Review

Folate and colorectal cancer: An evidence-based critical review

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Currently available evidence from epidemiologic, animal, and intervention studies does not unequivocally support the role of folate, a water-soluble B vitamin and important cofactor in one-carbon transfer, in the development and progression of colorectal cancer (CRC). However, when the portfolio of evidence from these studies is analyzed critically, the overall conclusion supports the inverse association between folate status and CRC risk. It is becoming increasingly evident that folate possesses dual modulatory effects on colorectal carcinogenesis depending on the timing and dose of folate intervention. Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established colorectal neoplasms. In contrast, folate deficiency in normal colorectal mucosa appears to predispose it to neoplastic transformation, and modest levels of folic acid supplementation suppress, whereas supraphysiologic supplemental doses enhance, the development of cancer in normal colorectal mucosa. Several potential mechanisms relating to the disruption of one-carbon transfer reactions exist to support the dual modulatory role of folate in colorectal carcinogenesis. Based on the lack of compelling supportive evidence and on the potential tumor-promoting effect, routine folic acid supplementation should not be recommended as a chemopreventive measure against CRC at present..

Keywords: Carcinogenesis / Chemoprevention / Colorectal cancer / Folate / Folic acid

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1 Folate and human health

Folate is a water-soluble B vitamin that is present naturally in foods such as green leafy vegetables, asparagus, broccoli, Brussels sprouts, citrus fruit, legumes, dry cereals, whole grain, yeast, lima beans, liver, and other organ meats [1]. Folate is the generic term referring to compounds that have similar chemical structures and nutritional properties. Folic acid is the fully oxidized monoglutamyl form of this vitamin that is used commercially in supplements and in fortified foods. Folic acid consists of three moieties: the pterin (or pteridine) ring, which is conjugated to *para*-aminobenzoic acid (PABA) by a methylene bridge, which is in turn joined to a glutamic residue *via* a peptide bond (Fig. 1) [2]. All naturally occurring folates found in food differ from the oxidized folic acid in the oxidation state of the pteridine ring and are typically reduced [2]. Furthermore, one-carbon units can be linked to tetrahydrofolate (THF) at the N-5 and N-10 positions, which confers folate the role of mediating the transfer of one-carbon units (Fig. 1) [2]. In addition, multiple glutamate residues of varying numbers (up to 9) can be added *via* a γ-peptide linkage [2]. Except for the *de novo* synthesis by intestinal microflora, some of which is incorporated into the host's tissues [3, 4], mammals are generally unable to synthesize folate [2]. They have the ability to synthesize all of the components but lack the enzyme required for coupling the pteridine ring to PABA [2]. Thus, the daily folate requirement must be obtained from dietary or supplemental sources.

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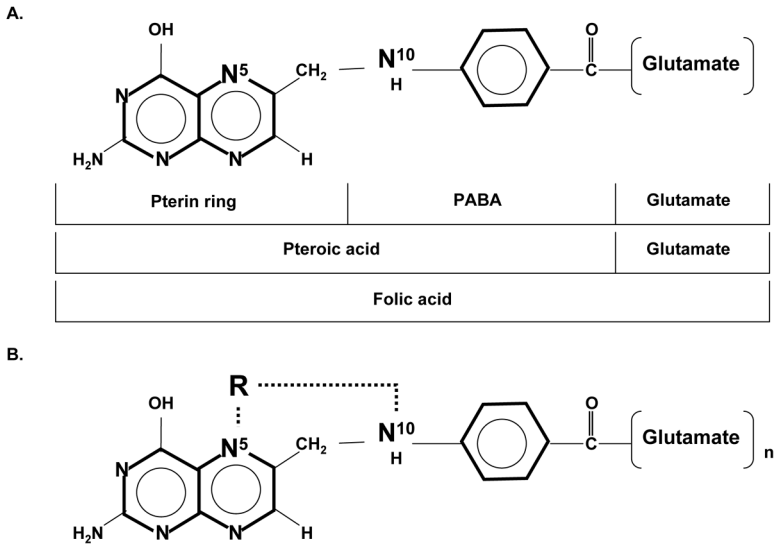
Abbreviations: ACF, aberrant crypt foci; **BDR,** basal daily requirement; **CI,** confidence interval; **CRC,** colorectal cancer; **DFE,** dietary folate equivalents; **DHFR,** dihydrofolate reductase; **DNMT,** DNA methyltransferase; **FPGS,** folylpolyglumate synthase; **FR,** folate receptors; **GGH,** γ-glutamyl hydrolase; **HR,** hazard ratio; **MTHFR,** methylenetetrahydrofolate reductase; **MTR,** methionine synthase; **NTD,** neural tube defects; **OR,** odds ratio; **PABA,** *para*-aminobenzoic acid; **RFC,** reduced-folate carrier; **RR,** relative risk; **SAM,** *S*-adenosylmethionine; **SEPB,** surrogate end point biomarkers; **SHMT,** serine hydroxymethyltransferase; **THF,** tetrahydrofolate; **TS,** thymidylate synthase; **UC,** ulcerative colitis



R = CH_3 (N^5), CHO (N^5 & N^{10}), CH=NH (N^5), CH_2 (N^5 & N^{10}) and CH= (N^5 & N^{10})

Figure 1. Chemical structures of folic acid (A) and folate (B). Folic acid consists of three moieties: the pterin (or pteridine) ring, which is conjugated to PABA by a methylene bridge, which is in turn joined to a glutamic residue *via* a peptide bond. Folic acid is the fully oxidized monoglutamyl form of this vitamin that is used commercially in supplements and in fortified foods. Folate is the generic term referring to compounds that have similar chemical structures and nutritional properties. All naturally occurring folates found in food differ from the oxidized folic acid in the oxidation state of the pteridine ring and are typically reduced. Furthermore, one-carbon units (R) can be linked to THF at the N-5 and N-10 positions, which confers folate the role of mediating the transfer of one-carbon units. In addition, multiple glutamate residues of varying numbers (up to 9) can be added *via* a γ-peptide linkage.

Naturally occurring folates are very unstable and rapidly lose their activity in foods over a period of days or weeks and are easily oxidized under low pH [1]. Approximately 50–75% of the original folate values are lost through food harvesting, storage, processing, and preparation [1]. Folate bioavailability varies widely depending on the food source and food preparation method [1]. In contrast, folic acid is highly stable for months or even years and has higher bioavailability compared with naturally occurring folates, especially when taken on an empty stomach [1]. The Recommended Dietary Allowance (RDA) for both men and women in North America is 400 μg/day of dietary folate equivalents (DFEs) [1]. DFEs adjust for the nearly 50% lower bioavailability of food folate compared with that of folic acid: 1 μg of DFE = 0.6 μg of folic acid from fortified food or as a supplement taken with meals = 1 μg of food folate = 0.5 μg of a supplement taken on an empty stomach [1].

The role of folate in human health and disease has been rapidly evolving beyond the prevention of macrocytic anemia. Folate deficiency appears to play an important role in the pathogenesis of several disorders in humans including atherosclerosis, neural tube defects (NTD) and other congenital defects, adverse pregnancy outcomes, neuropsychiatric and cognitive disorders, and cancer [5, 6]. The expanding role of folate nutrition in health and disease has major public health implications. For example, evidence from intervention trials and observational studies for a protective effect of folate supplementation on NTD [7–9] was considered to be sufficiently conclusive and led public health policy makers, including the US Public Health Service in 1992 and the Institute of Medicine in 1998, to recommend that all women who were of reproductive age or were capable of becoming pregnant consume daily 400 μg of folic acid from supplements or fortified foods in conjunction with consumption of folate-rich foods [1, 10]. This recommendation was followed by the US Food and Drug Administration to issue a regulation in 1996 requiring that all flour and uncooked cereal-grain products in the United States be fortified with folic acid (140 μg/100 g) by January 1998 [11]. Mandatory fortification was also implemented in Canada in 1998 [12]. Mandatory fortification is to provide on average 100 μg additional folic/day, with only a very small proportion of the population receiving > 1 mg [13], the tolerable upper intake level of folate arbitrarily chosen by the Institute of Medicine as unlikely to produce masking vitamin B_{12} deficiency [1]. The effectiveness of folic acid fortification in improving folate status has been quite striking, with a dramatic increase in blood measurements of folate and a substantial decrease in plasma homocysteine (an accurate inverse indicator of folate status) concentrations in the US and Canada [14–20]. In the National Health and Nutrition

Examination Survey 1999–2000, after folic acid fortification began, 23% of the US population, 43% of children aged ≤5 years, and 38% of the elderly persons had high serum folate concentrations (>45.3 nmol/L) [20]. The serum folate concentrations that should be considered excessively high and the potential health effects of these high concentrations are unclear at present. Preliminary reports suggest a significant reduction (~15–50%) in the prevalence and incidence of NTD in the US and Canada [21–26].

In the US and Canada, the average postfortification total folate intake is estimated to be ~400 μg/day in supplement nonusers with ~200 μg/day consumed a naturally occurring folate food and ~200 μg/day as folic acid provided in enriched products [16, 27]. For those taking multivitamins containing folic acid, the estimated total folate intake is ~800 μg/day. Several studies that assessed food composition and dietary intakes have suggested that the increased postfortification folate intake in the US population may be about twice that originally anticipated [16, 20, 27–29]. Furthermore, these estimates of folate intake based on food composition databases are likely to be underestimates because of limitations in the analytic methods previously used to analyze food folate [1]. From national surveys and large prospective studies, it is evident that up to 30–40% of the US and Canadian populations consume multivitamins containing ≥400 μg folic acid on a daily basis [30–35]. National Health and Nutrition Examination Survey reports that 63% of individuals over age 60 years take a dietary supplement: 40% folic acid containing multivitamins, 7% B-complex vitamins, and 2% folic acid supplements [35]. Higher supplemental levels of folic acid (1–5 mg/day) are routinely provided to certain subgroups of patients who are taking antifolate-based medications (*e.g.*, methotrexate for rheumatoid arthritis, psoriasis, or Crohn's disease, sulfasalazine for ulcerative colitis (UC)) to minimize or prevent adverse effects relating to folate depletion [36–39]. Even higher supplemental levels of folic acid in the range of 5–15 mg/day (in some cases even higher levels up to 40–50 mg/day) are given to patients with chronic renal failure on dialysis and renal transplant patients who are often hyperhomocysteinemic and are at high risk of developing premature atherosclerotic complications [40]. It has been reported that 30–60% of cancer patients use vitamin supplementation or megavitamin therapy [41, 42]. Studies have shown that ~10% of folic acid supplement users in the general population have daily intake of folate exceeding the Dietary Reference Intakes upper limit (1 mg/day) [43, 44].

2 Folate metabolism and biochemical function

The bioavailability of folate depends in part on the ability of the host to hydrolyze the polyglutamate chain because folate is unable to cross the cell membrane when the glutamate tail is longer than 3 [2]. In the human small intestine, where folate is absorbed, this hydrolysis is catalyzed by glutamate carboxypeptidase II (GCPII), an exopeptidase that is anchored to the intestinal apical brush border membrane [45]. This hydrolytic step is followed by membrane transport of monoglutamyl folate into cells by three mechanisms (Fig. 2) [46, 47]. The reduced-folate carrier (RFC) is a facilitative anion exchanger that mediates folate delivery into a variety of cells of different origin [46, 47]. RFC has a much higher affinity for reduced folates (Km = 1–5 μM), including the physiological substrate 5-methyltetrahydrofolate (5-methylTHF; the predominant folate found in serum), than folic acid (Km = 100–200 μM) [46, 47]. Folate receptors (FR), which are anchored to cell membranes through a glycosylphosphatidylinositol moiety, transport folates *via* an endocytotic process [46, 47]. These receptors have a very high affinity for folic acid (Km < 1 nM) and a lesser, but still high, affinity for 5-methylTHF (Km = 3 nM) [46, 47]. The third pathway for cellular folate transport *via* passive diffusion is only documented as a pharmacological effect [46, 47]. RFC is ubiquitously expressed in normal epithelial cells and in cancers of epithelial origin [46, 47]. Among several human FR isoforms, FR-α is primarily involved in folate transport in epithelial membranes; it is moderately expressed in certain normal epithelial cells and is markedly elevated in several carcinomas, particularly ovarian and uterine carcinomas [46, 47]. RFC and FR-α appear to localize to different regions in polarized cells (*i.e.*, apical *vs.* basolateral membranes), suggesting these spatial relationships may play a role in the vectorial transport of folate [48].

