# MULTIPLE MYELOMA

# RISK FACTORS, DIAGNOSIS AND TREATMENTS

STEVE HOLT

EDITOR

*Chapter 7*

# The Role of DNA Methylation in the Genetics and Epigenetics of Multiple Myeloma

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## Abstract

Multiple myeloma (MM) arises through an accumulation of multiple genetic and epigenetic changes, which play a significant role in tumorigenesis and tumor development. DNA methylation is often found in cancers including MM at the 5-carbon on cytosine residues within CpG islands of genes whose products are associated with the promoter regions of protein-coding genes. This methylation is an epigenetic alteration that leads to heritable changes in gene expression through the recruitment of histone deacetylases and histone methyltransferases. We and other researchers have reported the association of global and regional DNA methylation status with MM. Global DNA hypomethylation is the predominant early change during plasma cell oncogenesis from monoclonal gammopathy of undetermined significance to MM, while regional DNA hypermethylation occurs in tumor relapse and during disease progression. Thus, DNA methylation could be a useful biomarker of MM tumorigenesis and progression. In the current review, we discuss the role of DNA methylation changes; their potential application as epigenetic biomarkers to facilitate risk assessment, diagnosis, prediction of prognosis, and sensitivity to treatment; and epigenetic therapy in MM.

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# Introduction

Cancers, including multiple myeloma (MM), arise because of an accumulation of multiple genetic changes, which play a significant role in tumorigenesis and tumor development. In addition to genetics, recent studies revealed the role of epigenetics—heritable information that does not affect DNA sequence—in the pathogenesis of cancers, including MM [1-4]. Among epigenetic changes, DNA methylation and histone modification have been well-studied.

DNA methylation occurs at the 5-carbon on cytosine residues in cytosine-guanine pairs known as CpG dinucleotides. DNA methylation is catalyzed by three DNA methyltransferases, including DNMT1, DNMT3A, and DNMT3B, and is a crucial regulator in different biological processes, such as embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, genomic instability, and tumorigenesis [5]. Since transcriptionally active regions of the genome are usually CpG rich, methylation of CpG sites is a critical factor affecting gene transcription. DNA hypermethylation of the large clusters of CpG dinucleotides, referred to as CpG islands, at gene promoters and transcription start sites is an epigenetic alteration that can suppress gene expression through the recruitment of methyl-CpG binding domain proteins, histone deacetylases, and histone methyltransferases, thus causing chromatin condensation [6]. Genome-wide hypomethylation and regional hypermethylation are common events in tumors, including hematological malignancies. In MM, DNA hypomethylation was reported as the predominant early change during tumorigenesis that gradually transforms to regional DNA hypermethylation during disease progression [7-9].

In the current review, we discuss the role of alterations in DNA methylation, potential application of epigenetic biomarkers, and target therapeutics in MM.

# 2. Molecular Mechanism Involved in Tumorigenesis of MM

MM is a neoplastic plasma cell disorder that is characterized by the clonal proliferation of malignant plasma cells in the bone marrow, the presence of monoclonal immunoglobulin in the serum and/or urine in most cases, and associated organ dysfunction, including lytic bone lesions, compromised immunity, anemia, renal failure, and hypercalcemia [10-12]. Recent studies have shown that MM is consistently preceded by a premalignant stage of clonal plasma cell proliferation, termed monoclonal gammopathy of undetermined significance (MGUS) [13,14]. Approximately 1% of MGUS cases evolve to MM per year [15].

MM advances through a multistep transformation process of specific events, including somatic mutations, chromosomal copy-number changes, and non-random chromosomal translocations such as immunoglobulin gene rearrangements involved in cyclin D; furthermore, epigenetic changes drive progression from MGUS, to symptomatic MM, and ultimately to recurrent myeloma, including extramedullary disease and, in some cases, plasma cell leukemia [4,12,16,17] (Figure 1).
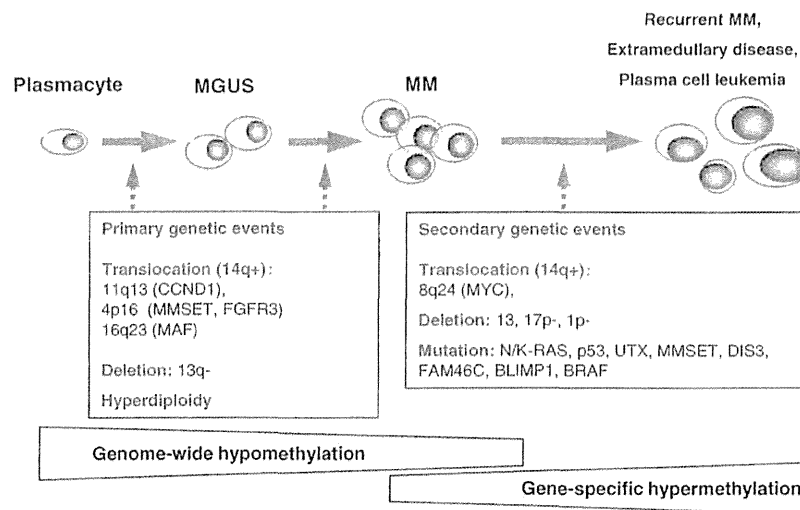
Figure 1. Multiple myeloma (MM) advances through a multistep transformation process due to specific events. These events include somatic mutations, chromosomal copy-number changes, and non-random chromosomal translocations, such as immunoglobulin gene rearrangements involved in cyclin D. Further, epigenetic changes drive progression from monoclonal gammopathy of undetermined significance (MGUS), to symptomatic MM, and ultimately to recurrent myeloma, including extramedullary disease and plasma cell leukemia. Genome-wide hypomethylation occurs during the events indicated by a blue wedge, and gene-specific hypermethylation occurs during the events indicated by a blue wedge.

Recently, several studies using high-throughput sequencing technologies demonstrated heterogeneity of MM genomic evolution and subclonal structure [18-22]. Chapman et al., reported that the analysis of somatic mutations by tumor-genome sequencing in MM cases revealed that, of the numerous genes mutated, identified genes are specifically involved in NFκB activation, protein homeostasis, and histone methylation, which are processes consistent with MM biology [18]. However, the key steps in MM oncogenesis remain unclear [3,4,23,24]. Recent findings also revealed that epigenetics, including DNA methylation and histone modification, is also important in MM pathogenesis. Global methylation analyses in MM have revealed the role of DNA methylation in MM pathogenesis and progress.

