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Promoter methylation status of the *MGMT*, *hMLH1*, and *CDKN2A/p16* genes in non-neoplastic mucosa of patients with and without colorectal adenomas

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Abstract. The aberrant methylation of CpG islands is a common epigenetic alteration found in cancers. The process contributes to cancer formation through the transcriptional silencing of tumor suppressor genes. CpG island methylation has been observed in aberrant crypt foci (ACF) and adenomas in the colon, implicating it in the earliest aspects of colon cancer formation. In addition, some investigators have identified an age-related increase in DNA methylation of the *ESR1* locus in the colon mucosa, suggesting that DNA methylation may be a pre-neoplastic change that increases the risk of colon adenomas and colon cancer. We investigated the methylation status in the promoter regions of the *CDKN2A/p16*, *hMLH1*, and *MGMT* genes in human non-neoplastic rectal mucosa and evaluated whether these methylation markers may predict the presence of adenomatous polyps in the colon. The promoter methylation patterns of these genes were examined in rectal biopsies (mucosa samples) of 97 colorectal adenoma cases and 94 healthy controls using methylation-specific PCR (MSP) assays. Methylation of the *MGMT* and *hMLH1* genes was present in both cases and controls, with a frequency of 12.4% and 18.1% for the *MGMT* gene and 12.4% and 11.7% for the *hMLH1* gene. The frequency of *CDKN2A/p16* promoter methylation was very rare in normal colorectal tissue with a frequency of ~2%. Overall, no apparent case-control difference was identified in the methylation status of these genes, either alone or in combination. *hMLH1* methylation was more frequently observed among overweight or obese subjects (BMI≥25) with an adjusted OR of 3.7 (95% CI=1.0-13.7). Methylated alleles of the *hMLH1* and *MGMT* genes were frequently detected in normal rectal mucosa, while the frequency of *CDKN2A/p16* methylation detected was very low. The methylation status of these genes in rectal mucosa biopsies detected by MSP assays may not distinguish between patients with and without adenomas in the colon.

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Introduction

Methylation of cytosines within CpG islands in the 5' region of genes is associated with loss of gene expression via repression of transcription (1-3). Several studies have demonstrated that certain tumor suppressor and DNA repair genes are often aberrantly hypermethylated and silenced in colorectal cancer and adenomatous polyps (3-7). More specifically, O^6-methylguanine-DNA methyltransferase (*MGMT*), the human DNA mismatch repair gene mutator L homologue 1 (*hMLH1*), and the cyclin-dependent kinase inhibitor 2A (*CDKN2A/p16*) are frequently methylated in the adenoma step of the adenoma-carcinoma sequence (5,8-10). CpG island methylation of *MGMT* reduces *MGMT* expression and has been associated with K-ras mutations (9,11-13). The methylation status of *hMLH1*, which encodes a DNA mismatch repair protein, has been found to contribute to 75% of sporadic colorectal carcinomas that display the microsatellite instability (MSI) phenotype (14). In addition, age-related methylation of *hMLH1* has been observed in normal mucosa associated with the development of colon tumors (14-16). Inactivation of *CDKN2A/p16* by methylation leads to disruption of cell-cycle regulation, potentially providing a growth advantage for affected cells. Epigenetic *CDKN2A/p16* inactivation has been observed in both adenomas and colorectal cancer (4,5,16,17). It has been consistently reported that the CpG islands of the *hMLH1*, *MGMT* and *CDKN2A/p16* genes are frequently methylated in colorectal cancer at a rate of 10~20%, 26~38% and 28~55%, respectively (7,14-16). Intriguingly, in a recent study of colorectal cancer, Shen *et al* found that 50% of patients whose cancer had *MGMT* promoter methylation also had substantial

Table I. Primer sequences and PCR conditions for methylation-specific PCR.

