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Molecular markers for colorectal cancer screening

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Abstract

Colorectal cancer (CRC), although a significant cause of morbidity and mortality worldwide, has seen a declining incidence and mortality in countries with programmatic screening. Fecal occult blood testing (FOBT) and endoscopic approaches are the predominant screening methods currently. The discovery of the adenoma→carcinoma sequence and a greater understanding of the genetic and epigenetic changes that drive the formation of CRC have contributed to innovative research to identify molecular markers for highly accurate, non-invasive screening tests for CRC. DNA, proteins, messenger RNA, and micro-RNA have all been evaluated. The observation of tumor cell exfoliation into the mucocellular layer of the colonic epithelium and proven stability of DNA in a harsh stool environment make stool DNA a particularly promising marker. The development of a clinically useful stool DNA test has required numerous technical advances, including optimization in DNA stabilization, the development of assays with high analytical sensitivity, and the identification of specific and broadly informative molecular markers. A multi-target stool DNA (MT-sDNA) test, which combines both mutant and methylated DNA markers and a fecal immunochemical test (FIT), recently performed favorably in a large cross-sectional validation study and has been approved by the US Food and Drug Administration (FDA) for the screening of asymptomatic, average risk individuals. The ultimate way in which molecular marker screening assays will be used in clinical practice will require additional studies to determine optimal screening intervals, factors affecting compliance, management of false positive results, and the use of these assays in high-risk populations, as well as other considerations.

I: Introduction

Colorectal cancer (CRC) will cause over 600,000 deaths globally¹; however, the incidence and mortality appear to be steadily declining in countries with programmatic screening². The predominant screening tools used to date have included fecal occult blood testing (FOBT),

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flexible sigmoidoscopy, and colonoscopy. For some patients, screening by computed tomography (CT) colonography has been a more recently introduced CRC screening modality. Although screening has clearly been shown to reduce the risk of colorectal cancer associated mortality³, even among prosperous nations, screening effectiveness is compromised by limitations of test performance, lack of access to CRC screening tests and suboptimal screening compliance. Consequently, the majority of patients in the United States, for example, present with regionally advanced or metastatic disease⁴.

The opportunity to improve the impact of current CRC screening programs on colorectal cancer mortality has driven innovative research to identify molecular markers for the development of highly accurate, non-invasive screening tests for CRC. Several marker classes have been evaluated for their use in CRC screening: DNA^{5,6}, proteins⁷, messenger RNA (mRNA)⁸, and micro RNA (miRNA)^{9,10}, and have all shown potential in early phase biomarker studies¹¹; however, aside from fecal hemoglobin, to date, only DNA-based markers have undergone the full spectrum of development and clinical testing required for an assessment of their performance in clinical practice. In fact, a multi-target stool DNA (MT-sDNA) test has been recently shown to have superior sensitivity, although with lower specificity, to fecal hemoglobin by immunochemical testing for the detection of curable-stage CRC and advanced adenomas and to have an overall cancer detection similar to colonoscopy¹². As a result, stool DNA testing was approved in the United States for population-wide screening of average risk, asymptomatic individuals in 2014.

The development of stool DNA tests has resulted from advances in our understanding of several important biological principles, including: 1) the seminal discovery of the adenoma→carcinoma sequence^{13,14,15,16}; 2) recognition of the genetic and epigenetic changes that drive the formation of CRC and the molecular pathways affected by such changes; 3) observation that tumor cells and constituents exfoliate into the mucocellular layer over the colonic epithelium¹⁷; and 4) recognition of the stability of DNA in the harsh stool environment¹⁸. In this article, we will focus on these principles and discuss the development and validation of a multi-target stool DNA test for CRC screening that has recently been approved by the US Food and Drug Administration (FDA) for CRC screening. The ultimate way in which stool DNA based molecular marker screening assays will be used in clinical practice will require additional studies to determine optimal screening intervals, factors affecting compliance, management of false positive results, and the use of these assays in high-risk populations, as well as other considerations.

II: The molecular pathogenesis of colorectal cancer

An understanding of the genetic and epigenetic landscape across colorectal neoplasms informs the rational design, affects performance, and guides interpretation of molecular screening tests. In the colon, the transformation of normal epithelial cells into adenocarcinoma is believed to follow a predictable progression of histological and concurrent epigenetic and genetic changes that alter the morphology and function of the epithelial cells and the surrounding stroma (Figure 1). In this classic tumorigenesis model, CRC arises via a polyp→cancer progression sequence that begins with an aberrant crypt focus that first evolves into an early adenoma (less than 1 cm in size, tubular or

tubulovillous histology), then progresses into an advanced adenoma (>1 cm in size, villous histology), and finally becomes a CRC. This process is driven by mutations and epigenetic alterations and usually takes 10–15 years to occur but can occur more rapidly in certain settings (e.g. Lynch syndrome, possibly microsatellite unstable tumors)¹⁹. Notably, although the histology of conventional tubular adenomas is fairly homogeneous, there is obvious molecular heterogeneity among polyps that have similar histologic appearance, which may determine which adenomas have the highest probability to progress to CRC^{20,21}. The potential to progress to CRC is particularly relevant to CRC screening. Although there is considerable uncertainty regarding our understanding of the potential for any specific polyp to progress to CRC, some studies have estimated that only 10% of all polyps will progress to CRC and that approximately 25% of advanced polyps will progress to CRC²².

