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Research Article

Cancer Prevention Research

Relative Distribution of Folate Species Is Associated with Global DNA Methylation in Human Colorectal Mucosa

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Abstract

Folate exists as functionally diverse species within cells. Although folate deficiency may contribute to DNA hypomethylation in colorectal cancer, findings on the association between total folate concentration and global DNA methylation have been inconsistent. This study determined global, LINE-1, and Alu DNA methylation in blood and colon of healthy and colorectal cancer patients and their relationship to folate distribution. Blood and normal mucosa from 112 colorectal cancer patients and 114 healthy people were analyzed for global DNA methylation and folate species distribution using liquid chromatography tandem mass spectrometry. Repeat element methylation was determined using end-specific PCR. Colorectal mucosa had lower global and repeat element DNA methylation compared with peripheral blood ($P < 0.0001$). After adjusting for age, sex and smoking history, global but not repeat element methylation was marginally higher in normal mucosa from colorectal cancer patients compared with healthy individuals. Colorectal mucosa from colorectal cancer subjects had lower 5-methyltetrahydrofolate and higher tetrahydrofolate and formyltetrahydrofolate levels than blood from the same individual. Blood folate levels should not be used as a surrogate for the levels in colorectal mucosa because there are marked differences in folate species distribution between the two tissues. Similarly, repeat element methylation is not a good surrogate measure of global DNA methylation in both blood and colonic mucosa. There was no evidence that mucosal global DNA methylation or folate distribution was related to the presence of cancer *per se*, suggesting that if abnormalities exist, they are confined to individual cells rather than the entire colon. *Cancer Prev Res; 5(7); 921–9. ©2012 AACR.*

Introduction

Folate is an important methyl donor and essential for DNA methylation. Mathematical modeling (1) and *in vivo* studies (2) have shown that DNA methylation competes with DNA synthesis pathways for folate species. In proliferative tissues such as colorectal epithelium, adequate folate is important in maintaining normal 5-methylcytosine levels. Reduction in genome-wide methylation (global DNA hypomethylation) is an early event in colorectal cancer development (3, 4) and is said to predispose to genomic instability (5). Hence it has been hypothesized that folate deficiency may contribute to colonic DNA hypomethylation and increased colorectal cancer risk (6). Arguably, this effect could occur within individual epithelial cells or through "field defects" generated within apparently normal colorectal mucosa (7).

The study of folate in colorectal mucosa has proven challenging because of the inherent difficulty in obtaining this tissue. Furthermore, the microbiologic assay for folate, long-established as the gold standard (8), has significant limitations in this context. In fact, most human studies to date have used dietary folate assessment and/or blood folate concentration as surrogates for colorectal folate status (6, 9, 10). However, the relationship between blood and colonic mucosal folate concentration is unclear because of discrepancies in the results from previous studies (11–14).

Folate exists *in vivo* as functionally distinct coenzyme species with distinct biochemical roles. Understanding the distribution of these species is critical to an understanding of folate biology. For example, 5-methyltetrahydrofolate (5-CH_3-H_4folate) donates the methyl groups required for DNA methylation, whereas 5-formyltetrahydrofolate (5-CHO-H_4folate) and 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) are required for DNA synthesis. Thus, variations

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in folate species distribution may influence the rate of folate-dependent reactions such as DNA methylation (15, 16). Recently, we have developed a biochemical assay based on liquid chromatography tandem mass spectrometry (LC-MS/MS), which accurately measures these individual folate species in diverse biologic samples, including colorectal mucosa (17). More generally, biochemical assays are now seen as the preferred system for measuring folate (18).

Changes in DNA methylation of potential relevance to colorectal carcinogenesis include global hypomethylation, hypomethylation of LINE-1 and Alu repeats, and CpG island hypermethylation. Some studies have reported the extent to which global DNA methylation varies between colorectal mucosa and peripheral blood (19, 20). Other studies have used methylation of LINE-1 and ALU repeat elements as a surrogate for global DNA methylation in tumors and normal tissues (21–24). However, none of those studies that compared repeat element methylation in blood and mucosa used paired samples from the same individual, and sample sizes in these studies were small (21–24).

A number of studies have shown a direct relationship between DNA methylation and folate concentration in the peripheral blood, both in terms of global (16, 25) and LINE-1 methylation (10). However, the relationship between folate and global DNA methylation in the colonic mucosa remains uncertain. Most folic acid supplementation trials have not found an increase in colonic mucosa DNA methylation following supplementation (reviewed in ref. 9), although only one of these studies confirmed that folic acid supplementation actually increased colonic folate concentration (26). In a prospective cohort study, Schernhammer and colleagues showed that tumoral LINE-1 hypomethylation was more common in colorectal cancers from individuals with a low dietary folate intake (10). The interpretation of this result needs to be qualified by the inherent heterogeneity of LINE-1 methylation in colorectal tumors of diverse stage and molecular background (24).

