Aberrantly Methylated *CDKN2A*, *MGMT*, and *MLH1* in Colon Polyps and in Fecal DNA from Patients with Colorectal Polyps

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ABSTRACT

Colon cancer is the third leading cause of cancer-related death in the United States, affecting ~147,000 people each year. Most colon cancers arise from benign neoplasms and evolve into adenocarcinomas through a stepwise histologic progression sequence that starts from adenomas or hyperplastic polyps/serrated adenomas. Genetic alterations and, more recently, epigenetic alterations have been associated with specific steps in this polyp-adenocarcinoma sequence and likely drive the histologic progression of colon cancer. Consequently, we have assessed in colon adenomas and hyperplastic polyps the methylation status of *MGMT*, *CDKN2A*, and *MLH1* to determine the timing and frequency of these events in the polyp-carcinoma progression sequence and subsequently to analyze the potential for these methylated genes to be molecular markers for adenomas and hyperplastic polyps. We have found that methylated *MGMT*, *CDKN2A*, and *MLH1* occur in 49%, 34%, and 7% of adenomas and in 5%, 10%, and 7% of hyperplastic polyps, respectively, and that they are more common in histologically advanced adenomas. Furthermore, analysis of fecal DNA from persons who have undergone colonoscopics revealed methylated *CDKN2A*, *MGMT*, and *MLH1* in fecal DNA from 31%, 48%, and 0% of individuals with adenomas and from 16%, 27%, and 10% of individuals with no detectable polyps, respectively. These results show that aberrant methylated genes can be detected frequently in sporadic colon polyps and that they can be detected in fecal DNA. Notably, improvements in the specificity and sensitivity of the fecal DNA-based assays will be needed to make them clinically useful diagnostic tests for polyps.

INTRODUCTION

Colorectal cancer affects ~147,000 people in the United States each year and is most effectively treated when diagnosed at an early stage (1). Colon cancers develop as the result of the transformation of normal colon epithelium to cancer via a progression of histologic changes and concurrent molecular changes that has been termed the adenoma to carcinoma progression sequence and the hyperplastic polyp-serrated adenoma to carcinoma sequence (2, 3). The adenoma to carcinoma sequence is characterized by recognizable histologic changes that start with dysplastic aberrant crypt foci and benign tubular adenomas. These lesions then have the potential to progress to advanced adenomas (characterized by size >1 cm, villous histology, and high-grade dysplasia), which have a significant potential to transform into invasive adenocarcinomas (4). The evidence that supports a hyperplastic polyp-serrated adenoma to carcinoma sequence suggests that these polyps, like adenomas, can evolve through a histologic progression sequence that culminates in adenocarcinomas of the colon, and that hyperplastic polyps that occur in the right colon and in the setting of hyperplastic polyposis have the greatest potential to transform into adenocarcinomas (5, 6).

Genetic alterations and epigenetic alterations are believed to play a pathogenic role in driving colon neoplasms through the polyp-carcinoma progression sequence (2). The most extensively studied epigenetic alteration in neoplasms, CpG island DNA methylation, has been shown to affect tumor suppressor genes during the adenoma to carcinoma and hyperplastic polyp-serrated...
adenoma to carcinoma sequences. CpG island DNA methylation is an epigenetic mechanism that represses gene transcription in a variety of normal cellular processes including imprinting and X-chromosome inactivation, but it becomes excessive and aberrant in many neoplasms. The aberrant DNA methylation affects CpG-rich regions, called “CpG islands,” in the 5′ region of genes and results in transcriptional silencing through complex effects on transcription factor binding and associated changes in chromatin structure. The aberrant methylation of certain tumor suppressor genes, such as CDKN2A, has been reported in colonic adenomas and aberrant crypt foci (7–9). These hypermethylated genes are not only probable pathogenic events in the polyposis-cancer progression sequence but also are neoplasm-specific molecular events that have the potential to be used as molecular markers for pre-malignant tumors in the colon. In fact, a novel approach to colon cancer early detection that has the potential to be less expensive and better accepted by patients than currently available screening methods is the use of fecal DNA-based tests that can detect tumor-specific DNA alterations in patients with cancer or colonic adenomas (10, 11). Pilot studies have shown the feasibility of an approach using tumor-derived DNA mutations for the early detection or prevention of colon cancer; however, the use of assays based on methylated genes for the detection of colon polyps, which is the histologic stage of colon neoplasm that is most easily treated, has not been well studied to date (11, 12).

