

Accelerated Age-related CpG Island Methylation in Ulcerative Colitis¹

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Abstract

CpG island hypermethylation is a mechanism of gene silencing that can be usurped by neoplastic cells to inactivate undesirable genes. In the colon, hypermethylation often starts in normal mucosa as a function of age and is markedly increased in cancer. To test the hypothesis that subjects at increased risk of colon cancer have higher levels of methylation in their nonneoplastic mucosa, we studied methylation patterns of five genes in the normal and dysplastic mucosa of patients with ulcerative colitis (UC), a condition associated with a marked increased risk of colon cancer. One gene (*Mlh1*) was unmethylated in all tissues examined. All four remaining genes had low but detectable levels of methylation in the epithelium of UC patients without evidence of dysplasia, and this methylation was not different from non-UC controls. By contrast, all four genes were highly methylated in dysplastic epithelium from high-grade dysplasia (HGD)/cancer patients with UC; methylation in HGD versus controls averaged 40.0% versus 7.4% ($P = 0.00003$) for *ER*, 44.0% versus 3.0% ($P < 0.00003$) for *MYOD*, 9.4% versus 2.4% ($P = 0.03$) for *p16* exon 1, and 57.5% versus 30.6% ($P = 0.01$) for *CSPG2*. Importantly, three of the four genes were also highly methylated in the normal appearing (nondysplastic) epithelium from these same HGD/cancer patients, indicating that methylation precedes dysplasia and is widespread in these patients. Compared with controls, methylation averaged 20.1% versus 7.2% ($P = 0.07$) for *ER*, 18.4% versus 3.0% ($P < 0.008$) for *MYOD*, and 7.9% versus 2.4% ($P = 0.007$) for *p16* exon 1. These results are consistent with the hypothesis that age-related methylation marks (and may lead to) the field defect that reflects acquired predisposition to colorectal neoplasia. Furthermore, the data suggest that chronic inflammation is associated with high levels of methylation, perhaps as a result of increased cell turnover, and that UC can be viewed as resulting in premature aging of colorectal epithelial cells.

Introduction

Transcriptional silencing is a physiological process common to many organisms and often required for proper development (1). In human cells, silencing usually involves methylation of CpG-rich sequences (CpG islands) in the promoters of affected genes and is critical for imprinting and chromosome X inactivation. Methylation, in turn, results in the recruitment of a protein complex that includes methylated-DNA binding proteins and histone deacetylases, eventually resulting in the formation of a closed chromatin structure with exclusion of transcription factors (2). Such silencing is clonal and thought to be physiologically irreversible in somatic cells. Neoplastic cells often display aberrant methylation and silencing of multiple genes, including genes that regulate critical processes such as cell cycle control, DNA repair, and angiogenesis (3). The cause(s) of aberrant promoter methylation in neoplastic cells remains to be elucidated.

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In the colon, CpG islands methylated in cancer have been divided into two groups: those that display cancer-restricted methylation (type C), and those that are methylated initially in aging normal epithelial cells (type A; Ref. 4). It has been proposed that age-related methylation identifies and contributes to an acquired predisposition to colorectal neoplasia because it parallels age-related increased cancer incidence, and it has the potential to alter the physiology of aging cells and tissues (5). This hypothesis predicts that higher levels of age-related methylation are associated with a heightened susceptibility to developing colorectal cancer, and that it may be present in conditions of rapid cell turnover that mimic premature aging.

UC³ is a disease that is characterized by chronic inflammation, rapid cell turnover, and a substantial risk of colon cancer (6, 7). From a molecular standpoint, UC-associated colon cancer differs from sporadic colon cancer in many ways. We and others have shown that UC tumorigenesis is distinguished by: (a) infrequent *K-Ras* mutations; (b) the presence of *p53* mutations and aneuploidy in nondysplastic mucosa surrounding areas of dysplasia and cancer; and (c) genetic instability, as measured by comparative genomic amplification and fluorescence *in situ* hybridization, in both the neoplastic and the nonneoplastic mucosa of UC patients with dysplasia/cancer (8, 9). We now report that both the dysplastic and nondysplastic mucosa of UC patients with neoplasia have significantly elevated levels of age-related methylation compared with UC patients without dysplasia and non-UC controls. Thus, UC patients with HGD or neoplasia have pan-colonic genetic instability that is accompanied by widespread abnormalities in age-related methylation.