## 2. DNA Hypomethylation in MM

Genome-wide DNA hypomethylation is a common epigenetic alteration in cancer cells. Low levels of DNA methylation in cancer cells is substantially due to the loss of methylation at repetitive sequences such as long interspersed nuclear element-1 (LINE-1; a kind of a retrotransposon), which accounts for 17% of the human genome [19,25]. The following mechanisms have been suggested for DNA hypomethylation in tumorigenesis and tumor development: increased instability of the genome and reactivation of transposable elements (transposons) that can move in DNA [26-28]. Importantly, we and others reported that global methylation levels of DNA repetitive sequences, including LINE-1, progressively decline during the development of MM from MGUS to aggressive myeloma such as plasma cell

leukemia [8,9]. We also reported that there is a significant inverse correlation between the degree of genomic loss and LINE-1 methylation levels, and MM cases with LINE-1 hypomethylation had a significantly poor prognosis [9]. Regarding the pathogenesis of MM plasma cells, microarray data examining genome-wide differences in CpG methylation patterns revealed that genome-wide hypomethylation occurs at the transition from MGUS to MM [7].

# 3. DNA Hypermethylation in MM

DNA hypermethylation at gene promoters and transcription start sites is an epigenetic alteration that suppresses gene expression. Global DNA hypomethylation is the predominant early change during plasma cell oncogenesis from MGUS to MM, while regional DNA hypermethylation occurs in tumor relapse and during disease progression [7]. We and others studied regional DNA hypermethylation in MM and identified certain key genes as targets for epigenetic inactivation (Table 1).

### Table 1. Epigenetically silenced genes in multiple myeloma (MM)

| Gene | Chromosome | Function | Frequency of DNA hypermethylation of patient MM samples (n > 50) |
|---|---|---|---|
| CDKN2A (p16) | 9p21.3 | Cell cycle | 34% [29] |
| DAPK1 | 9q21.33 | Apoptosis | 52.7% [35] |
| BNIP3 | 10q26.3 | Apoptosis | 5% [36] |
| RASD1 | 17p11.2 | Cell growth | 6–8% [33,40] |
| SPARC | 5q33.1 | Cell-extracellular matrix interaction | 8–18.2% [36,40] |
| CD38 | 4p15 | Ectoenzyme | 45.9% [40] |
| GPX3 | 5q23 | Glutathione peroxidase | 7.5% [40] |
| NCAM1 (CD56) | 11q23.1 | Cell adhesion | 5% [40] |
| PDK4 | 7q21.3 | Regulation of metabolism | 15.1% [40] |
| RBP1 | 3q23 | carrier protein involved in the transport of retinol | 16.3% [40] |
| TGFBI | 5q31 | Inhibition of cell adhesion | 18.2% [40] |

These genes include the following: cell-cycle regulators, such as cyclin-dependent kinase inhibitor 2A (CDKN2A) [29] and 2B (CDKN2B) [30] and checkpoint with fork head and ring finger domains (CHFR) [31]; genes involved in cell signaling, such as Ras association

(RalGDS/AF-6) domain family member 1 (*RASSF1*) [32], RAS, dexamethasone-induced 1 (*RASD1*) [33], and transforming growth factor, beta receptor II (*TGFBR2*) [34]; genes involved in apoptosis, such as death-associated protein kinase 1 (*DAPK1*) [35] and BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*) [36,37]; genes involved in antigen presentation, such as class II, major histocompatibility complex, transactivator (*CIITA*) [38]; genes involved in cell-extracellular matrix interaction, such as secreted protein, acidic, cysteine-rich (*SPARC*) [36]; and genes involved in polycomb repressive complexes, such as enhancer of zeste, drosophila, homolog 2 (*EZH2*) [39].

Recently, Kaiser et al., investigated the association between DNA methylation and MM prognosis using a genome-wide DNA methylation array of 159 patients treated in the Medical Research Council Myeloma IX trial [40]. They identified the following 8 epigenetically regulated genes with changes in DNA methylation status that were significantly associated with prognosis: *CD38*, *RASD1*, *SPARC*, glutathione peroxidase 3 (*GPX3*), neural cell adhesion molecule 1 (*NCAM1*), pyruvate dehydrogenase kinase 4 (*PDK4*), retinol-binding protein 1 (*RBP1*), and transforming growth factor, β induced (*TGFBI*). Importantly, multivariate analysis confirmed that *GPX3*, *RBP1*, *SPARC*, and *TGFBI* are associated with survival, and methylation of the genes is independent of established risk factors for MM. Methylation levels of these 4 genes is low in MGUS, and then increasing methylation is associated with more aggressive MM cellular phenotypes. Walker et al., investigated DNA methylation patterns associated with MM subtypes [7]. They found specific profiles with increased hypermethylation in clinically aggressive subtypes, such as plasma cell leukemia, and in the prognostically unfavorable t(4;14) cytogenetic subtype with overexpressed *MMSET*, which encodes a histone methyltransferase. These findings suggest that methylation changes affect disease biology.

Recent reports correlated hypermethylation of promoter-associated CpG islands with silencing of microRNAs (miRNAs), which are small 18–22 nucleotide RNAs that regulate many intracellular functions [41]. Dysregulation of miRNA genes has been implicated in MM. Moreover, several reports of MM described the role of hypermethylation of tumor-suppressor miRNA genes, including *miR-34b/c* [42], *miR-194-2-192* [43], and *miR-203* [44]. Combined genome-wide analysis of miRNA methylation and miRNA expression profiling is warranted to clarify the role of epigenetic regulation of miRNA in MM.

## 4. DNA Methylation As an Epigenetic Biomarker in MM

The current prognostic factors in MM include cytogenetic aberrations, such as the nonhyperdiploid, cytogenetically detected chromosomal 13q deletion, t(4;14), t(14;16), 1q gain, and del(17p) detected by fluorescence *in situ* hybridization [10]. Novel therapeutics, such as the proteasome inhibitor bortezomib, can partially overcome adverse outcomes conferred by these abnormalities [45]. However, there has been much less progress in the development of predictive biomarkers for specific treatments [46]. To identify predictive biomarkers for the effect of myeloma therapeutics, appropriate clinical trial designs are necessary. Since some novel MM therapeutics in development have specific molecular

targets, the identification of biomarkers that also characterize drug sensitivity is a promising therapeutic strategy [45].

