

The stool *syndecan2* methylation test is more robust than blood tests for methylated *septin9*, CEA, CA19-9 and CA724: a diagnostic test for the early detection of colorectal neoplasms

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Background: Methylated *syndecan2* (mSDC2) in stool samples has been found to be associated with colorectal cancer (CRC) and precancerous lesions. However, the available data are limited, and no previous studies have compared the analysis of mSDC2 with other diagnostic tests. Thus, we aimed to evaluate the clinical performance of a stool mSDC2 test and compare its performance with that of blood-based tests for methylated *septin9* (mSEPT9), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and carbohydrate antigen 724 (CA724) in detecting colorectal neoplasms.

Methods: The gold standard diagnostic technique that was used was colonoscopy combined with a pathological analysis of biopsied tissue. Stool DNA was extracted from 1,002 stool samples (445 from CRCs, 115 from adenomas, and 442 from controls) and then bisulfite-converted, followed by real-time quantitative methylation-specific polymerase chain reaction. Blood mSEPT9 levels were quantified by the Epi proColon 2.0 assay, and serum CEA, CA19-9 and CA724 levels were measured by electrochemiluminescence. The main indexes used during the evaluation were sensitivity, specificity and the area under the receiver operating characteristic curve (AUC).

Results: Stool mSDC2 detected 69.7% of CRCs, which was significantly higher than 53.8% by plasma mSEPT9, 37.2% by CEA, 13.1% by CA19-9 and 17.5% by CA724; for adenoma, the detection rates were 31.3%, 11.1%, 2.3% and 11.9%, respectively. The AUC of mSDC2 in detecting CRC was 0.83, compared to 0.72, 0.75, 0.63 and 0.54 for mSEPT9, CEA, CA19-9 and CA724, respectively. mSDC2 identified patients with stage I–III CRC with a sensitivity of 71.6%, which was significantly higher than that of mSEPT9, CEA, CA19-9 and CA724 (54.2%, 35.5%, 11.9%, and 15.0%, respectively); for stage IV CRC, the sensitivities of mSDC2, mSEPT9, CEA, CA19-9 and CA724 were 75.9%, 82.6%, 79.3%, 36.0% and 56.5%, respectively. SDC2 and CEA had a significantly higher sensitivity for distal CRC than for proximal CRC.

Conclusions: The stool SDC2 methylation test had a better performance in detecting nonmetastatic CRC and adenoma than evaluations of mSEPT9, CEA, CA19-9 and CA724 in blood. Our findings could be used to modify approaches for CRC prevention and early detection.

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Introduction

Although colorectal cancer (CRC) is the second leading cause of death due to cancer (1), decreased mortality has been observed in a large number of countries, especially high-income countries, over the last decade (2). This reduction can be primarily attributed to early detection efforts and improved treatments (3,4). Most CRCs develop from adenomas, with a very slow progression from benign adenomas to invasive carcinoma (5). As one of the most preventable cancers (6), CRC can be successfully treated if detected early; and screening substantially reduces the risk of CRC (5,7).

Currently, colonoscopy is the gold standard for the early diagnosis of CRC. However, due to its invasiveness, need for bowel preparation and complications, compliance in the public remains very low, which impedes screening efforts. A recent CRC screening program based on more than a million participants in China reported a colonoscopy participation rate of only 14.0% among high-risk individuals (8). Sigmoidoscopy or computed tomography (CT) colonography, other high-sensitivity screening methods, are semi-invasive tests that also have limitations (9). The fecal occult blood test (FOBT) and fecal immunochemical test (FIT) are the two most widely used noninvasive stool tests for CRC due to their convenience and relatively low costs (10). However, both the FOBT and FIT have relatively limited sensitivities (11-13). Thus, a noninvasive, highly accurate screening method to detect CRC at an early stage is urgently needed, especially for those who are reluctant to undergo colonoscopy examinations.

Aberrant DNA methylation is the most prevalent epigenetic alteration that occurs in all stages of carcinogenesis, and it can be successfully detected in several types of biological samples (blood, tissue, stool) (14,15). Due to their biological rationality and user-friendly nature, DNA methylation-based biomarkers are valuable tools in the early detection of CRC (16). To date, few studies have assessed the performance of stool methylated *syndecan2* (mSDC2) analysis in detecting colorectal neoplasms (17-22),

with a sensitivity ranging from 77.4% to 90.2% and a specificity ranging from 88.2% to 98.0%. However, results regarding the sensitivities of analyses performed according to tumor stages were inconsistent, with some showing a higher sensitivity for the detection of early stages I/II (20,21), while others showed contrary results (18,22); the performance of this analysis for the assessment of various tumor locations also remains unclear. In addition, it has been suggested that levels of SDC2 methylation are increased in other types of cancer tissues, such as in glioma and gastric cancer (23,24). However, few studies have explored the potential factors associated with the false-positive rate of stool SDC2 methylation tests.

Methylated *septin9* (mSEPT9) is thought to be released from apoptotic cells shed from solid tumors into the plasma in CRC patients (25,26). Increased concentrations of circulating mSEPT9 in the blood have been reported to be positively correlated with tumor burden (27), yielding a sensitivity of 52% to 73% and a specificity of 84% to 91% for CRC detection (27-30). Whereas, the evidence on its performance in detecting precancerous lesions remains limited and unclear, given the histological definition of precancers that do not invade the basement membrane or underlying blood vessels.

Serum tumor biomarkers, including carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and carbohydrate antigen 724 (CA724), have been widely used for the screening, diagnosis and surveillance of gastrointestinal (GI) cancer but have insufficient sensitivity and specificity (31-33). Thus, developing a more reliable method is urgently needed. To date, no previous studies have compared the performance of the stool SDC2 test with that of analyses of these serum biomarkers.

Thus, we aimed to evaluate and compare the clinical performance of a stool SDC2 methylation test and blood tests for mSEPT9, CEA, CA19-9 and CA724 in the identification of patients with CRC or adenoma. We present the following article in accordance with the STARD reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-1710/rc>).

Methods

Study population

Stool samples were obtained from patients in Tianjin Union Medical Center between February 2021 and March 2022. The target population was enrolled, including patients with a definitive or suspected diagnosis of CRC, those carrying benign gastroenterological lesions such as hemorrhoids and polyps, those with other types of cancers, and those with no evidence of disease (NED). All stool samples were collected prior to colonoscopy. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Tianjin Union Medicine Center (IRB number: 2021-B37). Written informed consent was obtained from all participants for obtaining blood or stool samples.

