Circulating Methylated SEPT9 DNA Analyses to Predict Recurrence Risk and Adjuvant Chemotherapy Benefit in Stage II to III Colorectal Cancer

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None declared

Background: We aimed to evaluate the ability of circulating cell-free methylated SEPT9 DNA (mSEPT9) to identify recurrence and to determine its clinical utility in adjuvant chemotherapy (ACT) regimen decisions.

Material/Methods: This study enrolled 426 patients with stage II-III CRC who received radical resection between January 8, 2018, and November 30, 2020. The median follow-up duration was 15.8 months (range, 8.1-43.4 months). The primary endpoint was recurrence-free survival (RFS). A propensity score matching model was used to minimize potential confounding covariates and to confirm our findings.

Results: In stage II-III CRC patients, postoperative (within 1 month after surgery) mSEPT9 positivity was significantly correlated with worse RFS (HR=6.21, P<0.001), and it remained the strongest independent predictor in multivariate Cox regression analysis (HR=5.83, P<0.001), which was significantly superior to CEA, CA19-9, and CA242. During disease surveillance, mSEPT9 positivity preceded radiographic recurrence by a median of 5.0 months. The postoperative mSEPT9-positive patients benefited more from CAPEOX compared to FOLFOX (HR=3.97, P=0.017), while for mSEPT9-negative patients, CAPEOX and FOLFOX resulted in similar RFS (HR=1.70, P=0.322). Furthermore, 3 months of CAPEOX was more effective than 3 and 6 months of FOLFOX (HR=4.40, P=0.065; HR=1.56, P=0.073, respectively).

Conclusions: Our results revealed that mSEPT9 detection after radical resection could identify minimal residual disease (MRD) and could predict a high risk of recurrence in patients with stage II-III CRC. Furthermore, we show pioneering work that mSEPT9 detection could be used to guide the selection of an adjuvant chemotherapy regimen to improve RFS.

Keywords: Colorectal Neoplasms • Neoadjuvant Therapy • Prognosis • SEPTIN9 Protein, Human

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Background

Colorectal cancer (CRC) is the third leading cause of cancer death [1]. The 5-year survival rate of CRC with all stages is 64%, but with the presence of distant metastasis, it drops to 14% [2]. Surgical radical resection remains the primary treatment choice for nonmetastatic colorectal cancer [3]. It is highly significant that routine postoperative surveillance with regular clinical assessments and radiologic imaging is recommended. Despite this, a large proportion of recurrence events are detected late, and distant relapse is the major cause of mortality [4].

A potential source for early recurrence and metastasis is the existence of postoperative minimal residual disease (MRD), which is difficult to detect by traditional radiological imaging and serum tumor biomarkers [5]. The ability of imaging modalities to identify MRD is still far from optimal due to their limited resolution [6,7]. The serum tumor biomarkers currently used for surveillance, such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), and carbohydrate antigen 242 (CA242), have limited sensitivity and specificity [7,8]. Thus, it is essential to identify reliable indicators to detect MRD, serving as a harbinger for recurrence or metastasis before becoming clinically evident.

Aberrant DNA methylation has been well characterized in tumor progression and widely described in cancer patients [9-11]. A variety of methylated DNA have been identified as biomarkers for cancer diagnosis and prognostic prediction [12-14]. Among these epigenetic biomarkers, methylated Septin9 (mSEPT9) was confirmed to be highly specific to carcinogenesis in cancers, particularly in colorectal cancer [15,16]. Plasma mSEPT9 is thought to be released from apoptotic cells shed from solid tumors [16,17], and several studies have revealed the potential value of mSEPT9 in recurrence monitoring and prognostic prediction for patients with CRC [18-22]. However, given that previous studies mainly focused on the association between single detection of mSEPT9 status after surgery and prognosis of patients, the role of continuous surveillance of mSEPT9 lasting for 12 months in predicting prognosis has not been well investigated. Furthermore, these studies focused on MRD detection of mSEPT9 in patients administered adjuvant chemotherapy (ACT), while whether mSEPT9-defined MRD status could guide the selection of ACT regimens in CRC has not been reported.