Materials and Methods

Patient Samples. We studied a total of 33 samples from 23 patients. Each sample was obtained at colonic resection according to approved Institutional Review Board guidelines at the University of Washington. The 23 patients included 12 UC patients with either HGD or cancer, 6 UC patients without dysplasia or cancer, and 5 non-UC controls. From those UC patients with HGD/cancer, we studied HGD/cancer samples, as well as, when available, nondysplastic samples far from the site of dysplasia. A total of 7 UC patients had paired HGD/nondysplastic mucosa samples, and 4 patients had paired HGD/indefinite for dysplasia mucosal samples. In the latter 4 patients, indefinite samples were used because nondysplastic sites were not available in these patients.

Epithelial Isolation. We performed epithelial cell isolation using EDTA release as described previously (8). The tissue is attached with Superglue to a wooden stick, mucosal side up, and incubated in PBS with 5 mM EDTA for 5 min. The end of the stick with the tissue is then immersed in a 15-ml tube containing 2 ml of EDTA buffer, and the tube is vortexed for 1 min. This procedure releases epithelium in sheets, including most of the crypts. Purity has been established previously by analysis of this procedure in regions of aneuploid epithelium in UC (in which aneuploidy serves as a marker of epithelial cells) with routine purity achieved in excess of 90%.

Methylation Analysis. To allow for quantitative methylation determination, we used the method of bisulfite conversion followed by gene-specific PCR and methylated allele-specific restriction enzyme analysis (referred to

³ The abbreviations used are: UC, ulcerative colitis; HGD, high-grade dysplasia.

Table 1 Primers and restriction enzymes sites used in this study

Gene	Primers	Restriction enzyme
<i>Mlh1</i>	F TAGTAGTYGTTTTAGGGAGGGA R TCTAAATACTCAACRAAAATACCTT	<i>RsaI</i>
<i>ER</i>	F GGTTTTGAGTTTTTGTGTTTTG R AACTTACTACTATCCAAATACACCTC	<i>BstUI</i>
<i>MYOD</i>	F TGTGGGAGAGGTTTGGAAAGG R AAAATCCRAAACCAATAAAAAACAC	<i>RsaI</i>
<i>p16</i> exon1	F AGTTTTYGGTTGATGTTGGTTGG R TACAACCTTCRTCTCCAAAATC	<i>MaeII</i>
<i>p16</i> upstream	F GGTTTTGGYGAGGTTGTTTT R ACCCTATCCCTCAAATCCTCTAAAA	<i>TaqI</i>
<i>CSPG2</i>	F TTATTAYGTTTTTATGTGATT R ACCTTCTACCAATTACTTCTTT	<i>MaeII</i>

here as bisulfite/PCR; Ref. 10). Bisulfite treatment of DNA was performed as described (11). Briefly, 1–2 μ g of genomic DNA were denatured with 2 M NaOH at 37°C for 10 min, followed by incubation with 3 M sodium bisulfite (pH 5.0) at 50°C for 16 h in darkness. After treatment, DNA was purified using the DNA Cleanup kit (Promega) as recommended by the manufacturer, incubated with 3 M NaOH at room temperature for 5 min, precipitated with 5 M ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 30 μ l of distilled water. Two to 3 μ l of the aliquot were used as a template for each bisulfite-PCR, as described previously.

Primers and PCR conditions used for amplifying specific DNA fragments of various target genes are listed in Table 1. Fig. 1 shows CpG maps of the genes analyzed, along with the location of the regions amplified. The following criteria were emphasized for designing primers: (a) to minimize bias in amplification of the methylated allele, primers contained no or a minimal number of CpG sites. For those primers that contain CpG sites, a mixture of C/T nucleotides for the sense primer and a mixture of G/A nucleotides for the antisense primer were used; (b) to prevent amplification of nonconverted genomic DNA, primers contained as many thymidines converted from cytosines as possible; and (c) restriction sites that were created by bisulfite treatment were used preferentially for restriction enzyme digestion. After PCR amplification, 20–80% of the PCR products was digested with appropriate restriction enzymes (listed in Table 1). In this analysis, only DNA that initially contained methylated alleles would be cut by the specific restriction enzymes and yield smaller DNA products on the gel. The DNA fragments were then precipitated with ethanol and separated by 6% PAGE. Gels were stained with ethidium bromide for analysis. For quantitation of methylated alleles, gel pictures were scanned, and the methylation-specific bands were quantitated by densitometry using the ImageQuant software (Molecular Dynamics).

