Research Article

Stool DNA Test of Methylated *Syndecan-2* for the Early Detection of Colorectal Neoplasia

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Abstract

Background: Although the incidence of colorectal cancer is steadily increasing, screening for colorectal cancer with conventional approaches is not routinely performed in China. Noninvasive screening methods are attractive options to resolve this issue. *Syndecan-2 (SDC2)* is frequently methylated in colorectal cancer. However, the value of a stool test of methylated *SDC2* for the detection of colorectal cancer is unknown.

Methods: Methylation status of *SDC2* was tested in cell lines and 398 colorectal tissue samples and further evaluated with 497 stool samples, including 196 from colorectal cancer patients, 122 from adenoma patients, and 179 from normal individuals, using real-time methylation-specific PCR. The impacts of one quantitative partial stool sampling device and 17 potentially interfering substances on the performance of fecal methylated *SDC2* were also analyzed. *SDC2* expression was also measured.

Results: *SDC2* methylation level was higher in 96.8% (120/124) of colorectal cancer tissues compared with paired adjacent normal epithelia. Stool test of methylated *SDC2* detected 81.1% (159/196) of colorectal cancer and 58.2% (71/122) of adenomas at a specificity of 93.3% (167/179). No significant difference was found between partial and whole stool collection on colorectal cancer detection ($P > 0.05$, $R^2 = 0.80$). Among 17 interfering substances, only berberine at high concentrations inhibited fecal detection of methylated *SDC2*. *SDC2* was overexpressed in colorectal cancer tissues compared with normal epithelia.

Conclusions: Fecal methylated *SDC2* is a valuable biomarker for the noninvasive detection of colorectal neoplasms.

Impact: Stool DNA test of methylated *SDC2* would serve as an alternative method for screening colorectal neoplasms. *Cancer Epidemiol Biomarkers Prev; 26(9); 1411–9. ©2017 AACR.*

Introduction

Colorectal cancer is one of the five most common cancers in China, and its incidence is still steadily increasing. In 2014, there were more than 370,000 new cases in China, and more than 190,000 of them died of colorectal cancer (1). The 5-year survival rate of Chinese colorectal cancer patients was only 47.2% (1). Screening has been proved to dramatically decrease the incidence and mortality of colorectal cancer in western countries (2–5). Chinese colorectal cancer screening guidelines recommend fecal occult blood test (FOBT) and colonoscopy as screening methods.

Although colonoscopy is accurate for the diagnosis of colorectal cancer, its compliance in screening setting is low (~20%) in China. In addition to invasiveness and bowel preparation (6, 7), lack of knowledge about colorectal cancer screening (8), poor doctor–patient communication (9), and no insurance coverage are also important factors responsible for low compliance of screening colonoscopy. FOBT is noninvasive, but its accuracy is relatively low. Because of their limitations, both colonoscopy and FOBT are not ideal approaches for screening colorectal neoplasms. Thus, alternative noninvasive tests may be an attractive option to increase colorectal cancer screening uptake.

Stool DNA test has emerged as a new method for screening colorectal neoplasms. For example, ColoGuard stool DNA test (sDNA, Exact Science) was approved by the FDA for clinical use in 2014 (10), and further included in Colorectal Cancer Screening Guideline published by the U.S. Preventive Services Task Force in 2016 (11). sDNA detects genetic and epigenetic DNA alterations, such as mutant *KRAS* (12), methylated *NDRG4* and *BMP3* (12–14), in tumor cells sloughed into stools. Various DNA markers have been studied in stool. However, no officially approved stool DNA test is currently available for Chinese patients.

SDC2 is also called fibroglycan, encoding a transmembrane (type I) heparan sulfate proteoglycan. Hypermethylation of *SDC2* had been reported in malignant glioma (15). Recently, methylated *SDC2* was detected at high frequency in blood from patients with colorectal cancer (16, 17). As exfoliation of tumor cells into colorectal lumen occurs earlier than vascular invasion during colorectal carcinogenesis (18), stool is theoretically a more suitable specimen than blood for the early detection of colorectal

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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doi: 10.1158/1055-9965.EPI-17-0153

neoplasms. However, a stool test of methylated *SDC2* for colorectal cancer detection has not been developed and evaluated.

In this study, we evaluated the performance of a stool DNA test of methylated *SDC2* for the detection of colorectal neoplasms, designed and tested one quantitative stool sampling device, and analyzed 17 substances potentially interfering fecal assay of methylated *SDC2*. In addition, we explored the impact of promoter methylation on the expression of *SDC2* gene.

Materials and Methods

Colorectal cancer cell lines

Eight human colorectal cancer cell lines, including WiDr, SW480, HCT116, HCT15, HT-29, DLD1, KM12, and Caco-2, were used in this study. WiDr, SW480, HCT116, HCT15, HT-29, DLD1, and Caco-2 were obtained from Guangdong Institute of Gastroenterology, the Sixth Affiliated Hospital, Sun Yat-sen University (Guangdong, China) in 2014 to 2015. KM12 was purchased from ATCC in 2013. Cell lines were either grown in RPMI1640 (Thermo Fisher Scientific) or in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS. All cell lines were authenticated at the VivaCell Biosciences and Beijing Microread Genetics Co., Ltd. using short tandem repeat analysis.