Gene	Forward primer sequence (5'-3')[b]	Reverse primer sequence (5'-3')[b]	Annealing temperature (˚C)	Product size (bp)
CDKN2A/p16	u[a] GTTATGGTTGTGGTTTGGGGTTG	CCACCTAAATCAACCTCCAACCA	65.1	146
	m GGTTACGGTCGCGGTTCG	CTAAATCGACCTCCGACCG	65.1	143
hMLH1	u AATGAATTAATAGGAAGAGTGGATAGT	TCTCTTCATCCCTCCCTAAAACA	57.5	136
	m CGGATAGCGATTTTTAACGC	CCTAAAACGACTACTACCCG	58.5	94
MGMT	u TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	62.0	121
	m TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	62.0	133

u[a], unmethylated sequence; m, methylated sequence; [b]the sequence 5'-GCGGTCCCAAAAGGGTCAGT-3' was added each primer in the 5 end'.

MGMT promoter methylation in normal adjacent mucosa. This indicated that *MGMT* promoter methylation might occur early in multi-step carcinogenesis, even before the emergence of morphologic changes in colorectal mucosa (18,19). Limited data, however, are available on the methylation status of these genes in the non-neoplastic mucosa of patients with colorectal tumors, and the results from previous studies have been conflicting (4,15,20,21).

Carcinogenesis is a complex process, with multiple genetic or epigenetic alterations that provide tumor cells with a selective advantage to expand their clones. It is possible that multiple lesions may develop throughout the colorectal mucosa after years of carcinogenic insults. Because CpG island hypermethylation is an early event in colorectal tumorigenesis, some epigenetic alterations may be detectable in colorectal mucosa that is histologically normal but 'primed' to become dysplastic. We hypothesize that promoter methylation in the *MGMT*, *hMLH1* and *CDKN2A/p16* genes in normal mucosa may reflect an underlying predisposition to develop colon adenomas and would be more common in cases with adenomatous polyps than in controls. We evaluated this hypothesis using non-neoplastic rectal tissue from patients with and without adenomas.

Materials and methods

Study participants. Colorectal adenoma cases and controls were participants in the Tennessee Colorectal Polyp Study, an ongoing colonoscopy-based case-control study being conducted in Nashville, Tennessee. Eligible participants were between 40 and 75 years old and were identified from patients scheduled for colonoscopy at the Vanderbilt University Hospital endoscopy suite and the Nashville campus of the Veteran's Affairs Tennessee Valley Health Care System. Patients with a prior history of inflammatory bowel disease, genetic colorectal cancer syndromes, or any cancer other than non-melanoma skin cancers, were excluded from our study. The study was approved by the institutional review boards of both hospitals and written informed consent was obtained from all participants. Eligible participants were asked to provide biological samples, including blood, urine, rectal biopsy tissue and polyp tissue. A telephone interview was also conducted to obtain information on lifestyle, medication use, demographics, and medical history. Participants included in this analysis were the first 100 adenoma cases who provided rectal biopsy samples and 100 polyp-free patients who were frequency-matched to cases by race (white/non-white), sex and age (within 5 years).

Sample collection and processing. Consenting participants presented for scheduled colonoscopy having fasted the previous day and undergone bowel preparation using either a polyethylene glycol or a sodium phosphate solution. Standard optical colonoscopy was performed on all participants, with polyp status being determined by the attending gastroenterologist. Any identified polyps were removed using biopsy forceps or snare techniques. Adenoma status was determined by an attending pathologist from standard H&E-stained slides. Four rectal pinch biopsies of non-neoplastic epithelium were obtained from the mid-rectum using jumbo biopsy forceps. One rectal biopsy was obtained from each of the four quadrants of the rectum. The rectal biopsies were immediately snap frozen and stored at -80˚C until DNA extraction.

DNA extraction. Genomic DNA was extracted using a QIAmp DNA mini kit® (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. The concentration of DNA was measured by the DNA Quant™ 200 Fluorometer (Amersham Biosciences, Piscataway, NJ). Human placental DNA (Sigma, St. Louis, MO) was used as a negative control and CpGenome® universal methylated human DNA (Intergen, Temecula, CA) was used as methylation-positive control DNA.

