Featured Article

The Folate Pool in Colorectal Cancers Is Associated with DNA Hypermethylation and with a Polymorphism in Methylenetetrahydrofolate Reductase

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Abstract

Purpose: **Aberrant DNA methylation occurs in a subset of colorectal cancers and is characterized by regional areas of hypermethylation at CpG islands. The aims of this study were firstly to evaluate the levels of folate intermediates (FIs) in tumors with aberrant DNA methylation and secondly to determine whether these levels are affected by polymorphisms in key genes involved in folate metabolism.**

Experimental Design: **The concentrations of two major intracellular FIs, 5,10-methylenetetrahydrofolate and tetrahydrofolate (FH_4), were measured in 103 surgically resected colorectal cancers. DNA hypermethylation at seven different CpG islands was measured using the MethylLight assay. Genotyping for polymorphisms in the thymidylate synthase, cystathionine β-synthase, methionine synthase, and methylenetetrahydrofolate reductase (MTHFR) genes was carried out using PCR and PCR-RFLP.**

Results: **Significantly higher levels of FH_4 were found in tumors from the proximal colon compared with those originating in the distal colon and rectum. Tumors with aberrant DNA methylation of CpG islands within promoter regions of the *hMLH1*, *TIMP3*, and *ARF* genes also contained higher levels of both 5,10-methylenetetrahydrofolate and FH_4. In contrast, patients who were homozygous for the C667T polymorphism of the MTHFR gene had significantly lower concentrations of both these FIs in their tumor tissue.**

Conclusions: **The concentrations of FIs in colorectal tumors are directly related to the presence of frequent DNA hypermethylation and inversely related to the presence of a common polymorphism in the MTHFR gene. FIs could serve as biochemical markers for the risk of developing this disease, as well as for the prediction of toxicity and efficacy of fluorouracil-based treatments.**

Introduction

DNA methylation patterns are altered frequently in human cancers, and include genome-wide hypomethylation as well as regional hypermethylation at CpG islands (1). The CIMP+[6] in CRCs is characterized by the frequent and concurrent methylation of specific CpG sites, including those present in the promoter regions of *p16* and *hMLH1* (2). CIMP+ tumors are frequently associated with origin in the proximal colon, older patient age, female gender, and poorly differentiated histological grade (3, 4). The majority of sporadic CRCs with the MSI+ phenotype show methylation-induced transcriptional silencing of the *hMLH1* DNA repair gene (5). It is not surprising then to find that MSI+ and CIMP+ phenotypes share many biological features (3–5). Depending on the definition used, approximately 20–30% of sporadic CRCs can be classified as CIMP+ (3, 4). This phenotype may be an indicator of more widespread aberrations in methyl-group metabolism in cancer cells including global DNA hypomethylation and altered concentrations of FIs. Whereas a recent study found no relationship between CIMP+ and global hypomethylation (6), there have been no reports to date on the level of FI in CIMP+ tumors. Additional characterization of CIMP+ tumors is important not only to gain a better understanding of their etiology but also to determine whether this phenotype is associated with toxicity and/or response to the antifolate therapies commonly used in the treatment of CRC.

The enzyme MTHFR catalyzes the reduction of CH_2FH_4 to 5-methyltetrahydrofolate. This molecule is a substrate for the conversion of homocysteine to methionine, which, in turn, is a precursor to *S*-adenosylmethionine. The latter is an important methyl group donor for intracellular methylation reactions, in particular the methylation of DNA. A common germ-line variant in *MTHFR* (C677T, alanine to valine) is associated with lower enzymatic activity compared with wild-type and has been implicated in the risk of developing CRC (7–10). Although the mechanism has yet to be determined, possible links could be with the aberrant DNA global

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[6] The abbreviations used are: CIMP+, CpG island methylator phenotype; CRC, colorectal cancer; MSI+, microsatellite instability; FI, folate intermediate; CH_2FH_4, 5,10-methylenetetrahydrofolate; FH_4, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; TS, thymidylate synthase; CBS, cystathionine β-synthase; 5FU, 5-fluorouracil.

hypomethylation or CpG-island-specific hypermethylation that are often observed in CRC. In support of this, the MTHFR C677T polymorphism has been associated with DNA hypomethylation in normal tissues (11–13). In addition to being a possible risk factor for CRC, the MTHFR C677T polymorphism has also been implicated in the toxicity to antifolate therapies used in cancer patients (14–16) and in the response to this treatment (17).

