Improved Fecal DNA Test for Colorectal Cancer Screening

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Background & Aims: **Fecal DNA testing has shown greater sensitivity than guaiac-based occult blood tests for noninvasive colorectal cancer (CRC) screening. The prototype assay (version 1), which analyzed 22 gene mutations and DNA integrity assay (DIA), showed a sensitivity of 52% for CRC detection and a specificity of 94% in average-risk individuals. The present study was conducted to determine the sensitivity and specificity of a second-generation assay (version 2) that uses improved DNA stabilization/isolation techniques and a new promoter methylation marker.** ***Methods:*** **Forty patients with CRC and 122 subjects with normal colonoscopy provided stool samples to which DNA preservation buffer was added immediately. DNA was purified using gel-based capture, and analyzed for the original panel of 22 mutations, DIA, and 2 new promoter methylation markers.** ***Results:*** **By using DNA that was optimally preserved and purified from stool, the sensitivity of the prototype version 1 assay increased to 72.5% because of enhanced performance of DIA. Vimentin gene methylation alone provided sensitivity and specificity of 72.5% and 86.9%, respectively. The optimal combination of vimentin methylation plus DIA resulted in 87.5% sensitivity and 82% specificity; cancers were detected regardless of stage or location. False-positive vimentin methylation was associated with older age.** ***Conclusions:*** **An improved fecal DNA test that incorporates only 2 markers shows much higher sensitivity for CRC. The new assay is easier to perform and should be less costly, thereby facilitating its use for noninvasive CRC screening.**

Screening for colorectal cancer (CRC) is arguably the most effective intervention for preventing any cancer. Unfortunately, despite the recommendations of all major medical societies, fewer than half of eligible individuals older than age 50 have undergone CRC screening.[1–3] In the United States, colonoscopy is being used increasingly as a primary screening tool because of its excellent diagnostic accuracy and ability to remove precancerous and early cancerous lesions. However, the availability of an accurate, noninvasive screening test might increase compliance with CRC screening guidelines by individuals who are reluctant to undergo more invasive tests, or situations in which colonoscopy screening is not feasible or readily available.

Several studies have shown the feasibility of detecting colon tumor–specific products in the stool.[4] The markers in these *K-ras*), the microsatellite instability pathway (*Bat-26*), and markers of abnormal apoptosis. Studies using stool samples from patients already known to have colon cancer, adenomas, or a normal colon report sensitivities of 62%–91% for CRC, 27%–82% for advanced adenomas, and specificities of 93%–96% in individuals with a normal colonoscopy.[4,5] These encouraging data prompted a large, prospective, multicenter study in more than 4000 average-risk, asymptomatic individuals older than age 50. The results showed a higher sensitivity for detecting cancer with the fecal DNA test compared with Hemoccult II (Beckman Coulter, Fullerton, CA) (51.6% vs 12.9%, $P = .003$), with comparable specificity (94.4% vs 95.2%, respectively).[6] Despite superior sensitivity over Hemoccult II, the prototype fecal DNA test (version 1) revealed lower than expected sensitivity, which was owing to an unexpectedly low rate of positivity for the DNA integrity assay (DIA) component. In retrospect, it was learned that the suboptimal performance of DIA was a result of DNA degradation during transit of specimens to the laboratory, despite precautions such as immediate chilling of samples and rapid delivery by express courier.

Since that time, pilot studies have shown that several technical and conceptual advances could improve fecal DNA testing. First, adding a DNA-stabilizing buffer to the stool immediately on defecation was shown to prevent DNA degradation for several days and enhance the performance of DIA.[7] Second, a gel-based DNA capture approach, rather than the original bead-based technology, allowed for enhanced extraction of DNA from stool.[8] Finally, promoter methylation has become recognized as a key pathway by which colon cancers develop.[9] This epigenetic alteration is not detected by approaches that analyze for gene mutations. Vimentin, a gene that typically is considered a product of mesenchymal cells, is not methylated in normal colonic epithelial cells, but becomes highly methylated in colon cancer cell lines and in 53%–83% of colon cancer tissues.[10] Vimentin methylation also has been detected in the stool from 43 of 94 (46%; 95% confidence interval [CI], 36%–56%) patients with CRC vs 20 of 198 (specificity, 90%; 95% confidence interval [CI], 85%–94%) with a normal colonoscopy,[10] suggesting that methylation markers might contribute to a fecal DNA assay panel.