Until the last 5–10 years, it was believed that only tubular and tubulovillous adenomatous polyps had the potential to progress to CRC; however, it now also appears that 5–30% of all CRCs may evolve from a subset of polyps called sessile serrated polyps and traditional serrated adenomas, which account for roughly 5–10% of all polyps. Sessile serrated polyps and traditional serrated adenomas appear to arise through a series of molecular as well as histologic events that are distinct from the adenoma→CRC progression sequence^{23,24,25}. Serrated polyps appear to have the potential to transform into CRCs through a hyperplastic polyp→serrated polyp→adenocarcinoma progression sequence^{23,26}. Furthermore, serrated polyps that arise from the right colon commonly display a form of genetic instability characterized by excessive aberrant CpG island DNA methylation, termed CpG Island Methylator Phenotype (CIMP), whereas those that arise in the left colon are typically microsatellite stable (MSS) and frequently carry mutations in *KRAS* and have an attenuated form of CIMP^{24,25,27}. In comparison, CRCs that arise from the adenoma→CRC pathway usually display a form of genomic instability called chromosomal instability (CIN) (>90%) and are initiated by mutations in *APC*. Although advances have been made in our understanding of the pathogenesis of CRC, it is important to recognize that the different polyp→CRC pathways noted above are still speculative and based on incomplete and imperfect data.

With the growing appreciation that the molecular changes in polyps and CRCs are primary drivers of the specific behaviors of the tumors, at the present, CRCs have been classified into at least four subgroups based on their molecular features: 1) Hypermutable-Microsatellite unstable (Hyp-MSI); 2) Hypermutable-Microsatellite Stable (Hyp-MSS); 3) Microsatellite Stable (aka Chromosome Unstable) (MSS or CIN); and 4) CpG Island Methylator Phenotype (CIMP) cancers^{28,29}. The frequency of specific mutations can vary dramatically between the molecular subclasses of CRC suggesting each subclass has its own set of cooperating driver genes²⁸. The driving mutations and epigenetic alterations in these different CRC molecular subgroups are not fully characterized although some mutant genes, such as *APC* and *TGFBR2/SMAD4*, are common among all the molecular subgroups, suggesting a central role for these genes and the signaling pathways that they regulate in CRC in general, while others appear to be restricted to one subclass of CRC (e.g. *BRAF* in CIMP CRC)^{30,31}.

In addition to gene mutations, epigenetic alterations occur commonly in polyps and CRCs and appear to cooperate with gene mutations to drive the polyp→CRC sequence^{32,33,34}. Modifications in DNA methylation related to the development of cancer include two fundamental changes: 1) hypermethylation of CpG islands in gene promoters, which can silence tumor suppress genes; and 2) hypomethylation of repetitive genetic elements, which may lead to genomic instability or oncogene activation³⁵. DNA methylation affects CpG rich regions, called ‘CpG islands’, in the 5’ region of genes and results in transcriptional silencing through effects on transcription factor binding and changes in chromatin structure³⁶. Notably, from the aspect of molecular marker development, aberrantly methylated genes have proven to be a particularly promising class of markers. The methods for assessing aberrantly methylated DNA are robust and sensitive, which has led to their use in many clinically used assays currently^{5,30,33}.

With regards to the specific mutations and epigenetic alterations present in CRCs, there is substantial heterogeneity between CRCs, although the mutations appear to cluster in epistatically related groups^{31,37,38}. The most common alterations seen in CRC include *APC*, *CTNNB1*, *KRAS*, *BRAF*, *SMAD4*, *TGFBR2*, *TP53*, *PIK3CA*, *ARID1A*, *SOX9*, *FAM123B*, and *ERBB2*, which appear to promote colorectal tumorigenesis by perturbing the function of key signaling pathways, including the WNT-β-catenin, EGF-MAPK, PI3K and TGF-β signaling pathways, or by affecting genes that regulate central behaviors of cells such as DNA repair, proliferation, etc³⁹. CRC appears to be most frequently initiated by alterations that affect the WNT signaling pathway. The initiated neoplastic cells then progress as the result of the deregulation of other signaling pathways, including the RAS-RAF-MAPK pathway, TGF-β pathway, and the PI3K-AKT pathway^{38,40}. Epigenetic alterations appear to arise very early in the polyp→CRC sequence, making them appealing CRC screening molecular markers. Furthermore, there is substantial evidence to suggest that they occur in histologically normal appearing mucosa and predispose to CRC formation (i.e. field cancerization)⁴¹.

