MGMT Promoter Methylation and Field Defect in Sporadic Colorectal Cancer

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Background: **Sporadic colorectal cancers often arise from a region of cells characterized by a "field defect" that has not been well defined molecularly. DNA methylation has been proposed as a candidate mediator of this field defect. The DNA repair gene O^{6}-methylguanine-DNA methyltransferase (MGMT) is frequently methylated in colorectal cancer. We hypothesized that MGMT methylation could be one of the mediators of field cancerization in the colon mucosa.** ***Methods:*** **We studied MGMT promoter methylation by three different bisulfite-based techniques in tumor, adjacent mucosa, and nonadjacent mucosa from 95 colorectal cancer patients and in colon mucosa from 33 subjects with no evidence of cancer. Statistical tests were two-sided.** ***Results:*** **MGMT promoter methylation was present in 46% of the tumors. Patients whose cancer had MGMT promoter methylation also had substantial MGMT promoter methylation in apparently normal adjacent mucosa. This methylation was seen with a quantitative assay in 50% (22/44; 95% confidence interval [CI] = 34% to 65%) of normal samples with MGMT promoter methylation in the adjacent tumors, 6% (3/51; 95% CI = 1% to 16%) of samples without MGMT methylation in adjacent tumors, and 12% (4/33; 95% CI = 3% to 28%) of control samples (*P*<.001 for comparison between each of the latter two groups and the first group). MGMT methylation was detected with a more sensitive assay in 94%, 34%, and 27% of these samples, respectively (*P*<.001). In grossly normal colonic mucosa of colon cancer patients, methylation was detected 10 cm away from the tumor in 10 of 13 cases. Tumors with MGMT promoter methylation had a higher rate of G-to-A mutation in the KRAS oncogene than tumors without MGMT promoter methylation (10/42 versus 3/46, *P* = .03). Using a sensitive mutant allele-specific amplification assay for KRAS mutations, we also found KRAS mutations in 12% (3/25; 95% CI = 2.5% to 31%) of colorectal mucosas with detectable MGMT methylation and 3% (2/64; 95% CI = 0.4% to 11%) of colorectal mucosas without MGMT methylation (*P* = .13).** ***Conclusion:*** **Some colorectal cancers arise from a field defect defined by epigenetic inactivation of MGMT. Detection of this abnormality may ultimately be useful in risk assessment for colorectal cancer. [J Natl Cancer Inst 2005;97:1330–8]**

In carcinogenesis, the "field defect" (also known as field cancerization) is recognized clinically because of the high propensity of survivors of certain cancers to develop other malignancies of the same tissue type, often in a nearby location (*1,2*). In colorectal cancer, the field defect is also characterized by the simultaneous occurrence of multiple but distinct tumors, which are either separate malignancies or a single malignancy accompanied by multiple preneoplastic lesions. The molecular basis for this phenomenon is relatively simple to understand when it occurs in patients who have a genetic predisposition for cancer development (e.g., patients with familial adenomatous polyposis) or massive exposure to a carcinogen, such as the lungs of chronic smokers. Indeed, patches of lung tissue bearing genetic alterations have been described in chronic smokers (*1*). In sporadic carcinogenesis, however, the molecular nature of the field defect remains elusive.

In principle, the molecular abnormalities that are responsible for a field defect should be detectable at high frequency in patients with cancer but at low frequency in patients without neoplasia. Furthermore, these abnormalities should be detectable in healthy individuals who have conditions that put them at risk for the cancer as well as in the neoplastic lesions themselves, and they should occur early in the neoplastic process. Ideally, the abnormalities should also be functionally involved in neoplasia. Age-related epigenetic defects have been proposed as potential sources of the field defect in colon carcinogenesis (*3,4*). However the identification of genes that clearly identify individuals at (high) risk for colon cancer has been lacking.

The DNA repair gene O^{6}-methylguanine-DNA methyltransferase (MGMT) is frequently methylated in colorectal cancer. The DNA repair protein encoded by the MGMT gene is involved in defending cells against alkylating agents (*5,6*). Alterations in the MGMT gene impair the ability of the MGMT protein to remove alkyl groups from the O^{6}-position of guanine, thereby increasing the mutation rate and the risk of cancer (*7*). To date, no published studies have reported an association between genetic defects in the MGMT gene, such as mutations and/or deletions,

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DOI: 10.1093/jnci/dji275

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and human cancer. However, several studies have reported that transcriptional silencing of this gene in multiple tumor types is associated with hypermethylation of the CpG island in its promoter *(8,9)*. Silencing of MGMT has been shown to be associated with and to precede the appearance of G-to-A point mutations in the KRAS gene during colorectal tumorigenesis *(10,11)*. We hypothesized that MGMT methylation could be one of the mediators of field cancerization in the colon mucosa. To test this hypothesis, we studied MGMT methylation quantitatively in the neoplastic tissues, adjacent mucosa, and distal mucosa of patients with colorectal cancer, as well as in the colonic mucosa from patients without evidence of malignancies.

