

Analysis of Promoter Methylation in Stool: A Novel Method for the Detection of Colorectal Cancer

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Background & Aims: Detection of tumor-derived DNA alterations in stool is an intriguing new approach with high potential for the noninvasive detection of colorectal cancer (CRC). Because of heterogeneity of tumors, usually multiple markers distributed throughout the human genome need to be analyzed. This is labor intensive and does not allow for high through-put screening. Therefore, markers with high sensitivity and good specificity are needed. We explored the potential of a single epigenetic marker in comparison with fecal occult blood testing (FOBT) for the discrimination of patients with CRCs and adenomas from those without. **Methods:** Methylation-specific polymerase chain reaction (PCR) was performed to analyze hypermethylated in cancer 1 (HIC1) promoter methylation status in a blinded fashion in stool samples from 26 patients with CRC, 13 with adenoma ≥ 1 cm, 9 with hyperplastic polyps, 9 with chronic inflammatory bowel disease, and 32 with endoscopically normal colon. **Results:** Ninety-seven percent of the stool samples contained amplifiable DNA. Forty-two percent of the samples from patients with CRC and 31% of the samples from patients with colorectal adenoma ≥ 1 cm were positive for HIC1 promoter methylation. No methylated HIC1 promoter DNA was detected in the fecal DNA from patients with endoscopically normal colon or hyperplastic polyps. **Conclusions:** The epigenetic marker HIC1 promoter methylation carries high potential for the remote detection of CRCs. We postulate that a panel of merely a few genetic and epigenetic markers will be required for the highly sensitive and specific detection of CRCs and adenomas in fecal samples from affected patients.

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality.¹ About 40% of patients die within 5 years of being diagnosed. This mainly is attributable to late presentation with locally advanced or metastatic disease in one third of cases, precluding curative surgery.² The majority of CRCs follow the adenoma-carcinoma sequence, requiring a time period of usually more than 10 years.³ Detection

of early disease and precancerous adenomatous lesions leads to a decrease of CRC-related mortality.⁴⁻⁷ Therefore, there is a strong rationale for screening programs. Sigmoidoscopy, colonoscopy, and fecal occult blood test (FOBT) alone or in combination with endoscopy, are recommended for screening of the average-risk population.⁸ However, FOBT, when applied regularly, can decrease CRC mortality by only 15%–33%.⁵⁻⁷ Compliance with endoscopic screening is not satisfactory. Overall, in 1998, only 37% of eligible adults in the United States had been screened for CRC in the previous 3 years.⁹

Detection of tumor-derived genetic changes in stool is a promising new approach for CRC screening. Studies published so far focus on the detection of mutations in oncogenes,¹⁰⁻¹⁶ and tumor-suppressor genes,¹⁷⁻¹⁹ as well as changes in microsatellite markers.²⁰ Two recent studies required the analysis of up to 15 different genetic markers²¹ or 144 aliquots from each stool¹⁸ for the detection of 91% and 61% of cancers and 82% and 50% of adenomas ≥ 1 cm, respectively. This precludes their application for routine high throughput screening owing to high labor intensity and costs. The identification of new markers with high sensitivities and specificities is needed. So far, little attention has been paid to DNA hypermethylation as a potential stool marker for CRC.

Methylation of CpG islands of promoters leads to silencing of transcription of the affected gene.^{22,23} Methylation-specific polymerase chain reaction (PCR) (MSP)²⁴ has been used successfully to detect DNA methylation in primary tumors and in various body fluids.²⁵ The feasibility of amplification of methylated

Abbreviations used in this paper: CI, confidence interval; CRC, colorectal cancer; FOBT, fecal occult blood test; HIC1, hypermethylated in cancer 1; MSP, methylation-specific PCR; PCR, polymerase chain reaction.

