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A Simplified, Noninvasive Stool DNA Test for Colorectal Cancer Detection

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BACKGROUND: As a noninvasive colorectal cancer (CRC) screening test, a multi-marker first generation stool DNA (sDNA V 1.0) test is superior to guaiac-based fecal occult blood tests. An improved sDNA assay (version 2), utilizing only two markers, hypermethylated *vimentin* gene (hV) and a two site DNA integrity assay (DY), demonstrated in a training set (phase 1a) an even higher sensitivity (88%) for CRC with a specificity of 82%.

AIM: To validate in an independent set of patients (phase 1b) the sensitivity and specificity of sDNA version 2 for CRC.

METHODS: Forty-two patients with CRC and 241 subjects with normal colonoscopy (NC) provided stool samples, to which they immediately added DNA stabilizing buffer, and mailed their specimen to the laboratory. DNA was purified using gel-based capture, and analyzed for hV and DY using methods identical to those previously published.

RESULTS: Using the same cutpoints as the 1a training set (N = 162; 40 CRCs, 122 normals), hV demonstrated a higher and DY a slightly lower sensitivity, for a combined sensitivity of hV + DY of 86%. Optimal cutpoints based on the combined phase 1a + 1b dataset (N = 445; 82 CRCs, 363 normals) yielded a CRC sensitivity of 83%. The vast majority of cancers were detected regardless of tumor stage, tumor location, or patient age. Assay specificity in the phase 1b dataset for hV, DY, and hV + DY was 82%, 85%, and 73%, respectively, using the phase 1a cutpoints. Optimal cutpoints based on the combined phase 1a + 1b dataset yield a specificity of 82%.

CONCLUSIONS: This study provides validation of a simplified, improved sDNA test that incorporates only two markers and that demonstrates high sensitivity (83%) and specificity (82%) for CRC. Test performance is highly reproducible in a large set of patients. The use of only two markers will make the test easier to perform, reduce the cost, and facilitate distribution to local laboratories.

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INTRODUCTION

Screening for colorectal cancer (CRC) is a highly effective intervention that substantially reduces cancer-specific mortality by detecting early stage CRC and premalignant lesions. Despite the recommendations of all major medical societies, fewer than 60% of eligible individuals over age 50 have undergone CRC screening (1–3). Although several CRC screening tests are available, colonoscopy is the most widely used as the primary screening tool because of its excellent diagnostic accuracy and ability to remove precancerous and early cancerous lesions. However, the invasive nature of the procedure itself, as well as the many physician, patient, and organizational barriers, limit its effectiveness. This has spawned efforts to develop an accurate noninvasive screening test that would increase adherence with CRC screening guidelines by individuals who are reluctant to undergo inva-

feasible. Indeed, the recently updated guidelines endorsed by the American Cancer Society, the U.S. Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology encourage all average risk individuals over age 50 to undergo CRC screening with either a structural or a noninvasive screening test (4).

Several studies have demonstrated the feasibility of extracting and detecting human DNA from stool (reviewed in Ref. 5). The DNA markers in these studies comprise mutations of genes involved in the predominant chromosomal instability pathway (such as *APC*, *p53*, and *K-ras*) and DNA alterations reflecting the microsatellite instability pathway (*Bat-26*) and abnormal apoptosis. Initial studies of stool DNA (sDNA) using stool samples from patients already determined by colonoscopy to have colon cancer, adenomas, or a normal colon, reported sensitivities of 62–91% for CRC, 27–82% for advanced adenomas, and specificities of 93–96% (5, 6). These encouraging data prompted a large, prospective, multi-center screening study of more than 4,000 average-risk, asymptomatic individuals over age 50. The results demonstrated fourfold greater sensitivity for detecting CRC with the sDNA test compared to Hemoccult II fecal occult blood test (51.6% *vs* 12.9%, $P = 0.003$), with comparable specificity (94.4% *vs* 95.2%) (7). Despite its superiority over Hemoccult II, the prototype sDNA test (version 1.0) exhibited lower than expected sensitivity, due to an unexpectedly low rate of positivity for the DNA integrity assay (DIA) component of the assay. Despite precautions such as immediate chilling of samples and rapid delivery to the laboratory by express courier, subsequent research demonstrated that the cause of the suboptimal performance of DIA was a result of DNA degradation during the transit of specimens to the laboratory (8).

