

Research Article

Multitarget Stool DNA Test Versus Fecal Occult Blood Test for Colorectal Cancer Screening

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Abstract

Background: Colorectal cancer (CRC) screening is the cornerstone in the prevention of this disease. Our study aimed to compare the multitarget stool DNA (mt-sDNA) test with the fecal occult blood test (FOBT) for CRC screening.

Methods: A total of 151 individuals were screened by colonoscopy, mt-sDNA, and FOBT, respectively, for the detection of CRC and adenoma. The results of mt-sDNA were compared with colonoscopy to acquire the sensitivity and specificity. Then comparison of the sensitivity and specificity of mt-sDNA with FOBT were performed in CRC and advanced precancer.

Results: Stool samples were collected from patients with CRC (n = 50) or large adenoma (n = 51), and normal controls (n = 50). The mt-sDNA test outperformed FOBT in detecting CRC with a sensitivity of 90% (45/50) versus 42% (21/50), advanced adenoma with 70.6% (36/51) versus 19.6% (10/51), stage I–III CRC with 94.6% (35/37) versus 29.7% (11/37), and stage IV CRC with 84.6% (11/13) versus 76.9% (10/13). In addition, mt-sDNA had a specificity of 94% (47/50) in detecting CRC, which is superior to FOBT with specificity of 90% (45/50).

Conclusions: The mt-sDNA test has higher sensitivity and specificity in diagnosing both CRC and advanced adenoma in comparison with FOBT.

Keywords: mt-sDNA; FOBT; Adenoma; Colorectal cancer; Sensitivity; Specificity

Introduction

Colorectal Cancer (CRC) is a common malignancy associated with mutations in multiple genes. It may take 10 years to progress from a benign tumor to CRC in 80% of the affected people. Therefore, CRC screening is critical for early detection and treatment of this disease. The Fecal Occult Blood Test (FOBT) and colonoscopy are the mainstay of CRC screening. However, the FOBT has a low diagnostic performance especially for colorectal adenoma [1-3]. Colonoscopy is the gold standard for diagnosing CRC with good sensitivity and specificity but is associated with a high risk of complications and low compliance [3]. Cancer cells from early-stage CRC are continuously shed into the colonic lumen and mixed into stool. Tests for genetic and

epigenetic alterations in fecal DNA have been considered a possible method for the early detection of CRC [4]. KRAS is an oncogene and is common in malignant tumors. About 30% to 40% of CRC tumors have KRAS gene mutations. The 7 mutation hotspots account for more than 90% of the KRAS gene mutations, including Gly12Asp, Gly12Val, Gly12Ser, Gly12Cys, Gly12Ala, Gly12Arg, and Gly13Asp [5]. It has been shown that mutations in the 12 and 13 codons in the exon 2 of KRAS are closely related to the development of CRC, and mutations in the 12 codons are associated with worse prognosis in comparison with that in the 13 codons [6].

Hypermethylation of the CpG islands in the gene promoter regions suppresses specific gene expression and promotes tumorigenesis in various cancers [7]. The early occurrence of CRC has a close relationship with the methylation of CRC-related gene promoter regions [8-10]. NDRG4 is a member of the NDRG gene family of tumor suppressor genes [11]. Previous studies have demonstrated that the 5' regulatory region of the gene contains CpG islands, which are often methylated during the development of colorectal cancer [11]. Methylation of the NDRG4 gene is considered to be important biological characteristics for CRC [12]. Thus, NDRG4 is a potential biomarker of candidate tumor suppressor genes and rectal cancer.

Syndecan-2 (SDC2), also called fibroglycan, encodes a transmembrane (type I) heparan sulfate proteoglycan and regulates

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adhesion and proliferation of the colon carcinoma cells [13]. Hypermethylation of SDC2 has been detected at high frequency in blood from people with CRC [14]. As a molecular marker of potential colorectal cancer, the SDC2 gene methylation shows a high degree of specificity for the diagnosis of early tumors [15]. TFPI2 belongs to a recently described group of embryonic cells Polycomb group (PcG)-marked genes that may be predisposed to aberrant DNA methylation in the early stage of colorectal carcinogenesis [16]. It has been shown that TFPI2 in fecal DNA testing is associated with CRC recurrence and early stage CRC [17]. In this study, we designed a multitarget stool DNA (mt-sDNA) test including quantitative molecular assays of the KRAS mutations, aberrant NDRG4, SDC2, and TFPI2 methylation for the diagnosis of CRC. The diagnostic performance of the mt-sDNA test was compared with a commercially available FOBT in the detection of colorectal cancer and large adenoma with a diameter ≥ 1 cm in diameter.