Stool sample collection

Every participant was required to provide a 1.5 to 10 g (average 4.5 g) stool sample in a semiquantitative stool collection device prefilled with 15 mL of preservative buffer (Creative Biosciences, Guangzhou, China). All buffered stools were immediately transported to our laboratory and homogenized and centrifuged subsequently. The supernatant was aliquoted and frozen at -80°C for subsequent processing.

Stool SDC2 methylation test

A methylation-specific detection kit (Creative Biosciences) was used to qualitatively assess the methylation of the human SDC2 gene promoter region in the stool samples. The tests were performed strictly in accordance with the manufacturer's protocol, which has been previously described in detail (17). Briefly, the test consisted of two steps: DNA extraction and transformation and real-time quantitative methylation-specific polymerase chain reaction (PCR) (qMSP). The SDC2 gene and β -actin (ACTB) gene were simultaneously captured by a magnetic bead. Stool DNA was purified and enriched using sequence-specific capture technology as reported previously with minor modifications (34). The unmethylated DNA was then transformed with sulfites, whereas the methylated SDC2 genes were not. qMSP was performed to quantitatively detect SDC2 and ACTB methylation status in stool samples. PCR amplification was performed under the

following cycling conditions: 95°C for 5 min, 48 cycles at 95°C for 20 s, 58°C for 60 s, and 72°C for 30 s, and a final cooling step at 37°C for 30 s. All valid samples satisfied the required cycle threshold (Ct) value of ACTB levels less than or equal to 36. Positivity for SDC2 methylation was defined as a Ct value less than or equal to 38, while negativity for SDC2 methylation was defined as a Ct value greater than 38 or undetected Ct values.

Blood tests for mSEPT9, CEA, CA19-9 and CA724 levels

All these tests were performed in the Clinical Laboratory of Tianjin Union Medical Center. The blood-based *Septin9* gene methylation assay was performed with an improved SEPT9 kit (Epigenomics AG for Epi proColon 2.0) following the provided instructions (35). The levels of serum tumor biomarkers CEA, CA19-9 and CA724 were measured by electrochemiluminescence. Positive values were defined by widely accepted cutoffs (CEA: 5 ng/mL, CA19-9: 37 U/mL, CA242: 20 U/mL). Positivity for SETP9 methylation was defined as a Ct value less than or equal to 41, while negativity for mSEPT9 methylation was defined as a Ct value greater than 41 or undetected Ct values.

Colonoscopy examinations

The gold diagnosis was colonoscopy combined with pathology report of tissue biopsy. All endoscopic examinations were performed in our medical center by experienced endoscopists who had at least 5 years of experience and were all board certified to perform endoscopy. All abnormal findings were confirmed by expert gastrointestinal (GI) pathologists following up-to-date clinical guidelines. Data from only high-quality colonoscopies were included, with adequate bowel preparation, photo documentation of cecal landmarks, and a withdrawal time greater than 6 min. Tumor-node-metastasis (TNM) stage was defined according to the guidelines of the 7th edition of the Cancer Staging Manual from the American Joint Committee on Cancer. To evaluate the performance of mSDC2 analysis in detecting colorectal neoplasms, colonoscopy findings were categorized into 3 groups: CRC, adenoma and normal colonoscopy, with the latter referring to a colonoscopy during which no adenoma or CRC was found. When a participant was diagnosed with two or more types of colorectal lesions, only the most advanced lesion was used for classification.

Statistical analysis

If mSEPT9 and mSDC2 levels were not detected, the Ct value of mSEPT9 levels was set to 42.0 (the maximal number of PCR cycles for mSEPT9), and the Ct value of mSDC2 levels was set to 48.0 (the maximal number of PCR cycles for mSDC2).

The chi-square test was employed to compare the qualitative methylation levels and clinicopathological features among patients. Sensitivity (equal to positive detection rate of CRC or adenoma) is defined as true positives/(true positives + false negatives), which implies the probability to identify diseased persons correctly using colonoscopy combined with pathology report as gold standard. Likewise, the specificity is defined as true negatives/(true negatives + false positives), which implies the probability to identify non-diseased person correctly. McNemar's chi-squared test with continuity correction was performed to compare sensitivity and specificity between the two methods. The Wilcoxon test was performed to compare levels between two testing subjects. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were used to assess predictive performance, and DeLong's test was performed to compare two correlated ROC curves. A multivariable logistic regression model was performed to further explore clinicopathological features associated with stool SDC2 methylation, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. All analyses were performed using R software (V4.1.2). Results with two-sided P values less than 0.05 were considered statistically significant.

Results

Patients and lesion characteristics

A total of 1,002 eligible patients with valid stool SDC2 methylation tests prior to colonoscopy were included in the present study, among whom 457 (45.6%) were tested for plasma mSEPT9, 601 (60.0%) were tested for serum CEA, 516 (51.5%) were tested for serum CA19-9, and 507 (50.6%) were tested for serum CA724. The demographic and lesion characteristics are shown in *Table 1*. There were a total of 445 (44.4%) CRC patients, including 24 patients with stage 0, 51 with stage I, 151 with stage II, 164 with stage III, 29 with stage IV and 26 patients with an unknown stage. In addition, 115 (11.5%) patients were diagnosed with adenoma, 357 (35.6%) patients with nonneoplastic GI diseases (including 34 polyps, 224 hemorrhoids, 55 perianal

fistulas, 14 abscesses and 30 other diagnoses), 16 (1.6%) patients with non-CRC cancers (including 6 GI cancers and 10 non-GI cancers), 7 (0.7%) patients with other non-GI disorders, and 62 (6.2%) patients with NED.

Notably, as the detection of CRC was the primary goal, patients who had adenoma, nonneoplastic GI diseases, non-CRC cancers, and NEDs were all grouped together and treated as control cases in the analyses. For the detection of adenoma, patients with nonneoplastic GI diseases, non-CRC cancers, and NED were combined and classified as "controls".

