

Review article: the evolution to stool DNA testing for colorectal cancer

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SUMMARY

Despite a variety of screening strategies and recent trends showing death rate stabilization, colorectal cancer still remains the second leading cause of overall cancer death. Current screening tools suffer from performance limitations, low patient acceptability, and marginal reliable access within the health care system. Noninvasive strategies present the lowest risk with the highest potential for patient satisfaction. However, serious implementation barriers exist requiring consistent programmatic screening, strict patient adherence, and poor sensitivity for adenomas. Colonoscopy remains an invasive screening test with the best sensitivity and specificity, but faces large financial costs, manpower requirements,

patient access and adherence. Development of advanced molecular techniques identifying altered DNA markers in exfoliated colonocytes signify early or precancerous growth. Stool-based DNA testing provides an entirely noninvasive population-based screening strategy which patients can perform easier than faecal occult blood testing (FOBT). Large-scale prospective randomized control trials currently pending should help characterize accurate test performance, screening intervals, cost-effectiveness, direct comparison to FOBT and analysis of patient adherence. As tumour development pathways and potential target genes are further elucidated, refinements in multi-assay stool-based DNA testing portend enhanced test characteristics to detect and treat this genetically heterogeneous disease.

INTRODUCTION

Colorectal cancer (CRC) remains the second leading cause of overall cancer death in the United States approaching an estimated 150 000 new cases and 60 000 deaths per year.¹ An average lifetime risk in the population of 6%, a 90% cure rate in early disease (stage 1), and a highly accessible target organ comprise sensible reasons mass CRC screening reduces cancer mortality. Early detection of CRC reduces CRC mortality.² Finding and removing precursor adenomas reduces CRC incidence and, in high risk groups, CRC mortality.³ However, only 37% of cases are diagnosed while still localized,⁴ suggesting that current screening options are

ineffective either because of inadequate sensitivity, low acceptability to patients, or high resource demands limiting widespread availability. The lack of consensus on a single best screening option reflects the limitations of all currently available strategies.

As molecular understanding of disease has enhanced approaches to modern medicine, population-based screening for CRC will similarly evolve. In addition to point mutations in the oncogene *k-ras*, allele losses of tumour suppressor genes are involved in the pathogenesis of CRC. These and other specific DNA alterations further identified in CRC progression became known as the adenoma-carcinoma sequence proposed in 1990.⁵ Thereafter, several investigators recovered mutated DNA from the stool of patients with advanced colorectal neoplasia.⁶ The inability to identify one single mutation expressed uniformly across all colorectal neoplasia

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reflects the genetic heterogeneity of the disease. Targeting multiple DNA mutations has been essential in improving detection rates and false-positive rates have been lowered by using neoplasm-specific markers. Developments in understanding the molecular pathways of colorectal tumorigenesis will lead to modifications that can only further enhance the utility of such a screening modality.

Proponents of stool-based DNA testing describe the sound biological rationale for targeting DNA markers in stool.^{7–12} This review will briefly examine limitations of current screening techniques, summarize the development of stool-based DNA testing and present the data from early clinical studies.

CURRENT SCREENING STRATEGIES

Performance limitations of existing screening practices have resulted in a limited overall impact on CRC mortality. Reliance on symptoms suggestive of CRC can delay diagnosis and have a very low predictive value.¹³ Programmatic faecal occult blood testing (FOBT) every 1–2 years leads to a 15–33% reduction in cumulative CRC mortality.^{2, 14, 15} However, FOBT sensitivity for advanced adenomas and early-stage lesions limits its impact on mortality. Adenomas rarely bleed placing FOBT sensitivities in the low 20% range.¹⁶ False-positive or indeterminate results lead to costly unnecessary and invasive tests. This may explain the failure of doctors to recommend colonoscopy in greater than half of the patients with positive FOBT, markedly reducing screening effectiveness.

Compared with guaiac FOBTs, immunochemical FOBTs (iFOBTs) have higher sensitivity and equivalent specificity.¹⁷ Advantages of iFOBT include fewer specimens needed, less stool handling, and the test specifically detects human haemoglobin requiring no dietary restrictions.¹⁸ Blood released from a supra-colonic source will not cause a falsely positive test. An automated tracking system is used along with this test to ensure that participants are reminded to screen continuously on an annual basis.

