

Detection of Circulating Tumor DNA Methylation in Diagnosis of Colorectal Cancer

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INTRODUCTION: Emerging evidence has demonstrated the potential of the circulating tumor DNA (ctDNA) methylation in the application of cancer diagnosis.

METHODS: Three genes including Septin9, Syndecan-2 (SDC2), and branched-chain amino acid transaminase 1 (BCAT1), which have been well demonstrated to have aberrant expression in colorectal cancer (CRC) as tumor suppressors, were selected for detection. A total of 234 peripheral plasma samples from 104 patients with CRC and 130 patients with colorectal polyps, and 60 plasma samples from healthy controls, were collected before any treatment. A real-time polymerase chain reaction-based gene panel was used to detect the methylation of Septin9, SDC2, and BCAT1. The composite score (P) was calculated according to the cycle threshold values of the 3 methylated genes using the logistic regression equation.

RESULTS: The ctDNA methylation of the 3 genes had a significantly higher level in patients with CRC, compared with patients with colorectal polyps and healthy controls. The composite score (P) showed association with tumor stages in CRC but not with the tumor location (colon or rectum). In addition, BCAT1 and Septin9 showed better performance for CRC diagnosis, by which CRC was able to distinguish from polyps with sensitivity of 83.7%, specificity of 93.9%, and area under the curve of 0.908. The diagnostic efficiency was significantly improved by combining composite score (P), carcinoembryonic antigen, and fecal immunochemical test for hemoglobin (area under the curve = 0.962).

DISCUSSION: The composite score (P) derived from the ctDNA methylation levels of Septin9, SDC2, and BCAT1 can be used for CRC diagnosis with high sensitivity and high specificity. A combination of ctDNA methylation, carcinoembryonic antigen, and fecal immunochemical test for hemoglobin was proved to be the most effective approach to diagnose CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common diseases all over the world. Around 1.2 million patients are diagnosed with CRC each year, causing ~600,000 deaths directly or indirectly (1,2). Early detection through physical examination screening would greatly increase the viability of CRC (3). As the gold standard and the most commonly used modality for CRC screening, colonoscopy still has shortcomings, such as invasiveness and unpleasantness (4,5). Although carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and fecal immunochemical test for hemoglobin (FIT) have been widely used for cancer diagnosis, the sensitivity and specificity for early CRC are unsatisfactory (6). There is an urgent need to develop other approaches for early detection of CRC to prolong the overall survival of the patients.

Recently, serum-based analysis of circulating tumor DNA (ctDNA), which was released from primary or metastatic tumor cells, has become a breakthrough in early detection of cancer (7–9). This approach is called “liquid biopsy” with advantages of high efficiency and noninvasiveness. Contemporary epigenetic research on CRC has made big progress in recent decade, especially on DNA methylation (10,11). Previous studies have shown that methylation regulation in the promoters of some genes can distinguish patients with cancer from healthy people using tissue biopsy and/or blood samples (12,13). Three tumor suppressor genes including Septin9, Syndecan-2 (SDC2), and branched-chain amino acid transaminase 1 (BCAT1) have been demonstrated to have hypermethylation at the promoter regions in patients with CRC. Methylation of Septin9, SDC2, and BCAT1

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Table 1. Clinical characteristics of patients and controls

Variables	CRC, N (%)	Colorectal polyps, N (%)	Healthy persons, N (%)
Sex	104	130	60
Male	65 (62.5%)	90 (69.2%)	37(61.7%) 23(38.3%)
Female	39 (37.5%)	40 (30.8%)	
Age			
Median	64.7	61.2	56.0
Range	26.0–85.0	18.0–83.0	18.0–83.0
Basic characteristics			
BMI			
Mean	22.8	23.9	22.8
Range	15.5–30.1	15.9–32.0	16.3–30.5
History of cholecystectomy			
Sedentary	13 (1.3%)	26 (2.00%)	3(5.00%)
High-fat diet	18 (17.3%)	18 (13.9%)	16 (26.6%)
Family history of colon cancer	7 (6.7%)	7 (5.4%)	4 (6.7%)
Tumor site			
Colon	50 (48.1%)	—	—
Rectum	54 (51.9%)	—	—
Lymph node metastasis			
Yes	32 (30.8%)	—	—
No	72 (69.2%)	—	—
Dukes staging			
A	22 (21.2%)	—	—
B	34 (32.7%)		
C	30 (28.8%)		
D	18 (17.3%)		
Pathological type			
Tubular adenoma	—	84 (64.6%)	—
Hyperplastic polyp	—	28 (21.5%)	—
Tubular villous adenoma	—	15 (11.5%)	—
Serrated adenoma	—	3 (2.3%)	—
Polyp size			
≤1 cm	—	93 (71.5%)	—
>1 cm	—	37 (28.5%)	—
Polyp number			
Single	—	45 (34.6%)	—
Multiple	—	85 (65.4%)	—
Advanced adenoma			
No	—	83 (63.8%)	—
Yes	—	47 (36.2%)	—

