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Fecal DNA–Based Detection of Colorectal Neoplasia

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Abstract

Human DNA fragments, as well as whole colonocytes, can be found in stool and interrogated for mutations associated with carcinogenesis. Multiplexed panels of point mutations in fecal DNA improved the sensitivity of cancer detection (51.6%; 95% CI, 34.8%–68%) compared with fecal occult blood testing (12.9%; 95% CI, 5.1–28.9). However, the multiplex panels are not more sensitive than fecal occult blood testing in detecting advanced adenomas. Markers of epigenetic events, such as DNA methylation of gene promoters in genes not previously known to be associated with carcinogenesis or colorectal cancer, have recently been shown to be potential markers for early detection. Future approaches to improve sensitivity of fecal DNA detection of colorectal adenocarcinoma may require the inclusion of epigenetic biomarkers, the use of colonocytes isolated from feces, or both.

Introduction

Colorectal cancer remains the second leading cause of cancer deaths in the United States and the leading cause in nonsmokers [1]. Although colorectal cancer is preventable, screening rates are lower than those for common solid tumors with established effective screening and early detection tests (breast, cervical, or prostate cancer) [2,3]. When not prevented, colorectal cancer is curable if identified at an early stage. Yet it is estimated that in 2007 only about 50% of eligible persons in the United States will undergo any screening evaluation for colon cancer and fewer than 20% will undergo colonoscopy.

Current colorectal cancer screening recommendations of both the American Cancer Society and the US Preventive Health Services Task Force (USPHSTF) include a “menu of options” for those aged 50 years or older to choose from: yearly fecal occult blood testing (FOBT), flexible sigmoidoscopy every 5 years, a combination of the first two options, colonoscopy every 10 years, or barium enema every 5 years [4,5]. The USPHSTF endorses colonoscopy as the most sensitive and specific test for colorectal cancer screening but says it is “not certain whether the potential added benefits of colonoscopy relative to the screening alternatives are large enough to justify added risks and inconvenience for all patients.” Colonoscopy is the final common pathway that provides the benefit for all these menu options. Colonoscopy has been proposed as first-line screening for colorectal cancer by the American Gastroenterology Association (AGA) and the American Society for Gastrointestinal Endoscopy (ASGE). If the US population fully adhered to the current

screening guidelines, however, the cost, burden, and morbidity would outstrip the health care system's ability to meet the needs [6•].

The currently recommended noninvasive colorectal cancer screening test (FOBT) is limited by several issues:

1. A high false-positive rate, which results in large numbers of unneeded follow-up tests (colonoscopy)
2. A substantial false-negative rate, which results in undetected disease
3. A tendency for the specificity (true negatives divided by true negatives and false positives) to be skewed because the large number of negative subjects without disease (true negatives) overwhelms the relatively high false-positive rate in an environment of low cancer prevalence
4. Low sensitivity (true positives divided by true positives and false negatives) because the true positives are few and even a low rate of false negatives will degrade the sensitivity.

Flexible sigmoidoscopy is minimally invasive but has not been well accepted or utilized. Flexible sigmoidoscopy has an impressive level of performance for detection of colorectal cancers in the distal colon (sensitivity 97% and specificity 94%) [7]. It is credited with 50% to 95% reduction in mortality from left-sided colorectal cancers, and this reduction in mortality may show benefit for up to 16 years. Unfortunately it screens only about one third of the tissue at risk and can miss up to 48% of advanced proximal lesions [8].

Unlike flexible sigmoidoscopy, colonoscopy screens all of the tissue at risk for visible lesions. It has a sensitivity of 97% and a specificity of 98% for the detection of colorectal cancers and a specificity of 90% for adenomas larger than 1 cm [9,10•]. It fails as an ideal first-line screening test because of its poor public acceptance, risk of complications, high cost, and technical difficulty [9•,10•]. Barium enema, although able to screen all the tissue at risk, has a lower sensitivity than colonoscopy, especially for adenomas. Like colonoscopy, it suffers from poor public acceptance as a first-line screening tool [11].

The weaknesses of noninvasive methods in current colorectal screening, combined with adherence and cost limitations of invasive screening procedures, provide a strong rationale for the development of early colorectal cancer detection biomarkers that are simple and cost-effective. Such biomarkers should be able to detect both preinvasive and invasive neoplasms with high sensitivity (low false-negative rate) and modest specificity (an acceptably high false-positive rate). A successful biomarker panel coupled with colonoscopic follow-up only for patients with positive results would reduce the burden and morbidity of screening colonoscopy and most likely would enhance adherence to colorectal screening.

