Biomarkers for Early Detection of Colorectal Cancer: The Early Detection Research Network, a Framework for Clinical Translation

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ABSTRACT

Early detection by screening significantly reduces mortality from colorectal cancer, but 40% of guideline-eligible patients are not screened as recommended in the United States. Novel strategies to improve screening uptake overall and efforts to deploy best practices to underserved populations are a high priority for health care. This review focuses on existing biomarkers in practice and those in development with clinical relevance to early detection of colorectal neoplasia, with an emphasis on those developed by investigators of the NCI's Early Detection Research Network. Aberrantly methylated DNA markers (blood and stool), stool-based markers (including fecal immunochemical test-DNA), and a variety of blood-based marker assays in development (protein markers, glycoproteins including mucins, and cell-free DNA tests) are reviewed. Individual markers and biomarker panels, sample resources, and barriers to translating biomarkers to clinical practice are discussed.

See all articles in this *CEBP Focus* section, "NCI Early Detection Research Network: Making Cancer Detection Possible."

Introduction

Colorectal cancer is the second leading cause of cancer-related death in industrialized nations, accounting for 10% of the total cancer burden with an individual lifetime risk of approximately 6% in western countries (1–4). Although early detection by screening significantly reduces mortality and numerous screening options exist (5–7), 40% of guideline-eligible patients are not screened as recommended in the United States. Globally 1.4 million new colorectal cancer cases and 700,000 related deaths occur yearly, and compliance with screening may even be lower. Universal implementation of colonoscopy for colorectal cancer screening for all persons ages 50–74 would require a capacity to screen 250 million individuals over a 10-year period in North America and Europe. Adherence to or uptake of colorectal cancer screening is especially poor among underserved populations, including those with low income and African American and Hispanic populations (8–11). Novel strategies to improve screening uptake overall, and efforts to deploy best practices to underserved populations is a high priority for healthcare and reducing colorectal cancer–related morbidity and mortality.

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Screening for colorectal cancer and precursor adenomatous polyps and sessile serrated lesions takes multiple forms, the most widely used approach in the United States is optical colonoscopy. However, colonoscopy requires sedation, carries a risk of adverse events, and involves bowel cleansing prior to the procedure, which together discourage adherence and contribute to the one-third of guideline-eligible individuals who do not access colorectal cancer screening as recommended. In addition, the paradigm for colorectal cancer screening world-wide outside the United States is often a two-step process whereby an initial positive screening test then triggers colonoscopy as a diagnostic test. While this is especially important in resource-limited countries, it is also the model in many countries with organized colorectal cancer screening programs such as the United Kingdom, Canada, Australia, Denmark, and many other countries in Europe and Asia. As a result, development of and clinical interest in noninvasive screening tests for colorectal cancer using "biomarkers" has been high, including various forms of stool- and blood-based tests. Patient-friendly approaches which improve patient uptake are needed to achieve the colorectal cancer screening goal of high compliance, but must also demonstrate high performance characteristics (sensitivity and specificity) for detection of early-stage cancer and high-risk precursor lesions, as well as broad acceptability to the general population, health care providers, regulatory agencies, and third-party payers. Consistent with this goal, adoption of cost-effective noninvasive methodologies designed to reduce complications and improve overall acceptance of the screening process would be highly desirable.

This review will focus on existing biomarkers in practice and those in development with clinical relevance to early detection of colorectal neoplasia, with an emphasis on those developed by investigators of the NCI's Early Detection Research Network (EDRN).

Barriers to Translating Biomarkers for Early Detection of Colorectal Cancer to Clinical Practice—The Right Samples and Enough of Them

Numerous barriers exist to clinical translation of biomarkers for early detection of colorectal neoplasia discovered in the laboratory to

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Table 1. Challenges to translating biomarker research to clinical practice.

- Lack of sensitivity for early-stage CRCs and advanced adenomas.
  - Depends on point of view (how good is "good enough?")
- Availability of relevant high-quality samples and funding for phase II/III studies.
  - EDRN, http://edrn.nci.nih.gov/resources/sample-reference-sets/edrn-pre-validation-reference-set-specimen-sharing-guidelines.
- Laboratory results versus clinically relevant validated assays.
- Cost of phase IV trials (impact on health and mortality).
  - Do not have for FIT/colonoscopy.
  - Comparison with accepted test adequate?
- Meaning of false positives?
- Path to commercialization long and expensive.
- Regulatory pathways and barriers (FDA vs. LDTs).
- Guideline inclusion.
- CMS and third-party payer acceptance

Abbreviations: CRC, colorectal cancers; LDT, laboratory developed tests.

clinical practice [**Table 1**; see also Ren and colleagues (12) and Feng and Pepe (13) in this *CEBP Focus*]. Indeed, few laboratory-discovered biomarker assays proceed to clinical practice. Translating a biomarker (or a panel of markers) from the laboratory to the clinic is a long and expensive multiphase process, with the ultimate goal of producing a clinical assay which will impact human health and disease management. Going beyond the research laboratory to phase I, II, and III clinical trial implementation (and eventually to phase IV assessment of clinical impact) requires rigorously controlled and unbiased study designs, with strict standard operating procedures (SOP) for biospecimen collection (collection, processing, storage, and retrieval) and adequate samples (14–17). The target population and intended setting for clinical use dictates the setting for collection of biospecimens, with outcomes of interest defined and prespecified. Biospecimens must be representative of the target population and reduce bias as much as possible. Many biomarker studies, for example, include overrepresentation of biospecimens from patients with late-stage cancers, a population unsuitable for a marker used for screening and early detection. Mechanisms must be in place to blind biospecimen handling, assays, and reporting of results. The effects of handling and storage must be taken into account. The limited availability of relevant high-quality samples and funding for such studies is a substantial challenge to validation and successful translation to clinical practice (including acceptance by regulatory agencies). Several EDRN-sponsored trials and collaborative trials involving EDRN members have sought to overcome these barriers in producing adequate high-quality samples for biomarker discovery and validation aimed at early detection of colorectal neoplasia (**Table 2**).