Abbreviations used in this paper: CI, confidence interval; CRC, colorectal cancer; DIA, DNA integrity assay; DY, locus D (5p21) and locus Y (LOC91199); HLTF, Helicase-like Transcription Factor; MSP, methylation-specific polymerase chain reaction; NC, normal colonoscopy; PCR, polymerase chain reaction.

These improvements of better DNA stabilization, enhanced DNA extraction, and use of gene-specific methylation have been incorporated into a second-generation fecal DNA test (version 2). The purpose of the present study was to determine the sensitivity and specificity of the newer version 2 assay for detection of CRC.

Methods

Study Design

This study was designed in 2 phases. Phase 1 involved analyzing stool samples from approximately 50 patients with CRC and 200 patients with normal colonoscopy (NC) to define suitable DIA cut-off values and to determine optimal markers for the new assay. Phase 2, which is ongoing, was designed as a validation set in which an additional 125 patients with CRC and 200 patients with NC will be analyzed using the optimal marker panel from phase 1. Without knowing the performance of the new assay, we decided to analyze specimens from phase 1 after 45 CRC and 150 NC patients were enrolled, which had a negligible effect on the initial estimations for setting cut-off points for the DIA assay. The findings presented herein represent the results of phase 1.

Source of Clinical Material

Seven centers participated in this study, representing a spectrum of academic medical settings (community based to tertiary care). Each center obtained local institutional review board approval before beginning the study. The number of patients contributed by each site varied depending on when institutional review board approval was obtained, with a mean number of 24 stool samples per site (range, 8–42). Between January and September 2005, subjects who were 50–80 years of age were eligible for the study if they were found at the time of colonoscopy to have either CRC or NC. The latter group consisted of individuals in whom the bowel preparation was classified as very good to excellent, the colonoscopy was complete to the cecum, and the mucosa was free of any type of mucosal lesion or polyps. Although they were younger than age 50, 4 subjects (3 CRC, 1 NC) between the ages of 44 and 50 were included because they fulfilled all other eligibility criteria. Individuals were excluded if any of the following conditions applied: any contraindication to colonoscopy or conscious sedation; personal history of, or coexistent, cancer except basal and squamous cell carcinomas of the skin; active therapy with chemotherapy or radiation therapy for a concurrent cancer; high-risk conditions such as familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, inflammatory bowel disease, and strong family history of CRC (2 or more first-degree relatives with CRC, or 1 or more first-degree relatives with CRC younger than age 50), personal history of colorectal polyps, prior colorectal resection for any reason, current pregnancy, or lactation. The presence of gastrointestinal symptoms was not an exclusion criterion, although patients with NC were almost all asymptomatic and presented for routine screening. The preparation for, and performance of, colonoscopy was performed according to standard operating procedures at each site. The histologic diagnosis of CRC was verified by a board-certified pathologist. Cancers were staged according to the

Sample Collection

To avoid any possible effect of the colonoscopic bowel preparation on test results, each subject provided a single stool sample approximately 6–14 days after colonoscopy. In the case of patients with CRC, the sample was provided before beginning the presurgical bowel preparation. Subjects were given detailed instructions and a special stool collection kit that is mounted on the toilet bowl. Immediately after defecation, subjects added 250 mL of a DNA-stabilizing buffer[7] to a stool specimen of at least 50 g. Only 10 patients provided less than 50 g of stool, and, of these, 3 subsequently provided an adequate second specimen. The specimen was shipped at room temperature overnight using a coded identifier provided by an external clinical research organization (Carestat Inc., Newton, MA) to keep the laboratory blinded to the clinical source. The clinical research organization was responsible for maintaining all of the clinical data files. The collection interval was defined as the number of hours from the time of defecation until the specimen arrived in the laboratory. Stool samples were processed and analyzed without knowledge of clinical information. The details of sample processing and human DNA purification have been described previously.[7]

Version 1 Assay

Samples were processed for 22 specific mutations according to Whitney et al[8] using a gel-based DNA capture approach (Effipure; Exact Sciences Corporation, Marlborough, MA) with the following modifications: (1) DNA amplifications were increased to 60 cycles; (2) single base extension reactions included internal controls, that is, 0.5-umol/L internal control primers and 25 ng (mutant reactions) or 5 ng (wild-type reactions); (3) acyclopol enzyme was increased to 0.027 U/reaction; and (4) extension reactions were treated with 0.1 uL of shrimp alkaline phosphatase (SAP; Promega, Madison, WI) at 37°C for 30 minutes before analysis by capillary electrophoresis (Applied Biosystems 3100 instrument; Applied Biosystems, Foster City, CA).