Comparison of clinical performance in detecting neoplasms

The AUC for stool mSDC2 test in detecting CRC among all subjects was 0.83 (95% CI: 0.80–0.85), whereas for testing mSEPT9, CEA, CA19-9 and CA724 in blood were 0.72 (95% CI: 0.67–0.77), 0.75 (95% CI: 0.71–0.79), 0.63 (95% CI: 0.58–0.68) and 0.54 (95% CI: 0.48–0.59), respectively (*Figure 1A*). DeLong's test showed that the differences between stool mSDC2 and all other biomarkers exhibited P values that were less than 0.05. In the detection of adenoma, the AUC for mSDC2 was 0.65 (95% CI: 0.59–0.70), which was greater than that for mSEPT9 (AUC: 0.55; 95% CI: 0.46–0.65; $P=0.014$) and CA724 (AUC: 0.48; 95% CI: 0.38–0.58; $P=0.01$) but was not different from that for CEA (AUC: 0.55; 95% CI: 0.45–0.65; $P=0.072$) and CA19-9 (AUC: 0.52; 95% CI: 0.42–0.62; $P=0.08$) (*Figure 1B*).

The levels of mSDC2 in stool and mSEPT9 in plasma and serum levels of CEA, CA19-9, and CA724 in the CRC, adenoma and control groups are shown in *Figure 2*. The levels of all five markers were significantly higher in CRC patients than in the control group, but only stool mSDC2 could be used significantly to distinguish adenoma patients from controls.

The positive detection rates in the CRC, adenoma and control groups are shown in *Figure 3A*. The stool mSDC2 test detected 69.7% of CRCs, which was a significantly higher rate than that detected by mSEPT9, CEA, CA19-9 and CA724 (53.8%, 37.2%, 13.1% and 17.5%, respectively, all $P<0.001$). In the detection of adenoma, the detection rate of the stool mSDC2 test was 31.3%, which was significantly higher than that of CEA (11.1%, $P=0.022$), CA19-9 (2.3%, $P=0.002$) and CA724 (11.9%, $P=0.027$) and higher than the 23.3% of plasma mSEPT9, although the difference was not significant.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of

Table 1 Demographic and lesion characteristics

| Characteristic | Patients tested with stool mSDC2 (n=1,002) | Patients tested with plasma mSEPT9 (n=457) | Patients tested with CEA (n=601) | Patients tested with CA19-9 (n=516) | Patients tested with CA724 (n=507) |
|-----------------------------------|--|--|----------------------------------|-------------------------------------|------------------------------------|
| Age, n (%) | | | | | |
| <60 years | 455 (45.4) | 149 (32.6) | 195 (32.4) | 164 (31.8) | 164 (32.3) |
| ≥60 years | 547 (54.6) | 308 (67.4) | 406 (67.6) | 352 (68.2) | 343 (67.7) |
| Sex, n (%) | | | | | |
| Male | 630 (62.9) | 298 (65.2) | 391 (65.1) | 332 (64.3) | 323 (63.7) |
| Female | 372 (37.1) | 159 (34.8) | 210 (34.9) | 184 (35.7) | 184 (36.3) |
| Lesion, n (%) | | | | | |
| CRC | 445 (44.4) | 340 (74.4) | 414 (68.9) | 352 (68.2) | 338 (66.7) |
| TNM stage | | | | | |
| 0 | 24 (5.4) | 20 (5.9) | 23 (5.6) | 20 (5.7) | 20 (5.9) |
| I | 51 (11.5) | 35 (10.3) | 44 (10.6) | 37 (10.5) | 37 (10.9) |
| II | 151 (33.9) | 115 (33.8) | 139 (33.6) | 117 (33.2) | 112 (33.1) |
| III | 164 (36.9) | 125 (36.8) | 154 (37.2) | 132 (37.5) | 125 (37.0) |
| IV | 29 (6.5) | 23 (6.8) | 29 (7.0) | 25 (7.1) | 23 (6.8) |
| Unknown | 26 (5.8) | 22 (6.5) | 25 (6.0) | 21 (6.0) | 21 (6.2) |
| Location [†] | | | | | |
| Right colon | 55 (12.4) | 46 (13.5) | 50 (12.1) | 43 (12.2) | 39 (11.5) |
| Left colon | 151 (33.9) | 111 (32.6) | 140 (33.8) | 112 (31.8) | 111 (32.8) |
| Rectum | 231 (51.9) | 177 (52.1) | 217 (52.4) | 190 (54.0) | 181 (53.6) |
| Unspecific | 8 (1.8) | 6 (1.8) | 7 (1.7) | 7 (2.0) | 7 (2.1) |
| Differentiation | | | | | |
| Well | 72 (16.2) | 55 (16.2) | 68 (16.4) | 64 (18.2) | 58 (17.2) |
| Moderate | 243 (54.6) | 187 (55.0) | 222 (53.6) | 184 (52.3) | 178 (52.7) |
| Poor | 39 (8.8) | 29 (8.5) | 36 (8.7) | 30 (8.5) | 29 (8.6) |
| Unknown | 91 (20.4) | 69 (20.3) | 88 (21.3) | 74 (21.0) | 73 (21.6) |
| Adenoma, n (%) | 115 (11.5) | 30 (6.6) | 45 (7.5) | 43 (8.3) | 42 (8.3) |
| Non-neoplastic GI diseases, n (%) | 357 (35.6) | 31 (6.8) | 60 (10.0) | 48 (9.3) | 50 (9.9) |
| Non-CRC cancers, n (%) | 16 (1.6) | 12 (2.6) | 16 (2.7) | 11 (2.1) | 13 (2.6) |
| Other non-GI disorders, n (%) | 7 (0.7) | 4 (0.9) | 6 (1.0) | 4 (0.8) | 4 (0.8) |
| NED, n (%) | 62 (6.2) | 40 (8.8) | 60 (10.0) | 58 (11.2) | 60 (11.8) |

[†], right colon was defined as proximal to splenic flexure, while left colon was defined as from splenic flexure to the rectosigmoid junction. mSDC2, methylated *syndecan2*; mSEPT9, methylated *septin9*; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724; CRC, colorectal cancer; TNM, tumor-node-metastasis; GI, gastrointestinal; NED, no evidence of disease.