Several technical and conceptual improvements have now been incorporated into a newer assay version (version 2). First, the addition of a DNA-stabilizing buffer to the stool immediately upon defecation prevents DNA degradation during transport for several days and enhances the performance of DIA (8). Second, a gel-based DNA capture approach, rather than the original bead-based technology, permits better extraction of DNA from stool (9). Third, a new marker, hypermethylated *vimentin* gene, has been included in the sDNA assay. The use of this new marker is based on the evidence that the epigenetic phenomenon of promoter methylation is a key pathway by which colon cancers develop (10). Vimentin protein is not normally expressed by colonic epithelial cells but is typically expressed by mesenchymal cells. The *vimentin* gene is minimally methylated in normal colonic epithelial cells, but was found to be highly methylated in colon cancer cell lines and in 53–83% of colon cancer tissues (11). In a recent study, hypermethylated *vimentin* was detected in the sDNA of 43/94 (46%) patients with CRC versus 20/198 (10%) with a normal colonoscopy (NC) (11).

To test the performance of the version 2 assay for CRC, we conducted a two-phase study. The study was designed to sDNA from CRC patients and those with NC. The first set (phase 1a) would be the training set; the second set (phase 1b) would be the validation set. The results of the training set (phase 1a) demonstrated a sensitivity of 88% for CRC, and a specificity of 82% (12). The purpose of the present study was to validate in an independent set of patients the performance of the version 2 assay with respect to the sensitivity and specificity for CRC.

METHODS

Source of Clinical Material

The same seven centers that participated in the phase 1a study also participated in the present phase 1b study. These centers represent a spectrum of academic medical settings (community based to tertiary care). Each center obtained local Institutional Review Board approval prior to beginning the study. Phase 1b was originally planned to include 125 new patients with CRC and 200 new subjects with NC. The quota of normal subjects was readily achieved, but CRC enrollment was slower than expected. Therefore, upon the advice of the statisticians from an independent contract research organization (Battelle CRO, Needham, MA), phase 1b was terminated with the enrollment of 50 CRC patients.

Between June 2005 and February 2006, subjects 50–80 yr of age were eligible for the study if they were found at the time of colonoscopy to have either CRC, or a NC. The latter group consisted of individuals in whom the bowel preparation (prep) was classified as very good to excellent (deemed adequate to exclude polyps >5mm), the colonoscopy was complete to the cecum, and the mucosa was free of any type of mucosal lesion or polyps. We offered entry to consecutive presenting patients with CRC at each site. 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 CRC, inflammatory bowel disease, and strong family history of CRC (two or more first degree relatives with CRC, or one or more first-degree relatives with CRC younger than age 50), personal history of colorectal adenomas or CRC, prior colorectal resection for any reason, current pregnancy or lactation. Gastrointestinal symptoms were not an exclusion criterion and were reported by 66/82 (80.5%) subjects with CRC, but only 40/363 (11%) subjects with NC. The preparation for, and performance of, colonoscopy was done 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 TNM (Tumor-Nodes-Metastasis) classification. Left-sided cancers were defined as those arising at, or

Sample Collection

To avoid any possible effect of the colonoscopic bowel prep or any biopsies done at the time of exam on test results, each subject was asked to provide a single stool sample approximately 6–14 days after colonoscopy. For patients with CRC, the sample was provided prior to beginning the presurgical bowel prep. Subjects were given a special stool collection kit that mounts on the toilet bowl along with detailed instructions. Immediately following defecation, subjects added 250 mL of a DNA stabilizing buffer (8) to the passed stool. Acceptable specimens were at least 35 g, with no upper limit of quantity. The specimen was shipped overnight at room temperature using a coded identifier to keep the laboratory blinded to the clinical source. 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 (12).

DNA Integrity Assay

The DIA assay was performed using real-time PCR as described previously (12). The assay has been converted to a multiplex format where four primer/probe pairs simultaneously interrogate the presence and quantity of 200, 1300, 1800, and 2400 bp human DNA fragments at two loci: 5p21 (Locus D); LOC91199 (Locus Y). For the four different-sized fragments at each of these two loci, a numerical cutpoint was determined based on the genome equivalents amplified for each fragment, above which the assay was considered positive for that fragment size. Any sample that returned values above the established cutoffs for at least three of the eight fragments was considered a positive (abnormal) DIA-DY test.

