Biomarkers in Colorectal Cancer Screening

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Abstract

Colorectal cancer (CRC) is the third most common cause of cancer death in men and women in the United States. The main goals of screening are to prevent carcinogenesis (via adenoma detection and removal) and detect cancer at an early, curable stage. CRC mortality is steadily dropping in the United States, partly because of greater screening utilization. However, nearly 1 in 3 average-risk people are not up to date with standard CRC screening recommendations. This review surveys a wide range of CRC biomarkers in various stages of development, which may offer attractive risk stratification tools; a few have reached the commercial stage. If widely accepted, these tools may contribute to shift CRC screening practices away from 1-step colonoscopy to a 2-step risk stratification process of predictive biomarker measurements followed by colonoscopy for lower-risk patients with a positive result. Such strategies could potentially increase the rate of CRC screening.

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe advances in biomarker technology as risk stratification tools for CRC screening
- Evaluate the efficacy, safety, convenience, and cost of stool, imaging, and endoscopy-based CRC screening methods

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Colorectal cancer (CRC) is the third most common cause of cancer death in men and women in the United States.¹ The lengthy process of adenoma-to-carcinoma progression facilitates the positive impact of CRC screening through 2 mechanisms: (1) prevention of cancer through adenoma detection and subsequent removal, and (2) detection of CRCs that are typically at an earlier stage than those detected mainly by symptoms. Various screening strategies, including fecal occult blood testing (FOBT), sigmoidoscopy, or colonoscopy, reduce CRC incidence by 52% to 81% and mortality by 65% to 84%.² Screen-detected CRC has an approximately 90% 5-year survival rate.³

Despite improving over time, only 65% of the average-risk US population is regularly in compliance with screening recommendations.⁴ The ideal cancer screening test would be effective, safe, convenient and inexpensive. No currently available stool, imaging, or endoscopy-based method of CRC screening fits all of these criteria. Because colonoscopy is relatively expensive and carries more risks than other screening tests, models have been developed to stratify CRC risk in asymptomatic individuals.⁵ These scores identify lower-risk patients for 2-step screening strategies (positive biomarkers then colonoscopy) and higher-risk patients for direct 1-step colonoscopy. Advances in biotechnology offer an array of methods to detect minute amounts of potentially relevant DNA, RNA, or protein disease markers in various bodily fluids in asymptomatic patients. This review explores how some of these new methods might serve as risk stratification tools relevant to CRC screening.

Stool-Based Biomarkers

Hemoglobin Detection

Despite their low sensitivity (30%–50%) and relatively low specificity (80%–90%) for advanced polyps and cancers, stool guaiac-based tests (gFOBT) are still widely used.⁶ Their simplicity, low cost, and demonstrated benefit when used programmatically make gFOBT an alternative included in all national and international CRC screening guidelines. Fecal immunochemical testing (FIT), which uses a different reaction to specifically identify human hemoglobin from the lower gastrointestinal tract⁷ in stool, has largely supplanted gFOBT because of increased sensitivity without loss of specificity.⁶ For CRC, the

overall sensitivity was 89% with a specificity of 91% based on a FIT cutoff value of 20 mcg/g.⁸ Generally, patients prefer FIT to gFOBT because only one stool sample is required and no diet or medication alterations are needed.⁷ FIT results can be quantified, which is useful when colonoscopy follow-up capability is limited.

The sensitivity of FIT is higher for distal cancers than proximal ones and is lower than that of colonoscopy.^{9,10} Although FIT performed less well than colonoscopy in the detection of advanced adenomas, its greater acceptance rate by patients may offset this difference.^{11,12} Patient choice is an important contributor to screening compliance. Overall, screening rates are highest when patients self-select a screening method (FOBT vs colonoscopy) versus when they are prescribed only one option.¹³

Genetics of CRC

Other types of fecal biomarkers—DNAs, RNAs, and nonheme proteins—have been evaluated as test components to identify colonic neoplasia. So far, only DNA biomarkers have been extensively tested and approved for clinical use.

