

Review

Folate and DNA Methylation: A Mechanistic Link between Folate Deficiency and Colorectal Cancer?

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Abstract

Epidemiological, clinical, and animal studies collectively indicate that dietary folate intake and blood folate levels are inversely associated with colorectal cancer risk. Folate plays an essential role in one-carbon transfer involving remethylation of homocysteine to methionine, which is a precursor of S-adenosylmethionine, the primary methyl group donor for most biological methylations. DNA methylation is an important epigenetic determinant in gene expression, maintenance of DNA integrity and stability, chromosomal modifications, and development of mutations. Dysregulation and aberrant patterns of DNA methylation are generally considered to be mechanistically involved in colorectal carcinogenesis. Aberrant DNA methylation has been considered as a leading mechanism by which folate deficiency enhances colorectal carcinogenesis. However, currently available data pertaining to the effects of folate deficiency on DNA methylation are

inconsistent and incomplete. The portfolio of evidence from animal, human, and *in vitro* studies suggests that the effects of folate deficiency on DNA methylation are highly complex; appear to depend on cell type, target organ, and stage of transformation; and are gene and site specific. In addition, the pattern of site- and gene-specific DNA methylation induced by folate deficiency may not be in concert with the direction of changes in genomic DNA methylation. Collectively, currently available evidence indicates that genomic DNA hypomethylation in the colorectum is not a probable mechanism by which folate deficiency enhances colorectal carcinogenesis. However, there is still a possibility that sequence-specific alterations of DNA methylation in critical cancer-related genes might be mechanistically involved in the folate deficiency-mediated colorectal carcinogenesis. (Cancer Epidemiol Biomarkers Prev 2004;13(4):511–519)

Folate and Colorectal Cancer Risk

An accumulating body of evidence over the past decade suggests that folate status (assessed by dietary folate intake or by the measurement of blood folate levels) is inversely related to the risk of sporadic and ulcerative colitis-associated colorectal cancer (CRC) or its precursor, adenoma (1–5). Although the results from epidemiological and clinical studies are not uniformly consistent, the portfolio of evidence indicates ~20–40% reduction in the risk of CRC or adenoma in subjects with the highest dietary intake or blood levels of folate compared with those with the lowest intake or blood levels (1–5). The role of folate in colorectal carcinogenesis has been further strengthened by the observations that genetic polymorphisms in the folate metabolic pathway [*e.g.*, the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism] modify CRC risk (6, 7). Although there is no definitive evidence supporting the protective effect

of folate supplementation on colorectal carcinogenesis from human experiments at present, several small intervention studies have demonstrated that folate supplementation can improve or reverse surrogate end point biomarkers of CRC (8–14) and some epidemiological studies have shown a beneficial effect of multivitamin supplements containing ≥ 400 μg folic acid on CRC risk and mortality (15–17). The data from animal studies generally support a causal relationship between folate depletion and CRC risk and an inhibitory effect of modest levels of folate supplementation on colorectal carcinogenesis (18). However, animal studies have also shown that folate supplementation may increase CRC risk and accelerate CRC progression if too much is given or if it is provided after neoplastic foci are established in the colorectum (18).

Mechanistic Understanding of the Folate Deficiency-Mediated Colorectal Carcinogenesis

The mechanisms by which folate deficiency enhances and supplementation suppresses colorectal carcinogenesis have not yet been clearly elucidated. However, several potential mechanisms relating to the disruption

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Table 1. Potential mechanisms of the folate deficiency-mediated colorectal carcinogenesis

DNA damage, uracil misincorporation, impaired DNA repair
Increased mutagenesis
Aberrant genomic and site-specific DNA methylation
Hyperproliferation
Abnormal apoptosis
MTHFR polymorphism and related gene-nutrient interactions

of the known biochemical function of folate have been proposed and investigated (Table 1; Refs. 1, 19–23). Folate is a water-soluble B vitamin that plays an important role in mediating the transfer of one-carbon moieties (Fig. 1; Ref. 24). In this role, folate is an essential cofactor for the *de novo* biosynthesis of purines and thymidylate (Fig. 1; Ref. 24). 5,10-Methylenetetrahydrofolate, an intracellular coenzymatic form of folate, is required for conversion of deoxyuridylate to thymidylate and can be oxidized to 10-formyltetrahydrofolate for *de novo* purine synthesis (Fig. 1; Ref. 24). Thus, folate is an important factor in DNA synthesis, stability and integrity, and repair (Fig. 1), aberrations of which have been implicated in colorectal carcinogenesis (1, 19–23). A growing body of evidence from *in vitro*, animal, and human studies indicates that folate deficiency is associated with DNA strand breaks, impaired DNA repair, and increased mutations and that folate supplementation can correct some of these defects induced by folate deficiency (1, 19–23). Indeed, induction of DNA damage, disrupted DNA integrity, impaired DNA repair, and hypermutability are generally considered to be the primary mechanisms by which folate deficiency enhances colorectal carcinogenesis (1, 19–23). In contrast, although genomic and site-specific DNA hypomethylation has previously been considered as a leading mechanism by