Patients, Materials, and Methods

Patient Selection and Sample Storage

We collected samples of primary colorectal tumors and samples of the corresponding adjacent normal-appearing tissue from 95 patients who had undergone surgery at the Johns Hopkins Hospital (n = 54; Baltimore, MD), the M. D. Anderson Cancer Center (n = 8; Houston, TX), the University of Arizona (n = 29; Tucson, AZ), and the Southern Arizona Veterans Affairs Health Care System (n = 4; Tucson, AZ) in accordance with institutional policies. All patients provided written informed consent. Tumors were selected solely on the basis of availability. All samples of normal-appearing mucosa were derived from sites adjacent to, but at least 1 cm away from, the tumors. We also obtained two additional samples of normal-appearing colonic mucosa from 36 patients: The two samples originated from tissue located 1 cm and 10 cm away from the cancer. Clinicopathologic data were available for most of the 95 colorectal cancer patients; for some patients we were missing information on sex (n = 4), age at surgery (n = 5), the location of the tumor in the colon (n = 12), and tumor stage (n = 22). We also obtained colonic biopsy specimens from 33 individuals who had no family history of colorectal cancer and who had no colonic lesions at screening colonoscopy. These healthy control specimens were selected on the basis of availability. All tissue samples were fresh-frozen and stored at −80 °C. CpG island methylator phenotype (CIMP) and p53 mutation status were available for 54 and 51 patients respectively from previous studies *(12,13)*.

Cell Lines and Culture Conditions

We isolated DNA from five colon cancer cell lines: SW48, RKO, HCT116, SW480, and $CaCO_2$. HCT116, $CACO_2$, and RKO cells were grown in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. SW48 and SW480 cells were grown in L-15 medium containing 10% fetal bovine serum in plastic tissue culture plates in a humidified atmosphere containing 5% CO_2 at 37 °C. Cell lines were obtained from the American Type Culture Collection (Manassas, VA). Media were purchased from Invitrogen (Carlsbad, CA).

Bisulfite Polymerase Chain Reaction Analysis of DNA Methylation

Genomic DNA was extracted from patient samples and from the cell lines using a standard phenol–chloroform method. Bisulfite treatment of 2 μg of genomic DNA was performed as previously described *(14)*. We used combined bisulfite restriction analysis (COBRA) and methylation-sensitive polymerase chain reaction (PCR) (MSP) to examine MGMT promoter methylation in all DNA samples *(15)*. For COBRA, we used the following oligonucleotide primers to amplify a 161-bp region of the promoter cytosine guanine dinucleotide (CpG) island: 5′-TTGGTAAATTAAGGTATAGAGTTTT-3′ (sense primer) and 5′-CTAAAACAATCTACGCATCCTC-3′ (antisense primer). PCRs were carried out in a volume of 50 μL. Each reaction contained 2 μL of bisulfite-treated DNA,1.25 m*M* deoxynucleoside triphosphate, 6.7 m*M* $MgCl_2$, 5 μL of PCR buffer, 1 nmol of each primer, and 1 U *Taq* polymerase. For PCR amplification, we used both hot-start and touchdown PCR. PCR cycling conditions were 95 °C for 5 minutes, followed by five cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds, followed by five cycles of 95 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 30 seconds, followed by five cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, followed by 20 cycles of 95 °C for 30 seconds and 51 °C for 30 seconds, and a final incubation at 72 °C for 4 minutes. The restriction enzyme *Taq*$^{\alpha}$I (New England Biolabs, Beverly, MA) was used to examine the methylation status of the amplified region. In brief, 20–40 μL of the amplified products were digested with the restriction enzyme *Taq*$^{\alpha}$I, which digests methylated DNA but not unmethylated DNA. The digested DNA was then ethanol precipitated, electrophoresed on 6% acrylamide gels, and visualized by ethidium bromide staining. We used a Geldoc 2000 imager (BioRad, Hercules, CA) to perform densitometric analysis of all the bands. Methylation level (%) was defined as the sum of the density of the shifted bands divided by the density of all bands in each lane. The identity of the amplified fragment was verified by restriction enzyme digestion. Each PCR assay included positive and negative controls (DNA from SW48 and RKO cells, respectively). Mixing experiments were performed to rule out PCR bias. All experiments were repeated twice to assess the reproducibility of results.

For MSP, we used the following oligonucleotide primers: 5′-GTAGGTTGTTTGTATGTTTGT-3′ (sense primer) and 5′-AAC CAATACAAACCAAACA-3′ (antisense primer) for amplification of unmethylated DNA (PCR product size 121 bp) and 5′-GGTC GTTTGTACGTTCGC-3′ (sense primer) and 5′-GACCGATA CAAACCGAACG-3′ (antisense primer) for amplification of methylated DNA (PCR product size 118 bp). PCR cycling conditions for both methylated and unmethylated primers were 95 °C for 5 minutes, followed by 35 cycles or 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds, and finally, 72 °C for 4 minutes. MSP provides qualitative data, and a sample was called positive for methylation if a band was seen in DNA amplified by the methylated reaction primers. MSP PCR products were also visualized on acrylamide gels as described above.

Bisulfite Sequencing

In six normal-appearing mucosa samples (four adjacent to methylated tumor and two adjacent to unmethylated tumor), bisulfite sequencing (performed at the M. D. Anderson Core Sequencing Facility) of cloned PCR products was used to confirm methylation of CpG sites within the MGMT promoter. For this analysis, we cloned the 161-bp PCR products into the TA vector

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pCR2.1 (Invitrogen) and extracted plasmid DNA from the resulting clones with the use of a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).

KRAS and TP53 Gene Mutations

In 91 tumors and 89 adjacent normal mucosa samples (DNA was no longer available for four tumors and six normal samples), we used mutant allele–specific PCR amplification of genomic DNA to analyze samples for activating mutations in codons 12 or 13 of the KRAS oncogene as previously described *(16)*. This method allows the detection and verification of a single mutant allele in a background of 10^6–10^7 copies of the wild-type allele. Data on TP53 gene mutations for 51 of the tumor samples, which were available from a prior study *(12)*, had been obtained by using single-stranded conformational analysis followed by sequencing of shifted bands.