Whereas monoglutamates are the only circulating forms of folate in blood and is the only form of folate that is transported across the cell membrane, once taken up into cells, cellular folate exists primarily as polyglutamates (Fig. 2) [2]. Intracellular folate is converted into polyglutamates by folylpolyglumate synthase (FPGS), while γ-glutamyl hydrolase (GGH) removes the terminal glutamates that are attached to the proximal glutamate residue (Fig. 1) [2]. The polyglutamylation of cellular folates is a form of metabolic trapping, allowing the retention of folate that would otherwise be lost to efflux from cells [2]. Polyglutamylated folates are better retained in cells and are better substrates than monoglutamates for intracellular folate dependent enzymes [2].

The sole biochemical function known for folate is mediating the transfer of one-carbon units involved in nucleotide biosynthesis, methionine cycle, and biological methylation reactions (Fig. 2) [2]. In the methionine cycle, 5-methylTHF transfers one methyl group to homocysteine to synthesize methionine, thereby ensuring the provision of *S*-adenosylmethionine (SAM), the primary methyl group donor for most biological methylation reactions, including that of DNA [49, 50]. The remethylation of homocysteine to methionine is catalyzed by methionine synthase (MTR),

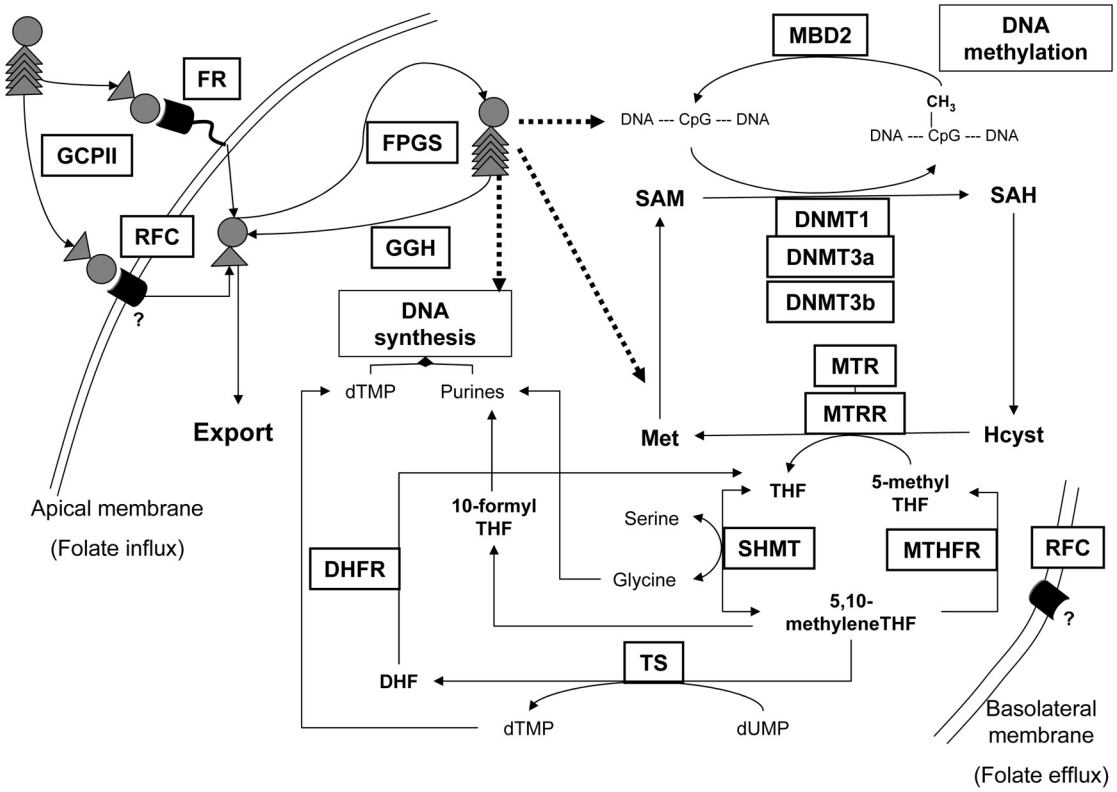


Figure 2. Simplified scheme of intracellular folate metabolism and one-carbon transfer reactions in epithelial cells, highlighting the genes that are involved in intraluminal folate hydrolysis (GCPII, glutamate carboxypeptidase II), intracellular folate uptake (FR-α, folate receptor; RFC, reduced folate carrier), intracellular folate retention (FPGS, folylpolyglutamyl synthase) and hydrolysis and efflux (GGH, γ-glutamyl hydrolase), methionine cycle (MTR, methionine synthase; MTRR, methionine synthase reductase; MTHFR, methylenetetrahydrofolate reductase), maintenance of intracellular folate pool (DHFR, dihydrofolate reductase; SHMT, serine hydroxylmethyltransferase) and nucleotide biosynthesis (TS, thymidylate synthase), DNA methylation (DNMT1, 3a, 3b, CpG methyltransferases), and DNA demethylation (MBD2, DNA demethylase). Intracellular folate exists primarily as polyglutamates. Intracellular folate is converted to polyglutamates by FPGS, while GGH removes the terminal glutamates. Polyglutamylated folates are better retained in cells and are better substrates than monoglutamates for intracellular folate dependent enzymes involved in one-carbon transfer reactions. Folate mediates the transfer of one-carbon units necessary for DNA synthesis, methionine cycle, and biological methylation reactions. CH_3, methyl group; CpG, cytosine – guanine dinucleotide sequence; dTMP, deoxythymidine-5-monophosphate (thymidylate); dUMP, deoxyuridine-5-monophosphate; Hcyt, homocysteine; Met, methionine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate. Filled circle represents a pteridine ring conjugated to PABA. Each filled triangle represents a glutamate, which is linked *via* a peptide bond to form various chain lengths of polyglutamylated folate.

a vitamin B_{12} (cobalamin) dependent enzyme [51]. The reductive methylation of the cobalamin cofactor of MTR to its active state is catalyzed by methionine synthase reductase (MTRR) [52]. After donating the methyl group, 5-methyl THF is converted to THF and is subsequently converted to 5,10-methylene THF by serine hydroxymethyltransferase (SHMT). SHMT catalyzes the reversible interconversion of serine and THF to glycine and 5,10-methylene THF and serves as a major entry point for one-carbon units into the folate pathway [53, 54]. 5,10-MethyleneTHF is a key substrate in folate metabolism, which can be directed toward nucleotide (thymidylate and purines) biosynthesis or toward methionine regeneration [55]. The cellular concentration of 5,10-methyleneTHF appears to regulate the flux of this substrate into these different pathways [55]. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF [56]. The substrate 5,10-methyleneTHF is the methyl donor for the nonreversible methylation, catalyzed by thymidylate synthase (TS), of deoxyuridine-5-monophosphate (dUMP) to deoxythymidine-5-monophosphate (dTMP; thymidylate), a precursor for DNA synthesis [2]. The synthesis of thymidylate results in the oxidation of 5,10-methyleneTHF to the inactive dihydrofolate, which can be converted back to THF by dihydrofolate reductase (DHFR) [2]. Both THF and 5,10-methyleneTHF can enter the purine biosynthesis pathway by the addition of a formyl group [2].

Several lines of evidence indicate that folate metabolism is regulated such that SAM synthesis has a metabolic prior-

ity over thymidylate biosynthesis. Scott *et al.* [57] proposed that limited methyl group availability, caused by either folate or methionine deficiency, shifts the flux of one-carbon units among folate-dependent pathways such that folate cofactors are preferentially shuttled to the methionine cycle to protect methylation reactions and thereby suppress DNA synthesis. Similarly, Green *et al.* [55] predicted that folate coenzymes are preferentially directed toward SAM-dependent methylation reactions at low cellular folate concentrations. These authors also projected that MTHFR enzyme would be insensitive to changes in 5,10-methyleneTHF availability, whereas TS activity would be highly dependent on them [55]. Therefore, this model assumes that these two enzymes directly compete for a common cellular pool of 5,10-methyleneTHF [55].

3 Folate and cancer risk

Perhaps one of the most speculative and provocative new medical applications of folate nutrition is the potential role of folate as a cancer preventive agent [5, 58]. The concept that folate deficiency enhances, whereas folate supplementation reduces, the risk of neoplastic transformation appears counterintuitive and contradictory to our conventional understanding of folate biochemistry. Folate is an essential cofactor for the *de novo* biosynthesis of purines and thymidylate, and in this role, folate plays an important role in DNA synthesis and replication [58, 59]. Consequently, folate deficiency in tissues with rapidly replicating cells results in ineffective DNA synthesis. In neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, interruption of folate metabolism causes ineffective DNA synthesis, resulting in inhibition of tumor growth [58, 59]. Indeed, this has been the basis for cancer chemotherapy using a number of antifolate agents (*e.g.*, methotrexate) and 5-fluorouracil [58, 59]. Furthermore, folate deficiency has been shown to induce regression and suppress progression of pre-existing neoplasms in experimental models [60 – 62]. In contrast to the inhibitory and promoting effect of folate deficiency and supplementation, respectively, on established neoplasms, however, folate status appears to have an opposite effect in normal tissues. An accumulating body of epidemiologic, clinical, and experimental evidence suggests that folate deficiency in normal tissues appears to predispose them to neoplastic transformation, and folate supplementation suppresses the development of tumors in normal tissues [5, 58]. Epidemiologic studies collectively suggest an inverse association (in some cases dose-dependent) between folate status (measured by either folate intake (dietary and supplemental) or blood levels of folate) and the risk of several malignancies including cancer of the colorectum, oropharynx, esophagus, stomach, pancreas, lungs, cervix, ovary, and breast and neuroblastoma and leukemia [58, 59]. The precise nature and magnitude of the inverse relationship between folate status and the risk of these malignancies, however, have not been clearly established [58, 59].

The role of folate in carcinogenesis has been best studied for colorectal cancer (CRC) [9, 58, 63].

