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TOPIC HIGHLIGHT

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Fecal DNA testing for colorectal cancer screening: Molecular targets and perspectives

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Abstract

The early detection of colorectal cancer with effective screening is essential for reduction of cancer-specific mortality. The addition of fecal DNA testing in the armamentarium of screening methods already in clinical use launches a new era in the noninvasive part of colorectal cancer screening and emanates from a large number of previous and ongoing clinical investigations and technological advancements. In this review, we discuss the molecular rational and most important genetic alterations hallmarking the early colorectal carcinogenesis process. Also, representative DNA targets-markers and key aspects of their testing at the clinical level in comparison or/and association with other screening methods are described. Finally, a critical view of the strengths and limitations of fecal DNA tests is provided, along with anticipated barriers and suggestions for further exploitation of their use.

Key words: Colorectal cancer; Screening; Fecal DNA; Cologuard®; Adenoma

Core tip: The molecular DNA targets from genetic and epigenetic alterations hallmarking colorectal carcinogenesis are reviewed here in the context of fecal testing. Also, comparison with other screening methods in terms of limitations, advantages and future perspectives of fecal DNA tests are discussed.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and women and accounts for 8% of all cancer-related deaths[1]. The incidence of CRC varies within different geographic locations and racial/ethnic groups. These differences may be related with different dietary and environmental exposures in association with a different genotype-driven susceptibility[2]. Screening for CRC plays a key role in reduction of CRC-related mortality, and the observed decline in the incidence of CRC since the mid-1980s is a striking proof of this effect, along with changes in risk factors[1].

CRC screening may be divided into two main categories: (1) biological sample-based tests, including fecal, blood and urine tests, as well as (2) colon structure-based and image-based tests, including flexible sigmoidoscopy, total colonoscopy, CT colonography and double-contrast barium enema[3,4]. Stool-based tests, including guaiac-based fecal occult blood test (g-FOBT), and the newer ones, fecal immunochemical test (FIT) and stool DNA test are already included in the American Cancer Society recommendations for CRC screening[4].

MOLECULAR RATIONAL FOR FECAL DNA TESTING

The detection of altered DNA from cancerous and pre-cancerous lesions of the colonic mucosa is based on the natural exfoliation of these cells and is further facilitated by their high degree of "integrity" compared to DNA from stools of healthy patients. Accumulating data on key mutations occurring during the early stages of colon carcinogenesis including K-Ras, adenoma polyposis coli (APC), and p53, as well as epigenetic changes such as microsatellite instability (MSI), has guided the targeted development of clinically relevant detection tests[5].

The genetic heterogeneity of CRC is essentially the reason underlying the concept of targeting multiple DNA markers. K-Ras encodes a RAS family protein which is a GTPase involved in many downstream signal transduction pathways[6]. The mutation is found in 13%-95% of CRC patients and is one of the initial mutations in colon carcinogenesis[6]. APC is an important tumor suppressor gene product involved in the Wnt/β-catenin signaling pathway, which in turn is a transcription regulator of several growth-controlling genes, including the oncogene *MYC*[7]. Thus it is not surprising that mutation or inactivation of the APC protein is a driver of inherited (familial adenomatous polyposis) and sporadic forms of CRC, occuring in the early stages of transition from adenoma to carcinoma[7]. Another tumor suppressor gene, *p53* is found deleted or mutated in 30%-60% of CRC tumors[8]. Given its critical role in cell cycle control, apoptosis, and DNA damage response, p53 aberrations ultimately promote the development of increased genomic instability which facilitates transformation of colorectal adenomas to cancer[7].

MSI is a condition of genetic hypermutability within tandem repeats of short nucleotide sequences, the microsatellites, that results from impaired DNA mismatch repair (MMR) and is a frequent event in cancers, including 15% of all CRC[9]. The most common cause of sporadic MSI is epigenetic silencing of *MMR* genes, such as MLH1 due to promoter hypermethylation[7] and there are several MSI markers (BAT25, BAT26, D2S123, D5S346, and D17S2720) for detection of MSI with polymerase chain reaction. The clinical relevance of MSI lies in the fact that patients with MSI positive tumors have better prognosis and longer overall survival compared with non-MSI tumors[9].

Epigenetic methylation of gene promoters is a central mechanism that can promote carcinogenesis in the appropriate context and several preclinical studies have identified hypermethylated genes in stool samples from CRC patients, which are strikingly un-methylated in normal epithelial cells[9]. Characteristic examples include the genes secreted frizzled-related protein (SFRP), vimentin, MGMT, FBN1, and p16[7]. In addition, the panel of methylated genes varies depending on the different stages of carcinogenesis, involving (1) SLC5A8, SFRP1, SFRP2, CDH13, CRBP1, RUNX3, MINT1 and MINT31 from normal colon mucosa to aberrant crypt focus formation; (2) p14, HLTF, ITGA4, p16, CDH1, and ESR1 from aberrant crypt focus to adenoma formation; and (3) TIMP3, CXCL12, ID4, and IRF8 from adenoma to carcinoma formation and metastatic progression of CRC[7].