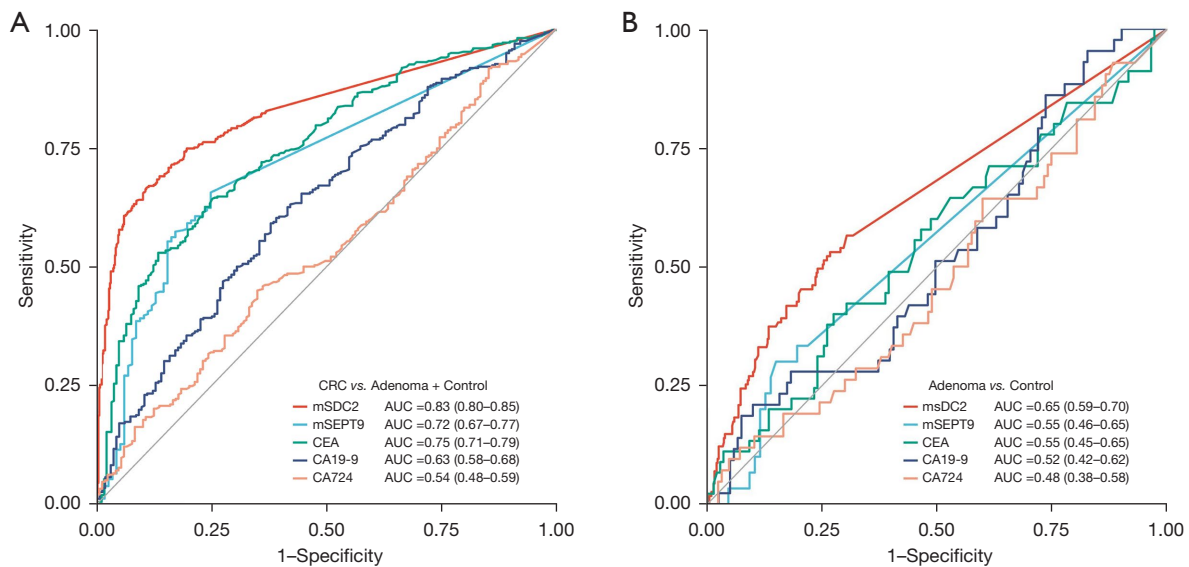


Figure 1 ROC curves of stool mSDC2, plasma mSEPT9, serum CEA, CA19-9 and CA724. (A) ROC curves for the detection of CRC. (B) ROC curves for the detection of adenoma. CRC, colorectal cancer; mSDC2, methylated *syndecan2*; AUC, area under the curve; mSEPT9, methylated *septin9*; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724; ROC, receiver operating characteristic.

these five biomarkers in detecting CRC and adenomas are shown in Table 2. The NPVs of mSDC2, mSEPT9, CEA, CA19-9 and CA724 for the detection of CRC were 78%, 39%, 40%, 34% and 35%, respectively, with accuracies of 78%, 62%, 55%, 39% and 42%, respectively.

Rates of the detection of various CRC stages

Among the 419 patients staged based on the surgically resected specimens, the respective detection rates by stool mSDC2 for patients with stage 0, I, II, III, and IV CRC were 50.0%, 66.7%, 70.9%, 73.8% and 75.9%, respectively, and did not differ significantly among stages ($P=0.166$). Regarding the stage-specific sensitivities, stool mSDC2 performed better than other biomarkers except for stage IV, followed by plasma mSEPT9 (Figure 3B). When patients with stages I–III cancer were combined, stool mSDC2 detected 71.6% of CRCs, which was significantly higher than 54.2% by mSEPT9, 35.3% by CEA, 11.9% by CA19-9 and 15.0% by CA724 (all $P<0.001$). However, detection rates for stage IV CRC did not differ significantly between mSDC2 and mSEPT9, CEA or CA724 (82.6%, 75.9%, 79.3% and 56.5%, respectively, all $P>0.05$), with mSDC2 exhibiting an advantage only over CA19-9 (36.0%, $P=0.016$). Interestingly, mSEPT9 and CEA showed even

higher detection rates than mSDC2 for stage IV CRC.

All tests except for stool mSDC2 had significantly higher sensitivities for detecting stage IV CRC than for detecting stage I–III CRC (Figure 3C). However, stool mSDC2 could distinguish patients with stage I–III CRC from those with stage 0 CRC well, while other biomarkers could not. The levels of mSDC2 in stool, mSEPT9 in plasma, and CEA, CA19-9, and CA724 in serum stratified according to CRC stage are shown in Figure S1. Similar to the sensitivity results, the methylation level of SDC2 increased with stage but was not higher in stage IV CRC, while the levels of biomarkers assessed in other tests were significantly higher in patients with stage IV cancer than in patients at any other stage.

Detection rates in various locations

A multivariable logistic regression model was created to assess the association between stool SDC2 methylation and clinicopathological features (Table 3). After adjusting for age (<60 and ≥ 60 years), sex, tumor stage, location, and differentiation, we found that only location was significantly associated with a positive result from the stool mSDC2 test. With the proximal colon as the reference, the ORs were 4.54 for detecting cancer in the distal colon ($P<0.001$) and 6.26