The Great Lakes New England Clinical Validation Center of the EDRN (GLNE) is a multicenter consortium dedicated to translating biomarkers for early detection of colorectal neoplasia to clinical practice. This includes development of sample resources for use by members of the EDRN and others through cross-sectional and prospective collection of high-quality biospecimens. Prevalidation and reference sets are available through a biospecimen-sharing process (http://edrn.nci.nih.gov/resources/sample-reference-sets/edrn-pre-validation-reference-set-specimen-sharing-guidelines).

EDRN GLNE protocol 007 represents a cross-sectional collection of biospecimens designed to permit convenience, training, and testing of promising biomarkers. Initial enrollment took place from January 2006 to June 2010. GLNE 007 included recruitment of subjects in the following "bins" 262 colonic adenomas (54 advanced); 191 invasive colonic adenocarcinomas; 65 high risk, colonoscopy normal; and 164 colonoscopy normal screens. GLNE investigators collected 30 sera, 30 plasma, five stool, and 20 5-mL urine aliquots/subject. The group shipped a reference sample set to NCI of 50 subjects each: invasive colonic adenocarcinoma, advanced adenomas, and normal colonoscopy. In addition, 12,600 aliquots of various types including 1,050 tissue samples were distributed for collaborative research. This protocol has recently been reactivated and subjects are actively being recruited. The revised protocol proposes to recruit 1,200 new subjects (400 subjects with diagnosed colorectal cancer, 200 subjects with adenomas, 200 higher risk subjects with endoscopically normal colons, 200 average-risk subjects with endoscopically normal colon for controls, and 200 subjects ≥60 years of age). Blood (all subjects), stool [fecal immunochemical test (FIT) all subjects plus archived stool collection from patients with cancer], and tissue (large adenomas and cancers) will be collected, handled, transported, processed, and stored according to detailed SOPs. Selected subjects, on the basis of future biomarker requirement, will have normal colonic epithelium collected during colonoscopy for future biomarker research. Biospecimens are curated through an independent data monitoring center sponsored by the EDRN, which is also responsible for statistical analysis. Renewing this collection will expand this valuable collection to 1,882 total subjects.

EDRN GLNE protocol 010 is a prospective PRoBE-compliant (15) validation trial of stool-based and serum-based tests for the detection of colorectal neoplasia. The trial was powered to evaluate tests for detecting early-stage colorectal adenocarcinoma (this is the most stringent, conservative approach to the early diagnosis of colonic neoplasia and addresses the important endpoint of identifying individuals with curable, early-stage cancer), as well as screen-relevant neoplasia (cancer plus advanced adenomas) and precursor adenomas. Blood [serum, plasma, and white blood cells (WBC)], stool, and urine were collected according to prespecified SOPs. This prospective screening trial successfully enrolled 6,820 subjects with 90% evaluable. The event rate for screen-relevant neoplasia (advanced adenomas plus cancer) was 13% (yielding ∼800 high-risk subjects), however, the event rate for invasive colorectal cancer was substantially lower than expected (0.2%), and the study was closed to new enrollment in March 2019. Per protocol, subjects will continue to be followed for 1 year to obtain interval information regarding health and cancer history. Biospecimens are available from those subjects completing the trial. Biospecimens were again curated through an independent data monitoring center sponsored by the EDRN, which was also responsible for statistical analysis.

The EDRN has sought to include interested international members and collaborators. Colorectal cancer is the third most common cancer in Denmark where its epidemiology and natural history closely resembles that of the United States. Specimens from several protocols directed by EDRN member H.J. Nielsen at the University of Copenhagen (Copenhagen, Denmark)/Hvidovre Hospitals (Hvidovre, Denmark) are being used by EDRN collaborators to develop biomarker panels for early detection of colorectal neoplasia (**Table 2**). Endoscopy II was designed to identify blood-based, cancer-associated biomarkers that may be used for early detection of colorectal cancer (18–21). Subjects were those undergoing diagnostic first-time colonoscopy due to symptoms attributable to colorectal cancer. While this was not a screening population, strict SOPs were used to collect samples from 4,698 subjects. The entire sample set is under analysis by EDRN investigators and industrial collaborators for early validation and to

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Table 2. Protocols for sample collection (CRC trials) EDRN investigators.

