish an association between folate status and colorectal adenoma risk.

Another prospective study conducted as a part of a multicenter chemoprevention trial of β-carotene, vitamin C, and vitamin E investigated the effect of dietary and supplemental folate on the recurrence of adenomas in 709 subjects after colonic adenomas had been removed[103] (*Table 5*). After adjustment for caloric intake, dietary folate intake had a significant protective association with the risk of recurrence of colonic adenomas (P trend = 0.04).[103] However, this inverse association was attenuated by further adjustment for intake of dietary fiber and fat (P trend = 0.69).[103] Use of folate supplementation was not associated with a reduced risk.[103] This study, therefore, provides only modest support for the protective effect of dietary folate on the recurrence of colonic adenomas.

Dietary folate intake and the risk of colorectal cancer. To date, eight case-control[101,106–112] studies and one prospective[113] study that examined the association between folate intake and the risk of colorectal cancer have been published (*Table 6*). Seven case-control studies indicate, on average, a 35% reduction in the risk of developing colorectal cancer in subjects with the highest dietary folate intake compared with those with the lowest intake,[101,106–112] an identical magnitude of the risk reduction observed with colorectal adenoma. Five of these studies showed a statistically significant reduction in subjects with the highest dietary folate intake compared with subjects with the lowest intake,[106–109,111] whereas three studies showed no significant reduction.[101,109] However, one of the three negative studies showed an 80% reduction in the risk of developing colorectal cancer when subjects with high folate–low alcohol–high methionine intake were compared with those with low folate–high alcohol–low methionine intake.[110] Some of these studies showed gender[107,108] and site specificity (i.e., colon vs. rectum).[107] Five of the eight case-control studies also demonstrated a significant inverse relationship between dietary folate intake and the risk of colorectal cancer in a dose-dependent manner.[106–109,111]

One prospective study that involved 47,931 men in the Health Professionals Follow-up Study showed a nonsignificant 15% reduction in the risk of developing colorectal cancer in subjects with the highest folate intake compared with those with the lowest intake, as well as a nonsignificant inverse dose-response relationship[113] (*Table 6*). However, when individuals with the combination of high folate–low alcohol–high methionine were compared with those with low folate–high alcohol–low methionine, the risk reduction was 70% (RR = 0.30, 95% CI, 0.14–0.63) with the predominant protective effect on the distal colorectal cancer (RR = 0.13, 95% CI, 0.03–0.58)[113] (*Table 6*).

Blood and colonic mucosal folate concentrations and the risk of colorectal neoplasia. The relationship between blood levels of folate and the risk of colorectal cancer and adenoma is less well defined (*Table 7*). Three prospectively conducted clinical studies showed that serum folate concentrations are not significantly different between subjects with and without colonic adenoma or cancer.[114–116] Only one of the three studies showed that RBC folate concentrations were significantly lower in patients with colorectal adenoma than in those without these lesions.[114] Although the mean RBC concentration of the patients with adenoma was 28% lower than that of the controls, it still fell well within the normal range (536 ng/mL),[114] confirming the earlier observations that had suggested that a modest reduction of folate status is sufficient to enhance colorectal carcinogenesis.[57,94] However, the sample size of these studies was small, and hence, the finding of no difference in serum and RBC folate concentrations between cases and controls might have resulted from a type II error. One interesting, and potentially important, observation from one of these studies was that the serum concentration of homocysteine, which is known to rise in the setting of folate deficiency[117] and is considered to be a more sensitive indicator of cellular folate depletion than blood folate levels,[118] was significantly higher (by 22%) in subjects with adenoma than in the controls.[115] This observation suggests that a modest reduction in systemic folate status in subjects with colorectal neoplasia may not be apparent with conventional blood measurements of folate; rather, a more sensitive indicator such as serum or plasma homocysteine is required to demonstrate this degree of diminished folate status (*Table 7*).

Two published population-based case-control studies that examined the relationship between blood folate levels

and colorectal cancer risk have produced conflicting results[99,110] (*Table 7*). One study from the United States showed that both plasma and RBC folate concentrations are significantly lower (by 10–12%) in subjects with colonic adenoma than those without, although this was observed only in men.[99] This study also demonstrated a significant inverse dose-responsive relationship between RBC folate levels and colorectal adenoma risk.[99] Another case-control study nested within the Alpha-Tocopherol Beta-Carotene Study cohort of male smokers aged 50 to 69 years in Finland failed to show any significant difference of serum folate concentrations between subjects with and without colorectal cancer.[110]

Two small clinical studies have examined the relationship between the actual folate concentrations in the colonic mucosa and colorectal cancer risk.[115,116] One study showed that the mean folate concentration in the normal rectal mucosa of human subjects harboring colorectal adenoma was significantly lower (by 34%) than that of subjects harboring nonneoplastic polyps in the absence of a significant depletion of serum and RBC folate concentrations.[115] This implies that, even in the absence of overt folate deficiency, localized folate deficiency exists in the colorectal mucosa of individuals at risk of developing colorectal adenoma, predisposing it to a subsequent neoplastic transformation. However, another recent human study observed that, although folate concentrations were significantly lower in neoplastic colonic epithelial cells than in adjacent normal cells, folate concentrations were not significantly different between normal colonic epithelial cells adjacent to neoplasms and colonic epithelial cells from normal controls.[116] Because these studies used different methods to determine colonic folate concentrations, the issue of localized folate deficiency in the colonic mucosa in individuals at risk of developing colorectal cancer is still unsettled.

