Analysis of DNA methylation in bowel lavage fluid for detection of colorectal cancer

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Abstract

Objective: Aberrant DNA methylation could potentially serve as a biomarker for colorectal neoplasms. In the present study, we assessed the feasibility of using DNA methylation detected in bowel lavage fluid (BLF) for colorectal cancer (CRC) screening.

Design: A total of 508 BLF specimens were collected from patients with CRC (n = 56), advanced adenoma (n = 53) or 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.

Results: After analyzing BLF specimens in a training set (n = 345), we selected the three genes showing the greatest sensitivity for CRC detection (*miR-124-3*, 71.8%; *LOC386758*, 79.5%; *SFRP1*, 74.4%). A scoring system based on 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 clinicopathological features of the CRCs.

Conclusions: Our results demonstrate that DNA methylation in BLF specimens may be a useful biomarker for detection of CRC. BLF methylation tests could potentially improve the diagnostic performance of other screening methods, including the fecal occult blood test and computed tomographic colonoscopy.

Significance of this study

What is already known about this subject?

- Molecular stool analysis is considered to be a promising approach to noninvasive colorectal cancer (CRC) screening, although it requires further improvement.
- Aberrant DNA methylation is a common event in CRC, and methylation of many genes is a potentially useful marker for stool-based CRC screening.
- Methylation of DNA in mucosal wash fluid obtained during colonoscopy could be a useful biomarker predictive of CRC invasiveness.

What are the new findings?

- This is the first study to show that aberrant DNA methylation is detectable in bowel lavage fluid (BLF) obtained through oral bowel preparation.
- Sufficient bowel preparation enables sensitive detection of tumor-derived DNA methylation in BLF specimens.
- Aberrant methylation of tumor-related genes in BLF could be a potentially useful biomarker for CRC screening.
- Methylation of a panel of marker genes in BLF showed high sensitivity and specificity for detection of CRC.

How might this impact clinical practice in the foreseeable future?

- A BLF methylation test could complement other CRC screening methods, including the fecal occult blood test.
- Combination with other CRC screening methods that require bowel preparation, such as sigmoidoscopy and computed tomographic colonoscopy, would be the best clinical application of the BLF methylation test.

Introduction

Colorectal cancer (CRC) 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 CRC 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 CRC screening in Japan and Europe. However, the FOBT continues to have limitations, especially for detection of early stage CRCs. 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 CRC screening.

Fecal DNA tests are a noninvasive and potentially effective means of screening for both early colorectal lesions and advanced CRCs [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 CRCs. Because of its high frequency and the wide variety of affected genes, aberrant DNA methylation has emerged as a new biomarker for stool-based CRC screening. For instance, *SFRP2* methylation occurs in approximately 90% of primary CRCs [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, 11, 12, 13, 14, 15].

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 CRC screening. To test that idea, in the present 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 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. Prior to colonoscopy, patients were pretreated with 2 liters of polyethylene glycol lavage solution and 10 ml of BLF specimens were collected from the rectum at the beginning of the colonoscopy (Figure 1A). BLF samples were initially classified into 4 groups according to the Boston bowel preparation scale (BBPS) (Figure 1B) [17]. Then on the basis of colonoscopic and histological findings, the BLF samples were divided into 4 groups: CRC patients, advanced adenoma patients, minor polyp patients 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 CRC, 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 CRC patients. BLF and tissue specimens were suspended in ThinPrep PreservCyt solution (Hologic, Bedford, MA, USA) 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 CRC patients. 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, Hilden, Germany), 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, Foster City, CA, USA). 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 s at 95°C followed by 40 cycles of 3 s at 95°C and 30 s 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* are listed in Supplementary Table 1; those used for *APC* are described elsewhere [20].

Statistical analysis

Quantitative variables were analyzed using Student's t test and one-way analysis of variance (ANOVA) with a post hoc Tukey's test. Fisher's exact test and the χ^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 CRC were constructed on the basis of the methylation levels, followed by calculation of the area under the curve (AUC). The best cut-off 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:

(1) methylation status of marker genes in BLF was assessed; (2) the number of methylated genes was determined, which we termed the Methylation score (M-score); and (3) 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 SPSS Statistics 18 (IBM Corporation, Somers, NY, USA) and GraphPad Prism ver. 5.0.2 (GraphPad Software, La Jolla, CA, USA).