DNA Integrity Assay

The DIA was performed using real-time polymerase chain reaction (PCR) as described previously.[8] The assay was converted to a multiplex format in which 4 primer/probe pairs simultaneously interrogated the presence and quantity of 200-, 1300-, 1800-, and 2400-bp human DNA fragments at 4 loci: 5p21 (locus D), 17p13 (locus E), HRMT1L1 (locus X), and LOC91199 (locus Y).

Methylation Assay

Stool samples were processed for vimentin and Helicase-like Transcription Factor (HLTF) analysis according to Whiney et al[8] by using the following capture sequences: vimentin (Vimcp50a: 5′- GGCCAGCGAGAAGTCCACCGAGTCCTGCAGGAGCCGC -3′; Vimcp29b: 5′- GAGCGAGAGTGGCAGAGGACTGGACCCCGCCGAGG -3′), and HLTF (methylation-specific polymerase chain reaction [MSP]5cp: 5′-CAAATGAACCTGACCTTCCCGGCGTTCCTCTGCGTTC-3′). Bisulfite conversion of DNA was performed as previously described.[11,12] MSP PCR reactions were performed using 0.5-umol/L armed primers for either

Table 1. Comparison of CRC Detection for Stool Samples Obtained Precolonoscopy and Postcolonoscopy (Version 1 Assay)

	Precolonoscopy[6]		Postcolonoscopy	
	No. positive/total	% (95% CI)	No. positive/total	% (95% CI)
Total	16/31	51.6 (34.8–68.0)	20/47	42.6 (29.5–56.7)
Stage I	8/15	53.3 (30.1–75.2)	7/16	43.8 (23.1–66.8)
Stage II	5/8	62.5 (30.6–86.3)	2/10	20.0 (5.7–51.0)
Stage III	3/8	37.5 (13.7–69.4)	7/11	63.6 (35.4–84.8)
Stage IV	0	—	3/7	42.9 (15.8–75.0)
Unknown	—	—	1/3	33.3 (6.2–79.2)

fied HLTF MSP-5 methylation-specific forward primers 5′-GACGTCTAACTAAACTCGCGA-3′ and reverse primers 5′-TTTTAGGTCGTTAGATCGAGC-3′ were extended by a 5′ tag sequence 5′-GCGGTCCCAATAGGGTCAGT-3′, which is not derived from the HLTF sequence, but which allows for more robust sequence-specific template amplification. Vimentin MSP-29 primer sequences have been reported previously.[10] Primers were combined with 1× HotStar buffer, 1.25 U HotStar polymerase (Qiagen, Alameda, CA), 200 μmol/L deoxynucleoside triphosphate (Promega), and 10 μL (capture stool) DNA in a final volume of 50 μL. Cycling conditions were 95°C for 14.5 minutes followed by 40 cycles of 94°C for 30 seconds, 57°C (HLTF), 68°C (vimentin methylated) or 62°C (vimentin unmethylated) for 1 minute, 72°C for 1 minute, with final 72°C for 5 minutes. Samples were visualized on 4% NuSieve 3:1 agarose (FMC, Rockland, ME) gels using a Stratagene EagleEye II (Stratagene, La Jolla, CA) still-image system. Samples were scored as positive if the PCR band intensity exceeded a previously determined level. Positive samples were repeated in duplicate to confirm methylation status.

Patient Satisfaction Questionnaire

Individuals submitting a stool sample were asked to complete a brief, 6-item satisfaction questionnaire designed by the authors to assess satisfaction with the new stool collection kit. The questionnaire was made available to all co-investigators, who then distributed it to participants without tracking. Completed questionnaires were mailed back anonymously without identifiers, so it was not possible to determine the response rate.