The relationship between conventional blood measurements of folate and colorectal cancer risk is less consistent than that observed between dietary folate intake and colorectal cancer risk. The relative insensitivity of blood measurements of folate in predicting colorectal cancer risk may be related to the observations from epidemiologic studies that indicate that mild folate depletion, rather than the development of overt systemic folate deficiency, is a sufficient condition to enhance the risk of colorectal neoplasia.[57,94,102] In this regard, a recent study suggests that the determination of serum homocysteine concentrations might be a more sensitive reflection of colorectal cancer risk.[115] Recent studies have produced conflicting results in regard to colonic mucosal folate concentrations and colorectal cancer risk.[115,116]

Folate intervention studies in humans. Accumulating epidemiologic, clinical, and experimental data have provided an impetus for a few centers to initiate chemoprevention trials of colorectal adenomas and adenocarcinomas using a pharmacologic dose of folic acid. A recent randomized, double-blind, placebo-controlled study from the New England Medical Center in Boston ($N = 20$) has demonstrated that folate supplementation of 5 mg/d for 1 year significantly increases serum, RBC, and colonic mucosal folate concentrations, and significantly decreases serum homocysteine levels in subjects with colonic adenoma, indicating that oral supplementation can modulate concentrations of folate in the target organ.[119]

To date, seven small randomized, placebo-controlled intervention studies have been published[120–126] (*Table 8*). Cravo et al.[120] have shown that folate supplementation at 10 mg/d for 6 months in patients with either colonic adenoma or cancer ($N = 22$) following removal of these lesions can significantly reverse a purported biomarker of colorectal cancer (genomic DNA hypomethylation[127]) in the normal rectal mucosa. During the washout period, it was shown that DNA methylation values moved toward the initial values in most cases.[120] In another study from Greece, 60 subjects with colorectal adenoma were randomized to receive either folate 1 mg/d or placebo for 2 years after removal of these polyps.[121] Although adenoma recurrence was reduced by 40% and 46% in those who had received folate compared with the placebo group at 1- and 2-year colonoscopic follow-ups, respectively, this difference fell short of statistical significance.[121] In another randomized study from Portugal, Cravo et al.[122] investigated the effect of a 3-month supplementation of folate 5 mg/d on genomic DNA methylation in 20 subjects after removal of colonic adenoma. When the study groups in this trial were examined as a whole, there was no significant effect of folate supplementation on genomic DNA methylation of the colonic mucosal.[122] However, when the subjects were subdivided into those harboring only one adenoma versus those harboring multiple adenomas, the data indicate that subjects harboring one adenoma responded to folate with a 40% increase in genomic DNA methylation ($P = 0.05$); in contrast, subjects harboring multiple adenomas did not respond.[122] Another study from Boston found that both folate supplementation (5 mg/d) and placebo for 1 year in 20 subjects with colonic adenomas increased genomic DNA methylation and decreased strand breaks in exons 5 through 8 of the p53 tumor suppressor gene to a similar extent during the study period ($P < 0.01$).[123] This observation suggests that factors other than folate supplementation were responsible for the parallel increase in DNA methylation and p53 stability in the two groups.[123] A recent Australian study investigated the effect of the combination of folate and vitamin B_{12} on genomic DNA methylation and DNA damage as measured by micronucleated cell (MNC) frequency in healthy, young (18–32 years of age) volunteers.[124] The supplemented group received 700 μg folic acid and 7 μg vitamin B_{12} in cereal per day for 12 weeks followed by one table containing 2 mg folic acid and 20 μg of vitamin B_{12} per day for an additional 12 weeks.[124] Genomic DNA methylation status was not altered in the supplemented group.[124] MNC frequency was significantly reduced during the intervention by 25.4% in those subjects with initial MNC frequency in the high 50th percentile ($P < 0.01$) but there was no change in those subjects in the low 50th percentile for initial MNC frequency.[124]

Two other studies investigated the chemopreventive role of folate in patients with chronic ulcerative colitis utilizing two intermediate biomarkers of colorectal cancer as the end point of the trial.[125,126] In a study from Portugal, folate supplementation of 5 mg/d for 6 months failed to reverse DNA hypomethylation.[125] This study, however, was limited

Table 8 Randomized, double-blind, placebo-controlled intervention trial of folate and colorectal neoplasia

Study	Location (year)	No. subject	Case diagnosis	Folate dose (mg/d)	Duration	Primary end point	End point modification
Cravo et al.[120]	Portugal (1994)	22	Cancer Adenoma	10	6 months	DNA methylation	Significant increase in DNA methylation
Paspatis et al.[121]	Greece (1995)	60	Adenoma	1	2 years	Adneoma recurrence	40% reduction at 1 year (P = NS) 46% reduction at 2 years (P = NS)
Cravo et al.[122]	Portugal (1998)	20	Adenoma	5	3 months	DNA methylation	Overall: no change Subgroup analysis: significant increase in DNA methylation only in those with single polyp
Kim et al.[123]	USA (1998)	20	Adenoma	5	1 year	DNA methylation	Significant increase in both groups compared with baseline
						p53 exons 5–8 Strand breaks	Significant decrease in both groups compared with baseline
French et al.[124]	Australia (1998)	63	Healthy volunteers	700 μg folate + 7 μg B_{12} in cereal then 2 mg folate + 20 μg B_{12} in tablet	3 months 3 months	DNA methylation	No significant change
						Micronucleated cell frequency	Significant reduction in the treatment group by 25%
Cravo et al. [125]	Portugal (1995)	25	Chronic ulcerative colitis	5	6 months	DNA methylation	No significant change
Biasco et al.[126]	Italy (1997)	24	Chronic ulcerative colitis	15*	3 months	Rectal cell proliferation	Significant reduction in cell proliferation in the upper 40% of crypts

*Folinic acid was used.
NS–not significant. B_{12}–vitamin B_{12}.

because folate supplementation failed to significantly increase blood and colonic mucosal folate concentrations and because the majority of the study subjects had been taking folate 10 mg/d and sulfasalazine, a folate antagonist, on entry to the trial.[125] Another study from Italy has shown that folinic acid at 15 mg/d can significantly reduce the cell proliferation in the upper 40% of the colonic crypts.[126] An expansion of the proliferative compartment from the lower 60% into the upper 40% of the crypts in the apparently normal colorectal mucosa is observed in patients with colorectal cancer and in individuals at high risk of developing colorectal cancer, and therefore, it is considered to be either one of the early steps in the multistep process of colorectal carcinogenesis or biomarkers of these early steps.[128]