Our primary aim was to evaluate the role of postoperative mSEPT9 in recurrence risk prediction and ACT regimen selection for patients with stage II-III CRC. Furthermore, we aimed to explore the value of continuous surveillance of mSEPT9 in CRC prognosis prediction.

Material and Methods

Study Design and Participants

This retrospective study was based on a database of 687 patients who underwent surgical treatment at Tianjin Union Medical Center in China between January 8, 2018, and November 30, 2020. A total of 261 patients were excluded from subsequent analysis due to loss to follow-up (n=70), diagnosis with non-CRC (n=31), nonprimary CRC (n=5), neoadjuvant therapy before surgery (n=61), no radical resection (n=15), insufficient imaging for recurrence (n=6), and stage 0 (n=4), stage I (n=46), or stage IV (n=23) CRC (Figure 1). Thus, a total of 426 evaluable patients who presented with histologically confirmed primary CRC were enrolled in the present study, and all of them subsequently underwent radical resection with or without ACT. The regimen and duration delivered were determined at the discretion of clinicians and patients and included single-agent (5-FU/capcitabine), FOLFOX (fluorouracil, leucovorin, and oxaliplatin), CAPEOX (capcitabine and oxaliplatin), and FOLFIRI (irinotecan, fluorouracil, and leucovorin). If patients received more than 1 treatment regimen, the regimen with the longest duration was included in the analysis. All patients had previously consented for their data to be used in this study. The study was approved by the Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

The test results of mSEPT9, CEA, CA19-9, and CA242 levels in plasma before surgery (preoperative), within 1 month after surgery (postoperative), and at various time points during disease surveillance were collected for analysis. Longitudinal mSEPT9 positivity was defined as random mSEPT9 positivity during surveillance. All patients received treatment and follow-up according to the guidelines of the National Comprehensive Cancer Network and Chinese Society of Clinical Oncology. RFS was measured from the date of surgery to the verified first radiographic recurrence (including local recurrence and distant metastasis) or the date of death caused by CRC. Patients provided oral and written informed consent before blood collection.

Plasma Methylated SEPT9 Quantification

A 10-ml peripheral blood sample was collected in a 10-ml K2EDTA anticoagulant tube, and a 3.5-ml plasma sample was collected from it upon centrifugation for the SEPT9 assay (BioChain [Beijing] Science and Technology, Inc., Beijing, PR China). Briefly, the SEPT9 gene methylation assay (PCR fluorescence probe method) was composed of 2 steps. First, total cell-free DNA was extracted from 3.5-ml plasma samples using a plasma processing kit manufactured by BioChain (Beijing) Science and Technology, Inc. and then incubated with bisulfite, leaving unmethylated cytosine converted to uracil, whereas
methylated cytosines were not. In the second step, double PCR amplification was performed on bisulfite-converted DNA. PCR blocking oligonucleotides and probes could distinguish between methylated and unmethylated sequences, leading to preferential amplification of methylated DNA sequences. The methylated SEPT9-specific fluorescent probes could specifically detect the methylated sequence. PCR was performed in a 60.0-μL reaction system. The sequences of the primers, blockers, and probes for SEPT9 detection used in methylation-specific PCR amplification were as follows:

- **Forward primer**: 5'-CCCACCAACCATCATAT-3';
- **Reverse primer**: 5'-GTAGTAGTTAGTTTAGTATTTATTTT-3';
- **Blocker**: 5'-CATCATATCAAACCCCACAATCAACACACAAC-3';
- **Probe 1**: 5'-GTTCGAAATGATTTTATTTAGTTGC-3';
- **Probe 2**: 5'-CGTTGATCGCGGGGTTC-3'.