which folate depletion enhances colorectal carcinogenesis (1, 19–22), the following discussion indicates that this hypothesis remains highly controversial and unresolved.

DNA Methylation and Cancer

The inheritance of information based on gene expression levels is known as epigenetics, as opposed to genetics, which refers to information transmitted on the basis of gene sequence (25, 26). The main epigenetic modification in humans is the methylation of cytosine located within the cytosine-guanine (CpG) dinucleotide sequences (25, 26). The pattern of methylation at cytosine residues in the CpG sequences is a heritable, tissue- and species-specific, postsynthetic modification of mammalian DNA (25, 26). DNA methylation is an important epigenetic determinant in gene expression (an inverse relationship), maintenance of DNA integrity and stability, chromatin modifications, and development of mutations (25, 26). Neoplastic cells simultaneously harbor widespread genomic hypomethylation and more specific regional areas of hypermethylation (25, 26). Genomic hypomethylation is an early, and consistent, event in colorectal carcinogenesis (25, 26) and is associated with genomic instability (27) and increased mutations (28). In addition, site-specific hypermethylation at promoter CpG islands of tumor suppressor and mismatch repair genes is an important mechanism in gene silencing in colorectal carcinogenesis (29, 30).

DNA Methylation Assays

The understanding of the role of DNA methylation in cancer has been greatly enhanced by recent technical

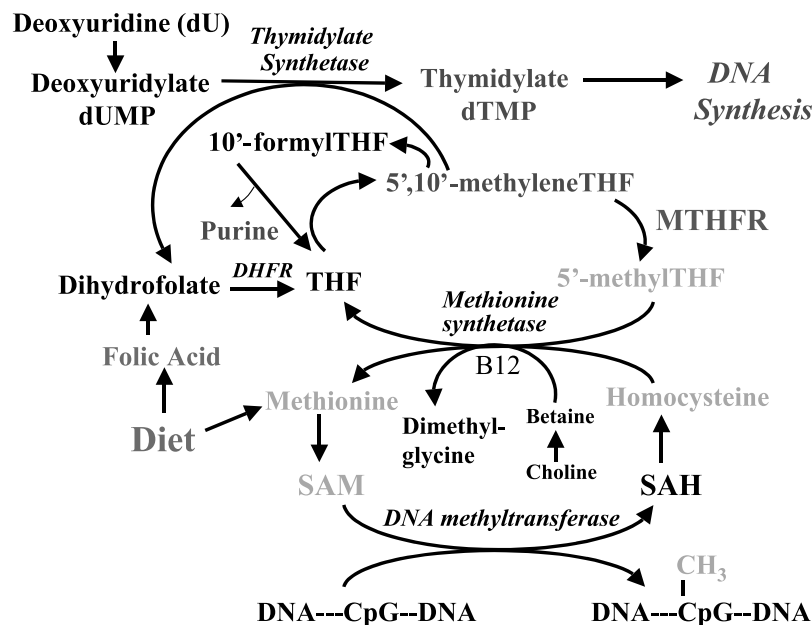


Fig. 1. Simplified scheme of folate metabolism involving DNA synthesis and methylation. B12, vitamin B₁₂; DHFR, dihydrofolate reductase; CH₃, methyl group; THF, tetrahydrofolate.