To interpret the results of fecal DNA tests requires an understanding of the genetic and epigenetic changes that drive colorectal carcinogenesis. Stool DNA testing is based on the detection of genetic and epigenetic changes in tumor cells that exfoliate into the mucocellular layer in the colonic lumen.^{14,15} There are at least 3 distinct pathways to colon cancer, each with genetic and epigenetic components: the chromosomal instability pathway (CIN) and the microsatellite instability (MSI) pathway, both of which demonstrate the standard adenoma-carcinoma transformation; and the serrated polyp or CpG island methylator phenotype (CIMP) pathway. This third pathway explains why some polyps, typically in the right colon, progress from sessile serrated adenoma (SSA) to carcinoma.

Most CRC (65%–70%) arises via the CIN pathway, with adenomas as precursor lesions. The pathway is characterized by defects in chromosomal segregation, telomere stability, and the DNA damage response, leading to chromosomal imbalance (aneuploidy) and a high frequency of loss of heterozygosity (LOH). In 1990, Vogelstein et al¹⁶ first proposed a specific sequence of mutations in the APC, Kras, and p53 genes in colon carcinogenesis. However, mul-

multiple alternative CIN pathways exist involving even more complex and heterogeneous patterns of gene mutations.¹⁷

MSI reflects the loss of DNA mismatch repair capacity and is found in 10% to 15% of all CRCs. In Lynch syndrome (hereditary nonpolyposis colorectal cancer), mutations in crucial DNA repair enzyme genes, such as *MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EP-CAM*, can allow DNA copy errors to go uncorrected. These errors will sometimes affect growth-regulating genes, leading to cancer development.

SSAs are the precursor lesions of the serrated neoplasia pathway. This pathway develops through the accumulation of insertion or deletion mutations throughout the genome, leading to genomic instability, which in most cases leads to MSI-high (MSI-H) adenocarcinomas.¹⁸ The *BRAF* mutation and hypermethylated *SLC5A8* play crucial roles. Progressive loss of cell cycle control and decreased and altered cytoplasmic differentiation produce the characteristic morphologic changes of SSAs.¹⁹

Despite attempts to delineate separate pathways for CRC, overlapping features of multiple pathways are often found in one tumor.^{20,21} Mutant genes such as *APC* and *TGFBR2/SMAD4* are common in most CRCs, suggesting a central role in colon carcinogenesis.¹⁵

Epigenetics of CRC

Epigenetics broadly refers to heritable alterations in gene expression not due to DNA mutations. Together with gene mutations, epigenetic mechanisms are common contributors to the formation of colon polyps and cancer. Epigenetic mechanisms currently believed to be important in CRC are (1) DNA methylation of cytosine bases in CpG Islands; (2) posttranslational modifications of histones, which are proteins tightly interwoven with DNA to form chromatin; (3) microRNAs (miRNAs) and other noncoding RNAs; and (4) nucleosome positioning.²²

Aberrant DNA methylation, the most extensively studied form of epigenetic deregulation, is found in virtually all CRCs. The average CRC methylome has thousands of abnormally methylated genes. Common epigenetic alterations in *APC*, *CTNNB1*, *KRAS*, *BRAF*, *SMAD4*, *TGFBR2*, *TP53*, *PIK3CA*, *ARID1A*, *SOX9*, *FAM123B*, and *ERBB2* genes disturb key signaling pathways or affect genes that regulate DNA repair and cellular proliferation.^{23,24} Epigenetic changes appear to occur

very early in the polyp-to-cancer sequence, making them ideal screening biomarkers. In addition, these epigenetic alterations can occur in histologically normal-appearing mucosa, offering the potential for early identification of higher-risk individuals.²⁵

Stool DNA Methylation Markers Alone

Stool DNA methylation biomarkers have multiple advantages over similar blood biomarkers. Aberrant DNA methylation in the blood may signal the presence of different cancers at the same time.²⁶ In addition, early CRC or precursor lesions may not yet release markers into the bloodstream. Thus, fecal DNA markers may be more useful in CRC screening. Methylated fibrillin-1 (*mFBN1*)²⁶ and vimentin (*VIM*)²⁷ in feces can be identified in early-stage CRC. In addition, numerous hypermethylated genes have been detected in human stool more readily than from blood samples. Methylated progesterone receptor (*mPGR*) and O⁶-methylguanine-DNA methyltransferase (*mMGMT*) are 2 examples of exclusive fecal biomarkers.²⁶

Stool DNA Mutation Markers Alone

The initial proof-of-concept study looking for abnormalities in exfoliated cells collected from stool samples used a DNA panel with 21 mutations (3 in *Kras*, 10 in *APC*, and 8 in *p53*) along with the MSI marker BAT-26 and a marker (long DNA) thought to reflect disordered neoplastic apoptosis.²⁸ When compared with gFOBT, the fecal DNA panel outperformed by detecting advanced colonic lesions, including cancer in 40.8% versus 14.1% ($P < .001$) of cases without compromising specificity (95%). Despite this difference, most neoplastic lesions identified by colonoscopy were not detected by either noninvasive test.