It is now also appreciated that the tumor microenvironment (i.e. gut microbiome, inflammatory state of adjacent tissue, etc.) modulates the way these mutations affect CRC formation. Thus, our current understanding of the pathogenesis of CRC is that it results from the accumulation of alterations in genes that then drive the formation of CRC in the context of tumor promoting factors derived from the adjacent tissue.

III: Development of non-invasive molecular screening tests for colon polyps and CRC

Fecal occult blood (FOBT) and the fecal immunochemical test (FIT)

Our understanding of the molecular pathogenesis of the polyp→CRC progression sequence has led to the recognition that the molecular alterations found in polyps and CRC have the potential to be neoplasm specific molecular markers for these lesions. The concept of using these molecular markers for CRC screening is essentially the next step in the evolution of a well established non-invasive detection method for CRC that has been in clinical use for decades that is based on fecal hemoglobin. In the mid-1960’s, guaiac-based methods for

fecal occult blood testing (gFOBT) were reported^{42,43} and soon commercialized as Hemoccult and later Hemoccult II. The gFOBT method of CRC screening has been shown in randomized controlled clinical trials (RCT) to reduce mortality by 11–33% over 20 years of follow up^{44,45,46,47,48}. The 17–20% reduction in CRC incidence demonstrated in the same studies is more modest, presumably because of the relative insensitivity of gFOBT for pre-cancerous polyps compared to CRC. Notable characteristics of gFOBT as a CRC screening test are its modest specificity for CRC, which generates many false positive test results when used in population-based screening programs and its modest sensitivity for colon polyps⁴⁹.

These limitations of gFOBT have led to the development of fecal immunochemical tests (FIT), which detect blood by a human hemoglobin specific immunoassay. FIT assays can detect both the presence and quantity of fecal hemoglobin¹⁷, which permits the use of different thresholds to modify the sensitivity and specificity of the assays for detecting polyps and CRC⁴⁹. In multiple studies, FIT assays have superior sensitivity and specificity for CRC and advanced adenomas compared to gFOBT^{18,42}. FIT also has the advantage over gFOBT in that only one sample is required for analysis, as opposed to three, and the process of sample collection is more acceptable to the general public, which have both been shown to increase compliance with screening compared to gFOBT based tests⁴³.

However, even with the more sensitive fecal immunochemical test, when used for CRC screening, the detection of adenomas >1 cm in diameter is only 20–30%^{49,50}. Moreover, occult blood testing detects significantly more lesions in the left than right colon⁵⁰, which is a significant issue given the increased incidence of right-sided CRCs that has developed over the last two decades⁵¹. This bias towards left-sided lesions is not unique to fecal hemoglobin testing; in RCTs, invasive flexible sigmoidoscopy reduced mortality and incidence of CRC but only in the left colon, as would be expected from this test since it only assesses the left colon^{52,53}. Although ongoing RCTs are not yet completed for colonoscopy, large observational studies have revealed the same trend; CRC related mortality or risk reduction from right-sided colon cancer after colonoscopy is unaffected or less substantially reduced for right-sided CRC compared to left-sided CRC (risk reduction of 27% vs 76%)^{54,55,56}.

Another drawback of fecal hemoglobin testing is that a number of non-neoplastic factors can affect the performance of the test and associate with an increased likelihood of false positive results. These factors include, but are not limited to, the use of anti-platelet drugs, relative decreased specificity in first time participants and in those people with a history of CRC, and benign bleeding disorders, all of which may lead to unnecessary diagnostic tests^{49,57,58}.

Brief history of the development of molecular marker assays for the detection of colon polyps and CRC

The lack of an ideal, non-invasive test for CRC screening created an opportunity for the development of tests based on the detection of specific molecular alterations (e.g. abnormal protein or mRNA expression, gene mutations, abnormally methylated genes, etc.) present in body fluids (blood, urine, stool, etc.) for the identification of individuals with asymptomatic colon polyps or CRC. The feasibility of this approach was first demonstrated by Sidransky

et al when they detected mutant *KRAS* in the stool of people with CRC⁵⁹. This success led to hundreds of studies that have assessed a myriad of molecular alterations as molecular CRC screening markers, and to the first commercialized molecular screening assays for CRC (e.g. ColoSure (Exact Sciences); methyl SEPT9 (Epigenomics and ARUP labs))^{60,61,62}. Reviews of the development of molecular screening assays has been covered previously and will not be discussed further in this review^{5,62,63}. Although virtually every class of molecular alteration and body fluid source has been evaluated for use in CRC screening assays, only those tests based on DNA have proven robust enough to result in assays approved for clinical use.