Immunohistochemistry

We performed immunohistochemical staining for MGMT protein on paraffin-embedded tissue sections as previously described *(17)*. Briefly, 5-µm-thick sections were deparaffinized, rehydrated, incubated with 0.3% H_2O_2 to block endogenous peroxidase activity, and incubated with normal mouse serum to block nonspecific antibody binding. The sections were incubated at 4 °C overnight with a monoclonal antibody against human MGMT protein (MAB16200, 1 : 100 dilution; Chemicon, Temecula, CA). The sections were incubated with biotinylated goat anti–mouse IgG antibodies (AP124B, 1 : 500 dilution; Chemicon, Temecular, CA). The antigen–antibody complexes were visualized using streptavidin–horseradish peroxidase conjugate (LSAB kit, DAKO, Los Angeles, CA) and diaminobenzidine as a chromogen. The slides were counterstained with hematoxylin. Normal-appearing epithelium and stromal cells in each section provided positive internal controls for binding of the primary antibody. MGMT expression in nuclei was scored as present or absent.

Genomic Sequencing

Eight colorectal cancer samples and adjacent normal-appearing tissues and three cancer cell lines (SW48, SW480, and HCT116) in which the MGMT promoter had been found to be methylated were further examined for the presence of genetic defects in the promoter region of MGMT. The promoter region was amplified by PCR from genomic DNA by using the forward primer (5′-GGGCCCACTAATTGATGGCT-3′) and the reverse primer (5′-CTCACCAAGTCGCAAACGG-3′). The 951-bp PCR product was directly sequenced using the same primers in the M. D. Anderson Core Sequencing Facility.

Statistical Analysis

All statistical analyses were done using S-Plus software for Windows (version 6.0; Insightful Corporation, Seattle, WA). Methylation status of MGMT as determined by COBRA was analyzed initially as a categorical variable (negative: methylation level <3%, positive: methlyation level ≥3%). The 3% cutoff was selected because lower values could not be easily distinguished from background staining of the gels. Associations between MGMT methylation status and clinicopathologic variables were analyzed by Fisher's exact test. In parallel, for samples classified as methylation positive, we also analyzed methylation level as a continuous variable and computed means, medians, and ranges. Associations between methylation level analyzed continuously and clinicopathologic variables were analyzed by a Wilcoxon rank-sum test. We examined possible correlations between MGMT methylation levels in normal-appearing mucosa and patient age at diagnosis expressed on a log scale by calculating Pearson's and Spearman's correlation coefficients (r and ρ, respectively). All reported P values were two-sided, and $P \leq .05$ was considered statistically significant.

Results

MGMT Promoter Methylation and Clinicopathologic Features

We measured MGMT promoter methylation in colorectal cancer samples and samples of the corresponding adjacent non-neoplastic mucosa from 95 patients. The locations of CpG sites within the MGMT promoter region and the regions of the promoter that were amplified by the COBRA and MSP primers are shown in Fig. 1, A. By using the quantitative COBRA assay, we found that the mean level of MGMT promoter methylation in the colorectal cancer samples was higher than that in the corresponding adjacent mucosa (20.0% versus 4.3%; difference = 15.7%, 95% CI = 11.2% to 20.3%; $P<.001$). Among the 95 paired samples, the MGMT promoter was methylated (i.e., methylation level of at least 3%) in 46% of colorectal tumor samples and in 26% of the corresponding samples of adjacent normal-appearing mucosa (see examples in Fig. 1, B).

We also performed an immunohistochemical analysis of MGMT protein expression on 24 colorectal cancer samples for which paraffin-embedded tissues were available and found a statistically significant association between the presence of MGMT promoter methylation and the lack of MGMT protein expression (as determined by the lack of staining with an antibody to MGMT protein) (examples in Fig. 1, C). Specifically, the 10 tumor samples that lacked MGMT promoter methylation all (100%) stained positive with an antibody to the MGMT protein; by contrast, of the 14 tumor samples with MGMT promoter methylation, six (43%) expressed MGMT protein (difference = 57%, 95% CI = 17% to 88%; $P<.01$, Fisher's exact test). The six tumors that had a methylated MGMT promoter and expressed MGMT protein had generally low levels of methylation, which probably reflects methylation of only a few cancerous cells, or methylation of one copy but absence of methylation of the other copy of the gene. MGMT staining was also examined in normal-appearing mucosa from regions adjacent to tumors. While many crypts were positive, a few were negative. However, it was not possible to rule out that the lack of staining of some of the crypts was an artifact.

Next, we examined the association between MGMT promoter methylation and clinicopathologic features of patients with primary colorectal cancer. We initially analyzed the data using methylation status as a categorical variable; samples with detectable methylation by COBRA (i.e., those with a methylation level ≥3%) were considered positive for MGMT promoter methylation (Table 1). We found that the MGMT promoter methylation