Bisulfite modification. Bisulfite modification was conducted based on the principle that bisulfite treatment of DNA converts unmethylated cytosine residues into uracil, whereas methylated cytosine residues remain unmodified (1,5,8). Thus, methylated and unmethylated DNA sequences after bisulfite conversion can be distinguished using sequence-specific primers. Genomic DNA was modified with sodium bisulfite according to the

reviously described by Herman JG *et al* with minor ions (22). Briefly, 150 ng of genomic DNA and 1 μg of salmon sperm DNA (Sigma) in a volume of 50 ml were denatured by NaOH (final concentration, 0.2 M) for 10 min at 37˚C. Thirty microliters of 10 mM hydroquinone (EM Science, Gibbstown, NJ) and 520 ml of 3 M sodium bisulfite (Mallinckrodt Baker Inc., Paris, KY) at pH 5.0, both freshly prepared, were added and mixed and samples were incubated under mineral oil at 53˚C for 16 h. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer's protocol (Promega, USA) and eluted into 50 μl of water. The DNA was then desulfonated with 0.2 M NaOH for 10 min at room temperature, followed by precipitation in 100% ethanol overnight at -80˚C following the addition of 17 μl of 10 M NH_4AC and 40 μg of glycogen (Roche Diagnostics, Florence, SC). The samples were then centrifuged at 13,000 rpm for 20 min to pellet and were then washed with 70% ethanol. The samples were repelleted by centrifugation, air-dried and then resuspended in 30 μl UV-treated, distilled water. The samples were stored at -80˚C for up to 8 weeks until they were used. The set of known methylated and unmethylated control DNA samples used in the MSP assays was included in each round of bisulfite treatment.

Methylation specific PCR (MSP). Methylation of the 5'-CpG promoter region of three genes, *MGMT*, *hMLH1* and *CDKN2A/p16*, were determined by MSP. Forward and reverse primers were synthesized, which corresponded to the predicted sequence of methylated or unmethylated genomic DNA after sodium bisulfite treatment (Table I). For each PCR run, a master mixture was prepared on ice with 1X PCR buffer; 4 mmol/l $MgCl_2$; 200 mmol/l dATP, dCTP, dGTP and dTTP; 200 nmol/l each primer; and 1 U of HotStarTaq DNA polymerase (Qiagen). Two μl of sodium bisulfite-treated DNA were added to 18 μl of PCR mixture. Thermocycling conditions used were: initial denaturation and hot start at 95˚C for 15 min; 40 cycles consisting of 30 sec at 92˚C, the specific annealing temperature for 30 sec and 30 sec at 72˚C, followed by a final extension at 72˚C for 10 min. Four control samples were included in each MSP assay run for both the methylated and unmethylated reactions and included the following: Human placental DNA (Sigma), CpGenome® universal methylated human DNA (Intergen), a bisulfite-treated water control, and a no template control for cross-contamination assessment. MSP reactions were analyzed by electrophoresis on 2.5% agarose gels and the ethidium bromide-stained PCR products were imaged with a UV transilluminator. The samples were considered methylation-positive only when methylated PCR products were shown in gel electrophoresis regardless of the status of unmethylated PCR products. Methylation-negative was defined as amplified DNA that was shown only with unmethylated primers but not methylated primers. If both the methylated and unmethylated primers were negative, the quality of the DNA was assumed to be inadequate and a repeat experiment was performed. A result was final when both observations were in agreement. In the case of a discordant interpretation, the analysis was repeated on two further occasions and, if concordance was not obtained, the result was considered nonassessable at that locus. Of the 100 cases and 100 controls included in the study, methylation data were obtained from 97 cases and 94 controls.

Table II. Demographic and descriptive characteristics of study participants.