Data Analysis

Descriptive statistics were used to characterize the data. The sensitivities and specificities with 95% confidence intervals were computed for all markers. *t* tests and χ^2 tests comparing the CRC with the NC group were used to examine associations between patient characteristics (eg, sex, age, time since colonoscopy) or markers. *P* values less than .05 were considered significant. SPSS (version 14; SPSS, Chicago, IL) was used for all analyses.

Results

Rationale for Postcolonoscopy Stool Samples

In the previous multicenter study using version 1, precolonoscopy stool samples were collected from an asymptomatic average-risk population undergoing screening colonoscopy. Subsequently, we also participated in a parallel study to collect postcolonoscopy stool samples from patients with CRC. Both sets of samples were processed in a blinded fashion alongside 1423 CRC-negative samples using the version 1 assay. At that time, stools were collected without DNA stabilization buffer and DNA was extracted by a bead-capture technique. As shown in Table 1, CRC detection for the precolonoscopy and postcolonoscopy stool groups was 51.6% (95% CI, 34.8%–68.0%) and 42.6% (95% CI, 29.5%–56.7%), respectively (not statistically significant). There was also no significant difference in detection frequency according to tumor stage, although the number of patients was small. If anything, postcolonoscopy stool samples showed a lower sensitivity, suggesting that such samples may underestimate the assay's sensitivity. Thus, tumor manipulation or other potential factors at the time of colonoscopy do not appear to bias in favor of molecular CRC detection, suggesting that analysis of postcolonoscopy stool samples is adequate for estimating the performance of a fecal DNA test.

Patient Population

Initially, 45 patients in the CRC group and 150 subjects in the NC group were enrolled. Of the patients in the CRC group, 5 were excluded because of age younger than 40 years (n = 2), carcinoma in situ (n = 1), history of colitis (n = 1), and the finding of an adenoma instead of cancer (n = 1). Of the

Table 2. Demographic Characteristics of the Study Population

	Colon cancer (N = 40)	NC (N = 122)
Male, N (%)	24 (60)	62 (51)
Mean collection interval, h (±SD)	38.9 (21.6)	27.4 (5.2)
Days since colonoscopy (mean ± SD)	12.4 (6.2)	10.2 (5.5)
Age, *y* (mean ± SD)	65.6 (10.3)	58.5 (7.2)[a]
Stage I	71.0 (7.4)	N/A
Stage II	67.1 (10.6)	N/A
Stage III	64.9 (10.0)	N/A
Stage IV	56.8 (11.3)	N/A
Stage of cancer, N (%)		
I	8 (20)	N/A
II	10 (25)	N/A
III	17 (43)	N/A
IV	5 (12)	N/A
Location of cancer, N (%)		
Left-sided	29 (73)	N/A

Table 3. Sensitivity and Specificity of Version 1 Assay

	Imperiale et al[6]		Present study[a]	
	No. positive/total	% (95% CI)	No. positive/total	% (95% CI)
Sensitivity				
All version 1 markers	16/31	51.6 (34.8–68.0)	29/40	72.5 (57.2–83.9)
MuMu22	16/31	51.6 (34.8–68.0)	17/40	42.5 (28.5–57.8)
DIA	1/31	3.2 (0.6–16.2)	26/40	65.0 (49.5–77.9)[b]
Specificity				
All version 1 markers	79/1423	94.4 (93.1–95.5)	13/122	89.3 (82.6–93.7)
MuMu22	65/1423	95.4 (94.2–96.4)	5/122	95.9 (90.8–98.2)
DIA	18/1423	98.7 (98.0–99.9)	10/122	91.8 (85.6–95.5)

[a]Using stabilization buffer + gel capture (postcolonoscopy stool samples).
[b]$P < .0001$.

subjects in the NC group, 28 were excluded because of inadequate colonoscopy preparation (n = 13), insufficient stool sample (n = 7), strong family history of CRC (n = 2), personal history of polyps (n = 3), polyp found on colonoscopy (n = 2), and patient withdrawal (n = 1).

Table 2 lists the demographic characteristics of the 40 CRC and 122 NC subjects studied. There were no significant differences between the 2 groups in terms of sex, collection interval, or number of days after colonoscopy that the stool sample was collected. The NC group was younger than the CRC group ($P = .03$). Among those with CRC, there was no difference in mean age according to cancer stage. Almost half of all cancers were early stage (I and II), and approximately three quarters of the CRCs were left-sided.