As mentioned above, hypermethylation of *TGFB1*, *SPARC*, *RBP1*, and *GPX3* is associated with significantly shorter overall survival, independent of age, international staging system score, and adverse cytogenetics [40]. Future prospective studies will verify these genes as prognostic MM biomarkers.

We identified *RASD1* as a possible biomarker in MM [33]. *RASD1*, located on chromosome 17p11.2 with frequent loss of heterozygosity in various human tumors, encodes a Ras GTPase with tumor suppressor functions induced by dexamethasone [47,48]. Importantly, MM cells that show *RASD1* methylation are resistant to dexamethasone, and combined treatment with dexamethasone and the hypomethylating agent decitabine (5-aza-2'-deoxycytidine), which inhibits DNA methyltransferase, restores the cytotoxicity of dexamethasone to tumor cells. While the hypermethylation of *RASD1* was observed in approximately 10% of primary MM samples, the methylation levels of *RASD1* were elevated in all of the MM cases that had pair DNAs after repeated antimyeloma therapy, including dexamethasone.

Limited studies have addressed the antitumor effects of the hypomethylating agents decitabine and azacitidine (5-azacytidine) in MM, demonstrating significant *in vitro* antimyeloma activity. The mechanisms involve changes in gene expression and induction of DNA damage [49,50]. Recently, a gene expression-based DNA methylation score was reported, which relates the expression of methylation-regulated genes to predict the efficacy of hypomethylating agents—decitabine and azacitidine—in human MM cell lines and in patient MM cells *in vitro* [51,52]. Phase I/II clinical trials are ongoing to study the side effects and best dose of azacitidine in combination with lenalidomide and dexamethasone in MM; therefore, an investigation regarding association of the methylation score and the response of MM patients could provide promising information [53]. Taken together, these findings suggest the involvement of epigenetic gene silencing in MM progression and drug resistance and the usefulness of demethylation therapy for MM treatment.

# Conclusion

In summary, DNA methylation functions in MM tumorigenesis and progression. Several reports have suggested that DNA methylation could be a useful biomarker to predict prognosis and sensitivity to treatment. A further comprehensive analysis using a genome-wide approach with high-throughput sequencing technologies will be necessary to clarify the molecular mechanisms of MM oncogenesis and progression. Epigenetics has become to an essential research area where important challenges should be resolved through further investigations of MM.

# Acknowledgments

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Cancer
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# Analysis of DNA Methylation in Bowel Lavage Fluid for Detection of Colorectal Cancer

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## Abstract

Aberrant DNA methylation could potentially serve as a biomarker for colorectal neoplasms. In this study, we assessed the feasibility of using DNA methylation detected in bowel lavage fluid (BLF) for colorectal cancer screening. A total of 508 BLF specimens were collected from patients with colorectal cancer ($n = 56$), advanced adenoma ($n = 53$), minor polyp ($n = 209$), and healthy individuals ($n = 190$) undergoing colonoscopy. Methylation of 15 genes (*miR-1-1, miR-9-1, miR-9-3, miR-34b/c, miR-124-1, miR-124-2, miR-124-3, miR-137, SFRP1, SFRP2, APC, DKK2, WIF1, LOC386758,* and *ZNF582*) was then analyzed in MethyLight assays, after which receiver operating characteristic (ROC) curves were analyzed to assess the diagnostic performance of BLF methylation. Through analyzing BLF specimens in a training set ($n = 345$), we selected the three genes showing the greatest sensitivity for colorectal cancer detection (*miR-124-3,* 71.8%; *LOC386758,* 79.5%; and *SFRP1,* 74.4%). A scoring system based on the methylation of those three genes (M-score) achieved 82% sensitivity and 79% specificity, and the area under the ROC curve (AUC) was 0.834. The strong performance of this system was then validated in an independent test set ($n = 153$; AUC = 0.808). No significant correlation was found between M-score and the clinicopathologic features of the colorectal cancers. Our results demonstrate that DNA methylation in BLF specimens may be a useful biomarker for the detection of colorectal cancer. *Cancer Prev Res; 7(10); 1002–10. ©2014 AACR.*

## Introduction

Colorectal cancer is one of the most commonly occurring malignancies worldwide, and early detection is essential for its successful treatment. Large population studies have shown that the fecal occult blood test (FOBT) is a highly cost-effective screening method that reduces colorectal cancer–related mortality (1). Moreover, the performance of the immunochemical FOBT (iFOBT or Fecal Immunochemical Test, FIT) has been improved (2, 3) and is now widely used for colorectal cancer screening in Japan and Europe. However, the FOBT continues to have limitations, especially for detection of early-stage colorectal cancers. Several other methods, including colonoscopy and barium enema, have been available for years, but none of these methods has been established as a gold standard for colorectal cancer screening.

Fecal DNA tests are a noninvasive and potentially effective means of screening for both early colorectal lesions and advanced colorectal cancers (4, 5). As such, the feasibility of detecting genetic mutation of oncogenes or tumor-suppressor genes, such as *APC, KRAS, TP53,* and *BAT-26,* has been extensively tested, but the diagnostic performance of these assays remains unsatisfactory (6, 7). Epigenetic alterations are also commonly observed in colorectal cancers. Because of its high frequency and the wide variety of affected genes, aberrant DNA methylation has emerged as a new biomarker for stool-based colorectal cancer screening. For instance, *SFRP2* methylation occurs in approximately 90% of primary colorectal cancers (8), and was one of the first epigenetic markers reported in fecal DNA (9). More recently, a variety of other genes have been identified as potential biomarkers for stool-based methylation testing, including *VIM, GATA4, TFPI2, PHACTR3, AGTR1, WNT2,* and *miR-34b/c* (10–15).