4 Folate and UC-associated colorectal carcinogenesis

The role of folate in colorectal carcinogenesis was first suggested in the setting of chronic UC. Chronic UC is associated with a 10 – 40-fold increased risk of developing CRC compared with the general population [64]. A recent *meta*-analysis of all published studies reporting a CRC risk in UC since 1925 has reported the risk for any patients with UC to be 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease [65]. Although megaloblastic anemia is rare, patients with UC often demonstrate depressed blood concentrations of folate due to the frequent use of sulfasalazine, a known folate antagonist, inadequate nutritional intake and intestinal losses from inflammation [58]. Lashner *et al.* [66] first reported that individuals with long standing UC taking folate supplementation had a nonsignificant 62% lower incidence of colorectal dysplasia and cancer compared with those not receiving folate supplementation (odds ratio (OR), 0.38; 95% confidence interval (CI), 0.12 – 1.20). In another study, the risk of colorectal dysplasia and cancer was found to be significantly decreased by 18% for each 10 ng/mL increase in red blood cell folate concentrations in patients with UC (OR, 0.82; 95% CI, 0.68 – 0.99) [67]. In another retrospective study, folic acid supplementation was inversely related to the risk of colorectal neoplasia in subjects with long standing UC in a dose-dependent manner (relative risk (RR), 0.54 and 0.76 for 1.0 and 0.4 mg folic acid/day, respectively) [68]. Although these studies included small sample sizes and were associated with inherent limitations associated with retrospective study designs, these studies nevertheless suggested a provocative inverse relationship between folate status and the risk of UC-associated CRC. These studies were the major impetus for the subsequent research activities in the field of folate and carcinogenesis.

The potential effect of folate supplementation on colorectal carcinogenesis in chronic UC was investigated in a recently developed and characterized genetically predisposed murine model, the IL-2 and β_2-microglobulin deficient (IL-$2^{null} \times \beta_2 m^{null}$) mouse [69]. IL-$2^{null} \times \beta_2 m^{null}$ mice develop mild-to-moderate colitis with diarrhea, mild wasting, and some rectal prolapse, usually between 8 and 12 wk, and most mice recover with normal stool, weight gain, and normal appearance and survive beyond 6 months, suggesting active disease followed by remission from colitis [70]. Histologically, 75% of these mice have mild-to-moderate colonic inflammation of the entire colon and 25% have no

inflammation at the time of necropsy [70]. Some of the IL-$2^{null} \times \beta_2m^{null}$ mice develop well to moderately differentiated adenocarcinoma in the proximal half of the colon between 6 and 12 months [71]. The molecular genetics of CRC arising in this model are sufficiently similar to those of human sporadic and UC-associated CRC [72]. Weaning IL-$2^{null} \times \beta_2m^{null}$ mice were randomly assigned to receive 0 (mild deficiency), 2 (basal dietary requirement, control), or 8 mg (supplemented) folic acid/kg diet for 32 wk [69]. The incidence of high-grade lesions (high-grade dysplasia, carcinoma *in situ*, and invasive adenocarcinoma) in the folate-supplemented group was 46% lower than that in the control group (35.3 *vs.* 65.1%, $p = 0.009$). Interestingly, the incidence of high-grade lesions in the folate-deficient group was also 49% lower than that in the control group (33.3 *vs.* 65.1%, $p = 0.007$). The higher mortality rate in the folate-deficient group than in the other two groups (25 *vs.* 6.5 and 5.6%, respectively, $p < 0.02$) partially accounted for the low incidence of high-grade lesions in this group. These data indicate that dietary folate supplementation at four times the basal dietary requirement significantly suppresses colorectal carcinogenesis associated with UC in this model. These data also suggest that folate deficiency may inhibit colorectal carcinogenesis in chronic UC, although the high mortality observed in the folate-deficient group precludes a definitive conclusion.

5 Epidemiologic evidence for the role of folate in sporadic colorectal carcinogenesis

Among >20 published retrospectively conducted epidemiologic studies that investigated the relationship between folate intake (dietary and/or total folate intake including supplemental folic acid) and the OR of CRC or its precursor, adenoma, the majority showed either a significant or equivocal inverse relationship that was not statistically significant, that became nonsignificant after adjustment, or that could not be distinguished from other factors in their relation to risk [9, 58, 63]. Some of these studies demonstrated site (*i.e.*, colon *vs.* rectum) and sex specificity and a dose-dependent association [9, 58, 63]. The relationship between blood levels of folate and the OR of CRC and adenoma is less well defined than that between folate intake and the OR of colorectal neoplasms [9, 58, 63]. Collectively, these retrospective studies suggest an ~40% reduction in the OR of colorectal neoplasms in subjects with the highest folate intake compared with those with the lowest intake [9, 58, 63]. These studies also suggest that a modest reduction in folate status without overt clinical evidence of folate deficiency is sufficient to enhance CRC [9, 58, 63]. The interpretation of results from case-control studies are often limited because of inherent problems associated with retrospective analyses, including the accuracy with which intake of dietary factors or supplementation can be established and inability to adequately control or correct potential confounding factors.

Several large prospective studies (*e.g.*, the Health Professional Follow-up Study, the Nurses' Health Study, the First National Health and Nutrition Examination Survey Epidemiologic Follow-up Study, the Iowa Women's Health Study, the Netherlands Cohort Study on Diet and Cancer, the Canadian National Breast Screening Study, the Breast Cancer Detection Demonstration Project Follow-up Study, and the Swedish Mammography Cohort Study) that avoid most of the methodological problems of case-control studies and that can control and correct confounding factors more adequately than can case-control studies have also confirmed the inverse association between folate consumption and the risk of CRC [31, 32, 73–80]. Collectively, these prospective studies suggest a 20–40% reduction in the RR of CRC and adenomas in those with the highest intake of folate compared with those with the lowest intake [31, 32, 63, 73–80]. A recent *meta*-analysis of 11 prospective epidemiologic studies from the US, Canada, Netherlands, and Sweden including more than 500 000 subjects demonstrated a significant inverse association between folate intake (dietary and supplemental) and the RR of CRC (David Hunter, PhD, presented at the 2003 Environmental Mutagen Society Colon Cancer Conference, Miami Beach, FL, May 14–16, 2003) [81]. This *meta*-analysis also showed a 20% reduction in the RR of CRC in subjects with the highest folate intake compared with those with the lowest intake.

Another recent *meta*-analysis of seven prospective and nine retrospective studies also confirmed the inverse relationship between folate intake and CRC risk [82]. In prospective studies, the association between folate consumption and CRC risk was stronger for dietary folate (RR for high *vs.* low intake = 0.75; 95% CI = 0.64–0.89) than for total folate intake (folate from food and supplements; RR for high *vs.* low intake = 0.95; 95% CI = 0.81–1.11] [82]. There was no significant heterogeneity between prospective studies. Among case-control studies, the overall OR for high *versus* low dietary folate intake was 0.76 (95% CI = 0.60–0.96) but there was significant heterogeneity between studies [82]. For total folate from food and supplements, the OR was 0.81 (95% CI = 0.62–1.05) among case-control studies [82].

One of the most supportive pieces of epidemiologic evidence comes from a prospective study involving 88 756 female nurses in the Unites States (the Nurses' Health Study), which has shown a 75% reduction in CRC risk in women using multivitamin supplements containing ≥400 μg folic acid for ≥15 years compared with those not taking folic acid after all the known confounding factors were controlled for [33]. Furthermore, the Cancer Prevention Study I, which examined the association between daily multivitamin use and colon cancer mortality among 806 397 US men and women in the Cancer Prevention Study II cohort

who completed a questionnaire at enrollment in 1982 and were followed for mortality through 1998, showed that the use of multivitamin supplements containing ≥400 μg folic acid for ≥15 years significantly decreased CRC mortality after multivariate adjustment (RR = 0.89; 95% CI = 0.80–0.99) [34]. This association was stronger among participants consuming two or more alcoholic drinks *per* day (RR = 0.71; 95% CI = 0.56–0.91) [34].

Although all the published epidemiologic studies to date have not reported a harmful effect of folate on CRC risk, a recently published study suggests for the first time a potential harmful effect of high folate status on CRC risk [83]. This population based nested case-control study in the Northern Sweden Health and Disease Cohort reports that plasma folate concentrations are significantly related to the risk of CRC in a bell-shaped manner; multivariate ORs were 2.00 (95% CI = 1.13–3.56) for the middle *versus* lowest quintile and 1.34 (95% CI = 0.72–2.50) for the highest *versus* the lowest quintile. Furthermore, in subjects followed for longer than the median of 4.2 years, plasma folate concentrations were strongly positively related to CRC risk; multivariate OR for the highest *versus* lowest quintile was 3.87 (95% CI = 1.52–9.87; *p*-trend = 0.007). The main and novel finding of this study is that low folate status might inhibit colorectal carcinogenesis and that high folate status may promote colorectal carcinogenesis. Therefore, this study contradicts the findings of other epidemiologic studies that showed the inverse relationship between folate status and CRC risk [84]. However, this study supports the findings from animal studies and clinical observations that suggest that folate status might promote colorectal carcinogenesis depending on the dose and timing of folate intervention [84], which will be discussed in more details in the following sections. The widely accepted notion that the folate status is inversely related to the risk of developing other cancers has begun to be challenged in recent epidemiologic studies. For example, the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (n = 25, 400 postmenopausal women) has reported that women consuming supplemental folic acid ≥400 μg/day have a 20% increased risk of developing breast cancer compared with subjects reporting no supplemental intake (hazard ratio (HR), 1.19; 95% CI, 1.01–1.41; *p*-trend = 0.04) [85]. Although food folate intake was not significantly related to breast cancer risk (HR, 1.04; 95% CI, 0.83–1.31; *p*-trend = 0.56), total folate intake, mainly from folic acid supplementation, significantly increased the risk by 32% (HR, 1.32; 95% CI, 1.04–1.68; *p*-trend = 0.03) [85].

In summary, a growing body of observational epidemiologic studies suggests that folate deficiency increases whereas folate supplementation decreases the risk of CRC. Although the results from these studies are not uniformly consistent, the portfolio of evidence strongly supports the inverse association between folate status and CRC risk. However, these observational epidemiologic studies only suggest this association and cannot definitively establish a causal relationship between folate status and CRC risk.

6 Localized folate deficiency and CRC risk

Each tissue expresses differential susceptibility to folate depletion, with the gastrointestinal mucosa being among the tissues most prominently affected by folate deficiency [86, 87]. Animal studies have shown that dietary folate deficiency significantly reduces colonic mucosal folate levels and that the extent of folate depletion and repletion in the colonic mucosa significantly correlated with dietary folate levels [88–96]. In human studies, blood measurements of folate and plasma homocysteine concentrations appear to accurately reflect colorectal mucosal folate concentrations [97, 98]. However, significant correlations between colorectal mucosal folate concentrations and systemic indicators of folate were observed only in individuals not ingesting supraphysiological quantities of folate [98]. Furthermore, some studies have suggested that plasma homocysteine may be the best systemic marker to accurately predict folate concentrations in the colorectum [97, 99].

One important observation from epidemiologic studies is that the relationship between conventional blood levels of folate and the risk of CRC and adenoma is less consistent than that compared with that between dietary intake and the risk of colorectal neoplasms [9, 58, 63]. The relative insensitivity of blood measurements of folate in predicting CRC risk may be related to observations indicating that mild folate depletion, rather than the development of overt systemic folate deficiency, is a sufficient condition to enhance the risk of CRC [58, 100]. Also, the effect of folate on the colorectal mucosa may not be derived entirely from blood folate, because the colorectal mucosa is exposed to intestinal luminal folate that is synthesized by intestinal microflora or which has escaped small intestinal absorption [4].