Statistical Analysis. All methylation data were generated without knowledge of the clinical data pertaining to the cases under study. After methylation analyses were complete, the laboratory and clinical data were entered on an Excel (Microsoft) spreadsheet and analyzed using built-in software. Most analyses were conducted using a *t* Test, and all *P*s reported are two-sided.

Results

Genes and Patients Studied. To determine age-related methylation in the normal colonic epithelium of patients with UC compared with controls, we examined three genes determined previously to be affected by such age-related methylation: *ER*, *MYOD*, and *CSPG2* (Versican; Refs. 12–14). In addition, the *p16* gene exon 1 region had been reported previously to be hypermethylated in UC (15). We have found recently that *p16* exon 1 behaves as a type A CpG island in the colon (*i.e.*, displays age-related methylation), whereas the *p16* upstream region (critical for transcription) behaves as type C (*i.e.*, methylated in neoplasms only).⁴ Both regions were analyzed separately here. Finally, we also studied the *Mlh1* mismatch repair gene that has been reported recently to be hypermethylated in normal colon (16). Fig. 1 shows CpG maps of the genes analyzed along with the location of the areas amplified.

The patients studied included 18 patients with UC and 5 controls. Of these 18 cases, 12 had evidence of HGD or cancer in their colons. In these patients, epithelial cells showing HGD (UC-HGD) were studied separately from epithelial cells without dysplasia from the same patient, when available. Another group of 6 patients had UC without evidence of any dysplasia in their colonic mucosa. The non-UC control group consisted of 5 patients without evidence of chronic colonic inflammation or neoplasia. Epithelial cells were isolated from colon biopsies using the epithelial shake-off method that provides for >90% purity of colonocytes.

Type C Methylation. *Mlh1* methylation was determined by bisulfite-PCR using sites close to the transcription start site, the methylation of which correlates highly with microsatellite instability in colon cancer. Unlike a previous report (16), we found no evidence of *Mlh1* methylation in the 23 patients studied, regardless of age, dysplasia, or UC status (Fig. 2). These results are consistent with our previous studies (4). Similarly, *p16* was studied for methylation close to its transcription start site. A very low level of methylation (<5%) indistinguishable from background was observed in all patients, with no difference between non-UC controls, UC patients without dysplasia, and UC patients with HGD/CA (data not shown). In a subset of the cases, we also studied the methylation status of MINT2, another type C CpG island, and also found it to be completely unmethylated in these tissues (Fig. 2). These results confirm our previous findings that type C methylation is exclusively seen in colorectal tumors.

Type A Methylation. Four genes associated with age-related methylation were studied: *ER*, *MYOD*, *p16* exon 1, and *CSPG2*. We began studying type A methylation in samples from patients with UC and controls by studying *ER*, the first CpG island for which age-related methylation was demonstrated (Fig. 2; summarized in Table 2 and Fig. 3). *ER* methylation averaged 7% in controls, 3% in UC patients without evidence of dysplasia ($P > 0.1$ compared with control), 20% in the normal appearing epithelium of patients with UC-associated dysplasia ($P = 0.07$ compared with controls), and 40%

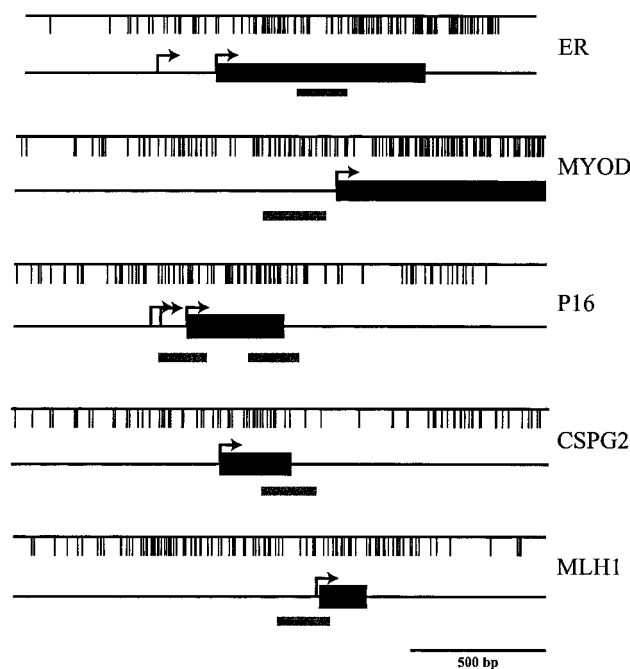


Fig. 1. Maps of the genes analyzed. Shown are CpG maps of 2 kb of sequence around exon 1 of the five genes analyzed in this study. For each gene, the CpG density is indicated by short vertical bars on the top of each panel, whereas exon 1 is indicated by black rectangles on the bottom of each panel. Arrows, known or presumed transcription start sites. Small gray boxes, areas analyzed for methylation. In the case of P16, two areas were studied (see text).