Flexible sigmoidoscopy (FS) identifies 50–70% of patients with advanced neoplasms. Four case-control studies found a reduction in distal CRC mortality with the protective effect persisting up to 10 years.^{19–22} Current recommendations for screening CRC include an option for FS every 5 years; however, repeat performance in this unsedated procedure is less preferred over

other methods²³ and refused in up to 10% of patients²⁴ who perceive discomfort. In most series, 50% of significant proximal neoplasms were not associated with a distal marker lesion detectable by FS.^{16, 25} The combination of a single application of FOBT and FS has sensitivity for advanced colonic neoplasia of 77%.²⁶ Repeated annual applications of FOBT would improve sensitivity, but depends on patient and doctor adherence.

Colonoscopy is typically recommended to be performed at 10-year intervals for screening.²⁷ The data supporting this interval come indirectly from sigmoidoscopy studies.¹⁹ Undisputed as the diagnostic gold standard, the ability to additionally perform intervention on pre-malignant lesions seems to make colonoscopy an ideal screening modality. Unfortunately, the available resources, cost, risk, considerable expertise required and inconvenience to the patient limit colonoscopy's appeal as a mass screening tool for CRC. Evidence from several polyp prevention trials demonstrates higher yields for subsequent cancer than would be expected despite a relatively high use of surveillance procedures in follow-up.^{28–30} It is unclear whether these data represent a higher miss rate in the community than that seen in expert series or variable tumour biology with aggressive interval tumours.

Radiographic modalities for screening CRC include double-contrast barium enema (DCBE) and virtual colonoscopy (VC). The DCBE has been recommended as a screening option, but is becoming less commonly utilized by practitioners. DCBE is a cost-effective option, but the low sensitivity for polyps and need for colonoscopic confirmation limits its acceptability for screening.³¹ The main role of DCBE is to visualize the right colon in cases where endoscopic visualization is not attained. VC has attracted some interest with its non-invasive nature and safety. The potential of VC to detect extracolonic disease is a double-edged sword as it adds cost and risk to subjects without any clear-cut evidence of benefit. Initial studies with VC showed mixed results for polyp detection and did not evaluate an average-risk asymptomatic population.^{32–35} Methods using prep-less cleansing techniques may improve acceptability, but sensitivity may be compromised.³⁶ Recent results using a primary three-dimensional approach have shown improved polyp detection 6–10 mm in size prompting serious consideration for mass population screening.³⁷ The appropriate patient population and optimal means of training examiners requires further investigation.

Multi-slice CT scanners and special software present highly resource-intensive implementation obstacles not available in excess supply at this time.

STOOL-BASED DNA TESTING

Rationale

Clearly, there is a need for a more cancer-specific, non-invasive and targeted screening test to directly assess colonic epithelium changes. A successful test must reflect the entire length of the colorectum and be exempt from limiting factors such as patient-perceived discomfort, dietary or medicinal restrictions, and intermittent leaking of markers. If the test was acceptable enough to patients, and cost low, it could be administered more frequently, possibly allowing detection of interval aggressive tumours.

Renewal of colonocytes results in exfoliation of at least 10^{10} cells every 24 h from the colonic surface. These cells resist degradation and can be isolated from dispersed human faeces.³⁸ Stool samples obtained from CRC patients may contain more exfoliated cells or DNA than material from healthy people.³⁹ Known specific DNA mutations previously characterized^{5, 40} in exfoliated cells have been targeted to screen for precursor lesions and malignant disease. Advances in powerful molecular biology techniques, such as polymerase chain reaction (PCR), allow DNA to be amplified a billion-fold. Selection of characteristic markers will continue to evolve as the carcinogenetic pathways are better understood. Interestingly, the amount of faecal human DNA in cancer patients does not correlate with tumour size suggesting either the presence of a field effect or degradation-resistant DNA.⁴¹