Some epidemiologic studies showed that subjects with colorectal neoplasms have only modestly, albeit statistically significant, depressed blood folate levels compared with those without colorectal neoplasms [9, 58, 63, 101]. These studies also suggested that depressed blood folate levels associated with increased CRC risk were still well within the ranges conventionally considered to be normal [9, 58, 63, 101]. These observations suggest that, even in the absence of overt systemic folate deficiency, localized folate deficiency may exist in the colorectum in subjects at increased CRC risk, predisposing it to a subsequent neoplastic transformation. In support of this, 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 non-neoplastic polyps in the absence of a significant depletion of serum or red blood cell folate concentrations [97]. The mean concentration of serum homocysteine, a

sensitive inverse indicator of folate status, was 22% higher in those with adenomas than in controls ($p = 0.04$) [97]. This study, therefore, suggests that localized folate depletion in the colorectal mucosa, regardless of the systemic folate status may be an important factor in colorectal carcinogenesis. However, another 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 [102]. 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 CRC remains unsettled. Nevertheless, the possibility that individuals at risk of developing CRC may have localized folate deficiency in the colorectal mucosa opens an important opportunity for further mechanistic studies. One hypothesis is that in predisposed individuals, intracellular uptake, retention and export, and metabolism of folate may be deranged, with consequent intracellular folate depletion in the colorectum.

Several studies have investigated correlations between systemic indicators of folate status and purported biomarkers of CRC in the colorectum. Two recent studies have shown that colonic DNA methylation was positively correlated with serum and red blood cell folate concentrations and negatively with plasma homocysteine concentrations in individuals with colonic adenomas and adenocarcinomas [103] and in those without these lesions [104]. In the Netherlands Cohort Study on Diet and Cancer, the prevalence of CpG island promoter hypermethylation was higher, albeit nonsignificant, in CRCs derived from patients with low folate/high alcohol intake compared with CRCs from patients with high folate/low alcohol intake for each of the six tested genes (*APC*, *p14*, *p16*, *hMLH1*, *O*^{6}*-MGMT*, and *RASSF1A*) [105]. The number of CRCs with at least one gene methylated was higher (84%) in the low folate intake/high alcohol intake group compared with the high folate intake/low alcohol intake group (70%; $p = 0.085$) [105]. However, a recent clinical study failed to demonstrate significant correlations between rectal mucosal folate concentrations and the number of rectal aberrant crypt foci (ACF), the probable earliest precursor of CRC [106], detected by magnifying chromoendoscopy in 83 subjects undergoing screening colonoscopy [99].

7 Epidemiologic evidence for folate–gene and folate–nutrient interactions in modulating sporadic colorectal carcinogenesis

In some epidemiologic studies, the observed inverse association between folate status and CRC risk was further modified by the intake of alcohol, a known folate antagonist [107], and other methyl group donors (*e.g.*, methionine, vitamins B_6 and B_{12}) that are involved in the folate metabolic pathway [58, 63, 101, 108].

Recent molecular epidemiologic studies also showed that the C677T polymorphism in the MTHFR gene may modulate CRC risk and that the direction and magnitude of the risk modification are influenced by folate status, alcohol consumption, and the supply of methyl group donors [56, 58, 63, 101, 108, 109]. Intracellular folate homeostasis depends on 5,10-MTHFR, a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methyleneTHF to 5-methylTHF (Fig. 2) [56, 109]. 5,10-MTHFR is critical to maintaining the balance of the nucleotide pool and to DNA synthesis while 5-methylTHF plays an important role in the provision of SAM necessary for most biological methylation reactions including that of DNA (Fig. 1) [56, 109].

The MTHFR C677T polymorphism, which results in an alanine to valine substitution [110, 111], is a common mutation with an allele frequency of about 35% in the general North American population [111, 112], and occurs frequently among Caucasian and Asian populations, with rates of ~12–15% for individuals who are homozygous (TT) for the mutation and up to 50% for individuals who are heterozygous (CT) [56]. However, there is considerable ethnic and geographic variation in the frequency of the MTHFR C677T variant [113, 114]. The TT prevalence ranges from around 1% in Black populations in the US, sub-Saharan Africa, and South America to >20% in US Hispanics, Columbians, and Amerindians in Brazil [114]. TT genotype frequency in White populations in Europe, North America, and Australia ranges from 8 to 20% [114].

This mutation is associated with reduced MTHFR activity and increased thermolability of MTHFR [110, 111], which lead to decreased 5-methylTHF and an accumulation of 5,10-methyleneTHF in red blood cells [115] and to increased plasma homocysteine concentrations in individuals with marginal folate status [111, 112, 116]. The MTHFR C677T polymorphism has been shown to be associated with the risk of several cancers in a site-specific manner [56, 109]. It appears to decrease the risk of CRC, hepatocellular carcinoma, and certain leukemias and lymphomas [56, 109]. In contrast, this polymorphism seems to increase the risk of cancer of the breast, endometrium, cervix, esophagus, stomach, pancreas, and bladder [56, 109]. For CRC, the portfolio of epidemiologic evidence indicates the protective effect of the MTHFR C677T polymorphism on CRC risk (~20% reduction in CRC risk) as well as the gene–nutrient interactions in modifying this risk [113, 117, 118]. For example, the protective effect of the MTHFR C677T was observed only in individuals with adequate or high status of folate and other nutrients involved in the folate metabolic pathway (*e.g.*, vitamins B_6 and B_{12}, riboflavin, and methionine) [63, 108, 113]. In those with an inade-

quate status of folate and related nutrients, the protective effect conferred by the MTFHR C677T polymorphism was completely abolished and in some cases, an increased risk of CRC was observed [63, 108, 113]. Also, there is a growing body of evidence that suggests that alcohol and smoking, which can antagonize folate absorption and metabolism, can interact with the MTHFR C677T polymorphism to modulate CRC risk [63, 108, 113]. For instance, high intake of alcohol abolished the reduced risk of CRC associated with the TT genotype to the extent that subjects with the TT genotype who consumed the largest quantities of alcohol were at the greatest risk of CRC (greater even than those without the T allele who were in the highest alcohol group) [119, 120].

The mechanisms by which the MTHFR C677T polymorphism could modulate the risk of CRC depending on the supply of folate and other nutrients and factors that are involved in the folate metabolic pathway have not yet been elucidated. One proposed mechanism suggests that when the dietary supply of folate and related nutrients is high, individuals with the MTHFR C677T polymorphism might be at reduced risk of CRC because higher intracellular levels of 5,10-methyleneTHF might prevent imbalances of nucleotide pool during DNA synthesis, thereby ensuring DNA replication with a high fidelity, stable DNA integrity, and decreased mutagenesis (Fig. 2) [63, 108, 113]. In contrast, when 5-methylTHF is depleted by low intake of folate and related nutrients, benefit of the MTHFR C677T polymorphism is offset. In this instance, nucleotide pool imbalance and abnormal DNA synthesis that result from depleted intracellular 5,10-methyleneTHF might become the primary mechanisms of carcinogenesis (Fig. 2). Furthermore, in this instance, DNA methylation might be affected because of reduced levels of 5-methylTHF resulting from insufficient supply from diet and reduced *de novo* methionine synthesis as a result of the MTHFR C677T polymorphism. DNA and chromosomal damages, genomic instability, and impaired DNA repair resulting from nucleotide pool imbalance are important mechanisms of colorectal carcinogenesis [121 – 123]. Both genomic DNA hypomethylation and promoter CpG island hypermethylation of tumor suppressor and mismatch repair genes are also important epigenetic mechanisms of carcinogenesis [50, 124].

In vitro studies using immortalized human lymphocytes have shown that the MTHFR C677T polymorphism does not significantly affect uracil misincorporation, chromosome damage, and other makers of genomic instability [125, 126], although these negative observations might have resulted from supraphysiologic levels of riboflavin and methionine in culture medium [127]. The effect of the MTHFR C677T polymorphism on uracil misincorporation, chromosome damage and other makers of genomic instability in peripheral lymphocytes or leukocytes in human studies has been conflicting [128 – 131]. In contrast, although not uniformly consistent [128], the portfolio of evidence suggests that the MTFHR C677T polymorphism induces genomic DNA hypomethylation in human leukocytes or lymphocytes, in particular in conjunction with low folate status [132 – 135]. The extrapolation of these observations made in lymphocytes or leukocytes to target organs of interest is questionable because the MTHFR C677T polymorphism appears to modulate cancer risk in a site-specific manner [56, 109]. In this regard, studies investigating the effect of the MTHFR C677T polymorphism on genomic instability in the colorectum are lacking, whereas few studies investigating the effect of the MTHFR C677T polymorphism on genomic and gene-specific DNA methylation in the colorectum have produced conflicting results [136 – 139].

8 Folate intervention studies in humans

Folic acid supplementation has been shown to significantly modulate several functional biomarkers of folate and one-carbon metabolism including DNA stability, DNA repair capacity, and DNA methylation in human lymphocytes or leukocytes [140 – 145]. Several small randomized, placebo-controlled intervention studies have examined the effect of folate supplementation on surrogate end point biomarkers (SEPB) of CRC in colorectal mucosa (Table 1). In a randomized, double-blind, placebo-controlled study ($n = 20$), folic acid supplementation at 5 mg/day for 1 year after removal of adenomas significantly increased serum (by six- to seven-fold), red blood cell (by two-fold) and colonic mucosal (by two- four-fold) folate concentrations and significantly decreased serum homocysteine levels (by 35%), indicating that oral folic acid supplementation can modulate concentrations of folate in the target organ [146]. Furthermore, folic acid supplementation significantly accelerated improvement in genomic DNA methylation and *p53* strand breaks in exons 5 – 8 in the normal rectal mucosa at 6 months compared with placebo, but these effects were no longer significantly different at 1 year [146]. Cravo *et al.* [147] observed that folic acid supplementation at a dose of 10 mg/day for 6 months in 22 patients with resected colonic adenoma or cancer significantly reversed genomic DNA hypomethylation in the normal rectal mucosa. During the washout period, DNA methylation values moved toward the initial values in most cases. In a subsequent study, Cravo *et al.* [148] observed that 3 months of folic acid supplementation (5 mg folate/day) significantly reversed genomic DNA hypomethylation in the normal rectal mucosa in 20 patients with single, but not multiple, resected colorectal adenomas compared with placebo. A study from the United Kingdom [149] demonstrated that folic acid supplementation at 2 mg/day for 3 months in 11 subjects with previously resected colorectal adenomas significantly decreased rectal mucosal cell proliferation, another purported SEPB of CRC [150]. Another study demonstrated that folic acid supplementation (5 mg/day) for 1 year significantly prevented

Table 1. Summary of randomized, double-blind, placebo-controlled clinical trials of folic acid chemoprevention of CRC

Study (reference)	Subjects (*n*)	Dose	Duration	End point	Outcome
Kim *et al.* [146]	Adenomas ($n = 20$)	5 mg/day	6 months and 1 year	Rectal mucosal genomic DNA methylation	57% increase in DNA methylation at 6 month ($p = 0.001$) but no difference at 1 year
				53 exons 5–8 strand breaks	No significant difference in p53 strand breaks compared with placebo
Cravo *et al.* [147]	CRC or adenomas ($n = 22$)	10 mg/day	6 months	Rectal mucosal genomic DNA methylation	93% increase ($p<0.002$)
Cravo *et al.* [148]	Adenomas ($n = 20$)	5 mg/day	3 months	Rectal mucosal genomic DNA methylation	37% increase in patients with 1, but not multiple, adenoma ($p = 0.05$)
Khosraviani *et al.* [149]	Adenomas ($n = 11$)	2 mg/day	3 months	Rectal mucocal cell proliferation (LI)	20% decrease in overall LI; most pronounced decrease in the upper 1/3 of the crypt
Nagothu *et al.* [151]	Adenomas ($n = 20$)	1 mg/day	1 year	LOH of DCC, APC, p53	100% protection of LOH of DCC (no effect on APC or p53)
				DCC protein expression	Significant increase in DCC protein expression ($p < 0.02$)
				Colonic mucosal cell proliferation (LI)	16% reduction in LI ($p< 0.05$)
Pufulete *et al.* [152]	Adenomas ($n = 31$)	400 μg/d	10 wk	Rectal mucosal genomic DNA methylation	25% increase ($p = 0.09$)
Bruce *et al.* [153]	CRC or adenomas ($n = 98$)	3 mg/day + calcium carbonate + Ω–3 fish oil	28 days	Biomakers of insulin resistance, fecal calprotectin, C-reactive protein	18% decrease in free fatty acid ($p = 0.013$); no effects on other markers
Biasco *et al.* [154]	Chronic UC ($n = 24$)	15 mg/day (folinic acid)	3 months	Rectal mucosal cell proliferation (LI)	44% decrease in LI in the upper 40% of the crypt
Cravo *et al.* [155]	Chronic UC ($n = 25$)	5 mg/day	6 months	Rectal mucosal genomic DNA methylation	No change
Paspatis and Karamanolis [162]	Adenomas ($n = 60$)	1 mg/day	2 years	Adenoma recurrence	40 and 46% reduction at 1 and 2 years, respectively (p = NS)
Jaszewski *et al.* [163]	Adenomas ($n = 93$)	5 mg/day	3 years	Adenoma recurrence	Significant reduction (0.456 *per* patient *vs.* 0.851 *per* patient ($p<0.05$))
Cole *et al.* [164]	Adenomas ($n = 1021$)	1 mg/day	6 years	Adenoma recurrence	Incidence: RR = 1.04 (95% CI = 0.90–1.20) Number: RR = 1.44 (95% CI = 1.03–2.02) Incidence of advanced adenomas: RR = 1.31 (95% CI = 0.90–1.89)

