Colorectal cancer screening: are stool and blood based tests good enough?

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Abstract: Colorectal cancer (CRC) is the third commonest cancer worldwide. As many CRC patients were identified at advanced stages, screening asymptomatic individuals has substantial clinical benefit. Most CRC arises through recognizable early stage. With the improved understanding of the biology of CRC and precancerous lesion, testing molecular aberrations in stool and blood promises novel screening approaches that are noninvasive, sensitive, and more affordable compared with traditional structural examinations.

Key Words: Colorectal cancer; screening; biomarkers; stool

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Colorectal cancer

Epidemiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. Over 1.2 million new cancer cases and 600,000 deaths were estimated to have occurred in 2008 (1). The lifetime risk of CRC is approximately 6%. Risk factors for CRC include family history, male gender, smoking, alcohol consumption, physical inactivity, obesity, and red and processed meat consumption. The risk of CRC increases with age, particularly after 50. Death rates of CRC have been decreasing in several Western countries largely because of improved treatment, increased awareness and early detection (2-4). However, both the incidence and death rates of CRC are increasing in Asia because of the lack of guideline for screening and public awareness (5).

Around 15% of CRCs are inherited. The most common forms are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). HNPCC arises because of mutations in mismatch-repair genes, including MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2 (6), leading to DNA instability, such as in the length of microsatellite sequences, and results in microsatellite instability (MSI) (7). HNPCC is characterized by the early onset of colorectal tumors, particularly in proximal colon.

Around 85% of CRCs are sporadic. Based on pathological data, most sporadic CRCs are developed from adenomas (8-10). Adenomas are masses that protrude into the gut lumen, which can either be pedunculated or sessile. Adenomas can be flat or even depressed. The epithelium of adenomas can form glands (tubular adenomas), finger-like structures (villous adenomas), or a combination of both (tubulovillous adenoma). Adenomas that are larger than 1 cm, or those with severe dysplasia or a villous architecture are referred to as advanced adenomas. The development of CRC from adenoma is estimated to require 5 to 10 years, as referred to the adenoma-carcinoma sequence.

Screening

Patients with early stage CRC or precancerous lesion are mostly asymptomatic. By the time patients present with symptoms such as anemia, abdominal pain, weight loss, change in bowel habit, and rectal bleeding, the disease is likely to have reached an advanced stage. The survival from CRC is closely related to the stage of cancer when diagnosed, with late CRC having the worst outcome (11).

Since most CRC develops from precancerous lesions, screening has substantial clinical benefits to patients.

Based on the guidelines from the United States, there are several options for CRC screening (12-14). Flexible sigmoidoscopy and colonoscopy are more invasive but offer the opportunity for removal of detected lesions. Stool based test represents a noninvasive approach; the most widely used is fecal occult blood test (FOBT) that tests the presence of blood in stool. With the progress in the understanding of the biology of CRC, tests based on detecting molecular abnormalities in stool offer new strategies for screening.

Using a flexible fibre-optic instrument inserted through the anus, colonoscopy allows direct visual examination of the entire colorectum, and is regarded as the gold standard for detecting colorectal lesions. It allows the option of removal and treatment of screen-detected lesions. However, colonoscopy imposes a risk of bowel perforation and bleeding, and a very low mortality risk of 1-3 death per 10,000 (14). Many patients find the procedure and the bowel preparation unpleasant. Due to its invasive nature, the cost of equipment and the demand for skilled operators, colonoscopy is not widely used as a first-line screening tool.

Stool based and blood based tests are the mainstream platforms for noninvasive CRC test. Compared to colonoscopy, both means are less sensitive and do not offer the option of immediate removal and treatment of the lesion. However, with the increased understanding in CRC biology, improved methods in stabilizing and purifying biomolecules from biological samples, these tests provide an excellent platform for testing various molecular abnormalities for CRC screening.

Stool based tests

Neoplastic features of intestinal lumen can be consistently detected in stool. Theoretically, stool based tests enable screening of the entire length of the colorectum, require no bowel preparation, and the specimens are easily transportable, which means that these tests can be obtained without the need to visit their doctors. These properties are likely to increase patient acceptability.

