Research Article

Performance of a Novel Blood-Based Early Colorectal Cancer Screening Assay in Remaining Serum after the Blood Biochemical Test

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Background. Combination of multiple biomarkers was an effective strategy to improve sensitivity in cancer diagnosis and screening. However, the performance of the combination of methylated SEPT9 and SDC2 for detection of colorectal cancer (CRC) has yet to be reported. Methods. A new qPCR-based assay combining the detection of methylated SEPT9 and SDC2 was used. Methylation statuses of SEPT9 and SDC2 were examined in 19 sets of cancer tissues and paired adjacent tissues and further evaluated with 225 serum samples, including 111 CRC patients and 114 no evidence of disease individuals. Results. SEPT9 and SDC2 methylation levels were higher in 94.7% and 100.0% of cancer tissues than in their paired adjacent tissues. The sensitivities for detecting CRC by SEPT9 methylation alone and SDC2 methylation alone were 73.0% (95% CI: 63.6–80.8%) and 71.2% (95% CI: 61.8–79.2%), respectively, with the same specificity of 95.6% (95% CI: 89.6–98.4%). However, when SEPT9 methylation was combined with SDC2 methylation to detect CRC, the sensitivity was improved to 86.5% (95% CI: 78.4–92.0%) with a specificity of 92.1% (95% CI: 85.1–96.1%). Conclusion. The combination of methylated SEPT9 and SDC2 detection in serum has the potential to be a noninvasive strategy for CRC screening.

1. Introduction

Colorectal cancer (CRC) is among the three most common cancer types worldwide [1]. It is also one of the five most common cancer types in China [2]. And due to the changes towards a more westernized lifestyle of Chinese population, its incidence has seen steady increase in recent years. During the past decade, the 5-year relative survival rate of Chinese CRC patients has increased from 47.2% to 56.9%, which, however, was still more than 8% lower than that of the developed countries [3, 4].

The Chinese CRC screening guideline recommends screening adults of 40–74 years old with the guaiac fecal occult blood test (gFOBT) followed up with digital rectal exam and colonoscopy [1]. However, it has been very difficult for the program to reach the entire population, resulting in a very low screening uptake. Due to invasiveness and bothersome bowel preparation, colonoscopy has rarely been used despite being the gold standard for CRC screening [5]. Moreover, it is hardly a primary CRC screening method in developing countries with limited resources including China where gFOBT is the most widely used CRC screening
method albeit with low accuracy [6]. Hence, a noninvasive and more accurate screening method needs to be developed to promote early CRC screening.

DNA methylation, the addition of methyl groups to the cytosine residues of DNA, is a form of epigenetic modification that mainly occurs in CpG islands usually present in promoter regions [7]. Abnormal hypermethylation of certain CpG islands may lead to transcriptional silencing and inactivation of cancer suppressor genes [8]. In recent years, several studies have reported the application of DNA methylation biomarkers in CRC diagnosis and screening [5, 9]. SEPT9 methylation, the only blood-based biomarker approved by FDA for CRC screening, has been used clinically for nearly 10 years [10, 11]. It has been proven an accurate, reliable, fast, and convenient method for CRC early screening [12]. However, the sensitivity of SEPT9 methylation for CRC detection was relatively low, especially for early stage cancers [3, 4]. Syndecan-2 (SDC2) gene was also found to be hypermethylated in the feces or blood samples of most CRC patients [10, 13, 14].

Biochemical tests are the most common medical testing program in clinics; they measure sodium, potassium, chloride, bicarbonate, blood urea nitrogen, and so on. For the majority of biochemical tests, blood is usually obtained from the patient’s vein, and serum or plasma is obtained from the blood sample for the next step test. However, most of the serum or plasma is discarded after the biochemical test is completed. Therefore, use of the remaining serum or plasma after the blood biochemical test might be an effective approach to detect CRC.

Combination of multiple biomarkers and/or methods has become a trend in cancer diagnosis and screening to improve sensitivity [15]. For example, it was reported that the sensitivities for CRC detection were 72.2% and 68.0%, respectively, for SEPT9 methylation and the fecal immunochemical test (FIT) individually, and the specificities were 81.5% and 97.4%. When test results for SEPT9 methylation and FIT were combined, the CRC detection rate was 88.7% with a specificity of 78.8% [16]. However, the combination of SEPT9 and SDC2 methylation for CRC screening has never been reported. In this study, we evaluated the performance of a new blood-based early CRC screening assay (ColoDefense), which combined the detection of SEPT9 and SDC2 methylations in a single qPCR reaction, with the remaining serum samples after the blood biochemical test. Such an approach also has an added advantage of avoidance of repeated blood draw.