LI, labeling index; LOH, Loss of heterozygosity.

loss of heterozygosity of the DCC tumor suppressor gene and significantly increased the DCC protein expression compared with placebo in 20 patients with resected colonic adenomas [151]. Folic acid supplementation had no significant effect on allelic status of either the APC or p53 tumor suppressor genes [151]. This trial also showed that folic acid supplementation significantly reduced the mucosal cell proliferation by 16% [151]. A recent study showed that a physiologic dose of folic acid (400 μg/day) for 10 wk increased genomic DNA methylation in lymphocytes (by 31%; $p = 0.05$) and in colonic mucosa (by 25%; $p = 0.09$) compared with placebo in patients with colorectal adenomas [152]. In this study, this dose of folic acid supplementation was sufficient to significantly increase serum (by 81%) and red blood cell (by 57%) folate concentrations and significantly decrease plasma homocysteine concentrations (by 12%). A recent pilot trial randomized 98 subjects with previous colonic adenomas or intramucosal carcinomas to a combined treatment of supplementary folic acid (3 mg/day), omega-3 fish oil and calcium carbonate, or placebo for 28 days [153]. This supplemental strategy significantly increased serum folate by two-fold and nonsignificantly decreased plasma homocysteine by 3% [153]. Furthermore, this strategy modestly improved some of the biomarkers of insulin resistance but had no effect on those associated with colon-specific or generalized inflammation [153].

Folate chemoprevention trials were also performed in patients with chronic UC at risk of colorectal dysplasia and

cancer (Table 1). In one trial, folinic acid (leucovorin or 5-formylTHF, a precursor of 5,10-methyleneTHF) significantly reduced cell proliferation in the upper portion of the colonic crypts [154]. In a study done in Portugal, however, folic acid supplementation at 5 mg/day for 6 months failed to reverse genomic DNA hypomethylation in patients with chronic UC [155]. In a case report, folic acid supplementation at 5 mg/day for 6 months was shown to partially correct microsatellite instability, the hallmark of DNA mismatch repair defects observed in hereditary, sporadic, and UC-associated colorectal carcinogenesis [156, 157], in a patient with chronic UC [158]. In a case-control study, Lashner *et al.* [39] showed that folic acid supplementation protected against the development of *p53* mutations, a common and early event in UC-associated colorectal carcinogenesis [159], in subjects with chronic UC.

The number of subjects studied in the aforementioned trials was too small, the duration of follow-up was relatively short, and most of these studies used less well-established SEPB of CRC instead of adenoma or cancer occurrence or recurrence as the endpoint of the trial. Therefore, it is difficult to draw any definitive conclusions about the chemopreventive role of folate supplementation in colorectal carcinogenesis from these small trials. In theory, large, randomized, double-blind, placebo-controlled folate chemoprevention trials in humans should provide definitive support for the purported causal relationship between folate status and CRC. However, even these trials may not be able to provide definitive conclusions about the chemopreventive role of folate supplementation in colorectal carcinogenesis for several reasons. First, these intervention studies attempt to intervene in incompletely understood biological pathways in special populations of adults at high risk of developing CRC (*i.e.*, individuals with previously resected colorectal adenomas) who therefore may be at a late, although preclinical, stage of colorectal carcinogenesis. Second, the time between the change in the level of folate intake and any expected change in the incidence of CRC (*i.e.*, relevant induction time) is usually uncertain and trials of 3 – 5 years may not be sufficiently long for any significant effect associated with folate supplementation to be apparent. Third, these trials use SEPB of CRC as the outcome instead of using occurrence or recurrence of cancer. All SEPB have limitations and most have not been conclusively validated in clinical studies [160]. Except for a few biomarkers (*e.g.*, adenomas [161]), modulating any of these SEPB has not yet unequivocally been shown to reduce CRC incidence and mortality [160]. Even for adenomas, the risk of progression to adenocarcinoma depends on the histologic type, size, and number and hence all adenomas cannot be considered to possess a similar CRC risk [161].

Folate chemoprevention trials using colorectal adenoma as the endpoint have also been conducted (Table 1). Investigators from Greece reported a study involving 60 subjects with colorectal adenomas: folic acid supplementation (1 mg/day for 2 years) after polypectomy decreased adenoma recurrence by 46% compared with placebo, although this difference was not statistically significant [162]. A preliminary report of another double-blind, placebo-controlled trial showed that folic acid supplementation at 5 mg/day for 3 years in subjects with resected adenomas ($n = 93$) significantly reduced the number of recurrent adenomas (0.456 *per* patient in the folic acid supplemented group *vs.* 0.851 *per* patient in the placebo group; $p < 0.05$) [163]. However, the final results of this trial have not been reported or published as yet. The Aspirin-Folate Polyp Prevention Study has recently reported that folic acid supplementation (1 mg/day) for up to 6 years in subjects with previous colorectal adenomas ($n = 1021$) did not significantly prevent the recurrence of colorectal adenomas (RR = 1.04) [164]. However, folic acid supplementation significantly increased the number of adenomas by 44% (RR = 1.44; 95% CI = 1.03 – 2.02) and nonsignificantly increased the incidence of advanced adenomas with a high malignant potential compared with placebo [164]. One explanation for this unexpected observation is that folic acid supplementation might have promoted the progression of already existing, undiagnosed preneoplastic lesions (*e.g.*, ACF or microscopic adenomas), or adenomas missed on initial colonoscopy in these predisposed patients at high risk of developing adenomas and CRC. This hypothesis is supported by prior observations that addition of folate to established tumors causes an "acceleration phenomenon" in humans. In the 1940s, children with acute leukemia treated with folate supplementation experienced an accelerated progression of leukemia [165].

In addition, several randomized studies have investigated the effect of folic acid supplementation on cancer risk as a secondary endpoint. Two recently published large, randomized, placebo-controlled intervention trials designed to test the effect of folic acid supplementation in conjunction with other B vitamins on primary and secondary prevention of cardiovascular events have reported a nonsignificant trend toward an increased risk of total cancer (RR, 1.22; 95% CI, 0.88 – 1.70) in the Norwegian Vitamin (NORVIT) trials ($n = 3749$; 800 μg folic acid/day for 40 months) [166] and of colon cancer (RR, 1.36; 95% CI, 0.89 – 2.08) in the Heart Outcomes Prevention Evaluation (HOPE) II trial ($n = 5522$; 2.5 mg folic acid/day for 5 years) [167]. Among participants in a large ($n = 2928$) trial of folic acid supplementation during pregnancy, women who received 5 mg folic acid/day had a 70% increased risk of total cancer mortality compared with those not on supplementation (HR = 1.70; 95% CI = 1.07 – 2.72) [168].

In summary, although small human intervention trials have suggested potential beneficial effects of folic acid supplementation on functional biomarkers of folate and one-carbon metabolism and on SEPB of CRC including colorectal adenomas, more recent large intervention trials using colorectal adenomas or CRC as either the primary or secondary

endpoint do not support this earlier observation. Furthermore, there is a suggestion that folic acid supplementation may promote the progression of colorectal and other neoplasms. At present, therefore, no conclusive evidence from human experiments supports the protective effect of folate supplementation on colorectal carcinogenesis.

9 Evidence from animal studies

The role of folate in colorectal carcinogenesis has been investigated in several animal studies using chemical carcinogens and in genetically predisposed rodent models of CRC [5, 58, 169]. Collectively, these studies have corroborated the inverse association between folate status and CRC risk observed in epidemiologic studies. These studies, however, have also shown that the dose and timing of folate intervention are critical in providing safe and effective chemoprevention [5, 58, 169].

Two animal studies using the well-established chemical carcinogen (dimethylhydrazine, DMH) rat model of CRC showed that a moderate degree of folate deficiency promoted, whereas modest levels of folic acid supplementation up to 4 × the basal daily requirement (BDR) for rodents inhibited, the development of CRC [90, 93]. There was a suggestion that a very high supplemental dose of folic acid (20 × the BDR] might promote the progression of microscopic neoplastic foci to CRC [93]. In support of this latter finding, animal studies using a metabolite of DMH, azoxymethane (AOM), showed that folic acid supplementation exceeding the BDR by 1000–10 000 × enhanced colorectal carcinogenesis in rats [170–174]. Therefore, it appears that folate modulates colorectal carcinogenesis in chemical carcinogen rodent models over a wide range of dietary intakes. Folate deficiency of a moderate degree enhances colorectal carcinogenesis whereas modest levels of folate supplementation above the BDR suppress colorectal tumorigenesis. Supraphysiologic levels of folate supplementation do not appear to confer additional protection and, in some cases, may enhance colorectal carcinogenesis. The implication of this issue is important because the optimal dose of folate supplementation must be determined for folate chemoprevention to be effective and safe in humans. Although some similarities do exist, tumor development in chemical rodent models of CRC differs in several important histological, clinical, and molecular genetic aspects from that observed in humans [175, 176]. Therefore, any extrapolation of the observations from these models to human situations should be made very cautiously. Whether or not the supplemental doses of folic acid used in these animal studies can be directly extrapolated to intake levels in humans is a highly contentious and controversial issue at present because of inherent differences in folate absorption and metabolism between rodents and humans [177, 178]. Recent evidence suggests that animals, unlike humans, have a comparatively high DHFR activity [177, 178]. Consequently, if assessing the impact of systemic exposure of unmetabolized folic acid, animals would have to be orally dosed with a much greater than prorata amount of folic acid in order to elicit the same circulating serum/plasma concentrations of unmetabolized folic acid [177, 178]. Therefore, it may be a gross mistake to dismiss the effects of folic acid exposure in animal studies on the grounds that the experimental folic acid intake (multiple of animal BDR) would translate to an unlikely intake in humans [177, 178]. Therefore, arguably, a 10–20 × exposure in a small animal model may turn out to hardly equate to an extra 1–2 × RDA in humans [177, 178]. In a recent study using the *Apc*^{Min} mouse model of CRC, both semisynthetic diets with low and high vitamin contents (1/3 of the BDR and 5 × BDR, respectively), which also contained 1/3 of the BDR and 2 × BDR folic acid, respectively, significantly increased the number of small intestinal polyps [179].