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Table 1. Clinicopathologic Data and HIC1 Methylation Status of Patients With CRC

No.	Sex	Age (y)	UICC stage	Localization	Symptoms ^a	HIC1 methylation	FOBT
1	M	71	IV	Sigmoid colon/rectum	1	—	—
2	F	75	III	Sigmoid colon	1,3	—	—
3	M	66	IV	Descending/sigmoid colon	0	—	—
4	M	69	II	Ascending colon	0	+	—
5	M	65	III	Transverse colon	0	+	+
6	M	56	IV	Rectum	1	+	—
7	M	63	IV	Ascending colon	2	—	—
8	F	72	III	Cecum	2	+	+
9	M	74	III	Sigmoid colon/rectum	0	—	+
10	M	68	IV	Rectum	3	+	+
11	M	66	IV	Rectum	1	—	+
12	M	75	III	Cecum	0	—	—
13	M	71	IV	Sigmoid colon	0	+	—
14	M	67	II	Hepatic flexure	3	+	—
15	F	72	IV	Sigmoid colon	1,2	+	—
16	M	59	II	Rectum	0	+	—
17	M	59	II	Rectum	0	—	+
18	M	53	III	Descending colon	0	—	—
19	F	55	IV	Rectum	2	—	+
20	F	61	IV	Sigmoid colon	2,3	—	—
21	M	61	IV	Rectum	0	—	—
22	M	80	II	Transverse colon	2	+	—
23	F	53	IV	Ascending colon	1,3	—	—
24	M	63	I	Cecum	4	NA	+
25	M	76	III	Transverse colon	2	—	+
26	F	60	IV	Ascending colon	1	+	—

UICC, International Union Against Cancer; M, male; F, female; NA, no amplification.

^a0, no symptoms; 1, change in bowel habits; 2, blood admixed with stool; 3, abdominal pain; 4, anemia.

DNA from stool samples of patients with CRC has been reported.^{26,27} In one study evaluating several potentially methylated genes, SFRP2, SFRP5, and PGR were found to be methylated differentially in the stool of patients with CRC.²⁷ Of these markers, SFRP2 was found to be the most sensitive fecal methylation marker, detecting 77%–90% of CRCs. However, specificity of SFRP2 methylation was quite poor, at 77%. Therefore, identification of both sensitive and highly specific methylation markers is required before MSP can be evaluated as a stool-based screening procedure for CRC in prospective studies.

We studied the potential of hypermethylated in cancer 1 (HIC1) promoter methylation as a stool-based DNA marker. The promoter of HIC1, a candidate tumor-suppressor gene localized on 17p13.3 and the first gene cloned based on the finding of CpG island hypermethylation in cancer,^{28,29} frequently is methylated in CRC,³⁰ but not in normal or aging colonic tissue.³¹ We show that HIC1 promoter methylation can be detected frequently and with high specificity in stool samples from patients with CRCs. The combination of HIC1 methylation analysis with FOBT allowed for the detection of two thirds of CRCs.

Materials and Methods

Patients and Stool Samples

The investigation was approved by the ethical committee of the Medical Faculty of the University of Munich. Stool samples were collected preoperatively from patients with verified CRCs (Table 1), and before endoscopy from patients with adenomas ≥ 1 cm (Table 2), endoscopically normal colons, hyperplastic polyps, and chronic inflammatory bowel disease (Table 3). Most patients with CRCs had been referred with a known diagnosis, all persons with adenomas and normal colons were asymptomatic and underwent colonoscopies for surveillance reasons.

Stool samples were collected before cathartic preparation for scheduled surgery or colonoscopy. A period of at least 12 days was respected between previous biopsy examinations of colorectal tumors before stool sampling. Samples were received within 10 hours after defecation at the laboratory, aliquoted, and stored at -80°C .

Isolation of DNA

Stool samples were thawed on ice and DNA was isolated using the QiaAmp DNA Stool Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, DNA was isolated from stool samples of approximately 200 mg, and purified DNA was eluted from the column in 50 μL of deionized water. The total yield of

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Table 2. Clinicopathologic Data and HIC1 Methylation Status of Patients With Adenomatous Polyps