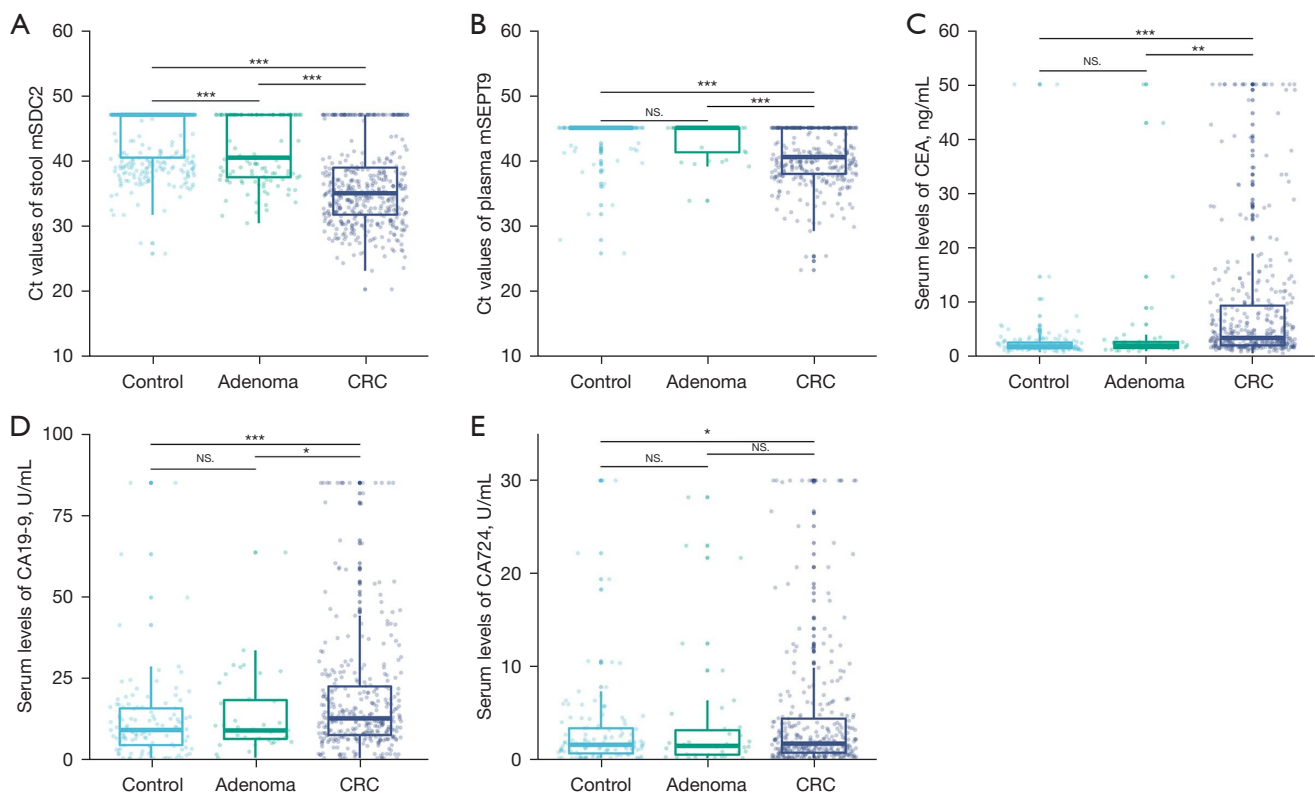


Figure 2 Levels of five biomarkers in the control, adenoma and CRC groups. (A) Levels of mSDC2 in stool. (B) Levels of mSEPT9 in plasma. (C) Serum CEA levels. (D) Serum levels of CA19-9. (E) Serum CA724 levels. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$. NS., non significant; CRC, colorectal cancer; mSDC2, methylated *syndecan2*; mSEPT9, methylated *septin9*; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724.

for detecting cancer in the rectum ($P<0.001$). Sex, age, stage and differentiation had no effect on positive or negative test results (all $P>0.05$).

The positive detection rates according to tumor location are shown in Figure 3D. The sensitivities did not vary significantly between CRCs in the rectum and distal colon (77.1% vs. 68.9%, $P>0.05$), but both had significantly higher sensitivities than the proximal colon (77.1% vs. 47.2%, $P<0.001$; 68.9% vs. 47.2%, $P=0.007$). CEA showed a similar trend to SDC2, and the sensitivities of the other biomarkers were not significantly influenced by location.

False-positive rates

The positive detection rates of mSDC2, mSEPT9, CEA, CA19-9 and CA724 in the control group were 11.1%, 12.6%, 4.9%, 5.3% and 9.4%, respectively, with no significant variation. The positive rates of mSDC2 and mSEPT9 were high, which may have been partly due to

the presence of interfering diseases. Details regarding the types of interfering diseases and the outcomes of the tests are shown in Table S1. Stool mSDC2 detected 6/16 (37.5%) non-CRC cancers (including one small intestine cancer, one prostate cancer, three lymphomas and one lung cancer) and 42/357 (11.8%) nonneoplastic GI diseases. Plasma mSEPT9 detected 4/12 (33.3%) non-CRC cancers (including three prostate cancers and one lung cancer) and 4/31 (12.9%) nonneoplastic GI diseases.

Discussion

In a clinical setting, earlier detection of CRC is still the most effective method to reduce morbidity and mortality (1,36). A high-risk factor questionnaire plus the FOBT is currently the preliminary CRC screening strategy in China, but accumulated data showed a low PPV for selecting high-risk individuals (37,38). Moreover, even when identified as high-risk individuals, only 14.0% underwent a colonoscopy