advances in DNA methylation assays. As extensively discussed in an excellent recent review article, currently available techniques can detect the extent and patterns of DNA methylation at the genomic, gene-specific, and site-specific levels with varying degrees of certainty (31). For most parts, however, it has been notoriously difficult to accurately measure the level of methylation at given CpG sites within target areas of genes (31). The following approaches have been used in most studies that investigated the effect of folate deficiency on DNA methylation. First, the total content of methylated cytosines or those within the CpG sites in the genome can be detected by high-performance liquid chromatography, immunostaining, *in vitro* methyl acceptance capacity assay, or cytosine extension assay (31). Although global genomic DNA methylation content might have an important role in carcinogenesis, its measurement in cancer or normal cells has little to offer as a molecular marker either in sensitivity or in informational content (31). Second, methylation status within specific CpG sites in a given gene can be determined by the use of methylation-sensitive restriction enzymes followed by Southern blotting or PCR-based semiquantitative analysis (31). This experimental approach is reliable but cumbersome and requires a substantial amount of high-quality DNA. The use of postdigestion PCR to circumvent these limitations is prone to false-positive results that occur because of incomplete enzyme cleavage (31). More importantly, this type of DNA methylation assay is limited by the number of CpG dinucleotides that could be assessed because only those CpG sites present in the recognition sequences for specific methylation-sensitive restriction enzymes could be analyzed (31). Third, cytosine methylation status of individual CpG sequences can be determined using techniques based on the conversion of unmethylated but not methylated cytosines to uracil by bisulfite treatment (31). These techniques include (a) sequencing of individual molecules of DNA (the bisulfite sequencing assay) that can obviate the problem of allele-specific methylation; (b) very sensitive assays for determining methylation in given areas based on primers specific for converted DNA (methylation-specific PCR); (c) techniques that allow a quantitative assessment at the level of methylation at individual CpG dinucleotides (methylation-sensitive single nucleotide primer extension and combined bisulfite-restriction analysis); and (d) automated, high throughput MethyLight assay that relies on fluorescence-based real-time PCR for the quantitative determination of the prevalence of hypermethylated alleles and that combines the advantages of quantitative accuracy of combined bisulfite-restriction analysis with the sensitivity of methylation-specific PCR (31). All of the techniques described above are associated with significant potential pitfalls and limitations (31). No one technique or general approach is universally superior, as the competing goals of quantitative accuracy, sensitive detection, high local or global informational content, and compatibility with automation are not all found in a single technique (31). Therefore, the method of choice depends on the desired application (31).

Folate and DNA Methylation

Folate, in the form of 5-methyltetrahydrofolate, is involved in remethylation of homocysteine to methio-

nine, which is a precursor of *S*-adenosylmethionine (SAM), the primary methyl group donor for most biological methylations including that of DNA (Fig. 1; Ref. 32). After transfer of the methyl group, SAM is converted to *S*-adenosylhomocysteine (SAH), a potent inhibitor of most SAM-dependent methyltransferases (Fig. 1; Ref. 32). Cravo and Mason first proposed that a mechanism by which folate deficiency enhances colorectal carcinogenesis might be through an induction of genomic DNA hypomethylation based on the biochemical function of folate in mediating one-carbon transfer and on evidence from animal experiments that demonstrated methyl group donor deficiency-induced DNA hypomethylation (33).

The Effect of Methyl Group Deficiency on DNA Methylation in Rodents

Diets deficient in methyl group donors (choline, folate, methionine, and vitamin B₁₂) are associated with spontaneous and chemically induced development of hepatocellular carcinoma in rats (34). Diets deficient in different combinations of methyl group donors have been consistently observed to induce genomic and proto-oncogene (*c-myc*, *c-fos*, and *c-Ha-ras*) DNA hypomethylation and elevated steady-state levels of corresponding mRNA (35–40) and site-specific *p53* hypomethylation (40–42) in rat liver. Therefore, DNA hypomethylation was proposed as one of the possible mechanisms for the development of hepatocellular carcinoma associated with methyl-deficient diets (35–42). Methyl group donor deficiency has also been shown to up-regulate DNA methyltransferase (Dnmt) in rat liver (36, 37, 41–43). However, a recent study reported that a diet deficient in choline, methionine, and folate, which increased a 30% increase in DNA strand breaks, did not induce a significant degree of genomic DNA hypomethylation in rat colon, suggesting that the colorectum may be resistant to the hypomethylating effect of methyl group deficiency (44).