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 (Figure 1A). To determine the best time to obtain the BLF specimens, we scored the BLF samples using the BBPS (Figure 1B) [17]. Among the 268 BLF samples 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) (Figure 1C). However, MethyLight assays revealed that the endogenous control Alu element was detected at lower Ct values in BLF specimens with high BBPS scores than in those with residual stool (Figure 1C). This suggests 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 CRC patients, comparing the detectability of DNA methylation between specimens with lower and higher BBPS scores. As shown in Figure 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 (Figure 1D). For these reasons, we only used BLF specimens with BBPS scores of 2 or 3 for analysis.

Selection of marker genes for CRC detection

Our training set consisted of 355 BLF specimens obtained from patients with CRC (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 CRC (*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*). 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 CRC (Table 2, Supplementary Figure 1). Among the candidate marker genes analyzed, we found that *miR-124-3*, *LOC386758* and *SFRP1* were highly discriminative between patients with CRC and those without CRC (Table 2). The most discriminating PMR cut-offs for *miR124-3*, *LOC386758* and *SFRP1* were 11.1, 0.0003 and 1.1, while the most sensitive setting (PMR > 0) also achieved high sensitivity and specificity (Table 2).

To develop a more efficient diagnostic system for detection of CRC, 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 (Figure 2A). A ROC curve was then constructed to evaluate the ability of the scoring system to distinguish samples obtained from CRC patients by plotting the sensitivity over 1-specificity at each point (Figure 2B). We then validated the diagnostic system by analyzing an independent test set (Table 1, Figure 2A,B). AUCs in the training and test sets were 0.834 and 0.808, respectively, confirming the accuracy of our system for detecting CRC.

The association between the clinical characteristics and M-scores are summarized in Tables 3 and 4. Higher M-scores were significantly associated with CRC, but their association with advanced adenomas or minor polyps was limited (Figure 2C, Table 3). M-scores were not

significantly associated with tumor location, size or stage (Table 4). These results suggest the M-score system can detect CRCs, irrespective of the tumor's location, size or clinical stage.

Although the results summarized above demonstrate the clinical utility of BLF methylation for CRC screening, the system failed to detect 5 of the 56 CRC patients (Table 3). 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 CRC patients with different M-scores (score 3, n = 20; score 2, n = 10; score 1, n = 8; score 0, n = 3), and found that the majority of the tumors exhibited methylation of all 3 genes (*miR-124-3*, *LOC386758* and *SFRP1*), irrespective of the M-score (Supplementary Figure 2). MethyLight assays revealed that the Ct 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 (GI) tract cancers. Among the individuals enrolled in this study were 294 who underwent upper GI endoscopy; of those, 21 were found to have a gastric cancer (GC). BLF methylation was detected in 12 of the 21 GC patients, 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; score 3, n = 3). Five of the 8 GC patients 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 GC patients with high M-scores (> 2) were also found to have CRCs, while the remaining 2 patients

showed no colorectal lesions. These results suggest that BLF methylation could be a potential biomarker of upper GI cancers, but its sensitivity is far less than for CRCs.

BLF methylation and fecal occult blood test

FIT was performed in 349 of the study participants, including 17 CRC patients (Table 5). Most of the CRC patients were positive on the FIT (14 of 17), while a significant number of CRC-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 CRC patients, all the CRC patients were detected using the M-score system (Table 5). In the FIT-positive group, most of the CRC patients (12 of 14) exhibited BLF methylation (M-score $\geq$ 1), while a majority of the BLF methylation-negative subjects were CRC-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 CRC [21], we examined the feasibility of using BLF methylation testing to complement the diagnostic performance of CTC. Among the subjects enrolled in this study, 9, including 5 CRC patients, were examined using CTC (Table 6). CTC detected 4 CRCs, while all 5 CRC patients were positive for BLF methylation (M-score > 2). Notably, one 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 polyploid

component, so the lesion was diagnosed as a minor polyp (Table 6, Supplementary Figure 3). Although the number of patients in this study is limited, our results suggest that the BLF methylation could improve the diagnostic performance of CTC.