In summary, several important questions remain unanswered about the relationship between folate and DNA methylation in the context of colorectal cancer. These include whether global DNA methylation in normal colonic mucosa differ between healthy people and those with colorectal cancer, whether folate and DNA methylation in blood can be used as a surrogate for their levels in colonic mucosa, and whether in different tissues the distribution of folate species correlates with the level of DNA methylation.

To address these important questions, we used recently developed assays to systematically profile folate distribution and DNA methylation (global, LINE-1, and Alu) in blood and mucosal samples obtained from colorectal cancer patients and from disease-free individuals.

Materials and Methods

Study subjects

We conducted a prospective study between October 2008 and April 2011 for the recruitment of colorectal cancer patients and healthy individuals. Informed consent was obtained from 112 patients presenting to St Vincent's and Prince of Wales Hospitals, Sydney, for primary colorectal cancer resection (cancer participants). Exclusion criteria included a hereditary cancer syndrome, inflammatory bowel disease, neoadjuvant radiotherapy or chemotherapy, and high-dose folic acid supplementation ($\geq$5 mg/d). We also obtained consent from 324 individuals referred for colonoscopy at 3 endoscopy clinics in metropolitan Sydney. From this group, we used criteria outlined in Fig. 1 to select a control group (healthy participants) of 114 individuals with normal colonoscopies. In addition, otherwise healthy individuals were excluded if they were taking medications that inhibited folate metabolism (methotrexate, trimethoprim, and sulfasalazine). Information on tobacco use was obtained at recruitment, and clinicopathologic information was obtained from medical records. Blood and normal colorectal tissue were obtained from all individuals in both participant groups.

This study also used frozen fresh samples of macroscopically normal mucosa collected between April 2005 and September 2006 from 19 colorectal cancer patients and 23 individuals who underwent bowel resection for noncancer–related conditions, including constipation, diverticular disease, and adhesions. Exclusion criteria were identical to those described above for the cancer participants.

Ethical approval for all aspects of this study was obtained from St Vincent's Hospital, South Eastern Illawarra Area Health Service and the NSW Population and Health Services Human Research Ethics Committees.

Sample collection and storage

Macroscopically normal colorectal mucosa was collected at least 10 cm from the tumor, then snap frozen in liquid

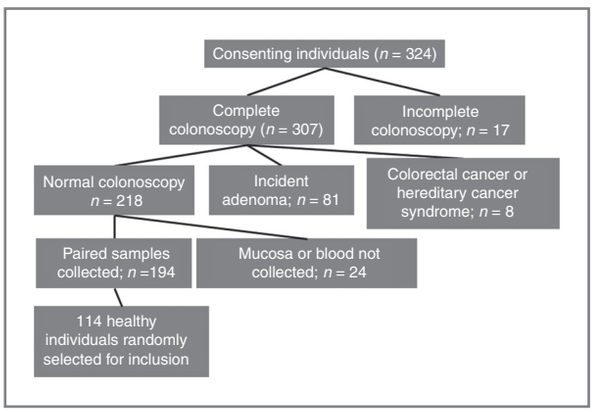

Figure 1. Selection process for the healthy participants from screening colonoscopy. In cases in which the endoscope was unable to be advanced to the terminal ileum, the individual was considered to have an "incomplete colonoscopy." Individuals found to have a histologically confirmed neoplasm (of any type with the exception of hyperplastic polyps) as well as colitis at the time of recruitment were excluded. Paired samples refer to both blood and colonic mucosa collected from the same individual.

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nitrogen or dry ice and stored at −80°C. The microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) status of the tumors from cancer participants was determined as previously described (27). For endoscopic collection of tissue from healthy participants, 5 biopsies of normal colorectal mucosa (~20 mg) were frozen on dry ice within 30 minutes and stored at −80°C. Although global and repeat element DNA methylation analysis was carried out on endoscopic biopsies from healthy participants, there was insufficient material for tissue folate species analysis.

Fasting blood samples were collected in EDTA tubes, the hematocrit determined (Auto Haematology Analyser), and aliquots of whole blood and plasma (separated by centrifugation at 400 *g* for 10 minutes) were stored at −80°C under nitrogen in amber microcentrifuge tubes. DNA was extracted from peripheral blood mononuclear cells (PBMC) and mucosal samples using phenol:chloroform:isoamyl alcohol and quantified by UV absorbance at 260 nm (NanoDrop 1000; NanoDrop Technologies Inc.).