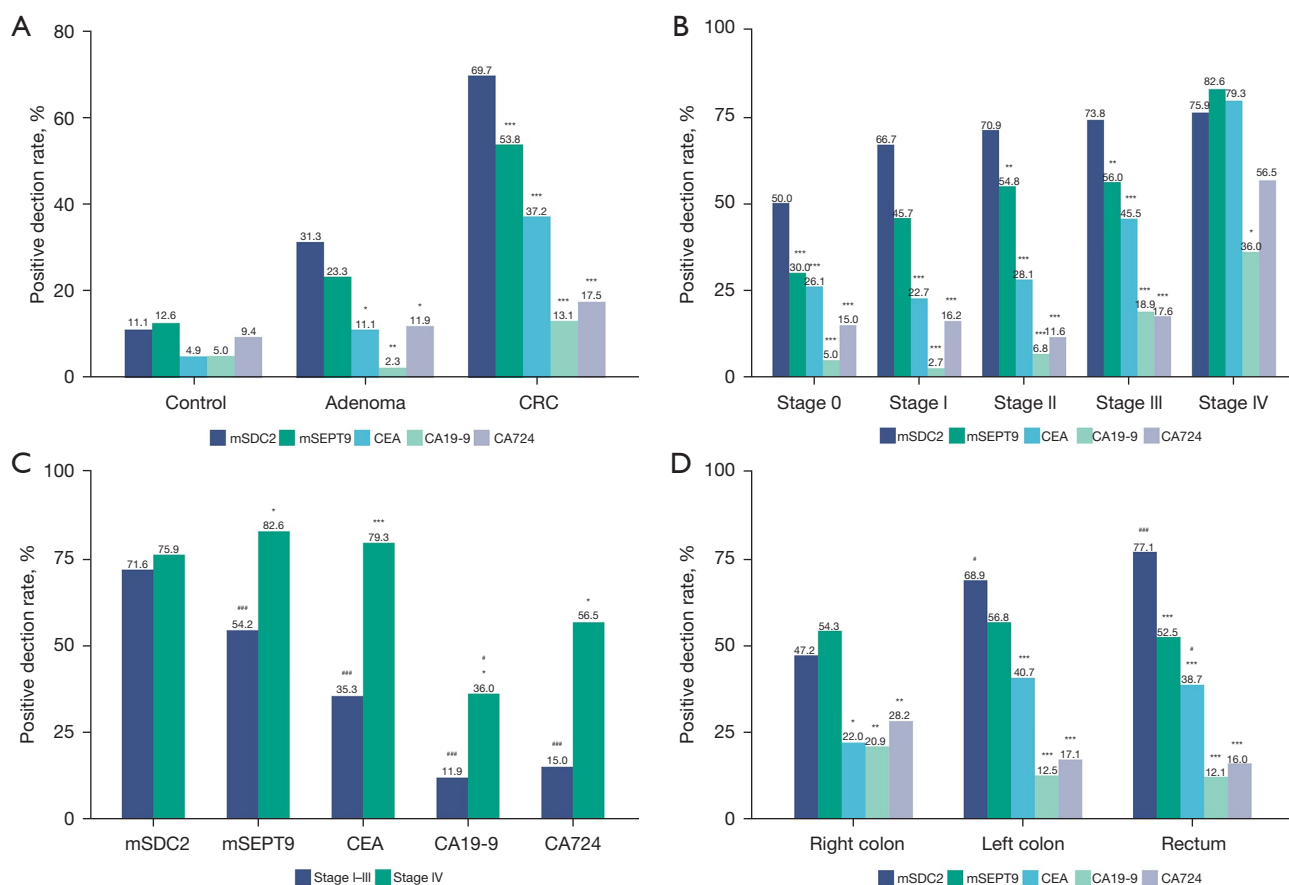


Figure 3 Positive detection rates by stool mSDC2, plasma mSEPT9, serum CEA, CA19-9 and CA724 testing. (A) Positive detection rates in the control, adenoma and CRC groups. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ vs. mSDC2. (B) Positive detection rates in stages 0, I, II, III and IV. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ vs. mSDC2. (C) Positive detection rates in combined stage I–III vs. stage IV. ***, $P < 0.001$; *, $P < 0.05$ vs. stage I–III; ###, $P < 0.001$; #, $P < 0.05$ vs. mSDC2. (D) Positive detection rates in the right colon, left colon and rectum. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ vs. mSDC2; ###, $P < 0.001$; #, $P < 0.05$ vs. right colon. CRC, colorectal cancer; mSDC2, methylated syndecan2; mSEPT9, methylated septin9; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724.

as recommended (8). Thus, a noninvasive screening method with high sensitivity in detecting early-stage CRC and precancerous lesions is urgently needed. In this comparison of testing methods using inpatient cases, we found that both nonmetastatic CRCs and colorectal adenomas were detected at significantly higher rates by the stool SDC2 methylation test than by blood tests for mSEPT9, CEA, CA19-9 or CA724.

Although both methylated SDC2 and SEPT9 are broadly expressed in adenoma tissue (17,27,28), the detection rate by plasma mSEPT9 remained low. In the present study, the detection rate for adenoma by plasma mSEPT9 was only 23.3%, which was comparable to the 14–18% reported by previous studies (28,30,39,40). Moreover, plasma mSEPT9

detected significantly fewer early-stage CRC patients than stool mSDC2; however, for stage IV CRC, mSEPT9 had a higher detection rate than stool mSDC2, although the difference was not significant.

This result can be explained by the key differences in how the markers are released into the blood and stool (40). While methylated SDC2 is released into the stool through luminal exfoliation, circulating methylated SEPT9 is released into plasma from apoptotic cells that are shed from the tumor (25,26), with the latter appearing to be associated with vascular invasion. Thus, based on the histological definition, which is that there is no invasion of the basement membrane or underlying blood vessels in adenomas, there is no route for mSEPT9 entry into the blood. Moreover, exfoliation

Table 2 Performance characteristics of stool mSDC2, plasma mSEPT9, CEA, CA19-9 and CA724 test for the detection of colorectal neoplasia

| Test | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | Accuracy (95% CI) |
|----------------------|----------------------|----------------------|--------------|--------------|-------------------|
| Detection of CRC | | | | | |
| mSDC2 | 70% (65–74%) | 85% (81–88%) | 78% (74–82%) | 78% (74–81%) | 78% (75–80%) |
| mSEPT9 | 54% (48–59%) | 85% (77–91%) | 91% (86–95%) | 39% (33–45%) | 62% (57–66%) |
| CEA | 37% (33–42%) | 94% (89–97%) | 93% (88–96%) | 40% (36–45%) | 55% (51–59%) |
| CA19-9 | 13% (10–17%) | 96% (91–98%) | 87% (75–95%) | 34% (30–38%) | 39% (35–44%) |
| CA724 | 17% (14–22%) | 90% (84–94%) | 78% (67–86%) | 35% (31–40%) | 42% (37–46%) |
| Detection of adenoma | | | | | |
| mSDC2 | 31% (23–41%) | 89% (86–92%) | 42% (32–54%) | 83% (80–87%) | 77% (73–80%) |
| mSEPT9 | 23% (10–42%) | 87% (79–94%) | 39% (17–64%) | 77% (67–85%) | 71% (62–79%) |
| CEA | 11% (4–24%) | 95% (90–98%) | 42% (15–72%) | 77% (70–83%) | 75% (68–81%) |
| CA19-9 | 2% (0–12%) | 95% (90–98%) | 14% (0–58%) | 73% (66–80%) | 71% (63–78%) |
| CA724 | 12% (4–26%) | 91% (84–95%) | 29% (10–56%) | 76% (68–82%) | 71% (64–78%) |

mSDC2, methylated *syndecan2*; mSEPT9, methylated *septin9*; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; CRC, colorectal cancer.