Fecal occult blood

The most widely used stool based test is the fecal occult blood test (FOBT). It detects blood in the stool that has leaked from disrupted vessels on the tumor or adenoma surface. FOBT has a low sensitivity as not all colorectal adenomas and tumors bleed, and those that do bleed do so intermittently (15). There is evidence that large adenomas and tumors bleed more frequently than smaller lesions (16). Asymptomatic tumors, which are the intended target of screening, also bleed less than symptomatic tumors (17). The classical FOBT involves a guaiac test for the peroxidase-like activity of heme in haemoglobin. Since heme is present in red meat, and peroxidase activity is present in fresh fruits and vegetables, false positive rate is high using this test. A diet or medication restriction is needed to optimize test performance. Sensitivity of FOBTs is typically around 50% for CRC and lower than 20% for adenomas. Despite its low sensitivity, FOBT is the only form of noninvasive test with proven efficacy in reducing CRC mortality. In three randomized controlled trials from the United States (18,19), Denmark (20,21), and the United Kingdom (22) using FOBT with annual or biennial testing has demonstrated a moderate (15-33%) reduction in CRC mortality after 10-14 years of follow-up.

A more advanced version of FOBT is the fecal immunochemical tests (FITs). FITs use antibodies specific to human hemoglobin or other blood components independent of peroxidase activity. They could be more specific in detecting blood of human origin and can eliminate the need of diet and medication restriction. Furthermore, FITs enable automated analysis for reading the test results, removing human error associated with interpretation. FITs have demonstrated a higher sensitivity towards CRC compared to guaiac based tests but its sensitivity remains low for precancerous lesions (23). In a study consisting of more than 20,000 subjects, FIT showed a sensitivity of 27% for advanced neoplasms and 66% for invasive cancer (24).

Stool DNA

Molecular alterations found in tumors can be detected in the stool because colonocytes exfoliate consistently into the lumen. The stool DNA test represents the most established noninvasive test for CRC. Various DNA mutation and methylation have been reported to be useful in discriminating CRC patients from healthy individuals. A study in an average-risk population showed that the individual marker of *APC*, *TP53*, *KRAS*, MSI and DNA integrity has a sensitivity ranging from 3.2% to 25.8% for the detection of CRC; a combined panel of these DNA markers has a sensitivity and specificity of 52% and 94%, respectively, for the detection of CRC (15). Technology used to detect DNA mutation continues to improve and the DNA panels continue to refine. Pilot studies have demonstrated the use of more sensitive approaches in testing stool based DNA mutation, such as BEAMing (which

derives its name from its principal components: beads, emulsion, amplification, and magnetics) (25) and digital melt curve (26). Better stool based DNA recovery was achieved by using EDTA-containing buffer to stabilize the stool sample (27). The addition of vimentin into the marker panel had also greatly improved the panel's performance (28). A new generation of stool DNA panel was described recently (29). It combined 4 methylation markers (*BMP3*, *NDRG4*, *vimentin*, and *TFPI2*), 7 reference mutations in *KRAS*, β-*actin* and a hemoglobin assay, achieved a sensitivity of 85% for CRC, and 54% for adenoma ≥1 cm. Each component marker typically yielded an area under the curve (AUC) value ranging from 0.61 to 0.75 towards CRC. This version of DNA test is currently seeking approval from the U.S. Food and Drug Administration.

Stool messenger RNA and protein

Stool based messenger RNA (mRNA) is another frequently exploited analyte. Several reports have shown that detecting stool based mRNA such as cyclin (30), cyclo-oxygenase 2 (COX-2) (31-34), or matrix metalloproteinase 7 (MMP-7) was able to discriminate CRC patients from healthy individuals. Notably, COX-2 mRNA was reported to be able to detect 26 out of 29 CRC cases (90% sensitivity) with 100% specificity in a Japanese study (32). Although some mRNA markers could achieve high sensitivities, the lack of stability of mRNA in stool samples has limited its application. In addition, neoplasm-derived proteins such as minichromosome maintenance proteins (35), carcinoembryonic antigen (32,36), M2 pyruvate kinase (37) and secreted clusterin isoform (38) in stool samples were also reported to be able to discriminate CRC patients from controls. Among them, stool carcinoembryonic antigen showed a sensitivity of 86% and a specificity of 93% for CRC (36). Compared with the stool DNA test, testing for RNA or protein in stool is less established. Validations in larger numbers of patients, including patients with adenomas, are warranted.