BMI, body mass index; CRC, colorectal cancer.

was considered as promising biomarkers in CRC diagnosis (14–16). Septin9 was used for CRC detection with sensitivity of 65% and specificity of 90% in Western countries (12,17). However, another study reported that the sensitivity of Septin9

application for CRC diagnosis was only 35.0% for stage I and 63.0% for stage II (13,18). SDC2 functions as an integral membrane protein to participate in cell proliferation, cell migration, and cell-matrix interactions and also showed hypermethylation

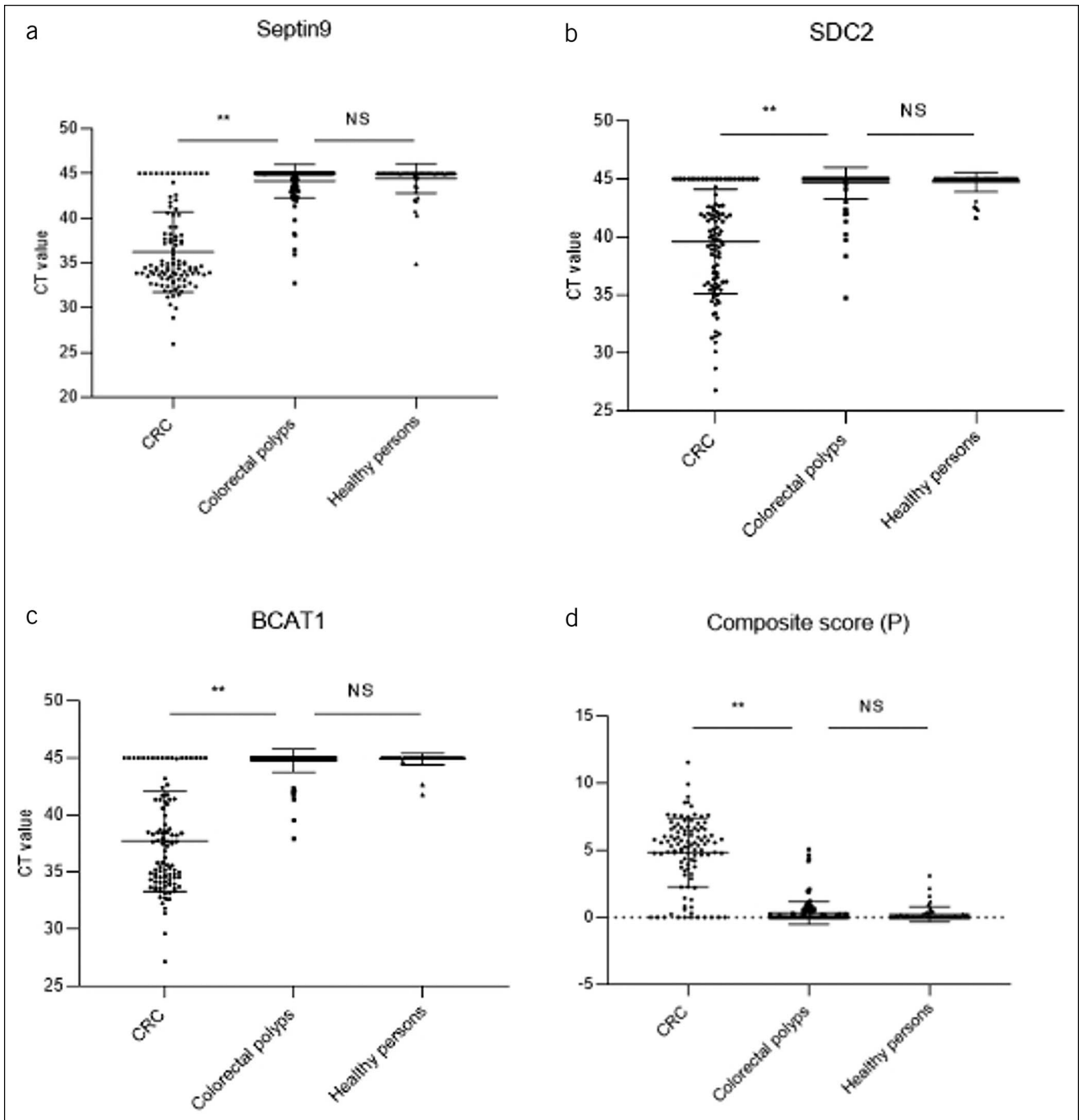


Figure 1. Levels of methylated Septin9, SDC2, BCAT1, and composite score (P) in the plasma of patients with colorectal cancer, colorectal polyps, and healthy persons. Data are presented as mean \pm SD. * $P < 0.05$, ** $P < 0.001$. CRC, colorectal cancer; NS, no significance; BCAT1, branched-chain amino acid transaminase 1; SDC2, Syndecan-2.