2. Materials and Methods

2.1. Sample Collection. Fresh-frozen cancer tissues (n = 19) and paired adjacent paracancer tissues (n = 19) from CRC patients were collected at the time of surgery at the Affiliated Hospital of Xuzhou Medical University. Serum specimens were collected from 111 CRC patients undergoing colonoscopy at the same hospital and histologically confirmed by a pathologist. Control serum specimens were collected from 114 subjects with no evidence of diseases (NED). The remaining serum specimens after the blood biochemical test were collected and frozen at -80°C until used. The study was approved by the Institutional Review Board of the Affiliated Hospital of Xuzhou Medical University (Ethics Committee reference number: XYFY2017-KL105-02), and the informed consent was obtained from all participating patients and healthy control subjects.

2.2. DNA Extraction, Bisulfite Treatment, and Quantitative Real-Time PCR. Genomic DNA was isolated from tissue specimens using a DNA mini kit (Qiagen). For serum samples, the volume was adjusted to 3.5 mL with PBS buffer if the serum volume was less than 3.5 mL, and each sample was extracted using a cfDNA extraction kit (Suzhou VersaBio Technologies Co. Ltd.). Subsequently, bisulfite conversion of purified DNA and purification of the converted product were performed with a bisulfite conversion kit (Suzhou VersaBio Technologies Co. Ltd.). All the kits were used according to the manufacturers’ instructions.

Purified DNA obtained from the above steps was tested by real-time methylation-specific qPCR using the ColoDefense test (Suzhou VersaBio Technologies Co. Ltd.), a new blood-based methylation assay for colorectal cancer screening. For the ColoDefense assay, methylated SEPT9, methylated SDC2, and an internal control (ACTB) were performed in the same reaction. Three separate PCR replicates were prepared with purified bisulfite-converted DNA from each serum sample, and single PCR reaction was performed with purified bisulfite-converted DNA from each tissue sample. Real-time PCR was performed on the LC480-II thermal cycler (Roche Diagnostics) using the following cycling conditions: activation at 95°C for 30 minutes, 50 cycles at 95°C for 10 seconds and 56°C for 30 seconds, and final cooling at 40°C for 30 seconds.

2.3. Data Analysis. ΔCp was used to determine the tissue methylation status of SDC2 and SEPT9; ΔCp was defined as the difference between the Cp values for the target (methylated SDC2 or methylated SEPT9) and the internal control gene (ACTB). The results for serum specimens were “invalid” if the ACTB Cp was greater than 35.0, and methylated SEPT9 and methylated SDC2 were “detected” if their Cp values were less than 50.0. Methylated SEPT9 was analyzed by using a 1/3 rule in which a serum sample was scored positive if one of the three PCR replicates had a valid amplification curve (1/3 algorithm). And methylated SDC2 was analyzed by using a 2/3 rule, whereby to be called positive, two of three PCR replicates of a serum sample must have valid amplification curves (2/3 algorithm). The serum sample would be considered as positive if either methylated SEPT9 or methylated SDC2 was positive. Data were subjected to statistical analysis by using IBM SPSS for Windows version 22.0, and the t test was used for comparison between two samples at the significant level of p < 0.01. Data from sensitivity and specificity were used to plot the receiver operating characteristic (ROC) curve. Because most Cp values from normal individuals were not detected in the qPCR reaction, we had to set the Cp values to 50.0 (the maximal number of PCR cycles) for those not detected samples to plot the curve [11].
Disease Markers

3. Results

The ColoDefense assay was used to quantify methylation levels of SEPT9 and SDC2 genes in 19 colorectal cancer tissues and paired adjacent paracancer tissues. The SEPT9 and SDC2 methylation levels were higher in 94.7% (18/19) and 100.0% (19/19) of cancer tissues than in their paired adjacent paracancer tissues ($p < 0.001$, Figure 1), thus making the ColoDefense assay a candidate method for CRC screening.

To examine the feasibility of applying the ColoDefense assay in CRC screening, 225 remaining serum samples after the blood biochemical test were collected from the Affiliated Hospital of Xuzhou Medical University, of which 111 were from CRC patients. The ages of all CRC patients ranged from 25 to 89 with a mean age of 61 and a median age of 62. The ages of NED individuals ranged from 19 to 60. The volumes of all serum samples ranged from 0.5 to 3.5 mL. The median serum volumes for both CRC patients and NED individuals were 1.8 mL (Table 1).

When the serum samples were subjected to the ColoDefense assay, the positive detection rate for each stage of CRC is shown in Figure 2(a). The sensitivity for detecting CRC by methylated SEPT9 alone was 73.0% (95% CI: 63.6–80.8%) with a specificity of 95.6% (95% CI: 89.6–98.4%). The sensitivity by methylated SDC2 alone was 71.2% (95% CI: 61.8–79.2%) with the same specificity of 95.6% (95% CI: 89.6–98.4%). However, when methylated SEPT9 was combined with methylated SDC2 to detect the CRC, the overall sensitivity was improved to 86.5% (95% CI: 78.4–92.0%) with a specificity of 92.1% (95% CI: 85.1–96.1%).