The Effect of Folate Status on DNA Methylation in Humans

There are some observations in humans suggesting that altered folate status can affect genomic DNA methylation. Folate depletion in healthy human volunteers in a metabolic unit setting has been observed to diminish genomic DNA methylation in leukocytes (Table 2; Refs. 45, 46). Rampersaud *et al.* showed that lymphocyte genomic DNA methylation significantly decreased by 10% in response to moderate (118 µg/day folate) folate depletion for a period of 7 weeks in elderly women aged 60–85 years (46). No significant changes in leukocyte genomic DNA methylation were detected during the 7-week period of folate repletion with either 200 or 415 µg/day folate (46). Another study by Jacob *et al.* housed healthy, postmenopausal women aged 49–63 years in a metabolic unit and fed them folate-depleted diets (56–111 µg/day folate) for 9 weeks (45). This resulted in a significant (by 120%) degree of lymphocyte genomic DNA hypomethylation, which was reversed

Table 2. Summary of dietary folate deficiency and supplementation on DNA methylation in human studies

Study (reference)	Subjects	Dose	Duration	DNA methylation	Effect
Folate deficiency					
Rampersaud <i>et al.</i> (46)	Women 60–85 yr	118 µg/day	7 wk	Leukocytes genomic	10% decrease ($P = 0.0012$)
Jacob <i>et al.</i> (45)	Women 49–63 yr	56–111 µg/day	9 wk	Lymphocytes genomic	120% decrease ($P < 0.05$)
Jacob <i>et al.</i> (47)	Men	25 µg/day	30 days	Methylation capacity (not DNA)	No change
Folate supplementation					
Cravo <i>et al.</i> (8)	Patients with CRC and adenoma	10 mg/day	6 mo	Rectal mucosa genomic	93% increase ($P < 0.002$)
Cravo <i>et al.</i> (9)	Patients with colonic adenoma	5 mg/day	3 mo	Rectal mucosa genomic	37% increase in patients with 1 adenoma ($P = 0.05$) and no change in those with >1 adenomas
Kim <i>et al.</i> (11)	Patients with colonic adenoma	5 mg/day	6 mo	Rectal mucosa genomic	57% increase ($P = 0.001$)
Cravo <i>et al.</i> (52)	Patients with inflammatory bowel disease	5 mg/day	1 yr	Rectal mucosa genomic	No change
Fenech <i>et al.</i> (48)	Normal subjects	2 mg/day	6 mo	Lymphocytes genomic	No change
			12 wk		

during the 3-week period of folate supplementation (285–516 µg/day folate). However, an earlier study by the same group using healthy males aged 33–46 years failed to show a change in *in vivo* methylation capacity (as measured by the ability to methylate *p.o.* administered nicotinamide as detected in the urine as methylated metabolites) in response to dietary folate and methyl group restriction (Table 2; Ref. 47). In this study, male subjects housed in a metabolic unit were placed on a folate-deficient diet (25 µg/day folate) for 30 days (47).

Several human studies have investigated correlations between DNA methylation and folate status. In human subjects with normal folate status, no significant correlations between genomic lymphocyte DNA methylation and RBC folate and plasma homocysteine concentrations were observed (48). A recent study, however, has shown that colonic DNA methylation was positively correlated with serum and RBC folate concentrations and negatively correlated with plasma homocysteine concentrations in individuals with colonic adenomas and adenocarcinomas and in those without these lesions, while such correlations with leukocyte DNA methylation were observed only for serum folate (49). 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*, *O⁶-MGMT*, and *RASSF1A*; Ref. 50). The number of CRCs with at least one gene methylated was higher (84%) in the low folate/high alcohol intake group compared with the high folate/low alcohol intake group (70%; $P = 0.085$; Ref. 50). In one human study, serum and cervical tissue folate concentrations correlated inversely, albeit weakly, with

cervical genomic DNA methylation (51). The interpretation of these two studies is, however, limited by the measurement of folate concentrations (51) and DNA methylation (50, 51) in premalignant and malignant cervical and colorectal tissues instead of normal tissue alone.

In some human intervention studies, folate supplementation at 12.5–25 times the daily requirement for 3–12 months significantly increased the extent of colonic genomic DNA methylation in subjects with resected colorectal adenoma or cancer (8, 9, 11), whereas no such effect was observed in patients with chronic ulcerative colitis who were given folate supplementation at 12.5 times the daily requirement for 6 months (Table 2; Ref. 52). Folate supplementation at five times the daily requirement, which was sufficient to correct a marker of DNA damage, failed to modulate genomic DNA methylation in lymphocytes in healthy volunteers (Table 2; Ref. 48). Data from these human studies collectively raise a possibility that the effect of folate status on genomic DNA methylation may be site- and tissue-specific and may depend on the degree of folate depletion and supplementation. However, there is no conclusive data suggesting that folate deficiency of a physiologically and clinically relevant degree induces significant genomic DNA hypomethylation and/or site- and gene-specific aberrant DNA methylation in the colorectum.