Materials and Methods

Participants and stool collection

Our study included 151 participants aged between 45 and 75 years old who underwent colonoscopy during 2016-2017. The participants included 51 patients with adenoma (≥ 1 cm in diameter, smaller or diminutive polyps excluded) and 50 patients with CRC. Fifty participants who were free of colorectal polyps or tumors were selected from people who were receiving medical examination. The stools were collected from all participants before bowel purgation and colonoscopy, otherwise were collected 1 week after the colonoscopy but before neoplasm resection. The FOBT was performed before addition of the preservative buffer to the stool. Then the homogenized stools were stored at -20°C for the subsequent mt-sDNA test.

FOBT

The FOBT was performed using an immunochemical kit (Wanhuaupuman, China). The test results were classified as follows, depending on whether a large adenoma or tumor was found by colonoscopy. A positive result was a true positive if a neoplasm was found or a false positive if no neoplasm was found. A negative result was a false negative if a neoplasm was found or a true negative if no neoplasm was found. Sensitivity and specificity were expressed as percentages in the standard manner.

DNA extraction

A total of 211 frozen or fresh colorectal tissues, including 64 pairs of CRC and adjacent normal tissues and 83 colorectal adenomas (≥ 1 cm), were used to determine whether the mutated KRAS and hypermethylated NDRG4, SDC2 and TFPI2 could detect CRC and advanced precancer. DNA was extracted using a TIANamp DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instruction. The genomic DNA was isolated from the tumor tissues and treated with bisulfite. The methylation levels of NDRG4, SDC2, and TFPI2 were detected using real-time methylation-specific PCR.

Stool samples were thawed at room temperature and homogenized. The aliquots were transferred to tubes and centrifuged. The supernatant was used as the source of mt-sDNA. The mt-sDNA markers were enriched using the sequence-specific DNA captures, magnetic beads-based oligonucleotides, and purified using magnetic separation.

Bisulfite treatment

The tissue DNA and stool DNA were treated with bisulfite using

an EZ DNA methylation kit (Zymo Research, Irvine, CA) according to the manufacturer's instruction. For tissue DNA, 600 ng genomic DNA was added into the bisulfite reaction and eluted out in 30 μl TE buffer. For stool DNA, 30 μl captured DNA was added into the reaction and eluted out in 20 μl TE buffer.

mt-sDNA test

The test panel included three methylated genes (mNDRG4, mSDC2, and mTFPI2). The mutant forms of KRAS and the β -actin gene were used as references. The mt-sDNA for the methylation assay was treated with bisulfite. The genomic DNA was used for the KRAS mutation assay. Multiplex real-time PCR (qPCR) was used to detect the mutation and methylation (7500 Real-Time PCR System, ABI Biosystems, USA). Each run consisted of sDNA samples, positive controls (methylated NDRG4, SDC2 and TFPI2 and mutant KRAS and internal control), and negative controls (water blanks). The cycle threshold (Ct) value of each gene was used to evaluate the result of each sample. All assays were performed in a blinded fashion.

Statistical analysis

The strand number of each marker output from the ABI 7500 was quantified by the Ct value. It was considered the maximum amplification cycle number (Ct = 45) if there was no amplification. A logistic regression with specific boundary conditions was developed to evaluate the performance of each biomarker. The single marker cutoff was identified by a logistic regression algorithm that produce dichotomous (positive/negative) results for each sample. A threshold was defined for each marker in the mt-sDNA panel that optimally separates the cases from the control samples. The logistic regression assigned a weight to each component assay result and then aggregated these individual marker results to obtain a logistic score. Boundary conditions for each of the methylation and mutation markers were defined on the basis of a single value for each marker above which a positive result can be inferred. A positive result for the logistic score or a value exceeding any of the boundary conditions resulted in a positive result for the mt-sDNA test. Colonoscopy-based findings were compared with the mt-sDNA test results. Sensitivity and specificity were defined in the standard manner for comparison with FOBT.