Stool Multitarget Marker Panel

Two large multicenter case-control studies evaluated multitarget stool DNA (MT-sDNA) performance. The first-generation prototype test detected mutant *KRAS*; aberrantly methylated *VIM*, *NDRG4*, *BMP3*, and *TFPI2*; β -actin (reference gene for human DNA quantity); and hemoglobin. Its sensitivity for early-stage CRC was 87%, and for adenomas it was 54% to 92%, depending on size.²⁹ A later, more-refined automated MT-sDNA, which detected mutant *KRAS*, aberrantly methylated *BMP3* and *NDRG4*, β -actin,

and hemoglobin, has a sensitivity of 95% to 97% for CRCs and 57% to 83% for adenomas, depending on size.³⁰

These favorable results eventually led to FDA approval for the refined MT-sDNA test in 2014, with a 3-year testing interval based on a large cross-sectional study in 90 centers in North America.³¹ The overall sensitivity was 93% for curable CRC, and 42% to 66% for adenomas and SSAs, depending on polyp size and grade, with a specificity of 87% to 90%. Clinically, MT-sDNA outperforms gFOBT/FIT but is considerably more costly. Currently, there are no firm recommendations about the evaluation of patients with positive results but a negative colonoscopy, or compelling data suggesting that this test is cost-effective compared with alternative screening strategies.

Stool Protein and RNA Tests

The use of fecal nonheme proteins and RNAs as markers for early detection and prediction of CRC outcome has gained increasing attention. Fecal proteins such as calprotectin³² and tumor M2 pyruvate kinase (M2-PK)^{33,34} have been studied as CRC markers, but neither has been found to be superior to FIT. These studies have thus far remained at the proof-of-principle level.³⁵

Gut Microbiome

Microbial dysbiosis, a pathologic imbalance in the gut flora, has been implicated in colonic tumorigenesis. Gut bacterial composition in patients with CRC differed significantly from that in healthy patients.^{36,37} Certain types of bacterial species, such as *Fusobacterium*, *Bacteroides*, *Parvimonas*, and *Solobacterium*, appeared to have a strong co-occurrence network in patients with CRC.³⁸ These microbial networks can be detected across ethnic and international boundaries. Some of their genes were enriched in early-stage CRC, suggesting a potential role as CRC biomarkers.^{38,39} A causal relationship between continual alterations in the gut microbiome and CRC development was demonstrated in a colitis-driven colon cancer mouse model.⁴⁰ Despite these promising findings, host–bacterial interactions remain ill-defined. Mucosa-associated microbiome and fecal microbiome differ, and currently there is no consensus regarding which one to use in CRC screening.

Blood-Based CRC Biomarkers

A clinically effective blood test for CRC screening would represent a substantial advance. It could be performed with minimal risk and potentially greater patient acceptance. Like any CRC risk stratification tool, a blood test would reduce over-reliance on colonoscopy, an expensive and riskier procedure. The main problem is to identify molecules that are sensitive and specific enough for CRC detection. Unlike stool-based tests, in which marker detection uses exfoliated cells in feces, detection of abnormal cells and associated markers in the systematic blood supply is more complicated. Blood-based tests require either blood vessel invasion (usually a characteristic of later stage cancers) or phagocytosis by inflammatory cells of neoplasia-derived markers.

DNA Markers

Aberrant DNA Methylation Markers: Methylated *SEPT9* is a frequently studied marker in CRC screening. The gene is a member of the septin family involved in cytokinesis and cell cycle control. Hypermethylation of *SEPT9* in adenoma and CRC specimens is confined to one of several CpG islands of this gene. For CRC detection, its sensitivity was 77% and specificity was 80%,⁴¹ which was not better than FIT.⁴² In 2016, this test was narrowly FDA-approved.