Rationale for Stool-Based Biomarkers for Detection of Colorectal Cancer

Molecular diagnostic tests performed on fecal samples are emerging as potentially important new approaches that may provide accurate and cost-effective early detection of colonic neoplasia. Details of many of the currently employed and novel approaches have been

recently reviewed [12•]. The concept of fecal screening is to exploit the regional advantage of sampling colonic epithelial cells that appear in the stool by either intact shedding, necrosis, or apoptosis. Using contemporary DNA or protein degradation technologies, these products, some of which are from neoplastic cells, can be detected and assumed to be of colorectal epithelial origin, unlike gene or protein products detected from blood or urine, which could have arisen from any systemic epithelial source.

Stool testing has many important advantages over other screening strategies. It is noninvasive and samples can be collected in privacy without inconvenience. With mail-in next-day delivery, geographic availability and access are no longer barriers. Patients surveyed about the commercially available versions of stool-based DNA screening tests have found them easy to use and acceptable [13]. Therefore the development of accurate fecal colonocyte DNA and mRNA markers has the potential to revolutionize screening for colorectal cancer.

Cellular Trafficking in the Colonic Crypt

Colonocytes in the crypts have 3-day to 5-day turnover, resulting in the potential shedding into stool of more than 10 billion adult colonic epithelial cells per day, some unknown percentage of which remain viable and trapped in the surface mucous. In the right colon, shed colonocytes in surface mucosa mix with the unformed stool, whereas in the left colon, colonocytes adhere to the surface of the stool, perhaps as the result of physical propulsion along the mucous layer [11]. Many investigators consider normal epithelial renewal to involve apoptosis of normal colonocytes at the top of the colonic crypts or along the luminal surface between crypts [14]. There is some evidence of phagocytic recycling by macrophages and other evidence of cellular invasion through small colonic pores [15]. The percentage of senescent crypt cells that undergo apoptosis, phagocytic recycling, or cellular involution versus the percentage of crypt cells released into the mucous layer of the lumen remains unknown. Similarly, if viable colonocytes populate the mucous layer, their ability to escape into the lumen of the colon remains unknown (Fig. 1).

In contrast, colorectal neoplasms are more prone to exfoliation because of increased proliferation, less apoptosis, and the decreased cell-cell adhesion inherent in the neoplastic transformation process [16]. The frequent luminal projection and irregularity of polyps and colon cancers increases the surface area and susceptibility to trauma from passing liquid and solid stool. Some argue that in the presence of a neoplasm in the colon, normal colonocytes are outnumbered by neoplastic colonocytes among the shed colonocytes in the stool [12•], but data to support this argument are limited. Despite the higher turnover of colonocytes in a neoplasm, the limited size and surface area of such a lesion compared with the vast expanse of normal mucosa suggests that crypt epithelial turnover most likely contributes the bulk of cells—or at least cell fragments—in stool. Nevertheless, colonic neoplasms shed sufficient DNA into the colon to permit detection of mutations from stool DNA fragments among the sea of DNA from normal colonocytes, food sources, and bacteria [17].

Reliable methods have recently been developed to isolate colonocytes and colorectal cancer cells from spontaneously evacuated stool (not laxative-induced) [18]. New methods for

human colonocyte mRNA quantification also have been developed, allowing quantification of transcriptional gene expression from these exfoliated colonocytes [19]. These techniques involve the use of reverse transcription real-time polymerase chain reaction (PCR) and can be combined with DNA mutation tests and DNA methylation.

Because exfoliation is likely to be a continuous process, examination of fecal colonocytes will be less prone to sample variation than FOBT and will result in improved sensitivity. It also would eliminate the problem of confounding nonhuman DNA in feces.