Results

Patients characteristics

To determine the performance of biomarkers (NDRG4, SDC2, TFPI2, and KRAS) in detecting CRC and adenoma, a blind independent tissue study was performed. A total of 211 frozen or fresh colorectal tissues, including 64 pairs of CRC and adjacent normal tissues [median age 63 (range 43-79), 48.4% women] and 83 colorectal adenoma (≥ 1 cm) [median age 57 (range 39-72), 41% women] were included in the study. Age and sex distributions were similar between the three groups. In the carcinoma and adenoma tissues, 38% (19/50) and 43.1% (22/51) neoplasms were located in the colon, respectively. In the carcinoma and adenoma stool samples, 45.3% (29/64) and 36.1% (30/83) neoplasms were located in the colon, respectively. The demographic and clinical characteristics of the subjects are shown in Table 1.

Detection of the DNA markers

Methylated NDRG4 was detected in 92.2% (59/64) of the carcinomas and 64% (53/83) of the adenoma, and in only 3.1% (2/64) of the adjacent normal tissues (Figure 1). Methylated SDC2 was detected in 96.9% (62/64) of the carcinomas and 78% (65/83) of the adenoma at a specificity of 95.3% (61/64). Methylated TFPI2 was

Table 1: Clinical characteristics of the participants.

	CRC (n = 50)	Adenoma (n = 51)	Control (n = 50)	p
Median age, year(range)	66 (51-75)	62 (48-72)	56 (45-75)	>0.05
Female, n (%)	52% (26/50)	60.8% (31/51)	46% (23/50)	>0.05
Colon neoplasms	38% (19/50)	43.1% (22/51)	/	>0.05
Rectum neoplasms	62% (31/50)	56.9% (29/51)	/	>0.05

CRC: Colorectal Cancer

detected in 95.3% (61/64) of the carcinomas and 71% (59/83) of the adenoma at a specificity of 93.8% (60/64), respectively. Since KRAS mutation was more distributed to the right colon, it was detected to further increase sensitivity, especially to reduce the missed detection of the ascending colon tumors. KRAS mutation was detected in 45.3% (29/64) of the carcinomas, 25% (21/83) of the adenoma, and 96.9% (62/64) of the adjacent normal tissues (Figure 1). The multitarget DNA test including mutation and methylation assays detected 100% of the carcinomas and 81% of the adenoma.

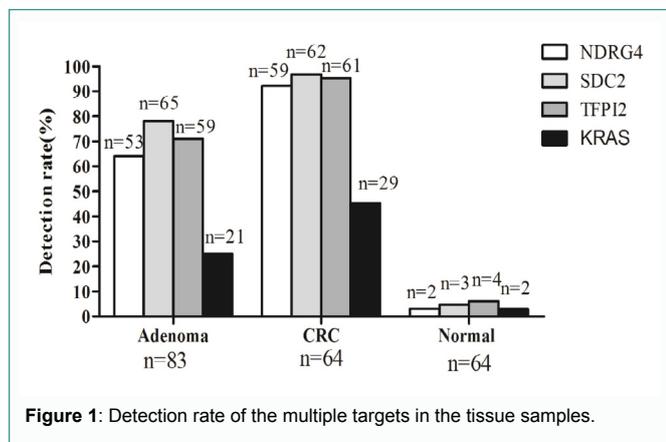


Figure 1: Detection rate of the multiple targets in the tissue samples.

Receiver Operating Characteristic (ROC) curves were constructed for each of the 4 genes (Figure 2). Comparing the cancer to the adjacent normal tissues, the area under the curve (AUC) values were 0.95, 0.99, 0.97, and 0.79 for NDRG4, SDC2, TFPI2, and KRAS, respectively. Comparing the adenoma to the adjacent normal tissues, the AUC values were 0.84, 0.88, 0.83, and 0.64 for NDRG4, SDC2, TFPI2, and KRAS, respectively.