Furthermore, in two genetic models of CRC (*Apc*^{Min} and *Apc*+/– × Msh2–/–mice), moderate dietary folate deficiency enhanced, whereas modest levels of folic acid supplementation (four to ten times the BDR) suppressed, the development and progression of CRC, if folate intervention was started before the establishment of neoplastic foci in the intestine [180, 181]. If, however, folate intervention was started after the establishment of neoplastic foci, the same degree of folate deficiency inhibited the progression and induced regression of the established neoplastic foci [180, 181]. A potential tumor promoting effect of folic acid supplementation on the established neoplastic foci could not be clearly determined in these studies because of the inherent limitations associated with these genetic models [180, 181]. Therefore, these observations suggest that the timing of folate intervention is critical in providing an effective and safe chemopreventive effect on colorectal carcinogenesis. Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established colorectal neoplasms. In contrast, folate deficiency in the normal colorectum appears predispose it to neoplastic transformation, and modest supplemental levels of folate suppress the development of neoplasms in the normal colorectum. Some animal studies have also shown that dietary folate deficiency inhibits, and not suppresses, the development of breast cancer in rats [182–184] in contrast to the inverse association between folate status and breast cancer risk observed in epidemiologic studies [185].

10 Dual modulatory role of folate in CRC development and progression: Biologically plausible mechanisms

Data from animal studies and clinical observations suggest that folate possesses dual modulatory effects on CRC devel-

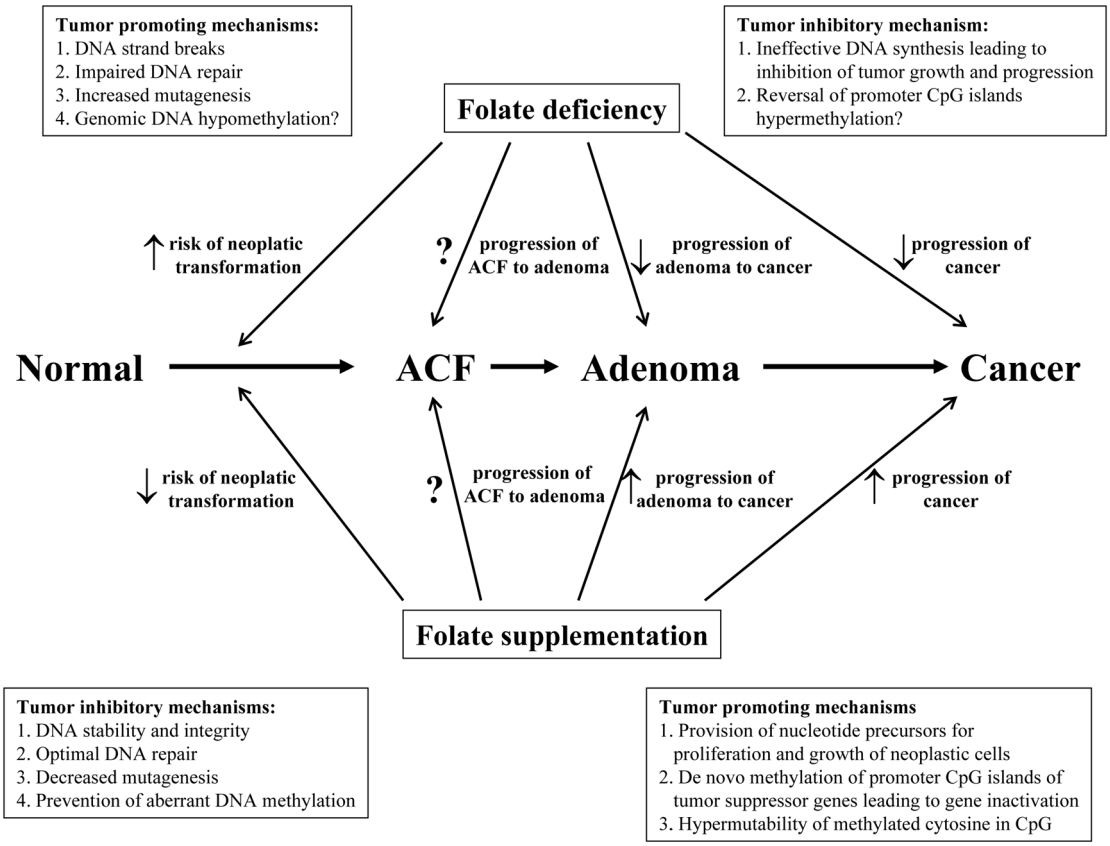


Figure 3. Dual modulatory role of folate in carcinogenesis: cancer develops over decades, if not lifetime, through different stages of premalignant lesions in the target organ. Folate deficiency in normal tissues predispose them to neoplastic transformation, and modest supplemental levels suppress, whereas supraphysiologic doses of supplementation enhances, the development of tumors in normal tissues. In contrast, folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established neoplasms. It is unknown at present the effect of folate deficiency and supplementation on the progression of early precursor or preneoplastic lesions of CRC (*e.g.*, aberrant crypt foci, ACF) to adenoma and to frank cancer. The mechanisms by which folate exerts dual modulatory effects on carcinogenesis depending on the timing and dose of folate intervention relate to its essential role in one-carbon transfer reactions involved in DNA synthesis and biological methylation reactions.

opment and progression depending on the timing and dose of folate intervention (Fig. 3) [5, 6, 58, 84, 169]. Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established colorectal neoplasms (Fig. 3). In contrast, folate deficiency in normal colorectal mucosa appears to predispose it to neoplastic transformation, and modest levels of folic acid supplementation (4 – 10 times above the BDR) suppress, whereas supraphysiological supplemental doses enhance the development of CRC in normal colorectal mucosa (Fig. 3). Are there biologically plausible explanations for these seemingly paradoxical and contradictory epidemiologic, animal, and clinical observations concerning the dual role of folate in CRC development and progression?

10.1 The effects of folate deficiency and supplementation in the normal colorectum

There exist several biologically plausible mechanisms by which folate deficiency increases, whereas folate supplementation reduces, the risk of CRC in normal colorectal epithelial cells [50, 58, 59, 123]. As an essential cofactor for the *de novo* biosynthesis of purines and thymidylate (Fig. 2), folate plays an important role in DNA synthesis, stability and integrity, and repair, aberrations of which have been implicated in colorectal carcinogenesis [58, 59, 123]. Indeed, a large body of evidence from *in vitro*, animal and human studies indicates that folate deficiency is associated with DNA strand breaks, chromosomal and genomic instability, uracil

misincorporation, impaired DNA repair, and increased mutations [58, 59, 121 – 123, 186]. Furthermore, this body of evidence indicates that folate supplementation can correct some of these defects induced by folate deficiency, and ensures DNA fidelity, maintains DNA integrity and stability, and optimizes DNA repair by providing nucleotide precursors for DNA synthesis and replication [58, 59, 121 – 123, 186]. Therefore, the effect of folate deficiency and supplementation on the DNA synthesis pathway in the normal colorectum have been generally considered to be the primary mechanism by which folate deficiency predisposes it to neoplastic transformation and folate supplementation prevents or suppresses neoplastic transformation, respectively (Fig. 3) [58, 59, 121 – 123, 186].

Another proposed mechanism by which folate deficiency enhances the development of cancer in the colorectum is through an induction of genomic DNA hypomethylation [90]. It has been proposed that a mechanism by which folate supplementation may protect against the development of cancer in the colorectum is through a protection against genomic DNA hypomethylation [90]. This mechanism is based on the biochemical function of folate in mediating one-carbon transfer for the provision of SAM, the primary methyl group donor for most biological methylation reactions, including that of DNA (Fig. 2) and on evidence from animal experiments that demonstrated that diets deficient in different combinations of methyl group donors (choline, folate, methionine, and vitamin B_{12}) consistently induce genomic and site and gene-specific DNA hypomethylation [49, 50].

DNA methylation of cytosine located within the cytosine-guanine (CpG) dinucleotide sequences is an important epigenetic determinant in gene expression (an inverse relation), in the maintenance of DNA integrity and stability, in chromosomal modifications, and in the development of mutations [124, 187]. In contrast to methylated CpG sites in the CpG-poor bulk of the genome and unmethylated CpG islands in normal cells, cancer cells simultaneously harbor widespread loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gains in methylation of CpG islands in gene promoter regions (Fig. 4) [124, 187].

Global hypomethylation is an early, and consistent, event in colorectal carcinogenesis [124, 187]. Global hypomethylation of the coding and noncoding regions and demethylation of repetitive DNA sequences contribute to the development of cancer through the following mechanisms: chromosomal instability; increased mutations; reactivation of intragenomic parasitic sequences that could be transcribed and moved to other sites where they could disrupt normal cellular genes; mitotic recombination leading to loss of heterozygosity and promotion of rearrangements; aneuploidy; loss of imprinting; and up-regulation of protooncogenes (Fig. 4) [188]. However, animal studies have shown that genomic demethylation may protect against some cancers

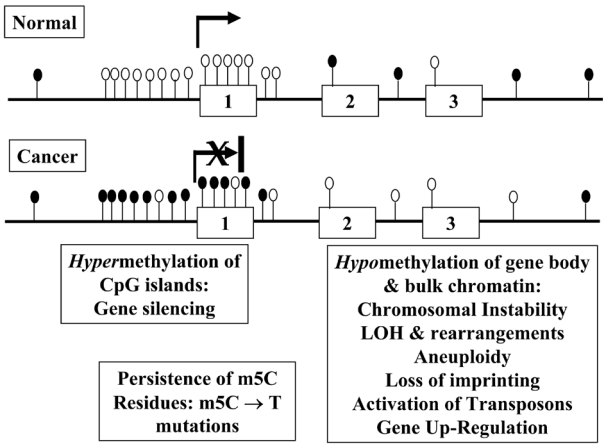


Figure 4. Distribution of CpG dinucleotides in the human genome and CpG methylation patterns in normal and tumor cells. In contrast to methylated CpG sites in the CpG-poor bulk of the genome and unmethylated CpG islands in normal cells, cancer cells simultaneously harbor widespread loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gains in methylation of CpG islands in gene promoter regions. Open circles represent unmethylated CpG sites whereas filled circles are methylated CpG sites. Boxes 1, 2, and 3 represent exons and line between exons are introns. X at the transcription start site represents transcriptional silencing.

(*e.g.*, intestinal tumors) [189, 190] but may promote chromosomal instability and increase the risk of cancer in other tissues (*e.g.*, lymphoma, sarcoma) [191, 192].

Methylation at promoter CpG islands is an important mechanism of silencing transcription in carcinogenesis; the affected genes are silenced and their function is stably lost in a clonally propagated fashion (Fig. 4) [187, 188, 193]. Many genes inactivated by promoter CpG methylation in carcinogenesis have classic tumor-suppressor function or play critical roles in cell cycle control, repair of DNA damage, apoptosis, differentiation, angiogenesis, metastasis, growth – factor response, drug resistance, and detoxification [187]. Promoter CpG islands of over 60% of tumor suppressor and mismatch repair genes have been observed to be methylated in cancer [187].

Another means by which CpG methylation may contribute to carcinogenesis is the hypermutability of methylated cytosine. CpG dinucleotides within certain genes are not only the sites of DNA methylation but also mutational hot spots for human cancers [194]. The majority of mutations observed in CpG sites are cytosine-to-thymine transitions mediated by the spontaneous deamination of 5-methylcytosine to thymine, by the enzymatic deamination of 5-methylcytosine to thymine by DNA methyltransferase (DNMT), and by the enzymatic deamination of unmethylated cytosine to uracil and subsequent methylation of uracil to thymine by DNMT [194]. CpG sites have been shown to act as hot spots for germline mutations, contributing to 30% of all

point mutations in the germ line, and for acquired somatic mutations that lead to cancer [195]. For example, methylated CpG sites in the p53 tumor suppressor coding region contribute to as many as 50% of all inactivating mutations in CRC and to 25% of cancers in general [195].