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In an earlier study, we demonstrated that DNA methylation is detectable in the mucosal wash fluid from colorectal tumors, which can be collected during colonoscopy (16). Importantly, wash fluid from invasive cancers exhibited significantly higher levels of methylation of tumor-related genes than noninvasive tumors. This prompted us to postulate that wash fluid from invasive tumors contained greater numbers of exfoliated tumor cells, and that the methylation was a potential biomarker predictive of tumor invasiveness. Our results also suggested that a DNA methylation test might complement the diagnostic performance of colonoscopy and that intestinal wash fluid could be a useful source for analysis of tumor-derived DNA methylation. We therefore hypothesized that oral bowel lavage fluid (BLF) might contain tumor-derived DNA, and thus molecular alteration in BLF specimens could be a useful biomarker for colorectal cancer screening. To test that idea, in this study, we analyzed DNA methylation of tumor-related genes in BLF specimens from patients with colorectal tumors and healthy individuals, and examined its clinical utility for cancer detection.

## Materials and Methods

### Patients and BLF specimens

All samples were collected from Japanese patients who underwent colonoscopy at Akita Red Cross Hospital (Akita, Japan) because of abdominal symptoms or a positive FOBT. Informed consent was obtained from all patients before collection of the specimens. Approval for this study was obtained from the Institutional Review Board of Akita Red Cross Hospital and Sapporo Medical University (Sapporo, Japan). Before colonoscopy, patients were pretreated with 2 L of polyethylene glycol lavage solution and 10 mL of BLF specimens were collected from the rectum at the beginning of the colonoscopy (Fig. 1A). BLF samples were initially classified into four groups according to the Boston bowel preparation scale (BBPS; Fig. 1B; ref. 17). Then, on the basis of colonoscopic and histologic findings, the BLF samples were divided into four groups: patients with colorectal cancer, patients with advanced adenoma, patients with minor polyp, and individuals without colorectal lesions. Advanced adenomas were defined as being 1 cm or more in diameter, and/or with villous components, and/or with high-grade dysplasia. Minor polyps were defined as being adenomas that did not satisfy the above criteria. A total of 508 BLF samples from 56 patients with colorectal cancer, 53 patients with advanced adenoma, 209 patients with minor polyp, and 190 individuals with a normal colon were collected. In addition, biopsy specimens were collected from 44 of the 56 patients with colorectal cancer. BLF and tissue specimens were suspended in ThinPrep PreservCyt solution (Hologic) and stored at 4°C until DNA extraction. Genomic DNA was extracted using the standard phenol–chloroform procedure. FIT was performed in 349 individuals, including 17 patients with colorectal cancer. Samples were randomly sorted into two groups (training set and test set) for validation analysis (Table 1).

### Methylation analysis

Genomic DNA (1 µg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen), after which methylation analysis was carried out as described previously (18). PCR for MethyLight assays was run in a 20-µL volume containing 50 ng of bisulfite-treated DNA, 625 nmol/L each primer, 250 nmol/L TaqMan-MGB probe, and 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Fast real-time PCR was done using a 7500 Fast Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems). The PCR protocol entailed 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. The Alu repetitive element was used as an endogenous control, and the percentage of methylated reference (PMR) was calculated as described previously (19, 20). Sequence information for the primers and probes used for *miR-1-1, miR-9-1, miR-9-3, miR-34b/c, miR-124-1, miR-124-2, miR-124-3, miR-137, SFRP1, SFRP2, DKK2, WIF1, LOC386758,* and *ZNF582* is listed in Supplementary Table S1; those used for *APC* is described elsewhere (20).

### Statistical analysis

Quantitative variables were analyzed using the Student $t$ test. The Fisher exact test and the $\chi^2$ test were used for analysis of categorical data. The Pearson correlation coefficient was used to evaluate correlations between continuous data. Receiver–operating characteristic (ROC) curves for the diagnosis of colorectal cancer were constructed on the basis of the methylation levels, followed by calculation of the area under the curve (AUC). The best cutoff PMR value for each gene was defined as the point on the ROC curve closest to the upper left corner. A diagnostic scoring system using a panel of selected marker genes was constructed by analyzing a training set using the following three-step algorithm: (i) methylation status of marker genes in BLF was assessed; (ii) the number of methylated genes was determined, which we termed the methylation score (M-score); and (iii) the samples were classified into four groups based on the M-score. Values of $P < 0.05$ (two-sided) were regarded as significant. All statistical analyses were performed using the SPSS Statistics 18 (IBM Corporation) and GraphPad Prism ver. 5.0.2 (GraphPad Software).