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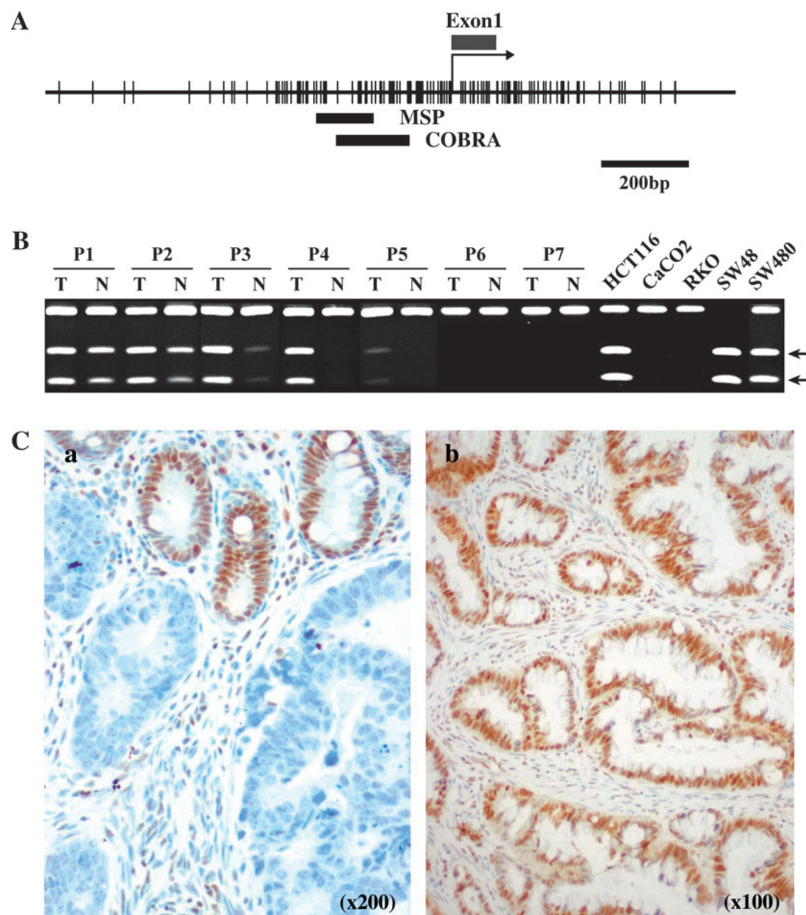

Fig. 1. MGMT promoter methylation and expression in colorectal cancer. **A**) Diagram of the CpG island of MGMT promoter. Each **vertical line** represents a single CpG site. The location of exon 1 of the MGMT gene is shown at the top. The **arrow** indicates the putative transcription initiation site. **Thick bars** indicate the location of regions amplified by the methylation-specific polymerase chain reaction (MSP) and combined bisulfite restriction analysis (COBRA) primers. **B**) Methylation of the MGMT promoter in primary colorectal cancer (T) and adjacent normal-appearing mucosa (N) from seven patients (P1–P7) and in five human colon cancer cell lines (right) by COBRA. The primer set was designed to amplify a 161-bp fragment from nucleotides −270 to −109 relative to the putative transcription start site. The restriction enzyme *Taq*αI was used to determine the methylation status of this region. **Arrows** point to digested bands (97 bp and 64 bp), which indicate the presence of methylated alleles. The band at the top corresponds to undigested DNA. **C**) Immunohistochemical staining of primary colorectal cancers with a mouse monoclonal antibody against human MGMT protein. **a**) A tumor positive for MGMT promoter methylation (T3 in panel B) shows no staining in cancer cells but clear staining in the nuclei of normal cells. **b**) A tumor with no detectable MGMT promoter methylation (T6 in panel B) has staining for the MGMT protein in tumor cell nuclei.

status of cancer samples was not associated with patient age at diagnosis, with the location of the tumor in the colon, or with tumor–node–metastasis (TNM) stage. However, statistically significantly more tumors from women than from men had MGMT promoter methylation (16/24 [67%] versus 26/67 [39%], P = .03). We had previously determined the CpG island methylator phenotype (CIMP) status of 54 of the 95 tumors in this study *(13)*. CIMP is a molecular property of some colorectal cancers whereby many genes are hypermethylated simultaneously. Of the 54 tumors whose CIMP status had been determined, 24 were CIMP negative; of these, four (17%) were MGMT promoter methylation positive. Of the 30 CIMP-positive tumors, 20 (67%) were MGMT promoter methylation positive (P<.001). This difference suggests that the MGMT promoter can be affected by CIMP in colorectal cancer. We also analyzed MGMT methylation level as a continuous quantitative variable, limiting the analysis to the 44 tumors that were MGMT promoter methylation positive by the categorical classification. In this group of 44 cancers, there was no association between the degree of methylation and patient age, patient sex, tumor stage, or the location of the tumor in the colon (data not shown).

We used similar methods to examine the degree of methylation of the MGMT promoter in normal-appearing mucosa from the same patients whose tumors were analyzed. As shown in Table 2, MGMT promoter methylation (≥3% by COBRA) was detectable in normal-appearing mucosa from 25 out of the 95 patients studied. There was no association between the presence of MGMT promoter methylation and any of the clinicopathologic variables examined. Results of a quantitative analysis of methylation level in the 25 normal colon samples that were positive for MGMT promoter methylation are shown in Table 3. Mean MGMT promoter methylation level in normal mucosa adjacent to tumors was higher in patients who were 60 years or older at surgery than in patients who were younger than 60 years (17.6% versus 7.0%; P = .002 by the Wilcoxon rank-sum test). In this group of samples, methylation level was positively correlated with age overall (Spearman correlation test on a log scale with age: r = 0.4, P = .048. Pearson correlation test on a log scale with age: r = 0.42, P = .039) (Fig. 2). There was also an indication that MGMT promoter methylation levels were higher in men than in women, but the difference was not statistically significant (Table 3).