Vimentin Methylation Assay

Bisulfite conversion of DNA was performed as previously described (13). Methylation specific-PCR (MS-PCR) reactions were performed using 0.5 μM armed primers for *vimentin* (IDT, Coralville, IA), 1X HotStar buffer, 1.25 U HotStar polymerase (Qiagen, Alameda, CA), 200 μM dNTP (Promega, Madison, WI), and 10 μL (capture stool) DNA in a final volume of 50 μL. Cycling conditions were 95°C for 14.5 min followed by 40 cycles of 94°C for 30 s, 68°C (*vimentin* methylated) or 62°C (*vimentin* unmethylated) for 1 min, 72°C for 1 min with final 72°C for 5 min. 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 positive (hypermethylated *vimentin* present) if MS-PCR band intensity exceeded a previously determined level. Positive samples were repeated in duplicate to confirm methylation status. Primer sequences are available on request.

Data Analysis

To ensure adequate blinding of data, Battelle CRO maintained had been completed and scored. Descriptive statistics were used to characterize the data. Positivity for each marker (hV and DY) is reported separately. The sDNA test, which combines both markers, is considered positive if either hV or DY is positive. Sensitivity and specificity with 95% confidence intervals (CI) were computed for all markers. The *t*-tests and χ^2 tests, comparing the CRC to the NC group, were used to examine associations between patient characteristics (*e.g.*, gender, age, time since colonoscopy) or markers. *P* values less than 0.05 were considered significant. SPSS (version 14) (SPSS, Inc., Chicago, IL) was used for all analyses.

RESULTS

Patient Population

Initially, 51 patients with CRC and 248 subjects in the NC group were enrolled. Of the patients in the CRC group, nine were excluded: three because of a positive family history or personal history of CRC, and six because the cancer was interpreted as high-grade dysplasia (HGD) and not invasive cancer. Of the subjects in the NC group, seven were excluded because no collection date was indicated (N = 1), there was inadequate stool weight (N = 2), or because of a previous history of cancer (one CRC, one breast, one leukemia, one larynx). Five subjects with CRC between ages 42 and 50 were included because they fulfilled all other eligibility criteria. Thus, the phase 1b subject set consisted of 283 patients (42 CRC, 241 NC).

Table 1 lists the demographic characteristics of the subjects studied. There were no significant differences between the two groups in terms of gender or collection interval. The number of days between colonoscopy and stool sample collection was longer in the CRC group, perhaps owing to factors related to patients adjusting to their new diagnosis. The NC group was younger than the CRC group. Among those with CRC, there was no difference in mean age according to cancer stage. Almost two-thirds of all cancers were early stage (I and II), and two-thirds of CRCs were located distal to the splenic flexure.

Assay Sensitivity

The same markers used in the phase 1a study were analyzed using the identical sample collection kit, DNA stabilization buffer, and gel-based DNA purification. Table 2 shows the sensitivity and specificity of the markers in the phase 1a study, phase 1b study, and the combined dataset. Using cutpoint values derived from phase 1a, the sensitivity of hV as a single marker in phase 1b was 81% (95% CI = 66.7–90.0%), higher than the value found in the phase 1a study. The sensitivity of DY was 60% (95% CI = 44.5–73.0%), slightly lower than phase 1a results. Combining both markers yielded a sensitivity of 86% (95% CI = 72.2–93.3%), a value almost identical to the phase 1a result of 88%. Optimal cutpoints based on the combined phase 1a + 1b dataset yielded a sensitivity for hV + DY of 83% (95% CI = 73.4–89.5%). The positive

Table 1. Demographic Characteristics of the Study Population (Phase 1b)

	Colorectal Cancer (N = 42)	Normal Colonoscopy (N = 241)	*P* value
Male, N (%)	15 (35.7%)	90 (37.3%)	0.841
Collection interval, h (mean ± SD)	30.31 ±7.43	28.18 ±7.38	0.091
Time since c'scopy, days (mean ± SD)	16.07 ±10.95	10.68 ±2.92	0.003
Age, yr (mean ± SD)	67.44 ±11.21	56.90 ±6.33	0.0
Stage I	69.12 ±10.91	N/A	
Stage II	68.11 ±12.66	N/A	
Stage III	65.33 ±11.16	N/A	
Stage IV	67.93 ±9.00	N/A	
Stage of cancer, n (%)			
Stage I	11 (26.2%)	N/A	
Stage II	14 (33.3%)	N/A	
Stage III	14 (33.3%)	N/A	
Stage IV	3 (7.1%)	N/A	
Size of cancer, cm (mean ± SD)			
Stage I	3.40 ±1.23	N/A	
Stage II	3.81 ± 2.11	N/A	
Stage III	3.77 ± 1.76	N/A	
Stage IV	7.33 ± 5.80	N/A	
Location of cancer, n (%)			
Distal	28 (66.7%)	N/A	
Proximal	14 (33.3%)	N/A	

Six (14%) of the 42 CRC cases were not associated with detectable hV or DY. There were no apparent distinguishable clinicopathological features of these few tumors. The mean age of this group was 70.7 yr; four were stage III, two were stage II, and four were in the proximal colon.