Stool DNA based molecular marker assays: biology and rationale

Due to these limitations in the commonly used methods for CRC screening, there has been intense effort to develop a more optimal, non-invasive CRC screening test that rely on specific molecular alterations observed in colon polyps and CRCs (e.g. gene mutations, aberrantly methylated DNA loci, micro RNAs, etc.). The detection of these biomarkers in blood and urine from people with colon polyps and CRC has been assessed, but the most accurate tests for the detection of colon polyps and CRCs to date are based on stool samples. Stool-based assays have been the most successful assay type likely because of several important biological factors. Direct histologic observations show that CRCs and polyps abundantly exfoliate neoplastic cells and their debris into the mucocellular layer of the colonic lumen at a continuous rate¹⁷. (Figure 2) In contrast, normal colonic epithelial cells often turn-over by *in situ* apoptosis and subsequent phagocytosis by sub-epithelial macrophages⁶⁴. The proportionately greater exfoliation from colonic neoplasms compared to the normal colon mucosa is likely due to increased proliferation and escape from anoikis, a programmed cell death after loss of contact with the basement membrane or adjacent cells⁶⁵. Unlike cellular exfoliation, hemoglobin enters the fecal stream by hemorrhage, which is often intermittent and infrequent from adenomas and early stage cancer⁶⁶. Despite this theoretical advantage of markers based on exfoliated cells from colon neoplasms, even with continuous release, it was not initially clear that cellular or molecular analytes from a focal source would be in sufficient concentrations to allow reliable detection of adenomas as small as 1 cm in diameter. However, this has now been shown to be feasible likely due to topographic involutions in the surface architecture of cancers and polyps, the actual unfolded surface area of the epithelial monolayer may be 200 times larger than predicted by the gross polyp dimensions⁶⁷.

Despite abundant cellular exfoliation from a large target surface area, and the presence of viable neoplastic cells in the mucocellular layer over the colonic epithelium¹⁷, intact colonocytes are difficult to recover when shed from the right colon⁶⁸, likely reflecting a degree of intra-luminal lysis. This has led to the development of assays that depend on the detection of components of the exfoliated cells that are present even after cell lysis. DNA is such a constituent: 1) specific genetic and epigenetic alterations are present in colon polyp and CRC DNA; 2) DNA is very stable, especially compared to most proteins and mRNAs; and 3) technically sensitive and robust assays exist for the detection of DNA.

From concept to clinic: the technical development of clinical stool DNA based molecular marker CRC screening assays

Despite strong biological plausibility, the development of a clinically useful stool DNA test has required numerous technical advances, including optimization in DNA stabilization, the development of assay methods with adequate analytical sensitivity, and the identification of specific and broadly informative molecular markers. Although exfoliated colon polyp and CRC cells are enriched in the feces, stool is a harsh and complex milieu from which to extract and analyze tumor-specific DNA. Bacterial DNAase enzymes can degrade DNA but can be inhibited by the addition of EDTA-based buffer to the stools immediately after collection. The addition of EDTA to stool collection buffers was shown to significantly improve DNA recovery from stool specimens, even after prolonged time without refrigeration or freezing^{18,69}. In addition, inhibitors to the polymerase chain reaction (PCR), the most commonly used method for detecting DNA, are present in stool and need to be removed to optimize the performance of stool DNA molecular marker assays. While the addition of collection buffer initially dilutes PCR inhibitors, homogenization of buffered stools and treatment of supernatants with polyvinylpolypyrrolidone further reduces inhibitor concentration, enhancing assay detection limits⁷⁰.

In addition to overcoming barriers related to the stool milieu, assays that detect tumor-specific DNA sequences in a background of normal DNA are required. Tumor-specific DNA must be able to be detected in the background of total stool DNA, the vast majority of which is bacterial and dietary. In fact, human DNA may make up only 1 part per 100,000 total DNA⁷¹ and may be fragmented into sequence lengths of 150 base pairs or less⁷¹. Even after enriching the isolated DNA for the gene fragment of interest by magnetic bead capture techniques, DNA from stool of patients with CRC may contain a single mutant or methylated target among a background of 200 total copies (0.5%) of the same gene⁷². Early generation stool DNA assays could detect a target signal of 1% from the normal background, which limited the analytical sensitivity of the assays⁶⁰. Fortunately, technological breakthroughs such as digital melt-curve PCR⁷², emulsion & bead-based PCR (BEAMing)⁷³ and quantitative allele-specific real-time target and signal amplification (QuARTS) assays⁷⁴ have improved analytical sensitivity to 0.01%, sufficient to detect DNA from polyps and small CRC equally from both the left and right colon⁷². Furthermore, newer generation assay platforms such as QuARTS have now been automated in order to meet the demands of high throughput and high reliability of clinical laboratories (Figure 3). The automated platform is operator-independent and has been validated by blinded comparison to manual methods⁷⁵.