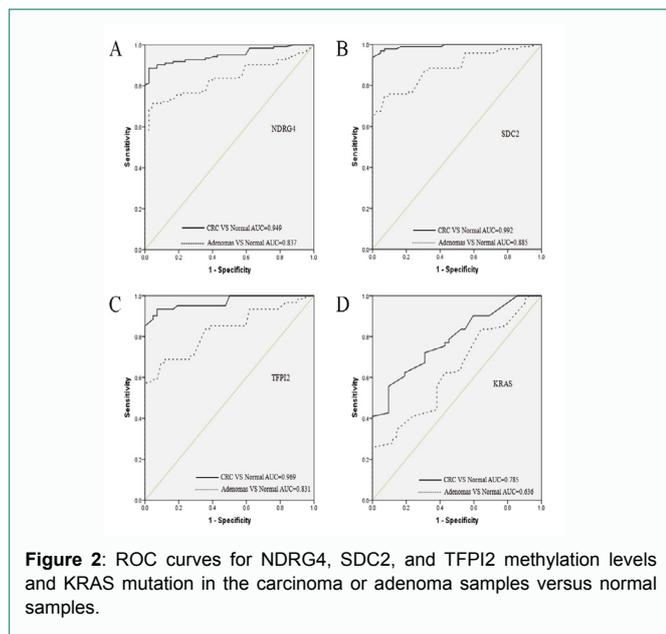


Figure 2: ROC curves for NDRG4, SDC2, and TFPI2 methylation levels and KRAS mutation in the carcinoma or adenoma samples versus normal samples.

Comparison of mt-sDNA test with FOBT

Fifty subjects with CRC received the mt-sDNA test. The mt-sDNA test detection rate for CRC was 90% (45/50), including 91.9% (34/37) of stage I-III cancer and 84.6% (11/13) of stage IV cancer (Figure 3). The FOBT had a sensitivity of 42% (21/50) for CRC in the same batch samples, a 29.7% (11/37) sensitivity for stage I-III cancer, and a 76.9% (10/13) sensitivity for stage IV cancer. The results showed that the mt-sDNA test outperformed the FOBT in detecting colorectal cancer. In addition, the specificity of mt-sDNA [94% (47/50)] was superior to the FOBT [90% (45/50)].

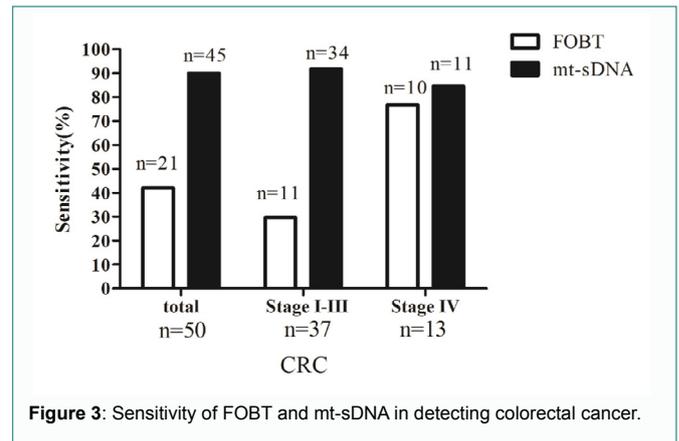


Figure 3: Sensitivity of FOBT and mt-sDNA in detecting colorectal cancer.

Comparison of mt-sDNA test with FOBT in detecting advanced precancer

A total of 51 participants were diagnosed with advanced adenoma by colonoscopy. The size of the adenoma was 1-2 cm in 12 cases, 2 cm - 3 cm in 21 cases, and 3 cm - 5 cm in 18 cases. The mt-sDNA test detected 7 1 cm - 2 cm adenoma, 15 2 cm - 3 cm adenoma, and 14 3 cm - 5 cm adenoma. The FOBT detected 21 cm - 2 cm adenoma, 52 cm - 3 cm adenoma, and 43 cm - 5 cm adenoma (Figure 4). The mt-sDNA test outperformed the FOBT in detecting advanced adenoma with a sensitivity of 70.6% (36/51) versus 19.6% (10/51). For the mt-sDNA test, the area under the ROC curve was 0.95 [95% Confidence Interval (CI), 0.98-1] for detecting colorectal cancer and 0.84(95% CI, 0.83-0.93) for detecting adenoma (Figure 5A). For FOBT, the area under the ROC curve was 0.78 (95% CI, 0.69-0.87) for detecting colorectal cancer and 0.64 (95% CI, 0.53-0.74) for detecting adenoma (Figure 5B).