The Effect of Isolated Folate Deficiency on Genomic DNA Methylation in Rodent Liver and Colon

Although isolated folate deficiency has been shown to reduce SAM levels and SAM/SAH ratios and increase

SAH concentrations in rat liver (53–57), conflicting data exist for the effect of isolated folate deficiency on DNA methylation in rodent liver (Table 3). One study reported a significant 20% decrease in genomic DNA methylation associated with a severe degree of dietary folate deficiency of 4 weeks' duration in rat liver (58), while another study showed a paradoxical 56%, albeit nonsignificant, increase in genomic DNA methylation associated with the same severe folate deficient diet of 6 weeks' duration in rat liver (57). A prolonged (15–24 weeks), moderate degree of dietary folate deficiency failed to induce significant genomic DNA and *c-myc* proto-oncogene hypomethylation in rat liver (56). Recently, however, the same moderate folate-deficient diet for 5 weeks in mice was shown to induce a significant 56% increase in genomic DNA methylation in the liver followed by the return of genomic DNA methylation value to that of the baseline by 8 weeks (59). Taken together, the results from these studies suggest that folate deficiency of a moderate degree or short duration appears to induce genomic DNA hypermethylation in rodent liver, likely due to compensatory up-regulation of Dnmt, and that the effect of sustained or severe folate deficiency on genomic DNA methylation in rodent liver is not consistent. One intriguing observation from one of these animal studies was that severe folate deficiency produced significant hypomethylation (by 40%) within mutation hotspot (exons 6–7), but not in exon 8, of the *p53* tumor suppressor gene despite a 56% increase in genomic DNA methylation in rat liver (57). This observation raises a possibility that the effect of folate

deficiency on DNA methylation may be site and gene specific and suggests that the changes in genomic and site-specific DNA methylation in response to folate deficiency may not be in the same direction.

The effect of isolated folate deficiency on DNA methylation in the colorectum, the primary target tissue that is particularly susceptible to the folate deficiency-induced carcinogenic effect, has not yet been clearly elucidated (Table 3). A moderate degree of folate deficiency for 15–24 weeks failed to induce significant genomic and *c-myc*-specific DNA hypomethylation rat colon (56). The same degree of moderate folate deficiency for 20 weeks in conjunction with an alkylating colon carcinogen, dimethylhydrazine (DMH), did not cause significant genomic DNA hypomethylation in rat colon (60). No significant DNA hypomethylation was observed in rat colon in two other animal studies using a similar degree of moderate folate deficiency in conjunction with azoxymethane (AOM), a metabolite of DMH (61, 62). However, these studies (56, 60–62) were limited by the fact that the degree of folate deficiency used in these studies failed to significantly alter colonic SAM or SAH concentrations as well as by the use of DMH or AOM, which can alter tissue SAM and SAH levels (63) and the extent of DNA methylation (64) independent of the effect of folate. A recent study showed that moderate folate deficiency for 13 weeks in conjunction with DMH injection did significantly increase colonic SAH concentrations but did not change colonic SAM levels or the degree of genomic DNA methylation in rats (65). Another recent study showed that a moderate degree

Table 3. Summary of the effect of isolated folate deficiency on DNA methylation in rodents

Study (reference)	Folate deficiency	Duration (wk)	Species	Organ	DNA methylation	Effect	
Balaghi <i>et al.</i> (58)	Severe	4	Rat	Liver	Genomic	20% decrease ($P = 0.032$)	
Kim <i>et al.</i> (57)	Severe	6	Rat	Liver	Genomic	60% increase ($P = 0.1$)	
Kim <i>et al.</i> (56)	Mild	15 and 24	Rat	Liver	Genomic	No effect	
				Colon	Genomic	No effect	
Song <i>et al.</i> (59)	Mild	5	Mouse	Liver	<i>c-myc</i>	No effect	
					Genomic	56% increase ($P < 0.005$)	
Kim <i>et al.</i> (60)	Mild + DMH	8	Rat	Colon	Genomic	No effect	
Kim <i>et al.</i> (66)	Mild + DMH	20	Rat	Colon	<i>p53</i> (exon 8)	25% decrease ($P = 0.038$)	
Le Leu <i>et al.</i> (62)	Mild + AOM	26	Rat	Colon	Genomic	No effect	
Le Leu <i>et al.</i> (61)	Mild + AOM	13	Rat	Liver	Genomic	No effect	
Davis <i>et al.</i> (65)	Mild + DMH	13	Rat	Colon	Genomic	No effect	
				Liver	Genomic	No effect	
				Colon	Genomic	No effect	
Duthie <i>et al.</i> (44)	Mild	10	Rat	Colon	Genomic	No effect	
Sohn <i>et al.</i> (67)	Severe	5	Rat	Colon	Genomic	30% increase at wk 3 ($P = 0.022$) and no effect at other time points	
Choi <i>et al.</i> (70)	Mild	8 and 20	Rat (young and old)	Colon	Genomic	<i>p53</i> (promoter and exons 6–7)	Highly variable; decrease at CpG site 1 in exons 6–7 at wk 5
						No effect	