No.	Sex	Age (y)	Diagnosis	HIC1 methylation	FOBT
27	M	72	Tubular adenoma, ascending colon, 3 cm	+	—
28	F	63	Tubulovillous adenoma, ascending colon, hepatic flexure, sigmoid colon, 1–1.5 cm	—	—
29	M	63	Tubulovillous adenoma, ascending colon, 2 cm	—	—
30	M	76	Tubulovillous adenoma, ascending and transverse colon, 1, 3, 4 cm	+	+
31	M	83	Tubulovillous adenoma, transverse colon, 3 cm	—	—
32	F	65	Tubulovillous adenoma, sigmoid colon, 1.5 cm	—	—
33	M	72	Tubulovillous adenoma, ascending colon, 1 cm	—	—
34	F	60	Tubular adenoma, sigmoid colon, 1.5 cm	—	—
35	M	69	Tubular adenoma, 1.5 cm, ascending colon	—	—
36	M	70	Tubulovillous adenoma, sigmoid colon, 1 cm	—	—
37	M	64	Tubulovillous adenoma, rectum, 1 cm	—	—
38	M	72	Adenoma, cecum, 2 cm	+	—
39	F	63	Tubulovillous adenoma, sigmoid colon, 2 cm	+	—

M, male; F, female.

DNA was determined by ultraviolet absorbance at 260 nm; the range of DNA concentrations was 120–300 ng/μL. Leukocyte DNA was extracted with the Qia-Amp Blood Mini Kit (Qiagen) according to the manufacturer's recommendations. Microdissected paraffin-embedded sections of normal colon and CRCs were treated with lysis buffer containing .5% Tween 20 (Sigma, Taufkirchen, Germany), 20 μg proteinase K (Sigma), 50 mmol/L trizma base at pH 8.9, and 2 mmol/L ethylenediaminetetraacetic acid. After overnight incubation at 56°C, proteinase K was heat inactivated. Samples were subjected to phenol:chloroform/chloroform DNA extraction twice, chloroform extraction, and ethanol precipitation. Precipitated DNA was dissolved in Tris-ethylenediaminetetraacetic acid buffer and stored at 4°C.

Methylation-Specific Polymerase Chain Reaction

MSP is based on a 2-step process.²⁴ First, DNA is treated with sodium bisulfite, which efficiently converts unmethylated cytosine to uracil but leaves methylated cytosines unchanged. Consequently, after treatment, methylated and unmethylated alleles have different sequences. Second, allele-specific primers allow for the specific amplification of methylated and unmethylated alleles.

A total of 2 μg DNA from peripheral blood leukocytes, microdissected colonic tissues, and stool was diluted to 50 μL of distilled water. Then 2 μg of salmon sperm DNA was added as a carrier to stool and tissue DNA. DNA was denatured by .2 mol/L NaOH for 15 minutes at 37°C. A total of 30 μL of 10 mmol/L hydroquinone (Sigma) and 520 μL of 3 mol/L sodium bisulfite (Sigma) at pH 5 were added, and the samples were incubated for 16 hours at 50°C. After bisulfite treatment, DNA was purified using the Wizard DNA Clean-up System (Promega, Mannheim, Germany), following the manufacturer's protocol, and incubated for 5 minutes at room temperature with .3 mol/L NaOH. DNA was ethanol-precipitated and resuspended in 50 μL of deionized water and stored at –80°C for up to 8 weeks.

Primers were designed to discriminate between methylated and unmethylated alleles. Primers were designed to hybridize to the CpG-rich region of the HIC1 promoter 1b (Figure 1A), which is affected by methylation in carcinomas.³¹ All PCR reactions were performed in a blinded fashion. The person performing the reactions was not aware of the diagnosis of the patients. A 2-step nested PCR was established to improve sensitivity. The methylated and unmethylated primer pair sequences for the first-step PCR were as follows: 5'-TTAGG-TATTGAAGTTGTGAAGTTGT-3' (unmethylated forward), 5'-ACCATAACAACCAATAAAATACACC-3' (unmethylated reverse), 5'-GTTAGGTATCGAAGTCGTGAAGTC-3' (methylated forward), and 5'-TTACCATAACAACCGATA-AAATACG-3' (methylated reverse). The second-step PCR was performed with the following primers: 5'-GAAGTTGT-GAAGTTGTTTGTGTAG-3' (unmethylated forward), 5'-ACTTAACAACCACACTCACCA-3' (unmethylated reverse), 5'-GAAGTCGTGAAGTCGTTTGCCTAG-3' (methylated forward), and 5'-ACTTAACAACCGCGCTCGCCG-3' (methylated reverse). The sizes of the first- and second-round PCR products were 231 and 181 bp, respectively.