in most patients with CRC. The sensitivity of SDC2 at the stage I CRC reached $\sim 92.3\%$, indicating the strong potential of SDC2 methylation for early detection of CRC (15,19). BCAT1 plays an important role in regulating cell growth, apoptosis, and differentiation. Epigenetic silencing of BCAT1 caused activation of Wnt signaling, inducing tumorigenesis (20–22). Circulating DNA in the plasma samples of patients with CRC has a significantly higher fraction of methylated BCAT1, compared with healthy controls (23).

In this study, a novel DNA methylation panel containing Septin9, SDC2, and BCAT1 was used to detect plasma samples from patients with CRC and colorectal polyps. The sensitivity and specificity were evaluated for cancer prognosis. Comparisons with the currently used markers were performed, including CEA, CA19-9, and FIT. A combination of ctDNA methylation, CEA, and FIT was tested as well, showing the improved efficiency to diagnose CRC.

Table 2. Associations between the biomarkers and clinicopathological parameters in the CRC group

CRC	CEA	CA19-9	FIT	Septin9	SDC2	BCAT1	Composite score (P)
Location							
Colon (N = 50)							
Mean ± SD	45.2 ± 165.4	78.0 ± 349.8	2.2 ± 1.0	36.4 ± 4.8	39.5 ± 4.6	37.6 ± 4.7	4.8 ± 2.8
Positive (%)	27 (54.0)	11 (22.0)	33 (66.0)	41 (82.0)	39 (78.0)	41 (82.0)	41 (82.0)
Rectum (N = 54)							
Mean ± SD	12.3 ± 18.07	78.5 ± 338.9	2.3 ± 1.0	36.1 ± 4.2	39.7 ± 4.4	37.8 ± 4.2	4.8 ± 2.4
Positive (%)	26 (48.1)	6 (11.1)	36 (66.7)	46 (85.2)	41 (75.9)	46 (85.2)	45 (83.3)
Dukes staging							
A + B (N = 56)							
Mean ± SD	10.5 ± 16.0	17.5 ± 26.7	2.1 ± 1.0	37.3 ± 4.5	40.6 ± 3.7	38.5 ± 4.2	4.2 ± 2.5
Positive (%)	24 (42.9)	3 (5.4)	36 (64.3)	44 (78.6)	41 (73.2)	46 (82.1)	43 (76.8)
C + D (N = 48)							
Mean ± SD	48.0 ± 166.9	482.9 ± 69.0	2.4 ± 1.0	35.1 ± 4.2*	38.5 ± 5.0*	36.8 ± 4.5*	5.5 ± 2.5*
Positive (%)	29 (60.4)	14 (29.2)**	33 (68.8)	43 (89.6)	39 (81.3)	41 (85.4)	43 (89.6)
CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CRC, colorectal cancer; FIT, fecal immunochemical test for hemoglobin. * <i>P</i> < 0.05, ** <i>P</i> < 0.001.							

MATERIALS AND METHODS

Patient enrollment

The SOP for sample collection and methylated DNA detection was established before the beginning of the trial. In this study, patients with CRC and colorectal polyps diagnosed by colonoscopy from March 2019 to August 2019 in Shanghai East Hospital were included. After a series of exclusion criteria including absence of CEA or CA19-9 detection and cytology examination or without exact detection, 104 patients with CRC, 130 patients with colorectal polyps, and 60 healthy persons were finally enrolled in our research. The age of all subjects was between 18 and 85 years. All enrolled patients underwent colonoscopy to exclude other intestinal diseases that may affect the outcome of the FIT, including inflammatory bowel disease, ischemic bowel disease, and non-specific colonic ulcer. Controls were selected by standard CRC screening with colonoscopy negative for either polypoid changes or CRC, together with other exclusion criteria including (i) currently having any types of cancers, (ii) being pregnant, (iii) taking folic acid and vitamin B for more than 3 months (24,25), and (iv) recurrence of CRC after operation. All the colorectal polyps were removed completely using biting or endoscopic mucosal resection or endoscopic submucosal dissection and sent for pathological examination. Colorectal polyps were classified into advanced and non-advanced adenomas. Advanced adenoma is defined if 1 of the 3 items was met: (i) diameter of polyp or lesion \geq 10 mm; (ii) villous adenoma or mixed adenoma, with the villous structure \geq 25%; and (iii) high grade of intraepithelial neoplasia (26,27).