MGMT Promoter Methylation as a Field Defect in Normal-Appearing Colorectal Mucosa

Patients who had MGMT promoter methylation–positive cancers also had detectable methylation of the MGMT promoter in the apparently normal-appearing mucosa adjacent to the cancer (Fig. 3). When analyzed as a categorical variable, methylation in normal colon was positive in 22 (50%) of 44 normal-appearing mucosa samples taken from regions adjacent to MGMT promoter methylation–positive cancers compared with only three (6%) of 51 normal-appearing mucosa samples taken from regions adjacent to MGMT promoter methylation–negative

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Table 1. Clinicopathologic features of patients by tumor MGMT promoter methylation status*

Characteristic	No. without promoter methylation	No. with promoter methylation	P†
Sex			
Female	8	16	.03
Male	41	26	
Missing	2	2	
Age at diagnosis, y			
<60	12	8	.61
≥60	36	34	
Missing	3	2	
Tumor location in colon			
Proximal	26	16	.27
Distal	20	21	
Missing	5	7	
Tumor stage‡			
I or II	22	17	.82
III or IV	21	13	
Missing	8	14	
KRAS mutation			
G to A	3	10	.03
Other	12	12	
No mutation	34	20	
Missing	2	2	
TP53 mutation			
G to A	9	2	.13
Other	6	5	
No mutation	13	16	
Missing	23	21	
CIMP			
Positive	10	20	<.001
Negative	20	4	
Missing	21	20	

*Methylation of tumor samples was measured by combined bisulfite restriction analysis, and a sample was considered positive for methylation if the methylation level was ≥3%. MGMT = gene encoding O6-methylguanine-DNA methyltransferase; CIMP = CpG island methylator phenotype.

†Fisher's exact test (two-sided).

‡See Winawer et al. *(29)*.

cancers (*P*<.001 by Fisher's exact test). We also examined the methylation status of the MGMT promoter in colonic biopsy specimens obtained at screening colonoscopy from 33 patients who had no family history of colorectal cancer and no colonic lesions (mean age of these patients was 54 years) and found that four specimens (12%) had detectable MGMT promoter methylation. When we used the more sensitive MSP assay at 35 PCR cycles, MGMT promoter methylation was detected in 66%, 19%, and 16% of the samples, respectively. When we used the MSP assay at 40 PCR cycles to increase sensitivity, MGMT promoter methylation was detected in 94%, 34%, and 26% of the samples, respectively. Similar results were obtained when methylation level was analyzed as a continuous variable. That is, MGMT methylation levels as measured by COBRA in the normal-appearing mucosa adjacent to an MGMT promoter methylation–positive cancer were statistically significantly higher than levels in the normal-appearing mucosa adjacent to an MGMT promoter methylation–negative cancer (8.8% versus 0.4%; difference = 8.4%, 95% CI = 5.1% to 12.1%; *P*<.001, Fisher's exact test).

We next used bisulfite sequencing to examine the pattern of CpG island methylation in six samples of normal-appearing mucosa adjacent to tumor tissue of known MGMT promoter methylation status (Fig. 4). All four samples that had detectable MGMT promoter methylation by COBRA had methylated alleles throughout the sequenced CpG island region. In the two samples that had no detectable MGMT promoter methylation by COBRA, we found the CpG island region to be essentially unmethylated by bisulfite sequencing analysis as well.

To evaluate the extent of this apparent MGMT promoter methylation field defect, we examined normal-appearing mucosal specimens that were located 1 cm and 10 cm away from the colon cancers of the 36 patients for whom tissue was available. Among these patients, 18 had detectable MGMT promoter methylation in their tumors by COBRA. Among these 18 patients, 13 (72%) were also positive for MGMT methylation in normal-appearing mucosa 1 cm away from the tumor, and 10 (77%) of those 13 patients were also positive for MGMT methylation in normal-appearing mucosa 10 cm away from the tumor. All of the tumors that had no detectable methylation in normal mucosa located 1 cm away from the cancer also had no detectable methylation in normal mucosa located 10 cm away from the cancer.

Finally, to rule out genetic mutations as a cause of the observed methylation, we sequenced the entire promoter region of the MGMT gene for eight patients with MGMT promoter methylation. We found no mutations or deletions in either their tumor DNA or in the paired normal mucosa DNA, suggesting that the observed methylation was not caused by genetic abnormalities.

MGMT Promoter Methylation and Molecular Characteristics of the Cancers

We examined the association between MGMT promoter methylation level and the presence of mutations in KRAS and TP53, two genes that are frequently mutated in colorectal cancer. We examined these associations in parallel analyses that used MGMT promoter methylation data as a continuous variable and as a

Table 2. Clinicopathologic features of patients by MGMT methylation status of normal-appearing mucosa adjacent to the tumor*

Characteristic	No. without promoter methylation	No. with promoter methylation	P†
Sex			
Female	14	10	.11
Male	52	15	
Missing	4	0	
Age at diagnosis, y			
<60	16	4	.57
≥60	49	21	
Missing	5	0	
Tumor location in colon			
Proximal	29	13	.81
Distal	30	11	
Missing	11	1	
Tumor stage‡			
I or II	29	10	.61
III or IV	23	11	
Missing	18	4	
KRAS mutation			
G to A or other	2	3	.13
No mutation	62	22	
Missing	6	0	

*Methylation of normal-appearing mucosa samples was measured by combined bisulfite restriction analysis, and a sample was considered positive for methylation if the result was ≥3%. MGMT = gene encoding O6-methylguanine-DNA methyltransferase.

†Fisher's exact test (two-sided).

‡See Winawer et al. *(29)*.