Protocol	Aim/design	Biospecimens	Comments
EDRN GLNE 007	Cross-sectional collection of biospecimens designed to permit convenience, training, and testing of promising biomarkers.	Blood (serum and plasma), stool, and urine collected before bowel preparation. 262 colonic adenomas (54 advanced); 191 invasive colonic adenocarcinomas; 65 high risk, colonoscopy normal; and 164 colonoscopy normal screens. 30 serum, 30 plasma, 5 stool, 20 5-mL urine aliquots/subject.	Original collection January 2006 to June 2010. Reactivated September 2019. Phase II will enroll 1,200 new subjects, (400 subjects with diagnosed colorectal cancer, 200 subjects with adenomas, 200 higher-risk subjects with endoscopically normal colons, 200 average-risk subjects with endoscopically normal colons for controls, and 200 subjects ≥60 years of age).
EDRN GLNE 010	Prospective PRoBE-compliant (15) validation trial of stool-based and serum-based tests for the detection of colorectal neoplasia in a screening/surveillance population.	Blood (serum, plasma, and WBCs), stool, and urine collected before bowel preparation. 6,820 subjects enrolled with 90% evaluable.	Event rate for screen-relevant neoplasia (advanced adenomas plus cancer) 13%; event rate for invasive colorectal cancer lower than expected (0.2%). Study was closed to new enrollment in March 2019. Samples being analyzed.
Endoscopy II	Objective: Identify blood-based, cancer-associated biomarkers that may be used for early detection of CRC. Subjects undergoing diagnostic first-time colonoscopy due to symptoms attributable to CRC.	$N = 4{,}698$ subjects. Distribution: 512 CRC (323 colon and 189 rectum), 177 extra-colonic cancers, 699 adenomas (398 high risk and 301 low risk), 1,176 with other benign bowel lesions (>95% diverticula), 1,014 no findings but with comorbidity, and 1,120 no findings, no comorbidity.	Entire sample set is under analysis by EDRN investigators (P.D. Lampe and R.S. Bresalier) and industrial collaborators for early validation and to develop optimal panels for detection of colorectal neoplasia.
Endoscopy III part 1	Screening population. Objective: to develop a combined blood-based biomarker panel for use as an alternative to FIT screening.	90 mL of peripheral blood collected after bowel preparation, but before colonoscopy. 13,215 subjects undergoing FIT screening for CRC. 8,110 FIT (+) (>100 ng/mL) and 5,105 FIT (−) at screening. FIT (+) subjects underwent colonoscopy.	Findings: 446 CRC [stage I: 210 (T1 = 52.1%); stage II: 97; stage III: 112; and stage IV: 27]. 1,074 with high-risk adenoma (≥5 lesions or one lesion ≥20 mm or one or more lesions removed by piecemeal technique); 1,406 with medium-risk adenoma (3–4 lesions or 10–19 mm or high-grade dysplasia or villous histology); and 1,651 with low-risk adenoma (<3 lesions or <10 mm or low-grade dysplasia or tubular histology). 3,533 had no neoplastic findings at colonoscopy. Samples undergoing analysis by EDRN and multiple industrial collaborators.
Endoscopy III part 2	Objective: to prospectively identify FIT (−) subjects at risk of developing CRC and other cancers.	Blood samples collected within 2 months after a FIT (−) result. Collections biennially for three rounds.	First round = 32,640; second round 15,996 returned to date for new blood samples. Currently >35 CRCs and >700 extra colonic cancers have been identified. Blood analyses to be performed.

Abbreviation: CRC, colorectal cancer.

develop optimal panels for detection of colorectal neoplasia. Endoscopy III (22) is a prospective screening trial designed to develop a combined blood-based biomarker panel for use as an alternative to FIT screening. In phase I of this trial, 90 mL of peripheral blood was collected before colonoscopy from 13,215 subjects undergoing FIT screening for colorectal cancer. There were 8,110 FIT-positive (+; >100 ng/mL) and 5,105 FIT-negative (−) subjects at screening. FIT (+) subjects underwent initial colonoscopy. Findings included 446 subjects with colorectal cancer [stage I, 210 (T1 = 52.1%); stage II, 97; stage III, 112; and stage IV, 27]. A total of 1,074 subjects with high-risk adenoma (≥5 lesions or one lesion ≥20 mm or one or more lesions removed by piece-meal technique); 1,406 with medium-risk adenoma (3–4 lesions or 10–19 mm or high-grade dysplasia or villous histology); and 1,651 with low-risk adenoma (<3 lesions or <10 mm or low-grade dysplasia or tubular histology). A total of 3,533 had no neoplastic findings at colonoscopy. Samples are undergoing analysis by EDRN members and multiple industrial collaborators. Phase II seeks to prospectively identify FIT (−) subjects at risk of developing colorectal cancer and other cancers. In phase II, blood samples were collected within 2 months after a FIT (−) result. Collections were performed biennially for three rounds. To date >35 colorectal cancers and >700 extra colonics have been identified.

Noninvasive Biomarkers for Early Detection of Colorectal Neoplasia

Tumor cells in the colon, as elsewhere, are characterized by heritable phenotypic changes that are the result of quantitative or qualitative

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alterations in gene expression. A large body of evidence demonstrates that colorectal cancers are associated with an accumulation of such genetic alterations. There has been a veritable explosion of knowledge regarding genetic alterations associated with colorectal cancer, in part driven by advances in modern next-generation sequencing and genome-wide association studies. A number of approaches have been employed in attempts to identify noninvasive markers for detection of colorectal neoplasia. "Bio-signatures" have been detected from neoplasm-generated genetic products (DNA and RNA markers), protein antigens, glycoproteins and other glycans, antibodies, and tumor cells shed in the stool or circulating tumor cells. Alterations in DNA methylation affect residues in regulatory portions of genes and also provide advantages in designing biomarker assays. Digital-based quantitative technologies improving upon bisulfite conversion while minimizing bisulfite-associated DNA fragmentation and single-molecule detection technologies permit cost-effective development of DNA hypermethylated gene biomarkers.