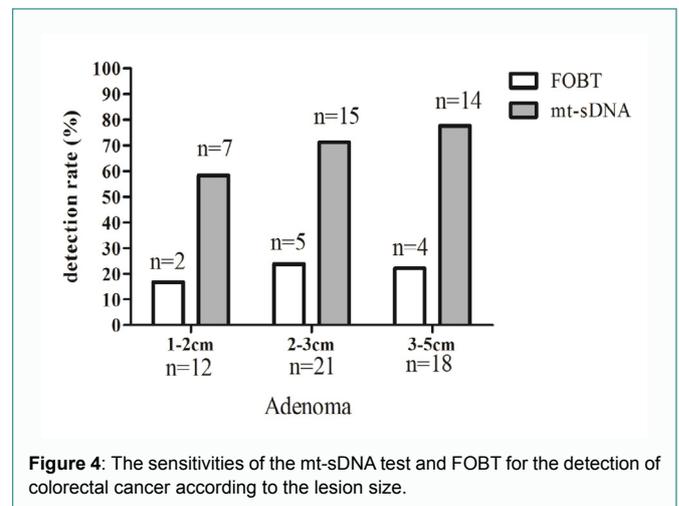


Figure 4: The sensitivities of the mt-sDNA test and FOBT for the detection of colorectal cancer according to the lesion size.

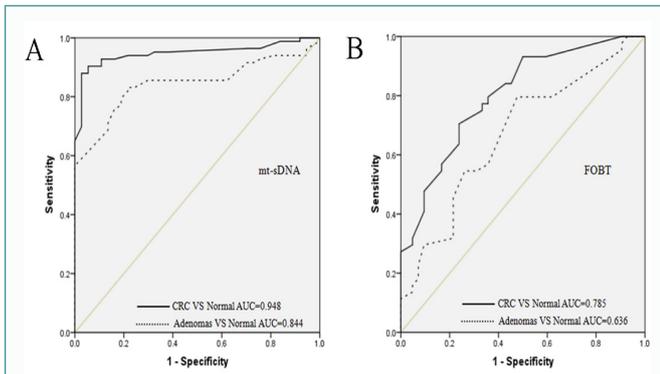


Figure 5: Receiver operating characteristic curves of the mt-sDNA test and the FOBT for the detection of colorectal cancer and adenoma.

Detecting CRC of different sites

Tumor location of the 50 CRC included 10 cases in the ascending colon, 5 cases in the transverse colon, 4 cases in the descending colon, and 31 cases in the rectum. The sensitivity of mt-sDNA test for detecting CRC was 90% (9/10) for the ascending colon, 60% (3/5) for the transverse colon, 75% (3/4) for the descending colon, and 96.8% (30/31) for the rectum. The sensitivity of FOBT for detecting CRC was 50% (5/10) for the ascending colon, 20% (1/5) for the transverse colon, 25% (1/4) for the descending colon, and 45.2% (14/31) for the rectum (Figure 6), 51 adenoma samples were consisted of 15 on ascending colon, 7 on transverse colon and 29 on rectum. As the result showed (Figure 7), there was no difference of the detection rate among neoplasms in different location, the sensitivity of FOBT for detecting adenoma was about 20%, 60% - 90% was in detection colorectal cancer. It should be explained that it is possible that the small sample size caused the difference in sensitivity of detecting the neoplasms located in different parts.

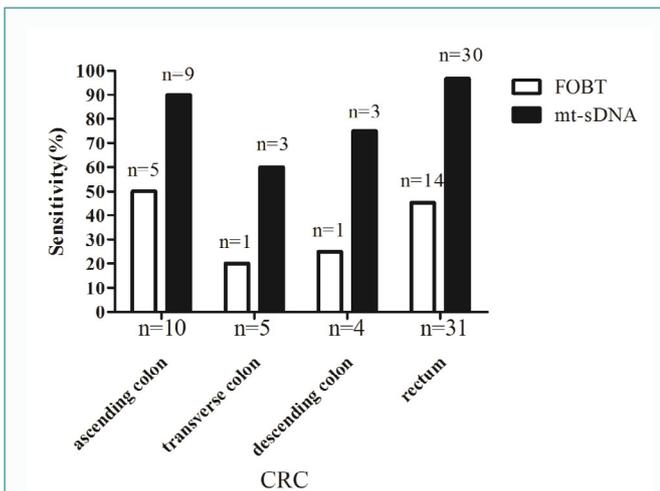


Figure 6: The sensitivities of the mt-sDNA test and FOBT for the detection of colorectal cancer according to the location in the colon and rectum.