Positive (methylated SEPT9 DNA) and negative controls (SEPT9 DNA) were run in parallel with samples each time as quality controls. β-actin was used as an internal reference to evaluate the plasma DNA quality and the validity of PCR amplification. The sequences of the primers and probes for β-actin detection used in PCR amplification were as follows:

- **Forward primer**: 5'-GTGATGGAGGAGGTTTAGTAAGTT-3';
- **Reverse primer**: 5'-CCAATAAAACCTACTCCTCCCTTAA-3'; and
- **Probe**: 5'-ACCACCACCCAACACAATACAACACACAAC-3'.

The PCR was programmed as follows: 94°C for 20 min; 45 cycles at 62°C for 5 s, 55.5°C for 35 s, and 93°C for 30 s; and cooling at 40°C for 5 s. According to the instructions, mSEPT9 positive was defined as cycle threshold (Ct) value ≤41, while mSEPT9 negative was defined as Ct value >41 or not detected (ND). For those not detected samples, Ct values were set as 45 (the maximal number of PCR cycles run in the mSEPT9 assay).

### Serum CEA, CA19-9, and CA242 Quantification

Serum CEA and CA19-9 were measured by the electrochemiluminescence immunoassay (ECLI) on a Cobas E601 immunoassay analyzer (Roche Diagnostics, GmbH, Mannheim, Germany). Serum CA242 was measured by CanAg CA242 enzyme immunometric assay (ELISA) kit (CanAg Diagnostics AB, Gothenburg, Sweden). The positive value was defined by widely accepted cutoffs (CEA: 5 ng/mL, CA19-9: 37 U/mL, CA242: 20 U/mL).

### Statistical Analysis

The chi-square test or Fisher’s exact test was used to compare categorical variables between the mSEPT9-positive and mSEPT9-negative subgroups, while the Wilcoxon rank test was used to compare continuous parameters. Kaplan-Meier estimates were performed to calculate the survival rate with the log-rank test to compare the differences. Hazard ratios (HRs)
and 95% confidence intervals (CIs) were calculated using Cox proportional hazards regression models. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were used to assess the predictive performance of mSEPT9, CEA, CA19-9, and CA242.

The propensity score model (PSM) was performed to match the potential bias of confounding covariates between subgroups (Supplementary Table 1). Logistic regression was run first to calculate propensity scores, and then clinicopathological factors, including age, sex, histology, differentiation, pT stage, pN stage, and localization, were used to match controls for the cases. A 1:1 nearest neighbor matching algorithm with a 0.02 caliper width added without replacement was performed in preoperative mSEPT9-positive and -negative matching, CAPEOX and FOLFOX matching, and other subgroups if appropriate. In addition, 1: n nearest neighbor caliper matching without replacement was performed in postoperative mSEPT9-positive and -negative matching, and longitudinal mSEPT9-positive and -negative matching. All analyses were performed using SPSS 26.0 and R 4.1.2. Two-sided P values <0.05 were considered to be statistically significant.

**Results**

**Clinicopathological Characteristics and mSEPT9**

A total of 426 patients with histologically confirmed stage II-III CRC who received radical resection were enrolled in the present study. The baseline characteristics based on the status of preoperative mSEPT9, postoperative mSEPT9 (within 1 month after surgery) and longitudinal mSEPT9 (within 12 months after surgery) are presented in Table 1.

Overall, the median age of the patients was 62 years (range, 23-83 years), and 253/426 (59.4%) were male. The proportions of patients with stage II and III disease were 180/426 (42.3%) and 246/426 (57.7%), respectively. With a median follow-up of 15.8 months (range, 8.1-43.4 months), 10 patients died from recurrence. Local recurrence and distant metastasis cases were 9/50 (12.0%) and 41/50 (82.0%), respectively.

Preoperative mSEPT9 was evaluable in 348/426 (81.7%) patients, while postoperative mSEPT9 detected within 1 month after resection was evaluable in 339/426 (79.6%) patients. The preoperative and postoperative mSEPT9-positive rates were 173/348 (49.7%) and 60/339 (17.7%), respectively.