Both PCRs were performed in 20 μL reaction volumes containing 1 × PCR buffer (Qiagen), 4 mmol/L MgCl₂, 250 μmol/L deoxynucleotide triphosphate mixture (Invitrogen), 1 μmol/L of each primer, 1 × Q-Solution (Qiagen), 2 μL bisulfite-treated DNA, and 1.7 units taq DNA polymerase (Hot Star Taq; Qiagen). Thermocycler conditions for the first-step PCR were as follows: 95°C for 15 minutes (95°C for 30 s; 60°C for unmethylated and 56°C for methylated primers for 30 s; 72°C for 30 s) for 20 cycles followed by a final extension at 72°C for 10 minutes. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and one tenth of the elution volume was subjected to the second-step PCR. Here the cycling conditions were as follows: 95°C for 15 minutes (95°C for 30 s; 54°C for unmethylated and 64°C for methylated primers for 30 s; 72°C for 30 s) for 40 cycles followed by a final extension at 72°C for 10 minutes. PCR products were analyzed by 3% agarose gel electro-

Table 3. HIC1 Methylation Status of Healthy Controls and Patients With Nonneoplastic Disease

No.	Sex	Age	Diagnosis	HIC1 methylation	FOBT
40	F	61	Normal	—	+
41	F	61	Normal	—	—
42	F	73	Normal	—	—
43	M	63	Normal	—	—
44	M	47	Normal	—	—
45	F	41	Normal	—	—
46	M	53	Normal	—	—
47	F	59	Normal	—	+
48	F	73	Normal	—	—
49	M	36	Normal	—	—
50	M	71	Normal	—	—
51	F	65	Normal	—	—
52	F	60	Normal	—	—
53	F	66	Normal	—	—
54	M	80	Normal	—	—
55	M	70	Normal	—	—
56	F	27	Normal	—	—
57	F	57	Normal	—	—
58	M	55	Normal	—	ND
59	M	23	Normal	—	—
60	F	73	Normal	—	—
61	F	79	Normal	—	—
62	M	68	Normal	—	—
63	F	78	Normal	—	—
64	F	38	Normal	—	—
65	F	62	Normal	—	—
66	M	76	Normal	—	—
67	F	61	Normal	—	—
68	M	23	Normal	NA	—
69	M	74	Normal	—	—
70	M	60	Normal	—	—
71	F	49	Normal	—	—
72	M	59	Hyperplastic polyp	—	—
73	F	66	Hyperplastic polyp	—	—
74	F	67	Hyperplastic polyp	—	—
75	F	65	Hyperplastic polyp	—	—
76	F	58	Hyperplastic polyp	NA	—
77	F	76	Hyperplastic polyp	—	—
78	F	54	Hyperplastic polyp	—	—
79	F	76	Hyperplastic polyp	—	—
80	F	60	Hyperplastic polyp	—	—
81	M	47	Ulcerative colitis	+	+
82	M	64	Ulcerative colitis	—	+
83	M	27	Ulcerative colitis	—	—
84	M	67	Ulcerative colitis	—	+
85	M	53	Ulcerative colitis	—	—
86	M	51	Ulcerative colitis	—	—
87	F	44	Crohn's disease	—	—
88	M	30	Crohn's disease	—	—
89	M	37	Crohn's disease	—	—

F, female; M, male; ND, not determined; NA, no amplification.

phoresis and ethidium bromide staining followed by visualization with ultraviolet illumination using a digital imaging system (Intas, Göttingen, Germany). The first-step MSP followed by the second-step MSP independently was performed twice for each sample. For the sensitivity assay, human leukocyte DNA was treated with the CpG methylase

SssI (New England Biolabs, Frankfurt, Germany). Indicated genome equivalents³² corresponding to the DNA content of 1 to 1000 human cells were spiked into normal stool DNA and the nested PCR was performed.

