What Should We Seek for in the Stool?
Blood, DNA, or Something Else?

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Introduction

Candidate stool markers are hemorrhaged, secreted, exuded and exfoliated into stool. Detection of the presence of hemoglobin in feces using a fecal occult blood test (FOBT) has proved effective; guaiac-based FOBTs are proven by multiple RCTs\(^1\)\(^-\)\(^4\) and fecal immunochemical tests (FIT) for hemoglobin, by case-control studies.\(^5\)\(^-\)\(^7\) DNA changes and the quantitative downstream effects on RNA and protein expression result in many candidate markers. A growing body of evidence suggests a potential link between the microbiota and colorectal carcinogenesis. CRC risk is determined by the influence of the diet on the microbiota to produce anti- or pro-neoplastic metabolites. Previous studies reported a profound differences in the microbiota and metabolic phenotype between African Americans and rural South Africans.\(^8\)\(^,\)\(^9\) I’d like to summarize candidate stool markers for CRC screening in this chapter.

Fecal Occult Blood Tests (FOBTs)

1. Guaiac-based FOBT (GT)

GT detect blood in the stool through the pseudoperoxidase activity of heme or hemoglobin. 3 RCTs\(^1\)\(^-\)\(^4\) demonstrated 15 to 21% reductions in CRC mortality, but they are much lower than expected. The reasons for lower mortality reduction can be explained by low compliance, limited test sensitivity, and positive tests are not followed up with colonoscopy. So GT is not performed anymore in Korea.

2. Fecal Immunochemical Test (FIT)

FIT detects human hemoglobin, so shows lower false positivity than GT. Lack or dietary or drug restriction increases compliance. The sample collection is less demanding. FIT is specific to colorectal bleeding. Many countries including Korea performed annual or biennial FIT for the national CRC screening program.\(^10\) This test has 10 to 12% gains in adherence and detects more advanced lesions.

However, a number of issues need to be clarified. The number of samples necessary for optimal sensitivity and specificity. The cutoff point of fecal hemoglobin for the best sensitivity and specificity. Stability of samples. The majority of FIT studies in average-risk populations did not have “gold standard” endoscopy results for
FIT-negative patients, so evidence of FIT’s effectiveness was insufficient. No FIT has been tested in a randomized trial where the outcome of interest is CRC mortality yet.

**Stool DNA (sDNA) Testing**

The mutated or aberrantly methylated copies of the tumor genes to be identified are only a small proportion of the minute fraction of stool DNA that is of human origin. Comparing with FIT, the advantage of sDNA testing is higher sensitivity for adenomatous polyp. However, considering the molecular heterogeneity of CRC, complementary genetic and epigenetic marker panels rather than single marker should be selected that cover essentially all forms of CRC and precancerous lesions. The 1st-generation multi-target sDNA test, SDT-1 analyzed complex marker panels Including 21 mutations in genes known to be involved in MSS CRCs, a marker of MSI and an assay that detected aberrant apoptosis. In the 1st study performed by Imperiale in an average risk screening population, SDT-1 was better than GT for detecting CRC. However, the sensitivity of 52% for CRC was lower than expected. A 2nd large-scale prospective study was performed by Dr. Ahlquist. SDT-1 provides no improvement over HemoccultSensa for detection of screen-relevant neoplasia. The new stool DNA test, SDT-2 which is consisted of 3 markers- KRAS mutation, scanning of APC mutator cluster regions, and vimentin gene methylation showed higher sensitivity for screen-relevant neoplasia than Hemoccult or HemoccultSensa. But showed higher false positivity than Hemoccult or HemoccultSensa. A recent Multi-center study in the US and Canada included almost 10 thousand asymptomatic average risk person aged between 50 and 84 who are undergoing screening colonoscopy. They performed quantitative molecular assays for KRAS mutation, aberrant NDGR4 and BMP3 methylation and generated with the use of logistic-regression algorithm, with values of 183 or more considered to be positive. The sensitivity for detecting CRC was 92.3% with sDNA testing and 73.8% with FIT. The sensitivity for detecting advanced precancerous lesions was 42.4% with sDNA testing and 23.8% with FIT. Specificities with sDNA testing and FIT were 89.8% and 96.4%, respectively. sDNA testing has been endorsed for CRC screening by many scientific organizations, however, US government concluded that the evidence from earlier sDNA tests was insufficient to assess the benefit and harms, deferring a final recommendation for the accumulation of additional evidence in 2008. Thereafter, several studies were published in an average-risk screening population, the FDA formally approved the MT-sDNA test for use in general CRC screening on Aug 11, 2014, and on the same date, CMS ruled that the test would be covered by Medicare at a frequency of every 3 years.