Sample collection

This study was approved by the Institutional Review Board at the Sixth Affiliated Hospital of Sun Yat-sen University. A total of 398 fresh-frozen colorectal tissues, including 124 pairs of colorectal cancer and adjacent normal tissues, 109 colorectal adenomas (≥1 cm), and 41 normal epithelia from colonoscopically normal individuals, were used in the study. Whole stools were collected and kept in a preservative buffer (19) from 497 individuals, including 196 colorectal cancer patients, 122 adenoma (≥1 cm) patients, and 179 normal individuals, before bowel preparation or 1 week after colonoscopy but before surgery. Of them, 39 people, including 19 colorectal

cancer patients and 20 normal individuals, also collected stool samples (average 5.5 g) using one quantitative partial stool collection device (Supplementary Fig. S1) we designed before whole stool collection. Fifteen milliliters of preservative buffer had been prefilled in the quantitative collection device. The impact of quantitative partial stool collection on marker performance was evaluated by comparing marker levels in stool samples provided by the abovementioned 39 people who collected both partial and whole stools. All buffered stools were immediately transported to our laboratory and stored at −80°C. Subjects included in this study were 100% Asian. Detailed demographic and clinical characteristics of the subjects were listed in Table 1.

Microdissection and DNA extraction

Tissue sections were examined by an experienced pathologist who circled out histologically distinct lesions to direct careful microdissection. Different types of DNA were extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instruction.

Sequence-specific capture

Target human genes in stool DNA were purified and enriched with a sequence-specific capture technology as reported before with some minor modifications (20). Briefly, each capture reaction was carried out by adding 300 µL of crude stool DNA to an equal volume of 6 mol/L guanidine isothiocyanate solution (Sigma) containing two biotinylated sequence-specific oligonucleotides (10 pmol total; Supplementary Table S1). After an incubation for 4 hours at room temperature, 50 µL prepared Dynabeads M-280 streptavidin (Thermo Fisher Scientific) was added to the solution and incubated for 1 hour at room temperature. The bead/hybrid capture complexes were then washed twice with 1× wash buffer (1.0 mol/L NaCl, 5 mmol/L Tris-HCl pH 7.5, and 0.5 mmol/L EDTA), and then eluted out in 50 µL nuclease-free

Table 1. Clinical characteristics of tissue and stool samples

	Tissue samples			Stool samples		
	Carcinoma (*n* = 124)	Adenoma (*n* = 109)	Normal (*n* = 41)	Carcinoma (*n* = 196)	Adenoma (*n* = 122)	Normal (*n* = 179)
Race	Asian	Asian	Asian	Asian	Asian	Asian
Age, y						
Median (range)	61 (26–82)	57 (16–87)	43 (6–81)	61 (43–79)	61 (45–76)	56 (43–77)
Sex, *n* (%)						
Male	78 (62.9)	75 (68.8)	20 (48.8)	121 (61.7)	76 (62.3)	70 (39.1)
Female	46 (37.1)	34 (31.2)	21 (51.2)	75 (38.3)	46 (37.7)	109 (60.9)
Stage, *n* (%)						
I/II	69 (55.6)			87 (44.4)		
III/IV	55 (44.4)			109 (55.6)		
Location, *n* (%)						
Proximal	27 (21.8)	19 (17.4)		43 (21.9)	46 (37.7)	
Distal	97 (78.2)	82 (75.2)		153 (78.1)	67 (54.9)	
Unknown	0 (0.0)	8 (7.4)		0 (0.0)	9 (7.4)	
Tumor size (mm)						
Median (range)	45 (2–120)	13 (10–75)		40 (8–120)	15 (10–50)	
Dysplasia, *n* (%)						
Low	2 (1.6)			14 (7.1)		
Moderate	69 (55.6)			105 (53.6)		
High	40 (32.3)			67 (34.2)		
Unknown	14 (11.3)			10 (5.1)		

water with 20 ng/μL transfer RNA (Sigma). Target gene *SDC2* gene and reference gene β-actin (*ACTB*) were captured together in one reaction. Capture probe sequences are listed in Supplementary Table S1.

Bisulfite treatment

DNA was bisulfite treated using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. For cell line and tissue DNA samples, approximately 500 ng genomic DNA was added into the bisulfite treatment reaction and eluted out in 30 μL TE buffer. For stool DNA samples, 50 μL captured DNA was added into the reaction and eluted out in 15 μL TE buffer.

Methylation-specific PCR

Methylation-specific PCR (MSP) was performed to determine the methylation status of *SDC2* in colorectal cancer cell lines as reported previously (21). Methylated and unmethylated primers were designed in the CpG islands of *SDC2* gene (Supplementary Table S1). Briefly, 2 μL bisulfite-treated DNA was amplified in a total volume of 25 μL containing 2 × iQ Supermix (Bio-Rad) and 40 nmol/L of each primer. Amplification included hot-start at 95°C for 12 minutes, denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles, and a final 10-minute extension step at 72°C. Bisulfite-treated human genomic DNA and CpGenome Universal Methylated DNA (EMD Millipore) were used as positive controls for unmethylation and methylation, respectively. Water was used as negative control. MSP products were verified by 2% agarose gel electrophoresis. Primers and annealing temperatures are shown in Supplementary Table S1.