Stool microRNA

microRNA (miRNA) is a relatively new class of biomolecules being exploited as disease markers. They are 18- to 25-nucleotide non-coding RNA molecules that regulate the gene translation (39). Binding of a miRNA-loaded RNA induced silencing complex (RISC) to a complementary sequence will lead to either translational repression or decay of the targeted mRNA (40). Through this, miRNAs regulate a variety of cellular processes including apoptosis (41-43), differentiation (44) and cell proliferation (45). Altered miRNA expression profiles were found in most tumor types including CRC (46-49).

In colorectal tumors, miRNA expression profile tends to show a typical signature aberration (50). Since in 2009, several pilot studies based on small cohorts have reported the feasibility of using stool based miRNAs as biomarkers for CRC screening (51,52). In a cohort of 197 CRC patients and 119 healthy controls, Koga *et al.* investigated the sensitivities of stool based miR-17-92 cluster members, miR-21 and miR-135 in discriminating CRC patients from healthy individual (53). They reported a combined sensitivity of 74% and a specificity of 79% towards CRC; however, sensitivity towards adenoma was not investigated in this study. Wu *et al.* demonstrated stool miRNAs were relatively stable in stool and the detection by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was highly reproducible (54). Notably, miR-92a showed a sensitivity of 72% for CRC and 56% for polyps (including hyperplastic polyps and adenomas), with a specificity of 73%. The level of stool miR-92a dropped significantly after the removal of tumor or advanced adenoma. miR-92a also had a higher sensitivity towards advanced adenoma than minor polyps, and a high sensitivity in detecting distal CRC than proximal CRC.

Blood based tests

For the markers released by the tumor to be detected in blood, the mechanism of vascular invasion is required. In precancerous lesions of which vascular invasion has not yet been involved, it is expected that the amount of blood entering bloodstream is negligible. But as the staging of the cancer advances, the amount of marker detected in blood will increase as the degree of vascular invasion progresses. Compared to stool based test, blood test could be less sensitive in detecting early stage lesions but easier to implement and comply with.

Blood protein

Carcinoembryonic antigen (CEA) is a glycoprotein involved in the process of cell adhesion. It was first described as a specific CRC marker in 1969 (55). Kuusela *et al.* demonstrated its value as a diagnostic marker, in a cohort of 111 CRC patients, serum CEA showed a sensitivity of 69% and specificity of 70%. In the same cohort, cancer antigen

19-9 (CA 19-9), a cancer marker more commonly used to detect pancreatic cancer, showed a sensitivity of 36% and a specificity of 97% for CRC (56). Until now, serum CEA level is still frequently used as a marker to monitor recurrence after surgery, but rarely as a marker in predicting the disease. Colon cancer-specific antigen (CCSA)-3 and CCSA-4 are nuclear matrix proteins. They were found to detect all 28 CRC patients (sensitivity =100%) in a study, with test specificities of 96% for CCSA-3 and 98% for CCSA-4 (57). Galectin-3 is a beta-galactoside binding protein relevant to tumor progression and metastasis. Bresalier *et al.* showed serum Galectin-3 level was able to discriminate patients with CRC from those with other colorectal diseases (hyperplastic polyps, adenomas, and inflammatory bowel disease). However, no sensitivity or specificity of Galectin-3 was reported in this study (58).