⁴ M. Toyota and J-P. J. Issa, unpublished observations.

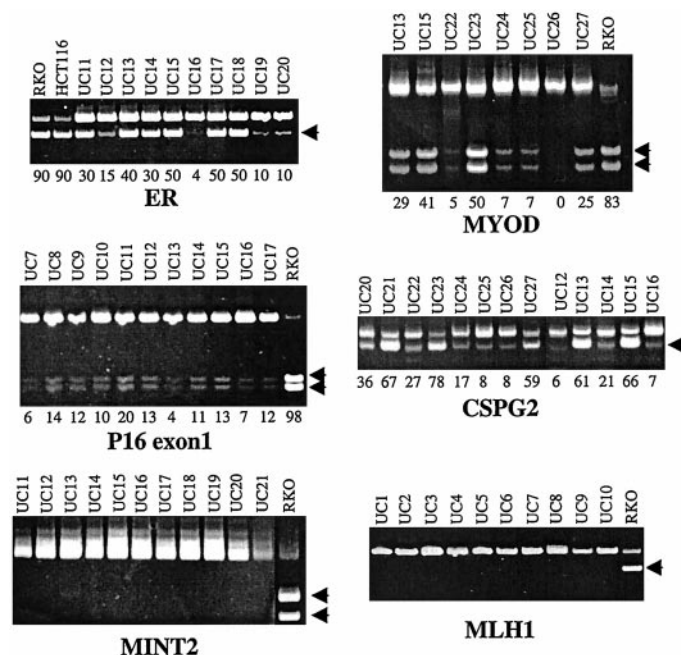


Fig. 2. Examples of bisulfite-PCR methylation analyses. Bisulfite-treated DNA was amplified with gene-specific primers, digested with an appropriate restriction enzyme (Table 1), and electrophoresed on 6% acrylamide gels. In each case, bands of lower molecular weight on the gels indicate methylation (arrows). The gene name is indicated on the *bottom* of each panel, and the percentage of methylation (determined by densitometry) is shown *below* each lane. Methylation analyses were done without prior knowledge of the clinicopathological status of the samples.

in the dysplastic epithelium of these same patients ($P = 0.00003$ compared with controls, $P = 0.01$ compared with nondysplastic epithelium from the same patients). These data suggest that patients with long-standing UC have elevated levels of *ER* methylation in both dysplastic and normal-appearing epithelium, indicating that methylation precedes dysplasia development.

Essentially identical results were obtained for the next gene studied, *MYOD* (Figs. 2 and 3; Table 2). *MYOD* methylation averaged 3% in controls, 3% in UC patients without evidence of dysplasia ($P =$ non-significant compared with control), 18% in the normal-appearing epithelium of patients with UC-associated dysplasia ($P = 0.008$ compared with controls), and 44% in the dysplastic epithelium of these same patients ($P = 0.00003$ compared with controls, $P = 0.00008$ compared with nondysplastic epithelium from the same patients).

We next examined *p16*, which had been reported previously to be hypermethylated in UC mucosa (Figs. 2 and 3; Table 2). As mentioned above, the *p16* upstream region (type C methylation target) had no detectable methylation. In contrast, *p16* exon 1 (type A methylation target) showed substantial levels of methylation in neoplastic and nonneoplastic mucosa of UC patients with HGD/CA. Similar to *ER* and *MYOD*, *p16* exon 1 methylation averaged 2% in controls, 3% in UC patients without evidence of dysplasia ($P = 0.6$ compared with control), 8% in the normal-appearing epithelium of UC patients with HGD/CA ($P = 0.007$ compared with controls), and 9% in the dysplastic epithelium of these same patients ($P = 0.03$ compared with controls, $P = 0.6$ compared with nondysplastic epithelium from the same patients).

Finally, we examined *CSPG2* methylation in UC patients *versus* controls (Figs. 2 and 3; Table 2). Similar to the other age-related methylation target genes, *CSPG2* methylation was higher in dysplastic mucosa compared with non-UC control mucosa (58% *versus* 31%, $P = 0.01$) or compared with adjacent uninvolved mucosa (58% *versus*

35%, $P = 0.06$). However, there was no difference between *CSPG2* methylation in the normal-appearing epithelium of patients with UC-associated dysplasia compared with non-UC controls, being substantially high in both.