**Association Between mSEPT9 and RFS**

Preoperative mSEPT9 had no association with RFS (HR=1.26, 95% CI=0.68-2.33, P=0.465) (Figure 2A), whereas postoperative mSEPT9 positivity was significantly associated with worse RFS (HR=6.08, 95% CI=3.27-11.31, P<0.001) (Figure 2B).

Furthermore, we evaluated the dynamic change in postoperative mSEPT9. Patients with persistent negativity for 12 months after surgery had a superior RFS, while patients with a conversion from mSEPT9 negativity to positivity had a worse RFS (HR=3.12, 95% CI=1.37-7.08, P<0.01, Supplementary Figure 1A). Patients with persistent positivity had the worst RFS, even compared to the group that changed from negative to positive (HR=6.70, 95% CI=2.72-16.50, P<0.001, Supplementary Figure 1A).

**Potential Utility of mSEPT9 in Guiding ACT Selection**

The majority of patients (403/426, 94.6%) received adjuvant chemotherapy, and the proportion of patients receiving the different chemotherapy regimens was as follows: 161/426 (37.8%) received FOLFOX, 177/426 (41.5%) received CAPEOX, and 36/426 (8.5%) received FOLFIRI; a single-agent capcitabine was administered to 26/426 (6.1%) patients; and 26/426 (6.1%) did not receive any adjuvant therapy. A total of 148/426 (34.7%) patients received at least 3 months of adjuvant chemotherapy, and 253/426 (59.2%) patients completed a full course (6 months) of treatment (Table 1). Although guidelines have already provided detailed recommendations for selecting treatment regimens, including drugs and cycles, the optimal regimens among the first-line recommendations remain to be determined.

We further explored whether postoperative mSEPT9 was correlated with RFS when the patients were stratified by 2 first-line therapies, FOLFOX and CAPEOX. There was no significant difference in RFS of the mSEPT9-negative subgroup (P=0.325, Figure 2C), whereas in the postoperative mSEPT9-positive subgroup, patients who received CAPEOX had a markedly better RFS than those who received FOLFOX (HR=4.43, 95% CI=1.27-15.38, P<0.05, Figure 2D), indicating that the difference in efficacy between regimens was correlated with mSEPT9.

For postoperative mSEPT9-positive patients, a surprising trend was observed in which 3 months of CAPEOX was more effective than 3 and 6 months of FOLFOX (3 months, HR=4.40, 95% CI=0.90-21.37, P=0.065; 6 months, HR=1.56, 95% CI=0.92-2.62, P=0.073) (Supplementary Figure 1B), but this difference was not statistically significant, probably owing to the limited number of patients. No significant difference between 3 and 6 months was found for either FOLFOX (HR=1.25, 95% CI=0.28-2.19, P=0.660) or CAPEOX (HR=1.20, 95% CI=0.07-9.18, P=0.878) (Supplementary Figure 1B).

**Serum CEA, CA19-9, CA242, and Recurrence**

The elevation rates of preoperative CEA, CA19-9, and CA242 levels were 181/404 (44.8%), 67/403 (16.6%), and 26/405 (6.4%), respectively.
Table 1. Baseline characteristics of patients with stage II-III colorectal cancer.