In the present study we have sought to obtain more information on colorectal tumors with aberrant methyl-group metabolism by measuring the concentrations of two major intracellular FIs: CH_2FH_4 and FH_4. These were correlated with various pathological features of the tumors, with the presence of DNA hypermethylation at seven different CpG islands, and with the presence of genetic variants in four enzymes involved in methyl-group metabolism.

Materials and Methods

Tissue samples from a consecutive series of 103 CRC patients undergoing elective surgery at the Colorectal Unit of the Royal Adelaide Hospital were snap frozen in liquid nitrogen within 20–40 min after resection and stored at −70°C. The clinical and pathological features of this series are shown in Table 1. Patients had been fasted for 24 h before surgery. MSI+ status was determined as described previously (18) by screening for instability at nine microsatellite loci that included both mononucleotide (Bat25, Bat26, and Bat40) and dinucleotide (D2S123, D10S197, D17S579, D18S34, D5S346, and D17S250) repeats. Tumors showing instability at two or more loci were considered to have the MSI+ phenotype.

Table 1 Associations between the concentration of folate intermediates and pathological features of colorectal cancer

Feature (*n*)	CH_2FH_4 (pmol/g tissue)	*P*	FH_4 (pmol/g tissue)	*P*
Total (103)	1.68 ± 1.36		2.18 ± 1.67	
Sex				
Male (60)	1.62 ± 1.31		2.08 ± 1.52	
Female (43)	1.76 ± 1.44	0.61	2.32 ± 1.86	0.49
Age				
≤72 years (52)	1.48 ± 1.02		1.96 ± 1.37	
>72 years (51)	1.87 ± 1.62	0.15	2.40 ± 1.91	0.18
youngest quartile (26)	1.30 ± 0.79		1.83 ± 1.05	
oldest quartile (26)	2.08 ± 1.98	0.074	2.60 ± 1.93	0.084
Site				
Proximal (49)	1.91 ± 1.56		2.58 ± 1.91	
Distal (51)	1.49 ± 1.15	0.19	1.83 ± 1.35	0.026
Grade				
Well/moderate (73)	1.74 ± 1.45		2.18 ± 1.77	
Poor (14)	1.66 ± 0.85	0.78	2.36 ± 1.22	0.51
Nodal status				
Negative (68)	1.81 ± 1.50		2.34 ± 1.72	
Positive (35)	1.42 ± 1.02	0.12	1.87 ± 1.53	0.16
Malignancy				
Adenoma (13)	1.36 ± 1.23		2.18 ± 1.58	
Adenocarcinoma (90)	1.72 ± 1.38	0.36	2.18 ± 1.69	1.0
Mucinous				
No (88)	1.63 ± 1.27		2.15 ± 1.62	
Yes (15)	1.97 ± 1.83	0.50	2.33 ± 1.96	0.74
TILs[a]				
No (89)	1.60 ± 1.30		2.12 ± 1.68	
Yes (14)	2.20 ± 1.66	0.21	2.58 ± 1.57	0.33
MSI				
Negative (73)	1.59 ± 1.37		2.06 ± 1.71	
Positive (18)	2.32 ± 1.44	0.06	2.87 ± 1.60	0.06

[a] Tumor-infiltrating lymphocytes.

Aliquots of each tumor were sent on dry ice to the Kanazawa University School of Medicine for measurement of CH_2FH_4 and FH_4 concentrations. This assay was carried out as described previously (19) with the exception that recombinant TS (gift from Dr. M. Fukushima, TAIHO Pharmaceutical Industry, Tokyo, Japan) was substituted for the TS solution obtained from Yoshida Sarcoma. Briefly, for the quantitation of CH_2FH_4, a cytosolic fraction of tissue was incubated with an excess of [^{3}H]fluoro-dUMP and recombinant TS to form a ternary complex that was then measured in a scintillation counter. FH_4 was converted to CH_2FH_4 with formaldehyde and then quantitated using the same procedure. Concentrations of CH_2FH_4 and FH_4 are expressed as pmol/g tissue and are shown as mean ± SD for each tumor subgroup.

Screening for the presence of CpG island methylation within the promoter regions of *hMLH1*, *p16*, *TIMP3*, *ARF*, *MINT2*, *APC*, and *DAPK* was carried out using the MethyLight assay and oligonucleotide primer sequences described previously (20, 21). The probe and primers for *MINT2* and *DAPK* were newly designed in this study and are as follows: MINT2 forward PCR primer TAACTAATCGAACCTACCGCCG; MINT2 reverse PCR primer AAATAAAAGAGAGCGCGAGGGG; MINT2 probe CTCGAATCCCAAACGCTCCCTCTCCC; DAPK forward PCR primer GGATAGTCGGATCGAGTTAACGTC; DAPK reverse PCR primer CCCTCCCAAACGCCGA; and DAPK probe CTACCGCTACGAATTACCGAATCCCCTCCG.