CLINICAL STUDIES OF FECAL DNA TESTS

An important limiting factor for developing a screening stool test with high sensitivity is the fact that only 0.01% of total fecal DNA is human and the tumor DNA is only a small percentage of the former[10].

K-RAS was the first gene tested for mutations in feces from CRC patients[11-13]. A comparative study assessed gFOBT and a fecal DNA test analyzing a panel of 21 gene mutations[14]. Imperiale *et al*[14] concluded that the multitarget fecal DNA test detected more invasive cancers plus adenomas with high-grade dysplasia than did gFOBT (40.8% *vs* 14.1%) without compromising specificity (94.4% *vs* 95.2%). In a blinded, multicenter, case-control study, with cases including CRC, advanced adenoma (AA), or sessile serrated adenoma $\geqslant$ 1 cm (SSA), an automated multitarget stool DNA assay was able to detect AA with high-grade dysplasia with 83% sensitivity[15]. Another blinded, multicenter, case-control study assessing a similar panel of DNA markers identified 85% of patients with CRC and 54% with AA,

Table 1 Fecal DNA markers for advanced adenoma and colorectal cancer *n* (%)

Ref.	Marker	Sensitivity		Specificity
		CRC	Adenoma > 1 cm	
[12]	Meth BMP3, hDNA, KRAS, APC	67 (91)	21 (78)	85 (85)
[13]	APC, KRAS, p53, long DNA	3 (25)	47 (8)	2246 (96)
[14]	APC, KRAS, p53, long DNA	16 (52)	84 (12)	1344 (94)
[15]	β-actin, KRAS, meth BMP3 and NDRG4, fecal hemoglobin	91 (98)	48 (57)	139 (90)
[16]	KRAS, a actina Meth NDRG4, BMP3, vimentin, TFPI2	214 (85)	72 (54)	264 (90)
[17]	KRAS, NDRG4, BMP3, β-actin, fecal hemoglobin	60 (92)	321 (42)	4457 (90)
[20]	Meth vimentin	9 (41)	9 (45)	63 (95)
[21]	Meth SFRP2	60 (87)	21 (62)	28 (93)
[22]	Meth TFPI2, long DNA	52 (87)	4 (44)	25 (83)
[23]	Meth SFRP2, HPPI, MGMT	50 (96)	15 (71)	23 (96)
[24]	Meth APC, ATM, hMLH1, sFRP2, HLTF, MGMT, and GSTP1	15 (75)	17 (68)	27 (90)
[25]	Meth vimentin, long DNA	68 (83)	6 (86)	298 (82)
[26]	Meth RASSF2 or SFRP2	63 (75)	25 (44)	101 (89)
[27]	Meth vimentin, MLH1, MGMT	45 (75)	31 (60)	32 (87)
[28]	Meth RARB2, p16INK4a, MGMT, APC	16 (62)	8 (40)	20 (100)

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without sensitivity differences based on location, but with tumor size affecting detection rates[16].

More recently, Imperiale *et al*[17] reported their results from comparison of fecal DNA to FIT in a huge patient population who had a complete screening colonoscopy (n = 9989). The sensitivity of fecal DNA test including evaluation of KRAS mutations, aberrant NDRG4 and BMP3 methylation, B-actin and a hemoglobin assay was superior to that of FIT (92.3% *vs* 73.8%). However, in addition to a lower specificity of fecal DNA and the lack of comparison with repeated FIT applications over time, a far higher number of patients (n = 689) were excluded due to problematic fecal DNA testing, compared to those who underwent FIT (n = 34)[18].

A systematic review of the literature for studies of biomarkers for early detection of colorectal cancer and polyps since 2007, disclosed overall sensitivities for colorectal cancer detection by fecal DNA markers ranging from 53% to 87%, with varying specificities above 76%[19]. The diversity and combinations of various fecal DNA markers with the corresponding sensitivities and specificities per study[12-17,20-28] are summarized in Table 1.

EVOLUTION OF FECAL DNA TESTING METHODOLOGY AND TECHNIQUES

Initially, the first fecal DNA tests were performed without stabilizing buffers, resulting in low sensitivities[13,14]. Upon incorporation of stabilizing buffers and introduction of more sensitive detection techniques such as the digital melt curve method and beads, emulsion, amplification, and magnetics (BEAMing), the initial detection threshold of 1% of mutated copies was decreased to less than 0.1%[10,12].

Furthermore, implementation of the allele-specific quantitative real-time target and signal amplification (QuARTS) technique led to detection of less frequent mutations, thus improving the sensitivity for AA[12]. Another technique termed fluorescent long DNA (FL-DNA), allows for identification of tumor DNA fragments longer than 150-200 base pairs, given that cancer cells evade apoptosis and subsequent DNA degradation. FL-DNA detects CRC with a sensitivity of 80%[29]. Other advances that have been introduced in different studies include neutralization of bacterial enzymes with EDTA[30], enrichment of the panel of DNA markers (*e.g.,* vimentin gene), and inclusion of hemoglobin detection in the same panel[16,31].