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Table 3. Clinicopathologic characteristics of the 25 normal-appearing colon mucosa samples that were positive for MGMT promoter methylation*

Characteristic	N	Median (%)	Mean (%)	Range (%)	*P*†
Age, y					
<60	4	4.5	7.0	3.7–15.2	.002
≥60	21	15.2	17.6	4.6–47.4	
Sex					
Female	10	8.2	9.7	3.7–17.9	.07
Male	15	17.9	19.9	4.6–47.4	
Location of tumor in colon					
Proximal	13	11.1	16.0	3.7–43.1	.98
Distal	11	9.2	15.76	4.2–47.4	
Missing	1				
Tumor stage‡					
I or II	10	9.1	14.4	4.6–47.4	.67
III or VI	11	7.3	14.2	3.7–43.1	
Missing	4				

*Samples with a methylation level ≥3% were considered MGMT promoter methylation positive.

†Wilcoxon rank-sum test (two-sided).

‡See Winawer et al. *(29)*.

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categorical variable, with identical results. We found that MGMT promoter methylation positivity in the tumor samples was associated with the presence of KRAS gene mutations, similar to what has previously been observed in a different set of tumors *(10)*. This association between MGMT methylation positivity and KRAS mutations was largely a result of a difference in G-to-A transitions, which were present in 10 (24%) of 42 cancers with MGMT promoter methylation versus three (6%) of 49 cancers without MGMT promoter methylation (P = .03) (Table 1). The frequency of KRAS gene mutations was 12% (3/25; one specimen had a G-to-A mutation and two specimens had G-to-T mutations) in mucosal specimens with detectable MGMT promoter methylation and only 3% (2/64; both specimens had G-to-T mutations) in mucosal specimens that lacked MGMT promoter methylation; however, the difference in proportions was not statistically significant (difference = 9%, 95% CI = 1% to 19%; P = .13; Table 2). It is interesting that the tumors and their corresponding mucosas did not have identical KRAS gene mutation patterns, suggesting multiple independent mutational events.

We used data obtained in a previous study *(6)* of these tumor and corresponding mucosa samples to examine the association between MGMT promoter methylation status and the presence

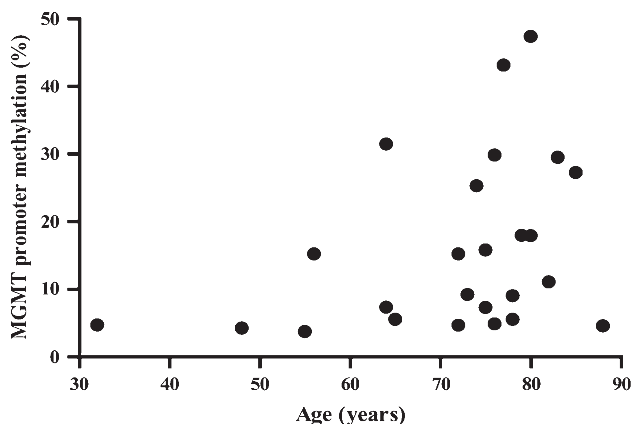

Fig. 2. Scatter plot of MGMT promoter methylation level versus patient age in the 25 normal-appearing colon mucosa samples (all from patients with colon cancer) that were positive for MGMT promoter methylation.

TP53 gene mutations. In contrast with our findings for the KRAS gene, we found an inverse association between the presence of TP53 gene mutations and MGMT promoter methylation in the tumor samples. The frequency of TP53 gene mutations was 30% (7/23; two cancers had a G-to-A mutation) among the MGMT promoter methylation–positive cancers, and 54% (15/28; nine cancers had a G-to-A mutation) among the MGMT promoter methylation–negative cancers; however, the difference was not statistically significant (P = .13). No TP53 gene mutations were detected in the corresponding normal mucosa samples.

Discussion

Here we examined whether MGMT promoter methylation might characterize normal-appearing cells that are precursors to cancer. We used three different methods to analyze MGMT promoter methylation: COBRA (a quantitative method), MSP (a more sensitive quantitative method), and bisulfite sequencing (a high-resolution method), and obtained consistent results. There was a statistically significant correlation between MGMT promoter methylation in normal-appearing colonic mucosa and MGMT promoter methylation in the adjacent tumors. When we used COBRA to detect methylation, the percentage of cases with detectable MGMT promoter methylation in normal mucosa adjacent to MGMT promoter methylation–positive cancer was 50%; when we used MSP with 40 PCR cycles, that percentage rose to 94%. Furthermore, we found that extended regions of the normal-appearing colonic mucosa (at least 10 cm away from the tumor) also had detectable MGMT promoter methylation in 77% of the cases.

One limitation of our study is that we were not able to analyze single colonic crypts for MGMT expression and promoter methylation simultaneously. Much of the tissue we currently have available for these studies is paraffin embedded, and we found that the quality of the immunohistochemistry varied substantially. Thus, although examining cancers in which large tracts of cells may express or lack expression of MGMT protein is relatively straightforward, it is difficult to use immunohistochemistry to examine MGMT protein expression in normal tissues, where an absence of expression in single cells or crypts could be related to promoter methylation or to technical issues. Given the large body of literature describing the association between