Molecular assays

MTHFR genotyping. Genotyping for *MTHFR c.677C>T* was carried out by PCR using 10 ng of peripheral blood genomic DNA followed by pyrosequencing (ref. 28; see Supplementary Methods).

DNA methylation

The absolute quantity of 5-methyl-2′-deoxycytidine (5mdC; global methylation) was determined as a percentage of 2′-deoxycytidine plus 5mdC in genomic DNA using LC-MS/MS based upon the method of Quinlivan and Gregory (ref. 29; see Supplementary Methods). Quantitative assessment of unmethylated LINE-1 and Alu was carried out using the end-specific PCR assay (30). Restriction of the amplicon by the methylation-sensitive enzyme AciI (for Alu) and HpaII (for LINE-1) was compared with that of the methylation-insensitive DraI. Unmethylated repeat elements were normalized relative to a reference normal blood DNA sample ("Reference DNA"; Affymetrix) using the formula: Relative degree of unmethylated repeats in sample = Quantity of unmethylated repeats in sample/Quantity of unmethylated repeats in control. Because global methylation is typically expressed as the quantity of methylated DNA, the degree of methylated LINE-1 and Alu repeats is also reported here by stating the reciprocal of the degree of unmethylated DNA.

Distribution and level of erythrocyte and colonic folate using LC-MS/MS

Folate was extracted from whole blood (31) and plasma (ref. 32; see Supplementary Methods), then subjected to solid-phase extraction (33) using LC-18 3 mL cartridges (Sigma-Aldrich). LC-MS/MS analysis was carried out as previously described (17). Given the potential of 5,10-CH^+-H_4folate to interconvert to 5-CHO-H_4folate during extraction and because these coenzymes are interconvertible *in vivo*, these 2 measurements were pooled into a single "formyl-H_4folate" value as previously described (31). Erythrocyte folate concentration was calculated using the formula {(whole blood folate × 100) − [plasma folate × (100 − hematocrit)]}/hematocrit (34).

We used our previously described method (17) to determine folate distribution in resected normal colorectal mucosa from the 19 colorectal cancer patients and 23 noncancer subjects collected in 2006, as well as in 67 of the cancer participants in whom sufficient tissue (>50 mg) was available for analysis. Assessment of tissue folate distribution was not possible on endoscopic mucosal biopsies from the healthy participants because of poor reproducibility of the assay when less than 50 mg of tissue was available.

The erythrocyte folate assay was validated as previously described (17), using whole blood and plasma pooled from 10 randomly selected colorectal cancer patients to create a quality control matrix. Because it is not possible to directly compare the absolute folate concentration in erythrocytes and colonic mucosa, the results were presented as folate species distribution (i.e., each folate species was expressed as a percentage of total folate, calculated as the sum of values for the 5 folate forms).

Statistical analysis

Global, LINE-1, Alu methylations, and folate were measured in quadruplicate and triplicate, respectively. Baseline characteristics were compared using one-way ANOVA, χ^2 tests, or *t* test as appropriate. Relationships between folate and DNA methylation were assessed using linear regression. Relationships between methylation assays were determined using bivariate correlation. Differences in folate species distribution and DNA methylation between the groups and between tissues within each group were examined using independent samples *t* test. Multiple linear regression was used to evaluate whether these DNA methylation comparisons between the groups were consistent after accounting for age, sex, and smoking. Adjustment had only a modest effect and did not significantly alter the study's conclusions; hence only unadjusted means were reported. Adjustment was not done on folate distribution analyses and subgroup analyses by CIMP and MSI status because of the smaller sample sizes in these comparisons. Statistical significance was defined at $P < 0.01$. Analyses were done using SPSS software (Version 18.0, SPSS Inc.) and verified by an independent statistician.

Results

Characteristics of study participants

Characteristics of the 226 subjects are shown in Table 1. Of the 112 cancer participants, 102 (91%) had stage I–III disease and 10 (9%) had metastatic disease. Twenty-two of 108 (20%) had MSI tumors, and 18 of 102 (18%) had CIMP+ tumors. Cancer participants were on average 13 years older than the healthy participants and had a lower

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Table 1. Characteristics of study groups

Characteristic		Healthy participants	Cancer participants	*P*[a]
Age	*n*	114	112	<**0.0001**
	Mean (SD)	54 (12.5)	67 (13.6)	
Male gender	*n* (% Male)	70/114 (61)	66/112 (59)	0.70
Hematocrit	*n*	102	81	<**0.0001**
	Mean (SD)	0.42 (0.04)	0.36 (0.05)	
Current smoker	*n* (% Yes)	41/114 (36)	57/94 (61)[b]	<**0.0001**
MTHFR C677T[b]				
CC	*n* (%)	43 (38)	50 (45)	0.57
CT	*n* (%)	53 (47)	47 (42)	
TT	*n* (%)	18 (16)	15 (13)	
Region of mucosa sampled				
Right-colon	*n* (%)	48/102 (47)	45/111 (40)	0.06
Left-colon	*n* (%)	37/102 (36)	32/111 (29)	
Rectal	*n* (%)	17/102 (17)	34/111 (31)	

[a]Statistical significance was calculated using *t* tests (mean age, hematocrit, and current smoker) and χ^2 tests for all other variables. Significant *P* values are indicated in bold.