Aberrantly methylated DNA

Over the last 20 years, aberrantly methylated DNA [see also Farooq and Herman (23) in this *CEBP Focus*] has evolved from a novel observation in cancer to clinically useful classes of biomarkers for cancer early detection. EDRN investigators have served a central role in the development of this class of epigenetic markers for colorectal cancer screening. Through support from the EDRN, these investigators have characterized the role and extent of aberrant DNA methylation in gastrointestinal cancer. These studies have led to clinically available noninvasive screening tests for colorectal cancer. After being first observed in the 1980s, our understanding of aberrant DNA methylation has advanced dramatically, particularly for colorectal cancer (24). EDRN principal investigators W.M. Grady and S.D. Markowitz provided some of the first evidence that the aberrant methylation and epigenetic repression of the tumor suppressor genes *MLH1* and *CDH1* act to drive the formation of gastrointestinal cancer (25, 26). The initial studies of aberrant promoter methylation demonstrated associated loss of gene expression in many human cancers. However, it was not clear whether in any given tumor the methylation of a specific gene was causal and not consequent to malignant transformation. W.M. Grady and S.D. Markowitz assessed the role of aberrant DNA methylation in the genesis of gastric cancers in individuals with hereditary diffuse gastric cancer. These individuals carry germline mutations in the gene encoding E-cadherin, *CDH1*, and these studies provided evidence that *CDH1* promoter methylation might function as the "second genetic hit" in the genesis of these cancers. Later studies have further advanced our understanding of the potential driver role for epigenetic alterations in colorectal cancer and led to insights regarding epigenetic-mediated deregulation of TGFβ signaling, the functional role of epigenetic alterations of the dependence receptors *NTRK1* and *RET* in colorectal cancer, and the epigenetic deregulation of miRNAs in colorectal cancer (27–30).

The characterization of DNA methylation in the normal colon, colorectal cancer initiation, and colorectal cancer progression has also been advanced by EDRN investigators. The DNA methylome of the colon has been shown to vary between regions in the colon, which significantly informs studies of colonic biomarkers. Furthermore, aberrant DNA methylation occurs early in the colorectal cancer initiation process and leads to unique molecular subclasses of colon adenomas and colorectal cancer (31–33). Luo and colleagues observed three molecular subclasses of colorectal cancers and two subclasses of adenomas based on their DNA methylation patterns. The adenomas separated into classes of high-frequency methylation and low-frequency methylation. Within the high-frequency methylation adenoma class, a subset of adenomas had mutant *KRAS*. In addition, the high-frequency methylation adenoma class had DNA methylation signatures similar to those of cancers with low or intermediate levels of methylation, and the low-frequency methylation adenoma class had methylation signatures similar to that of nontumor colon tissue (33).

Further work by these investigators employed bisulfite conversion and DNA-sequencing to identify aberrant DNA methylation in the first exon of the vimentin gene, finding this epigenetic event as a sensitive and specific biomarker of colorectal cancer. This led to development of a methylation-specific PCR assay for detecting vimentin DNA methylation, finding methylated vimentin present in 83% of colorectal cancer tissues but in only 5% of matched normal colon tissues (34). To develop a method for early detection of colorectal cancer, they collaborated with Exact Sciences to demonstrate that aberrant vimentin methylation could be robustly detected by methylation-specific PCR performed on DNA isolated from feces of patients with colorectal cancer, with initial studies showing positive stool DNA tests in 43% of patients with stage I and stage II colorectal cancer, and only in 10% of noncancer cases (34). Follow-on studies showed methylated vimentin tests of stool DNA as 77% sensitive and 83% specific for detecting colorectal cancer, with 83% sensitivity for detecting stage I and II disease, and with ability to also detect advanced adenomas (35). On the basis of these findings, stool DNA testing for vimentin methylation was brought forward commercially by Exact Sciences and LabCorp as the ColoSure test. This milestone demonstrated the scientific and clinical robustness of methylated DNA as a biomarker for early-colorectal cancer detection in stool DNA tests, and provided the scientific foundation for this technology, which has been further implemented in Exact Sciences FDA-approved Cologuard test (which incorporates stool DNA testing for methylated BMP3 and NDRG4 DNAs; see below).

In conjunction with and informed by the studies defining the role of aberrant DNA methylation in the pathogenesis of colorectal cancer, studies by EDRN principal investigators demonstrated the feasibility of serum-methylated *MLH1* to be a blood-based biomarker for colorectal cancer detection and also for aberrantly methylated genes to be used as stool DNA–based colon adenoma and colorectal cancer detection markers (11, 14). Advances in the techniques used for methylated DNA biomarkers has also been made including the development of methylation-specific droplet digital PCR, which has superior limits of detection and precision compared with quantitative methylation-specific PCR (e.g., Methylight) and better performance for detecting low level biomarker signals (36, 37).

In addition to the pioneering studies in the molecular biology of colorectal cancer and early detection biomarkers, EDRN investigators have identified and characterized novel predictive and prognostic biomarkers for colorectal cancer (38, 39). Shiovitz and colleagues, using colorectal cancer samples from the clinical trial CALGB9803, have shown that patients with stage III, CpG island methylator phenotype–positive, mutation mismatch repair intact colorectal cancers have longer survival times when irinotecan is added to combination therapy with fluorouracil and leucovorin (40).

Other EDRN investigators have studied circulating aberrantly methylated DNAs as biomarkers for early detection of colorectal neoplasia. These are discussed below (blood-based markers).

Stool-based biomarkers

Stool-based assays such as FITs and the FIT-DNA (Cologuard) test are relatively sensitive for detection of advanced-stage colorectal cancers, but detect fewer early-stage cancers and only 28% (FIT) to