Current understandings of carcinogenesis

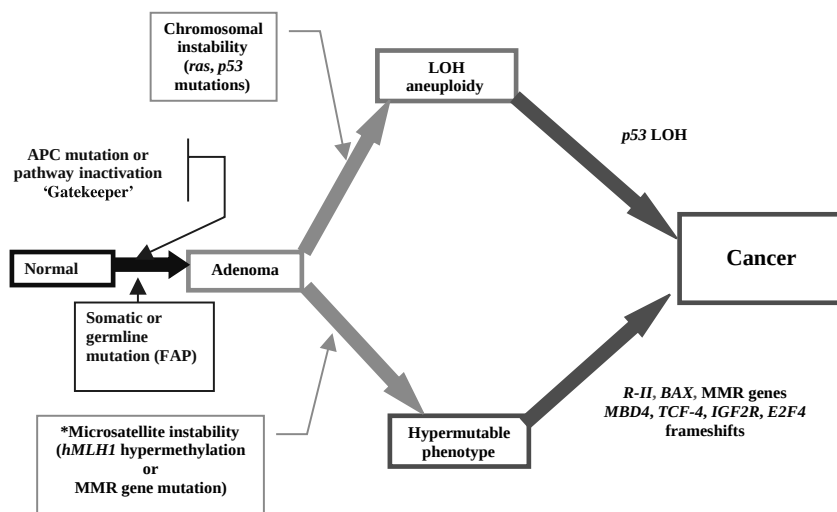
Genomic instability is seen in virtually all CRCs. This disruption of genomic replication creates the necessary alterations for carcinogenesis. While the responsible mechanisms remain unknown, three different pathogenetic pathways are described. Although potentially overlapping, each pathway gives rise to mutations that can be strategically targeted for screening purposes. These processes are likely to be initiated after the loss of a 'gatekeeper' gene, most likely the tumour suppressor APC gene. The APC gene inhibits β -catenin protein, an

important regulator of cellular proliferation and adhesion.

In 80–85% of colorectal neoplasms, point mutations at some loci produce a large number of loss of heterozygosity events. The chromosomal instability pathway was first conceptualized in relation to loss of both alleles in tumour suppressor genes, such as the APC gene. Sporadic mutation in one allele of the APC gene with inactivation of the second allele through genomic instability provides a growth advantage to a cell line allowing a polyp to undergo clonal expansion. Ninety per cent of the mutations occur in the first half of the APC-coding region,⁴² providing predictable specific targets for stool-based DNA testing. However, loss of APC function is not the sole determining factor in chromosomal instability in colorectal adenomas and the existence of multiple independent chromosomal instability pathways has been demonstrated.⁴³

The second pathway known as microsatellite instability (MSI) occurs in 12–15% of CRC patients. MSI was characterized in 1993 by loss of the DNA mismatch repair (MMR) system causing many point mutations and a large number of mutations at simple repeat sequences known as microsatellites. Several key growth regulators are altered in this process. MSI is known to cause mutations in large poly-A regions known as big adenine tracts (BAT) and dinucleotide repeat sequences known as CA-repeats. Targeting these sequences in DNA shed into stool has made it possible to detect MSI-containing cancers. Both of the above pathways lead to CRC and are illustrated in Figure 1.

A small minority of CRC, 2–3%, does not develop through the above two pathways (it may be higher – as the multi-target assay panel (MTAP) is only 80% sensitive on tumour tissue). In 1999, one group demonstrated hypermethylation in promoter regions composed of clusters of cytosine-guanosine residues (CpG islands) could inactivate the genes.⁴⁴ Methylation of tumour suppressor genes, especially APC, has been described in addition to a number of other genes including TIMP-3, HIC-1, PTEN, p16, p14, RARB and MGMT. Even as a distinct pathway, overlap exists when hypermethylation of the hMLH1 gene leads to MSI. Essentially, multiple pathways to CRC exist with each pathway containing its own mechanism of genomic instability. For example, the chronic inflammatory condition of ulcerative colitis may induce MSI through adaptive increases in the base excision repair enzymes.⁴⁵



*NOTE: Whether the two distinct pathways of Chromosomal Instability and Microsatellite Instability diverge before or after an initiating “gatekeeper” event is unclear. The diagram shows that this most commonly occurs with the mutational inactivation of both APC alleles, however APC is usually normal in the Microsatellite Instability pathway.