## Results

### Detection of DNA methylation in BLF specimens

After collecting 10-mL BLF specimens from the rectums of the study participants at the beginning of their colonoscopy, we successfully extracted sufficient amounts of genomic DNA to perform a methylation analysis (Fig. 1A). To determine the best time to obtain the BLF specimens, we scored the BLF samples using the BBPS (Fig. 1B; ref. 17). Among the 268 BLF samples initially collected, 58 were scored as 3, 154 were scored as 2, 46 were scored as 1, and 10 were scored as 0. BLF samples without residual stool (BBPS scores 2 and 3) contained significantly smaller amounts of genomic DNA than those with residual stool (BBPS scores 0 and 1; Fig. 1C). However, MethyLight assays revealed that the endogenous control Alu element was detected at lower threshold cycle
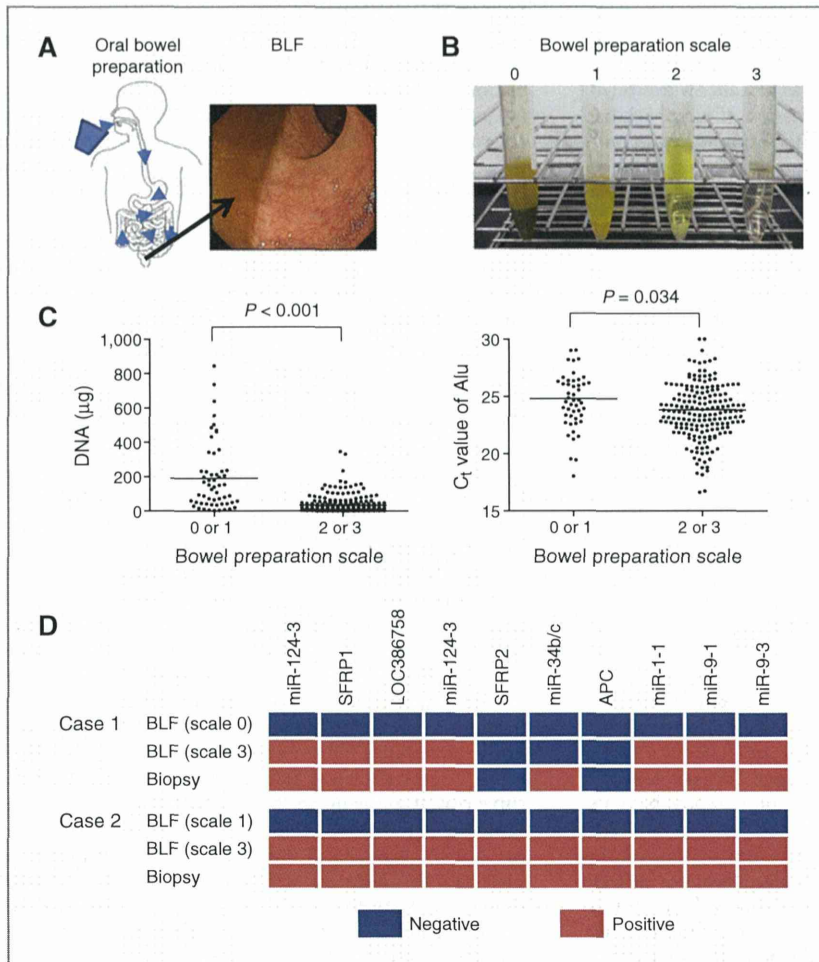
Figure 1. Collection of BLF and detection of DNA methylation. A, after oral bowel preparation, BLF specimens were collected from the rectum of individuals undergoing colonoscopy. B, BLF samples representative of the indicated BBPS scores. C, association between bowel preparation and the amount of extracted DNA (left) and Alu elements in MethyLight assays (right). Note that a larger amount of DNA is obtained from BLF specimens with a lower BBPS score, but human Alu element is more readily detectable in specimens with a higher BBPS score. D, MethyLight assay results for the indicated genes in BLF specimens with low and high BBPS scores and biopsy specimens from 2 representative patients with colorectal cancer.

($C_t$) values in BLF specimens with high BBPS scores than in those with residual stool (Fig. 1C). This suggests that the relative fraction of human genomic DNA is larger in higher BBPS score BLF, most likely because of the smaller amount of contaminating bacteria-derived DNA. We then analyzed BLF specimens from selected patients with colorectal cancer, comparing the detectability of DNA methylation between specimens with lower and higher BBPS scores. As shown in Fig. 1D, methylation of representative genes was readily detectable in BLF specimens with a higher BBPS score, whereas it was undetectable in specimens with a lower score (Fig. 1D). For these reasons, we collected BLF specimens after sufficient bowel preparation for the next analysis.

## Selection of marker genes for colorectal cancer detection

Our training set consisted of 355 BLF specimens obtained from patients with colorectal cancer ($n = 39$),

advanced adenomas ($n = 31$), or minor polyps ($n = 135$), as well as individuals with no colorectal lesions ($n = 150$; Table 1). Using these specimens, we performed quantitative MethyLight assays to assess the methylation status of 15 genes known to be frequent targets of aberrant CpG island methylation in colorectal cancer (*miR-1-1, miR-9-1, miR-9-3, miR-34b/c, miR-124-1, miR-124-2, miR-124-3, miR-137, SFRP1, SFRP2, APC, DKK2, WIF1, LOC386758,* and *ZNF582*; refs. 8, 21). The methylation levels of the respective genes were calculated as PMR values, and we generated ROC curves to assess their clinical utility for detection of colorectal cancer (Supplementary Table S2 and Supplementary Fig. S1). Among the candidate marker genes analyzed, we found that *miR-124-3, LOC386758,* and *SFRP1* were highly discriminative between patients with colorectal cancer and those without colorectal cancer (Supplementary Table S2). The most discriminating PMR cutoffs for *miR-124-3, LOC386758,* and *SFRP1* were 11.1, 0.0003, and 1.1, while the most

**Table 1.** Clinicopathologic features of the subjects in this study

|  | Training set (N = 355) | Test set (N = 153) | Total (N = 508) |
|---|---|---|---|
| Demographics |  |  |  |
| Median age, y (range) | 61 (28–93) | 61 (33–89) | 61 (28–93) |
| Male | 235 | 108 | 343 |
| Female | 120 | 45 | 165 |
| Colorectal cancer |  |  |  |
| Total N | 39 | 17 | 56 |
| Location (right/left/rectum) | 13/11/15 | 7/7/3 | 20/18/18 |
| Median size, cm (range) | 4.5 (0.7–11.5) | 4.8 (1.5–9.3) | 4.6 (0.7–11.5) |
| Dukes stage (A/B/C/D) | 9/16/11/3 | 4/8/4/1 | 13/24/15/4 |
| Advanced adenoma[a] |  |  |  |
| Total N | 31 | 22 | 53 |
| Location (right/left/rectum) | 16/12/3 | 14/7/1 | 30/19/4 |
| Median size, cm (range) | 1.7 (0.6–4.0) | 1.3 (0.6–2.6) | 1.5 (0.6–4.0) |
| Minor polyp[b] |  |  |  |
| Total N | 135 | 74 | 209 |
| Location (right/left/rectum) | 80/46/9 | 47/17/9 | 128/63/18 |
| Median size, cm (range) | 0.5 (0.1–0.9) | 0.5 (0.2–0.9) | 0.5 (0.1–0.9) |
| Normal colon |  |  |  |
| Total N | 150 | 40 | 190 |

[a]Advanced adenomas were defined as adenomas 1 cm or greater in diameter, and/or containing villous components, and/or with high-grade dysplasia.
[b]Minor polyps were defined as adenomas other than advanced adenomas.

sensitive setting (PMR > 0) also achieved high sensitivity and specificity (Supplementary Table S2).

To develop a more efficient diagnostic system for detection of colorectal cancer, we constructed a scoring system based on the methylation of *miR-124-3*, *LOC386758*, and *SFRP1*. Using the number of methylated genes (PMR > 0), we classified the samples into four groups based on their M-score (Fig. 2A). A ROC curve was then constructed to evaluate the ability of the scoring system to distinguish samples obtained from patients with colorectal cancer by plotting the sensitivity over 1 − specificity at each point (Fig. 2B). We then validated the diagnostic system by analyzing an independent test set (Table 1 and Fig. 2A and B). AUCs in the training and test sets were 0.834 and 0.808, respectively, confirming the accuracy of our system for detecting colorectal cancer.