In another example, Pedersen et al⁴³ showed that a combination of *mBCAT1* and *mIKZF1* detected CRC with a sensitivity 77% (higher than either alone) and a combined specificity of 92.4%. Because *BCAT1* and *IKZF1* genes are both involved in tumor growth and invasiveness, the degree of their hypermethylation and the frequency of their combined detection closely corresponded to CRC stages I–IV, at 50%, 68%, 87%, and 100%, respectively.⁴³

Other DNA Markers: Other circulating tumor-related molecules, such as aberrant DNA markers, microsatellite alterations markers, and mitochondrial DNA, are currently very difficult to detect against the background of all other circulating DNA,⁴⁴ especially in early-stage CRC, making them unsuitable as screening biomarkers.

RNA Markers

Various forms of RNA, such as messenger RNA (mRNA), noncoding miRNA, and long noncoding RNA (lncRNA), are very stable outside of cells and

Table 1. Comparison of CRC Screening

Test	Type	Preparation	Sensitivity	Specificity	References
gFOBT	Stool	No	30%–50%	80%	6,10,62,63
FIT	Stool	No	65%–70%	85%	7–10,12
Colonoscopy	Procedure	Yes	95%–98%	90%	44,64,65
CT colonography	Radiology	Yes	84% (AA ≥6 mm)	90%	66,67
Low-coherence enhanced backscattering spectroscopy	Procedure	Yes	88% AA 71% nAA	72%	61
MT-sDNA (heme, DNA methylation, and mutation panel)	Stool	No	85% CRC 55% AA	90% CRC 89% AA	31
mRNA 7-gene panel	Blood	No	78%	66%	46
miRNA 5-gene panel	Blood	No	≈83%–91%	≈70%–95%	44
lncRNA 1-gene	Blood	No	N/A	N/A	44
DNA methylation					
SEPT9	Blood	No	77%	80%	41,42
BCAT1/IKZF1	Blood	No	77%	92.4%	43
VIM	Stool	No	72%–77%	83%–94%	27

Abbreviations: AA, advanced adenoma; CRC, colorectal cancer; FIT, fecal immunochemical testing; gFOBT, guaiac fecal occult blood test; lncRNA, long noncoding RNA; MT-sDNA, multitarget stool DNA; N/A, not applicable; nAA, nonadvanced adenoma; VIM, vimentin.

can be extracted, safely stored, and studied in various bodily fluids. In cancer, RNA expression becomes highly dysregulated, and therefore RNA is more conducive to profiling studies.

The general experimental strategy initially consisted of mRNA expression microarray profiles, followed by validation using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). This technology is being gradually replaced by next-generation sequencing (NGS). NGS is not only fast and cheaper but it also enables researchers to perform direct RNA sequencing (RNA-seq) to sequence the whole transcriptome (ie, the entire set of coding and noncoding RNAs).⁴⁴

mRNA Markers: In a proof-of-concept study, Tsouma et al⁴⁵ demonstrated that extracted carcinoembryonic antigen (CEA), cytokeratin 20, and epidermal growth factor receptor (EGFR) transcripts from patients with CRC can indicate disease stage and prognosis.

In 2010, Marshall et al⁴⁶ introduced a blood-based 7-gene mRNA biomarker panel as a CRC risk assessment tool. These 7 genes (*ANXA3*, *CLEC4D*, *LMNB1*, *PRRG4*, *TNFAIP6*, *VNN1*, and *IL2RB*) are derived from a 196-gene expression profile using both patients with CRC at various disease stages and

healthy controls, and was later validated in a larger population. The test panel has a sensitivity of 78% and a specificity of 66% (Table 1), and provides a scale assessing a person's current relative risk of having CRC based on gene signature.