[b]The *MTHFR C677T* genotype distribution was confirmed to be in Hardy–Weinberg equilibrium in both healthy and cancer participants.

blood hematocrit ($P < 0.0001$). A total of 61% of the cancer participants were smokers compared with 36% of the healthy participants ($P < 0.0001$).

Global and repeat element DNA methylation in mucosa and blood of colorectal cancer patients is similar to healthy individuals

The global DNA methylation assay using LC-MS/MS had excellent linearity ($R^2 > 0.999$) and reproducibility with low intra-assay (3.2%, $n = 6$) and inter-assay (2.7%, $n = 6$) variability. Global DNA methylation in the normal mucosa of cancer participants (4.11% ± 0.29) was marginally higher than that observed in healthy participants (4.02% ± 0.26) after adjusting for age, sex, and smoking status (difference of 0.12%, $P = 0.008$, Supplementary Table S1A).

Colorectal mucosa had lower global DNA methylation than the blood of healthy participants (4.02% ± 0.26 vs. 4.37% ± 0.25; difference of 0.35%; $P < 0.0001$) and cancer participants (4.11% ± 0.29 vs. 4.45% ± 0.27; difference of 0.34%; $P < 0.0001$; Fig. 2A). The lower global DNA methylation in colorectal mucosa was accompanied by less methylation at LINE-1 and Alu repeats. LINE-1 methylation in the mucosa of healthy participants was only 36% that of the control normal blood, whereas Alu methylation in the mucosa of healthy participants was 59% of the level seen in the control DNA (Fig. 2B and C). Alu methylation was 6% lower in the right colon ($n = 117$, 59% ± 14) compared with the left colon and rectum ($n = 92$, 53% ± 14; $P < 0.0001$, pooled data from cancer and healthy participants), but there were no differences at LINE-1 repeats and global DNA methylation (data not shown). Consistent with the small differences in global DNA methylation, there were no significant differences in the degree of LINE-1 and Alu methylation in the colorectum of cancer participants compared with the healthy participants (Fig. 2). Alu and LINE-1 methylation remained identical after adjusting for age, sex, and smoking status (Supplementary Table S1). Furthermore, global, LINE-1, and Alu methylations in the colonic mucosa were not significantly different in healthy versus cancer participants after stratification by site of mucosal sampling (right sided vs. left sided/rectum; data not shown). Global and repeat element DNA methylation in the normal mucosa of healthy individuals was no different to that of cancer patients when stratified by CIMP status (Supplementary Table S2). Similarly, mucosal global or repeat element DNA methylation was not significantly different between individuals with MSI or MSS tumors (Supplementary Table S3). Using bivariate correlation analysis, there was no relationship between age and global or repeat element methylation, either in blood or normal colorectal mucosa (data not shown).

Repeat element methylation does not correlate with global DNA methylation

The relationship between global, LINE-1, and Alu methylations in blood ($n = 221$) and colorectal mucosa ($n = 217$) from both participant groups is shown in Fig. 3. Data from healthy and cancer participants were pooled for this analysis, as similar relationships were identified when they were analyzed separately. In the blood, there was no correlation between LINE-1 and Alu ($r = -0.05$; $P = 0.4$), LINE-1 and global ($r = 0.05$; $P = 0.5$), and Alu and global DNA methylation ($r = 0.05$; $P = 0.5$). Likewise in the colorectal mucosa, no correlation was found between LINE-1 and global methylation ($r = 0.08$; $P = 0.2$) or Alu and global methylations ($r = -0.15$; $P = 0.8$). LINE-1 methylation in