Briefly, tumor DNA was converted with sodium bisulfite before methylation analysis with the fluorescence-based, real-time PCR MethyLight assay. The results were analyzed to obtain a percentage of methylated reference value as described previously (21). For each of the CpG sites examined, hypermethylation was defined arbitrarily as a percentage of methylated reference value of >10.

Genotyping for the 28-bp tandem repeat in the enhancer region of TS and for the 68-bp insert variant of the CBS gene was carried out using 3% agarose gels to estimate PCR product size (22, 23). Screening for the C677T polymorphism of MTHFR and the A919G polymorphism of MS was carried out using PCR-RFLP (12, 23).

Statistical analyses were performed using the SPSS software package (Chicago, IL). The two-sided *t* test was used to compare CH_2FH_4 and FH_4 concentrations between different CRC subgroups defined by clinical, pathological, molecular, and genotypic features. Associations between DNA methylation and clinicopathological features were determined using the χ^2 test for independence and the Pearson statistic. Fisher's exact test was used when expected frequencies fell below five. Multinomial logistic regression analysis was used to identify factors that were independently associated with heavy DNA methylation. All of the *P*s given are two tailed with $P < 0.05$ taken as statistically significant.

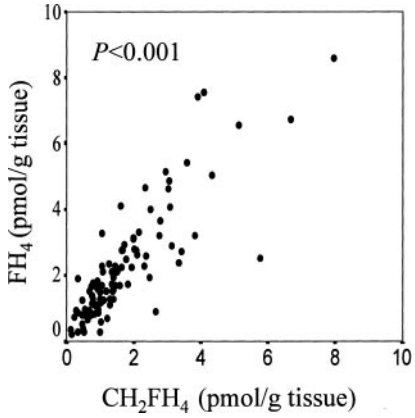

Fig. 1 Concentrations of the FIs CH_2FH_4 and FH_4 evaluated in 103 surgically resected CRC specimens.

Results

A strong correlation was observed between the concentrations of CH_2FH_4 and FH_4 in individual tumors ($P < 0.001$; Fig. 1). Wide variations were noted between samples, however, with ranges of 0.12–7.96 and 0.22–8.57 pmol/g tissue for CH_2FH_4 and FH_4, respectively. Tumors from patients older than the median age of 72 years showed a trend toward higher concentrations of FI, and this was more pronounced when comparing quartiles of youngest and oldest patient groups (Table 1). Tumors located in the proximal colon also showed a higher concentration of FI, although this reached significance only for FH_4. Gender, histological grade, tumor stage, mucinous appearance, and the presence of infiltrating lymphocytes were not associated with the level of FI, whereas MSI+ tumors showed borderline significance for association with higher concentrations of FI.

Proximal tumor location, older age, and MSI+ are all characteristics that have been associated previously with CIMP+ CRC (3–5). In light of the above observations one would, therefore, expect higher concentrations of FI in tumors showing DNA hypermethylation. This was indeed the case for three of the seven CpG sites examined: *hMLH1*, *TIMP3*, and *ARF* (Table 2). Tumors showing methylation at these sites contained significantly higher levels of both CH_2FH_4 and FH_4. Methylation at two other sites, *p16* and *MINT2*, were also associated with higher FI levels, although this did not reach statistical significance. The methylation of *DAPK* showed no association with FI levels, whereas methylation of *APC* showed trends for association with lower FI. Although there is as yet no consensus definition for CIMP+, in the present study methylation at zero or one of the seven CpG sites was considered to represent absent or light methylation, whereas methylation at four or more of the seven sites was defined as heavy methylation. Using this classification, tumors with heavy methylation were found to contain almost twice the concentrations of both CH_2FH_4 and FH_4 compared with tumors with light or absent methylation (Table 2). Of the 13 tumors with heavy methylation, 9 (69%) were in the highest quartile of CH_2FH_4 and FH_4 concentrations but none (0%) were in the lowest quartile ($P = 0.002$ and $P = 0.0007$, respectively).