Real-time MSP

Real-time MSP (qMSP) was used to detect *SDC2* methylation in DNA samples from tissues, stools, and cell lines. Primers and probe were designed in the CpG island of *SDC2* gene (Supplementary Table S1). *ACTB* gene was employed as a reference for bisulfite treatment and DNA input. PCR was done in a volume of 25 μL containing 400 nmol/L of each primer, 200 nmol/L of each probe, 5 mmol/L Mg^{2+}, 400 μmol/L dNTPs, 0.1 U/μL GoTaq Hot Start Polymerase (Promega), and 1× buffer. For cell line and tissue samples, 1 μL bisulfite-converted DNA was added to the PCR reaction, but for stool samples, 5 μL bisulfite-converted captured stool DNA was used. PCR reaction was performed in a LightCycler 96 under the following cycling conditions: 95°C for 5 minutes; 10 cycles at 95°C for 20 seconds, 62°C for 30 seconds, and 70°C for 30 seconds; 40 cycles at 95°C for 20 seconds, 58°C for 60 seconds, and 72°C for 30 seconds; and a final cooling step at 37°C for 30 seconds. Assays were performed in a blinded fashion. Plasmid DNA was diluted as standards for quantification. Each plate consisted of bisulfite-treated DNA samples, positive and negative controls, and water blanks. For cell line and tissue samples, the methylation level of *SDC2* gene was defined as the ratio of the copy number of *SDC2* to that of *ACTB* and multiplied by 100 (22). For stool samples, the quantified strand number of methylated *SDC2* was used to calculate marker performance.

Potentially interfering substances

On the basis of the clinical applications and the diet habits of Chinese, 17 potentially interfering substances were selected mainly from the following categories: (i) common lotions, creams, and common over-the-counter women products; (ii) stool softeners, antidiarrhea, and laxative products; (iii) antacids and stomach medicine; (iv) anti-inflammatory drugs and pain relievers; (v) animal and plant DNA; and (vi) fatty acid. These potentially interfering substances included 14 commonly prescribed and over-the-counter medicines, two mixed DNA extracts from vegetables, fruits, and meat, and one cup of vegetable oil. All 14 medicines were locally purchased in China and listed in Supplementary Table S2. The mixed animal tissue DNA was extracted from chicken, beef, and pork tissues, whereas the mixed plant DNA was extracted from grapes, watermelon, cantaloupe, apple, and cabbage. Vegetable oil was chosen to represent fatty acid.

Interfering substance test

Fifty-four stool samples from colorectal cancer patients were mixed together as one sample pool. The sample pool was redivided equally into 54 portions and regrouped into 18 groups with three portions in each group. One potentially interfering substance was spiked into each group except the control group. The theoretical concentration of each of 14 medicines in stool was determined according to clinically recommended dosage and drug metabolism in human body. The final concentration of each medicine spiked into stool was three times of its theoretical concentration. For food DNA and vegetable oil, a highest daily intake dosage was spiked into the stool samples. Detailed concentration of each interfering substance was shown in Supplementary Table S2. Target genes, *SDC2*, and *ACTB*, in these stool samples were captured, bisulfite treated, and quantified as above.

Deviation (d_{obs}) of the mean value of test samples from that of control samples was used to evaluate interference effect: $d_{obs} = \bar{x}_{test} - \bar{x}_{control}$. Here, $\bar{x}_{test}$ is the mean value of test samples, and $\bar{x}_{control}$ is the mean value of control samples. d_c was used to determine interference cutoff. The cut-off d_c can be computed as ±0.57 for a two-sided test using the equation $d_c = \frac{d_{null} + sz_{(1-\frac{\alpha}{2})}}{\sqrt{n}}$, where d_{null} is the value stated in the null hypothesis (usually is 0), n is 3 and means the number of replicates per sample, $z_{(1-\frac{\alpha}{2})}$ is the percentage of normal distribution for a two-sided test at 100 (1-α) % confidence level, and s is the SD of the measurement procedure (23). If $d_{obs} \leq |d_c|$, the deviation caused by this substance would be judged as acceptable, and this substance would not be classified as an interfering substance to our detection. Otherwise, this substance would be classified as an interfering substance (23).

IHC and Western blot analysis

IHC was used to detect *SDC2* expression in cell lines and tissues. Cells grown on slides and tissue sections were incubated in *SDC2* antibody (Genetex) and stained with Biotin-Streptavidin HRP Detection Systems (SP-9001, ZSGB-BIO Company). The immunostaining conditions had been optimized for multiple times. Previously confirmed positive and negative sections were stained at the same time as controls for each batch of slides.

Western blot analysis was also conducted to detect *SDC2* protein expression in cell lines. Total protein was extracted, electrophoresed, and transferred to nitrocellulose membranes. Membranes were incubated with *SDC2* and *GAPDH* primary antibodies (Proteintech) and then with appropriate fluorescent secondary antibodies (LI-COR Biosciences). Fluorescent signals were detected with Odyssey Infrared Imaging System (Thermo Fisher Scientific).