Sample collection and storage

Blood samples were collected from outpatients and inpatients with pathological information recorded in detail and obtained on

the day of colonoscopy. Five milliliter of peripheral blood sample from each subject was collected using K2EDTA-pretreated anti-coagulant blood collection tubes (BD Biosciences, Franklin Lakes, NJ). After centrifuging at the speed of 1,350g for 12 minutes at 4 °C, the plasma was prepared and stored at -80 °C until further analysis. Approximately 2 g of fresh feces from each subject was collected before colonoscopy and processed immediately in the laboratory.

Laboratory tests

For all plasma samples, CEA and CA19-9 were measured using a Cobase 801 automatic electrochemical luminescence instrument and supporting reagents (Roche, Switzerland). FIT (gold gel stripe) was measured using the diagnostic test kit (Wanhua Puman, Beijing, China). The subjects were considered abnormal if the values of CEA were more than 5.0 ng/mL or CA19-9 were more than 30 U/mL. The positive threshold of FIT was 0.2 μ g/mL.

DNA isolation and bisulfite conversion

DNA was extracted using the nucleic acid extraction kit (Excellen Medical Technology, Beijing, China) according to the instruction. Briefly, 2 mL of plasma was digested with 3 mL of lysis buffer for 20 minutes, and then DNA was extracted using magnetic particles. Thereafter, the unmethylated cytosines (C) in DNA were modified to uracils (U) using sodium bisulfite. After purification, the bisulfite-converted DNA (bisDNA) was stored in elution solution at -20 °C for further analysis.

DNA methylation analysis

Bisulfite-modified bisDNAs were applied as templates for gene methylation analysis using the diagnostic kit (Excellen Medical Technology). Briefly, gene amplifications were performed in

Table 3. Associations between the biomarkers and clinicopathological parameters in the colorectal polyps' group

Colorectal polyps	CEA	CA19-9	FIT	Septin9	SDC2	BCAT1	Composite score (P)
Number							
Single (N = 45)							
Mean ± SD	2.6 ± 2.0	13.3 ± 10.4	1.1 ± 0.3	44.3 ± 1.5	44.8 ± 1.1	44.9 ± 0.8	0.3 ± 0.7
Positive (%)	5 (11.1)	3 (6.7)	3 (6.7)	1 (2.2)	2 (4.4)	1 (2.2)	1 (2.2)
Multiple (N = 85)							
Mean ± SD	5.7 ± 22.7	10.5 ± 8.0	1.2 ± 0.4*	44.0 ± 2.1	44.6 ± 1.5	44.7 ± 1.1	0.4 ± 0.9
Positive (%)	17 (20)	4 (4.7)	17 (20)	6 (7.1)	10 (11.8)	7 (8.2)	3 (3.5)
Size							
≤1 cm (N = 93)							
Mean ± SD	5.4 ± 21.7	11.3 ± 9.1	1.1 ± 0.3	44.1 ± 2.0	44.7 ± 1.4	44.8 ± 0.8	0.4 ± 0.9
Positive (%)	18 (19.4)	6 (6.5)	10 (10.8)	4 (4.3)	7 (7.5)	5 (5.4)	3 (3.2)
>1 cm (N = 37)							
Mean ± SD	2.8 ± 1.7	11.9 ± 8.7	1.3 ± 0.5*	44.3 ± 1.7	44.6 ± 1.2	44.7 ± 1.32	0.3 ± 0.8
Positive (%)	4 (10.8)	1 (2.7)	10 (27)*	3 (8.1)	5 (13.5)	3 (8.1)	1 (2.7)
Advanced adenoma							
No (N = 83)							
Mean ± SD	3.0 ± 2.5	11.8 ± 10.0	1.1 ± 0.5	44.1 ± 2.1	44.6 ± 1.5	44.8 ± 0.9	0.36 ± 0.9
Positive (%)	13 (15.7)	6 (7.2)	7 (8.4)	4 (4.8)	7 (8.4)	5 (6.0)	3 (3.6)
Yes (N = 47)							
Mean ± SD	7.6 ± 30.4	10.9 ± 7.0	1.3 ± 0.3*	44.3 ± 1.6	44.7 ± 1.0	44.7 ± 1.2	0.3 ± 0.7
Positive (%)	9 (19.1)	1 (2.1)	13 (27.7)*	3 (6.4)	5 (10.6)	3 (6.4)	1 (2.1)
CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; FIT, fecal immunochemical test for hemoglobin. * <i>P</i> < 0.05.							