	Adenoma cases		Polyp-free controls	
	No. of patients	%	No. of controls	%
Age (years)				
<60	43	44.8	47	50.0
60-70	40	41.7	37	39.4
>70	13	13.5	10	10.6
	P=0.72			
Sex				
Male	71	73.2	69	73.4
Female	26	26.8	25	26.61
	P=0.97			
Study site				
Vanderbilt	59	60.8	51	54.3
VA	38	39.2	43	45.7
	P=0.36			
Purpose of colonoscopy				
Screening	63	64.9	53	56.4
Diagnostic	34	35.1	41	43.6
	P=0.23			

Statistical analysis. Statistical analyses were performed using SAS software (Version 9.1, SAS Institute, Cary, NC, USA). Chi-squared statistics and Fisher's exact test were used to evaluate case-control differences in the distribution of methylation status for the genes under study. A two-sided probability value of less than 0.05 was considered statistically significant. Multivariate logistic regression models were used to estimate odds ratios (ORs) and their 95% confidence intervals (95% CIs) as a measure of the strength of the association between methylation status and participant characteristics.

Results

Comparisons of selected demographic and descriptive characteristics of study participants are shown in Table II. Approximately 50% of the cases were older than 60 years of age at diagnosis. Consistent with the frequency-matching, cases and controls were similar in age, sex, study location and purpose of colonoscopy.

The methylation distribution of the *MGMT*, *hMLH1*, and *CDKN2A/p16* genes in cases and controls are presented in Table III. Preliminary results indicate that methylation of the *CDKN2A/p16* promoter was rare; approximately 2% in both cases and controls; therefore, only methylation of the *hMLH1* and *MGMT* genes was included in the final assay. Methylation of the *hMLH1* and *MGMT* genes was present in the non-neoplastic rectal mucosa from both cases and controls. However,

Table III. *hMLH1*, *CDKN2A/p16* and *MGMT* methylation status in study participants with and without colorectal adenomas.

Methylation status	Adenoma cases			Polyp-free controls	
	No.	%		No.	%
hMLH1 gene methylation					
No	85	87.6		83	88.3
Yes	12	12.4		11	11.7
			P=0.89		
MGMT gene methylation					
No	85	87.6		77	81.9
Yes	12	12.4		17	18.1
			P=0.27		
CDKN2A/p16 gene methylation					
No	47	97.9		49	98.0
Yes	1	2.1		1	2.0
			P=0.98		
Combined methylation status of *hMLH1/MGMT* gene					
No/no	76	78.3		69	73.4
No/yes	9	9.3		14	14.9
Yes/no	9	9.3		8	8.5
Yes/yes	3	3.1		3	3.2
			P=0.70		

Table IV. *hMLH1* and *MGMT* methylation status and adenoma characteristics.

Clinical characteristics	Total (n=97)	*hMLH1* and *MGMT* methylation		
		None (n=76) No. (%)	Any one (n=18) No. (%)	Both (n=3) No. (%)
Most advanced histology				
Tubular	81	64 (79.0%)	15 (18.5%)	2 (2.5%)
Tubulovillous	16	12 (75.0%)	3 (18.8%)	1 (6.2%)
			P=0.72	
Size of largest adenoma				
<1cm	68	52 (76.5%)	13 (19.1%)	3 (4.4%)
≥1cm	29	24 (82.8%)	5 (17.2%)	0 (0%)
			P=0.49	
Number of adenoma				
1	50	38 (76.0%)	11 (22.0%)	1 (2.0%)
≥2	40	31 (77.5%)	7 (17.5%)	2 (5.0%)
			P=0.66	
Site				
Proximal	15	11 (73.3%)	3 (20.0%)	1 (6.7%)
Distal	39	29 (74.4%)	9 (23.1%)	1 (2.5%)
Both	36	29 (80.5%)	6 (16.7%)	1 (2.8%)
			P=0.89	
Category of adenomas				
Single, small, tubular	41	33 (80.5%)	8 (19.5%)	0 (0%)
Multiple and/or advanced adenomas	54	41 (75.9%)	10 (18.5%)	3 (5.6%)
			P=0.31	