Polymorphisms in four genes encoding enzymes involved in methyl-group metabolism were examined for association with FI levels (Table 3). Variants in MS and CBS showed no associations with FI, the homozygous triple repeat in TS showed a trend for association with lower FI levels, whereas the homozygous MTHFR-TT genotype was associated with significantly lower concentrations of FI compared with the CC or CT genotypes. Of the 25 tumors in the quartile with highest concentration of CH_2FH_4, only 1 (4%) was MTHFR-TT genotype compared with 9 of 26 (35%) of those in the quartile with lowest concentration of CH_2FH_4 ($P = 0.006$).

The associations between DNA methylation, and the clin-

Table 2 Associations between the concentration of folate intermediates and DNA methylation

Methylated site (*n*)	CH_2FH_4 (pmol/g tissue)	*P*	FH_4 (pmol/g tissue)	*P*
hMLH1				
− (83)	1.47 ± 1.28		1.97 ± 1.56	
+ (20)	2.52 ± 1.40	0.005	3.05 ± 1.85	0.023
TIMP3				
− (84)	1.50 ± 1.24		1.98 ± 1.54	
+ (19)	2.45 ± 1.61	0.024	3.08 ± 1.95	0.028
ARF				
− (92)	1.57 ± 1.35		2.03 ± 1.58	
+ (11)	2.54 ± 1.19	0.022	3.46 ± 1.92	0.033
p16				
− (80)	1.55 ± 1.20		2.05 ± 1.58	
+ (23)	2.11 ± 1.53	0.12	2.64 ± 1.90	0.18
MINT2				
− (77)	1.51 ± 1.19		2.02 ± 1.60	
+ (26)	2.18 ± 1.69	0.069	2.65 ± 1.79	0.12
DAPK				
− (93)	1.67 ± 1.39		2.17 ± 1.71	
+ (10)	1.79 ± 1.08	0.76	2.26 ± 1.30	0.85
APC				
− (82)	1.76 ± 1.44		2.27 ± 1.76	
+ (21)	1.35 ± 0.95	0.13	1.85 ± 1.19	0.20
Sites methylated				
0 or 1 (74)	1.53 ± 1.28		1.99 ± 1.57	
2 or 3 (16)	1.31 ± 1.02		1.80 ± 1.34	
≥4 (13)	2.95 ± 1.53	0.007[a]	3.71 ± 1.87	0.007[a]

[a] ≥4 sites methylated versus 0 or 1 site methylated.

Table 3 Associations between the concentration of folate intermediates and polymorphisms in methyl group metabolism genes

Polymorphism (*n*)	CH_2FH_4 (pmol/g tissue)	*P*	FH_4 (pmol/g tissue)	*P*
TS				
2/2 or 2/3 (71)	1.78 ± 1.47		2.30 ± 1.82	
3/3 (32)	1.45 ± 1.06	0.20	1.91 ± 1.25	0.21
MTHFR				
CC or CT (85)	1.79 ± 1.42		2.34 ± 1.74	
TT (17)	1.10 ± 0.84	0.009	1.24 ± 0.67	<0.0001
CBS				
−68bp insert (91)	1.67 ± 1.39		2.22 ± 1.73	
+68bp insert (12)	1.72 ± 1.12	0.89	1.92 ± 1.11	0.42
MS				
AA (58)	1.63 ± 1.08		2.25 ± 1.57	
AG (41)	1.74 ± 1.72		2.13 ± 1.85	
GG (4)	1.68 ± 1.17	0.73[a]	1.68 ± 1.34	0.64[a]

[a] AA *versus* AG/GG.

Table 4 Clinical and pathological characteristics of methylated colorectal tumors