Development of Gene-Based Tests in Stool for Detection of Colonic Neoplasms

In 1992, Sidransky et al. [17] reported that *K-ras* mutations were detectable in DNA extracted from exfoliated cells in stool. Because *K-ras* is expressed in less than half of advanced colorectal neoplasia, the sensitivity of this test was low (< 35%), so a single-gene test based on *K-ras* detection is not feasible [17]. Mutations in the adenomatous polyposis coli (*APC*) gene or the *p53* gene are present in up to 70% of colorectal cancers and occur early in the transition from adenoma to cancer, so detecting common mutations in these genes may increase the true-positive rate in combination panels. Mutations were detected in the stool of seven (64%) of 11 patients with known *p53* tumor mutations; among these, three of five FOBT-negative samples had *p53* mutations in the stool [20]. Traverso et al. [21] found *APC* mutations in stool from 61% of patients with early colorectal cancers and 50% with advanced adenomas. No mutations were identified in any of the stool samples from 28 control patients with negative colonoscopies. These data demonstrate that *p53*, *APC*, and *K-ras* mutations from a colonic mutation are detected in DNA fragments from the stool. Unfortunately, true-positive rates are poor, suggesting that it may be necessary to look for different genetic events associated with carcinogenesis to achieve adequate sensitivity and specificity of neoplasm detection (Table 1). Because no single gene mutation has been found to exist in all colonic neoplasia, screening based only on single-gene mutation analysis will yield an unacceptably high false-negative rate. Moreover, the technical efficiency of detecting rare mutations in small DNA fragments from the stool matrix is likely to be less than 100%, further decreasing the diagnostic yield [12•].

Multiplexed-DNA Mutation Panel for Human Stool

The development of more efficient technologies to enable amplification of multiple genes in single-reaction, multiplex PCR technology [22,23] created new opportunities to detect mutations in multiple genes in a single assay (Table 1). Automated methods create the potential for cost-effective tools for mass screening by permitting analytic scale-up of multiple-gene mutation detection [12•]. Several multigene panels have been developed to capitalize on the heterogeneity of alterations in colonic neoplasia for early detection of colorectal cancer [23,24]. These tests required a large volume of stool, from which purified, naked human DNA is obtained using oligonucleotide-based hybrid capture. Realtime PCR is used to detect and quantify the chosen DNA mutation sites. Ahlquist et al. [24] reported a specificity of 93% with sensitivity of 91% for cancer and 82% for adenomas larger than 1 cm while using an assay that included 15 point mutations on *K-ras*, *p53*, and *APC* genes,

along with Bat26 (a marker of microsatellite instability) and long DNA, in 51 freezer-archived stool specimens [24].

Using a similar multitarget assay panel of 21 specific mutations in *K-ras*, *p53*, and *APC* with an assay for Bat26 and DNA integrity, Tagore et al. [25] found a specificity of 93.6%, a sensitivity of 63.5% for cancer, and a sensitivity of 57.1% for advanced adenomas in 132 patients who provided stool samples prior to colonoscopy. Syngal et al. [26••] showed that these stool DNA mutation abnormalities disappear after excision and treatment of colon cancer, further strengthening a potential role for these panels.

The results of these studies are very promising, but a screening diagnostic test that requires shipment of a bio-sample to a central laboratory poses major problems with logistics, cost, and quality control. To address these issues, a group at Exact Sciences (Marlborough, MA) undertook a population-based screening trial of a multigene fecal DNA diagnostic test ("PreGen-Plus™") compared with FOBT (Hemoccult II). The data of Imperiale et al. [27••] from 4404 patients screened for colorectal cancer report sensitivities for the detection of cancer of 51.6% (95% CI, 34.8%–68%) for the DNA panel and 12.9% (95% CI, 5.1%–28.9%) for Hemoccult II ($P = 0.003$); specificity was 94.4% for the DNA panel and 95.2% for Hemoccult II. When advanced adenoma detection was added to this analysis, the sensitivity of the fecal DNA panel dropped to 40.8% (95% CI, 30.2%–52.5%) while the Hemoccult II sensitivity remained the same at 14.1% (95% CI, 7.8%–24.6%, $P < 0.001$). The fecal DNA panel was not more sensitive than the Hemoccult test in detecting advanced adenomas alone: sensitivity was 15.1% (95% CI, 12.0%–19.0%) for the DNA panel and 10.7% (95% CI, 10.7%–14.1%) for Hemoccult II.

These data support the concept that fecal DNA tests could improve the sensitivity of FOBT and serve as an intermediate, noninvasive screening tool for colorectal adenocarcinoma. Although these tests are more sensitive than single-gene testing, FOBT, and no screening at all, when they were tested in a clinical screening environment of asymptomatic subjects, their sensitivity fell. Moreover, the fecal DNA test did not improve upon FOBT sensitivity for the detection of adenomas, a disappointing outcome. Non-invasive dysplastic cells from large adenomas are known to contain a spectrum of mutations similar to those found in invasive neoplasms [28]. Is the cellular turnover in large adenomas insufficient to generate a mutational signal from DNA in stool? Or are there insufficient DNA mutational events in large adenomas for detection by a limited number of multiplexed mutations available in the stool DNA assay?