Discussion

Our study demonstrated that the mt-sDNA test is superior to FOBT in detecting both CRC and advanced precancer with a specificity of 93% versus 91%. Our findings suggested that the mt-sDNA test is a feasible and promising approach for early detection of CRC, which is consistent with prior studies [24-28]. FOBT is a traditional screening

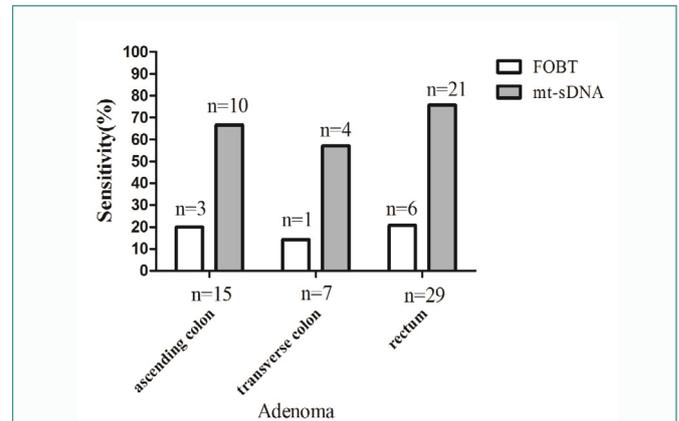


Figure 7: The sensitivities of the mt-sDNA test and FOBT for the detection of advanced precancerous lesions according to the location in the colon and rectum.

tool for CRC. However, it is not widely used for CRC screening in China, partially due to their inherent low sensitivity for detecting colorectal neoplasms especially advanced adenomas in asymptomatic patients [29-30]. Our study showed that the FOBT had a sensitivity of 19.6% for advanced adenoma and 29.7% for stage I-III CRC. The mt-sDNA test is 50% higher in the sensitivity for adenoma and 60% higher in the sensitivity for stage I-III CRC. Colonoscopy is considered the gold standard for CRC diagnosis, but its application in CRC screening has been hindered by some limitations, such as the requirement of a visible lesion, risk of complications, and invasiveness, which lead to low patient compliance [31]. The new multitarget panel in our study also has a better performance than other studies [32]. We have screened a dozen of exfoliated markers for gastrointestinal neoplasms, then high analytical sensitivity and discriminant markers, including mutated KRAS and hypermethylated NDRG4, SDC2 and TFPI2, were determined by both tissue and stool assays. Then the methylation and mutation levels were confirmed by pyrosequencing and Sanger sequencing. As we observed, NDRG4, SDC2, and TFPI2 were highly methylated in colorectal cancer tissues, which discriminated normal colon mucosa and KRAS mutated tumors were more likely to develop on the right of the colon, which coincided with informed researches [33]. Stool observations were consistent with the tissues study, the biomarkers provided high sensitivity and discrimination from colorectal cancer lesions to normal. In our study, the mt-sDNA panel performed no significant differences between the diverse site tumors with 90% higher sensitivity. CRC is the third most prevalent cancer worldwide (1.23 million annual cases), and the morbidity and mortality of CRC in China has gradually increased [3]. In the United States, the incidence and mortality of CRC have gradually decreased, mainly due to the large-scale population screening, intervention of precancerous lesions for primary prevention, and early detection of CRC. In China, screening rates for CRC remain low and there is a serious shortage of medical resources. In this study, we offered a noninvasive approach for CRC diagnosis and screening with high sensitivity and specificity. We have recruited hundreds average-risk participants to investigate the compliance with fecal DNA test by analysis of questionnaires, 90% higher of these participants are apt to do the mt-sDNA test, which indicate that the mt-sDNA test is patient-friendly to the average-risk population.

Conclusions

The mt-sDNA test has higher sensitivity and specificity in

diagnosing both CRC and advanced adenoma in comparison with FOBT. Considering the molecular diagnostics capability and the broad accessibility to clinical laboratories, the mt-sDNA could be a valuable addition to current CRC screening options.

Funding

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