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 studies represent alterations of genes involved in the predominant chromosomal instability pathway (such as *APC*, *p53*, and *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.

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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 TNM classification. Left-sided cancers were defined as those arising at, or distal to, the splenic flexure.

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 HLTF MSP-5 or vimentin MSP-29 (IDT, Coralville, IA). HLTF MSP-5 primer sequences have been reported previously.[13] Modi-