Figure 1. The chromosomal instability and microsatellite instability pathways for colorectal cancer.

Development

Tumour-derived mutations in oncogenes and suppressor genes provide specific tumour markers. The discovery of these genetic alterations has raised the possibility of detecting colorectal neoplasms through examination of stool DNA from shed colonocytes. The challenge has been to identify these genetic mutational targets by isolating faecal colonocytes and purifying sufficient quantities of human DNA diluted amongst microorganisms, food and mucus. Initial attempts were cumbersome and included cloning of PCR-amplified DNA products in a bacteriophage vector followed by hybridization to mutant-specific oligonucleotides.

Early studies focused on *k-ras*, a mutated intracellular signalling protein found in up to 50% of malignant and benign tumours.^{5–12} Mutations in *k-ras* stabilize the protein in the guanosine triphosphate bound form, creating an oncogene that sends an unremitting stimulus for cellular proliferation. More than 80% of *k-ras* mutations are confined to codons 12 or 13 of *k-ras*⁴⁶ allowing for technically easier gene PCR amplification producing large numbers of copies for examination. However, *k-ras* mutations are identified in controls,⁴⁷ in addition to nondysplastic aberrant crypt foci and small hyperplastic polyps which often contain *k-ras* mutations.^{48–50}

Attention turned to other mutational markers including *p53* mutations which occur in approximately 50%

of all human cancers.⁵¹ Activated with DNA damage, the tumour suppressor gene makes a *p53* protein to control the cell cycle, repair DNA, halt synthesis of mutant DNA and cause apoptosis. Known as the ‘guardian of the genome,’ the ultimate effect of *p53* to prevent clonal expansion of mutant cells. Missense mutations of this tumour suppressor gene occur in predictable portions in exons 5–9, leading to its use as a stool DNA marker. While mutations in *p53* are thought to be present only in the later stages of colorectal neoplasia, we have identified such changes in up to 64% of severely dysplastic polyps.⁵²

The ‘gatekeeper’ APC gene’s role in early cell transformation makes it most favourable for detecting early-stage colorectal neoplasia. Although large, this gene does have a good potential for isolating mutations as 83% occur in the first part of the coding sequence. Missense mutations producing stop codons lead to a truncated APC protein. However, the mutations are not as easily detected in faecal DNA due to a low presence of the APC gene. Traverso *et al.* amplified the main region where the majority of mutations take place within a single PCR product.⁵³ Magnetic capture beads were coated with oligonucleotides corresponding to the region between codons 1210 and 1581. This allowed for increased detection of the APC gene through identification of abnormal proteins produced from mutant genes, known as digital protein truncation.⁵³

The results showed that stools from over half the patients with CRC or polyps contained such genes, but normal subjects had no detectable mutant *APC* genes.

Proximal and distal tumours may have inherent and acquired differences that provide the right colon with a predilection to undergo DNA mismatch repair.⁵⁴ The inclusion of MSI in a panel of markers screening for CRC reflects the entire colorectum. Mutations in DNA-MMR produce changes in the coding of genes involved in the regulation of cell growth, including cell programmed death or apoptosis. Inactivation of DNA-MMR genes results in MSI in 15% of all CRCs. The phenotype can be detected as frequent alterations in certain microsatellite sequences such as BAT-26.⁵⁵

Colonocytes are sloughed in the intestinal lumen and undergo a process of physiological cell death termed apoptosis. Apoptotic cells shed from normal mucosa contain DNA cleaved by endonucleases in short fragment lengths of 180–200 bp. Observation of long PCR products, 'long' DNA (L-DNA) or high-molecular weight DNA, identifies the presence of non-apoptotic colonocytes which are characteristically exfoliated from neoplasms. Stools from patients with CRC contain subsets of both non-apoptotic L-DNA from dysplastic cells and 'short' DNA from normal mucosa. This longer template DNA is an epigenetic phenomenon consistent with the known loss of apoptosis that occurs with CRCs.⁴⁷ Differential and quantitative analysis of DNA fragments isolated from stools of patients with CRC have higher 'integrity' than DNA isolated from stools of patients with healthy colonic mucosa. Hence, the presence of L-DNA, as demonstrated by an assay of faecal DNA integrity, may be a sensitive and specific marker for detecting the presence of CRC.⁵⁶