miRNA Markers: The roles of miRNAs in regulating posttranslational gene expression in cancer development, growth, and metastasis have been well-established in a large body of research publications.⁴⁷ A single miRNA, a short 18- to 22-nucleotide-long noncoding molecule, can normally target many mRNAs. miRNAs lack complex transcriptional and translational modifications compared with mRNAs and proteins. Almost all cancer types present a unique profile of upregulated and downregulated miRNAs. Thus, these characteristics can potentially make miRNAs very useful cancer biomarkers.⁴⁷ miRNAs can behave as tumor suppressors, oncogenes, or both.⁴⁸

miR-17-3p, miR-92, miR-92a, miR-29a, miR-141, and miR-21 have been associated with advanced CRC and poor prognosis.^{44,49} miR-21 expression levels may be a special pan-cancer marker, because it has been shown to be upregulated in other cancers.⁵⁰ miR-15b, miR-17-3p, miR-18a, miR-20a, miR-21, miR-29a, and miR-92a have been studied by multiple investigators for CRC detection.⁴⁴

However, conflicting data limit their usefulness. Methods for specimen collection, processing, purification, and analysis of RNA must be standardized before miRNA tests can serve as reliable cancer biomarkers.

lncRNA Markers: lncRNAs, molecules 200 nucleotides or longer, regulate gene expression and cellular functions.⁵¹ They appear to play a role in CRC development.^{52–54} Existing data suggest that their modulation can result in anticancer effects.⁵⁵ Thus, they may be more important as a therapeutic rather than diagnostic strategy.

Endoscopy-Based Biomarkers

Although stool- or blood-based methods remain most attractive, substantial efforts continue to be devoted to endoscopy-based screening and/or risk stratification methods. The endoscopic identification of raised lesions in the colorectum is typically straightforward. However, white light–driven detection of flat polyps or lesions can be more difficult, especially in a field of chronically inflamed intestinal mucosa (eg, ulcerative colitis). Topical chromoendoscopy, in which color dyes are applied directly to highlight mucosal variation, or virtual chromoendoscopy, in which light processing software built into high-definition endoscopes accomplishes a similar task, are well described. Standard chromoendoscopy was recently supported as the approach of choice in screening patients with ulcerative colitis.⁵⁶ However, the feasibility of broad use has been questioned. Virtual chromoendoscopy implementation would be simpler; however, evidence to date is mixed about how much this technique augments standard high-definition endoscopy to identify polyps.^{57,58}

Novel endoscopic variations exploit other properties of light shined on intestinal mucosa. For example, light of certain wavelengths, in addition to being absorbed or reflected, can produce either autofluorescence⁵⁹ or stimulate a fluorescent response from exogenously introduced fluorophores.⁶⁰ Variation in fluorescence patterns between normal and abnormal intestinal mucosa, including neoplastic change, are well described. These types of molecular imaging represent areas of intense research, but to date have not yielded clinically important advantages. Finally, some groups have attempted to exploit the possibility of field carcinogenesis, the concept that endogenous

and exogenous factors conspire to create an environment permissive to widespread mutation (here, the entire colorectal mucosa). Techniques to evaluate mucosa for field defect could serve to stratify cancer risk across the entire bowel, not just the segment where sampling was performed. Early efforts with low-coherence enhanced backscattering spectroscopy have provided hopeful results that office-based interrogation of the rectal mucosa can predict the presence of neoplasia throughout the colorectum.⁶¹

Conclusions

This review surveys a wide range of CRC biomarkers in various stages of development. A few biomarker tests have reached the commercial stage. Identification of suitable risk stratification biomarkers could potentially increase the rate of CRC screening. If their predictive values are deemed adequate in specific clinical settings, they may offer attractive alternatives to colonoscopy-predominant screening strategies.

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Posttest Questions

1. CRC detected through screening has approximately a ____ 5-year survival rate.
 - a. 65%
 - b. 80%
 - c. 90%
2. Stool DNA methylation biomarkers have all of the following advances over similar blood biomarkers except:
 - a. Aberrant DNA methylation in the blood may signal the presence of different cancers at the same time
 - b. Early CRC or precursor lesion may not yet release markers into the bloodstream
 - c. mFBN1 and VIM in feces can be identified in early-stage CRC

- d. Numerous hypermethylated genes have been detected in blood samples more readily than in human stool
3. Next-generation sequencing:
 - a. Enables researchers to perform direct RNA-seq to sequence the whole transcriptome
 - b. Is less costly than mRNA expression microarray profiles and validation using RT-qPCR
 - c. Is convenient (eg, faster than RT-qPCR)
 - d. Both a and b
 - e. All of the above