of isolated folate deficiency for 10 weeks, which was associated with a significant degree of DNA strand breaks, did not induce significant genomic DNA hypomethylation in rat colon (44). In contrast, significant *p53* hypomethylation in exon 8, but not in exons 6–7, was observed in the DMH-treated rat colon in conjunction with folate deficiency, although it remains unclear whether this was due to the DMH, the folate deficiency, or the combination of the two, and this was effectively overcome in a dose-dependent manner by increasing levels of dietary folate (66).

A recent study investigated the time-dependent effects of dietary folate on genomic and *p53* (in the promoter region and exons 6–7) DNA methylation in rat colon and how these changes are related to steady-state levels of *p53* transcript (67). Despite a marked reduction in plasma and colonic folate concentrations, a large increase in plasma homocysteine, and a progressive decrease in colonic SAM/SAH ratio, isolated folate deficiency did not induce significant genomic DNA hypomethylation in the colon (67). Paradoxically, isolated folate deficiency significantly increased (by 30%) the extent of genomic DNA methylation in the colon at an intermediate time point (67), which is consistent with prior observations made in rodent liver (57, 59). Folate supplementation did not modulate colonic SAM, SAH, and SAM/SAH ratios and genomic DNA methylation at any time point (67). The extent of *p53* methylation in the promoter and exons 6–7 was variable over time at each of the CpG sites examined, and no associations with time or dietary folate were observed at any CpG site, except for a significant degree of hypomethylation with folate deficiency at the CpG site 1 in exons 6–7 at an extreme time point (67). Because this change was not evident at any earlier time point, however, its significance is questionable. Dietary folate deprivation progressively decreased, whereas supplementation increased, steady-state levels of *p53* transcript during the study period (67). However, steady-state levels of *p53* mRNA did not significantly correlate with either genomic or *p53* methylation within the promoter region and exons 6–7 (67). These data indicate that isolated folate deficiency, which significantly reduces steady-state levels of colonic *p53* mRNA, is not associated with a significant degree of genomic or *p53* DNA hypomethylation in rat colon. These observations suggest that isolated folate deficiency does not induce consistent and predictable changes in *p53* methylation in rat colon whereas it may produce *p53* hypomethylation in specific exons in rat liver and colon in conjunction with alkylating agents. In contrast, dietary depletion of combined methyl donors predictably induces *p53* hypomethylation within exons 6–7 of the *p53* gene in rat liver (40–42, 67). These observations suggest that *p53* methylation changes likely depend on the degree of methyl donor supply and consequent levels of methylation intermediates that are predictably and consistently achieved by combined methyl deficiency and not by isolated folate deficiency.

One critical factor that may explain the inability of folate deficiency to modulate genomic and site- and gene-specific DNA methylation in the colorectum appears to be SAM and SAH concentrations in the colorectum. In the above-mentioned study (67), although isolated folate deficiency progressively decreased colonic

mucosal SAM/SAH ratio during 5 weeks of dietary intervention, only in the extreme deficient state, associated with 20% growth retardation and a 22-fold rise in plasma homocysteine, was there a significantly elevated level of colonic mucosal SAH and a significantly reduced colonic mucosal SAM/SAH ratio compared with the control diet. Folate supplementation at four times the basal requirement did not modulate colonic mucosal concentrations of SAM, SAH, and SAM/SAH ratios at any time point. These data are consistent with previous observations made in the setting of a milder and more chronic state of folate deficiency (56, 60) and indicate that modulation of SAM and SAH in the colonic mucosa is particularly resistant to the level of dietary folate. In contrast, folate deficiency, even mild and moderate degrees, has been shown to modulate SAM and SAH in the brain (68), kidney (68), pancreas (69), and liver (53, 54, 56–58) in rats. The reason for this tenacious resistance to altered SAM and SAH levels in the colorectum compared with other tissues is unclear at present. A recent animal study using a moderate degree of dietary folate deficiency has suggested that the colon of old rats is more susceptible to changes in SAM and SAH in response to folate deficiency as compared with that of young rats, although these changes in SAM and SAH do not appear to induce genomic DNA hypomethylation (70).

