Abstract. Patient screening is important for early diagnosis of colorectal cancer (CRC). The present study aimed to compare the multitarget stool DNA (mt-sDNA) test with the fecal occult blood test (FOBT) for CRC screening. A total of 151 individuals were screened using colonoscopy, mt-sDNA and FOBT for the detection of CRC and adenoma. The results of the mt-sDNA test and FOBT were compared with colonoscopy to examine their sensitivity and specificity. Subsequently, the sensitivity and specificity of the mt-sDNA test were compared with those of FOBT in CRC and advanced adenoma. Stool samples were collected from patients with CRC (n=50) or large adenoma (n=51), as well as from normal controls (n=50). The mt-sDNA test outperformed FOBT in detecting CRC with a sensitivity of 90.0% (45/50) vs. 42.0% (21/50), advanced adenoma with a sensitivity of 70.6% (36/51) vs. 19.6% (10/51), stage I-III CRC with a sensitivity of 91.9% (34/37) vs. 29.7% (11/37), and stage IV CRC with a sensitivity of 84.6% (11/13) vs. 76.9% (10/13). In addition, the mt-sDNA test exhibited a specificity of 94.0% (47/50) in detecting CRC, which was superior to FOBT with a specificity of 90.0% (45/50). Therefore, the mt-sDNA test may have higher sensitivity and specificity compared with FOBT in diagnosing both CRC and advanced adenoma.

Introduction

Colorectal cancer (CRC) is a common malignancy associated with mutations in multiple genes, such as KRAS (1) and SDC2 (2). Progression from a benign tumor to CRC takes up to 10 years in 80% of affected individuals (3). Therefore, CRC screening is critical for early detection and treatment of CRC. The fecal occult blood test (FOBT) and colonoscopy are the mainstay of CRC screening. However, the FOBT has a low diagnostic performance, particularly for colorectal adenoma (3-5). Colonoscopy is the gold standard for diagnosing CRC with good sensitivity and specificity; however, it is associated with a high risk of complications and low compliance (5). Cancer cells from early-stage CRC are continuously shed into the colonic lumen and mixed into stool (6). Tests for genetic and epigenetic alterations in fecal DNA have been considered as a possible method for the early detection of CRC (7).

KRAS is a common oncogene in malignant tumors. KRAS mutations are detected in 30-40% of CRCs (1). There are seven mutation hotspots that account for >90% of the KRAS mutations, including Gly12Asp, Gly12Val, Gly12Ser, Gly12Cys, Gly12Ala, Gly12Arg and Gly13Asp (8). It has been demonstrated that mutations in codons 12 and 13 of exon 2 of KRAS are closely associated with the development of CRC, and mutations in codon 12 are associated with a less favorable prognosis compared with mutations in codon 13 (9).
Hypermethylation of CpG islands in gene promoter regions suppresses specific gene expression and promotes tumorigenesis in various types of cancer (1). The early occurrence of CRC is closely associated with the methylation of CRC-related gene promoter regions (10-12). The N-myc downstream-regulated gene (NDRG) 4 is a member of the NDRG family of tumor suppressor genes (13). It has been demonstrated that the 5' regulatory region of NDRG4 contains CpG islands, which are often methylated during the development of CRC (13). Methylation of NDRG4 is considered to be an important biological characteristic of CRC (14). Therefore, NDRG4 may be a potential diagnostic biomarker for CRC screening.

Syndecan-2 (SDC2), also known as fibroglycan, encodes a transmembrane (type I) heparan sulfate proteoglycan and regulates adhesion and proliferation of colon carcinoma cells (15). Hypermethylation of SDC2 has been detected at high frequency in the blood of patients with CRC (2). As a molecular marker of potential CRC, SDC2 methylation demonstrates a high degree of specificity for the diagnosis of early-stage tumors (16).