The number of subjects studied in these trials was too small and the duration of follow-up was relatively short, and hence, it is difficult to draw any definitive conclusions about the chemopreventive role of folate supplementation in colorectal carcinogenesis. Furthermore, six of the seven trials used less well established intermediate biomarkers of colorectal cancer instead of adenoma or cancer recurrence as the end point of the trial. There are three large, randomized, double-blind, placebo-controlled multi-center folate chemoprevention trials ongoing in the United Statea at present. These are: (1) the New England Medical Center Multicenter Study (primary investigator, Dr. J. B. Mason; folate 5 mg/d vs. placebo for 1 year followed by a washout period of 1 year; end point: molecular biomarkers); (2) the Dartmouth Medical Center Multicenter Study (primary investigator, Dr. J. Baron; a 2 × 2 factorial design with folate 1 mg/d and aspirin; end point: adenoma recurrence); and (3) the Harvard Nurses' Health Study and Health Professionals Follow-up Study (primary investigator, Dr. E. Giovannucci; folate 1 mg/d or placebo; end point: adenoma recurrence). It is hoped that these studies will clarify the effect of folate supplementation on colorectal adenoma and cancer recurrence.

Animal studies. Two animal studies support the role of folate deficiency in colorectal carcinogenesis[129,130] (*Table 9*). In the first study, 100% of male Sprague-Dawley rats fed a folate-depleted diet developed *microscopic* colorectal neoplasms 20 weeks after initiation of dimethylhydrazine (DMH), a colorectal carcinogen, injections compared with 29% of the rats fed a control diet.[129] The incidence of *macroscopic* colorectal neoplasms were 86% and 43% in the folate-deficient and control groups, respectively (P = 0.09).[129] This study, therefore, suggests that folate deficiency affects an early phase of colorectal carcinogenesis in this rodent model. Another important finding in this study was that no cancer was observed in the two control groups that received saline injections in conjunction with either a folate-depleted or control diet, thereby confirming observa-

Table 9 Summary of animal studies: Folate status and colorectal neoplasia

Study	Model	Folate dose (mg/kg diet)	Duration	End point	Outcome
Cravo et al.[129]	Sprague-Dawley rats Dimethylhydrazine	0 mg 8 mg	20 weeks	Tumor incidence: Microscopic: Macroscopic:	0 mg/kg vs. 8 mg/kg 100% 29% ($P = 0.005$) 86% 43% ($P = 0.09$)
Kim et al.[130]	Sprague-Dawley rats Dimethylhydrazine	0 mg 2 mg 8 mg 40 mg	15 weeks	Tumor incidence: Microscopic: Macroscopic:	 no difference 0 mg: 70% 2 mg: 40% 8 mg: 10% 40 mg: 50% ($P < 0.03$)
Shivapurkar et al.[131]	Fischer 344 rats Azoxymethane	3 mg + high fat+low fiber vs. high fat+low fiber	10, 14, 18 weeks	Aberrant crypt foci	No effect
Reddy et al. [132]	Fischer 344 rats Azoxymethane	2,000 mg vs. control (? amount of folate)	50 weeks	Tumor incidence Tumor size Tumor multiplicity	No effect Increased with folate Increased with folate
Wargovich et al.[133]	Fischer 344 rats Azoxymethane	2,500 mg 5,000 mg vs. 2 mg	2 weeks	Aberrant crypt foci	Increased compared with 2 mg No effect compared with 2mg

tions in cervical cancer that suggest that folate deficiency alone is not sufficient to initiate carcinogenesis but rather potentiates other risk factors for carcinogenesis.[57] The degree of folate depletion induced in this study was moderate enough to permit maintenance of good health of the animals and to prevent growth retardation, anemia, and premature death. This study, therefore, corroborates earlier epidemiologic and clinical observations that mild to moderate folate depletion is sufficient to enhance carcinogenesis.[57,94,102]

In a subsequent study utilizing the same DMH rodent model of colorectal cancer, a folate-deficient diet was associated with a potentiation of the development of *macroscopic* colorectal tumors, and dietary folate supplementation, up to four times the dietary requirement, was shown to retard the progression from microscopic colorectal neoplastic foci to *macroscopic* tumors in a dose-dependent manner.[130] This study, therefore, suggests that folate also has a modulatory effect in a later stage of colorectal carcinogenesis.[130] One interesting finding from this study was that levels of dietary folate greater than four times the dietary requirement did not convey further benefit.[130] In fact, there was a nonsignificant trend toward increased macroscopic neoplasms in the group that was fed a supraphysiologic dose of folate (20 times daily requirement) compared with the group that was fed four times the daily requirement.[130] This study suggests that supplemental folate may have two distinct actions: At lower levels of supplementation beyond the dietary requirement it seems to possess an inhibitory effect on genesis of microscopic foci of neoplasia[129,130] as well as the progression of macroscopic neoplasms from microscopic ones.[130] Independently, in a strongly procarcinogenic environment where the appearance of microscopic neoplasms is inevitable, exceptionally high supplemental folate levels may promote the growth of microscopic neoplasms.[130] This "acceleration phenomenon" has been observed in humans and animals that have well established cancers and that are given exceptionally large doses of folate.[19–22,85]

However, not all animal studies published to date have supported the protective role of folate supplementation in colorectal carcinogenesis[131–133] (*Table 9*). Three studies utilized Fischer-344 rats and Azoxymethane, a metabolite of DMH.[131–133] In one study,[131] dietary folate supplementation at 3 mg/kg did not have a significant inhibitory effect on aberrant crypt foci, probably the earliest recognizable precursor lesion and a well established intermediate biomarker of colorectal cancer.[134] However, the animals in this study were fed high-fat, low-fiber diet throughout the study period.[131] This diet is considered to promote colorectal cancer in humans and animals[135] and hence, might have masked any protective effect of folate supplementation. In another study,[132] which has been reported as an abstract only, folate supplementation at 2,000 mg/kg diet had no effect on colorectal tumor incidence but significantly increased size and multiplicity of colorectal tumors. In another study,[133] folate supplementation at 2.5 g/kg diet increased the development of aberrant crypt foci compared with the control diet (AIN-76A; 2 mg/kg diet). Folate supplementation of 5.0 g/kg diet had no effect on the development of aberrant crypt foci compared with the control diet.[133] However, the dietary folate levels used in this study exceeded physiologic ranges by 1,000-fold.[133]