| Variables                      | Total number (n=426) | Preoperative mSEPT9 (n=348) | Postoperative mSEPT9 (n=339) | Longitudinal mSEPT9 (n=426) |
|--------------------------------|----------------------|-----------------------------|-------------------------------|-----------------------------|
|                                |                      | Positive (n=173) | Negative (n=175) | p value | Positive (n=60) | Negative (n=279) | p value | Positive (n=110) | Negative (n=316) | p value |
| Age, n (%)                     |                      |                            |                              |            |                |                      |            |                |                      |            |
| ≤60 years                      | 186                  | 79 (45.7)          | 78 (44.6)          | 0.914     | 21 (35)       | 127 (45.5)         | 0.153     | 36 (32.7)       | 150 (47.5)         | 0.008    |
| >60 years                      | 240                  | 94 (54.3)          | 97 (55.4)          |           | 39 (65)       | 152 (54.5)         |           | 74 (67.3)       | 166 (52.5)         |          |
| Sex, n (%)                     |                      |                            |                              |            |                |                      |            |                |                      |            |
| Female                         | 173                  | 72 (41.6)          | 71 (40.6)          | 0.913     | 25 (41.7)     | 115 (42.1)         | 1.000     | 39 (35.5)       | 134 (42.4)         | 0.216    |
| Male                           | 253                  | 101 (58.4)         | 104 (59.4)         |           | 35 (58.3)     | 164 (58.8)         |           | 71 (64.5)       | 182 (57.6)         |          |
| Differentiation, n (%)         |                      |                            |                              |            |                |                      |            |                |                      |            |
| Well/moderate                  | 277                  | 113 (65.3)         | 110 (62.9)         | 0.656     | 31 (51.7)     | 183 (65.6)         | 0.055     | 62 (56.4)       | 215 (68)          | 0.036    |
| Poor                           | 149                  | 60 (34.7)          | 65 (37.1)          |           | 29 (48.3)     | 96 (34.4)          |           | 48 (43.6)       | 101 (32)          |          |
| Histology, n (%)               |                      |                            |                              |            |                |                      |            |                |                      |            |
| Adenocarcinoma                 | 393                  | 164 (94.8)         | 154 (88)           | 0.034     | 53 (88.3)     | 256 (91.8)         | 0.450     | 100 (90.9)      | 293 (92.7)         | 0.538    |
| Other types                    | 33                   | 9 (5.2)            | 21 (12)            |           | 7 (11.7)      | 23 (8.2)           |           | 10 (9.1)        | 23 (7.3)          |          |
| TNM stage, n (%)               |                      |                            |                              |            |                |                      |            |                |                      |            |
| II                             | 180                  | 78 (45.1)          | 74 (42.3)          | 0.666     | 18 (30.0)     | 122 (43.7)         | 0.060     | 36 (32.7)       | 144 (45.6)         | 0.019    |
| III                            | 246                  | 95 (54.9)          | 101 (57.7)         |           | 42 (70.0)     | 157 (56.3)         |           | 74 (67.3)       | 172 (54.4)         |          |
| Pathological T stage, n (%)    |                      |                            |                              |            |                |                      |            |                |                      |            |
| pT1                            | 1                    | 0 (0.0)            | 1 (0.6)            |           | 0 (0.0)       | 1 (0.4)            |           | 0 (0.0)         | 1 (0.3)            |          |
| pT2                            | 19                   | 3 (1.7)            | 15 (8.6)           | 0.190     | 3 (5.0)       | 12 (4.3)           | 0.899     | 5 (4.5)         | 14 (4.4)           | 0.400    |
| pT3                            | 262                  | 111 (64.2)         | 103 (58.9)         |           | 37 (61.7)     | 171 (61.3)         |           | 64 (58.2)       | 198 (62.7)         |          |
| pT4                            | 144                  | 59 (34.1)          | 56 (32.0)          |           | 20 (33.3)     | 95 (34.1)          |           | 41 (37.3)       | 103 (32.6)         |          |
| Pathological N stage, n (%)    |                      |                            |                              |            |                |                      |            |                |                      |            |
| pN0                            | 180                  | 78 (45.1)          | 74 (42.3)          | 0.398     | 18 (30.0)     | 122 (43.7)         | 0.031     | 36 (32.7)       | 144 (45.6)         | 0.030    |
| pN1                            | 176                  | 71 (41)            | 69 (39.4)          |           | 28 (46.7)     | 115 (41.2)         |           | 53 (48.2)       | 123 (38.9)         |          |
| pN2                            | 70                   | 24 (13.9)          | 32 (18.3)          |           | 14 (23.3)     | 42 (15.1)          |           | 21 (19.1)       | 49 (15.5)          |          |
Table 1 continued. Baseline characteristics of patients with stage II-III colorectal cancer.