triplicate in a volume of 25 μ L containing 12.5 μ L of reaction buffer, 2.5 μ L of primer mix, and 10 μ L of bisDNA. The methylated and unmethylated DNAs can be discriminated by the primers and probes. The methylated DNAs are preferred for amplification by the reaction system. Probes targeting specific gene methylation were designed using fluorescence FAM for Septin9, 4',5'-dichloro-2',7'-dimethoxy-fluorescein (JOE) for SDC2, fluorescence Texas Red for BCAT1, and fluorescence CY5 for beta-Actin. Amplifications were performed using the following procedure: 98 °C for 5 minutes; 45 cycles of 95 °C for 10 seconds and 63 °C for 5 seconds; 58 °C for 30 seconds using ABI7500 fast real-time polymerase chain reaction (RT-PCR) systems (Applied Biosystems, Waltham, MA). The cycle threshold (Ct) values of each methylated gene were analyzed using ABI7500 Fast RT-PCR System Sequence Detection Software v1.4.1 (Applied Biosystems), with appropriate setting of threshold and baseline.

Composite score

The result of composite score was analyzed using Lung Cancer Analysis Software v2.2 (Excellen Medical Technology) based on the Ct values of the 3 methylated genes by a constant marker-specific weighting factor. The calculated formula of the composite score was constructed by the company using the logistic regression model. The aggregate of these individually weighted marker results determined the composite score (P) (range of

0–28). The optimal cutoff point for each methylated gene was chosen as the value that maximized the Youden index according to the CRC and control groups (polyps and healthy person). The cutoff value for Septin9, SDC2, BCAT1, and composite score (P) was 41.9, 44.5, 45.0, and 2.15, respectively.

Statistical analysis

Statistical analysis was performed using software SPSS20.0 (SPSS, Chicago, IL). The associations between gene methylation and pathological features in CRC were analyzed using the Fisher exact method or χ^2 test. The maximum value of the Youden index was used to define the cutoff value of methylated genes and composite score (P). The data in 104 patients with CRC and 130 patients with colorectal polyps were used to plot the receiver operating characteristic curves. Combination analysis was calculated using binary logistic regression. All *P* values were 2-sided, and *P* < 0.05 was considered statistically significant.

All samples were collected from consenting individuals according to protocols approved by the Ethics Committee of Shanghai East Hospital, affiliated to Tongji University (Approval No.: 2018 [64]).

RESULTS

Clinicopathologic information

There were 104 CRC, 130 colorectal polyps, and 60 healthy persons confirmed by colonoscopy and/or tissue biopsy. All the

Table 4. The diagnostic efficiency of CEA, CA19-9, FIT, composite score (P), and combined analysis in the diagnosis of CRC vs polyp groups

Independent variable	Sensitivity	Specificity	PPV	NPV	AUC (95% CI)
CRC vs polyps					
CEA	51.0%	75.4%	62.4%	65.8%	0.731 (0.664–0.798)
CA19-9	16.4%	94.6%	70.8%	58.6%	0.683 (0.614–0.753)
FIT	66.4%	84.6%	77.5%	75.9%	0.781 (0.718–0.844)
Septin9	83.7%	94.6%	92.6%	87.9%	0.901 (0.854–0.947)
SDC2	76.9%	90.8%	87.0%	83.1%	0.855 (0.801–0.908)
BCAT1	83.7%	93.9%	91.6%	87.8%	0.908 (0.864–0.952)
Composite score (P)	82.7%	96.9%	95.6%	87.5%	0.914 (0.871–0.957)
CEA + CA19-9	66.4%	70.0%	63.9%	72.2%	0.743 (0.679–0.807)
Composite score (P) + CEA	81.7%	96.2%	94.4%	86.8%	0.944 (0.915–0.972)
Composite score (P) + CEA + CA19-9	78.9%	96.2%	94.3%	85.0%	0.930 (0.895–0.965)
Composite score (P) + FIT	83.7%	96.9%	95.6%	88.1%	0.952 (0.923–0.980)
Composite score (P) + CEA + FIT	84.6%	95.4%	93.6%	88.6%	0.962 (0.941–0.983)
Composite score (P) + CEA + CA19-9 + FIT	81.7%	94.6%	92.4%	86.6%	0.953 (0.928–0.978)

The cutoff value for CEA and CA19-9 was 5 and 30 ng/mL, respectively. The optimal cutoff point for each methylated gene was chosen as the value that maximized the Youden index according to the CRC and control group (polyps and healthy person). The cutoff value for Septin9, SDC2, BCAT1, and composite core (P) is 41.9, 44.5, 45.0, and 2.15, respectively.

AUC, area under the curve; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CI, confidence interval; CRC, colorectal cancer; FIT, fecal immunochemical test for hemoglobin; NPV, negative predictive value; PPV, positive predictive value.