Finally, even with assay technology that has a high analytical sensitivity, molecular markers that are present in a very high proportion of polyps and CRCs (ideally 100%) must be identified. As previously reviewed^{28,76}, DNA point mutations are heterogeneous among CRC making them suboptimal biomarker candidates because any individual point mutation is present in a small fraction of polyps and CRCs. As such, a marker panel assembled solely from mutant DNA might require assay that assesses for >100 point mutations, even across the most commonly altered genes in CRCs⁷⁷. A clear example of the challenge of developing a molecular marker assay based on DNA mutations is the first generation stool

DNA test developed, which assayed for 21 point mutations across *APC*, *KRAS* & *TP53*, as well as BAT-26 for MSI, and a marker of long DNA. While this panel showed superior cancer and advanced adenoma detection compared to Hemoccult II, the sensitivity for screen relevant neoplasia was only 41%⁶⁰. When examining tissues of screen-relevant neoplasms (i.e. advanced adenomas and early stage CRC), these markers were found in only 67% of cases⁷⁸. In contrast, a set of specific aberrantly methylated genes were present in almost all CRC and precursor lesions⁷⁹. The higher frequency (and thus potential sensitivity) of methylated genes in polyps and CRC, compared to specific gene mutations, has been shown in a variety of studies, including a recent study in which a panel of only 4 methylation markers detected in tissues of target cancer and adenoma lesions at nearly 100% specificity⁷⁴. Importantly, these markers in combination with DNA mutations, are also well-represented in stools from patients with sessile serrated polyps, which was shown in a blinded case-control analysis⁸⁰.

IV: Clinical performance of FDA approved molecular marker CRC screening assays

Over the last 20 years, numerous molecular assay approaches have been explored for potential use in CRC screening. However, few have achieved high clinical accuracy or become available for patient use⁸¹. Only the Cologuard MT-sDNA test (Exact Sciences) has been rigorously reviewed and approved by the US FDA, covered by the US Centers for Medicaid and Medicare (CMS), and been made available commercially. This test detects a combination of both mutant and methylated DNA markers and FIT, collectively called multi-target stool DNA (MT-sDNA), and has demonstrated the best clinical performance of CRC molecular marker screening assays to date. Two recent, large, multi-center case-control studies have evaluated MT-sDNA performance. The first measured sensitivity and specificity in training set (n=456) and test set (n=222) comparisons using a first-generation, non-optimized prototype MT-sDNA test, which used QuARTS to detect methylated *VIM*, *NDRG4*, *BMP3* & *TFPI2*, mutant *KRAS* and *CTNNB1* in addition to hemoglobin by FIT. Test cut-off values were established by the modeled 90% specificity threshold (95% confidence interval [CI], 85–94%) among the training set patients which included the 170 cancers, 89 advanced adenomas and 197 control patients with normal colonoscopies. The sensitivity for stage I and II CRC was 87% and adenoma detection ranged from 54%–92%, depending on size (54% for lesions ≤ 1 cm; 77% for >2cm, 86% for >3 cm and 92% for >4 cm (p < 0.0001 for trend))⁸². Importantly, the sensitivity for detection of either CRC or adenoma was not affected by location (proximal vs. distal colon)⁸². The second study used an optimized and automated MT-sDNA test in 459 asymptomatic individuals and 544 referred patients⁷⁵. Cases included 93 CRC, 84 with advanced adenoma, 30 with sessile serrated polyps ≤ 1 cm. Controls included 155 patients with non-advanced polyps and 641 with no colonoscopic abnormalities. The sensitivity for CRC was 98% (91 of 93) overall and 97% (74 of 76) for stages I-III at a nominal 90% specificity cut-off. Again, sensitivity for advanced adenomas and polyps was significantly correlated with size; sensitivity for adenomas and sessile serrated polyps ≤ 1 cm was 57% (65 of 114), >2 cm was 73% (27 of 37), and >3 cm was 83% (20 of 24). Lesions with high-grade dysplasia were detected at 83% (15 of 18) sensitivity; 94% (16 of 17, where size was recorded) of these were >2 cm.

Performance of MT-sDNA assay in a large cross-sectional validation study

The performance of the MT-sDNA test in the case-control studies described above led to its evaluation in a large cross-sectional study in asymptomatic patients undergoing routine screening colonoscopy, which served as the criterion standard. This study, named “DeeP-C”, compared MT-sDNA to FIT and colonoscopy in nearly 10,000 average-risk patients enrolled at 90 sites in North America¹². By MT-sDNA, the overall sensitivity for CRC was 92% (95% CI, 83–97.5%) and 93% (95% CI 83.8–98.2%) for stage I–III CRC, compared to FIT sensitivities of 74% (95% CI, 61.5–84%) and 73% (95% CI, 60.3–83.9%), respectively ($p=0.002$) (Figure 4). For advanced adenomas and sessile serrated polyps, the sensitivity of the MT-sDNA test increased proportionately with lesion size and grade. Detection of polyps with high grade dysplasia was 69% by MT-sDNA vs 46% by FIT ($p=0.004$). MT-sDNA was also significantly more sensitive than FIT for advanced adenomas: 42% (95% CI, 38.9–46%) vs 24% (95% CI, 20.8–27%), respectively, for those ≤ 1 cm and 66% vs 43% for those > 2 cm ($p < 0.001$). Sessile serrated polyps ≤ 1 cm were detected at a rate of 42% for MT-sDNA compared 5% for FIT ($p < 0.001$); MT-sDNA was 67% sensitive for those > 2 cm compared to 11% by FIT⁸³. Test specificity was based on the primary and secondary study endpoints, specifically the detection of CRC and advanced pre-cancers. For these endpoints combined, the specificity was 87%; however, when not including other lesions detected at colonoscopy, including polyps < 1 cm, the specificity was 90%.