| Variables                              | Total number (n=426) | Preoperative mSEPT9 (n=348) | Postoperative mSEPT9 (n=339) | Longitudinal mSETP9 (n=426) |  |
|----------------------------------------|----------------------|-----------------------------|-----------------------------|-----------------------------|---|
|                                        |                      | Positive (n=173) | Negative (n=175) | p value | Positive (n=60) | Negative (n=279) | p value | Positive (n=110) | Negative (n=316) | p value | |
| Tumor location, n (%)                  |                      | Positive (n=60) | Negative (n=279) | 0.524 | Positive | Negative | 0.718 | Positive | Negative | 0.781 | |
| Rectum                                 |                      | 227 (53.3) | 86 (49.7) | 94 (53.7) |          | 31 (15.7) | 138 (49.5) |          | 56 (13.0) | 171 (54.1) |          | |
| Left colon                              |                      | 115 (27.0) | 54 (31.2) | 45 (25.7) | 0.524 | 14 (23.3) | 79 (28.3) |          | 30 (27.3) | 85 (26.9) | 0.781 | |
| Right colon                             |                      | 84 (19.7) | 33 (19.1) | 36 (20.6) |          | 15 (25.0) | 62 (22.2) |          | 24 (11.8) | 60 (19.0) |          | |
| ACT administration, n (%)              |                      | 26 (6.1) | 13 (7.5) | 10 (5.7) |          | 4 (6.7) | 11 (3.9) |          | 6 (5.5) | 20 (6.3) |          | |
| ACT                                     |                      | 400 (93.9) | 160 (92.5) | 165 (94.3) |          | 56 (39.3) | 268 (96.1) |          | 104 (94.5) | 296 (93.7) |          | |
| ACT regimen, n (%)                     |                      | 26 (6.1) | 13 (7.5) | 10 (5.7) |          | 4 (6.7) | 11 (3.9) |          | 6 (5.5) | 20 (6.3) |          | |
| None                                    |                      | 161 (37.8) | 72 (41.2) | 73 (41.7) |          | 30 (50.0) | 112 (40.1) |          | 50 (45.5) | 111 (35.1) |          | |
| FOLFOX                                  |                      | 36 (8.5) | 13 (7.5) | 10 (5.7) |          | 4 (6.7) | 25 (9.0) |          | 7 (6.4) | 29 (9.2) | 0.399 | |
| FOLFIRI                                 |                      | 177 (41.5) | 60 (34.7) | 72 (41.1) | 0.649 | 20 (33.3) | 119 (42.7) |          | 41 (37.2) | 136 (43.0) |          | |
| CAPEOX                                  |                      | 26 (6.1) | 15 (8.7) | 10 (5.7) |          | 2 (3.3) | 12 (4.3) |          | 6 (5.5) | 20 (6.3) |          | |
| Single agent                            |                      | 148 (34.7) | 57 (32.9) | 61 (34.9) | 0.858 | 25 (41.7) | 94 (33.7) | 0.366 | 41 (37.3) | 107 (33.9) | 0.687 | |
| ACT duration, n (%)                    |                      | 253 (59.2) | 103 (59.5) | 104 (59.4) |          | 31 (51.7) | 174 (62.4) |          | 63 (57.3) | 189 (59.8) |          | |
| Recurrence at any site, n (%)          |                      | 50 (11.7) | 22 (12.7) | 19 (10.9) |          | 21 (35) | 19 (6.8) | <0.001 | 36 (32.7) | 14 (4.4) |          | |
| No                                      |                      | 376 (88.3) | 151 (87.3) | 156 (89.1) |          | 39 (65) | 260 (93.2) |          | 74 (67.3) | 302 (95.6) | <0.001 | |
| Site of recurrence, n (%)              |                      | 9/50 (12.0) | 3/22 (13.6) | 6/19 (31.4) | 0.260 | 3/21 (14.3) | 4/19 (21.1) | 0.689 | 6/36 (16.7) | 3/14 (21.4) | 0.697 | |
| Locoregional only                      |                      | 41/50 (82.0) | 19/22 (86.4) | 13/19 (68.4) |          | 18/21 (85.7) | 15/19 (78.9) |          | 30/36 (83.3) | 11/14 (78.6) |          | |
respectively, and postoperative rates were 44/353 (12.5%), 21/341 (6.2%), and 29/264 (11.0%), respectively. The Kaplan-Meier curves of preoperative and postoperative CEA, CA19-9, and CA242 are shown in Supplementary Figure 2A-2F, none of which showed a significant difference in RFS between the elevated group and the nonelevated group (all P>0.05).