The Effect of Folate Deficiency on DNA Methylation in *In Vitro* Systems

In one study by Duthie *et al.*, normal human colonic epithelial cells were immortalized by SV40 T antigen and cultured in folate-deficient (<1 ng/ml folic acid, 2.3 nM) and control (4 mg/l folic acid) medium for 14 days (71). Folate deficiency led to genomic DNA hypomethylation, increased uracil misincorporation, and inhibition of DNA excision repair in colonic epithelial cells (71). In contrast, a recent study, published only in abstract form, using two human colon adenocarcinoma cell lines, Caco2 and HCT116, have shown that the extent and direction of the changes of SAM and SAH in response to folate deficiency (0 μ M folic acid in the medium *versus* 2.3 μ M in control medium) are cell specific and that genomic and site- and gene-specific DNA methylation are not affected by the changes of SAM and SAH induced by folate depletion (72). In a similar experiment using the nontransformed cell lines, folate deficiency (0 μ M in the medium) was shown to induce significant genomic DNA hypomethylation in both mouse fibroblast cell line, NIH/3T3, and Chinese hamster ovarian cell line, CHO-K1, by cell-specific mechanisms as indicated by cell-specific differential effects of folate deficiency on intracellular SAM and SAH and Dnmt (73). In another study, human nasopharyngeal carcinoma KB cells grown in folate-depleted (2–10 nM folic acid) medium was associated with paradoxical hypermethylation in a 5' CpG island (by 40%) and consequent down-regulation of the H-cadherin gene compared with cells grown in folate-replete (2.0 μ M folic acid) medium (74). The results from these studies collectively suggest that the effects of folate deficiency on DNA methylation are site and gene specific. In addition, the direction of methylation changes may be cell, target organ, and stage of transformation specific and may not

be the same between genomic and gene or site-specific DNA methylation. These conclusions are supported by prior observations that suggest that cancers from different organs and histologically different subtypes of cancer within a given organ exhibit distinct global and gene-specific methylation patterns (29, 75). The major limitation of the *in vitro* system to study the effect of folate on DNA methylation is that the degree of folate deficiency and supplementation used in this system is not physiologically and clinically relevant and applicable to *in vivo* situations.

Can Isolated Folate Deficiency Cause DNA Methylation Changes?

A mechanistic understanding of how folate status modulates colorectal carcinogenesis further strengthens the case for a causal relationship and provides insight into a possible chemopreventive role of folate. Dysregulation and aberrant patterns of DNA methylation are mechanistically involved in colorectal carcinogenesis (25, 26). Because of the essential role of folate in one-carbon transfer, it has been proposed that folate deficiency may contribute to aberrant DNA methylation (1–3, 19, 20, 33, 58, 70, 76). The human feeding studies of folate deficiency in metabolic unit settings have shown a consistent, significant hypomethylating effect of folate deficiency in genomic lymphocytes DNA (45, 46). However, the effect of folate deficiency of a physiologically and clinically relevant degree on DNA methylation in the colorectum has not been studied in humans, although folate supplementation appears to be capable of increasing the degree of DNA methylation in the colorectum in some human studies (8, 9, 11). Currently available data pertaining to the effects folate deficiency on DNA methylation in the liver and colon in animal studies are inconsistent and do not support the hypothesis that folate deficiency induces DNA hypomethylation in the colon. Although some similarities do exist, these animal models differ in several important physiological aspects from humans including bioavailability, metabolism, and excretion of folate (18). Therefore, any extrapolation of the observations from these models to human situations should be made very cautiously. Furthermore, these animal models may produce variable results owing to species differences, different diet compositions, and variable dose, time, and duration of folate manipulations.

The portfolio of evidence from animal, human, and *in vitro* studies collectively suggests that the effects of folate deficiency on DNA methylation are highly complex and appear to depend on cell type, target organ, and stage of transformation and are gene and site specific. These studies also suggest that changes in DNA methylation induced by folate deficiency may be mediated via both SAM and SAH dependent and independent pathways. In addition, there is evidence suggesting that the pattern of site- and gene-specific DNA methylation may not be in concert with the direction of changes in genomic DNA methylation. Careful inspection of the portfolio of the currently available evidence indicates that genomic DNA hypomethylation in the colorectum is not a probable mechanism by which folate deficiency enhances colorectal carcinogenesis. However, there is still a possibility that

sequence-specific alterations of DNA methylation in critical cancer-related genes might be mechanistically involved in the folate deficiency-mediated colorectal carcinogenesis.