* $P < 0.05$, ** $P < 0.001$.

clinicopathologic data of these 294 cases are summarized in Table 1, including sex, age, histology subtypes, and tumor stage. There was no significant difference for age and sex between different groups ($P > 0.05$). In the group of CRC, 48.1% (50/104) had lesions in the colon and 51.9% (54/104) in the rectum. Stages of patients with CRC were evaluated according to the modified Dukes staging, with 21.2% (22/104) at stage A, 32.7% (34/104) at stage B, 28.8% (30/104) at stage C, and 17.3% (18/104) at stage D. In the group of colonic polyps, 34.6% (45/130) had single polyps, 65.4% (85/130) had multiple polyps, 63.8% (83/130) had nonadvanced polyps, and 36.2% (47/130) had advanced adenoma. For pathological types in colonic polyps, 64.6% are tubular adenoma, 21.5% are hyperplastic polyps, 11.5% are tubular villous adenoma, and 2.3% are serrated adenoma.

Hypermethylation detection of Septin9, SDC2, and BCAT1 in plasma

Quantitative RT-PCR analysis was performed in triplicate to detect the methylation of Septin9, SDC2, and BCAT1 genes in the plasma samples. The results were represented by the mean of 3 replicates (Figure 1). Based on the Ct values, the composite score (P) was derived from the ctDNA methylation levels. As shown in Figure 1, the ctDNA methylation levels in the CRC group were significantly higher than those in the polyp group and healthy controls ($P < 0.001$). No significant difference was observed in the level of the gene methylation between colorectal polyps' group and normal group ($P > 0.05$).

Correlation between ctDNA methylation and clinical parameters

The associations between the prognostic parameters and clinical parameters were analyzed in the groups of CRC (Table 2) and colorectal polyp (Table 3), respectively. In the CRC group, no significant difference was found in the levels and positive rates of CEA, CA19-9, FIT, Septin9, BCAT1, and SDC2 between colon and rectal cancer. Patients at the late stage (Dukes staging C and D) showed a higher positive rate of CA19-9 ($P < 0.001$) and higher levels of methylation in Septin9 ($P < 0.05$), BCAT1 ($P < 0.05$), SDC2 ($P < 0.05$), and higher level of composite score (P) ($P < 0.05$). In the early stage of patients with CRC (Dukes stage A and B), the diagnostic sensitivity of CEA, CA19-9, FIT, Septin9, BCAT1, SDC2, and composite score (P) was 42.9%, 5.4%, 64.3%, 78.6%, 73.2%, 82.1%, and 76.8%, respectively, whereas in the late stage of patients with CRC (Dukes staging C and D), the sensitivity of the biomarkers above was 60.4%, 29.2%, 68.8%, 89.6%, 81.3%, 85.4%, and 89.6%, respectively. In the colorectal polyps' group, the associations between FIT and clinical parameters including number, size, and progression were statistically significant. However, there was no significant association between the ctDNA methylation of Septin9, BCAT1, SDC2, and the analyzed clinicopathological parameters in the colorectal polyps' group (Table 3).

Evaluation of the ctDNA methylation of Septin9, SDC2, BCAT1, and composite score (P) in CRC diagnosis

To evaluate the potential of the ctDNA methylation of circulating Septin9, SDC2, and BCAT1 for CRC diagnosis, the values of composite score (P), CEA, CA19-9, and FIT in patients with CRC were compared each other, as shown in