5-aza-2′-deoxycytidine and trichostatin A treatment

To assess the impact of methylation on the expression of *SDC2* gene, demethylation agent 5-aza-2′-deoxycytidine (5-Aza-dC, Sigma) and histone deacetylase inhibitor trichostatin A (TSA, Selleck Chemicals) were used to treat all eight colorectal cancer cell lines as reported previously (24). The mRNA expression of *SDC2* in cell lines was quantified with RT-PCR. *GAPDH* (25) was used as an internal reference gene to normalize cDNA input. The RT-PCR primers of *SDC2* and *GAPDH* are listed in Supplementary Table S1.

Statistical analysis

Wilcoxon rank sum tests were performed to compare methylation levels between different types of sample groups. Paired *t* test was used in paired samples. χ^2 test was applied to evaluate the correlation of methylation levels with demographic and clinical characteristics, such as age, sex, tumor–node–metastasis (TNM) stage, tumor location, tumor size, and dysplasia. ROC curve was constructed to compare *SDC2* methylation levels between sample types. The associated AUC value was calculated for each ROC curve. Linear regression was used to evaluate the correlation of partial and whole stool collection. Statistical analyses were conducted with GraphPad Prism Version 5.0 (Graph Pad Software Inc.).

Results

Frequent methylation of *SDC2* in colorectal neoplasms

qMSP was used to quantify methylation levels of *SDC2* gene in 398 colorectal tissues. Median methylation levels of *SDC2* in 124 cancers, 109 adenomas, 124 paired adjacent normal epithelia, and 41 normal epithelia from normal individuals were, respectively,

6.7 (1.3–10.5), 0.8 (0–3.2), 0.1 (0–0.2), and 0 (0–0.2; Fig. 1A, $P < 0.0001$ across tissue types). ROC curves were constructed to evaluate the performance of methylated *SDC2* for detecting colorectal neoplasms. AUCs were 0.93 [95% confidence interval (CI), 0.89–0.98] for colorectal cancer and 0.84 (95% CI, 0.76–0.91) for adenomas when compared with normal epithelia from normal individuals (Fig. 1B). At a specificity of 90% (37/41), methylated *SDC2* detected 83.1% (103/124) of carcinomas and 56% (61/109) of adenomas.

The data of paired samples were further analyzed independently ($P < 0.0001$ for cancer vs. normal, Fig. 1C). The area under ROC curve was 0.92 (95% CI, 0.88–0.96; Fig. 1D) for colorectal cancer when compared with paired adjacent normal tissues. *SDC2* methylation levels were higher in 96.8% (120/124) of cancers than in their paired adjacent normal epithelia ($P < 0.0001$ for cancer vs. normal), including 97.1% (66/68) for stage I/II and 96.4% (54/56) for stage III/IV cancers. No significant association was observed between *SDC2* methylation and clinical features of cancer subjects, including age, sex, TNM stage, cancer location, tumor size, and dysplasia ($P > 0.05$, Table 2).

Fecal methylated *SDC2* for the detection of colorectal neoplasms

Methylated *SDC2* in 497 stool samples were quantified with qMSP. Median log-transformed methylated *SDC2* levels were, respectively, 8.4 (4.3–11.8), 2.3 (−0.2–6.1), and −3.3 (−12.5–0.2) for cancer ($n = 196$), adenoma (≥ 1 cm, $n = 122$), and normal subjects ($n = 179$, $P < 0.0001$; Fig. 2A). Areas under ROC curve were 0.92 (95% CI, 0.89–0.95) for colorectal cancer and 0.79 (95% CI, 0.74–0.85) for adenomas (≥ 1 cm, Fig. 2B). Fecal methylated *SDC2* detected 81.1% (159/196) of colorectal cancer
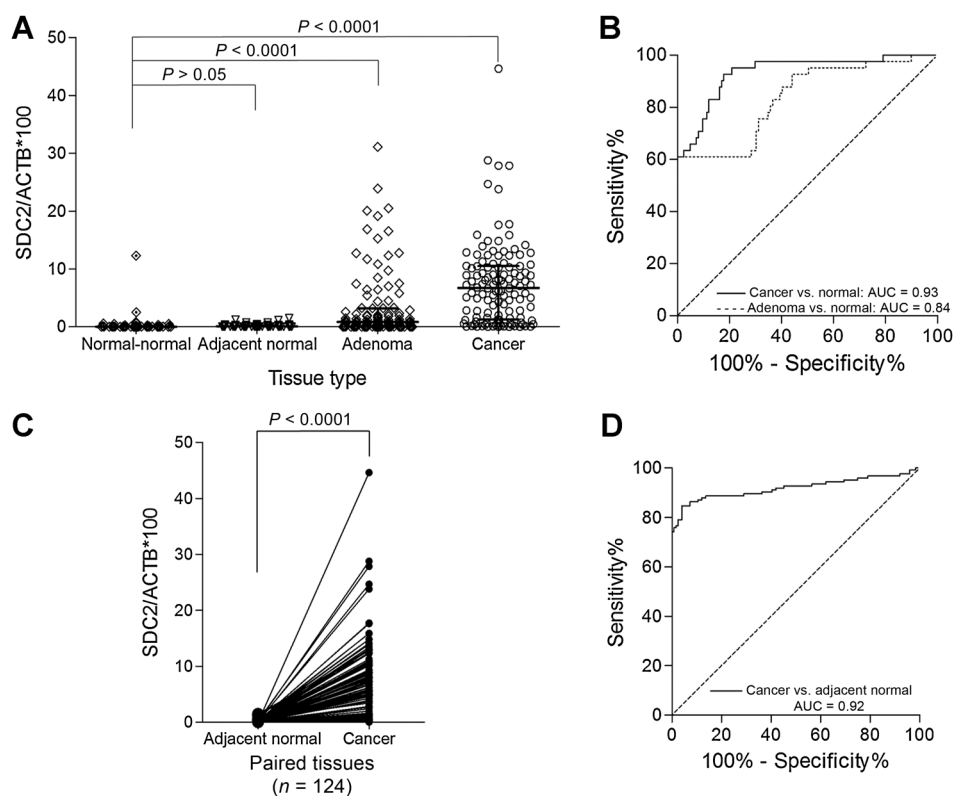