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40% (FIT-DNA) of advanced adenomas (41, 42). Successful use of these tests is also highly dependent on compliance. FIT is the most common currently used noninvasive stool test world-wide, and is often the first step in two-step population-based screening. This is to some extent due to its relatively low per-test cost. FIT is currently recommended as a first-tier test by the U.S. Multi-society Task Force (USMSTF) on Colorectal Cancer (5), and is the comparator for most studies aimed at validating other noninvasive markers. Single-application FIT test have moderate to high sensitivity and specificity for colorectal cancer depending on the threshold for positivity employed, but sensitivity for advanced adenomas is low, regardless of threshold (43). A molecular approach to colorectal cancer screening is attractive because it targets biological changes that are fundamental to the neoplastic process. The feasibility of detecting altered DNA in stool has been demonstrated using a multitarget assay panel of molecular markers. The DeeP-C study of stool DNA testing for colorectal cancer in almost 10,000 average-risk individuals showed sensitivity of a panel, which included several DNA markers plus FIT for detecting colorectal cancer, was 92.3% compared with 73.8% for FIT alone (with colonoscopy as the standard). The sensitivity for detecting advanced precancerous lesions [advanced adenomas or sessile serrated adenomas (SSA) ≥1 cm] was 42.4% for the panel compared with 23.8% for FIT alone. Specificities with DNA and FIT were 86.6% and 94.9%, respectively, among participants with non-advanced or negative finding on colonoscopy (41). The multi-targeted stool FIT-DNA test (Cologuard, Exact Sciences) is FDA and Center for Medicare and Medicaid Services (CMS) approved as a colorectal cancer screening test and is included as part of USMSTF guidelines (every 3 years; tier 2 recommendation). Modeling analysis conducted for the American Cancer Society (ACS) did not categorize it as a model-recommended test because of the high number of colonoscopies required per life-years gained. The GLNE 10 trial described above was designed to compare EDRN-discovered biomarkers with both FIT and FIT-DNA.

Blood-based markers

Serum or plasma markers have been considered an important "next step" for the successful implementation of population-based screening for colorectal neoplasia, but blood-based markers have, to date, proven inadequate for this purpose. While any screening test must demonstrate high positive and negative predictive values in clinically relevant settings, blood-based markers are attractive in several ways. Biospecimens are easy to sample and control for reproducibility, can be combined to enhance performance, and should lead to high-compliance, cost-effective tests. Blood-based biomarkers also have good potential for use in low-compliance or underserved populations, and in some cases may lead to easily administered point-of-care tests. Blood tests are also noninvasive compared with endoscopy, and are likely to prove more socially acceptable compared with stool-based tests. An integrated signal in blood should make it possible to detect both proximal (right sided) and distal lesions. Noninvasive tests have proven useful in individuals refusing other forms of screening, and may be valuable as reflex tests following inadequate colonoscopy. While existence of an acceptable blood-based biomarker would of course not result in 100% compliance by screen-eligible individuals, the target of unscreened individuals is substantial. If one were to invoke United States Preventive Services Task Force criteria for colorectal cancer screening (6) this represents approximately 30 million individuals in the United States. If such a blood test replaces other available noninvasive tests (e.g., FIT) this would rise to approximately 40 million individuals. This will likely increase due to revisions in ACS guidelines,

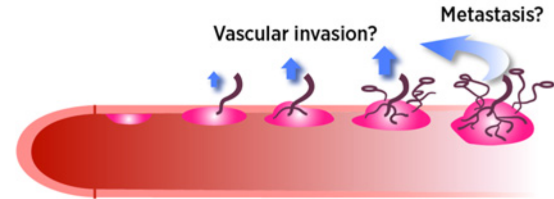

Figure 1.
Sources of tumor markers in the blood. Tumor-related biomarkers may reach the blood via a number of mechanisms, including tumor necrosis, apoptosis, lysis of circulating tumor cells, active release, secretion, or release of vesicles such as exosomes or apoptotic bodies. A variety of marker types may be detected in blood, including tumor cells, protein and glycoproteins, DNA, RNA, and miRNAs. Other markers may represent secondary effects, such as activated lymphocytes. It is unclear whether colorectal neoplasms require invasion to yield circulating markers.

which recommend average-risk screening begin at age 40 (7). There are compelling reasons to believe that these represent low estimates given the significant pressure from a cost perspective to reduce colonoscopy as an initial screening modality, and pressures to increase compliance in underserved populations.

It remains to be determined whether colorectal neoplasms must be invasive to yield detectable circulating markers (**Fig. 1**). This is relevant to the potential of blood-based markers to detect precursor lesions such as advanced adenomas or early-stage cancers. There are several potential mechanisms by which tumor markers may enter the blood stream. Cell surface markers may be released after proteolytic cleavage or cell lysis. Membrane bound proteins or glycoproteins may also be released after engagement by specific ligands, while other molecules may be actively secreted. Tumors may also shed vesicles as exosomes after outward budding of the plasma membrane and release into the extracellular space. Exosomes may contain tumor-derived proteins and transport oncogenes and onco- miRNAs. Other membrane vesicles include apoptotic bodies, created via bleb formation of the membrane of dying cells. Paramount to the use of blood-based markers for colorectal cancer screening and surveillance is the ability of relevant markers associated with advanced adenomas or early-stage cancers to reach the circulation.

A large number of blood-based markers have been proposed for early detection of colorectal neoplasia, but few have been evaluated in adequately powered prospective screening trials. Many reports utilize biospecimens from patients with predominantly late-stage cancers (44). In a pivotal prospective trial including almost 8,000 subjects, methylated Septin 9 (*SEPT9*) detected colorectal cancer with 48% sensitivity and 92% specificity (45). The sensitivity of this test decreased to 35% for stage I disease, 63% for stage II disease, 46% for stage III disease, and 11% for advanced adenoma. The overall sensitivity for detecting colorectal cancer was superior to guaiac-based fecal occult blood testing, but less than that of FIT. An assay for Septin 9 is commercially available and FDA approved for individuals refusing other screening tests, but is not guideline recommended (5–7).

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Several promising blood-based marker assays are on the horizon from EDRN investigators and collaborators.