Summary of the role of folate status and colorectal carcinogenesis. A growing body of epidemiologic, clinical, and animal studies has suggested that folate deficiency is an important nutritional factor in sporadic colorectal carcinogenesis. Although the results from epidemiologic studies pertaining to this issue are not uniform, dietary folate intake appears to be inversely associated with the risk of developing colorectal adenoma and cancer in a dose-dependent fashion, with a 35 to 40% reduction in the risk of developing colorectal neoplasia in those with the highest dietary folate

intake compared with those with the lowest intake.[98–103,106–113] The degree and consistency of this inverse association between dietary folate intake and colorectal adenoma/cancer risk are quite impressive. In contrast, conventional blood measurements of folate have not been consistently shown to be lower in subjects with colorectal adenoma or cancer.[99,110,114–116] The relative insensitivity of blood measurements of folate in correlating with colorectal cancer risk may be related to the observations from epidemiologic studies that indicate that mild folate depletion, rather than the development of overt systemic folate deficiency, is a sufficient condition to enhance the risk of colorectal neoplasia.[57,94,102] However, recent studies have suggested that serum homocysteine concentrations, a more sensitive inverse indicator of folate deficiency compared with conventional blood measurements of folate status, are significantly higher in subjects with colorectal neoplasia than in those without.[115] The issue of localized folate deficiency in the colonic mucosa in individuals at risk of developing colorectal cancer has not been settled yet.[115,116] Stimulated by promising results from small folate intervention trials,[120–122,124,126] three large multicenter trials are underway to determine the chemopreventive role of folate supplementation in colorectal carcinogenesis. Animal studies have provided considerable support for a cause-and-effect relationship between folate deficiency and colorectal cancer as well as a dose-dependent protective effect of modest levels of dietary folate supplementation above basal levels.[129,130] However, supraphysiologic levels of folate supplementation do not confer protection[130] and, in some cases, may enhance colorectal tumorigenesis.[132,133] The implication of this issue is important because the optimal dose of folate supplementation and the target populations must be determined for folate chemoprevention to be effective and safe in humans.

Potential mechanisms by which folate modulates carcinogenesis

A mechanistic understanding of how folate deficiency is involved in carcinogenesis might further strengthen the case for a causal relationship and provide insights into the possible chemopreventive role of folate. To date, however, the mechanisms by which folate deficiency enhances carcinogenesis have been speculative.[8,10,12]

The effect of folate on DNA methylation

A leading hypothesis pertains to alterations in DNA methylation. Mediating the transfer of one-carbon moieties is the sole biochemical function known for folate.[16] In this role, folate is critical for the synthesis of S-adenosylmethionine (SAM), a compound that serves as the methyl donor for over 100 biochemical reactions, including methylation of DNA.[118] It is in this manner that folate modulates DNA methylation (*Figure 1*). The pattern of methylation at cytosine residues in the cytosine-guanine (CpG) sequences is a heritable, tissue- and species-specific, postsynthetic modification of mammalian DNA.[136] It has been estimated that in normal differentiated eukaryotic cells, 70 to 90% of the cytosines in the CpG are methylated.[136] Genomic and gene specific DNA hypomethylation appears to be an early, and consistent, event in carcinogenesis[138,139] including that of colorectal cancer.[104,105] Although it is not known how alterations in DNA methylation are related to carcinogenesis, it is known that DNA methylation is an important determinant in gene expression,[139,140] conformational configuration and structural stability of DNA,[141–145] binding of transcription factors and other proteins,[143,145–148] mutations,[149] and imprinting (differential expression of parental allele in normal development),[150,151] all of which are considered to play a role in carcinogenesis. DNA methylation has been observed to be inversely related to gene expression; methylation at so called "CpG islands" at the 5′ end of many genes where the regulation of gene expression occurs has been shown to be associated with gene silencing.[139,140] Regional hypomethylation is associated with alterations in chromatin conformation and with alterations in interaction between DNA and methyl-specific proteins.[141–145] Each of these can enhance the accessibility of specific sequences to DNA damaging agents or endonucleases, thereby promoting genomic instability.[141–145] CpG sequences within certain genes (e.g., the p53 gene) are not only the sites of DNA methylation but also mutational hot spots for certain cancers including colorectal cancer.[104,105,149,152,153] The majority of mutations observed in CpG sequences within these genes are cytosine to thymine transitions.[149] Several different mechanisms are believed to be responsible for the hypermutability of the CpG dinucleotide: (1) the spontaneous deamination of 5-methylcytosine to thymine[154]; (2) the enzymatic deamination of 5-methylcytosine to thymine by methyltransferase;[155] and (3) the enzymatic deamination of unmethylated cytosine to uracil by methyltransferase, the methylation of uracil to thymine by methyltransferase, and the blockage of repair of DNA mismatches by the binding of methyltransferase.[156,157] The last mechanism is particularly pertinent to folate deficiency because it is believed to be operative when concentrations of the methyl group donor SAM are limited, as is the case with folate deficiency.

As previously discussed, in rodent models of hepatocarcinogenesis, diets deficient in all sources of methyl group donors (choline, folate, methionine, and vitamin B_{12}), in methionine and choline, and in choline alone have been shown to enhance the development of spontaneous and chemically-induced hepatocellular carcinoma.[81,82] Preceding the development of liver cancer in these rodent models of hepatocarcinogenesis is the appearance of genomic and gene-specific (e.g., c-myc, c-fos, c-Ha-ras protooncogenes) hepatic DNA hypomethylation, as well as elevated levels of the corresponding mRNAs.[158–162] Recently, a diet deficient in choline, methionine, and folate has been shown to induce DNA hypomethylation at specific sites within the p53 tumor suppressor gene in rat liver.[163] Only a few studies, however, have examined the effects of an isolated deficiency of folate on DNA methylation. Numerous experiments in intact animals have shown that folate deficiency significantly reduces hepatic SAM.[164–167] Whether such reductions in SAM result in altered patterns of genomic DNA methylation, however, remains unclear: One study[168] that used severe folate deficiency observed significant genomic hypomethylation in hepatic DNA, whereas another study[167]