Discussion

Numerous studies have shown that aberrant methylation of DNA in the stool is a promising biomarker suitable for noninvasive CRC 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 the present study, we demonstrated that aberrant DNA methylation is detectable in the wash fluid of oral bowel lavage collected from the rectum of CRC patients. Earlier studies showed that methylation of DNA in body fluids, including pancreatic juice [22], saliva [23] and gastric juice [24], 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 CRC. 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 the present study, we tested a set of genes known to be frequently methylated in CRC, and selected the three genes with the highest sensitivities for detection of CRC (*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 CRC and GC [25, 26]. *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 CRC, GC and esophageal cancer [27, 28, 29]. *LOC386758* is a frequent

target of aberrant methylation newly identified in our recent epigenome analysis in CRC, though its function remains unknown (manuscript in preparation). Although BLF methylation of each of these genes could be used to detect CRC 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 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 CRCs 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 previously showed that many of the 15 genes analyzed in this study are frequently methylated in colorectal premalignant lesions [30, 31]. 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 previously showed 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 the present 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 Ct (threshold cycle) values for Alu elements with

MethyLight, which is indicative of the relative abundance of human genomic DNA (Supplementary Figure 1). These results suggest that successful detection of BLF methylation is highly dependent on the amount of tumor-derived DNA in the BLF specimens.

Our findings also suggest that BLF methylation could be used to complement current CRC 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 CRC, even among FIT-negative individuals.

As compared to 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 CRCs is equivalent to colonoscopy, its ability to detect small or flat lesions is more limited [32, 33, 34]. 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 CRCs using CTC. In the present study, we compared BLF

methylation with CTC findings in nine individuals, including 5 CRC patients. Using CTC, 4 of the CRCs were successfully detected, but a flat type cancer was diagnosed as a minor polyp. By contrast, BLF methylation (M-score > 2) was detected in all 5 CRC patients. Further analysis to test the diagnostic performance of this combination is therefore warranted.

In sum, our results demonstrate the feasibility of using aberrant DNA methylation in BLF specimens for noninvasive CRC 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 CRC 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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Competing interest

None.

Patient consent

Obtained.

Ethics approval

This study was approved by the institutional review boards of all participating institutions.

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Tables

Table 1. Clinicopathological features of the subjects in this study

	Training set (N=355)	Test set (N=153)	Total (N=508)
Demographics			
Median age, years (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*			
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**			
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

*Advanced adenomas were defined as adenomas 1 cm or greater in diameter, and/or containing villous components, and/or with high-grade dysplasia.

**Minor polyps were defined as adenomas other than advanced adenomas.

Table 2. Results of ROC curve analysis for detection of CRC using BLF methylation