Galectin-3 ligand

The galectins are widely distributed and evolutionarily conserved carbohydrate-binding proteins characterized by their binding affinity for β-galactosides. Galectin-3 plays a functional role in the progression and metastasis of several forms of cancer, and is elevated in the blood of patients with colorectal cancer. The predominant galectin-3 ligand detected in serum is a 40 kDa glycoprotein identified by MALDI-MS as an aberrantly glycosylated form of haptoglobin produced by neoplastic cells in the colon (46). Aberrant glycosylation of common proteins is an increasingly recognized theme in biomarker development. Preliminary validation across a variety of disease states indicated that this protein is present at levels 30-fold higher in patients with colorectal cancer versus normal controls. Galectin-3 ligand is stable for long periods in blood and the assay can be performed on volumes of 5 μL or less. An independent set of blinded serum from the EDRN repository (protocol GLNE 007) were assayed for galectin-3 ligand using an ELISA developed in our laboratory as well as a companion Luminex-type bead assay run in an independent EDRN validation laboratory. Galectin-3 ligand differentiated normal individuals from those with all stages of cancer and advanced adenomas with blinded verification across sample sets (AUCs vs. normal for the EDRN 007 sample set include 0.80 all cancer, 0.84 stages III + IV cancer, 0.77 stages I + II cancer, and 0.78 advanced adenomas). These results were confirmed using blinded samples from the Endoscopy II trial described previously. In collaboration with EDRN investigators and industrial collaborators we evaluated several additional serum markers previously shown to be elevated in the blood of patients with colorectal cancer in combination with galectin-3 ligand (47). Pairwise correlation and modeling informed final markers chosen for a multiplex assay. With search of all combinations using logistic regression, the best models were developed for discriminating individuals with cancer from normal. A combined test score derived from individual markers higher than a minimally accepted value preset at an AUC of 0.89 for colorectal cancer was chosen to meet that obtained with FIT. A goal of 45% sensitivity at a threshold of 90% specificity was prespecified for detecting advanced adenomas on the basis of the reported 23.8% sensitivity (AUC 0.67) for FIT and 42.4% (AUC 0.73) for Cologuard for detecting "advanced precancerous lesions." Models with 2, 3, or 4 marker combinations performed similarly in differentiating cancer from normal and met prespecified criterion (AUCs ranged from 0.87 to 0.90). Sensitivities (at 90% specificity) were up to 70% for early-stage cancer and 88% for late-stage cancer. All models also met our prespecified criteria for detecting advanced adenoma and screen relevant neoplasia (cancer + advanced adenoma). The four marker panel for example yielded (at 90% specificity) sensitivities of 56% for advanced adenoma (64% at 85% specificity) and 68% for screen-relevant neoplasia compared with 23.8% for FIT and 42.4% for Cologuard. Optimal cutoffs were also determined for use in a binary (positive vs. negative) test. The galectin-3 ligand assay was recently converted to a lateral flow format toward developing a point-of-care assay which can be used in low-resource underserved populations. Two patents have been issued by the U.S. patent Office based on this work (UTSC.P1049US and UTSB.P1049USC1). Galectin-3 ligand is currently being evaluated using the large GLNE 10 sample set, and in collaborative studies using Endoscopy II and Endoscopy III samples (multimarker analyses).

Multimarker protein/glycoprotein panel

The Lampe laboratory created a high-density antibody microarray to detect differences in protein levels in plasma from individuals diagnosed with colon cancer up to 3 years after blood was drawn (i.e., prediagnostic from the Cardiovascular Health Study) and cancer-free, matched controls (48). Of the 78 significantly (t test, $P < 0.015$ and AUC > 0.6) increased proteins, 32 were confirmed using plasma samples from the EDRN collaborative group project 1 that were from people diagnosed with adenoma or cancer, compared with controls (i.e., GLNE 07). Components of an optimal five-marker panel (BAG4, IL6ST, VWF, and CD44 or EGFR) were tested via immunoblotting using a third sample set, and confirmed via Luminex assay in a large fourth sample set. Inclusion of EGFR and CD44 sialyl Lewis-A and –X content increased the panel performance. The protein/glycoprotein panel was statistically significantly higher in colon cancer samples, characterized by a range of AUCs from 0.90 [95% confidence interval (CI), 0.82–0.98] to 0.86 (95% CI, 0.83–0.88), for the larger second and fourth sets, respectively. IHC on tissue microarrays showed increased levels of BAG4, IL6ST, and CD44 in adenoma and cancer tissues. These results constitute the proposed markers contributed by the Lampe laboratory for the ongoing EDRN collaborative group project with members R.S. Bresalier (galectin-3 ligand), H.J. Nielsen (Endoscopy III), and industry collaborators.

Multimarker protein panel

Eight cancer-associated protein biomarkers in plasma from symptomatic subjects undergoing first-time colonoscopy (Endoscopy II) were assayed using the Abbott ARCHITECT platform (AFP, CA19-9, CEA, hs-CRP, CyFra21-1, ferritin, galectin-3, and TIMP-1; ref. 19). Multivariate regression was used to combine markers and develop models. Primary endpoints were colorectal cancer + high-risk adenomas and colorectal cancer. AUCs were 0.76 and 0.84, respectively, for a four-marker model. While the negative predictive values were >90%, positive predictive values were 25% for endpoint 1 and 18% for endpoint 2.

Mucins and mucin-associated glycoproteins

Aberrant posttranslational glycosylation is a common theme during carcinogenesis. Mucins are the most abundant glycoproteins in the colon and serve as a first-line of innate defense for the protection of the gastrointestinal tract against multitude of factors including digested food, acid, enzymes, and bacteria (49, 50). With over 20 identified members, the mucin family contains both secreted, that is, gel-forming and nongel forming and membrane-tethered proteins (50). Alterations in composition, expression, and localization of mucins are observed across both benign and malignant phenotypes in the colon, and have been a focus of EDRN member S.K. Batra and colleagues. In conjunction with expression and localization changes in members of mucins family, mucins differential expression and altered glycosylation facilitate tumor initiation, proliferation, and metastasis (51). Several studies have investigated the expression of mucins, including MUC1, MUC2, MUC4, MUC5AC, MUC6, and MUC17 and their associated glycans during the colon adenoma-carcinoma progression from both biomarker and mechanistic perspective.