In the 108 cases of CRC whose stages were identified based on the surgically resected specimens, methylated SEPT9 alone was positive in 38.5% of stage I (5/13), 81.6% of stage II (40/49), 69.2% of stage III (27/39), and 100% of stage IV (7/7). Methylated SDC2 alone was positive in 53.9% of stage I (7/13), 67.4% of stage II (33/49), 79.5% of stage III (31/39), and 85.7% of stage IV (6/7). However, for the methylated SEPT9 and methylated SDC2 panel (ColoDefense), the sensitivities for different stages of CRC were 69.2% for stage I (9/13), 85.7% for stage II (42/49), 89.7% for stage III (35/39), and 100% for stage IV (7/7) (Figure 2(a)).

The ROC curves for ColoDefense detecting CRC in serum are shown in Figure 2(b). The AUC for methylated SEPT9 alone was 0.854 (95% CI: 0.800–0.907), and the AUC for methylated SDC2 alone was 0.881 (95% CI: 0.835–0.928). However, the methylated SEPT9 and methylated SDC2 panel (ColoDefense) improved the AUC to 0.922 (95% CI: 0.883–0.961), indicating both high sensitivity and high specificity of the assay and a good performance in distinguishing the CRC subjects from NED subjects.

Due to the inconsistent volume of serum samples in this study, serum samples were divided into three groups by volume (<1.5 mL, 1.5–2.5 mL, and >2.5 mL) for further analysis (Table 2). There was no increase in the positive detection rate as the volume increased for different stages when detected by methylated SEPT9 alone, methylated SDC2 alone, or the methylated SEPT9 and methylated SDC2 panel (ColoDefense), and there seemed to be no significant difference among the positive detection rates of different volume groups ($p > 0.01$, data not shown).

Furthermore, there was no significant difference among the positive detection rates of methylated SEPT9 alone or methylated SDC2 alone or a combination of methylated SEPT9 and methylated SDC2 between different ages, genders,

![Figure 1: SEPT9 and SDC2 methylation levels in colorectal cancer tissues and paracancer tissues. ΔCp was defined as the difference between the Cp values for the target (methylated SEPT9 or methylated SDC2) and the internal control gene (ACTB): (a) methylation levels of SEPT9 and (b) methylation levels of SDC2.](image)

![Table 1: Characteristics of individuals examined by the ColoDefense assay.](table)

|               | CRC       | NED       |
|---------------|-----------|-----------|
| **Age (years)** | 25-89     | 19-60     |
| Min–Max       | 25-89     | 19-60     |
| Mean ± SD     | 61.0 ± 12.0 | 33.2 ± 8.3 |
| Median        | 62        | 32        |
| **Serum volume (mL)** | 0.5-3.5 | 0.7-3.5 |
| Min–Max       | 0.5-3.5   | 0.7-3.5   |
| Mean ± SD     | 2.16 ± 1.01 | 1.45 ± 0.85 |
| Median        | 1.8       | 1.8       |

CRC colorectal cancer; NED: no evidence of diseases.
or locations of the tumors ($p > 0.01$, Table 3). However, the positive detection rates seemed to increase with the increase of tumor sizes. The positive detection rates were 36.4%, 72.4%, and 94.7%, respectively, for tumors of $<3$ cm, 3-6 cm, and $>6$ cm in sizes by methylated SEPT9 alone, 54.6%, 69.7%, and 89.5% by methylated SDC2 alone, and 63.6%, 85.5%, and 100.0% by the methylated SEPT9 and methylated SDC2 panel (ColoDefense).

4. Discussion

CRC is a leading cause of cancer-related deaths worldwide, and late-stage CRC patients have low five-year survival rates. Blood-based screening strategies present the advantage of minimal invasiveness compared to endoscopies and are expected to have higher compliance rates than stool-based tests [10]. At present, a blood-based SEPT9 methylation assay
Colon 2.0 showed a sensitivity of 68.2% and a specificity of 73.3% to 81.0% [16, 17]. Epi proColon 2.0 assay, has been approved by FDA and Chinese FDA (CFDA) [17]. Epi proColon 2.0 for CRC screening, the Epi proColon 2.0 assay, has been found that serum volume was not the most important factor affecting CRC positive detection rates (Table 2).