Table 3 Association of stool SDC2 methylation with clinicopathological features of CRC

| Feature | Number (%) | Crude OR (95% CI) | Adjusted OR (95% CI) | P value |
|-----------------|------------|---------------------|----------------------|---------|
| Age | | | | |
| <60 years | 117 (26.3) | Reference | Reference | 0.345 |
| ≥60 years | 328 (73.7) | 0.92 (0.54–1.58) | 0.75 (0.42–1.36) | |
| Sex | | | | |
| Male | 295 (66.3) | Reference | Reference | 0.127 |
| Female | 150 (33.7) | 1.33 (0.80–2.20) | 1.54 (0.88–2.68) | |
| Location | | | | |
| Right colon | 55 (12.6) | Reference | Reference | <0.001 |
| Left colon | 151 (34.6) | 4.36 (2.12–8.95) | 4.54 (2.12–9.75) | |
| Rectum | 231 (52.9) | 5.72 (2.85–11.48) | 6.26 (2.99–13.1) | |
| Differentiation | | | | |
| Well | 72 (20.3) | Reference | Reference | 0.201 |
| Moderate | 243 (68.6) | 0.69 (0.36–1.30) | 0.64 (0.32–1.27) | |
| Poor | 39 (11.0) | 0.46 (0.19–1.10) | 0.54 (0.20–1.47) | |
| Stage | | | | |
| 0 | 24 (5.7) | Reference | Reference | 0.777 |
| I | 51 (12.2) | 4.57 (0.38–54.66) | 1.49 (0.09–23.76) | |
| II | 151 (36.0) | 5.25 (0.46–59.51) | 2.35 (0.16–34.04) | |
| III | 164 (39.1) | 5.49 (0.48–62.22) | 2.07 (0.14–30.18) | |
| IV | 29 (6.9) | 14.00 (0.58–338.78) | 4.05 (0.14–117.86) | |

CRC, colorectal cancer; OR, odds ratio; CI, confidence interval.

into stool generally occurs earlier than vascular invasion into blood during the progression of CRC, limiting plasma DNA testing for early-stage CRC (40). This explains why plasma mSEPT9 had higher sensitivity for detecting patients with distant metastasis than for those with early-stage CRCs (39,41). Therefore, plasma mSEPT9 is more suitable for monitoring recurrence than for early screening.

While the levels of mSEPT9, CEA, CA19-9 and CA724 in blood tended to be highest in patients with stage IV disease, there appeared to be an opposite trend for mSDC2 levels in stool. A similar trend was also reported by two previous studies targeting other methylated genes in stool (*BMP3*, *NDRG4*, *vimentin*, and *TFPI2*), in which the detection rates for stage IV CRC were significantly lower than those for stage I–III CRC (40,42). This finding might be explained by the fact that DNA hypomethylation occurs with late progression (43), surface necrosis occurs with reduced colonocyte exfoliation, or additional factors. Even so, prior studies focusing on mSDC2 as well as our study suggest that this marker has a superior sensitivity for more advanced stages, although the differences were not significant (18,20,22).

Although the FOBT is widely used in CRC screening, its sensitivity for the early screening of CRC is limited. With intermittent occult bleeding, repeated FOBTs are needed to guarantee its efficacy (44,45). In addition, unlike the FOBT, the stool mSDC2 test requires no diet or medication restrictions that might result in false-positive or false-negative outcomes (46), except for berberine, a Chinese herbal medicine (19).

It has been suggested that the SDC2 methylation level is elevated in some types of cancer tissues, such as in glioma and gastric cancer (23,24). However, it remains unclear whether interfering diseases influence the accuracy of stool SDC2 methylation tests in detecting CRC. A multicenter clinical study by Wang *et al.* reported that stool mSDC2 analysis could not be used to detect any of the cancers in the digestive tract (0/30) (20). Cooper *et al.* reassessed false-positive stool DNA tests in 30 patients and did not find non-colorectal lesions by repeat colonoscopy, upper endoscopy or a review of the medical records 11–29 months after the initial test (47). However, in another clinical trial by Han *et al.*, 30.4% of gastric cancer patients (7/23) and 30% of liver cancer patients (3/10) tested positive for SDC2 methylation in the stool (18). In our study population, tests for SDC2 methylation were positive in 37.5% (6/16) of patients with other types of cancers; for those with other nonneoplastic GI diseases, the detection rate was 11.8%

(42/357). However, Wang *et al.* reported only 1 positive mSDC2 test result among 21 patients (4.7%) with digestive tract ailments (20). This difference might have resulted from the distribution of diseases, with 90% of the patients in their study exhibiting nonneoplastic GI diseases located in the upper digestive tracts, but over 80% of patients had anal and rectal diseases in our study. Hence, further research is needed to define the effects of interfering diseases on SDC2 methylation in the stool.

Evidence suggests that plasma mSEPT9 is a potential biomarker for several types of cancer (48–50). In our study, 33.3% of non-CRC cancer patients and 12.9% of patients with nonneoplastic GI disease exhibited positivity for plasma mSEPT9 expression, which was consistent with other reports, with a rate of 30.8% (4/13) observed among patients with non-CRC cancers and a rate of 18.2% (2/11) observed among patients with non-CRC GI diseases (39,40). Given the high cost of the test and the presence of multiple potential contributors to elevated plasma levels, mSEPT9 analysis might be unsuitable for CRC screening.

This study had some limitations. First, this was a single-center study in only Asian patients. Second, the stool test for SDC2 methylation was performed on a hospital-based cohort with many interfering diseases; thus, the results may not be representative of the findings observed in asymptomatic individuals undergoing general screening. However, the test would be more effective if such differences hold, as this method is applied in a general screening setting. Third, the size of the adenomas and pathological information regarding villous and serrated adenomas were not collected, which further limited our analysis of their influence on the performance of the test.

Conclusions

In conclusion, this is the first study to compare a stool SDC2 methylation test with blood tests for mSEPT9, CEA, CA19-9 and CA724. We demonstrated that the stool SDC2 methylation test had a better performance for detecting adenoma and nonmetastatic CRC than the other biomarkers, confirming the clinical potential of this test as a new useful noninvasive screening tool for early-stage CRC. Further large-scale studies are needed to corroborate and expand upon these findings.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-1710/rc>

Data Sharing Statement: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-1710/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-1710/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Tianjin Union Medicine Center (IRB number: 2021-B37). Written informed consent was obtained from all participants for obtaining blood or stool samples.