The Cancer Genome Atlas database analyses of mucins across colon cancer has revealed differential expression of mucins MUC1, MUC2, MUC4, MUC5AC, MUC6, and MUC17. Forty- to 140-fold differential expression of secreted mucins MUC5AC and MUC6 is observed in colon cancer cases in comparison with normal tissues. Significant alterations in expressions of mucins including MUC2, MUC4,

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MUC5AC, MUC17, as well as mucins-associated glycans including Tn/STn-MUC1 have been observed across benign versus malignant pathologies of the colon using IHC on tissue microarrays (52). Interestingly, pathologic changes in the progression from normal to adenoma and adenocarcinoma are accompanied by the loss of normal colonic mucins including secreted mucin MUC2 and transmembrane mucin MUC4. Notably, adenoma and adenocarcinoma development is accompanied by elevated expression of MUC1 and MUC5AC (52). In addition to transmembrane mucin MUC4, transmembrane mucin MUC17 is a characteristic mucin of colon, and is highly expressed both on the surface epithelium as well as colonic crypts. In contrast to MUC1 and MUC5AC, loss of MUC17 is observed during inflammation, while its elevated expression is observed in colon adenomas and adenocarcinomas. Similarly, loss of MUC17 is observed during colorectal cancer progression and with inflammation (53). The human recombinant protein from extracellular regions of MUC17 containing two EGF-like Cys-rich domains (CRD1 and CRD2) exhibits antiapoptotic and promigratory activity, facilitates cellular restitution, and has potential as a therapeutic candidate for colitis-associated injury of colon (54). Elevated expression of MUC1-specific glycan-Tn/STn-MUC1 is also expressed in adenomas and adenocarcinomas compared with the normal colon (52). Patients with early-stage tumors (I and II), which express high levels of MUC4, have shorter disease-specific survival (log-rank, $P = 0.007$) than those with low expression (55).

Mechanistic analyses using colorectal cancer cell lines (LS180, HCT-8, and HCT116) indicate that suppression of Hath1 by the activated Wnt/β-catenin pathway causes suppression of MUC4 expression in colorectal cancer (56). Muc4-knockout (KO) mice are phenotypically normal and exhibit no visible changes on the glycan or carbohydrate content of colon (57). Muc4-KO C57BL/6J animals exhibit increased resistance to DSS-induced colitis in comparison with wild-type controls. AOM-DSS–treated Muc4$^{-/-}$ mice also exhibit fewer and smaller tumor nodules after carcinogen exposure, suggesting a role of Muc4 in chemical-induced colitis and carcinogenesis in the colon.

MUC1 and MUC5AC mucins and associated glycans Tn/STn on MUC1, and CA19-9 are more highly expressed in various precursor lesions in the colon compared with adjacent normal tissues. Reduced expression of MUC4 and elevated expression of MUC5AC and MUC17 has been observed in SSA/polyps (SSA/P) in comparison with hyperplastic polyps (HP; ref. 58). Similar to colon cancers, loss of MUC4 is observed across SSA/P's, while MUC17 and MUC5AC show significantly elevated expression. Tubular adenomas exhibit higher expression of MUC1 and lower expression of MUC17, MUC5AC, and CA19.9 in comparison with SSA/P. In addition to alterations in expression levels, mucins MUC1 and MUC4 also exhibit differential localization pattern across polyp types. MUC5AC expression is restricted to goblet cells in HP while both the goblet cells and the lumen of the colon crypt are positive for MUC5AC in the SSA/Ps. MUC4 expression is observed in the whole crypt except the luminal surface epithelium in normal colonic epithelium, while MUC4 expression is observed throughout colon crypt including luminal surface epithelium for HPs and tubular adenomas. Notably, MUC4 expression is restricted to the lower one-third of colonic crypts. In multivariate regression models in conjunction with ROC curve analysis, the combination of MUC17/MUC5AC effectively discriminated SSA/Ps from HPs. The three-marker combination (CA 19-9, MUC17, and MUC5AC) emerged as combinatorial panel for discriminating polyp subtypes and accurately classifying colorectal cancer polyps. Current surveillance recommendations depend on the histologic type of colonic polyps, suggesting that that CA 19-9, MUC17, and MUC5AC histologic analyses could provide unique tools for differentiating various polyp subtypes and reducing interobserver variability for classifying colorectal cancer polyps.

Alterations in protein glycosylation, including those associated with mucins can be detected in blood, and several EDRN investigators are currently examining whether the alterations associated with tumor progression observed in tissues described above, can be translated to noninvasive biomarkers for early-cancer detection.