Feature (*n*)	Methylated CpG sites (%) 0 or 1	2 or 3	≥4	*P*[a]
Total (103)	74 (72)	16 (15)	13 (13)	
Sex				
Male (60)	41 (68)	13 (22)	6 (10)	
Female (43)	33 (77)	3 (7)	7 (16)	NS[b]
Age				
≤72 years (52)	37 (71)	11 (21)	4 (8)	
>72 years (51)	37 (72)	5 (10)	9 (18)	0.13
Tumor site				
Distal (51)	43 (84)	3 (6)	5 (10)	
Proximal (49)	29 (59)	13 (27)	7 (14)	NS
Histological grade				
Well/moderate (73)	56 (77)	9 (12)	8 (11)	
Poor (14)	9 (64)	2 (14)	3 (21)	NS
Nodal status				
Negative (68)	44 (64)	12 (18)	12 (18)	
Positive (35)	30 (86)	4 (11)	1 (3)	0.032
Mucinous				
No (88)	67 (76)	12 (14)	9 (10)	
Yes (15)	7 (46)	4 (27)	4 (27)	0.076
Tumor-infiltrating lymphocytes				
Absent (89)	70 (79)	12 (13)	7 (8)	
Present (14)	4 (29)	4 (29)	6 (42)	0.0002
MSI				
Negative (73)	58 (80)	10 (14)	5 (6)	
Positive (18)	9 (50)	3 (17)	6 (33)	0.002
TS genotype				
2/2 or 2/3 (71)	50 (70)	11 (16)	10 (14)	
3/3 (32)	24 (75)	5 (16)	3 (9)	NS
MTHFR genotype				
CC or CT (85)	62 (73)	12 (14)	11 (14)	
TT (17)	11 (64)	4 (24)	2 (12)	NS
CBS genotype				
−68bp insert (91)	64 (70)	15 (17)	12 (13)	
+68bp insert (12)	10 (84)	1 (8)	1 (8)	NS
MS genotype				
AA (58)	41 (71)	10 (17)	7 (12)	
AG/GG (45)	33 (74)	6 (13)	6 (13)	NS

[a] 4 or more sites methylated *versus* <4 sites methylated.
[b] NS, not significant.

icopathological and genotypic features of this tumor series are shown in Table 4. The results should be interpreted with caution because of the few heavily methylated tumors ($n = 13$). Negative nodal status, presence of tumor-infiltrating lymphocytes, and MSI+ were all associated with heavy methylation. No associations were observed, however, between DNA methylation and genotype for any of the four methyl-group metabolism genes investigated here. In a multivariate model that included age, sex, tumor site, grade, mucinous histology, nodal status, infiltrating lymphocytes, MSI, MTHFR genotype, and the concentrations of either CH_2FH_4 or FH_4, only the latter were independent predictors of heavy DNA methylation ($P = 0.004$ and $P = 0.001$, respectively).

Discussion

We have evaluated the tumor concentrations of two major cellular FIs, CH_2FH_4 and FH_4, in relation to various pathological features of CRC, the presence of DNA hypermethylation, and to functionally important polymorphisms in methyl-group metabolism genes. Despite the relatively modest sample size ($n = 103$), strong trends were observed for higher FI levels in tumors from older patients, in tumors originating in the proximal colon, and in those with the MSI+ phenotype (Table 1). Similar associations with age, tumor site, and MSI+ have been reported for the CIMP+ CRC subgroup characterized by frequent and cancer-specific hypermethylation of CpG islands (2–5). Therefore, it was not surprising to find that higher FI levels were also present in tumors showing frequent methylation of genes classified previously by Toyota *et al.* (2) as type "C," or *de novo* methylated in cancer (Table 2). Methylation at the individual *hMLH1*, *TIMP3*, and *ARF* sites, and to a lesser extent *p16* and *MINT2* was associated with higher FI concentrations; however, this was not observed for the *APC* and *DAPK* genes (Table 2). Although the mechanism is unknown, these results suggest that there may be CpG island-specific differences in the association between FI levels and DNA hypermethylation.

In addition to the association between high FI levels and frequent DNA hypermethylation, the second major finding of this study was the link between MTHFR-TT genotype and low tumor levels of FI (Table 3). The enzymatic activity of the T variant is only 30% that of the wild-type form (reviewed in Refs. 24, 25). Because the substrate for MTHFR is CH_2FH_4, and this is itself produced from FH_4, it was somewhat intriguing to find the TT genotype associated with lower levels of these intermediates. One might expect higher FI concentrations to result as a consequence of the lower enzymatic activity of the variant. A previous study by our group on gastric and CRCs from Japanese patients found that MTHFR-CT heterozygotes contained higher levels of FI than MTHFR-CC wild-type homozygotes (26). This was not observed in the present investigation of Australian CRC cases and might be explained by different dietary habits between Japanese and Australian populations. Dietary factors involved in folate metabolism could modify the relationship between MTHFR genotype and tissue levels of FI in a manner similar to the interaction between MTHFR genotype and risk of CRC (8, 9, 24, 25), and TS polymorphism and risk of colorectal adenomas (27).

Lower levels of global 5-methyl cytosine in DNA from normal and tumor tissues of individuals harboring the MTHFR C677T polymorphism have been reported recently (12). Genomic DNA hypomethylation in the peripheral WBCs of MTHFR-TT individuals has also been described (11, 13). Our finding of lower FI levels in the tumor tissue of MTHFR-TT genotype CRC patients (Table 3) may be related to these observations. Although there is currently no direct evidence, one could hypothesize from the above observations that methyl-group metabolism in the normal colorectal mucosa of MTHFR-TT individuals is quantitatively different to that of –CC and –CT genotype individuals. If this is borne out by additional studies it could help to identify risk factors for CRC and, in particular, for the CIMP+ subgroup characterized by frequent DNA hypermethylation.