Figure 1.
SDC2 methylation in tissue samples. **A,** Methylation levels of *SDC2* measured by qMSP in 124 colorectal cancer, 109 adenomas, and 41 normal epithelia. Each dot represents one sample. The error bars in picture represent median with interquartile range. **B,** ROC curve for *SDC2* methylation levels in colorectal cancer or adenoma versus normal epithelia. **C,** Methylation of *SDC2* in 124 pairs of colorectal cancer and adjacent normal tissues. Each dot represents one sample. Paired samples from one patient were linked with a straight line. **D,** ROC curve for *SDC2* methylation levels in colorectal cancer versus paired adjacent normal tissues.

Table 2. The association of *SDC2* gene methylation with clinical variables in carcinoma samples

		SDC2 methylation of tissue samples				*SDC2* methylation of stool samples		
		+	−	*P*		+	−	*P*
Total	124	103	21		196	159	37	
Age	≤60 y	51	15	0.05	≤61 y	80	21	0.09
	>60 y	52	6		>61 y	79	16	
Sex	Male	62	16	0.13	Male	98	23	0.75
	Female	41	5		Female	61	14	
TNM stage	I/II	58	11	0.46	I/II	73	14	0.92
	III/IV	45	10		III/IV	86	23	
Location	Proximal	22	5	0.50	Proximal	28	15	0.0003
	Distal	81	16		Distal	131	22	
Tumor size	≤45 mm	61	11	0.37	≤40 mm	95	22	0.53
	>45 mm	42	10		>40 mm	64	15	
Dysplasia	Low	2	0	0.86	Low	10	4	0.56
	Moderate	57	12		Moderate	84	21	
	High	34	6		High	58	9	

and 58.2% (71/122) of adenomas (≥1 cm) at a specificity of 93.3% (167/179). No significant relationships were observed between *SDC2* methylation and clinical features, including age, gender, TNM stage, tumor size, and dysplasia ($P > 0.05$), except tumor location ($P = 0.0003$, Table 2).

Partial versus whole stool collection

Thirty-nine people, 19 colorectal cancer patients and 20 normal individuals, collected both partial and whole stool samples. The weights of samples collected by the quantitative partial stool collection device were shown in Fig. 2C. Levels of methylated *SDC2* in partial stool samples and whole stool samples were shown in Fig. 2D. Fecal methylated *SDC2* could detect 84.2% (16/19) of colorectal cancer at a specificity of 95% (19/20) for samples collected by both methods. Levels of methylated *SDC2* in partial stool samples significantly correlated with those in whole stool samples ($R^2 = 0.80$). There is no significant difference found in colorectal cancer detection rate between quantitative partial and whole stool collection ($P > 0.05$, Fig. 2E).

Substances interfering the detection of methylated *SDC2* in stool

Among the 17 potentially interfering substances tested, 16 substances did not interfere with the detection of methylated *SDC2* in stool samples ($d_{obs} \leq |d_c|$; Table 3; Fig. 2F). Berberine, a Chinese herbal medicine, showed an impact on colorectal cancer detection when three times of its normal metabolism concentration (27.69 mg/mL) was added into the stool sample (Table 3; Fig. 2F).

Impact of methylation on the expression of *SDC2*

Methylation-specific primers targeting promoter region were used to detect *SDC2* methylation in cell lines. *SDC2* methylation was detected in all eight colorectal cancer cell lines. The most heavily methylated cell lines were HCT116, SW480, and WiDr, whereas Caco-2 was the least methylated one (Fig. 3A). Demethylation with 5-Aza-dC and inhibition of histone deacetylation with TSA upregulated the expression of *SDC2* mRNA in all eight colorectal cancer cell lines, especially in the more densely methylated ones, such as HCT116, SW480, and WiDr (Fig. 3B).

SDC2 protein was abundantly expressed in the cytoplasm or on the membrane of colorectal cancer cell lines (Fig. 3C). Notably, the expression level of *SDC2* was significantly higher in both colorectal cancer and adenomas than in normal epithelia ($P < 0.05$, Fig. 3D). The expression status of *SDC2* in all eight colorectal cancer cell lines was also tested, as shown in Supplementary Fig. S2.