Other cell-free DNA tests

The term "liquid biopsy" [see also Cohen and colleagues (59) in this *CEBP Focus*] most often refers to the use of circulating cell-free DNA to detect neoplasia. Most cell-free DNA in plasma is derived from leukocytes, but cancers can release sufficient DNA into the circulation that can be detected by sensitive digital technologies such as massively parallel sequencing. Point mutations, small insertions and deletions, alterations in DNA fragment sizes or copy numbers, translocations, and epigenetic changes can be detected. Plasma from normal individuals or those with early-stage cancer contain between 3 and 9 ng of cell-free DNA per mL of plasma, while patients with advanced cancer may have as much as a 10-fold increase in cell-free DNA (60). The fraction of mutant DNA, however, is still less than 10% of the total templates. Tests employing alterations in circulating DNA (ctDNA) therefore tend to be highly specific but have limits with regard to sensitivity if used as single-marker stand-alone tests. Traditionally, relatively large blood samples have also been need, but this has improved with technological advances. Understanding the limitations of single marker ctDNA tests, EDRN member Dr. Kinzler and others developed a multianalyte test "Cancer-Seek" (61). Multiplex PCR analysis of ctDNA detects mutations at 1,933 genomic positions across 16 genes, and eight cancer-associated proteins (CA-125, CEA, CA-19-9, prolactin, hepatocyte growth factor, osteopontin, myeloperoxidase, and TIMP-1) are measured using immunoassays. This test was evaluated using samples from 1,005 patients with nonmetastatic cancers of the ovary, liver, stomach, pancreas, esophagus, colorectum, lung, and breast. CancerSEEK was positive in a median of 70% of the eight cancer types with sensitivities ranging from 69% to 98% (about 65% for colorectum). The specificity was greater than 99%. While the patient cohort studied was composed of individuals with known cancers, controls were comprised of health individuals, and test cases were not entirely independent, this multianalyte test shows great promise for translation to clinical practice. Additional studies are being performed involving several EDRN members.

BCAT1 and IKZF1

BCAT1 (branched chain amino acid transferase 1) and *IKZF1* (*IKAROS* family zinc finger) are methylated DNA markers involved in tumor growth and invasiveness of several cancers, including colorectal cancer. Methylation of *BCAT1* and *IKZF1* can be found in both tissue and serum of patients with colorectal cancer (62–64), and these markers comprise components of a blood-based panel currently being evaluated by corporate collaborator Clinical Genomics in conjunction with EDRN investigators, plans to use samples from GLNE 007 and GLNE 10.

Nucleosomes

Nucleosomes consist of small DNA chains of approximately 147 base pairs wrapped around a histone octamer that contains pairs of

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H2A, H2B, H3, and H4 proteins. Nucleosomes are bound together with linker DNA, linker histones, and other nonhistone proteins to form intracellular chromatin. Changes in the levels of cell-free circulating nucleosomes have potential as biomarkers for detection of colorectal cancer. These could serve as markers for early detection of colorectal cancer (65). Collaborative studies are ongoing using the EDRN biorepositories described previously.

Translation of Biomarkers to Clinical Practice: How Good Is Good Enough?

While a large number of blood-based markers have been proposed for colorectal cancer screening, few have been translated to clinical practice. Some of the difficulties in translating biomarkers to clinical practice have been discussed above (**Table 1**). The majority of markers which have shown promise in laboratory-based and small-clinical studies fall short when subjected to further scrutiny. Biomarker research is a phased process which includes a discovery (identifying promising markers), progressive validation (evaluate the performance of markers in the intended clinical setting), and a clinical impact phase (does the marker improve patient outcomes?; refs. 14, 15). The requirement for scientific stringency and the burden of proof increases with each phase. Compliance by eligible individuals is important to the success of any screening program, but acceptance by health care providers and the health care system is also a consideration. Colonoscopy remains the dominant screening modality for colorectal cancer in the United States, but may not be a practical "gold standard" elsewhere. The issue is "how good is good enough?" (**Fig. 2**). Population-based screening programs most often utilize a two-stage approach where an initial positive test (in most cases FIT) triggers a more definitive test (colonoscopy). A new noninvasive test, while unlikely to be as sensitive and specific as colonoscopy, as a first-step test may need only to be as good as other currently accepted alternatives. The potential role of a given biomarker-based test will depend on the setting in which it is offered, population rates of screening uptake and compliance, rates of recommended follow-up after an abnormal test, and of course cost.

As clinically relevant biomarkers emerge, several other questions will need to be answered. It seems clear that no one biomarker alone will have sufficient performance characteristics to be effective, and that combinations of biomarkers hold the most promise. What then is the optimal way to combine results from different biomarkers and different classes of biomarkers into a single discriminator? What is the optimal, or at least acceptable combination of sensitivity and specificity to be considered a "good" biomarker for early-cancer detection? Should the results of quantitative tests be reported as positive or negative based on a single cutoff? Will markers be generalizable to different molecular subtypes of colorectal cancer? How often should noninvasive markers be used as tests for colorectal cancer screening or surveillance? Should compliance be a factor in assessing the value of a screening test (efficacy = acceptability × accuracy)?

Figure 2.
Colorectal cancer screening: how good should a test be? The clinical utility of a screening test depends on a number of factors, including test performance (sensitivity and specificity), benefit as a test for opportunistic versus population-based programmatic screening, and resources (cost).

Conclusions

NCI's EDRN represents a model for discovery and validation of promising biomarkers for early detection of colorectal neoplasia. Efforts of EDRN investigators have led to biorepositories of high-quality biospecimens for both discovery and validation. Several promising markers and marker panels have been developed and studied by EDRN investigators, some progressing toward clinical validation. The EDRN represents evidence that publically funded agencies, working with academic investigators and industrial partners, can promote high-quality, unbiased, adequately powered research (including prospective trials), with collaboration toward a common goal of moving noninvasive biomarkers forward to clinical practice.

Disclosure of Potential Conflicts of Interest

W.M. Grady is an advisory board member for Freenome and SEngine, is a consultant for Guardant Health and DiaCarta, and reports receiving a commercial research grant from Janssen. S.D. Markowitz is a board member and consultant for Lucid Diagnostics, reports receiving a commercial research grant from Lucid Diagnostics, and has ownership interest (including patents) in Lucid Diagnostics and Exact Sciences. P.D. Lampe has ownership interest in a patent. No potential conflicts of interest were disclosed by the other authors.

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