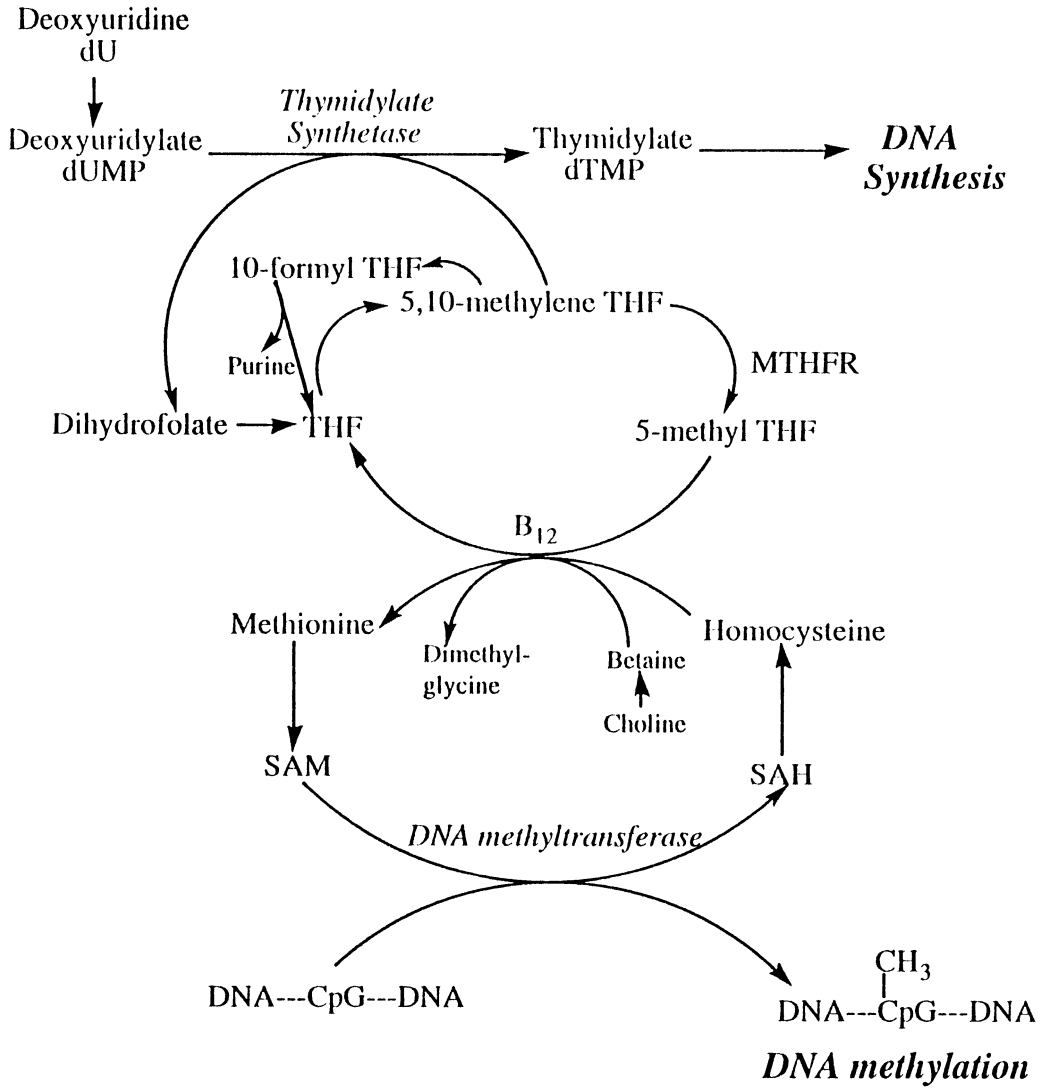

Figure 1 Simplified scheme of folate metabolism: Folate is an integral factor in the pathways leading to DNA methylation and synthesis. B_{12}, vitamin B_{12}; CH_3, methyl group; CpG, cytosine-guanine dinucleotide sequence; MTHFR, 5,10-methylenetetrahydrofolate reductase; SAH, S-adenosyl homocysteine; SAM, s-adenosylmethionine; and THF, tetrahydrofolate.

that used a more modest degree of deficiency observed neither genomic hypomethylation nor hypomethylation of the protooncogene c-myc in the liver or colon. More recently, a prolonged, severe degree of folate deficiency as in the previous study[168] failed to induce significant genomic DNA hypomethylation in liver DNA despite a significantly reduced hepatic SAM level.[169] In fact, there was a nonsignificant trend toward increased genomic DNA methylation in severely folate-depleted rats.[169] This may be related to the observations that states associated with diminished availability of SAM result in an enhancement of DNA methyltransferase activity, the enzyme responsible for DNA methylation.[170,171] Collectively, these data suggest that isolated dietary folate deficiency does not induce genomic or gene-specific protooncogene DNA hypomethylation in liver and colon DNA.

However, one recent study that examined the effect of isolated dietary folate deficiency on site-specific DNA methylation of the p53 tumor suppressor gene in rats has provided an exciting insight into how folate deficiency modulates DNA methylation.[169] Exons 5 through 8 of the p53 gene were examined in this study because this region contains unusually abundant numbers of CpG sites[152,153] and hence, these exons might be particularly susceptible to the hypomethylating effect of folate deficiency. Furthermore, this region is highly conserved and has been observed to be critically involved in carcinogenesis.[152,153] The methylation status of exons 5 through 8 within the p53 gene in rat liver was assessed by a well established quantitative HpaII-polymerase chain reaction (PCR) technique. The degree of DNA methylation within exons 6 and 7 of the p53 gene was reduced by 15% after 4 weeks and 40% after 6 weeks of folate deficiency relative to the controls ($P = 0.02$), indicating progressive hypomethylation within these exons with prolonged folate deficiency; in contrast, folate deficiency did not alter methylation status within exon 8 relative

to the controls.[169] This observation complements an earlier observation that has indicated that combined deficiencies of methyl group donors can induce DNA hypomethylation in exons 5 through 8 of the p53 gene in rat liver.[163] These data suggest that exons 5 through 8 of the p53 gene might be particularly susceptible to hypomethylating effects of deficiencies of folate and other methyl groups donors.