Overall, there were no significant differences in methylation by gender in the different groups, although the number of patients studied in each age group is too small to reach definitive conclusion. When combining the control and UC-ND group and averaging methylation values for all four type A genes examined (*ER*, *MYOD*, *p16* exon 1, and *CSPG2*), a regression analysis of age *versus* methylation revealed a positive association ($r = 0.7$; $P = 0.03$), as expected from our previous studies of age-related methylation (5).

Discussion

The data presented here demonstrate that methylation of several gene-associated CpG islands is substantially higher in dysplastic colorectal epithelium from UC patients when compared with epithelium from UC patients without dysplasia and non-UC control patients. Importantly, methylation abnormalities are present not just in the neoplastic mucosa but also in the normal-appearing epithelium from UC patients with HGD/cancer. These findings suggest that the increased levels of methylation are widespread and occur early in the process of tumorigenesis, preceding the histological appearance of dysplasia in these patients.

In UC patients with dysplasia/cancer, hypermethylation is limited to genes that are associated with age-related methylation (type A) and is not present in the genes associated with cancer-related methylation (type C). These results confirm our previous findings distinguishing these two types of methylation in the colon: methylation that starts in normal mucosa as a function of age and methylation restricted to tumors (4). Unlike previous reports, we found no evidence of *p16* upstream or *Mlh1* (type C) methylation in UC or non-UC control

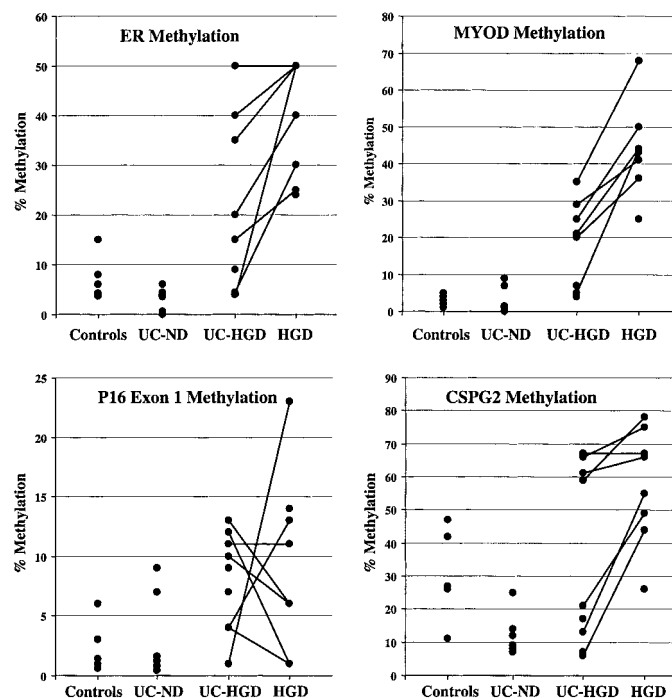


Fig. 3. Graphical representation of all methylation analyses. Each panel is a plot of the methylation densities of each gene (indicated on the *top*) in the three main groups studied: controls, UC without dysplasia (UC-ND) and UC with dysplasia/cancer (UC-HGD). Patients with HGD or cancer had at least two types of biopsies tested: one high-grade/cancer site (HGD) and one negative site distant to the site of dysplasia (UC-HGD). When paired samples were studied, they were joined by *straight lines*.

Table 2 Summary of methylation analyses in the various groups studied

Three main groups were studied: controls, UC without dysplasia (UC-ND), and UC with dysplasia/cancer (UC-HGD/CA). Whenever possible, patients with HGD or cancer had at least two types of biopsies tested: one high-grade/cancer site (HGD/CA) and one negative site distant to the site of dysplasia (NEG); if a negative site was not available, then an indefinite site (IND) was used.