**Stool RNA Testing**

During the last several years, investigation has turned to micro(mi)RNAs, which are 18-25 nucleotide non-coding RNA molecules that regulate gene expression and translation, indirectly affecting cell differentiation, cell cycle progression, and apoptosis. More stable in stool and plasma than other nucleic acid molecules, mi-RNAs play either an oncogenic or tumor-suppressor function in the multi-step process of carcinogenesis, and are thought to be cell-type and disease-specific. While a myriad of studies of single and panels of mi-RNAs have been published, the field is still nascent. Several technical and analytical issues affecting measurement stability remain to be addressed. The more recent use of digital PCR likely represents a technical im-
provement that could accelerate scientific advancement. Similar to the sDNA literature, studies on miRNA are largely case-control studies of subjects with CRC compared with either “healthy” or colonoscopy-negative subjects, with or without a third group with adenomas, and is quite heterogeneous in the specific single or panel of mi-RNA, study sample size, collection methods, and preparation, making comparisons challenging. Ahmed and colleagues have found differential expression of several mi-RNAs when comparing stool of cases with cancers to that of controls with no neoplasia\textsuperscript{15}; however, the results require independent validation. Of interest, only one differentially regulated mi-RNA (mi-106a) is common to these two works, suggesting the need for independent validation, followed by large-scale, population-based evaluation.

**Stool Protein Testing**

1. **Fecal calprotectin**

Calprotectin, a calcium binding protein found largely in neutrophils, has been the most widely studied of the non-cancer specific proteins. However, most of the studies are small case-control studies that were not performed within the target population of interest (i.e. average risk screening). One of the best evaluations of the marker was performed using stool samples obtained on individuals participating in the Norwegian Colorectal Cancer Prevention Screening trial (n = 2321) where the performance of calprotectin was compared with FIT.\textsuperscript{16} Calprotectin detected fewer cancers than FIT, and had lower overall specificity.

2. **Tumor M2 pyruvate kinase (M20PK)**

Of the cancer-related fecal proteins, tumor M2 pyruvate kinase (M2-PK) has received the most attention as a potential stool biomarker for cancer screening. A recently reported meta-analysis summarized 10 observational studies (6 case-control and 4 cohort) of M2-PK and only included those studies where colonoscopy was performed in all study participants.\textsuperscript{17} The pooled CRC sensitivity and specificity were 79% and 81%, respectively. The authors specifically looked at 4 studies that compared M2-PK to FIT and found that the diagnostic odds ratio favored FIT (67.2 vs. 9.5).

**Microbiota and Microbial Metabolites**

A growing body of evidence suggests a potential link between the microbiota and CRC carcinogenesis. Studies have demonstrated an enrichment of *Fusobacterium nucleatum, Campylobacter, Erysipelotrichaceae, Collinsella, Peptostreptococcus*, and *Anaerotruncus* in human CRC and adenomas compared with adjacent normal mucosa, opposite to a decrease of cluster IV and XIV *Clostridium* members, such as *Faecalibacterium prausnitzii* and *Roseburia*, both butyrate-producing bacteria.\textsuperscript{18,19} Experimental studies have shown that *F. nucleatum* activates the WNT signaling pathway in CRC cells and may promote colorectal tumor growth, and that *F. nucleatum* may inhibit T cell-mediated immune responses against colorectal tumors.\textsuperscript{20} The amount of *F. nucleatum* DNA in CRC tissue is associated with shorter survival and may potentially serve as a prognostic biomarker.\textsuperscript{21}

A diet rich in red meat and animal fat and higher counts of 7α dehydroxylating colonic bacteria will generate high levels of secondary bile acids in the bowel, which are cytotoxic to colonic epithelial cells, as well as muta-
genic with antiapoptotic properties. Bacterial activities and enzymes that have been linked to increased risk of CRC were described as glucuronidases, H2S generation, azoreductases, nitroreductases, alcohol dehydrogenases, arylsulfatases, and reactive oxygen intermediates-generating enzymes.

Conclusions

FIT is the most frequently used, stool-based test for CRC screening. DNA changes and the quantitative downstream effects on RNA and protein expression result in many candidate markers. Studies on gut microbiota and CRC are exciting and give insights on the understanding and management of colorectal neoplasia.

References