Discussion

This study demonstrated that fecal methylated *SDC2* is a promising marker for the detection/screening of colorectal neoplasms. Conventional methods, such as colonoscopy and FOBT, are not widely used for colorectal cancer screening in China partially due to their inherent weaknesses. Colonoscopy is considered as the gold standard for colorectal cancer diagnosis, but the compliance rate of colonoscopy in screening setting is low due to its invasive nature. In China, colonoscopy is usually performed without anesthesia, which further reduces its compliance in screening setting. Moreover, colonoscopy misses a significant percentage of neoplasms in proximal colon (26). FOBT is also commonly used for colorectal cancer screening, but its accuracy is quite low, especially for adenoma (≥1 cm; ref. 27). sDNA provided another accurate and noninvasive option for screening colorectal cancer. The value of sDNA for the early detection of colorectal cancer and advanced adenoma has been proved by many previous studies (28). However, the major breakthrough in the development of sDNA did not come until FDA-approved ColoGuard for clinical use based on one multicenter clinical trial showing that it could detect 92% of colorectal cancer and 42% of adenomas (≥1 cm) at a specificity of 87% (10). Although ColoGuard sDNA is now available commercially in the United States, it is quite expensive ($603 per test) and complicated as four markers in three different categories are analyzed in the test. In the current study with relatively small sample size, at a specificity of 93.3%, fecal methylated *SDC2* alone could detect 81.1% of colorectal cancer and 58.2% of adenomas (≥1 cm). Thus, stool test with methylated *SDC2* would serve as an attractive option for the early detection of colorectal neoplasms. Although the samples in the current study are all Asian, the value of fecal methylated *SDC2* deserves further validation in different ethnic groups through international collaboration. Mitchell and colleagues reported that *SDC2* was frequently methylated in colorectal cancer from Australian patients (17), which supports methylated *SDC2* is a promising biomarker across different ethnic groups.

By comparing partial stool collection with whole stool collection, we found out that the performance of stool DNA test was not
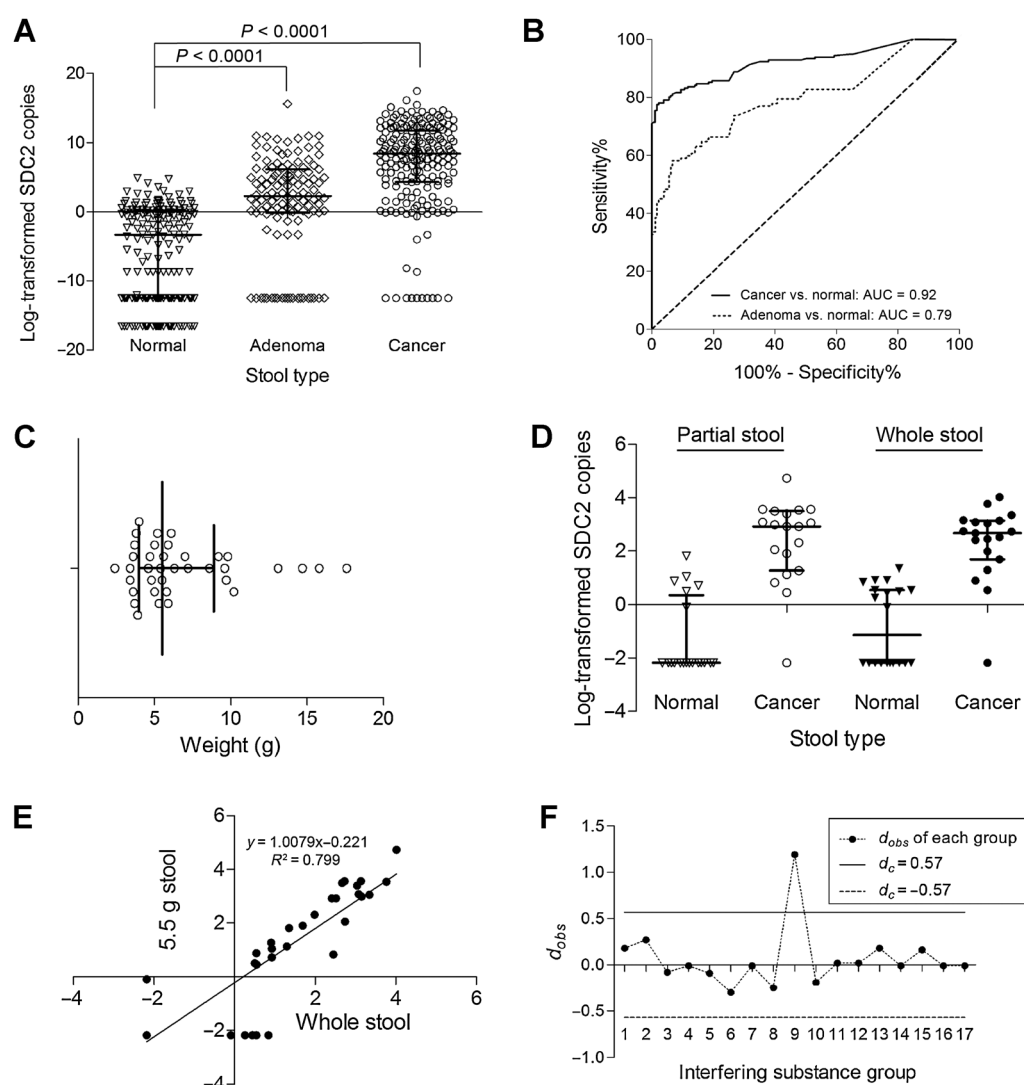
Figure 2.
SDC2 methylation in stool samples. **A,** Levels of methylated *SDC2* in 196 colorectal cancer, 122 adenoma, and 179 normal samples. **B,** ROC curves for *SDC2* methylation levels in carcinoma or adenoma versus normal samples. **C,** The sample weights collected by the quantitative collection device (average 5.5 g; range, 2.4–17.6 g). **D,** Levels of methylated *SDC2* in partial and whole stool samples from 19 colorectal cancer patients and 20 normal individuals. **E,** The correlation of two stool sampling methods ($y = 1.0079x - 0.221$, $R^2 = 0.80$). **F,** The impacts of potentially interfering substances on detection results. Each dot represents d_{obs} of one interfering substances group.