Version 1 Marker Performance Using Optimized Sample Collection and Purification Techniques

The same version 1 markers used in the previous multicenter study[6] were analyzed after the stool was collected using improved sample collection with DNA stabilization buffer[7] and a gel-based DNA purification method.[8] As shown in Table 3, the sensitivity of all version 1 markers was increased using the newer collection and purification methods from 51.6% (95% CI, 34.8%–68.0%) to 72.5% (95% CI, 57.2%–83.9%; not statistically significant). The sensitivity of DIA when using buffer with gel-based purification was increased markedly from 3.2% (95% CI, 0.6%–16.2%) to 65% (95% CI, 49.5%–77.9%) ($P < .0001$). Specificity was not affected significantly by the newer collection and purification methods.

Performance of the DNA Integrity Assay

The DIA was analyzed according to a previously determined requirement that 4 of 12 PCR fragments, excluding the 200-bp fragments, must be greater than the individual fragment thresholds for a sample to be positive. Additional analyses involved all combinations of fragments, including the 200-bp fragment, to determine the optimal combination for maximum sensitivity and specificity. All combinations of DIA markers were similar with regard to sensitivity (60%–65%), with speci-

Table 4. Sensitivity and Specificity of DIA Combinations

	Sensitivity (N = 40)		Specificity (N = 122)	
	No. positive	% (95% CI)	No. positive	% (95% CI)
DEXY without 200 bp	26	65.0 (49.5–77.9)	10	91.8 (85.6–95.5)
DEXY with 200 bp	25	62.5 (47.0–75.8)	6	95.1 (89.7–97.8)
DEX	24	60.0 (44.6–73.7)	13	89.3 (82.6–93.7)
DEY	25	62.5 (47.0–75.8)	12	90.2 (83.6–94.3)
DXY	25	62.5 (47.0–75.8)	10	91.8 (85.6–95.5)
EXY	26	65.0 (49.5–77.9)	15	87.7 (80.7–92.4)
DE	25	62.5 (47.0–75.8)	11	91.0 (84.6–94.9)
DX	26	65.0 (49.5–77.9)	14	88.5 (81.7–93.0)
DY	**26**	**65.0 (49.5–77.9)**	**9**	**92.6 (86.6–96.1)**
EX	24	60.0 (44.6–73.7)	11	91.0 (84.6–94.9)
EY	24	60.0 (44.6–73.7)	10	91.8 (85.6–95.5)
XY	26	65.0 (49.5–77.9)	10	91.8 (85.6–95.5)
D	25	62.5 (47.0–75.8)	12	90.2 (83.6–94.3)
E	24	60.0 (44.6–73.7)	8	93.4 (87.6–96.6)
X	25	62.5 (47.0–75.8)	11	91.0 (84.6–94.9)
Y	24	60.0 (44.6–73.7)	8	93.4 (87.6–96.6)

NOTE. DIA loci are D (5p21), E (17p13), X (HRMT1L1), and Y (LOC91199). DIA analysis was performed with and without the 200-bp fragment

Table 5. Sensitivity and Specificity of Different Marker Combinations

	Sensitivity (n = 40)		Specificity (n = 122)	
	No. positive/total	% (95% CI)	No. positive/total	% (95% CI)
Methylation (total):	31	77.5 (62.5–87.7)	19	84.4 (77.0–89.8)
HLTF	15	37.5 (24.2–53.0)	9	92.6 (86.6–96.1)
Vimentin	29	72.5 (57.2–83.9)	16	86.9 (79.8–91.8)
DIA-DY (from Table 4)	26	65.0 (49.5–77.9)	9	92.6 (86.6–96.1)
Vimentin + DY[a]	35/40	87.5 (73.9–94.5)	22/122	82.0 (74.2–87.8)
Stage I	6/8	75.0 (40.9–92.8)	—	—
Stage II	9/10	90.0 (59.6–98.2)	—	—
Stage III	16/17	94.1 (73.0–99.0)	—	—
Stage IV	4/5	80.0 (37.6–96.4)	—	—

[a]Vimentin + DY was the optimal marker combination.

ficities ranging from 87.7% to 95.1% (Table 4). The DY combination had the highest overall sensitivity (65%; 95% CI, 49.5%–77.9%) and specificity (92.6%; 95% CI, 86.6%–96.1%).