Consequently, we have developed modified, high-sensitivity methylation-specific PCR (MSP) assays for MLH1, CDKN2A, and MGMT, three genes previously shown to be methylated in pre-malignant neoplasms in the colon, and have applied these assays to DNA extracted from colon polyps to assess the prevalence of aberrant DNA methylation in neoplasms at different steps of the poly-p-cancer progression sequence and to analyze the feasibility of using these methylated genes as molecular markers for colon polyps (13). We have detected methylation of these genes in adenomas and hyperplastic polyps and have found increased frequencies of methylated CDKN2A, MGMT, and MLH1 in the subset of polyps with the greatest potential for malignant transformation. Furthermore, we have shown it is feasible to detect the methylated genes in fecal DNA using MSP-based assays.

MATERIALS AND METHODS

Patients and Collection of Tissue and Fecal DNA Samples. Sixty-six patients undergoing colonoscopy for routine clinical indications or for colon cancer screening in the Department of Veterans Affairs Tennessee Valley Health Care System (TVHCS) or at Vanderbilt University Medical Center (VUMC) at Nashville, Tennessee, were enrolled in this study over an 18-month time period using protocols approved by the Institutional Review Board of VUMC and the TVHCS. None of the patients had a clinically apparent polyposis syndrome or Hereditary Nonpolyposis Colon Cancer syndrome. Colon lavage effluent samples were collected from all patients during colonoscopy before polypectomy. After collection, the samples were kept at 4°C until being processed. Within 12 hours after collection, the samples were washed with 1× PBS and centrifuged at 1,800 rcf for 15 minutes to pellet the solid stool. The supernatant was discarded and the pellet was stored at −80°C.

Tissues from the polyps that were resected at the time of colonoscopy were obtained from the formalin-fixed, paraffin-embedded tissue blocks stored in the pathology archives. These tissues were used for DNA analysis after the cases had undergone pathologic review. In addition, 25 normal colon specimens that were resected for non-cancer-related indications were obtained from the VUMC pathology archives. DNA was extracted from the microdissected epithelial layer of formalin-fixed, paraffin-embedded tissue sections from these normal colon specimens.

Control Cell Lines. DNA from the colon cancer cell lines VACOs and RKO were used as controls in the studies described below. The establishment and maintenance of the cell lines has been described previously. Genomic DNA from the cell lines was extracted using previously published protocols (14, 15).

DNA Isolation from Tissue and Fecal Samples. DNA was extracted from 5 μL formalin-fixed, paraffin-embedded sections using InstaGene Matrix (Bio-Rad, Hercules, CA) as previously described (16). H&E-stained sections from each tumor sample were examined by an experienced pathologist to confirm the histological diagnosis, and the right-sided hyperplastic polyps were reviewed by an experienced gastrointestinal pathologist (M.K.W.).

For fecal DNA extraction, the fecal pellet was thawed at room temperature and resuspended in 7× TNE (EXACT Sciences, Maynard, MA). This fecal-TNE solution was centrifuged to pellet residual particulate matter, and then the supernatant was incubated at 37°C for 30 to 60 minutes after the addition of RNase (4 mg/mL; Sigma Chemical Co., St. Louis, MO). The DNA was precipitated in 1/10 volume 3 mol/L sodium acetate (Fisher Scientific, Pittsburgh, PA) and an equal volume of isopropyl-alcohol (EM Science, Gibbstown, NJ), then centrifuged and washed in 70% ethanol. After a final centrifugation, the pellet was air-dried and resuspended in 1× TE. The DNA solution was incubated at room temperature overnight and stored at −20°C.