The tissue factor pathway inhibitor (TFPI) 2 belongs to a previously described group in embryonic cells of polyclonal group-marked genes that may be predisposed to aberrant DNA methylation in the early stages of CRC carcinogenesis (17). It has been demonstrated that TFPI2 levels determined by fecal DNA testing are associated with CRC recurrence and early-stage CRC (17-23).

In the present study, a multitarget stool DNA (mt-sDNA) test was designed, including quantitative molecular assays for KRAS mutations and 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 carcinoma and large adenoma (≥1 cm in diameter).

Materials and methods

Participants and stool collection. The present study included 151 participants who underwent colonoscopy at Shanghai Jiao Tong University, School of Medicine, Ruijin Hospital North (Shanghai, China) between January 2016 and January 2017. The inclusion criteria were as follows: i) Colorectal adenoma (≥1 cm in diameter; smaller or diminutive polyps excluded); or ii) colorectal carcinoma; and iii) age ≥18 years. Patients with the following conditions were excluded: i) Contraindications to colonoscopy; ii) severe gastrointestinal bleeding; and iii) hemorrhoids. A total of 50 participants who were free of colorectal polyps or tumors were selected from individuals who were receiving routine medical examinations.

Stool was collected from all participants prior to bowel preparation and colonoscopy, or otherwise 1 week after the colonoscopy but prior to neoplasm resection. The FOBT was performed before addition of the preservative buffer to the stool. The homogenized stools were stored at -20°C for the subsequent mt-sDNA test. Colorectal adenoma or carcinoma tissues and adjacent normal tissues within 1 cm of the tumor were biopsied and stored in -196°C liquid nitrogen prior to DNA extraction.

FOBT. The FOBT was performed using a guaiac-based immunochemical kit according to the manufacturer's protocol (Hemosure T1-CK50, W.H.P.M. Bioresearch & Technology) with a minimum detection limit of 0.2 µg/ml. Depending on whether a large adenoma or tumor was identified during colonoscopy, the test results were classified as follows: A positive result was a true positive if a neoplasm was detected, or a false positive if no neoplasm was detected; a negative result was a false negative if a neoplasm was detected, or a true negative if no neoplasm was detected. Sensitivity and specificity were expressed as percentages.

DNA extraction. Frozen or fresh colorectal tissues were collected from 50 patients with CRC and 51 patients with colorectal adenoma, and were used to determine whether mutated KRAS and hypermethylated NDRG4, SDC2 and TFPI2 could be used to detect CRC and large adenoma. DNA was extracted using a TIANamp DNA kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. The methylation levels of NDRG4, SDC2 and TFPI2 were detected using quantitative (q)PCR.

Stool samples were thawed at room temperature and homogenized. The aliquots were transferred to tubes and centrifuged at 14,000 x g for 5 min at room temperature. The supernatant was used as the source of mt-sDNA. The mt-sDNA markers were enriched using sequence-specific DNA captures and 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 Corp.) according to the manufacturer's protocol. For tissue DNA, 600 ng genomic DNA was added into the bisulfite reaction and eluted in 30 µl Tris/EDTA (TE) buffer. For stool DNA, 30 µl captured DNA was added into the reaction and eluted in 20 µl TE buffer.

Methylation-specific qPCR. The test panel included three methylated (m) genes (mNDRG4, mSDC2 and mTFPI2). The mutant forms of KRAS and β-actin 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 qPCR was used to detect the mutation and methylation profile of candidate genes (ABI 7500 Real-Time PCR system; Applied Biosystems; Thermo Fisher Scientific, Inc.). Each run consisted of sDNA samples, positive controls (mNDRG4, mSDC2, mTFPI2, mutant KRAS and internal control), and negative controls (water blanks). Briefly, each multiplex PCR assay was performed with a final reaction mixture volume of 20 µl containing 0.5 U Phusion polymerase (Thermo Fisher Scientific, Inc.) in high-fidelity Phusion buffer with a final concentration of 200 µM deoxy-nucleotide triphosphates and 3 mM MgCl2. The primers were used at a final concentration of 0.2-0.4 µM. Primer sequences are listed in Table I. SYBR-Green I (Cambrex Bio Science Rockland, Inc.) was diluted as recommended by the manufacturer. The hot-start technique was used to prevent non-specific amplification (24). The amplification cycles consisted of incubation at 98°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec and 72°C for 10 sec. After 30 cycles, a melting curve was determined using SYBR-Green fluorescence with a ramp speed of 0.2°C/sec between 72 and 98°C.
with a reading every 0.2˚C. The cycle threshold (Ct) value of each gene was used to evaluate the result of each sample using the 2^ΔΔCq method (25). All assays were performed in a blinded manner.