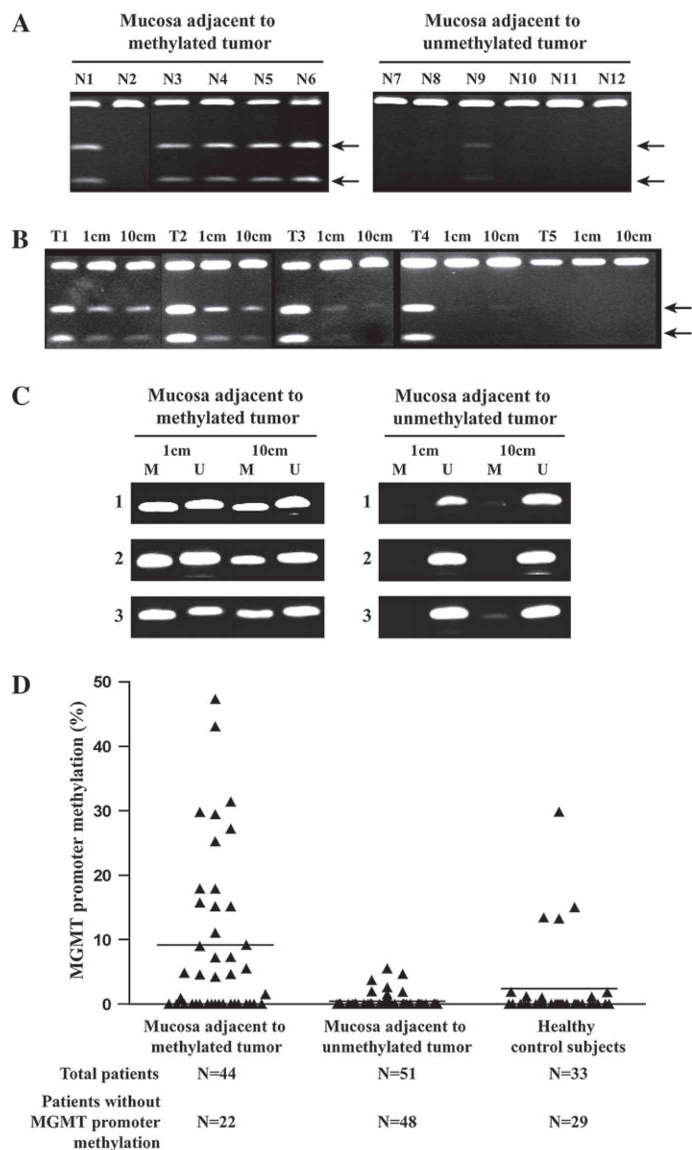

Fig. 3. MGMT methylation in normal-appearing colonic mucosa. **A**) Representative results from combined bisulfite restriction analysis (COBRA) of MGMT promoter methylation in mucosa samples located adjacent to MGMT promoter methylation–positive tumors (left, N1–N6) and adjacent to MGMT promoter methylation–negative tumors (right, N7–N12). **Arrows** point to bands that indicate the presence of methylated alleles. **B**) Representative results from COBRA of MGMT promoter methylation in five sample sets each consisting of tumor sample (T1–T5), normal-appearing mucosa located 1 cm away from the corresponding tumor (1 cm), and normal-appearing mucosa located 10cm away from the tumor (10 cm). **C**) Representative results from methylation-specific polymerase chain reaction (PCR) analysis of MGMT promoter methylation in normal colonic mucosa adjacent to MGMT promoter methylation–positive (left) and–negative (right) tumors (three examples of each). The distance from the tumor to the normal-appearing colonic mucosa sample is indicated. M = PCR products amplified by oligonucleotide primers specific for methylated DNA; U = PCR products amplified by primers specific for unmethylated DNA. **D**) Distribution of MGMT promoter methylation levels measured by COBRA in normal-appearing colonic mucosa. Each **triangle** represents the methylation level of normal-appearing mucosa from individual patients with MGMT promoter methylation–positive (left) and promoter methylation–negative (center) colorectal tumors and from patients without tumors (healthy control subjects; right). **Horizontal lines** represent mean methylation levels for each group.

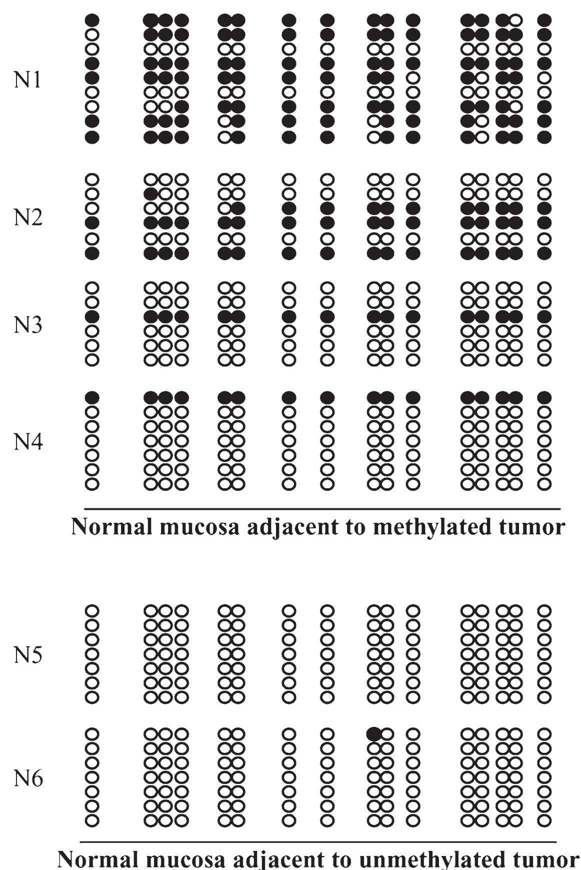

Fig. 4. Bisulfite sequencing of the MGMT promoter CpG island in normal-appearing colon mucosa. DNAs from six samples of normal-appearing colon mucosa were subjected to bisulfite sequencing: four samples were located adjacent to MGMT promoter methylation–positive tumors (N1–N4) and two samples were located adjacent to MGMT promoter methylation–negative tumors (N5, N6). Each row represents an individual cloned allele that was sequenced following sodium bisulfite DNA modification. **Circles** represent CpG sites and their spacing accurately reflects the CpG density of the region. This region corresponds to nucleotides 663–822 of GenBank entry X61657, which is at −293 bp to −133 bp relative to the putative transcription start site. **Black circle**, methylated CpG site; **white circle**, unmethylated CpG site.