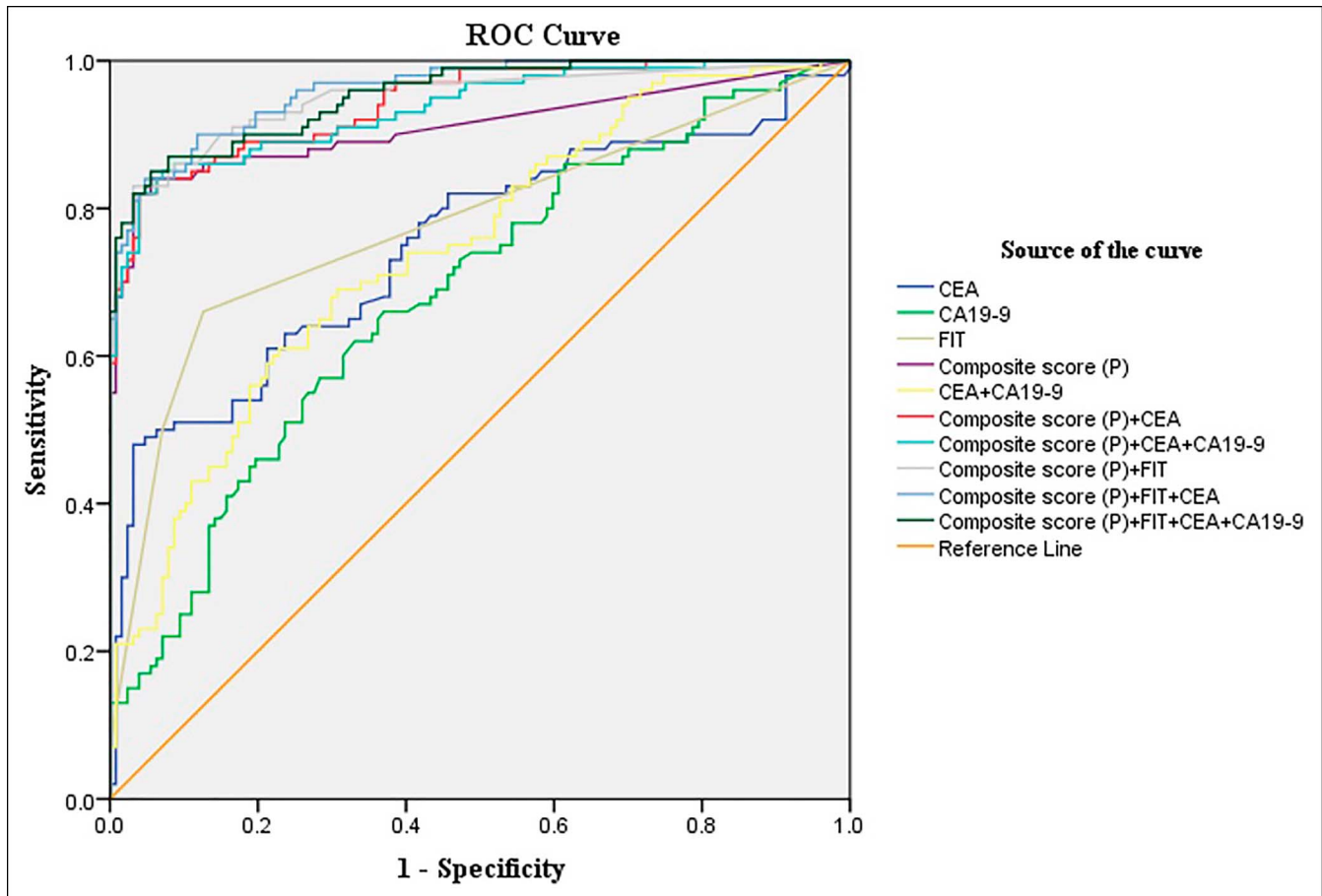


Figure 2. ROC curve for CEA, CA19-9, FIT, composite score (P), and combined analysis. CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; FIT, fecal immunochemical test for hemoglobin; ROC, receiver operating characteristic.

Table 4. The ctDNA methylation levels of Septin9, SDC2, BCAT1, and composite score (P) were significantly higher in the CRC group (83.7%, 76.9%, 83.7%, and 82.7%, respectively) than in the colorectal polyps' group (5.4%, 9.2%, 6.1%, and 4.4%, respectively). The receiver operating characteristic curve was implemented to evaluate the performance of single biomarker and multibiomarker panels (Figure 2). The ctDNA methylation of Septin9, SDC2, BCAT1, and composite score (P) showed an area under the curve (AUC) value of 0.901 (95% confidence interval [CI]: 0.854–0.947), 0.855 (95% CI: 0.801–0.908), 0.908 (95% CI: 0.864–0.952), and 0.914 (95% CI: 0.871–0.957), respectively, which were significantly higher than CEA (AUC value: 0.731, 95% CI: 0.664–0.798), CA19-9 (AUC value: 0.683, 95% CI: 0.614–0.735), and FIT (AUC value: 0.781, 95% CI: 0.718–0.844). The combination of CEA, CA19-9, and FIT with the composite score (P) was then performed by logistic regression analysis. As shown in Table 4 and Figure 2, the combination of composite score (P) and CEA and FIT showed the most effectiveness in the diagnosis of CRC (AUC value: 0.962, 95% CI: 0.941–0.983) with sensitivity of 84.6%, specificity of 95.4%, positive predictive value of 93.6%, and NPV of 88.6%.

DISCUSSION

Our prospective single-center study included a relatively small number of subjects. In this study, methylation of Septin9,

SDC2, and BCAT1 genes was detected in the plasma of patients with CRC in China before surgery, demonstrating the significantly higher level of ctDNA methylation of the 3 genes in patients with CRC compared with normal controls. This is a complementary addition to the literature about the methylation of Septin9 in CRC in Western countries (28,29). We further found a correlation between the gene methylation levels and the Dukes staging in CRC. The gene methylation in the early stage of CRC showed lower levels than that in the late stage. In addition, our study indicated a slightly superior sensitivity of BCAT1 than Septin9 and SDC2 (82.1% vs 78.6% and 73.2%) in the early stage of CRC (Dukes staging A and B). In advanced adenoma, all 3 methylated genes showed low sensitivity.