Statistical analysis. The strand number of each marker output from the ABI 7500 was quantified according to the Ct value. If there was no amplification, the maximum amplification cycle number (Ct=45) was considered. A logistic regression with specific boundary conditions was developed to evaluate the performance of each biomarker. The single marker cut-off was identified by a logistic regression algorithm that produced dichotomous (positive/negative) results for each sample. A threshold was defined for each marker in the mt-sDNA panel that optimally separated the tumor samples from the control samples. The logistic regression assigned a weight to each component assay result and subsequently 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 could 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 calculated as percentages for comparison with FOBT. Receiver operating characteristic (ROC) curves were constructed to analyze the diagnostic performances of the biomarkers.

Continuous data were presented as the mean ± SD and compared using the one-way ANOVA test followed by Tukey's post hoc test. Categorical data were presented as percentages and compared using the χ^2 test. All statistical analyses were performed using SPSS v21.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. To determine the performance of biomarkers (NDRG4, SDC2, TFPI2 and KRAS) in detecting CRC and adenoma, an 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 years; range, 43‑79 years; 48.4% women) and 83 colorectal adenomas (≥1 cm in diameter; median age, 57 years; range, 39‑72; 41% women) were included in the present study. Age and sex distributions were similar between patients with CRC, patients with colorectal adenomas and normal controls (Table II). In the carcinoma and adenoma tissues, 38.0% (19/50) and 43.1% (22 /51) of the neoplasms were located in the colon, respectively. The demographic and clinical characteristics of the subjects are shown in Table II.

Detection of the DNA markers. mNDRG4 was detected in 92.2% (59/64) of the carcinoma tissues, 63.9% (53/83) of the adenoma tissues and 3.1% (2/64) of the adjacent normal tissues (Fig. 1).

mSDC2 was detected in 96.9% (62/64) of the carcinoma tissues and 78.3% (65/83) of the adenoma samples, with a specificity of 95.3% (61/64).

mTFPI2 was detected in 95.3% (61/64) of the carcinoma tissues and 71.1% (59/83) of the adenoma samples, with a specificity of 93.8% (60/64).

KRAS mutations were detected in 45.3% (29/64) of the carcinoma samples, 25.3% (21/83) of the adenoma samples and 3.1% (2/64) of the adjacent normal tissues (Fig. 1).

ROC curves were constructed for each of the four genes (Fig. 2). When comparing the cancer tissues with the adjacent

Table I. Primer sequence for the DNA methylation and KRAS mutation assays.

| Name     | Primer sequences                                                                 |
|----------|-----------------------------------------------------------------------------------|
| NDRG4    | MS: 5'-TTTAGGTTCCGATCCGTTTGC-3' 3'-CGAATGTATAAGCATTACCCG-5' US: 5'-GATGATTATTAGTTGATTGTAAGTGT-3' 3'-AAAAGACACACTAAACATACACCA-5' |
| TFPI2    | MS: ATTTTTTAAGTTTTTCGTTGCC 5'-GCCTAACGAAAAATAACCGG-3' US: TTAGTTATTATTAGTTGTTTGT 3'-AAAAACACCTAAACAAATAACACCA-5' |
| SDC2     | MS: 5'-AAAGATTTCGGCACACCAAGCAGCTCAAACACTCGAAACACTCG-3' 3'-GACTCAAACTCGAAACACTCGAA-5' US: 5'-TTCGGGCGGTAGTTGCGGGCGG-3' 3'-TTTCGGGCGGTAGTTGCGGGCGG-5' |
| KRAS     | 5'-CTGTTGGTCAATTTGGATAGTTGTA-3' 3'-TGAATATGGGCTAGAAGAACCTTTA-5' |
| β-actin  | 5'-GCTAATGTTGGGCTGCTGTAGAT-3' 3'-GCTCTTTTCTTTGTTTGTCTCTTC-5' |