Fecal Occult Blood Testing

All stool samples were subjected to FOBT (Beckman Coulter, Fullerton, CA) immediately on receipt and before freezing. One drop of the peroxide catalyst was added to the reverse side of each window of the test cards 1 day after applying stool samples. A blue color reaction within 60 seconds was considered a positive result.

Statistical Analysis

Sensitivity and specificity were estimated relative to the results of colonoscopy in the usual manner. Exact binomial estimate was used to calculate 95% confidence intervals (CIs) for the estimated parameters.³³ *P* values were calculated with the Fisher exact test. Calculations were performed using SISA online statistics package (<http://home.clara.net/sisa/binomial.htm>; Daan G. Uitenbroek, Hilversum, The Netherlands).

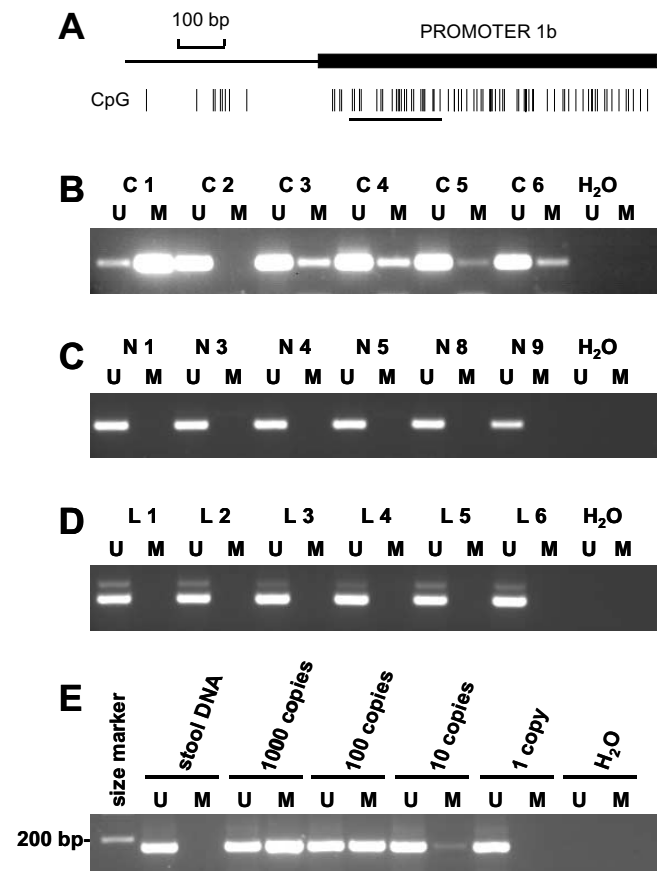


Figure 1. (A) Location of the amplified region (underlined) of the HIC1 promoter by MSP. (B) Detection of unmethylated (u) and methylated (m) HIC1 promoter DNA in primary CRCs, (C) normal colonic epithelium, and (D) human peripheral blood leukocytes. (E) Analysis of sensitivity of the HIC1 MSP assay. Indicated genome equivalents of fully methylated DNA were spiked into normal stool DNA.

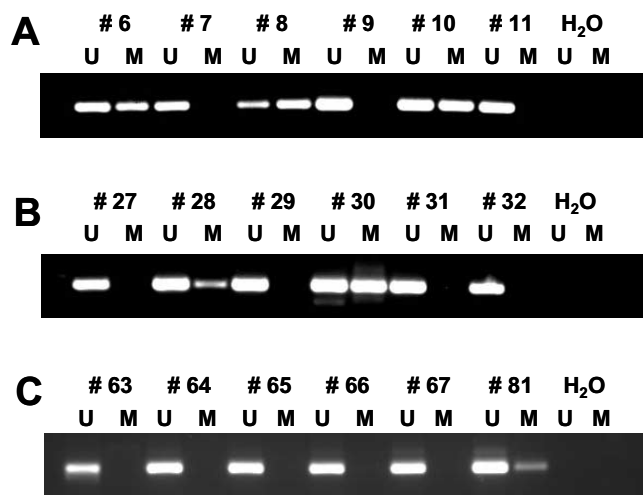


Figure 2. Detection of unmethylated (u) and methylated (m) HIC1 promoter DNA in stool samples from patients with (A) CRC, (B) adenoma, and (C) normal controls.