Blood messenger RNA and microRNA

Few studies had exploited blood based mRNA as CRC biomarkers. Identified by oligonucleotide microarray analysis on colorectal tissues, KIAA1199 was described as a CRC biomarker, however its function remains not clearly understood (59). Serum KIAA1199 mRNA level demonstrated a sensitivity of 74% for CRC and adenoma, and a specificity of 66%, based on a cohort of 20 CRC, 20 adenoma and 20 normal subjects. More studies had focused on plasma miRNAs, largely because they remained very stable in plasma and could be robustly quantified (60,61). Plasma based miRNA was first demonstrated to be useful as CRC biomarkers by Ng *et al.* (62). They reported plasma miR-92a, a candidate identified by miRNA array profiling, had a sensitivity of 89% and a specificity of 70% in discriminating CRC from control subjects. Notably, plasma miR-92a level dropped significantly upon the removal of tumor, showing the marker was likely to be derived from the colorectal lesions. Since then, more miRNA candidates were reported, including miR-29a (63), miR-221 (64), miR-21 (65), U2 small nuclear RNA (RNU2-1) (66), miR-601 and miR-760 (67). Among them, RNU2-1, a marker for both CRC and pancreatic ductal adenocarcinoma (PDAC), was found to have a sensitivity of 97.7% in detecting CRC and/or PDAC, at a specificity of 90.6%. But this has not yet been tested in another independent study.

Blood DNA

Because of the established mutation and methylation characterized in adenoma-carcinoma sequence, plasma DNA has been more robustly evaluated than other plasma based markers. Diehl *et al.* showed that mutant APC fragment has a 100% sensitivity in detecting Dukes D stage patients (n=6) and a sensitivity of 63% in detecting Dukes A and B stage (n=16). The test remained poor in detecting advanced adenoma (68). Hypermethylated Septin-9 is the most studied plasma DNA marker. Multiple studies had reported its sensitivity towards CRC, ranging from 52% to 73% at specificities ranging from 84% to 91%, while sensitivity towards advanced adenoma was less than 20% (69-72). Currently, Septin-9 test is the only commercially available plasma DNA test intended for CRC detection.

Blood fatty acid

Gastrointestinal tract acid-446 (GTA-446) is a long-chain polyunsaturated fatty acid. Its serum level can be detected by mass spectrometry. Serum GTA-446 level was found to be reduced in CRC patients. Ritchie *et al.* showed that among 4923 subjects who had undergone colonoscopy, 84 out of the 98 CRC cases were detected to have a low serum GTA-446 level (as defined by the lowest tenth percentile), with a test specificity of 90% (73). The reduction of serum GTA-446 level was proposed to represent a compromised ability to protect against abnormal cell growth and chronic inflammation.

Stool test *vs.* blood test

Tumor markers enter the stool and blood stream through different mechanisms. Theoretically, exfoliation of colonocytes into the lumen occurs earlier than vascular invasion. Stool based test should be more effective in detecting precancerous lesions. Ahlquist *et al.* compared two commercially available tests: the stool DNA panel test (Exact Sciences Corporation, Madison, Wisconsin) and plasma Septin-9 test (ARUP Laboratories, Salt Lake City, Utah) in the same cohort of CRC and adenoma samples (n=42) but using separate sets of normal controls (stool, n=46; plasma, n=49). They found that the stool test had a higher sensitivity in detecting CRC (87% *vs.* 60%) and large adenomas (82% *vs.* 14%) compared to the plasma Septin-9 test. The specificity for the stool test and plasma test was 93% and 73% respectively. Based on this study, the stool DNA panel test is more effective in detecting early stage lesion that the plasma Septin-9 test.

Conclusions

Colonoscopy remains to be the gold standard in detecting

CRC. Stool and blood based tests could serve as first line screening tests for the screening of asymptomatic individuals, in which only those tested positive will proceed to perform colonoscopy. Among the reported studies, many stool or blood markers had demonstrated very high sensitivity and specificity. And new biomarkers will also continue to emerge as we improve our understanding of CRC biology. However, it is always more important to validate the markers in multi-centered studies with large cohorts of samples. With vigorous testing and validation, it is foreseeable in the near future that highly sensitive noninvasive test could be achieved through combining markers of different classes of molecule (e.g., DNA, RNA, protein) sourced from different biological samples (stool, blood). Population-based CRC screening will become more common and effectively conducted.

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