Bisulfite Modification. Genomic DNA was modified with sodium bisulfite as previously described (13, 17). 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. The bisulfite-modified DNA was subject to MSP in a blinded manner using primer pairs designed to amplify specifically the methylated or unmethylated alleles for the respective genes (Table 1). Each PCR reaction mix consisted of a total volume of 20 μL containing 10× PCR buffer (Qiagen, Valencia, CA), 200 pmol/L deoxynucleotide triphosphate mix (Sigma), 500 pmol/L of each primer (Sigma Genosys, The Woodlands, TX), 1 unit of HotStar Taq enzyme (Qiagen), and bisulfite-modified DNA. The thermocycler conditions were in general as follows: 95°C for 15 minutes, 41 cycles (45 cycles for CLE DNA) of 92°C for 30 seconds, specific annealing temperature for 30 seconds, 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. All MSP assays were repeated at least twice to validate the results. Six control samples were included in each MSP assay run for both the methylated and unmethylated reactions and included the following: DNA from peripheral blood leukocytes, DNA extracted from colorectal cancer cell lines known to be methylated for the three target...
genes, a bisulfite treated water control, and a no template control for cross-contamination assessment. The MSP products were then subjected to horizontal gel electrophoresis through a 3% agarose gel, stained with ethidium bromide and visualized with UV transillumination using the Quantity One Image Analyzer system (Bio-Rad).

### Statistical Analysis
To compare characteristics of the different groups of patients and biologic samples, \( t \) tests, \( \chi^2 \) tests, and Fisher exact tests were used as appropriate. All statistical tests were two sided. All statistical tests were done using SAS software version 8.02 (SAS Institute, Inc., Cary, NC).

### RESULTS

#### Aberrantly Methylated CDKN2A, MGMT, and MLH1 Occur in Adenomas and Become More Frequent in Histologically Advanced Adenomas.
DNA from 42 separate adenomas obtained from 29 patients was analyzed by methylation-specific PCR (MSP) to analyze the prevalence of methylated CDKN2A, MGMT, and MLH1 in the adenomas. Methylated CDKN2A, MGMT, and MLH1 were detected in 34% \((n = 14/41)\), 49% \((n = 19/39)\), and 7% \((n = 3/41)\) of the adenomas, respectively. Fifty-nine percent \((n = 23/39)\) of the adenomas that were successfully analyzed for all three genes carried at least one methylated gene. The prevalence of methylated CDKN2A and MGMT was substantially higher in the tubulovillous and villous adenomas compared with the tubular adenomas. The prevalence of methylated CDKN2A and methylated MGMT increased from 13% \((n = 3/23)\) to 61% \((n = 11/18)\) and from 38% \((n = 8/21)\) to 64.7% \((n = 11/17)\; P = 0.0067\). Analysis of the frequency of the methylated genes in left and right sided adenomas revealed that 50% \((n = 11/22)\), 27% \((n = 6/22)\), and 4.5% \((n = 1/22)\) of right sided and 47% \((n = 8/17)\), 42% \((n = 8/19)\), and 10.5% \((n = 2/19)\) of left sided adenomas showed methylated MGMT, CDKN2A, and MLH1, respectively. There was no statistically significant association between any of the methylated genes and the location of the polyp(s). The overall prevalence of right sided and left sided adenomas with at least one methylated gene was 54.5% \((n = 12/22)\) and 64.7% \((n = 11/17)\; P = 0.74\), and the average number of methylated genes among right and left sided adenomas was 0.82 and 1.0, respectively.

In light of the detection of methylated CDKN2A, MGMT, and MLH1 in tubular adenomas, we determined the methylation status of these genes in normal colonic mucosa by analyzing DNA extracted from the normal mucosal layer of 25 people who had undergone partial colectomy for benign colonic disease. None of these samples had detectable methylated CDKN2A, MGMT, or MLH1. This observation shows that the MSP assays we have designed do not detect low-level methylation in normal colon mucosa, which has been observed for some genes, such as ESR, p14ARF, and DAPK (18, 19).