Although several case-control studies have shown a reduced risk of CRC for MTHFR-TT individuals, the protective effect appears to depend on an adequate level of dietary folate intake, gender, age, and location of the tumor in the proximal or distal colon (8, 10, 24, 25). In some circumstances the

MTHFR-TT genotype appears to increase the risk of CRC (4, 9, 28, 29). Careful molecular epidemiological studies will be required to better understand the relationships among MTHFR genotype, dietary folate intake, gender, and age on one side, and genomic hypomethylation, DNA hypermethylation, and level of FI in the normal colonic mucosa on the other. These studies should be facilitated by a recent report that for individuals not receiving folate supplementation, colonic mucosal concentrations of folate can be accurately predicted by blood measurements of folate status (30).

Aside from their possible significance as risk factors for CIMP+ CRC, the second important issue relating to FI levels in normal and neoplastic colorectal epithelia is whether they are predictive of toxicity to antifolate treatments such as 5FU. The MTHFR-TT genotype has been associated with toxicity to methotrexate/5FU-containing regimens used to treat breast and ovarian cancer patients (14, 15). However another study on patients with various solid cancers found that this genotype was associated with less toxicity to the antifolate raltitrexed (16). The MTHFR-TT genotype has also been linked to higher levels of overall toxicity to methotrexate in rheumatoid arthritis (31) and bone marrow transplant (32) patients. Although in the present study we did not observe gender differences in tumor levels of FI (Table 1), a recent study found that female CRC patients treated with 5FU suffered significantly more toxicity than males (33). Additional prospective clinical studies should establish whether MTHFR genotype, FI levels, and DNA methylation status in the normal and tumor tissues of CRC patients are predictive for toxicity to antifolate treatments.

A third issue arising from the study of FI levels in colorectal tumor tissues is whether these are predictive for response to antifolate treatments. MSI+ and proximal tumor location have been associated with good survival benefit from 5FU in CRC patients (34), and both were shown in this study to be associated with higher FI levels (Table 1). In line with this, we found recently that CRC patients with CIMP+ tumors obtained significant benefit from 5FU, but not those with CIMP− tumors (35). Although a direct link between high FI and good response to 5FU has yet to be confirmed, it might be expected that high concentrations of CH_2FH_4 enable more effective stabilization of the fluoro-dUMP-TS ternary complex and, thus, more efficient inhibition of thymidine synthesis. It could be extrapolated from this that CRC patients with the MTHFR-TT genotype might derive less benefit from 5FU because they have lower tumor FI levels. Interestingly, a small study by Wisotzkey *et al.* (17) found that of four stage III colon cancer patients treated with 5FU who were homozygous for the MTHFR-TT genotype, three (75%) died of their disease and one was alive with cancer. This compared with 42% and 44% survival for the MTHFR homozygous wild-type and heterozygous cases, respectively. Additional prospective clinical studies will be required to establish whether tumor FI levels and DNA methylation status have predictive value for response to antifolate chemotherapies commonly used in the adjuvant treatment of CRC.

Previous investigations in large, population-based CRC series have shown associations between CIMP+ and older age, female gender, proximal tumor location, and poor histological differentiation (3, 4). Significant associations with these features were not observed in the current study (Table 4); however, this was likely to be due to the much smaller sample size. Other characteristics of CIMP+ tumors are mucinous histology, tumor infiltrating lymphocytes, and MSI+ phenotype. Heavily methylated tumors in the current study showed a trend for association with mucinous histology and were strongly associated with the latter two features. The few heavily methylated tumors ($n = 13$) in this series meant that investigation into possible associations with polymorphisms in the TS, MTHFR, CBS, and MS genes should be regarded as exploratory.

In summary, our results show that CRCs with frequent DNA hypermethylation have high levels of FI, although it is unknown whether these features are causally linked. CRCs from MTHFR-TT individuals have low FI levels compared with –CC and –CT genotype individuals. Future studies should aim to determine whether FI levels in the normal colonic mucosa could serve as molecular-based risk factors for the development of CIMP+ tumors. FI levels in normal and neoplastic tissues may also be predictive for the toxicity and efficacy of antifolate therapies widely used in the treatment of CRC.

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