NDRG4, N-myC downstream-regulated gene 4; SDC2; syndecan-2; TFPI2, tissue factor pathway inhibitor 2; MS, methylated sequence; US, unmethylated sequence.
adenoma was 1-2 cm in 12 samples, 2-3 cm in 21 samples and with advanced adenoma by colonoscopy. The size of the large adenoma. A total of 51 individuals were diagnosed with adenoma samples, 25.3% (21/83) of the adenoma samples and 3.1% (2/64) of the adjacent normal tissues. The mt-sDNA test detected 92.2% (59/64) of the carcinoma tissues, 63.9% (53/83) of the adenoma tissues and 3.1% (2/64) of the adjacent normal tissues. mSDC2 was detected in 96.9% (62/64) of the carcinoma tissues and 78.3% (65/83) of the adenoma samples, with a specificity of 95.3% (61/64). mTFPI2 was detected in 95.3% (61/64) of the carcinoma tissues and 71.1% (59/83) of the adenoma samples, with a specificity of 93.8% (60/64). KRAS mutations were detected in 45.3% (29/64) of the carcinoma samples, 25.3% (21/83) of the adenoma samples and 3.1% (2/64) of the adjacent normal tissues. CRC, colorectal cancer; NDRG4, N-myc downstream-regulated gene 4; SDC2, syndecan-2; TFPI2, tissue factor pathway inhibitor 2.

Comparison of the mt-sDNA test with FOBT for the detection of CRC and an 84.6% (11/13) sensitivity for stage IV CRC (Fig. 3). The FOBT had a sensitivity of 42.0% (21/50) for CRC in the same samples, with a 29.7% (11/37) sensitivity for stage III CRC and a 76.9% (10/13) sensitivity for stage IV CRC (Fig. 3). These results demonstrated that the mt-sDNA test outperformed the FOBT in detecting CRC. In addition, the specificity of the mt-sDNA test (94.0%; 47/50) was higher than that of the FOBT (90.0%; 45/50) (data not shown).

Comparison of the mt-sDNA test with FOBT for the detection of large adenoma. A total of 51 individuals were diagnosed with advanced adenoma by colonoscopy. The size of the adenoma was 1-2 cm in 12 samples, 2-3 cm in 21 samples and 3-5 cm in 18 samples. The mt-sDNA test detected 7 adenomas of 1-2 cm, 15 adenomas of 2-3 cm and 14 adenomas of 3-5 cm, whereas FOBT detected 2 adenomas of 1-2 cm, 5 adenomas of 2-3 cm and 4 adenomas of 3-5 cm (Fig. 4). The mt-sDNA test outperformed the FOBT in detecting advanced adenomas with a sensitivity of 70.6% (36/51) vs. 19.6% (10/51) (data not shown).

For the mt-sDNA test, the area under the ROC curve was 0.948 (95% CI, 0.98-1) for detecting CRC and 0.844 (95% CI, 0.83-0.93) for detecting adenomas (Fig. 5A). For FOBT, the area under the ROC curve was 0.785 (95% CI, 0.69-0.87) for detecting CRC and 0.636 (95% CI, 0.53-0.74) for detecting adenoma (Fig. 5B).