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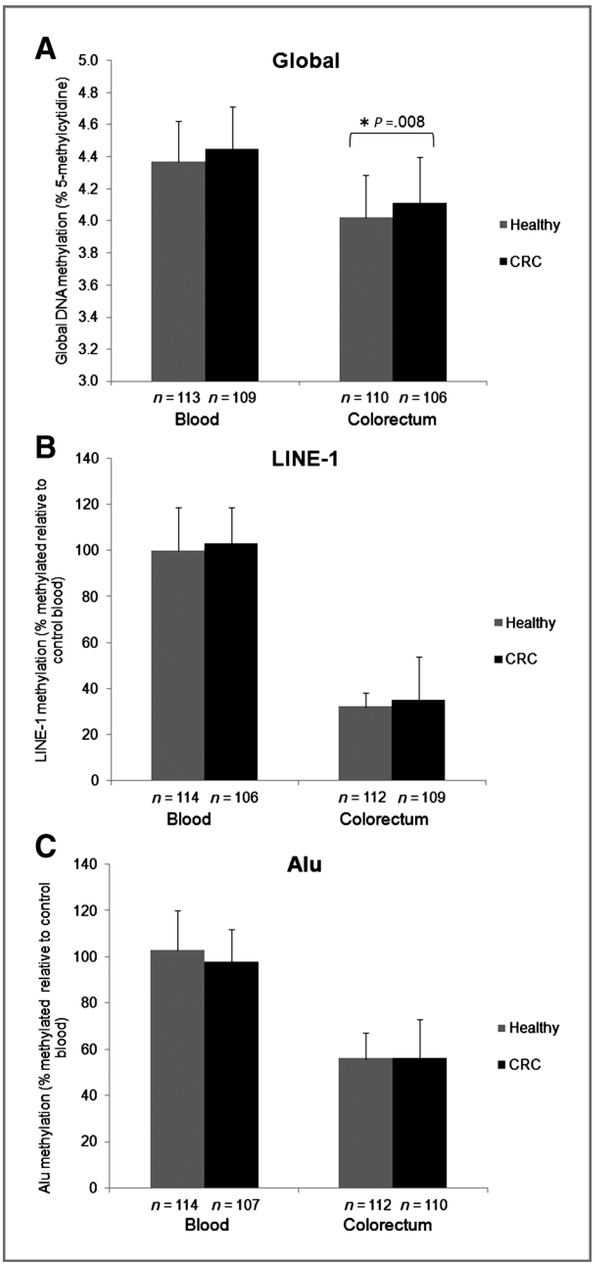

Figure 2. DNA methylation in cancer participants is similar to healthy participants. Data shown are mean values. Errors bars = SD. Significant *P* values after adjustment for age, sex, and smoking status differences between subjects in the cancer and healthy participants are reported. Where inconsistent measurements were seen for global ($n = 14$), LINE-1 ($n = 10$), and Alu ($n = 9$) methylation, these were excluded from the analysis.

the colorectal mucosa was directly correlated with Alu methylation ($r = 0.42$; $P < 0.0001$).

There was no correlation between blood and colonic mucosal DNA methylation, either globally ($n = 213$; $r = 0.13$; $P = 0.051$), at LINE-1 ($n = 215$; $r = 0.15$; $P = 0.026$), or Alu repeat elements ($n = 216$; $r = 0.07$; $P = 0.28$).

Distribution of folate in blood and colorectal mucosa

The erythrocyte folate assay was linear ($R^2 > 0.99$) and reproducible (inter-assay variability 3.8%–14.4%; intra-assay variability 2.2–6.0%), with excellent recovery of spiked folate standards (85.1%–125.5%).

By using previously collected mucosa derived from individuals with ($n = 19$) or without ($n = 23$) colorectal cancer, we showed that cancer status did not influence the observed distribution of folate species in colorectal mucosa (Fig. 4A) or total folate concentration (data not shown). With this issue clarified, all following data refer to results from the prospectively collected tissues from the cancer and healthy participants.

The distribution of folate species in the blood was identical in healthy and cancer participants (Fig. 4B). However, the colonic mucosa from cancer participants ($n = 67$) showed widespread differences in folate species distribution compared with the blood from the same individuals. These differences were significant for all species but are most notable in terms of a lower proportion of 5-CH_3-H_4folate (55% ± 13.6 vs. 88% ± 13.1) and a higher proportion of formyl-H_4folate (16% ± 12.1 vs. 9.3% ± 11.1) and H_4folate (25% ± 9.4 vs. 1.4% ± 3.1; Fig. 4B).

There were no differences in total erythrocyte folate levels between cancer and healthy participants (data not shown). In cancer participants, total erythrocyte folate correlated with total colorectal folate ($r = 0.38$; $P = 0.002$; Supplementary Fig. S1). There was a significant correlation in formyl-H_4folate distribution between colorectal mucosa and erythrocytes from the same individual ($r = 0.34$; $P = 0.005$, Supplementary Fig. S2), however no significant correlations in the other folate species were found (data not shown).

No difference in folate species distribution and total folate concentration were seen in the normal mucosa of patients with CIMP+ versus CIMP− tumors (Supplementary Table S2), nor those patients with MSI versus MSS tumors (Supplementary Table S3).