The results of the Deep-C study led to FDA approval of the Cologuard™ MT-sDNA test (Exact Sciences, Madison WI) in August of 2014. A simultaneous national coverage decision was announced by the Centers for Medicare and Medicaid Services (CMS). Cologuard has been approved for CRC screening in asymptomatic individuals, age 50–84. Those individuals with a personal history of advanced adenomas, family history of CRC, a known or suspected genetic CRC syndrome or known inflammatory bowel disease (IBD) should still be offered colonoscopy as a primary surveillance strategy as Cologuard has not yet been adequately assessed in these populations.

Plasma DNA based molecular marker screening assays: potential vs performance for CRC screening

As CRC screening using stool-based molecular markers has moved from a concept to reality, a parallel line of biomarker discovery studies and biomarker early phase performance studies has been underway for markers in blood plasma. Several tumor-associated DNA methylation markers have been tested in plasma for use in CRC detection; among these a plasma assay for methylated *SEPT9* has been clinically assessed in case-control studies^{84,85,86} and in average risk cohort studies⁸⁷. In asymptomatic patients undergoing CRC screening, a plasma DNA based assay of methylated *SEPT9* was 48% sensitive for CRC at 92% specificity⁸⁷. Sensitivity for advanced adenomas was low at 11%. The sensitivity improved with advancing cancer stage, such that 35% of stage I CRCs were detected compared to 77% of stage IV CRCs. This same trend was seen in a small case-control study, where MT-sDNA detected 91% of stage I–III CRCs vs 50% detected with a plasma assay for methylated *SEPT9* ($p=0.01$)⁸⁸. The stage-dependent sensitivity of plasma markers for CRC has generated the hypothesis that marker release from neoplasms into other media like blood plasma, or plasma ultrafiltrates such as urine, may depend on

vascular invasion. At this time, plasma DNA based assays have suboptimal sensitivity for screening for colon polyps and early stage CRC compared to currently available screening tests. There are a number of promising assays in development, but at this time none of them are suitable to be used in the clinic for colon polyp detection⁸⁹. However, it is noteworthy that a blood-based assay for methylated *SEPT9* (Epi proColon®, Epigenomics and BioChain) was recently approved by the Chinese FDA for CRC detection and is available for use in clinical care in China as well as in Europe for this purpose.

V: Unresolved issues related to the MT-sDNA test and other emerging molecular marker screening assays: implications for clinical practice

As mentioned, MT-sDNA testing was recently approved by the FDA for use in CRC screening. As MT-sDNA testing as a non-invasive method for CRC detection begins to be implemented, questions regarding screening intervals, populations most likely to benefit from MT-sDNA testing, cost, adherence, access to testing, and diagnostic evaluation of positive results, amongst others, have arisen. These issues are relevant for all the molecular marker screening assays that are under development.

False positive results

In the cohort of patients in the study performed by Imperial et al¹² previously mentioned, MT-sDNA testing had a higher sensitivity for the detection CRC and advanced adenomas when compared with FIT; however, this occurred at the expense of reduced specificity. Roughly 10% of the cohort had no polyps or CRC detected by colonoscopy after having a positive stool DNA result. The follow-up evaluation of such patients has yet to be defined. Such patients could undergo repeat colonoscopy, repeat DNA testing at a defined interval, or both as potential options. The higher cost and lower specificity of MT-sDNA testing compared to FIT make it unlikely that stool DNA testing would be performed annually⁹⁰. However, given the high “point-sensitivity”, sensitivity at a single point in time, of MT-sDNA testing, screening intervals can potentially be longer than that of FIT. Models using 3 year screening intervals predict a very high “program-sensitivity”, which is the cumulative detection of those polyps with high risk of progression to CRC with repeat testing over time. Within this screening model, the false positive rate per year of MT-sDNA is comparable to or lower than that of FIT⁹¹. Until further data become available on the diagnostic yield of follow-up colonoscopy and upper gastrointestinal interrogation in patients who are initially MT-sDNA positive but colonoscopy negative, clinical judgment will be required.

Adherence and access to screening

New noninvasive molecular tools, such as MT-sDNA, have potential to improve CRC screening effectiveness in three ways—through improved sensitivity, patient compliance, and test accessibility. At a screening frequency of every 3 years, the programmatic sensitivity of MT-sDNA for both CRC and highest risk precancers could compare favorably to that of colonoscopy performed every 10 years. However, the other factors of patient compliance and access may prove to be more important in determining the effectiveness of a test in population screening. Compliance with CRC screening remains suboptimal. In the US, only about half of adults have had a screening test for CRC⁹². It has been suggested that

improved compliance with screening alone could reduce mortality even more than improved treatment modalities or risk factor modification⁹³. Multiple studies have been done to explore patient preference as it pertains to screening for CRC with varying results. Study subjects have, however, consistently rated non-invasive screening modalities, such as stool-based DNA testing or FIT, over colonoscopy for their perceived lack of test discomfort, lack of cathartic preparation, lack of lost work time, and lower level of embarrassment. On the other hand, studies have demonstrated a consistent preference for colonoscopy over non-invasive methods amongst participants for its perceived increased accuracy of detection of CRC^{94,95,96}. It has also consistently been shown that patients are more likely to conform with screening recommendations if they feel they have played a role in choosing the screening method. Inadomi et al demonstrated this in a trial where 70% of participants who were given a choice between FOBT or colonoscopy completed screening as opposed to only 38% of participants where colonoscopy alone was recommended⁹⁷.