#### Aberrantly Methylated CDKN2A, MGMT, and MLH1 Also Occur in Hyperplastic Polyps.
In addition to the analysis of the adenomas, 44 hyperplastic polyps from 17 patients who had hyperplastic polyps were collected and analyzed. None of the individuals in this part of the study met the clinical criteria for hyperplastic polyposis. Interestingly, in the hyperplastic polyps, aberrantly methylated CDKN2A, MGMT, and MLH1 were detected in 5% \((n = 2/40)\), 10% \((n = 4/40)\), and 7.5% \((n = 3/40)\) of the polyps, respectively (Fig. 1). Twenty percent of all the hyperplastic polyps that could carry at least one methylated gene was 54.5% \((n = 12/22)\) and 64.7% \((n = 11/17)\; P = 0.74\), and the average number of methylated genes among right and left sided adenomas was 0.82 and 1.0, respectively.

In the detection of methylated CDKN2A, MGMT, and MLH1 in tubular adenomas, we determined the methylation status of these genes in normal colonic mucosa by analyzing DNA extracted from the normal mucosal layer of 25 people who had undergone partial colectomy for benign colonic disease. None of these samples had detectable methylated CDKN2A, MGMT, or MLH1. This observation shows that the MSP assays we have designed do not detect low-level methylation in normal colon mucosa, which has been observed for some genes, such as ESR, p14ARF, and DAPK (18, 19).

### Table 1

<table>
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<th>Gene</th>
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<th>Reverse primer (5’ → 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)*</th>
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<td>U</td>
<td>MGMT</td>
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<tr>
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<tr>
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<td>62.0</td>
<td>133</td>
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<tr>
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<tr>
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<td>57.5</td>
<td>136</td>
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*All of the primers were modified with a 20 bp GC-rich tail (5’-GGCGGTCCAAAAGGGTGACGT-3’) at their 5’ end.

![Fig. 1](file.png)

**Fig. 1** Prevalence of methylated CDKN2A, MGMT, and MLH1 in normal colonic mucosa, hyperplastic polyps (HP), tubular adenomas (TA), and tubulovillous and villous adenomas (TVA and VA) from patients undergoing colonoscopy for routine clinical indications, including colon cancer screening. The histologically normal colonic mucosa was obtained from surgical resection specimens from individuals with no neoplastic disease and showed no detectable methylation of the genes studied.
be analyzed for all three genes (n = 8/39) carried at least one methylated gene. Among the 17 patients with hyperplastic polyps, 35% (N = 6/17) had methylated gene(s) detected in their polyps. In the hyperplastic polyp group, the average size of the polyps with and without at least one methylated gene was 3.4 and 3.8 mm, respectively. These polyps were located throughout the colon but were almost exclusively from the left colon. Of note, the likelihood of identifying a polyp with a methylated gene was independent of the number of hyperplastic polyps present.

**Aberrantly Methylated Genes in Colon Polyps Can Be Detected in Fecal DNA.** Following the performance of the MSP assays on the DNA extracted from the polyps, we assessed fecal DNA from a group of study subjects who had matched fecal and polyp DNA samples to determine if we could detect methylated genes in the fecal DNA from individuals with colon polyps. Sixteen study subjects had an adenoma with methylated CDKN2A and eight (50%) of these cases had methylated CDKN2A in the fecal DNA as well. Nine subjects did not have methylated CDKN2A in either their adenomas or fecal samples, and one had methylated CDKN2A only in his fecal DNA but not in his adenoma. For MGMT, 12 of 17 study subjects had concordant methylated MGMT in their adenoma DNA and fecal DNA. Nine subjects had no methylated MGMT in either their adenomas or fecal samples, and two subjects had methylated MGMT only in the fecal DNA. None of the three individuals whose adenomas carried methylated MLH1 had detectable methylated MLH1 in their fecal DNA, and all of the remaining 25 subjects with adenomas that had unmethylated MLH1 showed unmethylated MLH1 in their fecal samples. Comparison of the performance characteristics of CDKN2A and MGMT MSP assays showed that the assays could detect 50% and 71% of individuals with adenomas that carried methylated CDKN2A or MGMT, respectively. Overall, methylation of at least one of the candidate genes was detected in the fecal DNA from 55% of patients with adenoma(s) (N = 16/29), and 14 of these individuals had at least one gene that showed matching methylation in the adenoma(s) and fecal DNA (Table 2; Fig. 2).