In another study, the effects of a more moderate degree of folate deficiency, folate supplementation, and DMH on p53 methylation were examined in the DMH rat model of colorectal cancer.[172] Rats were fed diets containing 0, 2, 8, or 40 mg folate/kg diet and injected weekly with DMH for 15 weeks. Colonic DNAs from rats that were free of macroscopic and microscopic neoplasms were selected. For control, colonic DNAs from the folate-depleted (0 mg folate/kg diet) and -replete (8 mg folate/kg diet) rats that had been fed the diets for the same duration as the DMH-treated rats but not injected with DMH were selected. The extent of p53 methylation was assessed by quantitative HpaII-PCR as described previously. The major findings of this study are: (1) moderate folate deficiency alone without DMH treatment decreases the extent of p53 methylation within exons 6 and 7 by 25% but has no effect in exon 8; (2) DMH treatment decreases the degree of p53 methylation in exons 6 and 7 by 30 to 36% in all DMH treated rats, independent of dietary folate levels, relative to the non-DMH-treated folate-replete controls; and (3) in exon 8, significant p53 hypomethylation was observed only in the DMH-treated folate-depleted rats compared with controls and was effectively overcome by increasing levels of dietary folate in a dose-responsive manner.[172] Thus, this study provides further evidence that dietary folate can modulate DNA methylation in the critical regions of the p53 tumor suppressor gene.

The functional ramifications of exon-specific DNA hypomethylation of the p53 tumor suppressor gene have not been clearly elucidated yet. As previously discussed, regional hypomethylation is associated with genomic instability due to its effects on chromatin conformation and on interaction between DNA and methyl-specific proteins.[141–145] This is supported by the observation that both hypomethylation and strand breaks in the p53 gene increased in parallel when the folate deficiency state was extended from 4 to 6 weeks.[169] In addition, in rats fed a methyl-deficient diet, the induction of DNA hypomethylation in the p53 gene paralleled the appearance of p53 strand breaks.[163] Furthermore, an increase in DNA methylation has been observed to confer partial resistance to calcium/magnesium-dependent endonuclease-induced strand breaks in the p53 gene.[163] This body of evidence suggests that the observed hypomethylation within the p53 gene may be mechanistically related to strand breaks, thereby affecting the stability of the p53 gene; this remains to be more clearly determined.

Although the inverse relationship between gene expression and DNA hypomethylation at "CpG islands" at the 5′ end of many genes where the regulation of gene expression occurs (i.e., promoter regions) has been well established,[139,140] the effect of DNA hypomethylation within exons 5 through 8 of the p53 gene on p53 gene expression is uncertain. There is some evidence that suggests that alterations in DNA methylation in coding regions can also modulate gene expression.[173–175] One study has shown that p53 hypomethylation within exons 6 and 7 result in an increased level of p53 mRNA.[175] Therefore, the functional significance of p53 hypomethylation within exons 5 through 8 needs to be more clearly defined in future studies. It would be of great interest to study the effect of folate deficiency on DNA methylation within the promoter region of the p53 gene. Unfortunately, however, the promoter region of the mammalian p53 gene does not contain CpG islands.[176]

Another possible functional significance of p53 hypomethylation in exons 5 through 8 might be its effects on p53 mutations. CpG sequences within certain genes, including the p53 gene, are not only the sites of DNA methylation but also mutational hot spots for certain cancers including colorectal cancer.[104,105,149,152,153] In some cancers, most notably colorectal cancer, the majority of mutations observed in CpG sequences within these genes are cytosine to thymine transitions[149] resulting from deamination of 5-methylcytosine.[154,155] However, recent studies have suggested that the occurrence of mutational hot spots at CpG sites may not always be due to spontaneous or enzymatic deamination of 5-methylcytosine, but also might be initiated by enzymatic deamination of unmethylated cytosine to uracil when SAM concentrations are limiting.[156,157] In this regard, deamination at nonmethylated cytosine in cells with lowered uracil glycosylase activity has been observed to increase the frequency of transitions at CpG.[177] Therefore, when SAM concentrations are limiting, as is the case with folate deficiency, and uracil glycosylase activity is overwhelmed, nonmethylated cytosine may be susceptible to transitional mutations.[156,157] The significance of the folate deficiency-induced p53 hypomethylation within the mutational hot spot must be clearly defined. Recently, folic acid supplementation has been observed to prevent p53 mutations, as documented by immunostaining in subjects with chronic ulcerative colitis at risk of developing colorectal cancer.[178]

Although the global level of DNA methylation is generally lower in tumor cells than in normal cells and some loci in several genes are hypomethylated in neoplastic transformation,[104,105,137,138] more recently, it has been suggested that site-specific *hypermethylation* also may be important in tumorigenesis.[179–182] For example, specific CpG islands within or upstream from the promoter region of certain tumor suppressor genes have recently been observed to be *hypermethylated* in several tumors including that of the colorectum,[179–182] thereby leading to gene inactivation or silencing. Moreover, reduction in DNA methyltransferase activity and DNA methylation by heterozygous DNA methyltransferase knockout combined with treatment with an inhibitor of DNA methyltransferase has been observed to suppress rather than enhance tumor formation in a genetically predisposed murine model.[183] Therefore, DNA methylation, as a mechanism of carcinogenesis, remains an ill-defined and tentative hypothesis as it pertains to either folate-related carcinogenesis and carcinogenesis as a whole. However, genomic and gene-specific DNA hypomethylation is still considered to be an important epigenetic phenomenon in neoplastic transformation.[104,105,137,138]

The effect of folate on DNA damage and repair

Another hypothesis pertains to how folate deficiency disrupts the integrity of DNA. Folate is an essential factor for the de novo biosynthesis of purines and thymidylate[16] (*Figure 1*). 5,10-Methylenetetrahydrofolate, an intracellular co-enzymatic form of folate, is required for conversion of deoxyuridylate to thymidylate and can be oxidized to 10-formyltetrahydrofolate for de novo purine synthesis[6] (*Figure 1*).