Results

Establishment of Hypermethylated in Cancer 1 Promoter Methylation Analysis and Determination of the Sensitivity of the Assay

DNA was extracted from microdissected tissues from primary CRCs, microdissected samples from normal colonic epithelium from individual patients, and peripheral blood leukocytes from healthy individuals. A nested MSP amplifying a CpG island within the HIC1 promoter 1b was established (Figure 1A). We found 7 of 9 cancers (78%) to contain both methylated and unmethylated and 2 cancers to contain unmethylated HIC1 DNA only (Figure 1B and data not shown). Furthermore, none of 8 samples from normal colonic epithelium containing analyzable DNA after bisulfite conversion revealed any detectable methylated HIC1 promoter DNA (Figure 1C and data not shown). In accordance with previously reported data,³⁰ we found that HIC1 pro-

moter methylation is frequent in primary CRCs and absent in healthy colorectal epithelium. Because stool of a significant fraction of patients with CRC and nonmalignant disease contains overt or occult blood we analyzed the HIC1 methylation status of peripheral blood leukocytes from 10 healthy individuals. MSP revealed that none of the 10 leukocyte samples contained any detectable methylated HIC1 DNA (Figure 1D and data not shown). Based on these data we were prompted to analyze the potential of HIC1 promoter methylation analysis as a stool-based marker for CRC detection.

To determine the sensitivity of the assay to detect tumor-derived methylated DNA in the presence of a surplus of normal stool DNA, 1–1000 genome equivalents of SssI-treated fully methylated leukocyte DNA were diluted into stool DNA from a healthy individual. MSP revealed that the equivalent of methylated HIC1 DNA from 10 cells could be detected with the assay (Figure 1E). This shows the high sensitivity of the assay because the vast majority of DNA is degraded during bisulfite conversion.³⁴ Tumors shedding less methylated DNA into the lumen of the colon would not be detected by this assay.

Analysis of Hypermethylated in Cancer 1 Promoter Methylation in Stool DNA

DNA was extracted and purified from small amounts of stool from 26 patients diagnosed with CRC before surgery (Table 1) and of 63 patients before colonoscopy (32 with confirmed healthy colons, 13 with adenomas >1 cm, 9 with hyperplastic polyps, and 9 with chronic inflammatory bowel disease; Tables 2 and 3). Amplification of the unmethylated allele, which was expected for all samples, was positive in 97% of cases and was used as a control for amplifiable DNA after bisulfite conversion. Five of 13 patients with cancers of the rectum or sigmoid colon tested positive for HIC1 methylation (sensitivity 39%; 95% CI, 14%–68%) (Figure 2A

Table 4. Positivity Rates of the Fecal DNA Assay, FOBT, and Combination of the Tests

	HIC1		FOBT		Combined	
	n (%)	95% CI	n (%)	95% CI	n (%)	95% CI
All cancers	11/26 (42)	23%–63%	9/26 (35)	17%–56%	17/26 (65)	44%–83%
Localized cancers	4/6 (67)	22%–96%	2/6 (33)	4%–77%	6/6 (100)	12%–188% ^a
Advanced cancers	5/20 (25)	9%–49%	7/20 (35)	15%–59%	11/20 (55)	32%–77%
Rectum and sigmoid colon	5/13 (39)	14%–68%	5/13 (39)	14%–68%	9/13 (69)	39%–91%
From descending colon to cecum	6/13 (46)	19%–75%	4/13 (31)	14%–68%	8/13 (62)	39%–91%
Asymptomatic cases	4/10 (40)	12%–74%	3/10 (30)	7%–65%	6/10 (60)	26%–88%
Symptomatic cases	7/16 (44)	20%–70%	6/16 (38)	15%–65%	11/16 (69)	41%–89%
Adenomas	4/13 (31)	9%–61%	1/13 (8)	.2%–36%	4/13 (31)	9%–61%