affected by partial stool collection. These results proved that the quantitative stool collection device is scientifically viable. A small sampling device offers additional advantages in miniaturizing and simplifying sample processing procedure and reducing cost. In the interfering substance test, we found no impact of 13 medicines, animal DNA, plant DNA, and fatty acid on the detection of methylated *SDC2*. Therefore, there are not many diet and medication restrictions to consider for stool DNA testing. Berberine, a Chinese herbal medicine, is the only exception. We will look further into other potential factors that would improve or restrict the compliance of stool DNA testing in the future.

Although methylated *SDC2* was detected as a frequent event in blood samples from patients with colorectal cancer, stool test could be more feasible than blood test for the early detection/

screening of colorectal cancer according to a previous report by Ahlquist and colleagues (18). In that head-to head comparison study, stool DNA testing showed much better performance than blood methylated *Septin9* for detecting colorectal cancer at stages I/II and advanced adenoma (18). They found that blood methylated *Septin9* could only detect 14% of advanced adenoma and further concluded that marker release into the bloodstream from precursor lesions is negligible (18). In the current study, fecal methylated *SDC2* detected 58% of advanced adenomas, which does support that stool is a suitable sample for detecting precursor lesions.

The current study also confirmed that *SDC2* gene was heavily methylated in cell lines and tissues from colorectal cancer, which is consistent with previous report (29). When paired samples were

Table 3. The impacts of potentially interfering substances on detection results

Group ID	Interfering substance group	d_{obs}	$|d_c|$	$d_{obs} \leq |d_c|$
0	Control	NA	NA	NA
1	Musk hemorrhoids ointment	0.18	0.57	No
2	Glycerol enema	0.27	0.57	No
3	Tetracycline tablets	−0.08	0.57	No
4	Phenoxymethylpenicillin potassium tablets	−0.01	0.57	No
5	Ibuprofen sustained release capsules	−0.09	0.57	No
6	Domperidone tablets	−0.3	0.57	No
7	Vitamin U, belladonna and aluminium capsules II	−0.01	0.57	No
8	Omeprazole enteric-coated capsules	−0.25	0.57	No
9	FufangHuangLianSuPian	1.19	0.57	Yes
10	Cefixime capsules	−0.19	0.57	No
11	Levofloxacin hydrochloride tablets	0.02	0.57	No
12	Cimetidine tablets	0.02	0.57	No
13	GanMaoLingJiaoNang	0.18	0.57	No
14	TongbianlingJiaonang	−0.01	0.57	No
15	Plant DNA	0.163	0.57	No
16	Animal DNA	−0.01	0.57	No
17	Vegetable oil	−0.01	0.57	No