In clinics, FIT is replacing the guaiac fecal occult blood test in CRC screening because of FIT's higher diagnostic performance (22). This is validated by our current study, in which the sensitivity and specificity of FIT were 66.4% and 84.6% in CRC diagnosis, respectively, much higher than CEA and CA19-9. In the colorectal polyps' group, the sensitivity and specificity of Septin9 (83.7%, 94.6%), BCAT1 (83.7%, 93.9%), and SDC2 (76.9%, 90.8%) were determined in the diagnosis of CRC. In addition, the composite score (P) showed minor better diagnostic ability (AUC = 0.914), compared with Septin9 (AUC = 0.901), SDC2 (AUC = 0.855), and BCAT1 (AUC = 0.908). The sensitivity and specificity of the

composite score (P) in the diagnosis of CRC reached to 82.7% and 96.9%, respectively. The positive predictive value (95.6%) of the composite score (P) showed higher level than single methylated genes.

In this study, a ctDNA panel was designed to detect 3 genes by a single test using only 2.0 mL of plasma sample. This is much better than commercially available test kits for DNA methylation detection in the diagnosis of CRC. For example, at least 3.5 mL of plasma sample is required to perform DNA methylation analysis of the Septin9 promoter region using an Epi proColon 2.0 test kit (Epigenomics, San Diego, CA) (20). We also determined the combination of ctDNA methylation and other screening approaches in the diagnosis of CRC. We found that the composite score (P) combined with CEA and FIT significantly improved the diagnostic efficiency of CRC (AUC = 0.962) and achieved a higher sensitivity of 84.6%, demonstrating its potential to be applied to the laboratory test in the diagnosis of CRC.

However, our study also has some limitations. Although the combined detection of Septin9, SDC2, and BCAT1 gene methylation in plasma achieved an efficient diagnostic ability in CRC diagnosis, more clinical trials would be needed to validate the clinical application of the model. More population-based investigations will be conducted to confirm the prediction model's performance in the diagnosis of CRC. In addition, the value of composite score (P) in prognosis prediction and follow-up examination of patients with CRC is unclear and needs to be validated by further studies.

In conclusion, our study not only demonstrated 3 novel ctDNA methylation gene markers in CRC but also proved an approach with improved sensitivity and specificity in CRC screening by the combination of multiple gene methylation detections and CEA/FIT tests.

CONFLICTS OF INTEREST

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Specific author contributors: Fei Xu, MD, Shanshan Yu, MD, and Junyi Han, MD, PhD, contributed equally to this work and should be considered as co-first authors. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. L.F.: had full access to all data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis. L.F. and X.Z.: were responsible for the study conception and design. F.X., S.Y., and J.H.: contributed to investigation, data analysis, made the tables and figures, and writing the original draft. F.X., S.Y., Q.T., M.Z., and X.Z.: made substantial contributions to the acquisition of the data.

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Study Highlights

WHAT IS KNOWN

- ✓ Early diagnosis of colorectal cancer (CRC) would greatly increase the viability of patients.
- ✓ Carcinoembryonic antigen (CEA), carbohydrate antigen 19-9, and fecal immunochemical test for hemoglobin (FIT) have been widely used for CRC screening, but the sensitivity and specificity are unsatisfactory.

WHAT IS NEW HERE

- ✓ In this study, a real-time polymerase chain reaction-based gene panel was used to detect the circulating tumor DNA methylation of Septin9, Syndecan-2, and branched-chain amino acid transaminase 1.
- ✓ Branched-chain amino acid transaminase 1 and Septin9 showed better performance for CRC diagnosis, by which CRC was able to distinguish from polyps with sensitivity of 83.7%, specificity of 93.9%, and area under the curve of 0.908.
- ✓ Composite score (P) was calculated according to the cycle threshold values of the 3 methylated genes using the logistic regression equation.
- ✓ Composite score (P) combined with CEA and FIT significantly improved the diagnostic efficiency of CRC (area under the curve = 0.962), demonstrating its potential to be applied in CRC diagnosis.

TRANSLATIONAL IMPACT

- ✓ Composite score (P) improved sensitivity and specificity in CRC screening by the combination of CEA/FIT tests.
- ✓ Follow-up of composite score (P)-positive patients would reveal the usefulness of composite score (P) as an early detection marker for recurrence.

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