^aEstimated by normal approximation of Poisson distribution.

and Table 4). Sensitivity for the 13 cancers of the cecum, and ascending, transverse, and descending colon was 46% (95% CI, 19%–75%) (Figure 2A and Table 4). Altogether the assay detected 42% (95% CI, 23%–63%) of all CRCs. Interestingly, the sensitivity was marginally higher for localized (International Union Against Cancer stages I and II) than advanced disease (67% vs. 25%, $P = .084$). Next to detecting CRCs, a stool-based screening assay also should detect relevant premalignant lesions (ie, adenoma). Thirteen stool samples from patients with adenomatous lesions ≥ 1 cm were collected before polypectomy. Four of the 13 patients (31%) with adenoma tested positive for methylated HIC1 DNA (Figure 2B, and Table 4).

To test for specificity, stool samples from 32 patients with normal colonoscopy results, 9 patients with hyperplastic polyps, and 9 patients with a known history of chronic inflammatory bowel disease were analyzed by MSP. One stool sample from a patient with ulcerative colitis who did not reveal any malignant process of the colon on colonoscopy contained methylated HIC1 DNA. All other samples from normal controls, and patients with hyperplastic polyps or chronic inflammatory bowel disease, tested negative for methylated HIC1 DNA (Figure 2C, and Table 3). Altogether only 1 of 50 samples (2%) from patients lacking neoplastic disease of the colon was positive, resulting in a specificity of 98% (95% CI, 92%–100%).

HIC1 MSP was compared with the best evaluated stool-based screening test to date: FOBT. Sensitivity and specificity of FOBT found in this study is comparable with published data.³⁵ The HIC1 assay was only slightly more sensitive for all cancers (42% vs. 35%; $P = .19$; Table 4). However, although there was no difference between both tests in detecting rectal and sigmoid cancers, symptomatic or asymptomatic cases ($P = .66$, $P = .26$, and $P = .33$, respectively), the HIC1 assay detected more localized cancers (67% vs. 25%, $P = .085$) and more adenomas (31% vs. 8%, $P = .14$). In addition, specificity of the HIC1 assay was higher (97.9% vs. 89.9%, $P = .102$). The combination of both assays resulted in increased detection rates for CRCs (65%; 95% CI, 44%–83%). Although the combined test detected all localized cancers, no increase in the sensitivity for adenoma was seen.

Discussion

Highly sensitive, specific, and easily analyzable markers are required for noninvasive stool-based CRC screening. We show here that DNA methylation can be detected in stool of CRC patients with high specificity.

The use of the DNA methylation marker, HIC1 promoter methylation, allowed for the highly specific detection of CRCs in 42% and of adenomas ≥ 1 cm in 31% of cases. Interestingly, sensitivity was quite high for early cancers and higher for adenoma than FOBT. The combination of HIC1 MSP with FOBT resulted in the detection of almost two thirds of CRCs.

DNA methylation was first described in primary tumors. More recently, tumor-derived methylated DNA has been detected in various remote media, including serum, urine, and sputum.²⁵ In 30%–70% of cases with methylation of a specific marker in the primary colorectal tumor, the same methylated marker also could be detected in serum.^{36–38} However, to date, methylation markers have not been assessed thoroughly in stool. Our data suggest that we detected at least 50% of cases expected to be methylated at the HIC1 locus, because methylation of HIC1 has been reported to occur in 60%–80% of CRCs.²⁸ Nonetheless, in at least 25% of expected cases with HIC1 methylation no methylation was detected by our assay. Because only small amounts of stool were used for DNA extraction and 84%–96% of DNA is lost during bisulfite conversion owing to degradation,³⁷ it is conceivable that tumors shedding only small amounts of DNA into the lumen of the colon will not be detected. The use of larger stool samples might increase sensitivity because only a fraction of the stool DNA is of human origin. It has been reported that the feces of healthy individuals contains 200–500 ng and the feces of CRC patients contains 1500–2000 ng human DNA per mg of stool.³⁹ The amount and quality of extracted and converted DNA is most likely also the reason for the failure to amplify an unmethylated product in 3 cases. Although our nested PCR allowed for the amplification of stool DNA in 97% of cases, another study using a single-step fluorescent PCR approach failed to amplify DNA after bisulfite conversion in more than 40% of cases.²⁷