The portfolio of evidence from animal, human, and *in vitro* studies collectively suggests that the effect of folate deficiency on DNA methylation is highly complex and variable. It appears to be gene and site-specific and depends on species, cell type, target organ, and stage of transformation as well as on the degree and duration of folate depletion [49, 50]. In particular, folate deficiency appears to be unable to induce genomic and gene-specific DNA hypomethylation in the colorectum on a consistent and predictable manner [49, 50]. This may be related to the fact that modulation of SAM and SAH in the colorectum is particularly resistant to folate depletion [49, 50]. Collectively, currently available evidence indicates that genomic DNA hypomethylation in the colorectum is not a probable mechanism by which folate deficiency enhances colorectal carcinogenesis [49, 50].

In contrast, folate supplementation appears to significantly increase the extent of genomic and site-specific DNA methylation in animal and human studies [49, 50]. Dietary folic acid supplementation up to 20 × BDR significantly reversed DMH-induced DNA hypomethylation within a coding region of the *p53* tumor suppressor gene in rat colon in a dose-dependent and site-specific manner [92] in the absence of change in genomic DNA methylation [93]. Folic acid supplementation (286 – 516 μg/day × 3 wk) [142] or 5-methylTHF (15 mg/day × 8 wk) [145] was able to normalize pre-existing genomic DNA hypomethylation in peripheral leukocytes in humans. Folic acid supplementation at 12.5 – 25 × BDR for 3 – 12 months significantly increased the extent of colonic mucosal genomic DNA methylation in subjects with resected colorectal adenomas or cancer [146 – 148]. Even a physiological dose of folic acid (400 μg/day) for 10 wk increased genomic DNA methylation in lymphocytes (by 31%; $p = 0.05$) and in colonic mucosa (by 25%; $p = 0.09$) in patients with colorectal adenomas [152]. Collectively, currently available evidence indicates that folate supplementation appears to be able to reverse pre-existing genomic DNA hypomethylation and to increase the extent of genomic DNA methylation above the pre-existing level [49, 50]. Therefore, prevention or reversal of genomic DNA hypomethylation may be a mechanism by which folate supplementation suppresses neoplastic transformation in the colorectum (Fig. 3).

10.2 The effects of folate deficiency and supplementation in preneoplastic and neoplastic foci in the colorectum

In preneoplastic and neoplastic cells where DNA replication and cell division are occurring at an accelerated rate, folate depletion causes ineffective DNA synthesis, resulting in inhibition of tumor growth and progression (Fig. 3), which is the basis for cancer chemotherapy using antifolate agents (*e.g.*, methotrexate) and 5-fluorouracil [58, 59, 123]. Thus, this is the most likely mechanism by which folate deficiency inhibits the progression of the established preneoplastic neoplastic foci in the colorectum.

Another possible mechanism is that folate deficiency may reverse CpG promoter methylation of tumor suppressor and other anticancer genes involved in colorectal carcinogenesis, thereby reactivating these genes. However, there is currently no experimental evidence to support this theoretical possibility. As discussed earlier, folate deficiency appears to be unable to induce genomic and gene-specific DNA hypomethylation in the colorectum [49, 50]. Furthermore, in a recent *in vitro* study, folate deficiency induced a significant reduction in genomic and site-specific DNA methylation in untransformed NIH/3T3 and CHO-K1 mammalian cells but not in HCT116 and Caco2 human colon adenocarcinoma cells [196]. In this study, folate deficiency did not produce significant changes in the promoter CpG island methylation of the p16 tumor suppressor gene and the MLH1 mismatch repair gene in HCT116 and Caco-2 cells [196]. However, certain sites in the promoter CpG island of the ER gene were associated with modest, albeit statistically significant, changes in CpG methylation in response to folate deficiency, which were not associated with significant functional consequences [196]. In line with this observation, another study showed that HCT116 cells lacking DNMT1 exhibited only a modest 20% decrease in the overall genomic DNA methylation despite the markedly decreased cellular DNMT activity [197]. In this model, although juxtacentromeric satellites became significantly demethylated, centromeric satellite loci, and the promoter CpG island of the p16 gene remained fully methylated [197]. Only when both the DNMT1 and DNMT3b genes were disrupted, genomic DNA methylation was reduced by >95% and significant hypomethylation of satellite sequences and several promoter CpG islands, including that of the p16 gene, was observed [198]. These observations suggest that it may be extremely difficult to reverse DNA methylation in cancer cell lines such as HCT116. The fact that an almost complete abolishment of DNMT activity by disruption of both the DNMT1 and DNMT3b genes is required to produce significant DNA hypomethylation in HCT116 cells [198] suggest that folate deficiency alone is unlikely to be a sufficient predisposing condition to produce significant DNA hypomethylation in colon cancer cells.

Mechanistically, the most likely mechanism by which folic acid supplementation may promote the progression of established preneoplastic and neoplastic lesions in the colorecutm is provision of nucleotide precursors to rapidly replicating neoplastic cells for accelerated proliferation and growth [58, 59, 123]. Another possible mechanism by

which folic acid supplementation may promote the progression of preneoplastic or neoplastic foci in the colorectum may be *de novo* methylation of promoter CpG islands of tumor suppressor genes and other critical genes involved in colorectal carcinogenesis with consequent gene inactivation leading to tumor progression (Fig. 3). This potential epigenetic mechanism of tumor progression is supported by recent animal studies using viable yellow agouti mice that unequivocally have demonstrated that maternal dietary methyl group supplementation containing folic acid permanently alters phenotypic coat color of the offspring *via* increased methylation at the promoter CpG site of the agouti gene [199–201]. However, it is unknown at present whether this *de novo* methylation of promoter CpG islands can happen with folic acid supplementation alone, whether it is operative in normal or neoplastic tissues or both, whether this effect is associated with folic acid supplementation provided in utero only, in postpartum period, or in adulthood, and whether it is tissue and gene-specific.

Another possible means by which folic acid supplementation may promote colorectal carcinogenesis may be through hypermutability of methylated cytosines in CpG dinucleotides (Fig. 3). Methylated CpG sites are mutational hot spots for human cancer as described earlier [194]. The majority of mutations observed in CpG sites are cytosine-to-thymine transitions mediated by the spontaneous deamination of 5-methylcytosine to thymine and by the enzymatic deamination of 5-methylcytosine to thymine by DNMT [194]. Therefore, there is a possibility that *de novo* methylation of cytosines in CpG sites in critical genes involved in colorectal carcinogenesis may create mutational hot spots, leading to inactivating mutations of these genes.

11 Folate and CRC risk: Portfolio of evidence

A cause-and-effect relationship between folate and CRC is difficult to establish. Because of inherent limitations associated with study design, the results from epidemiologic, animal, and interventional studies examining this relationship have been inconsistent and conflicting. In clinical medicine, the best evidence has been considered to come from well designed and executed double-blind randomized controls trials, which minimize a variety of biases. This has resulted in a clear hierarchy of evidence that is weighted heavily toward randomized controlled trials. Evidence from randomized controlled trials is thought to supersede evidence from other sources such as observational studies. The field of nutritional epidemiology has also followed this traditional approach and considered correlation, case-control, and prospective observational epidemiologic studies and intervention trials as a spectrum of increasing weight of evidence for or against a relationship between dietary factors and cancer risk [202]. Thus, general conclusions and recommendations regarding the effect of dietary factors on cancer risk have relied heavily on data from large prospective studies and randomized, controlled intervention human trials [202].

This traditional approach to grading epidemiologic evidence concerning the relationship between dietary factors and cancer risk has recently been challenged [203, 204]. As cancer develops over decades, if not a lifetime, single clinical trials, which normally last up to 5 years, cannot address the whole span of cancer development. In addition, randomized controlled trials tend to use uncharacteristic levels of exposure. Furthermore, the dietary, nutritional, and physical activity exposures involved are complex and interrelated, making them difficult to manipulate in a controlled fashion. Even if a difference in outcome followed such a clinical intervention, it would not necessarily indicate that reproducing the intervention under other conditions would cause similar outcomes. Thus, it has been argued that drawing a definitive conclusion concerning the effect of dietary factors on cancer risk mainly from randomized, controlled intervention human trials is probably not the right paradigm of nutritional epidemiology [203, 204]. Rather, it has been articulated that the totality or "portfolio" of evidence from observational and intervention studies as well as animal and *in vitro* experiments must be analyzed for this purpose [203, 204]. The portfolio approach does not set out a hierarchy of evidence. Instead, it recognizes that all types of evidence have advantages and disadvantages. This means that no single kind of study is considered to be definitive. Instead, all of the different types of studies that are used to investigate the link between nutrition and cancer are considered alongside each other, without favoring evidence from one type over another. In support of the portfolio approach, systematic comparisons of the results of randomized intervention studies with observational evidence in several clinical situations have shown that observational data from well-conducted studies do not appear to produce biased results compared to randomized interventions [205, 206].

Furthermore, the importance of experimental studies that contribute to understanding mechanisms that might underlie any observed association between a dietary factor and cancer and might bear on the inference of causation has been increasingly recognized and appreciated in the field of nutrition and cancer. Epidemiologic and experimental evidence indicating a causal association between a dietary factor and cancer is strengthened when a biologic pathway or mechanism by which colorectal carcinogenesis may be modified is identified and when this mechanism is biologically plausible [204]. It can be argued that epidemiologic data, however strong and consistent, are an inadequate basis for any definite judgment of causality unless supported by mechanistic evidence [204].

Recent advances in molecular epidemiology have added another dimension to the already complex field of nutrition and cancer. Recently identified and characterized single nucleotide polymorphisms and other genetic and epigenetic

variants of genes that are involved in absorption, transport, metabolism, and excretion of nutrients have been shown not only to modify cancer risk but also to significantly modulate the effect of nutrients and related compounds on cancer risk [207]. This emerging important topic in the field of nutrition and cancer, termed "gene–nutrient interactions" in carcinogenesis, has a very significant implication in designing and interpreting data from observational epidemiologic and intervention studies. Although individuals are subjected to the same level of nutritional exposure, systemic, and target tissue bioavailability of nutrients and their metabolites, as well as their functional effects in the target tissue, might be vastly different because of genetic and epigenetic variations. Genetic and epigenetic susceptibility to cancer and their interaction with diets and other environmental exposures have not been incorporated into the study design of and interpretation of data from previously published epidemiologic and intervention studies. The precise nature and magnitude of gene–nutrient interactions in carcinogenesis are yet to be clearly defined.

It appears that overall diet, rather than individual factors, plays the more important role in the development of CRC, thus underscoring the importance of as yet undetermined interactions among dietary components in the development of cancer. It is likely that dietary factors or components do not act in isolation but as part of a biological action package [208]. The major difficulty in establishing a relationship between diet and cancer and in translating observations from nutritional epidemiology into progress in cancer prevention has been due to inability to identify all relevant dietary components that act coordinately to modulate cancer risk and due to inability to identify the other relevant non-nutritional factors that interact with dietary components to modify cancer risk [208].