Another unexpected outcome in the trial by Imperiale et al. [27••] involved the poor predictive performance of long DNA. Phase II data suggested that long DNA, thought to reflect disordered apoptosis, was the most sensitive portion of the PreGen-Plus™ test. Yet in the prospective, multicenter screening trial, long DNA had the poorest predictive performance. These results might be explained by the much longer interval between collection and processing in the large multicenter screening trial compared with the phase II trials. Stool DNA, which can be easily digested by DNAases present in the feces, may have more time to fragment if processing of the sample takes a longer time. Enhanced preservation may reduce DNA degradation, but variable degradation in unformed and

formed fecal matter prior to defecation may undermine enhanced postdefecation stabilization. The problem of DNA degradation could be eliminated by using intact, viable colonocytes exfoliated into human stool as the source for DNA.

A cost-benefit Markov model by Song et al. [29] assumed the diagnostic sensitivity of the multigene fecal DNA panel to be 65% for adenocarcinoma and 40% for colorectal adenomas, at a base cost estimate of \$695 per PreGen-Plus™ test. The model generated a cost of \$47,700 per incremental life-year gained, assuming testing every 5 years. For a fecal DNA test to become cost-effective, the base cost would need to be reduced to \$195 per test, using sensitivity assumptions 10% higher than the recently published prospective screening trial [30]. On the basis of these data, it is concluded that fecal DNA testing is no more cost-effective than periodic colonoscopy. Given the superior diagnostic performance of colonoscopy, as well as its therapeutic value, routine testing with the currently available multigene fecal DNA panel is not recommended.

DNA Methylation-Based Stool Biomarkers

Epigenetic changes in cellular DNA during carcinogenesis play a major role in cellular transformation. Methylation of tumor suppressor gene promoter sites causes transcriptional silencing, and these genes have potential clinical use as biomarkers and therapeutic targets. At present, cancer-specific DNA methylation changes have focused on CpG islands. These are lengths of DNA about 1 kb long, containing clusters of CpG dinucleotides that are usually located at the 5' end of a gene and are unmethylated in normal cells. DNA hypomethylation remains largely unexplored in colorectal cancer and could represent another potential area for exploitation.

Aberrantly methylated *CDKN2A* and *MGMT* occur in advanced adenomas, whereas *MLH1* gene methylation remains stable in both tubular adenomas and higher-grade adenomas (Table 1) [31•]. Adenomas in which at least one of these genes was methylated were larger than those with nonmethylated genes (15.6 vs 7 mm; $P = 0.0067$). None of these three genes was methylated in DNA extracted from normal colonic mucosa. In 44 hyperplastic polyps from 17 patients, at least one of these genes was methylated in 20%. Aberrant methylation of these three genes also can be detected in DNA from colon lavage effluent. Unfortunately, the low sensitivity (55%) and specificity (37%) for these methylation assays limit their clinical utility as diagnostic biomarkers for colorectal cancer. This suboptimal performance may be explained by 1) lack of sensitivity of the PCR-based assays in detecting the small amount of polyp DNA in the samples or 2) loss of tumor DNA in the technique used to extract the fecal DNA in the samples that generate false-negative results.

Looking at colon cancers from two independently collected sources, Chen et al. [32••] found that vimentin exon-1 expression was methylated in 83% ($n = 46$) and 53% ($n = 107$). This same sequence was found to be unmethylated in 45 of 46 normal colonic tissues tested. In fecal DNA from a separate set of colon cancer patients ($n = 94$), vimentin methylation had a sensitivity of 46% (95% CI, 35%–56%) for detecting colon cancer and 43% (95% CI, 31%–57%) for detecting early-stage tumors. Vimentin methylation of fecal DNA from 198 subjects free of colon cancer was only 10%, giving a specificity of 90% (95% CI, 85%–

94%). Interestingly, vimentin is not expressed by normal colonic mucosa; the gene encodes a protein constituent of mesenchymal cells. These data suggest that methylation is a widespread event in dysplastic or transformed cells, even in gene promoters that are not specifically known to be important in tumor suppression or cellular differentiation.