Discussion

This study provides new evidence about the complex relationship between folate, DNA methylation, and colorectal carcinogenesis. It has done so by focusing on the analysis of colorectal mucosa, the tissue of direct biologic relevance to colorectal cancer development. Using LC-MS/MS assays, we have shown changes in folate species distribution in blood and colorectal mucosa from colorectal cancer patients and healthy individuals. We have also shown how folate distribution relates to DNA methylation in those tissues.

We found that although erythrocyte folate distribution was similar between healthy and cancer participants, there were significant differences in folate distribution in colonic mucosa from cancer participants when compared with their blood. Specifically, normal colorectal mucosa showed substantially lower 5-CH_3-H_4folate distribution and higher H_4folate and formyl-H_4folate distribution compared with

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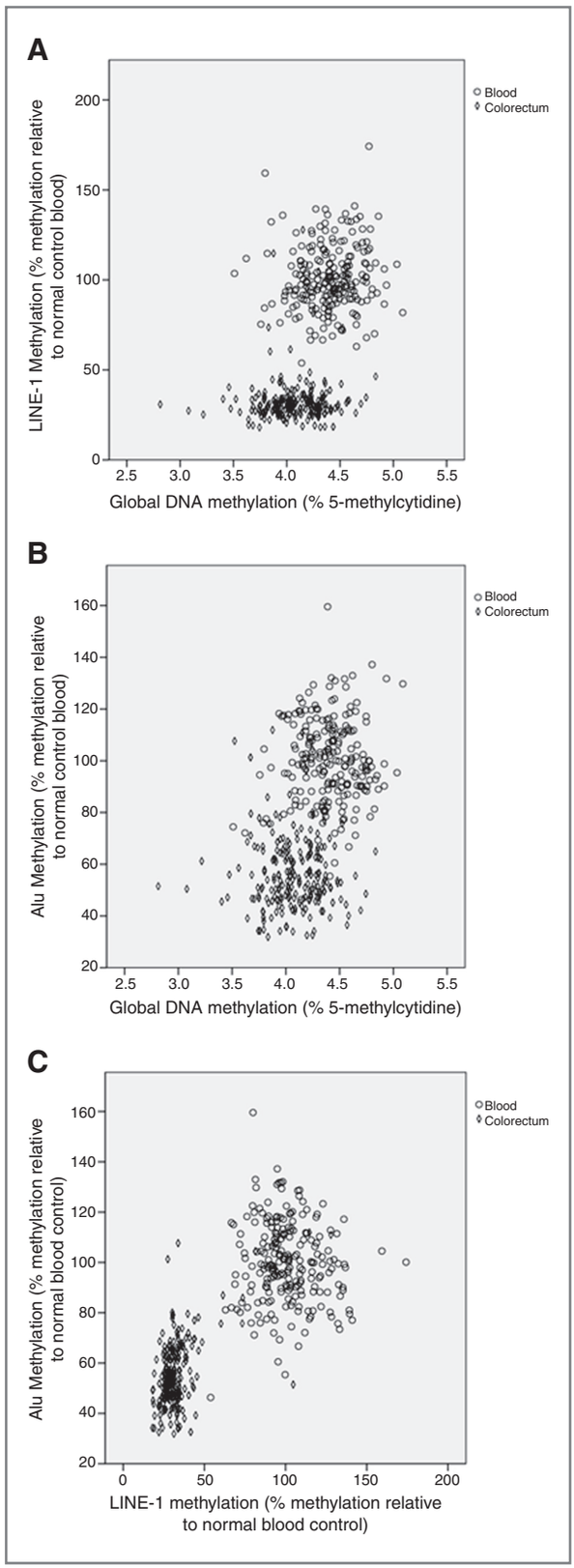

erythrocytes from the same patient. We were also able to show in both cancer and healthy participants that this redistribution of folate away from the methylation compartment and toward the DNA synthesis compartment was accompanied by a decrease in both global and repeat element DNA methylation in the colorectal mucosa, when compared with blood. This decrease in the degree of DNA methylation in the normal mucosa occurred to the same extent in both healthy and cancer participants.

The marked variation in folate species distribution in different tissues highlights the importance of determining individual folate species, rather than assessment of total folate. It is also noteworthy that the use of microbiologic assays to determine total folate concentration could produce significant experimental error because some bacteria respond differently to the different folate species (8).

Using by necessity mucosal samples from a previous collection, we were also able to tentatively show that folate species distribution and total folate concentration (calculated as the sum of the different folate species) did not differ in resected samples of normal colonic mucosa, regardless of whether the individual had a neoplastic or nonneoplastic colorectal disorder. This finding that normal mucosa of colorectal cancer patients does not harbor disease-specific changes in folate status is in agreement with previous observations that epithelial cells from normal mucosa had similar total folate concentration to cells from normal mucosa adjacent to tumors and adenomas (35).