**Detecting CRC at different sites.** In terms of tumor location, the 50 CRC samples included 10 samples in the ascending colon, 5 samples in the transverse colon, 4 samples in the descending colon and 31 samples in the rectum. The sensitivity of the mt-sDNA test for detecting CRC was 90.0% (9/10) for the ascending colon, 60.0% (3/5) for the transverse colon, 75.0% (3/4) for the descending colon and 96.8% (30/31) for the rectum, whereas the sensitivity of FOBT for detecting CRC was 50.0% (5/10) for the ascending colon, 20.0% (1/5) for the transverse colon, 25.0% (1/4) for the descending colon and 45.2% (14/31) for the rectum (Fig. 6). The 51 adenoma samples comprised 15 in the ascending colon, 7 in the transverse colon and 29 in the rectum. The sensitivity of the FOBT for detecting adenoma was 20.0% (3/15) for the ascending colon, 14.3% (1/7) for the transverse colon and 20.7% (6/29) for the rectum, whereas the sensitivity of the mt-sDNA test for detecting adenoma was 66.7% (10/15) for the ascending colon, 57.1% (4/7) for the transverse colon and 72.4% (21/29) for the rectum (Fig. 7).

**Discussion**

The present study demonstrated that the mt-sDNA test was superior to FOBT in detecting both CRC and large adenoma with a specificity of 93.0% vs. 91.0%. The current findings suggested that the mt-sDNA test may be a feasible and promising approach for early detection of CRC. FOBT is a traditional screening tool for CRC. However, it is not widely used for CRC screening in China, partially due to its inherent low sensitivity for detecting colorectal neoplasms, particularly advanced adenomas in asymptomatic patients (26,27). The present study demonstrated that FOBT had a sensitivity of 19.6% for advanced adenoma and 29.7% for stage I-III CRC. The mt-sDNA test had a 50% higher sensitivity for adenomas and a 60% higher sensitivity for stage I-III CRC. In addition, the sensitivity of the mt-sDNA test for detecting CRC was 90.0% (9/10) in the ascending

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**Table II. Clinical characteristics of the participants.**

| Variable                  | CRC (n=50) | Adenoma (n=51) | Control (n=50) | P-value  |
|---------------------------|------------|----------------|---------------|----------|
| Age, years (mean ± SD)    | 60.2±13.6  | 58.5±10.7      | 64.9±11.0     | >0.05    |
| Female                    | 52.0% (26/50) | 60.8% (31/51)  | 46.0% (23/50) | >0.05    |
| Colon neoplasms           | 38.0% (19/50) | 43.1% (22/51)  | -             | >0.05    |
| Rectum neoplasms          | 62.0% (31/50) | 56.9% (29/51)  | -             | >0.05    |

![Figure 1. Detection rate of the multiple targets in adenoma, CRC and normal tissue samples. mNDRG4 was detected in 92.2% (59/64) of the carcinoma tissues, 63.9% (53/83) of the adenoma tissues and 3.1% (2/64) of the adjacent normal tissues. mSDC2 was detected in 96.9% (62/64) of the carcinoma tissues and 78.3% (65/83) of the adenoma samples, with a specificity of 95.3% (61/64). mTFPI2 was detected in 95.3% (61/64) of the carcinoma tissues and 71.1% (59/83) of the adenoma samples, with a specificity of 93.8% (60/64). KRAS mutations were detected in 45.3% (29/64) of the carcinoma samples, 25.3% (21/83) of the adenoma samples and 3.1% (2/64) of the adjacent normal tissues. CRC, colorectal cancer; NDRG4, N-myc downstream-regulated gene 4; SDC2, syndecan-2; TFPI2, tissue factor pathway inhibitor 2.](image-url)
colon, 60.0% (3/5) in the transverse colon, 75.0% (3/4) in the descending colon and 96.8% (30/31) in the rectum. However, due to the small sample size, the current results did not support any conclusion concerning the performance of mt-sDNA for diagnosing CRC at any specific stage or location.

Colonoscopy is considered the gold standard for CRC diagnosis, but its application in CRC screening has been hindered by a number of factors, such as the requirement of a visible lesion, the risk of complications and the invasiveness of the procedure, resulting in low patient compliance (28).