Assay Specificity

Among the stool specimens from 241 subjects with normal colonoscopies in phase 1b, 18% contained hV and 15% demonstrated abnormal apoptosis by the DY assay, giving single marker specificities of 82% and 85%, respectively (Table 2). Combining both markers gave a specificity of 73% (95% CI = 67.1–78.2%). When the phase 1a and 1b datasets were combined, optimal cutpoints based on this more robust dataset (363 NC's) yielded specificities of 83%, 96%, and 82% for hV, DY, and hV + DY, respectively. The 95% CI of the specificity yielded by the phase 1a cutpoints applied to the 1a dataset, the 1a cutpoints applied to the 1b dataset and the cutpoints newly determined from the combined 1a + 1b dataset all overlap. The latter cutpoints were identical to the 1a cutpoint for hV but were adjusted with respect to DY to capture more fully the biologic variability represented by the larger combined NC data. The negative likelihood ratio for the combined dataset was 0.21.

The relative ability of cutpoints to separate the means of Gaussian distributions of a population of subjects with CRC from that of a population of subjects with normal colonoscopies is illustrated in Figure 1. The phase 1a training set cutpoint on the 1a dataset and the 1a + 1b cutpoint on the 1a + 1b dataset both cluster near 2 sigma of separation, with the 1a training set cutpoint on the 1b dataset somewhat below. The latter performance reflects the lowered specificity seen with the DY marker in the larger population of the 1b dataset (85%) compared to that of the smaller 1a dataset (93%). The 95% CI of the sensitivity and specificity generated by each of these three cutpoints on the three datasets overlap (Table 2).

Influence of Tumor Stage on Marker Expression

In phase 1b, hV was associated with the vast majority of cancers regardless of tumor stage (Table 3). A positive DY assay, however, was associated more frequently with earlier

Table 2. Sensitivity and Specificity of Version 2 Assay

	hV	DY	hV + DY [95% CI]
Phase 1b: (1a cutpoints)			
Sensitivity (N = 42 CRC)	81% [66.7–90.0%]	60% [44.5–73.0%]	86% [72.2–93.3%]
Specificity (N = 241 NL)	82% [76.4–86.1%]	85% [80.0–89.0%]	73% [67.1–78.2%]
Phase 1a: (Ref. 12)			
Sensitivity (N = 40 CRC)	73% [57.2–83.9%]	65% [49.5–77.9%]	88% [73.9–94.5%]
Specificity (N = 122 NL)	87% [79.8–91.8%]	93% [86.6–96.1%]	82% [74.2–87.8%]
Combined 1a and 1b dataset			
Sensitivity (N = 82 CRC)	77% [66.6–84.6%]	48% [37.1–58.2%]	83% [73.4–89.5%]