The results demonstrated that the CRC positive detection rates were significantly improved by the methylated SEPT9 and methylated SDC2 panel without significant impact on specificity. It suggested that the ColoDefense assay on such serum samples did show high sensitivity and specificity for CRC detection. In particular, by classifying serum samples by volume, it was found that the ColoDefense assay showed similar sensitivity with serum or plasma samples was 87.0% with a specificity of 95.2% [13] or 89.4% with a specificity of 81.1% [9], respectively. The sensitivities of methylated SDC2 from these studies were improved by nested PCR or calculation of the percentage of methylated SDC2 with ACTB as the reference gene. In this study, we introduced a new blood-based early CRC screening assay, ColoDefense, which combined two methylation biomarkers, SEPT9 and SDC2, in a single PCR reaction. Compared with Epi proColon 2.0, methylated SEPT9 alone from ColoDefense showed similar sensitivity and specificity, and both had relatively low positive detection rates for stages I and III CRC (35.0% [4] vs. 38.5% and 46.0% [4] vs. 69.2%). On the other hand, methylated SDC2 alone from ColoDefense showed higher positive detection rates for stages I and III CRC (53.9% and 79.5%). Taken together, the combination of methylated SEPT9 and methylated SDC2 significantly improved the positive detection rates for stages I and III CRC (Figure 2(a)). The overall sensitivity of methylated SEPT9 alone or methylated SDC2 alone was 73.0% or 71.2%, respectively, with specificity of 95.6% for both. In contrast, the combination of methylated SEPT9 and methylated SDC2 increased sensitivity to 86.5% with a specificity of 92.1%, resulting in 13.5% and 15.3% increases in sensitivity compared to methylated SEPT9 alone and methylated SDC2 alone whereas specificity only decreased 3.5% (Figure 2).

For Epi proColon 2.0, real-time PCR reactions are performed with 3.5 mL fresh plasma samples as initial input, which means that approximately 10 mL of blood is needed for each test. As the blood biochemical test is the most common test in the clinics and large amount of serum remains after each test, using the remaining serum samples for CRC screening may avoid repeated blood draw. In this study, we found that the ColoDefense assay on such serum samples did show high sensitivity and specificity for CRC detection. In particular, by classifying serum samples by volume, it was found that serum volume was not the most important factor affecting CRC positive detection rates (Table 2). Therefore, using less blood volume, such as 5 mL, or remaining serum samples after the blood biochemical test might be a promising CRC screening strategy for those patients who refuse repeated blood draw or are unwilling to have 10 mL blood drawn.

### Table 3: Results of ColoDefense in detecting colorectal cancer between different ages, genders, tumor locations, and tumor sizes.

| Age | SDC2+ (%) | p value | SEPT9+ (%) | p value | SDC2 or SEPT9+ (%) | p value |
|-----|-----------|---------|------------|---------|-------------------|---------|
| <60 (n = 38) | 86.8 (33/38) | 0.01 | 63.2 (24/38) | 0.09 | 89.5 (34/38) | 0.51 |
| ≥60 (n = 73) | 63.0 (46/73) | 78.1 (57/73) | 84.9 (62/73) | 0.50 |
| Gender | | | | | | |
| Male (n = 75) | 73.3 (55/75) | 0.47 | 74.7 (56/75) | 0.56 | 88.0 (66/75) | 0.50 |
| Female (n = 36) | 66.7 (24/36) | 69.4 (25/36) | 83.3 (30/36) | 0.85 |
| Location | | | | | | |
| Rectum (n = 60) | 68.3 (41/60) | 0.45 | 66.7 (40/60) | 0.15 | 86.7 (52/60) | 0.85 |
| Cecum (n = 48) | 75.0 (36/48) | 79.2 (38/48) | 85.4 (41/48) | 0.50 |
| NA (n = 3) | 66.7 (2/3) | 100.0 (3/3) | 100.0 (3/3) | 0.50 |
| Size | | | | | | |
| <3 cm (n = 11) | 54.6 (6/11) | 36.4 (4/11) | 63.6 (7/11) | 0.50 |
| 3-6 cm (n = 76) | 69.7 (53/76) | 72.4 (55/76) | 85.5 (65/76) | 0.50 |
| >6 cm (n = 19) | 89.5 (17/19) | 94.7 (18/19) | 100.0 (19/19) | 0.50 |
| NA (n = 5) | 100.0 (5/5) | 80.0 (4/5) | 100.0 (5/5) | 0.50 |

NA: not applicable.
that the methylated SEPT9 and methylated SDC2 panel might be the best combination for early CRC screening with high sensitivity and specificity.

Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
The authors declare no conflict of interest.

Authors’ Contributions
YC, ZW, and GZ performed the statistical analyses and drafted the manuscript. CS, YM, LZ, MZ, and HL participated in data analysis. YC, ZW, GZ, MZ, and HL conceived the study and participated in the design and coordination of the study. All authors read and approved the final manuscript. Ying Chen, Zhenzhen Wang, and Guodong Zhao contributed equally to this work.

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