Non-invasive tests can be made easily accessible to a wide population through distribution from clinics and by mailing, which would presumably increase the availability of screening to certain population who are resistant to the idea of more invasive screening options. However, accessibility alone does not guarantee compliance with, or success of, a screening method. For example, a small portion of participants in the stool DNA group from the study by Imperiale et al¹² were excluded due to problems with sample collection or assay application. This highlights the importance of a screening test not only being accessible, but also the necessity for having clear, practical, and simple collection instructions. The latest generation of non-invasive tests, FIT and Cologuard, have successfully addressed these issues.

Cost

The overall cost of a screening test is a function of the cost of the test itself, frequency of testing, and the cost of subsequent evaluation that is performed due to the result of the initial test. False positive results lead to unnecessary, and costly, further evaluation. While the screening interval for MT-sDNA testing has yet to be fully defined, current models predict three year screening intervals⁹¹ and CMS has approved coverage for this screening frequency. At a 3 year screening interval, the programmatic specificity of MT-sDNA (false positive rate of 3–4% per year) would be equal to or higher than that of FIT performed annually (false positive rate of 4–5% per year), as mentioned above. With biennial FIT testing (false positive rate would be 2–2.5% per year), the cumulative false positive rate after three cycles of FIT testing and two MT-sDNA cycles would be about 5% higher for MT-sDNA, assuming worst-case specificity estimates for both tests.

At least six Markov modeling studies have found stool DNA testing to be cost-effective when compared to no screening at all⁹⁸. While FIT appears to be more cost-effective than stool DNA testing in base-case analyses, the rate of screen compliance is a critical factor in sensitivity analysis^{98,99}. Given that screening compliance with FIT is only 52–62% in several recent European clinical trials^{100,101}, options to capture a greater number of participants may be of considerable value.

The cost of MT-sDNA testing includes related expenses for the collection and shipping of whole stools to the clinical laboratory. Because the stool is collected in a container suspended from a toilet seat, the patient is required only to swab the intact stool for FIT sampling and to cover the specimen with the included buffer solution. Patient acceptance of this collection protocol is favorable and survey data suggest that the simplicity of the collection process may be greater than that of guaiac-based FOBT⁹⁴. While a stool DNA test performed on a scoop or fragment of stool might lower costs associated with shipping, there is potential for lower acceptance with collection procedures if increased sample manipulation is required of patients. Test ergonomics and the interaction with patient preferences on test effectiveness are important areas for further study. Finally, whole stool collection may be most effective as it minimizes sampling error due to inhomogenous marker distribution within stool and to variation in patient sampling techniques. Future designs to miniaturize the sampling device for less expensive shipping will need to address sampling representativeness.

Expanded use of non-invasive tests

Stool DNA testing is currently being investigated as an approach to detect neoplasia in patient populations other than average-risk individuals. People with inflammatory bowel disease (IBD), specifically ulcerative colitis (UC) and Crohn's colitis are at increased risk of CRC¹⁰². Currently, patients with UC and Crohn's colitis are advised to undergo annual surveillance colonoscopy with random biopsies of the colon for CRC and dysplasia detection. Preliminary studies using stool-based DNA testing for the detection of dysplasia and CRC in IBD have been promising¹⁰³. Stool-based DNA molecular marker testing is also being explored as a method to detect cancers and pre-cancers throughout the digestive tract, including the pancreas^{104,105}. Other high-risk populations that could benefit from stool-based molecular marker testing include individuals with a family history of colorectal cancer, individuals with risk factors for CRC (i.e. diabetes mellitus, obesity, inactivity, tobacco use), etc. A highly specific non-invasive test has the potential to be used in screening programs directed at these groups that starts at a younger age than recommended for the general population.

VI: Conclusions

Advances in our understanding of the natural history of CRC and the molecular genetics and epigenetics of colon polyps and CRC has led to the development of molecular marker assays for CRC screening. The last two decades have seen studies that have demonstrated the feasibility of these assays and the discovery of a myriad of promising markers. The ultimate generation of a clinically robust and accurate molecular marker assay has been a slow and iterative process, which has now resulted in an FDA approved assay that is being implemented clinically. MT-sDNA can now be added to our CRC screening armamentarium. Research on the molecular detection of colorectal and other GI neoplasms is expanding and will likely lead to other high performance diagnostic or screening tools in the future.