Accumulating in vivo and in vitro evidence supports a role for folate in the maintenance of DNA integrity and stability. A diverse array of chromosomal anomalies, including gaps and breakage, are common in individuals with megaloblastic anemia due to folate deficiency, and the anomalies appear to be proportional to the severity of the megaloblastic changes.[184,185] On a biochemical level, the DNA of such individuals is noted to have a markedly elevated level of uridine misincorporation.[186] Appropriate vitamin repletion largely corrects these anomalies.[184–187] The in vitro proliferation of human cells in folate-deficient media also leads to breaks in chromosomes at particular "fragile sites" that are also observed in many cancers.[188] On a molecular level, mammalian cells exposed to antifolate agents such as methotrexate demonstrate DNA strand breaks.[189] Furthermore, the induction of DNA strand breaks by alkylating agents and irradiation in Chinese hamster ovary cells appears to be enhanced by media devoid of folate and nucleotide precursors.[190] Dietary folate deficiency in vivo has been observed to induce DNA strand breaks in rat lymphocytes.[191] More recently, dietary folate deficiency has been shown to induce DNA strand breaks within a highly conserved region (exons 5–8) of the p53 tumor suppressor gene in rat liver, whereas only a sustained, severe degree of dietary folate deficiency results in genomic DNA strand breaks.[169] Similarly, in rat colon, isolated dietary folate deficiency has been observed to produce progressive DNA strand breaks within exons 5 through 8 of the p53 gene in a time- and dose-dependent manner; this effect was not observed in other exons of the p53 gene, in a constitutive housekeeping gene (β-actin), or at the genomic level.[192] This study also has shown that dietary folate supplementation leads to a degree of p53 integrity greater than that observed with the basal diet.[192] The p53 tumor suppressor gene is one of the most frequently implicated genes in carcinogenesis and is integrally involved in transcription, DNA repair, genomic stability, senescence, cell cycle control, and apoptosis.[137,138] Therefore, dietary folate deficiency-induced DNA strand breaks within the highly conserved region of the p53 gene may lead to functional inactivation of the p53 with loss of tumor suppressor function. This issue remains to be more clearly defined.

The implications of the presence of unrepaired DNA strand breaks in vivo are not yet fully defined, but there is considerable in vitro evidence indicating that DNA strand breaks are integrally involved in the evolution of neoplastic transformation. DNA strand breaks create a site for potential chromosomal recombination.[193] The induction of strand breaks has been associated with chromosomal aberrations evident at mitosis,[194,195] loss of heterozygosity,[196] an increase in mutation rates,[190] and neoplastic transformation.[197–199] In addition, persistent DNA double strand breaks within human DNA contained in a yeast artificial chromosome (YAC) have been shown to result in YAC loss, deletion, or cell lethality if strand breaks are either not repaired or misrepaired.[200,201] DNA strand breaks also appear to be the necessary and sufficient condition for the activation of the DNA repair system,[202] thereby placing enhanced reliance on a repair system that depends on adequately proportioned pools of intracellular nucleotides. The latter is clearly disrupted in cells whose folate metabolism is impaired.[203–205] Increased ratios of uridylate and thymidylate concentrations, for example, are evident in folate-impaired cells, leading to the inappropriate insertion of uracil rather than thymine bases into DNA.[186,206,207] Recently, dietary folate deficiency has been shown to impair DNA excision repair in rat colonic mucosa.[208]

Methylenetetrahydrofolate reductase polymorphism

As shown in *Figure 1*, methylenetetrahydrofolate reductase (MTHFR) is a critical enzyme in folate metabolism. Its product, 5-methyltetrahydrofolate (5-methylTHF), is the predominant form of folate in plasma, whereas 5,10-methyleneTHF, is found mainly intracellularly. 5-MethylTHF provides the methyl group for de novo methionine synthesis and DNA methylation,[16] (*Figure 1*) whereas the substrate for MTHFR, 5,10-methyleneTHF, is required for conversion of deoxyuridylate to thymidylate and can be oxidized to 10-formylTHF for de novo purine synthesis[6] (*Figure 1*). Therefore, 5,10-methyleneTHF is critical in DNA biosynthesis as well as maintaining deoxynucleotide pool balance.[6] A common mutation (^{677}C to T, alanine-to-valine) has been identified in the MTHFR gene.[209,210] This mutation causes reduced enzyme activity,[210] leading to lower levels of circulating folate (5-methylTHF), an accumulation of 5,10-methyleneTHF, and increased plasma homocysteine levels.[210–213] This mutation has been proposed as a risk factor for cardiovascular disease and for neural tube defects associated with low folate intake.[210–213]

The relationship of a common polymorphism (^{667}C to T: alanine-to-valine) of the MTHFR gene and the risk of colorectal cancer was examined in two published studies.[214,215] In the first study, MTHFR genotypes were ascertained in 144 men previously diagnosed with colorectal cancer and 627 controls in the Health Professionals Follow-up Study.[214] Individuals with the MTHFR variant homozygous (*val/val*) genotype had a 40% reduction in the risk of developing colorectal cancer compared with those with the MTHFR heterozygous (*val/ala*) or normal (*ala/ala*) genotypes (OR = 0.57; 95% CI, 0.30–1.06) after adjustment for confounding factors.[214] However, high alcohol or low methionine intake abolished this reduction in the risk of colorectal cancer in individuals with the MTHFR *val/val* genotype.[214] In a subsequent nested case-control study within the Physicians' Health Study, the same group of investigators examined the association of MTHFR mutation and the risk of colorectal cancer in 202 male physicians with colorectal cancer and 326 cancer free controls.[215] In this study, men with the homozygous mutation had half the risk of colorectal cancer (OR = 0.49, 95% CI, 0.27–0.87)