Abbreviation: NA, nonapplicable.
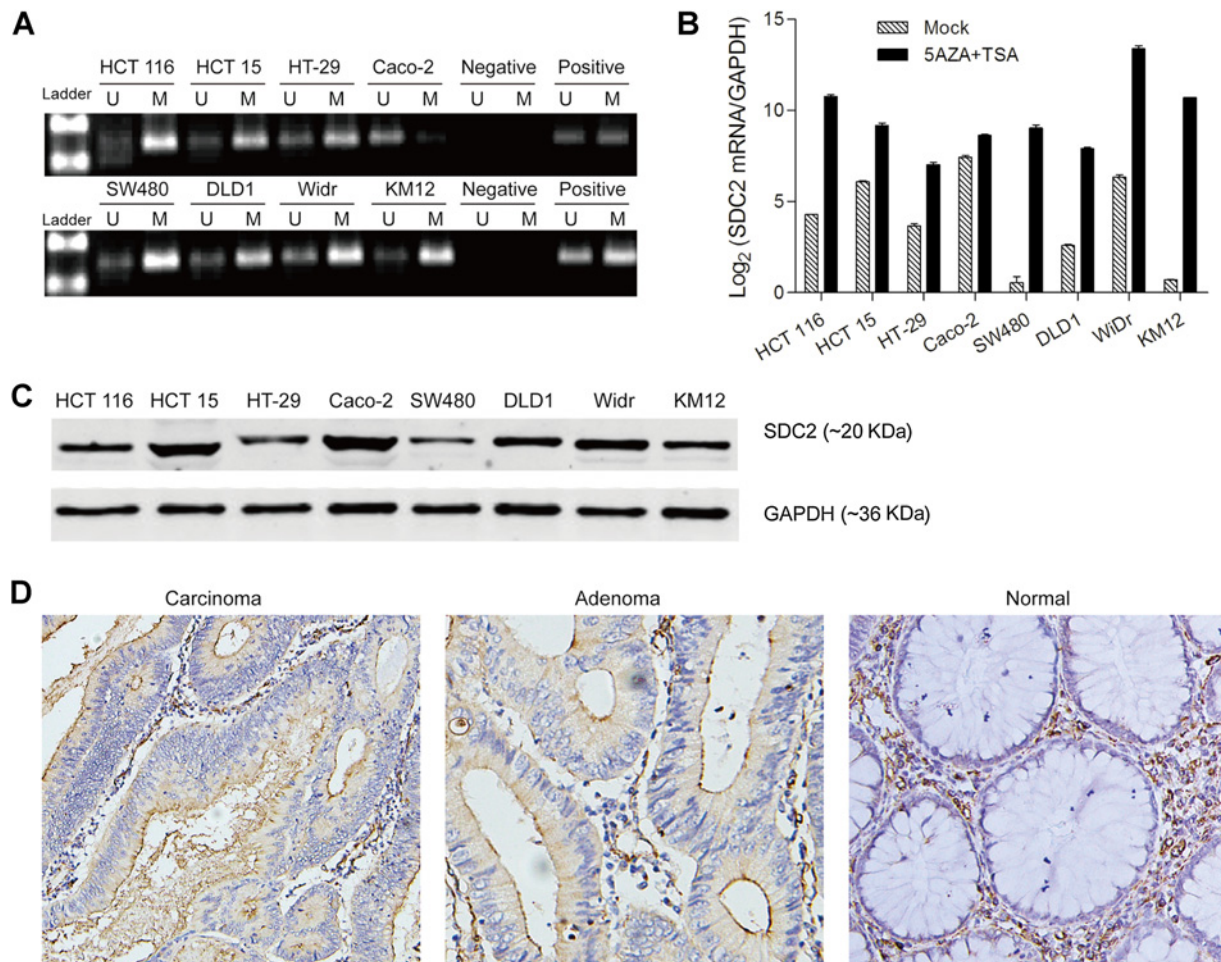
Figure 3.
A, *SDC2* methylation in colorectal cancer cell lines detected by MSP. MSP products in lanes U and M indicate the presence of unmethylated and methylated *SDC2*, respectively. **B,** Reexpression of *SDC2* in colorectal cancer cell lines by demethylation and inhibition of histone deacetylation. **C,** Abundant expression of *SDC2* protein was detected in colorectal cancer cell lines with Western blot analysis. **D,** IHC showed elevated *SDC2* expression in colorectal cancer and adenoma when compared with normal epithelium.

analyzed independently, *SDC2* methylation levels were higher in 96.8% (120/124) of colorectal cancer than in adjacent normal epithelia. When unpaired tissue samples were analyzed, the sensitivities of detecting carcinomas and adenomas were, respectively, 83.1% and 56% at a specificity of 90% (37/41). These results also support that *SDC2* is a valuable methylation biomarker for the detection of colorectal neoplasms.

Demethylation and inhibition of histone deacetylation upregulated the expression of *SDC2* in colorectal cancer cell lines with *SDC2* methylation, which indicates that *SDC2* expression was suppressed by aberrant promoter methylation. One could speculate based on common sense that *SDC2* expression in colorectal cancer tissues should be silenced by DNA methylation. Surprisingly, our experiments showed one contradictory phenomenon that overexpression and aberrant methylation of *SDC2* coexisted in colorectal cancer, which is consistent with previous reports (30, 31) and indicates one underlying unknown mechanism further regulating *SDC2* expression.

In conclusion, we have developed one stool DNA test with methylated *SDC2*. This test could be of high value for the non-invasive screening of colorectal neoplasms. However, there are limitations with the design of the current study. For example, this is a relatively small single-point verification study in Asian people only. We plan to initiate one multicenter clinical trial to further validate the performance of this test in this year. Furthermore, we will validate the performance of methylated *SDC2* in other ethnic groups within other existing screening guidelines through international collaboration, and further determine the cost-effectiveness of this test in screening setting through long-term follow-up in the future. The contradictory phenomenon of the coexistence of aberrant methylation and overexpression of *SDC2* also deserves deeper investigation.

Disclosure of Potential Conflicts of Interest

R. Zhao is a technologist at Creative Biosciences (Guangzhou) CO., Ltd. S. Wu is a technologist at Creative Biosciences (Guangzhou) CO., Ltd. H. Yu is a technologist at Creative Biosciences (Guangzhou) CO., Ltd. X. Zhao is the director of research at Creative Biosciences (Guangzhou) CO., Ltd. H. Zou is the founder of, reports receiving a commercial research grant from, and has ownership interest in Creative Biosciences (Guangzhou) CO., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: F. Niu, J. Wen, J. Wang, H. Zou
Development of methodology: F. Niu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Niu, J. Wen, X. Fu, R. Zhao, S. Wu, H. Yu, X. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Niu, J. Wen, X. Zhao
Writing, review, and/or revision of the manuscript: F. Niu, J. Wen, H. Zou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Li, S. Liu, X. Wang
Study supervision: J. Wang, H. Zou

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