The novel multitarget panel presented in the current study had an improved performance compared with previous findings (29). A dozen of exfoliated markers, including mutated KRAS and hypermethylated NDRG4, SDC2 and TFPI2, were

![Figure 2](image2.png)

**Figure 2.** Receiver operating characteristic curves. Areas under the curve of (A) NDRG4, (B) SDC2 (C) TFPI2 and (D) KRAS in the carcinoma or adenoma samples were compared with normal samples. CRC, colorectal cancer; NDRG4, N-myc downstream-regulated gene 4; SDC2, syndecan-2, TFPI2, tissue factor pathway inhibitor 2; AUC, area under the curve.

![Figure 3](image3.png)

**Figure 3.** Sensitivity of FOBT and mt-sDNA in detecting CRC. The sensitivity of the mt-sDNA test for CRC was 90.0% (45/50), with a 91.9% (34/37) sensitivity for stage I-III CRC and an 84.6% (11/13) sensitivity for stage IV CRC. The FOBT had a sensitivity of 42.0% (21/50) for CRC in the same samples, with a 29.7% (11/37) sensitivity for stage I-III CRC and a 76.9% (10/13) sensitivity for stage IV CRC. FOBT, fecal occult blood test; mt-sDNA, multitarget stool DNA; CRC, colorectal cancer.

![Figure 4](image4.png)

**Figure 4.** Sensitivities of the mt-sDNA test and FOBT for the detection of adenoma according to lesion size. The mt-sDNA test detected 7 adenomas of 1-2 cm, 15 adenomas of 2-3 cm and 14 adenomas of 3-5 cm, whereas FOBT detected 2 adenomas of 1-2 cm, 5 adenomas of 2-3 cm and 4 adenomas of 3-5 cm. FOBT, fecal occult blood test; mt-sDNA, multitarget stool DNA.
analyzed in tissue and stool assays in our study. *NDRG4*, *SDC2* and *TFPI2* were highly methylated in CRC tissues, distinguishing them from normal colon mucosal tissues, and *KRAS* mutated tumors were more likely to develop on the right side of the colon, in accordance with a previous study (30). Stool observations were consistent with the tissue assays, and the analyzed biomarkers exhibited high sensitivity and discrimination between CRC lesions and normal tissues. In the present study, the mt-sDNA panel exhibited no differences among the diverse tumor sites with 90% or higher sensitivity.

As of 2018, CRC is the third most prevalent cancer worldwide, and its morbidity and mortality in China has gradually increased (31). In the United States, the incidence and mortality of CRC have gradually decreased, mainly due to large-scale population screening, interventions for precancerous lesions for primary prevention and early detection of CRC (31). In China, screening rates for CRC remain low and there is a shortage of medical resources. The present study offered a non-invasive approach for CRC diagnosis and screening with high sensitivity and specificity. In a previous study, a number of average-risk participants were recruited to investigate their compliance with fecal DNA testing via questionnaires, with >90% of these individuals being prone to the mt-sDNA test, indicating that the mt-sDNA test is patient-friendly to the average-risk population (32). In addition, the mt-sDNA test may have the potential to detect CRC at an earlier stage of tumor development compared with FOBT. However, the relatively high cost of the mt-sDNA test may limit its popularity.

In conclusion, the mt-sDNA test had a higher sensitivity and specificity in diagnosing both CRC and advanced adenoma compared with FOBT. Considering its molecular diagnostic capability and its broad accessibility at clinical laboratories, the mt-sDNA test may be a valuable addition to current CRC screening options.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CY analyzed the samples and drafted the manuscript. WW, JS, XYa, KL, HY, YY, SJ, XYu, YS, YZ, SZ, YY, YD, LX, BC and XX collected the samples. PC, WZ and RZ analyzed the data and designed the study. YW conceived the study and critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine, Ruijin Hospital North. All participants provided written informed consent.

Patient consent for publication

All participants gave permission for publication.

Competing interests

The authors declare that they have no competing interests.

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