Taken together, these results suggest that the extent of DNA methylation is related to folate species distribution, rather than to tissue type or disease status *per se*. The results also offer insights into the way different tissues may prioritize folate for methylation versus DNA synthesis.

DNA hypomethylation is generally thought to result from incomplete copying of methylation patterns to the daughter strand after DNA synthesis, possibly as a consequence of a deficiency of methyl groups. Thus in the context of relative folate deficiency, DNA hypomethylation would occur only if cellular proliferation was sustained (via higher formyl-H_4folate distribution) at the expense of diminished availability of methyl groups (via decreased 5-CH_3-H_4folate distribution). In tissues such as the colonic mucosa that prioritize more folate for DNA synthesis, we would predict higher baseline formyl-H_4folate distribution and lower baseline DNA methylation levels. Although the relationship between folate species distribution, cell proliferation, and DNA methylation in different tissues has not been extensively studied in humans, methylenetetrahydrofolate

Figure 3. Levels of global, LINE-1, and Alu DNA methylation in blood (circles) and colorectal mucosa (diamonds). Data from healthy and cancer participants were pooled for this analysis. Panels show lack of correlation between global DNA methylation and LINE-1 (A: blood $r = 0.05$, $P = 0.5$; mucosa $r = 0.08$, $P = 0.2$), global DNA methylation and Alu (B: blood $r = 0.05$, $P = 0.5$; mucosa $r = -0.15$, $P = 0.8$) and Alu and LINE-1 (C: blood $r = -0.05$, $P = 0.4$). However, Alu and LINE-1 methylation were correlated in colorectal mucosa ($r = 0.42$, $P < 0.0001$). Alu and LINE-1 hypomethylation values are expressed as a percentage relative to the methylation content of a reference normal blood DNA (Affymetrix).

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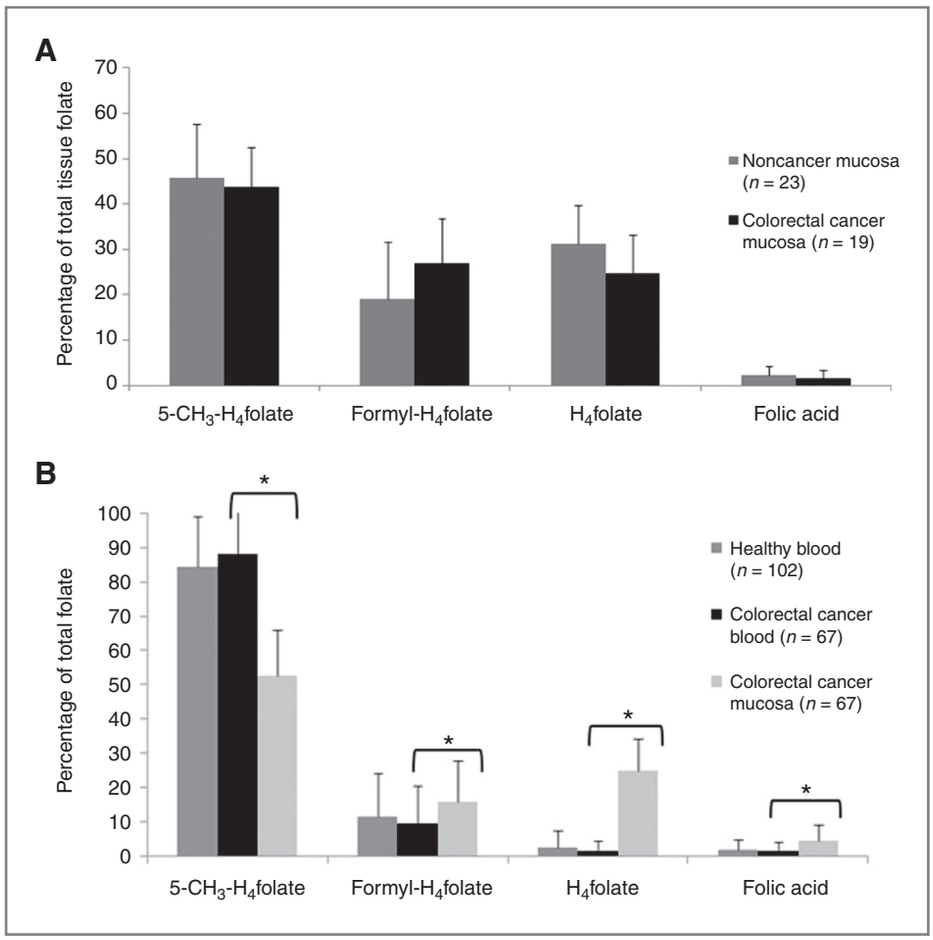