What can we conclude about the role of folate in CRC development and progression from the seemingly paradoxical and contradictory epidemiologic, animal, and clinical studies? Currently available evidence from epidemiologic, laboratory animal, and intervention studies does not unequivocally support the role of folate in the development and progression of CRC. Furthermore, the precise nature and magnitude of the relationship of CRC with folate have not been clearly defined. However, when the whole body or portfolio of evidence from these studies is analyzed critically, the overall conclusion supports the inverse association between folate status and CRC risk. Definitive answers to questions about folate and CRC are probably beyond the reach of both observational epidemiologic studies and randomized controlled trials [204]. It is clear that folate appears to possess dual modulatory effects on colorectal carcinogenesis depending on the timing and dose of folate intervention (Fig. 3). Folate deficiency has an inhibitory effect whereas folate supplementation has a promoting effect on the progression of established colorectal neoplasms (Fig. 3). In contrast, folate deficiency in normal colorectal mucosa appears to predispose it to neoplastic transformation, and modest levels of folic acid supplementation suppress, whereas supraphysiologic supplemental doses enhance the development of CRC in normal colorectal mucosa (Fig. 3). Several potential mechanisms relating to the disruption of the known biochemical function of folate (mediating the transfer of one-carbon moieties and consequent DNA synthesis and methylation) exist to support the dual modulatory role of folate in colorectal carcinogenesis (Fig. 3).

12 Public health issues concerning folic acid fortification and supplementation

As discussed briefly above, evidence for a protective effect of folate supplementation on NTD [7, 8] was considered to be sufficiently conclusive and led to mandatory folic acid fortification in the US [11] and Canada [12] in 1998. Folic acid fortification has already significantly improved folate status and has had a substantial beneficial effect on the original target, NTD, in the US and Canada [21–26]. Mandatory folic acid fortification is probably the most important science-drive intervention in nutrition and public health in decades [209]. However, the possibility remains that certain segments of the exposed population may benefit less and may even experience some adverse effects from an increased folic acid intake. Over the past few years, the US and Canadian populations have been exposed to a significant increase in folate intake, for which essentially no data on safety exist [13]. No studies have been done to look directly or even indirectly for the adverse effects of greatly increased folate intakes [13]. In addition to the drastic increase in dietary folate intake from mandatory folic acid fortification, 30–40% of the North American population consume supplemental folic acid for several possible but as yet unproven health benefits [35].

Whether or not possible deleterious effects of folic acid supplementation (*e.g.*, cancer-promoting effect on established preneoplastic and neoplastic lesions) outweigh the known and potential health benefits (*e.g.*, prevention of atherosclerosis and NTD; improvement of cognitive function; cancer prevention in normal tissues free of preneoplastic and neoplastic foci) is largely unknown at present. Folate is generally regarded as safe [210] and may become the ultimate functional food component for disease prevention [211]. The potential masking effect of folic acid on vitamin B_{12} deficiency, especially in the elderly, has been the only major concern of folic acid fortification and supplementation [13]. However, an emerging body of evidence suggests that folate supplementation may be associated with other potentially serious adverse effects [6]. These include: the occurrence of resistance or tolerance to antifolate-based chemotherapy and anti-inflammatory and antiseizure drugs; decreased natural killer cell cytotoxicty; accelerated

cognitive decline in older subjects; increased twin pregnancies; and genetic selections of disease alleles (*e.g.*, MTHFR C677T) that predispose individuals to chronic diseases if exposed to low folate status [6, 211–220].

Folic acid, the synthetic, fully oxidized form of folate used in fortification and supplementation, is normally reduced and methylated by the intestine before it is released into the circulation as 5-methylTHF; consequently, the latter form is the sole circulating form of folate under normal conditions [2]. However, studies show that this absorption and biotransformation process is saturated at doses in the region of 400 μg folic acid or less [221]. At higher doses, synthetic folic acid is also transported into the blood and may enter in large quantities. Consumption of folic acid >200 μg have shown to lead to the appearance of unmetabolized folic acid in the serum. Although compelling data about possible antagonistic activities of this fully oxidized form of folate in tissues is lacking, there nevertheless exist concerns about the effect of long-term exposure of cells to unmetabolized folic acid [222]. In this regard, Troen *et al.* [212] have recently reported that 78% of 104 postmenopausal women 60–75 years of age had detectable levels of folic acid in plasma, which was associated with an approximately 23% decrease in natural killer cell cytotoxicity independent of plasma 5-methylTHF and total folate concentrations. Among participants in a large (n = 2928) trial of folic acid supplementation during pregnancy, women who received 5 mg folic acid/day had a 70% increased risk of total cancer mortality compared with those not on supplementation (HR = 1.70; 95% CI = 1.06–2.72) [168]. In this study, the risk of death from breast cancer in women taking 5 mg folic acid/day was twice that of women taking no supplementation, albeit nonsignificant (HR = 2.02; 95% CI = 0.88–4.72) [168]. Furthermore, in line with this observation, a recent the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (n = 25, 400 postmenopausal women) has reported a significant positive association between total folate and supplemental folic acid intakes, but not food folate, and breast cancer risk [85]. Given the new evidence that cells of the absorptive mucosa in the small intestine may not be the primary site for folic acid biotransformation and that humans have an extremely low activity of DHFR, which converts folic acid through dihydrofolic acid to tetrahydrofolic acid, a form that can then enter the main folate metabolic cycle [177], potential adverse effects of the appearance of large quantities of folic acid in the circulation need to be clearly elucidated. Furthermore, given the new evidence of unmetabolized folic acid in the cord blood of newborn and in serum of 4-day-old infants, postformula-feeding [223], potential adverse effects of folic acid on developing fetus need to be determined as well.

Another potential adverse effect of folic acid fortification and supplementation is epigenetic instability. DNA methylation patterns are reprogrammed during embryogenesis by genome-wide demethylation, which erases significant parts of the parental DNA methylation, followed by *de novo* methylation, which established a new DNA methylation pattern, which is maintained postnatally [224, 225]. Therefore, during embryogenesis, epigenetics of developing fetus may be highly susceptible to environmental modifiers including dietary factors. In support of this, as discussed previously, studies using viable yellow agouti mice showed that maternal dietary methyl group supplementation containing folic acid permanently altered the phenotype of the offspring *via* increased CpG methylation at the promoter CpG site of the agouti gene [199–201]. Similarly, a methyl group rich maternal diet has been shown to significantly reduce the proportion of progeny with a kinked tail in the AxinFused mice by half *via* increased CpG methylation in the promoter of the $Axin^{Fu}$ gene [226]. These investigators speculated that "population-based supplementation with folic acid, intended to reduce the incidence of NTD and long presumed to be purely beneficial, may have unintended deleterious influences on the establishment of epigenetic gene-regulatory mechanisms during human embryonic development." [199] It has been recently shown that these diet-induced epigenetic changes can be transmitted to future generations [227, 228]. The possibility that folic acid fortification and supplementation during embryogenesis may establish and maintain "hypermethylated CpG islands" DNA methylation pattern in the offspring, leading to silencing of critical tumor suppressor genes is an important issue in the field of folate and cancer and should be investigated. Furthermore, folic acid supplementaion during embryogenesis may methylate cytosines within CpG sequences, rendering them mutational hot spots. However, the epigenetic effect of folic acid supplementation during embryogenesis may not all be detrimental because increased DNA methylation of CpG sites present in the coding and noncoding regions and in repetitive DNA sequences may protect against the development of cancer by genomic and chromosomal stability and by suppressing reactivation of intragenomic parasitic sequences (Fig. 4) [188]. The predominant epigenetic effect of folic acid supplementation on the developing colorectum is unknown at present.

Perhaps the most concerning potential adverse effect of folic acid fortification and supplementation is the cancer-promoting effect. Although folic acid fortification and supplementation may prevent the development of new cancers in persons without preexisting premalignant or neoplastic lesions, it may promote the progression of already existing, undiagnosed premalignant and malignant lesions including those in the colorectum [5, 6, 84, 169, 185]. Population-based folic acid fortification, intended to prevent NTD, and folic acid supplementation, long presumed to be purely beneficial and believed to provide several health benefits, may promote the development and progression of already existing, undiagnosed premalignant lesions (ACF, adenomas) in the colorectum to CRC in the vast majority of the US and

Canadian populations, who are not at risk of NTD but have been unintentionally exposed to high amounts of folic acid [6, 13]. In Canada, CRC is the fourth most frequently diagnosed cancer and the second most common cause of cancer-specific death [229]. In 2004 alone, 19 100 new cases of CRC were diagnosed, and ~40% of these are expected to die within 5 years [229]. In 2004, 8300 deaths were caused by CRC[229]. The lifetime risk of developing CRC is ~6% [161], and treatment costs nearly $6 billion annually in the US [230]. Colorectal adenomas are found in ~25 – 50% of people by 50 years of age in the US and Canada, and the prevalence increases with age [161]. It has been estimated that ~25% of adenomas progress to CRC over 5 – 10 years [161]. In contrast, NTD occur in ~1 of every 1000 births in the US and Canada [231], and spina bifida and anencephaly, the most common NTD, together affect ~4000 pregnancies resulting in 2500 – 3000 US births annually [231]. It is evident from these statistics that the potential effect of folic acid fortification and supplementation on adenoma progression to CRC and on CRC progression to metastasis far outweighs the effect on NTD risk reduction. Thus, the potential cancer-promoting effect of folic acid fortification and supplementation is a legitimate public health concern and needs a careful monitoring.

13 Folate chemoprevention of CRC: Conclusion

The role of folate has greatly evolved over the past two decades from the prevention of anemia to the prevention of cardiovascular disease and NTD. A large body of evidence suggests that folate may also play a role in the development and progression of cancer. In particular, the portfolio of evidence suggests an inverse association between folate status and the risk of CRC. Given the incidence and mortality of CRC in North America, determining the overall benefits of folic acid fortification and supplementation has major public health implications. As such, preclinical and population-based studies are needed to determine the efficacy, safety, and potential deleterious effects of folic acid fortification and supplementation on CRC and other health outcomes.

Although folate appears to be an ideal candidate for CRC chemoprevention given its proven safety and cost [210], the safe and effective dose range of folate supplementation and optimal timing of folate chemoprevention have not been clearly established in humans. An obvious inference from the above discussion is that for folate to be a safe and effective chemopreventive agent against CRC, modest doses of folic acid supplementation should be implemented before the development of precursor lesions in the colorectum or in individuals free of any evidence of neoplastic foci (Fig. 3). However, determining the presence of neoplastic foci in the general population is an almost impossible task. Furthermore, folate might prevent the progression of certain precursor or preneoplastic lesions to frank malignancy but promote the progression of other lesions. What constitutes safe precursor or preneoplastic lesions on which folate may exert a protective effect has not yet been established. For example, should folate chemoprevention be started before there is evidence of established premalignant lesions, such as ACF or microscopic adenomas in the colorectum or should folate chemoprevention be started even after these lesions are present? In this regard, animal studies investigating the effects of folic acid supplementation on the progression of ACF, microscopic adenomas, and adenomas are urgently needed. Furthermore, careful dose – response studies are warranted in both animal models and humans to determine safe dose and effective doses of folic acid supplementation.

Based on the lack of compelling supportive evidence and on the potential tumor-promoting effect, routine folic acid supplementation should not be recommended as a chemopreventive measure against CRC at present. A more logical approach to folate chemoprevention might be that of targeted chemoprevention in individuals at high risk of developing CRC without evidence of preexisting premalignant lesions or neoplastic foci. For instance, individuals with the MTHFR 677 TT genotype with inadequate folate intake or with significant alcohol consumption have been shown to have an increased risk of CRC [119, 120, 232, 233]. These individuals may therefore benefit from targeted folate chemoprevention, provided that they are free of preneoplastic or neoplastic foci in the colorectum and other target organs.

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