The association between the clinical characteristics and M-scores is summarized in Table 2 and Supplementary Table S3. Higher M-scores were significantly associated with colorectal cancer, but their association with advanced adenomas or minor polyps was limited (Fig. 2C and Supplementary Table S3). M-scores were not significantly associated with tumor location, size or stage (Table 2). We also did not find a correlation between M-scores and age in patients with colorectal cancer, though we observed a tendency for higher M-scores to be associated with older age in non–colorectal cancer individuals, perhaps due to age-dependent methylation (Supplementary Fig. S2). These results suggest that the

M-score system is able to reveal the presence of colorectal cancers, irrespective of the tumor's location, size, or clinical stage, but greater age may increase the false-positive rate.

Although the results summarized above demonstrate the clinical utility of BLF methylation for colorectal cancer screening, the system failed to detect 5 of the 56 patients with colorectal cancer (Supplementary Table S3). We therefore tested whether the apparent absence of methylation in those 5 BLF specimens actually reflects the unmethylated status of the genes in tumor tissues. For this purpose, we analyzed biopsy specimens from 41 patients with colorectal cancer with different M-scores (score 3, $n = 20$; score 2, $n = 10$; score 1, $n = 8$; and score 0, $n = 3$), and found that the majority of the tumors exhibited methylation of all three genes (*miR-124-3*, *LOC386758*, and *SFRP1*), irrespective of the M-score (Supplementary Fig. S3). MethyLight assays revealed that the $C_t$ values for the endogenous Alu tended to be higher in BLF specimens with low M-scores, indicating that the apparent absence of BLF methylation may be result of too little tumor-derived DNA in the sample.

## BLF methylation and upper gastrointestinal tract cancer

We next assessed whether BLF methylation could be used to detect upper gastrointestinal tract cancers. Among the individuals enrolled in this study were 294 who underwent upper gastrointestinal endoscopy; of those, 21 were found to have a gastric cancer. BLF methylation was detected in 12
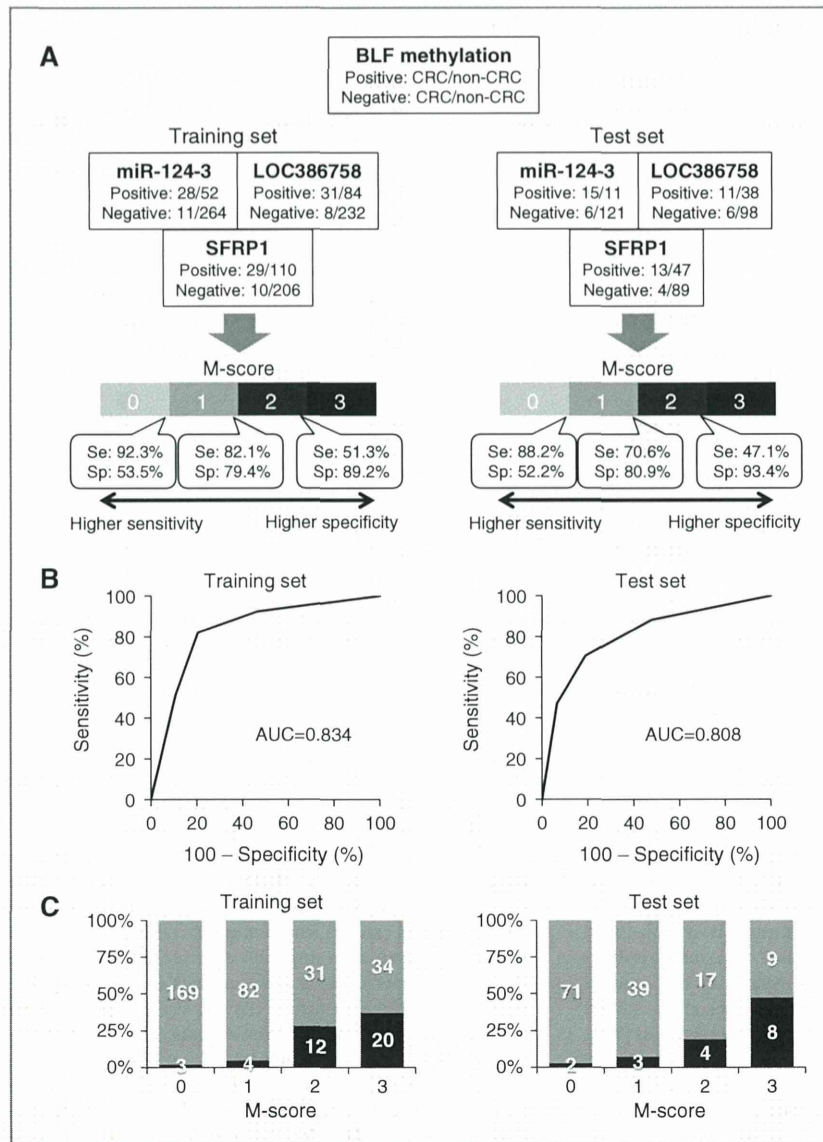
Figure 2. Diagnostic system for detecting colorectal cancer using BLF methylation. A, workflow of a system established on the basis of the ability to distinguish patients with colorectal cancer from colorectal cancer–free individuals. Results of the training set are shown on the left; those of the test set are on the right. A BLF M-score was determined from the number of methylation-positive genes, and samples were classified into four groups based on the M-score. The sensitivity (Se) and specificity (Sp) at each point are indicated below. B, ROC curve analysis of the training and test sets. The AUC is shown in the graphs. C, Percentages of patients with colorectal cancer in the respective M-score groups.

of the 21 patients with gastric cancer, and a majority of the positive cases showed only a minimal number of methylated markers (M-score 0, $n = 9$; score 1, $n = 8$; score 2, $n = 1$; and score 3, $n = 3$). Five of the 8 patients with gastric cancer with minimal methylation (M-score, 1) also had minor colorectal polyps, which could also have been the source of the methylated DNA. Interestingly, 2 of the 4 patients with gastric cancer with high M-scores (>2) were also found to have colorectal cancers, while the remaining 2 patients showed no colorectal lesions. These results suggest it would be difficult to use BLF methylation as a biomarker for upper gastrointestinal cancers.