With regards to the study subjects with hyperplastic polyps, we restricted our analysis to individuals who only had hyperplastic polyps to simplify the interpretation of our results. Ten study subjects with matched fecal and polyp DNA were found to only have hyperplastic polyps during colonoscopy. Two of these individuals had methylated MGMT in their polyps, and both of these individuals tested positive for methylated MGMT in their fecal DNA. Only two individuals had methylated CDKN2A in a hyperplastic polyp, and one of these two cases had detectable methylated CDKN2A in his fecal DNA. One additional subject had methylated CDKN2A in the fecal sample but had no detectable methylated CDKN2A in any of his polyps (Table 2).

After detecting methylated genes in the fecal DNA of individuals with colon polyps, fecal DNA samples from another 25 individuals without detectable polyps during colonoscopy were also tested for the presence of methylated CDKN2A, MGMT, or MLH1. The methylation analysis revealed that 37% (N = 7/19) of these individuals had methylated genes detected in their fecal DNA despite having no polyps detected during colonoscopy (Table 2). The MSP assays failed to generate products from the DNA of six subjects with normal colonoscopic exams, most likely because of an insufficient amount of DNA in their fecal samples.

**DISCUSSION**

We have identified aberrantly methylated genes in colorectal polyps and found that over 59% of adenomas carry at least one methylated allele of MGMT, CDKN2A, or MLH1. Notably, the prevalence of methylated MGMT and CDKN2A, but not MLH1, is higher in the more histologically progressed adenomas. We have also found that aberrant DNA methylation occurs in hyperplastic polyps although significantly less frequently than it does in adenomas. Finally, we have now shown for the first time to our knowledge the results of studies assessing the use of methylated genes as fecal DNA-based molecular markers for colon polyps.

Our results support the observation that the aberrant methylation of genes occurs early in the tumorigenesis process in the gastrointestinal tract, arising in the premalignant stage of these colon neoplasms. Consistent with our observation, aberrant CpG island methylation has been detected by some investigators in the precursor lesions for colon cancer, including aberrant crypt

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Fig. 2  Methylation analysis of CDKN2A, MGMT, and MLH1 in matched polyp-fecal DNA pairs by methylation-specific PCR using primers for methylated (M) and unmethylated (U) alleles of bisulfite-modified DNA. The gene analyzed and case numbers are indicated above each gel. DNA from lymphocytes (N1 and N2), and colon cancer cell lines [RKO and VACO5 (V5)] was used for unmethylated and methylated controls, respectively, and distilled UV light-treated water without DNA was used as a negative control. The unmethylated alleles detected in the polyp and fecal DNA are presumed secondary to contaminating DNA from normal cells.
Table 2  Correlation between colonoscopic findings and tissue and fecal DNA MSP assay results

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<tr>
<td></td>
<td></td>
<td>CDKN2A, tissue/feces</td>
<td>MGMT, tissue/feces</td>
<td>MLH1, tissue/feces</td>
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<td>--/U</td>
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<tr>
<td>(no polyps)</td>
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<td>--/U</td>
<td>--/U</td>
<td>--/U</td>
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<tr>
<td></td>
<td>3n</td>
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NOTE. MSP assay results from tissue samples and fecal DNA are shown respectively for study subjects with endoscopically detected polyps. Subjects were placed into the group with hyperplastic polyps if they only had hyperplastic polyps and were placed into the group with adenomas if any of the polyps they had detected on endoscopy included an adenoma. If an individual had more than one polyp and any of these polyps had a methylated allele, the polyp DNA was scored as positive. Subjects with normal colonoscopic findings have single methylation results obtained from analysis of DNA from the fecal material.

Abbreviations: U, unmethylated; M, methylated; CRC, colorectal cancer; na, no PCR product generated.
collection of sporadic hyperplastic polyps and, consistent with the
results of Bariol et al, we found methylated CDKN2A, MGMT,
and MLH1 in a subset of these hyperplastic polyps (20, 26). In
light of recent studies that have suggested that some serrated
polyps have the potential to transform into cancer and that
aberrantly methylated genes are commonly found in serrated
polyps, it is interesting to speculate that hyperplastic polyps
that have aberrantly methylated genes may have the potential
to transform (5, 6). Further studies will be needed to determine if
such a causal relationship exists between these two observations.