The gene for the p110 α catalytic subunit of phosphatidylinositol 3-kinase (*PIK3CA*), a key lipid kinase that regulates multiple cellular signaling pathways associated with tumorigenesis, has recently been shown to have a high rate of mutations in colorectal cancer [33•]. The high rate and clustering of mutations in the *PIK3CA* gene would make it a potential marker for early detection.

Recent technological advances have found many ways to identify mutations, including DNA micro-arrays, polymerase chain reaction/ligase detection reaction (PCR/LDR), primer extension assays, hybridization assays, high-performance liquid chromatography (HPLC), single-strand conformational polymorphism analysis, gradient gel electrophoresis, and heteroduplex analysis. Using these technologies to examine shed fecal colonocytes may prove useful in developing a noninvasive screening tool for colorectal cancer.

Conclusions

Fecal DNA testing on shed colonocytes has the potential of providing early detection of colorectal adenomas and cancers that will be accurate, noninvasive, cost-effective, and acceptable to the public. Experience with the Pre-Gen-Plus™ fecal DNA profile of common mutations associated with colorectal carcinogenesis supports the future likelihood that a screening biomarker panel will improve adherence and sensitivity in the early detection of colorectal cancer, but future approaches will require improved sample collection and logistics, as well as reduced cost. The panel of biomarkers needed to optimize sensitivity has yet to be identified.

The targeted population is likely to remain individuals aged 50 and older. The testing interval remains to be determined but probably will be no less than every 5 years, reflecting the current ability of colonoscopy to identify new lesions. The problem of individuals who are positive on screening but negative on colonoscopy will need to be resolved. Will false-positive fecal DNA screening mean that a small island of dysplastic or neoplastic cells is present in the colonic epithelium and was missed by colonoscopy? Or can morphologically normal colonocytes develop mutations with no current or future clinical importance? Can mutated DNA circulating from other organ sites (eg, the biliary tree) be detected in fecal DNA samples? Accurate interpretation of the results of future DNA panels will require clarification of these issues.

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Figure 1. Possible luminal cellular fluxes at the surface of a colorectal neoplasm (**A**) and normal colonic mucosa (**B**). AC—apoptotic cells; C—viable colonocytes; L—leukocytes; M—macrophages. (*Adapted from Osborn and Ahlquist [12•], with permission. Copyright © 2005 American Gastroenterological Association.*)

Table 1
Selected clinical validation trials of fecal DNA-based early detection tests for colorectal neoplasia

Study	Marker(s)	Colorectal cancer detection sensitivity, % (n)	Adenoma detection sensitivity, % (n)	Colorectal cancer detection specificity, % (n)	Adenoma detection specificity, % (n)
Sidransky et al. [17]	<i>K-ras</i>	37 (9/24)	NA	50 (3/6)	NA
Ahlquist et al. [24]	<i>APC</i> , <i>K-ras</i> , <i>p53</i> ; Bat26; long DNA	91 (20/22)	82 (9/11)	93 (26/28)	82 (9/11)
Tagore et al. [25]	<i>APC</i> , <i>K-ras</i> , <i>p53</i> ; Bat26; long DNA	63.5 (33/52)	57.1 (16/28)	94.5 (208/220)	98.2 (113/115)
Syngal et al. [26••]	<i>APC</i> , <i>K-ras</i> , <i>p53</i> ; Bat26; long DNA	54(49/91)	26 (6/23)	NA	NA
Imperiale et al. [27••]	<i>APC</i> , <i>K-ras</i> , <i>p53</i> ; Bat26; long DNA	52 (16/31)	33 (13/40)	99.3 (2460/2476)	94.4(1344/1423)
Petko et al. [31•]	Aberrantly methylated <i>CDKN2A</i>	NA	34(14/41)	NA	100(25/25)
	Aberrantly methylated <i>MGMT</i>	NA	49 (19/39)	NA	100 (25/25)
	Aberrantly methylated <i>MLH1</i>	NA	7(3/41)	NA	100 (25/25)
Chen et al. [32••]	Methylated vimentin	83 (38/46), 53 (57/107)	NA	90(178/198)	NA
Samuels et al. [33•]	<i>PIK3CA</i> mutation clustering	32 (74/119)	2.6 (2/76)	NA	NA

APC—adenomatous polyposis coli; NA—data not available; PIK3CA—phosphatidylinositol 3-kinase p110a catalytic subunit.