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Box 1**Fundamental issues that have led to the development of molecular marker assays for colorectal cancer screening**

1. Colorectal cancer (CRC) is a leading cause of cancer related death world-wide and results in over 600,000 deaths annually.
2. Colorectal cancer related death can be reduced with currently available screening tests, however, compliance with these tests is suboptimal.
3. The need to improve the prevention of colorectal cancer and CRC related death has led to studies to identify accurate noninvasive screening tests for colon polyps and early stage colorectal cancer.
4. Stool and blood based molecular marker assays are among the most promising accurate noninvasive screening tests for colorectal polyps and cancer.

Box 2**Key advances that have led to the development of molecular marker assays for colorectal cancer screening and issues that require further study**

1. Identification of common mutations and aberrantly methylated loci in colon polyps and CRC
2. Recognition of shed epithelial cells in feces
3. Improvement in methods for extraction and preservation of DNA from stool
4. Development of specific and sensitive assays for polyp and cancer specific molecular alterations.
5. Advances in the current molecular marker screening tests for colorectal polyps and cancer that would further optimize their use are reductions in their cost, improvement in their specificity for colorectal polyps and cancer, and improvement in their sensitivity for advanced colon polyps.

Figure 1.

Schematic diagram of the polyp to colorectal cancer sequences. Currently, two discrete normal colon to colorectal cancer sequences have been identified. Both sequences involve the progression of normal colon epithelial cells to aberrant crypt foci (ACF), followed by early and advanced polyps with subsequent progression to early cancer and then advanced cancer. The classic or traditional pathway was the pathway originally identified and involves the development of tubular adenomas that can progress to adenocarcinomas. An alternate pathway that involves serrated polyps and their progression to serrated colorectal cancer has been described in the last 5–10 years. The genes mutated or epigenetically altered are indicated by each pathway. Some genes are shared between the two pathways and others are unique (i.e. *BRAF* mutations and CIMP only in serrated pathway). The signaling pathways deregulated during the progression sequence are also shown with the width of the arrow reflecting the significance of the signaling pathway in tumor formation. (Figure reproduced from Grady WM Mechanisms of Colorectal Cancer, Nature Reviews Disease Primers 2015).

Figure 2. Molecular marker release from colorectal neoplasms into target media

This conceptual model shows proportional differences (illustrated by arrow sizes) expected in rates of marker release into the bloodstream via the mechanism of vascular invasion and into the stool via the mechanism of exfoliation during progressive phases of tumorigenesis. Marker release into the bloodstream from precursor lesions is negligible but increases progressively with advancing stages of cancer. In contrast, marker release by exfoliation into stool occurs at comparable rates from large precancers and all stages of cancer. (Used with permission from Ahlquist DA TW, Mahoney DW, Zou H, Domanico M, et al. The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. *Clinical Gastroenterology and Hepatology* 2012;10:272–7.)

Figure 3. The Exact Sciences Automated Analytic Platform

Multi-target sDNA analytic process. Patient samples are homogenized in the collection container, aliquoted, and centrifuged. DNA markers are captured with target-specific magnetic beads (capture incubator), washed (capture aspirator), and magnetically separated. Bead-bound sDNA biomarkers are transferred to a Hamilton Microlab STARlet (Reno, NV). The portion of sDNA for methylation assay is bisulfite treated, and the portion for KRAS mutation assay is pH neutralized. Treated DNA is then combined with reagents for quantitative allele-specific real time target and signal amplification (QuARTS) on an ABI 7500 FastDx (Carlsbad, CA) that generates results in log strands of DNA. Fecal hemoglobin samples are transferred to enzyme-linked immunosorbent assay plates with Hamilton Microlab STARlet liquid handler and then read (BioTek ELx808 plate reader (Winooski, VT)). Software algorithmically integrates results of assays to calculate a dichotomous “Positive” or Negative” result.

(Adapted and used with permission from Lidgard GP, Domanico MJ, Bruinsma JJ, Light J, Gagrut ZD, Oldham-Haltom RL, et al. Clinical Performance of an Automated Stool DNA Assay for Detection of Colorectal Neoplasia. *Clinical Gastroenterology and Hepatology* 2013; 11(10): 1313–1318.)

Figure 4.

Results of the multi-target sDNA test and commercial fecal immunochemical test in analyzed subgroups. Numbers in parentheses () are sample sizes. A. Sensitivity of assay to detect people with colorectal cancer of designated stages. B. Sensitivity of assay to detect polyps of designated sizes. Footnotes: 1. The stage of one colorectal cancer was not available; 2. The location of one advanced precancerous lesion was not available (Adapted and used with permission from Imperiale TF, Ransohoff DF, Itzkowitz SH, et al. Multi-target stool DNA test for colorectal cancer screening: prospective, multicenter assessment and comparison with fecal immunochemical testing in an average-risk population. *New England Journal of Medicine*. 2014 Apr 3;370(14):1287–97.)