Association of *hMLH1* and *MGMT* methylation status with selected demographic and lifestyle factors for adenoma

	hMLH1		OR (95% CI)	*MGMT*		OR (95% CI)	*hMLH1/MGMT*		OR (95% CI)
	No	Yes		No	Yes		No/no	Any one	
Age (years)									
<60	81	9	1.0[a]	74	16	1.0[a]	68	22	1.0[a]
60-70	67	10	1.4 (0.5-3.6)	67	10	0.6 (0.3-1.5)	59	18	0.9 (0.4-1.8)
>70	20	4	1.8 (0.5-6.5)	20	4	0.9 (0.2-3.4)	17	7	1.3 (0.4-3.7)
Sex									
Male	125	15	1.0[b]	116	24	1.0[b]	104	36	1.0[b]
Female	44	8	1.2 (0.4-3.7)	46	6	0.8 (0.2-2.9)	41	11	0.9 (0.3-2.4)
Family history									
None	70	9	1.0	62	17	1.0	56	23	1.0
CRC/CRAs	95	13	0.9 (0.4-2.4)	95	13	0.6 (0.3-1.3)	85	23	0.7 (0.4-1.5)
NSAID use									
None	106	19	1	106	19	1	92	33	1
Yes	59	3	0.3 (0.1-1.1)	51	11	0.9 (0.4-2.1)	49	13	0.6 (0.3-1.2)
Alcohol intake									
Never	132	14	1.0[c]	120	26	1.0[c]	111	35	1.0[c]
Former/current	30	7	2.4 (0.8-6.7)	35	2	0.3 (0.1-1.5)	29	8	1.0 (0.4-2.6)
Smoking status									
Never	55	7	1.0[c]	56	6	1.0[c]	51	11	1.0[c]
Former	68	8	1.3 (0.4-4.2)	62	14	1.5 (0.5-4.6)	56	20	1.4 (0.6-3.3)
Current	42	5	1.2 (0.3-4.2)	38	9	2.0 (0.6-6.7)	33	13	1.7 (0.6-4.5)
BMI, kg/m^2									
<25	52	3	1.0[c]	49	8	1.0[c]	46	11	1.0[c]
25	107	18	3.9 (1.0-14.7)	103	22	1.1 (0.4-2.8)	91	34	1.4 (0.7-3.2)

[a]Adjusted for sex, study site and case-control status; [b]adjusted for age, study site and case-control status; [c]adjusted for age, sex, study site and case-control status.

the case-control difference was not statistically significant in analyses performed for either a single gene or the combination of both genes.

Table IV contains the results of the evaluation of the methylation status of the *MGMT* and *hMLH1* genes in relation to the clinicopathological features of the cases. Only 3 cases were found to have both the *MGMT* and *hMLH1* genes methylated in their rectal mucosa, and all three of them had multiple and/or advanced adenomas. Perhaps due to a small sample size, none of the comparisons presented in Table IV was statistically significant.

The associations of gene methylation with some colorectal adenoma risk factors are summarized in Table V. Because no case-control differences were found in methylation, cases and controls were combined for these analyses. *hMLH1* gene methylation tended to be more frequently observed among subjects who were older (OR=1.8, 95% CI: 0.5-6.5 for >70 years), drank alcoholic beverages regularly (OR=2.4, 95% CI: 0.8-6.7), or had a high BMI (OR=3.9, 95% CI: 1.0-14.7). *MGMT* methylation status was positively associated with smoking status (OR=2.0, 95% CI: 0.6-6.7). However, the associations were not statistically significant, with the exception of the association of *hMLH1* gene methylation with BMI.