promoter hypermethylation and gene silencing, it is reasonable to infer that the MGMT promoter methylation we observed (as confirmed by bisulfite sequencing) was associated with MGMT gene silencing in some normal mucosas. Another potential limitation of our study is the possibility that the normal tissues were contaminated with malignant cells. We think this possibility is unlikely given that 1) normal tissue was confirmed histologically, 2) promoter methylation was present in tissue located 10 cm away from the tumor, and 3) promoter methylation was detectable in some patients who did not have tumors.

A field defect is an area of abnormal tissue that precedes and predisposes to the development of cancer *(1)*. Identification of such abnormal fields is important because they provide insight into the earliest stages of cancer and may provide markers for risk assessment. Accumulating evidence suggests that alterations in DNA methylation represent epigenetic phenomena that appear to be early events in tumorigenesis *(18,19)*. For example, it has been noted *(4)*, initially in the colon *(3)*, that normal-appearing tissues, in general, have aberrant methylation of promoter-associated CpG islands, albeit at low levels. Age-related increases in such methylation have been proposed to mark the field defect that accompanies sporadic colorectal tumorigenesis *(20,21)*, but clear markers to identify patients at risk of this disease have been lacking. In this study, we found that MGMT promoter methylation occurred frequently in the normal-appearing colonic mucosa of

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colorectal cancer patients whose tumors had MGMT promoter methylation and was much less frequent in the normal-appearing colonic mucosa of colorectal cancer patients whose tumors did not have MGMT promoter methylation and of healthy control subjects. Interestingly, we also observed an association between age and promoter methylation levels; among patients who had MGMT promoter methylation in their normal-appearing colonic mucosa, increasing age was associated with increased methylation density.

Because a loss of MGMT protein function is a plausible predisposing factor for cancer through the increased occurrence of mutations (such as G-A mutations in the KRAS gene, as shown herein), our data indicate that MGMT promoter methylation may qualify as a marker of the field defect in colorectal cancer. MGMT promoter methylation has also been detected in the sputum of patients who are at risk for lung cancer *(22)*, and it may represent a marker of the field defect in lung cancer as well. MGMT promoter methylation has been associated with G-to-A mutations in TP53 in lung cancer *(23)*, a finding that is also consistent with the field defect hypothesis. However, we found no association between MGMT promoter methylation and G-to-A mutations in TP53 in colon tumors—a reflection, perhaps, of different mechanisms of mutagenesis in these two tissues. It is interesting that MGMT promoter methylation is a frequent feature of a newly recognized evolutionary pathway for colon carcinogenesis, the hyperplastic polyp/serrated adenoma route *(11,24)*. Patients who have multiple hyperplastic polyps have a high degree of concordance in the methylation patterns of the different tumors *(25)*, supporting the existence of a field defect preceding such lesions. Our data indicate that MGMT promoter methylation in normal colon is a possible risk factor for developing tumors along the serrated adenoma pathway.

In this study, we found that MGMT promoter methylation can be clearly detected in the normal mucosa of healthy individuals. Variations in MGMT enzyme activity in the colon have been reported previously *(26)*, and it is possible that epigenetic inactivation of the MGMT gene, as described here, contributes to this variability. Given the high lifetime risk of colorectal tumor development in the U.S. population, it is reasonable to propose testing to determine whether healthy persons with MGMT promoter methylation in normal colorectal mucosa are at higher risk of developing a colon tumor than those without such methylation.

The causes of MGMT promoter methylation remain unclear. No mutations or deletions were apparent when we sequenced the MGMT promoter region in methylated samples, although a larger region would likely need to be sequenced to rule out the possibility of mutations or deletions. As noted above, we observed an association between age and MGMT promoter methylation that, unlike the relationship between age and the methylation status of other genes *(3,27)*, did not appear to be linear, implying that other factors accelerate MGMT promoter methylation. Chronic inflammation has previously been shown to accelerate DNA methylation in normal tissues *(28)*, and it would be interesting to examine whether inflammation plays a role in MGMT promoter methylation in colorectal carcinogenesis.

In conclusion, our data indicate that some sporadic colorectal cancers may arise from a field defect that is molecularly defined by epigenetic inactivation of MGMT and an increased rate of mutations in multiple genes, including KRAS. The discovery of a marker of a field defect, such as MGMT promoter methylation, in normal-appearing mucosa could be of great use, both for early detection of and risk assessment in colon cancer. Prospective clinical trials using this potential marker of risk are indicated.

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Notes

Supported by NIH grants CA41108 and CA89245. DNA sequencing in the Core Sequencing facility at the M. D. Anderson Cancer Center is supported by Core Grant CA16672 from the National Institutes of Health, Bethesda, MD.

Manuscript received August 27, 2004; revised July 1, 2005; accepted July 20, 2005.

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