Clinical studies

Combinations of the above markers as a panel have been applied in clinical situations to assess sensitivity and specificity. Estimates of usefulness of these markers for CRC screening can be obtained by collectively examining the results. The initial blinded pilot study of 33 tumours by Ahlquist *et al.* analysed point mutations at any of 15 sites on *k-ras*, *p53*, *APC* genes, BAT-26 and highly amplifiable DNA.⁴⁷ The study demonstrated an impressively high sensitivity of 91% for CRC and 82% for large adenomas from archived stool samples. Adenomas ≥ 1 cm initially had an equally impressive specificity of 93%. By excluding *k-ras* from the panel, cancer sensitivity remained unaffected while adenoma sensitivity decreased to 73% and adenoma specificity increased to 100%. BAT-26 was positive in over half the malignant neoplasms above the splenic flexure and L-DNA was positive in 83% of cancers distal to the splenic flexure.

Subsequent prospective studies have not been able to replicate such impressive results. Our study expanded the number of point mutations to a total of 21 sites and examined 80 *in vivo* neoplasms diagnosed at sigmoidoscopy with an overall cancer sensitivity of 63.5%.⁵⁷ Advanced adenomas ≥ 1 cm were detected in 57.1% of cases and 85.7% of polyps with high-grade dysplasia. The study was limited to mostly symptomatic patients, the investigators were not blinded, and no direct comparisons were made with the other main modality of stool screening (i.e. FOBT). Comparisons with other prospective studies are strikingly similar as shown in Table 1.

Calistri *et al.* found similar results using the same markers *k-ras*, *APC*, *p53*, BAT-26 and L-DNA.⁵⁸ Despite

Table 1. Performance of stool DNA markers in the detection of cancer and advanced adenomas

Authors	Sensitivity-invasive cancers	Specificity	Sensitivity for advanced adenomas
Ahlquist <i>et al.</i> ⁴⁷	20/22 (91)	26/28 (93)	9/11 (82)
Brand <i>et al.</i> ⁶⁷	11/17 (65)	N/A	N/A
Syngal <i>et al.</i> ⁶⁶	35/56 (62)	N/A	5/16 (31)
Tagore <i>et al.</i> ⁵⁷	33/52 (63)	204/212 (96)	16/28 (57)
Calistri <i>et al.</i> ⁵⁸	33/53 (62)	37/38 (97)	N/A
Imperiale <i>et al.</i> (pers. comm.)	16/31 (52)	1343/1423 (94)	61/403 (15)
Overall reported experience	148/231 (64)	1610/1701 (95)	91/458 (20)

Values in parentheses are expressed as percentage.

slightly different labelling and detection methods, molecular alterations were found in 62% of the samples. This study attempted a panel that would be less time-consuming and labour-intensive by only analysing four fragments on the *APC* gene. As suggested by Ahlquist, L-DNA amplification detected more than 50% of tumours alone. *K-ras* was the next most frequent alteration detected in stool leading the researchers to suggest that these two markers are potentially capable of detecting two-thirds of cancers. More sensitive techniques would need to be developed and employed, but such an approach could make the screening test much less labour-intensive and more cost-effective.

Preliminary data from a recent prospective trial presented at the 2003 American Society of Gastroenterology (ASGE) took 4022 average risk patients to stool DNA testing and FOBT prior to screening colonoscopy. Thirty-one advanced neoplasms were identified. DNA mutations were identified in 52% of tumour-bearing patients but only 13% were FOBT-positive (personal communication). Results from a similar-sized NIH-funded trial are pending.

Among the specific markers, L-DNA amplification has emerged as the most frequent event. Longer DNA represents exfoliated non-apoptotic neoplastic cells. This DNA has escaped the cleaving effects of endonucleases which normally create short DNA. Stool BAT-26 in most studies appears to be a marker of MSI which identifies the presence of half the advanced right-sided neoplasms. Performance characteristics of individual stool DNA markers are listed in Table 2.