Discussion

Epigenetic gene silencing is increasingly being recognized as a common mechanism through which cancer cells can inactivate tumor suppressor genes (2). However, only a few previous studies have evaluated the methylation status of non-neoplastic tissue in adenoma patients and normal controls. In a study of 64 colorectal adenomas, *hMLH1* was not detected in any paired normal colon tissues of adenomas (23). In a study with 24 normal colon epithelia and 95 colon adenomas, Lee *et al* found that methylation of *MGMT* was present in 21.1% of adenomas (4). However, the same study reported that *hMLH1* methylation was rarely present in adenoma tissue (1.1%) and was not at all present in normal controls. In another study in

which 34 controls with a normal colonoscopic examination were assayed, neither *hMLH1* nor *MGMT* gene methylation was detected (24). In contrast to all of these studies, we found a relatively high rate of methylation of both the *hMLH1* and *MGMT* genes in the rectal mucosa of cases and controls. We believe the higher frequency of methylation is a result of the sensitivity of the assay we employed to detect methylation. The MSP assays we employed can detect small clonal populations that would likely be below the detection threshold with other methylation assays (25). Methylation of these genes, however, was not related to the presence or absence of an adenoma in the colon or rectum. *CDKN2A/p16* showed infrequent methylation in both case and control tissue, as has been reported previously (4,15,17), indicating that *CDKN2A/p16* gene methylation is a rare event in the normal colorectal epithelium of adenoma patients.

Several previous studies evaluated the relationship between methylation and other clinicopathological characteristics of adenomas (1,4,5,24). Age has been reported to affect methylation overall and in a tissue-specific manner (4,15). Consistent with these reports, we found a suggestive, although non-significant increase in *hMLH1* methylation with increasing age, and no association between age and *MGMT* methylation. Gene methylation status in adenoma tissues has also been associated with the adenoma's size (25), histology (5), and degree of atypia (26). In our study, we evaluated gene methylation status in normal rectal mucosa and found no association with adenoma characteristics. Another salient finding of our analysis was the higher frequency of *hMLH1* methylation among subjects who were overweight or obese (BMI≥25). To our knowledge, this study is the first to report an association between overweight/obesity and an increased frequency of *hMLH1* methylation. These results are interesting and warrant investigation in future studies with a larger sample size.

Overall, this study provides little evidence that analysis of the methylation status of *MLH1*, *MGMT*, or *CDKN2A/p16* in non-neoplastic rectal mucosa will have substantial utility in predicting the presence of adenomas in the colorectum. Although the controls included in this study were all polyp-free, as confirmed by colonoscopy, some of them may have been at a higher risk for neoplasm because they were referred for active symptoms and were, thus, more likely to be referred for a colonoscopy. This may reduce the case-control difference in gene methylation (6,14). However, more than 50% of our controls had a colonoscopy simply for screening purposes. The MSP method used in this study can, in theory, detect the presence of methylation at the 0.1% level (27). However, this method only measures qualitative differences in methylation and it is possible that there are important quantitative differences between cases and controls that we were unable to identify. This issue also exists in almost all previous studies of methylation biomarkers (1,4,5,28). Our study was limited to individuals between the ages of 45 and 75. If differences are most salient at very young ages or very old ages, we were not able to evaluate these. It is also possible that the genes we assessed are not accurate indicators of early aberrant methylation. In fact, other authors have shown that other genes, such as *CRBP1*, *CDH13*, and *MINT31*, are aberrantly methylated in ACFs, and these may have been more appropriate genes to have assessed in non-neoplastic tissues (29). Although this is the largest methylation study of non-neoplastic rectal epithelia, the statistical power was limited and could only detect a strong association. However, for a marker of meaningful clinical significance, the OR should be 3.0 or above, and this study should have 80% power to detect such an association.

In summary, we found little difference in the promoter methylation status of the *hMLH1*, *MGMT*, and *CDK2NA/p16* genes in non-neoplastic rectal epithelium between patients with and without adenomas. These results suggest that the methylation status of these genes in rectal mucosa is unlikely to be a useful biomarker to predict the presence of colorectal adenomas.

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