Finally, based on our findings in the adenomas and
hyperplastic polyps, we have studied the use of aberrantly
methylated genes as fecal-based molecular markers for colon
adenomas, owing to these being the polyps that have the best-
demonstrated risk for transformation. We have shown that an
MSP-based assay panel employing two genes, CDKN2A and
MGMT, when applied to DNA from fecal material, identified
55% of the patients with adenomatous polyps. Notably, not all
adenomas carry the methylated gene being detected by the MSP
assays we used, suggesting that adding more MSP assays that
assess genes other than CDKN2A and MGMT may improve the
overall performance of the MSP assay panel to detect colon
adenomas (27). In light of the known heterogeneity of molecular
alterations in adenomas, this approach should improve the
sensitivity of MSP assays to detect adenoma DNA from fecal
material. However, it is important to note that the addition of
MSP assays to an assay panel carries the potential to decrease the
specificity of the assay panel and requires the selection of highly
specific individual assays to generate an assay panel with a high
positive predictive value for adenomas (28). Also, the positive
association that we and others have found between the frequency
of aberrant DNA methylation and advanced histological features
suggests that MSP assays may be more accurate for the detection
of advanced adenomas and colon cancer than for polyps in
general.

We also found that this assay panel only detected 55% of
patients with colon adenomas and detected methylated CDKN2A
or MGMT in 37% of people with no polyps found by
colonoscopy. We speculate that the MSP assays used in this
study have suboptimal sensitivity because the PCR-based assays
we have used either are not sensitive enough to detect the small
amount of polyp DNA in the fecal DNA samples of the false-
negative cases or because the technique we have used to extract
the fecal DNA has resulted in loss of the tumor DNA in the
samples that generated false-negative results. In addition, an
important question raised by our results is the significance of the
detection of methylated genes in 37% of the fecal DNA samples
from patients with normal colonoscopic exams, which is in
contrast with the complete lack of methylated genes detected in
the normal mucosal samples obtained from a separate control
group that was age-matched. Possible explanations for this
finding include technical errors, differences between the
populations from whom we obtained normal mucosa samples and
from whom we obtained fecal DNA samples after negative
colonoscopic exams, low-level DNA methylation in the normal
tissue of some people, and false-negative colonoscopic exams
due to colonoscopy having at least a 28% miss rate for small
adenomas (19, 29–33). Furthermore, it is possible that the
methylated genes we detected in the fecal DNA were derived
from the normal colon mucosa or aberrant crypt foci in the study
subjects rather than from the polyps, which could explain the
low sensitivity and specificity we observed (8, 19). The design of
our study did not permit us to investigate these possible causes
for the false-positive results.

In summary, we have shown that aberrantly methylated
CDKN2A, MGMT, and MLH1 occur in colon adenomas and
hyperplastic polyps and that the frequency of methylated genes
is higher in histologically advanced adenomas, which are
believed to have the highest likelihood of progressing to colon
cancer. We have also shown the feasibility of using aberrantly
methylated genes detected in fecal material as molecular markers
for these polyps. Our findings suggest that the detection of
aberrantly methylated genes in fecal material from colon lavage
eﬄuent, and likely from stool, has potential value in the
noninvasive and early diagnosis of colorectal neoplasms, but that
the low sensitivity and specificity of the assays we have used
necessitates further assay development before this approach can
be clinically useful as a diagnostic assay for colon polyps. The
identiﬁcation of additional genes that are frequently, and not
concurrently, methylated in polyps may improve the perfor-
man ce characteristics of the current assay panel. In light of the
success of molecular markers that detect DNA mutations and the
known genetic and epigenetic heterogeneity of colon cancer, it is
possible that molecular marker assays that employ both genetic
and epigenetic alterations could be effective for the early
detection of colon cancer (34–37).

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Zsolt Petko, Mahan Ghiassi, Anthony Shuber, et al.


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