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Table S1 Interfering disease types and the effect on the outcome of tests

| Disease types and distribution | Number of positive/total tested number | | | | |
|--|--|---------------|-----------|--------------|-------------|
| | Stool mSDC2 | Plasma mSEPT9 | Serum CEA | Serum CA19-9 | Serum CA724 |
| Non-CRC cancers | 6/16 | 4/12 | 3/16 | 4/11 | 2/13 |
| GI cancers | | | | | |
| Gastric cancer | 0/4 | 0/3 | 0/4 | 1/4 | 0/4 |
| Small intestine cancer | 1/2 | 0/1 | 1/2 | 1/2 | 0/2 |
| Non-GI cancers | | | | | |
| Prostate cancer | 1/4 | 3/4 | 1/4 | 2/2 | 0/2 |
| Lymphoma | 3/3 | 0/3 | 0/3 | 0/3 | 0/2 |
| Lung cancer | 1/2 | 1/1 | 0/2 | 0/0 | 1/2 |
| Cancer of the female reproductive system | 0/1 | 0/0 | 1/1 | 0/0 | 1/1 |
| Non-neoplasm GI disorders | 42/357 | 4/31 | 1/60 | 1/48 | 6/50 |
| Polyps | 3/34 | 0/8 | 1/14 | 0/12 | 1/11 |
| Inflammatory bowel disease | 1/7 | 0/1 | 0/6 | 0/6 | 1/6 |
| Gastritis | 0/1 | 0/0 | 0/1 | 0/0 | 0/0 |
| Hemorrhoids | 26/224 | 1/11 | 0/19 | 0/14 | 2/15 |
| Perianal fistulas | 8/55 | 0/2 | 0/9 | 0/6 | 2/8 |
| Abscesses | 1/14 | 1/2 | 0/1 | 0/1 | 0/1 |
| Colonic angiodysplasia | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 |
| Neuroendocrine tumor | 0/5 | 1/3 | 0/3 | 1/2 | 0/2 |
| Rectal prolapse | 0/3 | 0/1 | 0/1 | 0/1 | 0/1 |
| Intestinal tuberculosis | 0/1 | 0/1 | 0/1 | 0/0 | 0/0 |
| Cholecystitis | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 |
| Hairy sinus tract | 0/1 | 0/0 | 0/0 | 0/0 | 0/0 |
| Anal fissure | 3/7 | 0/0 | 0/0 | 0/0 | 0/0 |
| Lipomyoma | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 |
| Melanoma | 0/2 | 1/2 | 0/2 | 0/2 | 0/2 |
| Other non-GI disorders | 0/7 | 1/4 | 2/6 | 0/4 | 1/4 |
| Rheumatoid arthritis | 0/1 | 0/0 | 0/1 | 0/1 | 0/1 |
| Schwannoma | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Hernia | 0/2 | 1/1 | 2/2 | 0/2 | 1/2 |
| Presacral cyst | 0/1 | 0/1 | 0/1 | 0/0 | 0/0 |
| Pelvic benign tumor | 0/1 | 0/1 | 0/1 | 0/0 | 0/0 |
| Leiomyoma | 0/1 | 0/0 | 0/0 | 0/0 | 0/0 |

mSDC2, methylated *syndecan2*; mSEPT9, methylated *septin9*; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724; CRC, colorectal cancer; GI, gastrointestinal.

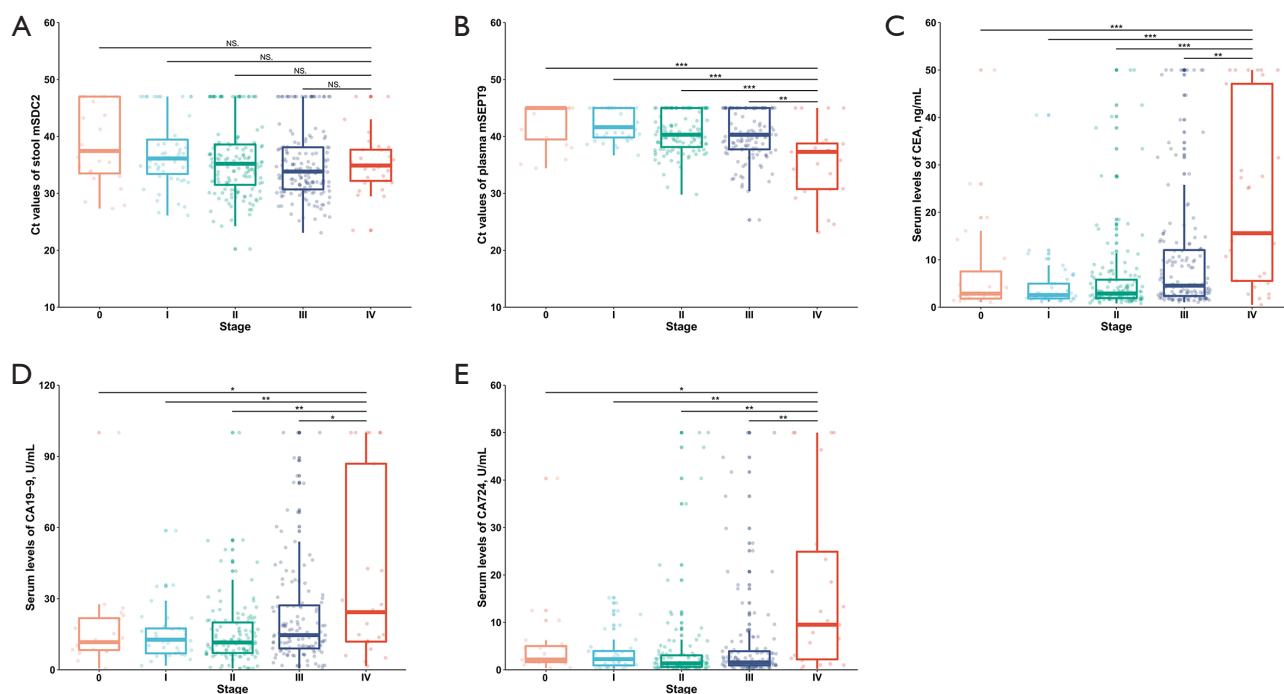


Figure S1 Levels of five biomarkers according to CRC stage. (A) Levels of methylated SDC2 in stool. (B) Levels of methylated SEPT9 in plasma. (C) Levels of CEA in serum. (D) Levels of CA19-9 in serum. (E) Levels of CA724 in serum. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. NS., nonsignificant; CRC, colorectal cancer; SDC2, *syndecan2*; SEPT9, *septin9*; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; CA724, carbohydrate antigen 724.