Future Studies

How folate deficiency and supplementation regulate Dnmts and demethylase as well as the epigenetic regulatory and transcriptional machinery involved in DNA methylation patterning and histone modifications (acetylation and methylation) that lead to changes in chromatin structure and conformation is largely unknown at present and clearly needs to be determined to understand the effect of folate on transcriptional regulation. In this regard, there is preliminary evidence suggesting that methyl donor deficiency have a profound effect on the methyl-CpG binding protein, MeCP2, in rat liver (77).

Recent studies have revealed that age-related methylation is a common event in human tissues and an important contributor to the promoter CpG island hypermethylation of several genes with consequent gene inactivation observed in CRC and other cancers (78, 79). Aging is a major determinant of CRC risk (80). It is therefore possible that age-related methylation of certain genes serves as a functional link between aging and CRC by providing selective advantage for normal colon cells through deregulating growth and differentiation (78). These cells may then be at higher susceptibility for acquiring genetic lesions such as mutations. The causes of age-related methylation changes are largely unknown, although some studies have suggested the role of environmental exposures or modifier genes (81). A recent study has demonstrated that the aging colon is highly susceptible to folate depletion and consequent changes in SAM and SAH compared with the young colon in rats (70). Therefore, folate deficiency in the aging colon may predispose it to changes in SAM and SAH and consequent DNA methylation changes more readily than that in the young colon, and folate status and DNA methylation changes may serve as a functional link between aging and CRC.

There is a need for the development of appropriate *in vitro* models of physiologically and clinically relevant folate deficiency. Folate levels can be accurately and consistently controlled in these models while adjusting for the levels of other methyl group donors. In addition, these *in vitro* systems allow for the direct impact on various cellular and molecular mechanisms to be determined in various cell types while eliminating the potential confounding factors found in animal models as described previously.

Interactions between folate and other methyl group donors in modulating colorectal SAM and SAH levels and DNA methylation patterns have not been clearly understood. In addition, potential interactions between folate and other nutrients that may affect methylation (e.g., selenium) have not been extensively studied. In this regard, a recent study has shown that selenium and folate interact to influence one-carbon metabolism, SAM and SAH levels and genomic DNA methylation in rat liver and colon, and CRC susceptibility (65).

Furthermore, there is evidence that folate status influences DNA methylation through an interaction with the MTHFR C677T polymorphism. MTHFR is a critical enzyme in folate metabolism that catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thereby playing an important role in DNA synthesis, maintenance of nucleotide pool balance, and DNA methylation (Fig. 1; Refs. 1, 82, 83). The MTHFR C677T polymorphism causes thermolability and reduced MTHFR activity, leading to lower levels of 5-methyltetrahydrofolate, an accumulation of 5,10-methylenetetrahydrofolate, increased plasma homocysteine levels (a sensitive inverse indicator of folate status), and changes in cellular composition of one-carbon folate derivatives (Fig. 1; Refs. 1, 82, 83). In a recent study, genomic DNA methylation in peripheral blood mononuclear cells was shown to directly correlate with folate status and inversely correlate with plasma homocysteine levels (84). MTHFR TT genotypes had a diminished level of genomic DNA methylation compared with those with the CC wild-type (84). When analyzed according to folate status, however, only the TT subjects with low levels of folate accounted for the diminished genomic DNA methylation (84). Whether the MTHFR C677T polymorphism in conjunction with marginal folate status affects DNA methylation in the colorectum is unknown at present. The results from this study (84) emphasize the importance of taking into consideration interactions between folate status and critical genes on the folate and one-carbon metabolic pathway when investigating the effect of folate on DNA methylation.

Studies examining the role of DNA methylation in cancer have been hampered by inability to precisely determine the extent and patterns of DNA methylation with currently available techniques. No one technique or general approach is universally superior as described earlier (31) and the most appropriate and accurate method needs to be applied to investigate the effect of folate on DNA methylation in colorectal carcinogenesis in future studies. Furthermore, the role of folate status on gene-specific DNA methylation has been traditionally investigated by candidate gene approaches focusing on promoter CpG islands or coding regions of specific genes that are biologically linked to the folate and one-carbon metabolic pathway. Although valuable information has been generated from this approach, as a further step toward a more comprehensive understanding of the underlying mechanisms, it is necessary to conduct a genome-wide analysis of CpG methylation as related to folate status and CRC.

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