Figure 4. A, no difference in folate species distribution was present in the normal colorectal mucosa of 23 individuals presenting for colorectal resection for noncancer causes compared with that of 19 colorectal cancer patients. Samples from both groups were collected in 2005–06 and have been in −80°C storage for 6 years. There were no differences in age, gender distribution, or colonic global or repeat element DNA methylation between these 2 groups (data not shown). B, mean folate species distribution in erythrocytes and colorectal mucosa. The distribution in erythrocytes of 5-CH_3-H_4folate (5-methyltetrahydrofolate) and formyl-H_4folate (formyltetrahydrofolate) was not different between subjects in the healthy and cancer participants $P = 0.97$ and 1.0, respectively, independent samples *t* test). Within the same individual, cancer participants showed lower 5-CH_3-H_4folate ($P < 0.0001$), higher formyl-H_4 folate ($P < 0.0001$), higher H_4folate ($P < 0.0001$), and higher folic acid ($P < 0.0001$) in colorectal mucosa than erythrocytes (paired samples *t* test). Error bars = SD. *, $P < 0.0001$. 5-CH_3-H_4folate, 5-methyltetrahydrofolate; formyl-H_4folate, formyltetrahydrofolate; H_4folate, tetrahydrofolate.

reductase (*MTHFR*) deletion in rats resulted in tissue-specific changes in folate species distribution (36). In addition, low colonic 5-CH_3-H_4folate distribution, but not total folate concentration, was associated with a 35% reduction in colonic DNA methylation in vitamin B_{12}–deficient rats (37).

To date, there has been conflicting evidence about the extent of global and repeat element hypomethylation in otherwise normal colorectal mucosa of patients with cancer. Regional or colon-wide hypomethylation has been reported in the macroscopically normal mucosa of colorectal cancer patients (19, 38), raising the possibility of a "field defect" that may predispose to tumor development. In contrast, others have found no significant difference in global (39) or repeat element methylation (40) in this setting, although folic acid supplementation or short-term depletion (sufficient to modify total colonic folate concentration) do not alter colonic global (26) or LINE-1 methylation (41). By analyzing mucosa from large numbers of colorectal cancer and healthy individuals using gold standard assays, this study has definitively showed that the normal mucosa of colorectal cancer patients is no more demethylated than mucosa from endoscopy-confirmed healthy individuals. When considered with our observations about folate distribution, these findings suggest that alterations in folate distribution and DNA methylation are a phenomenon occurring specifically within neoplastic cells.

The "field defect" concept has also been invoked in relation to CIMP+ tumors, a subgroup of colorectal cancers with widespread and tumor-specific CpG island hypermethylation and by inference to MSI tumors, which show MSI because of biallelic hypermethylation of the *MLH1* gene promoter (42). The bias of CIMP+ and MSI cancers to the right colon (43) and their increased synchronicity (44) would be consistent with a field defect, perhaps involving changes to DNA methylation or folate profiles. Indeed, higher erythrocyte folate has been associated with increased CpG island methylation in normal colonic mucosa (45), whereas altered folate distribution has been seen in CIMP+ cancers (46). In this study, both CIMP+ and MSI tumors were present at frequencies (18.2% and 20.4%, respectively) broadly consistent with previous reports. However, we found no significant differences in colonic DNA methylation or folate distribution in individuals with either CIMP+ or MSI tumors. Although based on relatively small numbers, this finding argues against an underlying field defect related to folate or methylation.

One limitation of this study relates to the measurement of colonic folate distribution in individuals without colorectal

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cancer. It is challenging to obtain mucosa from normal individuals in amounts suitable for folate distribution analysis, and individuals undergoing colorectal resection for noncancer causes may not be representative of a normal population. In addition, blood global and repeat DNA methylation and folate were measured in different cell populations (PBMCs and erythrocytes, respectively). At the same time, this study has a number of methodologic strengths. There was rigorous selection of healthy participants, including only those free of endoscopically identifiable colorectal neoplasms. State-of-the art biochemical assays were used to quantify folate and global DNA methylation. Finally, folate distribution and DNA methylation were measured in the same samples.

In summary, this study has shown that blood folate levels should not be used as a surrogate for the colorectal mucosa. We found no evidence that mucosal DNA methylation or folate distribution was related to the presence of cancer *per se*, suggesting that if abnormalities exist they are confined to individual cells rather than the entire colon.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Liu, L.B. Hesson, J.E. Pimanda, R.L. Ward

Development of methodology: J. Liu, L.B. Hesson, A.P. Meagher, K.N. Rand, P.L. Molloy, R.L. Ward

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Liu, L.B. Hesson, J.E. Pimanda, R.L. Ward

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