compared with the homozygous normal or heterozygous genotypes.[215] Among men with adequate folate levels, there was a threefold decrease in colorectal cancer risk (OR = 0.32, 95% CI, 0.15–0.68) among men with the homozygous mutation compared with those with the homozygous normal or heterozygous genotype.[215] The protection due to the mutation was absent in men with folate deficiency.[215] Again, alcohol consumption was shown to negate some of the protective effect of the homozygous mutation.[215] These investigators speculated that when dietary methyl supply is high, MTHFR *val/val* individuals may be at reduced risk of colorectal cancer because higher intracellular level of 5,10-methyleneTHF may prevent imbalances of nucleotide pools during DNA synthesis[214,215] (*Figure 1*). In contrast, when 5-methylTHF is depleted by alcohol consumption or low folate diet, benefit of MTHFR *val/val* genotype is offset. In this instance, abnormal DNA synthesis and deoxynucleotide pool imbalance resulting from depleted intracellular 5,10-methyleneTHF may become the primary mechanisms of colorectal carcinogenesis[214,215] (*Figure 1*). Furthermore, in this instance, DNA methylation may be affected because of reduced levels of 5-methylTHF resulting from insufficient supply from diet and reduced de novo methionine synthesis due to the MTHFR mutation[214,215] (*Figure 1*).

Other mechanisms

Several less well defined mechanisms have been proposed to be operative in folate deficiency-mediated carcinogenesis.[8–12] Folate supplementation has been observed to suppress carcinogen-induced ornithine decarboxylase activity, an enzyme associated with hyperproliferation in colorectal mucosal explants of rats,[216] suggesting possible antiproliferative properties associated with folate supplementation.

Adverse effects of folate deficiency on immune functions have been described. These include decreased number of T cells, reduced cytotoxic activity against foreign transplanted cells, a decrease in stimulation of T lymphocytes by phytohemagglutinin, and possibly a reduction in humoral immunity.[217] With respect to carcinogenesis, natural killer cells, a subset of lymphocytes, are considered to be responsible for the surveillance and destruction of arising clones of neoplastic cells.[218] Severe folate deficiency has previously been shown to suppress the ability of lymphocytes to kill heterologous cells to which the rodents had previously been sensitized.[219] However, in a rodent study recently conducted in our laboratory, severe, but not moderate, folate deficiency produced impaired natural killer cell activity compared with controls (unpublished data). Therefore, although severe folate deficiency may have adverse effects on immune functions, including natural killer cell activity, mild to moderate folate depletion, which is often associated with the increased risk of colorectal cancer, appears not to play a major role in immune dysfunction.

Another, yet unproven, mechanism by which diminished folate status may enhance the development of colorectal cancer is a secondary depletion of choline. Folate and choline are intimately interrelated in methionine biosynthesis from homocysteine by providing two separate, but closely related, transmethylation pathways (*Figure 1*). It has previously been shown that perturbations in one pathway apparently affect the other.[220,221] In this regard, it has been observed that severe folate deficiency causes secondary depletion of choline and its metabolites in rat liver.[166] Choline deficiency, in turn, is involved in activation of protein kinase C (PKC).[222] PKC is a secondary transcellular signal, an activation or compartmental shift of which is considered to play a role in carcinogenesis including that of the colorectum.[223,224] Furthermore, activation of PKC is associated with mitogenesis and enhanced expression of the c-myc protooncogene.[225,226] One of the most convincing pieces of evidence for PKC's role in carcinogenesis is that fibroblasts transfected with a mutant form of PKC that is continuously activated acquire a neoplastic phenotype.[227] However, the effects of folate deficiency on choline metabolism in tissues other than the liver and in species other than rats are unknown at present. It is uncertain whether a perturbation of folate metabolism or folate deficiency is capable of increasing the utilization of choline in such a way that it leads to depletion of choline in tissues other than the liver.

In summary, the proposed candidate mechanisms by which folate deficiency may enhance colorectal carcinogenesis provide biological explanations for a causal relationship between diminished folate status and the development of certain cancers. Some of these mechanisms have been extensively studied whereas others still remain speculative and untested. No one mechanism is likely responsible for the enhancement of carcinogenesis; rather, multiple factors interacting with one another are required to produce the effect.

Conclusion

A growing body of epidemiologic, clinical, and animal studies have implicated folate deficiency in carcinogenesis. The most compelling evidence in this regard exists for colorectal cancer. However, the association between folate deficiency and other cancers is significantly weaker and less certain at present. It appears that folate deficiency alone is not a sufficient causal factor for carcinogenesis, but it likely acts as a potentiator or co-carcinogen with other risk factors. In addition, only a modest reduction in folate status appears to be all that is necessary for the enhancement of carcinogenesis, even in the range conventionally accepted as normal. The association observed for folate in many of the epidemiologic studies is potentially confounded by other macro- and micronutrients in the same foods such as fruits and vegetables. Therefore, the cancer-inhibitory effects of folate may be a result of still undetermined interactions between folate and potentially anticarcinogenic agents that are found in these food sources including antioxidants, fiber, and other methyl donors (vitamin B_{12}, choline, methionine). Simultaneous consideration of, and adjustment for, several relevant macro- and micronutrients in dietary folate analyses would lead to a greater understanding of the interrelationships. Clinical trials involving supplementation provide the most direct evidence concerning the cancer-inhibitory effects of folate. Several potential mechanisms by which folate deficiency increases the risk of cancer have emerged and have been under intense investigation. It remains to be seen whether these mechanisms will be able

to account for this provocative association. What has been the most appealing in the area of folate and cancer is the potential application of folate as a chemopreventive agent. Folate is an ideal candidate for chemoprevention: It is inexpensive and has no side effects except possibly lowering the seizure threshold and masking the hematologic manifestations of vitamin B_{12} deficiency, enabling the neurologic sequelae to proceed.[228] The optimal dose of folate supplementation, however, must be determined for folate chemoprevention to be effective and safe in humans because animal studies have suggested that supraphysiologic levels of folate supplementation do not confer protection and, in some cases, may enhance carcinogenesis. Recent studies have revealed that up to 30% of healthy, ambulatory populations in the United States have a subclinical, but biochemically evident, degree of folate deficiency as indicated by elevated serum homocysteine concentrations.[229] Although the jury is still out, the implications for folate chemoprevention of cancer remain provocative.

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