Future of DNA testing

Population-based screening of average risk individuals will require a highly sensitive test capable of detecting early disease. Stool DNA mutations are inherently reliable markers of molecular carcinogenesis, which may provide to both patients and primary care doctors a

clear reason why screening and follow-up for CRC is necessary. While preliminary studies are promising, the problems of sensitivity and cost need to be addressed. As more markers are added to improve sensitivity, the cost of the assay increases. The current iteration of the EXACT assay costs between \$500 and 800. Compared with immunochemical or guaiac-based FOBTs which cost between \$5 and 30, the sensitivity benefit has yet to demonstrate cost-effectiveness.

Refinements in detection techniques, targeting multiple markers, and developing novel strategies of targeting new mutations may improve sensitivity and specificity. EffipureTM is a novel technique that allows five-fold greater amplification of stool human DNA using a highly efficient capture technique.⁵⁹ The new sample prep method gives an average 5.4-fold increase in the quantity of human DNA that can routinely be retrieved from faecal samples compared with the bead capture method. This resulted in a 16% increase (14 of 89) in detection of confirmed cancers, with no loss of specificity (personal communication). Preliminary studies show far greater tumour sensitivities on previously negative tumour samples. It is likely that future technological improvements will further enhance the sensitivity of stool DNA testing.

A plasma or serum test to detect sporadic colorectal neoplasia would involve DNA markers including *k-ras*,^{60–62} *APC*⁶³ and hypermethylated p16,⁶⁴ leaked into the bloodstream during tissue invasion. The overall detection rates in these studies have ranged from 15 to 70%, but show higher detection rates with more advanced stage neoplasia, limiting its value as a screening test. One recent report describes gynaecological and breast cancer detection rates of 62% overall and 50% for stage I cancers using an assay of L-DNA in serum.⁶⁵ If tumour marker exfoliation into the colon likely precedes tissue invasion, stool assay of DNA markers for adenomas and early-stage cancers will prove more sensitive than the plasma assay. No comparison

Table 2. Sensitivity performance of individual markers in the stool DNA assay panel for invasive cancers only

Authors	APC	K-ras	P53	BAT-26	L-DNA
Ahlquist <i>et al.</i> ⁴⁷	5/22 (23)	4/22 (18)	3/22 (14)	5/22 (23)	14/22 (61)
Dong <i>et al.</i> ⁶⁸	N/A	8/51 (16)	30/51 (59)	3/51 (6)	N/A
Tagore <i>et al.</i> ⁵⁷	7/52 (14)	9/52 (17)	17/52 (33)	2/52 (4)	19/52 (37)
Calistri <i>et al.</i> ⁵⁸	2/53 (4)	6/53 (11)	3/53 (6)	3/53 (6)	27/53 (51)
Range (%)	4–23	11–18	6–59	4–23	37–61

Values in parentheses are expressed as percentage.

studies have been performed of stool-based DNA tests with plasma-based DNA tests for detection of cancer.

The cost of the procedure is high and far exceeds that of FOBT. Rationalization of the test to one or two markers could significantly lower the cost. It appears likely that markers germane to all tumours will be more suitable than a large panel. Candidates for this streamlined approach include L-DNA and hypermethylation markers because of their sensitivity. Application as an interval test between endoscopies may be an attractive option for patients who prefer to avoid or minimize repeat colonoscopy. Up to 93% of follow-up post-polypectomy colonoscopies fail to find advanced adenomas and in the elderly the risks of the procedure outweigh the low benefit. To rationalize resources and minimize risk, this group could be better served by DNA screening.

Another potential area of utilization for stool DNA testing is for tumour follow-up and risk assessment. Syngal *et al.* did post-resection stool DNA testing for patients with colonic neoplasms.⁶⁶ Most mutational markers resolved post-resection with the exception of L-DNA suggesting the presence of a pervasive field effect. These findings suggest that stool DNA mutational analysis could have potential use in monitoring CRC patients after treatment.

Stool DNA testing offers a sensitive and non-invasive alternative for colon cancer screening likely to appeal to patients who have avoided screening because of fear or inconvenience. If the sensitivity and cost limitations can be overcome, DNA markers offer the potential for a less risky screening procedure which could target populations who are currently underserved by screening.

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