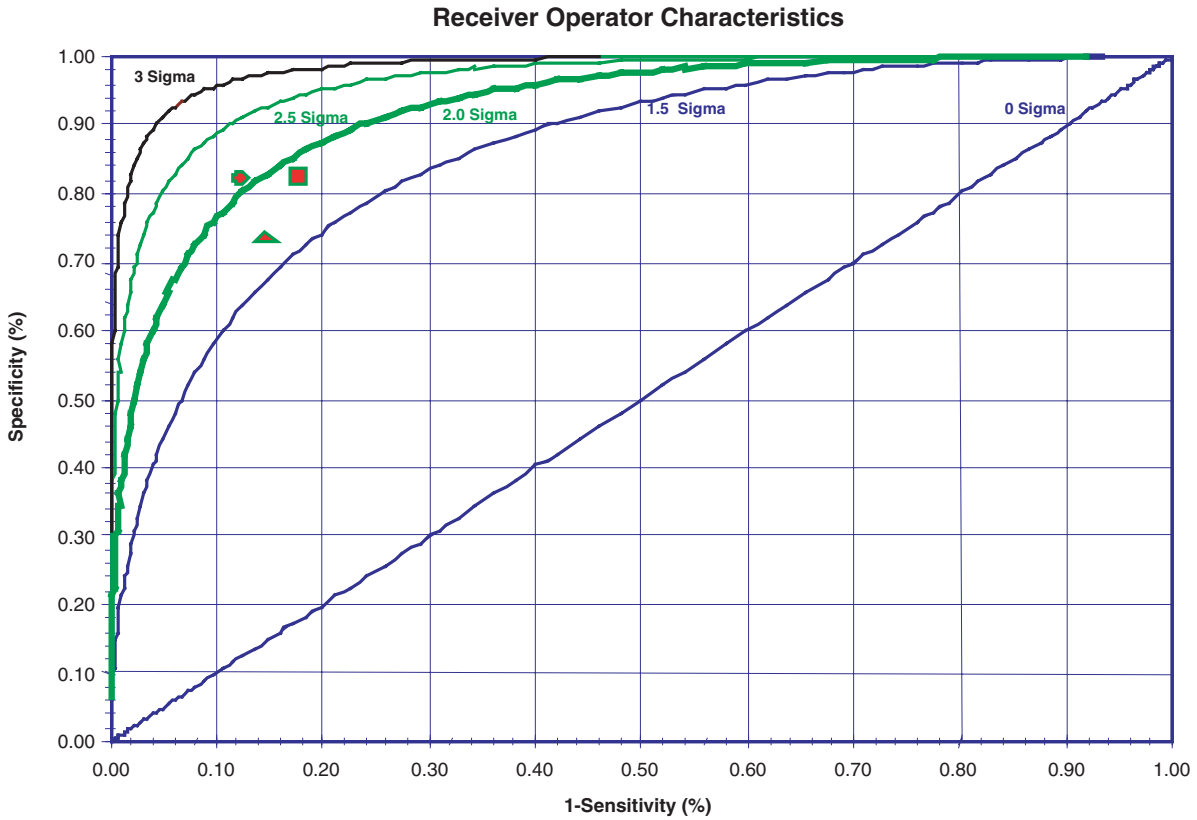

Figure 1. The receiver operator plot shows five curves of iso-performance ranging from 3 standard deviations of separation of the means of normal and affected populations (3 sigma) to no separation (0 sigma). These curves assume that the underlying distribution consists of two Gaussians, each having the same standard deviation, and a mean separated by the amount of discriminating power as measured in units of standard deviation. The 1a training set cutpoint on the 1a dataset (●) and the 1a + 1b cutpoint on the 1a + 1b dataset (■) cluster near 2 sigma of separation, the 1a training set cutpoint on the 1b dataset (▲) somewhat below. The diagonal represents the line of no discrimination.

stage cancers than with late stage disease. Taken together, DY and hV detected all stage I and stage IV cancers, and almost all stage II and III cancers. The 83% CRC sensitivity seen in the 82 cancers of the combined phase 1a + 1b dataset was independent of stage.

Influence of Tumor Location on Marker Expression

In the present study, distal cancers were more likely than proximal cancers to be positive for both the hV and DY markers (Table 4). However, this was statistically significant only for DY, so that the combination of the two markers detected cancers regardless of location. A similar finding was observed in phase 1a (12). When the results from phase 1a and 1b were combined, there was a slight trend toward detection of left-sided cancers that just reached statistical significance.

Influence of Patient Age on Sensitivity and Specificity

In phase 1a, we previously observed that hV positivity was associated with older age in both healthy controls and cancer patients (Table 5) (12). In the present study, there was a weaker (not statistically significant) association between hV and older age among NCs but not among cancer patients marker hV positivity remained associated with older age, but only among NCs. DY alone demonstrated no association with patient age. Combining hV and DY in both datasets revealed an association with older age among NCs but not patients with CRC.

Influence of Family History or Symptoms on Test Results

The possible effect of family history or symptoms was analyzed in the combined dataset (phase 1a + 1b), using the phase 1a cutpoints. Among the 82 subjects with CRC, none reported a family history of colon cancer or polyps, so this did not influence test results. Among the 363 subjects with NC, only 24 reported a family history of CRC or polyps and four (16.6%) had a positive sDNA test, which is not significantly different from those with a negative family history.

Among the 82 subjects with CRC, the frequency of a positive sDNA test was 57/66 (86.4%) among those with symptoms, compared to 14/16 (87.3%) among those without symptoms. Among the 363 subjects with NC, the frequency of a positive sDNA test was 12/40 (30%) among those with symptoms, compared to 73/323 (22.6%) among those without symptoms. In both cases, these differences were not statistically significant ($P > 0.9$ for subjects with CRC, and $P > 0.3$

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