## BLF methylation and FOBT

FIT was performed in 349 of the study participants, including 17 patients with colorectal cancer (Table 3). Most of the patients with colorectal cancer were positive on the FIT (14 of 17), while a significant number of colorectal cancer–free individuals also showed positive results (142 of 332). For that reason, we next tested whether the diagnostic performance of FIT could be improved by combining it with the BLF methylation test. In the FIT-negative group ($n = 193$), which included 3 patients with colorectal cancer, all the patients with colorectal cancer were detected using the M-score system (Table 3). In the FIT-positive group, most of the patients with

**Table 2.** Correlation between clinical features and BLF methylation in colorectal cancer

| | Total N | M-score | | | | P |
| | | 0 | 1 | 2 | 3 | |
|---|---|---|---|---|---|---|
| Location | | | | | | |
| Proximal colon | 20 | 3 | 3 | 4 | 10 | |
| Distal colon | 18 | 2 | 2 | 6 | 8 | |
| Rectum | 18 | 0 | 2 | 6 | 10 | 0.720 |
| Tumor size, cm | | | | | | |
| −2.0 | 8 | 1 | 0 | 2 | 5 | |
| 2.1–4.0 | 18 | 2 | 3 | 4 | 9 | |
| 4.1–6.0 | 17 | 1 | 2 | 8 | 6 | |
| 6.1– | 13 | 1 | 2 | 2 | 8 | 0.720 |
| Dukes stage | | | | | | |
| A | 13 | 3 | 1 | 5 | 4 | |
| B | 24 | 1 | 3 | 6 | 14 | |
| C+D | 19 | 1 | 3 | 5 | 10 | 0.410 |

NOTE: $P$ values were calculated using the $\chi^2$ test.

colorectal cancer (12 of 14) exhibited BLF methylation (M-score $\geq$ 1), while a majority of the BLF methylation-negative subjects were colorectal cancer–free (80 of 82). Thus, the combination of FIT and the BLF methylation test significantly improved the positive predictive value (PPV) in both the FIT-negative and FIT-positive groups.

**BLF methylation and computed tomographic colonography**

Because computed tomographic colonography (CTC) has emerged in recent years as a noninvasive screening method for colorectal cancer (22), we examined the feasibility of using BLF methylation testing to complement the

**Table 3.** Diagnostic performance of the FIT and BLF methylation test for detection of colorectal cancer

**FIT only**

| Study | Total N | CRC | CRC-free | Sensitivity | Specificity | PPV | NPV | |
|---|---|---|---|---|---|---|---|---|
| Allison et al. | 7,493 | 32 | 7,461 | 0.688 | 0.944 | 0.050 | 0.999 | |
| Current study | 349 | 17 | 332 | 0.824 | 0.428 | 0.090 | 0.984 | |

**FIT and BLF methylation test**

| M-score | Total N | CRC | CRC-free | FIT-negative group | | | | | |
| | | | | Cutoff | Sensitivity | Specificity | PPV | NPV | P |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 102 | 0 | 102 | | | | | | |
| 1 | 54 | 0 | 54 | $\geq$1 | 1.000 | 0.537 | 0.000 | 1.000 | 0.103 |
| 2 | 25 | 1 | 24 | $\geq$2 | 1.000 | 0.821 | 0.081 | 1.000 | 0.007 |
| 3 | 12 | 2 | 10 | 3 | 0.667 | 0.947 | 0.167 | 0.994 | 0.090 |

| M-score | Total N | CRC | CRC-free | FIT-positive group | | | | | |
| | | | | Cutoff | Sensitivity | Specificity | PPV | NPV | P |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 82 | 2 | 80 | | | | | | |
| 1 | 43 | 5 | 38 | $\geq$1 | 0.857 | 0.563 | 0.162 | 0.976 | 0.004 |
| 2 | 12 | 5 | 7 | $\geq$2 | 0.500 | 0.831 | 0.226 | 0.944 | 0.008 |
| 3 | 19 | 2 | 17 | 3 | 0.143 | 0.880 | 0.105 | 0.912 | 0.681 |

NOTE: $P$ value were calculated using the Fisher exact test.
Abbreviations: CRC, colorectal cancer; NPV, negative predictive value.

**Table 4.** Comparison of CTC and BLF methylation test

| Case | Colonoscopic finding | Location | Size, mm | Histologic diagnosis | Dukes' stage | CTC diagnosis | M-score |
|------|----------------------|----------|----------|----------------------|--------------|---------------|---------|
| 1 | CRC | Distal | 30 | Adenocarcinoma | B | CRC | 3 |
| 2 | CRC | Distal | 60 | Adenocarcinoma | B | CRC | 2 |
| 3 | CRC | Distal | 60 | Adenocarcinoma | B | CRC | 2 |
| 4 | CRC | Distal | 43 | Adenocarcinoma | A | CRC | 2 |
| 5 | CRC | Rectum | 11 | Adenocarcinoma in adenoma | A | Minor polyp | 3 |
| 6 | Minor polyp | Proximal | 3 | Tubular adenoma | | Normal | 0 |
| 7 | Minor polyp | Proximal | 4 | Tubular adenoma | | Normal | 1 |
| 8 | Minor polyp | Proximal | 3 | Tubular adenoma | | Minor polyp | 1 |
| 9 | Normal | | | | | Normal | 0 |

diagnostic performance of CTC. Among the subjects enrolled in this study, 9, including 5 patients with colorectal cancer, were examined using CTC (Table 4). CTC detected four colorectal cancers, while all 5 patients with colorectal cancer were positive for BLF methylation (M-score, > 2). Notably, 1 patient (case 5) developed a laterally spreading tumor (LST) that consisted of a histologically benign polypoid component and a flat adenocarcinoma component. CTC detected only the polypoid component, so the lesion was diagnosed as a minor polyp (Table 4 and Supplementary Fig. S4). Our results suggest that combining assessment of BLF methylation with CTC may improve diagnostic performance, though further study of a larger population will be necessary to confirm the clinical utility of this combination.