STRENGTHS AND LIMITATIONS OF FECAL DNA TESTS

A major advantage of fecal DNA tests as compared to either FOBT or colonoscopy is the fact that they are not affected by proximal location of tumors[32,33]. Another advantage is the lack of need for purging or dietary changes.

However, the sensitivity of fecal DNA tests appears to be lower for adenomas when compared to CRC detection (Table 1). In addition, although there is evidence of reductions in CRC incidence and mortality from randomized controlled trials of fecal occult blood test (FOBT) screening[34], similar data are lacking for fecal DNA tests.

Other technical difficulties may involve the burden of large volume stool collection and shipping for the patients undergoing screening[31]. In addition, the fact that in the latest study of Imperiale *et al*[17] the DNA tests had over twice as many abnormal results as FIT, with a higher rate of false-positive results implies that more colonoscopies would be needed to further evaluate for CRC in the former arm. Thus, the inevitably higher number of diagnostic testing would increase the costs and risks of screening. Only with the current screening method of gFOBT, 690011 colonoscopies for false positive screening tests result in an additional estimated annual cost of £800000000[19].

Cost-effectiveness *per se* seems to be a major disadvantage of fecal DNA tests as both older and newer studies, particularly based on a Markov model, have concluded that fecal DNA is cost-effective only when compared with no screening, but is essentially dominated by most of the other available screening options, including FOBT and colonoscopy[36,37]. This may necessitate the limitation of number of DNA markers to render their clinical use more reasonable[38].

CURRENT STATUS OF FECAL DNA TESTING (COLOGUARD®)

The United States Food and Drug Administration has recently approved Cologuard® (Exact Sciences Corporation, Madison, WI, United States), a multitarget stool DNA test in CRC screening[39]. The frequency of interval testing was determined to be every 3 years with adequate Medicare coverage[40]. Cologuard® incorporates molecular assays for aberrantly methylated *BMP3* and *NDRG4* gene promoter regions, mutant KRAS and β-actin as well as an immunochemical assay for human hemoglobin. It is based on the recent study of Imperiale *et al*[17] which showed a significantly better sensitivity for cancer detection compared to FIT. Further laboratory-based processing of the samples is necessary, entailing amplification and detection with the use of Quantitative Allele-specific Real-time Target and Signal Amplification (QuARTSTM) technology[41].

FUTURE PERSPECTIVES FOR FECAL DNA SCREENING TESTS

The combined use of screening tests would likely maximize the benefits of different biomarkers for early detection of CRC and adenomas. However, synchronous implementation of these tests in a mass screening program would not fulfill the cost-effectiveness requirement for clinical use.

Thus, there is a need for prospectively designed, systematic evaluations of the most promising fecal tests in a well-defined, large-scale screening population, with standardized sample collection, processing, and storage. This assessment should be combined with sigmoidoscopy or colonoscopy screening and ideally involve repeated testing and longitudinal monitoring of the screened population[19]. Another parameter that merits prospective evaluation is the clinical significance of fecal DNA-positive results in patients with negative colonoscopy results[40].

In the future, Imperiale and colleagues plan to "take this work forward by conducting a post-approval study, which will inform the important issue of test interval, that is, how often does the test need to be repeated". They will also conduct computer simulation studies that will inform comparative effectiveness and cost-effectiveness relative to other screening tests and strategies[42].

Given the high sensitivity for CRC that is unaffected by tumor location and its superior sensitivity over FIT for detection of SSA and AA with greatest risk of progression, Cologuard® may be a good candidate for interval testing after initial colonoscopy. For the same reason, in cases of poor preparation or incomplete colonoscopy, it might represent a convenient follow-up screening test alternative to repeat colonoscopy or other CT colonography, particularly for those patients who are either unable or unwilling to undergo repeat bowel preparation and invasive endoscopy[40].

In an expanding view, fecal DNA testing could be implemented as a screening in CRC predisposing conditions, such as inflammatory bowel disease, playing a role complementary to colonoscopy for early dysplasia detection and surveillance[40,43]. A relevant multicenter validation study has recently been initiated (Government-registered Trial: NCT01819766) and its results are eagerly awaited.

Finally, technological advancements in detection assays of small fragment DNA from stool may render the identification of altered DNA shed from upper GI pre-cancerous and malignant lesions feasible[44-46].

Discussion of screening tests involving non-DNA (*e.g.,* mRNA, miRNA) or non-fecal origin (*e.g.,* blood, urine) biomarkers was beyond the scope of this review. However, it is reasonable to assume that fecal shedding of tumor DNA is an earlier event compared to inner tissue and bloodstream invasion, and is also directly related to the natural, constant process of luminal colonic mucosa exfoliation; thus rendering fecal testing more timely sensitive for the purpose of screening.

Collectively, the accumulation of experience from clinical use of Cologuard® and the numerous ongoing studies on a plethora of biomarkers, as well as further technological advancement of colonoscopy with the full-spectrum endoscopy[47] are expected to further elucidate and expand the landscape of CRC screening research in the coming years, with the hope of further reducing CRC-specific mortality through earlier and accurate detection of pre-cancerous lesions.

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