DNA methylation of promoters has been shown to be an early event in colorectal carcinogenesis already occurring in aberrant crypt foci.⁴⁰ Promoters of some genes are affected more frequently by methylation than others.^{40–42} The reason for this currently is not very well understood. Promoters of several genes, including the estrogen receptor and insulin-like growth factor II, have been reported to be methylated in both CRCs and in aging colon,^{31,43} rendering these promoters unattractive markers for screening owing to low specificity for CRC. So far, no HIC1 promoter methylation has been found in the colon mucosa of healthy controls³¹ and our analysis of samples from normal colon did not reveal any HIC1

promoter methylation. One recent study proposed SFRP2 hypermethylation as a marker for stool-based detection of CRC.²⁷ However, this marker has low specificity for CRC. Although SFRP2 methylation was detected in 77%–90% of CRCs with amplifiable DNA, 23% of colonoscopically healthy controls revealed methylation of this locus as well. This low specificity is most likely owing to the SFRP2 methylation that occurs frequently in premalignant aberrant crypt foci.⁴⁴ In one study, 13 of 15 of these lesions revealed hypermethylation of SFRP2. Because aberrant crypt foci are not detected by routine colonoscopy and their progression rate to adenoma and CRC is not known, their detection by a screening assay does not seem to be favorable. Moreover, K-ras mutation, the best evaluated molecular stool marker to date, has a sensitivity for CRC detection in stool samples of 40%–60% of cases.^{10–16} But K-ras mutations also have been found in the stool of controls with normal colons. In one study, K-ras mutations were detected in 7% of healthy individuals.²⁰ Self-limiting hyperplastic polyps, nondysplastic aberrant crypt foci, and morphologically normal colonic mucosa,^{45–47} which do not progress to cancer, frequently contain K-ras mutations. This shows that methylation markers as well as genetic markers need to be selected carefully to achieve both high sensitivity and specificity. Choosing sensitive markers that already are positive in a premalignant situation will compromise specificity severely. In addition, low specificity of a test results in a low positive predictive value and consecutively in high rates of unnecessarily performed colonoscopies.

One of the stool samples from a patient with ulcerative colitis who did not reveal any detectable colonic malignancy contained methylated HIC1 DNA. The reason for this is not entirely clear. However, aberrant methylation of several promoters has been reported in normal and dysplastic colonic epithelium of patients with longstanding and extensive ulcerative colitis^{48,49} as well as in ulcerative colitis-associated CRCs.^{50,51} Alternatively, the patient could have an as yet unknown disease or malignant process somewhere else throughout the gastrointestinal tract, shedding methylated HIC1 DNA into the lumen, or a pathologic finding was overseen by the colonoscopy.

Although methylated DNA of several markers has been found in hyperplastic polyps from patients with multiple hyperplastic polyps as well as in patients with hyperplastic polyposis, no DNA methylation has been described in sporadic hyperplastic polyps.^{52,53} In accordance, none of the stools analyzed from patients with single hyperplastic polyps revealed methylated HIC1

DNA. Nonetheless, detection of hyperplastic polyps in patients with hyperplastic polyposis would be favorable because these lesions have been found to be associated with CRC.⁵³ Altogether, of the 50 stool samples from controls with either endoscopically normal colons, hyperplastic polyps, or inflammatory bowel disease, only one was MSP positive. This specificity of 98% emphasizes the value of HIC1 promoter methylation as a sensitive and specific marker for stool-based CRC screening.

In summary, this study shows HIC1 as a sensitive and specific marker well suited for stool-based CRC screening. Combination of HIC1 with a few additional sensitive and specific methylation markers may allow for the highly sensitive and specific stool-based detection of CRCs and adenomas. Evaluation of other genes known to be methylated in CRC such as hMLH1⁵⁴ and HPP1,⁵⁵ identification of other useful methylation markers, prospective screening studies, as well as further simplifications and automatization of the assays used, clearly are indicated.

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