## Discussion

Numerous studies have shown that aberrant methylation of DNA in the stool is a promising biomarker suitable for noninvasive colorectal cancer screening. For instance, *VIM*, *SFRP2*, and *TFPI2* are reported to be useful single-gene markers for a fecal DNA methylation test (9, 10, 12). In addition, other groups have shown that combinations of multiple markers improve the diagnostic efficacy of stool DNA methylation (14). In this study, we demonstrated that aberrant DNA methylation is detectable in the wash fluid of oral bowel lavage collected from the rectum of patients with colorectal cancer. Earlier studies have shown that methylation of DNA in body fluids, including pancreatic juice (23), saliva (24), and gastric juice (25), has the potential to serve as a biomarker for cancer detection and risk assessment, yet there have been no studies assessing the feasibility of using BLF for molecular screening for colorectal cancer. Importantly, we found that the utility of BLF depends on successful bowel preparation, and that residual stool may interfere with sensitive detection of tumor-derived DNA methylation. Although the total amount of extracted DNA is small, BLF specimens with sufficient bowel preparation appear to contain a greater proportion of tumor-derived DNA than those with insufficient treatment.

In this study, we tested a set of genes known to be frequently methylated in colorectal cancer, and selected the

three genes with the highest sensitivities for detection of colorectal cancer (*miR-124-3*, *SFRP1*, and *LOC386758*). The *miR-124* family consists of three members (*miR-124-1*, *miR-124-2*, and *miR-124-3*), all of which are reportedly methylated in multiple types of human malignancy, including colorectal cancer and gastric cancer (26, 27). *SFRP1* encodes secreted frizzed-related protein 1, a negative regulator of Wnt signaling, and the promoter CpG island of *SFRP1* is frequently methylated in various cancers, including colorectal cancer, gastric cancer, and esophageal cancer (28–30). *LOC386758* is a frequent target of aberrant methylation newly identified in our recent epigenome analysis in colorectal cancer, though its function remains unknown (manuscript in preparation). Although BLF methylation of each of these genes could be used to detect colorectal cancer with relatively high sensitivity and specificity, we found that combining them improved diagnostic accuracy. Importantly, BLF methylation was not affected by tumor size, location, or stage, suggesting that it could potentially serve as a biomarker for both proximal and distal colon cancers.

However, the BLF methylation system failed to detect a small number of colorectal cancers as well as more than half of the precancerous lesions (minor polyps and advanced adenomas). We confirmed that the negative result was not due to the absence of methylation in the tumor tissues. In addition, we and others have previously shown that many of the 15 genes analyzed in this study are frequently methylated in colorectal premalignant lesions (31, 32). Although the true reason for the false-negative finding remains uncertain, we suspect that the presence of a too small number of exfoliated cells in the BLF is the cause. We have previously shown that DNA methylation in colonoscopically obtained mucosal wash fluids could be a predictive biomarker of tumor invasiveness (16). By performing quantitative bisulfite-pyrosequencing, we detected elevated levels of DNA methylation of tumor-related genes (*miR-34b/c*, *SFRP1*, *SFRP2*, and *DKK2*) in the mucosa of invasive tumors, though these genes were equally methylated in noninvasive and invasive tumors. Early during this study, we found that we were unable to detect BLF methylation using bisulfite-pyrosequencing, so we switched to the more sensitive MethyLight assay. We therefore suggest that the numbers

of exfoliated cells and the amount of cell-free DNA in BLF specimens are far smaller than in the colonoscopically obtained mucosal wash fluid. Moreover, BLF specimens with high M-scores tended to show lower $C_t$ values for Alu elements with MethyLight, which is indicative of the relative abundance of human genomic DNA (Fig. 1C). These results suggest that successful detection of BLF methylation is highly dependent on the amount of tumor-derived DNA in the BLF specimens. In addition, we speculate that differences in the sample collection steps and the methods used for methylation analysis could be major reasons why the best marker genes differed between our early studies and the present one (16).

Our findings also suggest that BLF methylation could be used to complement current colorectal cancer screening methods. FIT is one of the most commonly used and cost-effective screening tests, but its low PPV may lead to a low compliance rate among FIT-positive individuals receiving medical advice to go for secondary screening. When combined with FIT, a BLF methylation test could significantly improve PPV and more effectively select individuals who should be strongly encouraged to undergo total colonoscopy. Moreover, our data demonstrated that BLF methylation of multiple genes could be an indicator of colorectal cancer, even among FIT-negative individuals.

As compared with stool DNA tests, the biggest disadvantage of the BLF methylation test is that it requires bowel preparation. Therefore, combination with endoscopies is another feasible clinical application of BLF methylation. For instance, when combined with sigmoidoscopy, a BLF methylation test may complement the diagnostic performance for detection of proximal colon cancers. Similarly, BLF methylation could provide supportive information for patients with unsuccessful total colonoscopy. In addition, we propose that BLF methylation may improve the diagnostic performance of CTC. Although the sensitivity of CTC for detection of some colorectal cancers is equivalent to colonoscopy, its ability to detect small or flat lesions is more limited (33–35). Moreover, it is sometimes difficult to distinguish between early-stage cancers and benign adenomas using CTC. The fact that BLF methylation is highly specific for malignant tumors indicates that it could increase the ability to detect colorectal cancers using CTC. In this study, we compared BLF methylation with CTC findings in 9 individuals, including 5 patients with colorectal cancer. Using CTC, four of the colorectal cancers were successfully detected, but a flat type cancer was diagnosed as a minor polyp. In contrast, BLF methylation (M-score, > 2) was detected in all 5 patients with colorectal cancer. These results suggest that combining assessment of BLF methyl-

ation with CTC may improve the diagnostic performance, but further prospective study of a larger number of patients will be necessary to evaluate the diagnostic performance of this combination.

In sum, our results demonstrate the feasibility of using aberrant DNA methylation in BLF specimens for noninvasive colorectal cancer screening. We also found that using a panel of several marker genes further improved the sensitivity and specificity of this diagnostic system. It is noteworthy that DNA methylation was readily detectable in BLF specimens with no purification or capture of human genomic DNA. Thus, combination with other colorectal cancer detection methods that require bowel preparation, including sigmoidoscopy or CTC, would be a suitable application of the BLF methylation test. Further technical refinements, including easier bowel preparation, single-molecule detection of methylated DNA, and identification of better marker sets, would also enhance the practicality of this test.

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