Gene name	AUC estimate (95% CI)	Cutoff (PMR)	Sensitivity (95% CI)	Specificity (95% CI)	Cutoff (PMR)	Sensitivity (95% CI)	Specificity (95% CI)
miR-124-3	0.812 (0.722-0.901)	11.1	0.667 (0.498-0.809)	0.940 (0.908-0.963)	>0	0.718 (0.498-0.809)	0.835 (0.908-0.963)
LOC386758	0.767 (0.688-0.847)	0.0003	0.795 (0.635-0.907)	0.734 (0.682-0.782)	>0	0.795 (0.635-0.907)	0.734 (0.682-0.782)
SFRP1	0.722 (0.635-0.809)	1.1	0.718 (0.551-0.850)	0.709 (0.655-0.758)	>0	0.744 (0.579-0.870)	0.752 (0.600-0.704)
miR-137	0.694 (0.604-0.783)	0.2679	0.667 (0.498-0.809)	0.731 (0.679-0.780)	>0	0.667 (0.498-0.809)	0.728 (0.675-0.776)
miR-34b/c	0.646 (0.548-0.744)	0.4080	0.513 (0.348-0.676)	0.782 (0.732-0.826)	>0	0.513 (0.348-0.676)	0.779 (0.729-0.823)
SFRP2	0.642 (0.547-0.736)	0.2388	0.513 (0.348-0.676)	0.807 (0.759-0.849)	>0	0.513 (0.348-0.676)	0.807 (0.759-0.849)
miR-9-1	0.622 (0.517-0.728)	0.02169	0.282 (0.150-0.449)	0.970 (0.939-0.983)	>0	0.282 (0.150-0.449)	0.965 (0.939-0.983)
miR-1-1	0.588 (0.506-0.670)	21.89	0.718 (0.551-0.850)	0.567 (0.510-0.622)	>0	0.718 (0.551-0.850)	0.551 (0.494-0.606)
APC	0.549 (0.452-0.646)	0.04522	0.385 (0.234-0.554)	0.731 (0.679-0.780)	>0	0.385 (0.234-0.554)	0.728 (0.675-0.776)
miR-9-3	0.538 (0.453-0.623)	2.558	0.744 (0.579-0.870)	0.462 (0.406-0.519)	>0	0.744 (0.579-0.870)	0.411 (0.357-0.468)

ROC, Receiver operating characteristic; AUC, area under the curve; CI, confidence interval.

Table 3. Correlation between colorectal lesions and BLF methylation

		M-score				
	Total N	0	1	2	3	p Value
Normal	190	102	44	20	24	
Minor polyp	209	115	62	20	12	
Advanced adenoma	53	23	15	8	7	
CRC	56	5	7	16	28	<0.001

p Values were calculated using the χ^2 test.

Table 4. Correlation between clinical features and BLF methylation in CRC

		M-score				
	Total N	0	1	2	3	p Value
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

p Values were calculated using the χ^2 test.

Table 5. Diagnostic performance of the FIT and BLF methylation test for detection of CRC

FIT only

Study	Total N	CRC	CRC-free	Sensitivity	Specificity	PPV	NPV
Allison et al.	7493	32	7461	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

FIT-negative group

M-score	Total N	CRC	CRC-free	Cut-off	Sensitivity	Specificity	PPV	NPV	p Value
0	102	0	102						
1	54	0	54	≥1	1.000	0.537	0.000	1.000	0.103
2	25	1	24	≥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

FIT-positive group

M-score	Total N	CRC	CRC-free	Cut-off	Sensitivity	Specificity	PPV	NPV	p Value
0	82	2	80						
1	43	5	38	≥1	0.857	0.563	0.162	0.976	0.004
2	12	5	7	≥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

PPV, positive predictive value; NPV, negative predictive value.

p Values were calculated using Fisher's exact test.

Table 6. Comparison of CTC and BLF methylation test

Case	Colonoscopic finding	Location	Size (mm)	Histological 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

Figure legends

Figure 1

Collection of bowel lavage fluid (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 Boston bowel preparation scale (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 CRC patients.

Figure 2

Diagnostic system for detecting CRC using BLF methylation. (A) Workflow of a system established based on the ability to distinguish CRC patients from CRC-free individuals. Results of the training set are shown on the left; those of the test set are on the right. A BLF methylation score (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) Receiver operating characteristic curve analysis of the training and test sets. The area under the curve (AUC) is shown in the graphs. (C) Percentages of CRC patients in the respective M-score groups.

Supplementary figure legends

Supplementary Figure 1

Receiver operating characteristic curve analysis of the respective marker genes.

Supplementary Figure 2

Comparison of the methylation results in paired BLF and biopsy specimens from primary tumors. The M-score and the Ct values of the endogenous control Alu repetitive element are indicated on the top. The methylation status of the three marker genes in the BLF and biopsy specimens is shown below.

Supplementary Figure 3

CTC and colonoscopic views of the CRC of case 5 in Table 6 are shown. The polypoid adenoma portion is indicated by the red arrow and the flat adenocarcinoma portion is indicated by the yellow arrow.