	Age			ER			MYOD			P16			CSPG2		
	Mean	Median	Range	Mean	Median	95% ^a	Mean	Median	95% ^a	Mean	Median	95% ^a	Mean	Median	95% ^a
Control	53.4	54.0	32–69	7.4	6.0	1.7–13.1	3.0	3.0	1.0–5.0	2.4	1.0	0–5.1	30.6	27.0	12.9–48.3
UC-ND	42.0	43.0	33–51	3.0	4.0	0.4–5.6	3.0	1.0	0–7.1	3.3	1.0	0–7.2	12.3	10.0	5.2–19.4
UC-HGD/CA															
NEG	53.2	54.0	34–71	20.1 ^b	15.0	6.7–33.5	18.4	21.0 ^b	8.6–28.2	7.9 ^b	9.0	4.7–11.1	35.2	21.0	14.4–56
IND	40.0	37.0	30–56	20.0	20.0	1.6–38.4	27.7	29.0	0–84.9	13.0	12.5	4.3–21.7	28.8	29.0	18.7–38.8
HGD/CA	54.0	55.0	34–71	40.0 ^b	45.0	30.3–49.7	44.0	44.0 ^b	33.8–54.2	9.4 ^b	8.5	3.2–15.6	57.5 ^b	60.5	42.9–72.1

^a Confidence intervals.

^b Statistically significant differences compared with control.

mucosa. These discrepancies most likely relate to our recent finding that, for some type C loci, age-related methylation can occur but is limited to the edges of the CpG islands with apparent strong protection against spreading to the transcription start sites.⁵ In fact, in both previous reports of *p16* and *Mlh1* methylation in normal and UC mucosa, the areas examined do not correspond to the transcription start sites of the genes.

What is the cause of age-related methylation, in general, and of its acceleration in UC in particular? Age-related methylation may result from interplay between local susceptibility factors and protection factors (17). It has been suggested that some CpG islands progressively lose protection against methylation because repetitive transcription of a gene weakens the putative DNA-protein interactions that may be protective (18). Given a certain rate of methylation spreading per cell division, one might expect that methylation would increase linearly with time in proliferating tissues, as we have in fact observed for the *ER* gene (12). In this model, one would predict that disorders characterized by increased cell turnover, such as the chronic inflammation associated with UC, might be accompanied by higher levels of age-related methylation—in short, UC could be a disorder of premature aging in the colon.

Another potential cause of methylation to be considered is damage by reactive oxygen species that could trigger or accelerate CpG island methylation. Renal cancers induced by oxidative damage in a rat model were found to have a relatively high rate of methylation-associated inactivation of *p16* (19). Oxidant-induced transformation of fibroblasts was associated with an unusual pattern of DNA methylation in an intronic region of the *c-abl* gene (20). However, the common oxygen radical adduct 8-hydroxy-guanine inhibits rather than enhances methylation in *in vitro* experiments (20). Thus, the relationship between oxidative DNA damage and CpG island methylation abnormalities remains to be elucidated.

Although all UC patients share the putative susceptibility factors of rapid cell turnover and oxidative injury attributable to chronic inflammation, not all UC patients have methylation abnormalities in their colonic mucosa. Our data revealed that only UC patients with dysplasia have abnormalities in age-related methylation, whereas UC patients who are dysplasia free do not. These findings support the concept that methylation abnormalities may be contributory in tumorigenesis, probably through gene silencing. In addition, these findings suggest that susceptibility to methylation is greater in certain individuals with UC. Is it possible that patients with dysplasia/cancer have a greater exposure to oxidative injury or more rapid cellular turnover, or are there protective factors such as environmental and/or genetic modifiers that would make some patients more susceptible to methylation than others? One intriguing modifier that could potentially influence methylation is folate,

a nutrient the deficiency of which is associated with colon cancer (21). Retrospective studies have revealed a trend of reduced risk for dysplasia in UC patients who take the highest doses of folate (22, 23). Supplementation of folate in UC patients has also been demonstrated to decrease cell proliferation (24), which could theoretically decrease age-related methylation. Folate measurements were not possible in this retrospective study but would be of interest in future research.

The finding that UC patients with dysplasia have widespread abnormalities in methylation, whereas UC patients who are dysplasia free do not, raises important clinical issues. UC patients with extensive disease >8 years require lifelong surveillance for colon cancer. Because UC dysplasia can often appear in endoscopically normal-appearing mucosa, numerous biopsies are required to have confidence that dysplasia will not be missed if present. This type of surveillance is expensive, time consuming, and requires repeated effort on the part of the physician and patient. Our preliminary data suggest that age-related methylation could be a biomarker of patients with dysplastic foci; theoretically, several rectal biopsies could be tested for age-related methylation, and colonoscopy with extensive mucosal biopsy sampling could be reserved for patients who test positive. Further studies are needed to determine the potential predictive power of methylation as a biomarker in the surveillance of UC patients. Lastly, inhibition of DNA methylation has shown promise as a potential chemoprevention strategy in the colon (25). If safe and effective compounds can be identified that slow down or reverse age-related methylation, they may be particularly appropriate to prevent neoplasia in UC patients.

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