Optimal Marker Combinations for Maximal Sensitivity and Specificity

Of the methylation markers, HLTF was 2-fold less sensitive than vimentin, and did not significantly improve overall methylation marker sensitivity and specificity when combined with vimentin (Table 5). Vimentin alone gave a sensitivity of 72.5% (95% CI, 57.2%–83.9%) and a specificity of 86.9% (95% CI, 79.8%–91.8%)—values that are nearly identical to the composite version 1 panel of MuMu22 plus DIA (see Table 3). Examples of vimentin methylation in normal and cancer specimens are shown in Figure 1. We explored marker combinations to determine which formulation would provide maximum sensitivity and specificity. As noted earlier, DIA-DY alone gave a sensitivity and specificity of 65% and 92.6%, respectively. The least complex assay consisted of hypermethylation of vimentin and DIA-DY (vim + DY), yielding a maximum sensitivity of 87.5% (95% CI, 73.9%–94.5%), with a specificity of 82.0% (95% CI, 74.2%–87.8%) (Table 5). Importantly, among the 40 cancers, vim+DY detected cancers regardless of stage.

Influence of Tumor Location and Patient Age on Marker Expression

There was a significant predilection for left-sided cancers to be DY positive (Table 6). However, vimentin detected cancers regardless of the location (Table 6). Thus, the combination of vim+DY detected cancers regardless of the location.

We observed that among individuals with NC, those with a positive methylation marker (either vimentin or HLTF) were significantly older than those with a negative test (Table 7). A similar trend was noted among the CRC patients. There were 22 subjects (18%) with false-positive vimentin and/or DY in the NC group (Table 5). Among these, 14 were positive for vimentin alone (specificity, 88.6%), 6 were positive for DIA alone (specificity, 95.1%), and 2 were positive for DIA and vimentin (specificity, 98.4%). The mean age of subjects in these groups was 65.1, 58.5, and 59 years, respectively, indi-

Patient Satisfaction

Forty-one percent of the respondents to the satisfaction questionnaire were men, and 40% were older than age 60. The percentage that found it easy or very easy to (1) perform the test, (2) open the preservative bottle, or (3) add the preservative to the specimen was 97%, 96%, and 100%, respectively, and 93% of respondents felt very comfortable performing the stool test. Importantly, 84% would repeat the test if recommended by their doctor.

Discussion

Screening is a cost-effective yet underused strategy for reducing CRC incidence and mortality. Concerns about test discomfort, invasiveness, embarrassment, and self-efficacy have been identified as important barriers to more effective screening.[14,15] The availability of a noninvasive screening test that is convenient, safe, and easy to perform at home without bowel preparation or dietary restriction has the potential to significantly increase participation. Prior studies clearly have shown that fecal DNA testing fulfills these criteria and has distinct advantages over existing screening strategies, including the fecal occult blood test.[16] In addition, because fecal DNA testing involves mailing of specimens, geographic access becomes less of a barrier, there is no loss of time from work, and no formal health care visit. Improved performance

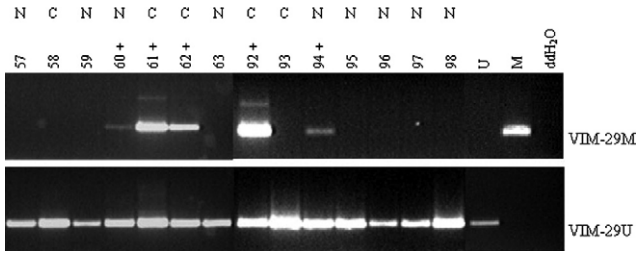

Figure 1. Detection of vimentin methylation in fecal DNA analyzed by VIM-29 PCR primers. Amplification of fecal DNA from 9 normal (N) and 5 colon cancer (C) patients. Upper panel (VIM-29M) shows vimentin gene methylation and the lower panel (VIM-29U) shows control wild-type amplification of unmethylated vimentin sequences derived from normal cells in all samples. Positive (+) vimentin methylation is noted next to the sample identification number. Assay controls include un-

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