For patients with recurrence, the mSEPT9-positive rates in elevated postoperative CEA, CA19-9, and CA242 were 5/6 (83.3%), 4/4 (100.0%), and 4/5 (80.0%), respectively. However, as shown in Supplementary Table 2, for recurrence patients with postoperative mSEPT9 positivity, the elevation rates of CEA, CA19-9, and CA242 were just 5/19 (26.3%), 4/19 (21.1%), and 4/18 (22.2%), respectively. Furthermore, postoperative mSEPT9 positivity remained strongly associated with worse RFS even for patients with nonelevated CEA (HR=7.05, 95% CI=3.26-15.25, P<0.001, Supplementary Figure 3A), CA19-9 (HR=6.24, 95% CI=2.97-13.13, P<0.001, Supplementary Figure 3B) and CA242 (HR=6.51, 95% CI=2.95-14.36, P<0.001, Supplementary Figure 3C).

Furthermore, among patients who had recurrence, the postoperative mSEPT9-positive rate was maintained at 40% to 60% during surveillance, which was higher than the levels of CEA, CA19-9, and CA242 (Figure 3A), and it increased before clinical relapse, suggesting that mSEPT9 could better reflect the existence of MRD. In contrast, for patients without recurrence, the mSEPT9-positive rate remained below 20% within 12 months after surgery (Figure 3B).

In ROC curve analysis (Figure 3C), mSEPT9 also showed the best ability to monitor recurrence (AUC=0.821, 95% CI=0.752-0.890), outperforming CEA (AUC=0.501, 95% CI=0.408-0.594), CA19-9 (AUC=0.573, 95% CI=0.488-0.659), and CA242 (AUC=0.531, 95% CI=0.430-0.632).

L2 Longitudinal mSEPT9 Performed Better in Predicting Recurrence and Guiding ACT Selection

Next, we explored whether longitudinal mSEPT9 analysis performed better as a monitoring biomarker. As mentioned above, longitudinal mSEPT9 positivity was defined as random mSEPT9 positivity occurring during surveillance. Of 50 patients with recurrence, 72.0% had longitudinal mSEPT9 positivity, while only 52.5% had postoperative mSEPT9 positivity.

The longitudinal mSEPT9-positive group had a worse RFS than the longitudinal mSEPT9-negative group (HR=8.55, 95% CI=4.61-15.85, P<0.001, Figure 4A), which was more significant than that in the postoperative mSEPT9 analysis. Among the longitudinal positive mSEPT9 patients, those treated with CAPEOX showed better RFS than those treated with FOLFOX (HR=2.98, 95% CI=1.32-6.74, P<0.01, Figure 4B). However, there was no difference in RFS between regimens under longitudinal negative mSEPT9 conditions (P>0.05, Figure 4C).

In addition, the longitudinal mSEPT9-positivity rate was 36/50 (72.0%) for patients with recurrence. In 33 of these 36 patients (91.7%), mSEPT9 was detected early ahead of the radiological evidence. The median lead time from positive mSEPT9 detection to radiological relapse was 5.0 months (Wilcoxon signed-rank test, Z=4.721, P<0.001; Figure 4D). Moreover, of 376 patients without recurrence, 302 (80.3%) patients had a longitudinal negative mSEPT9.

Additional analyses for longitudinal CEA, CA19-9, and CA242 are shown in Supplementary Figure 2G-2I, none of which was associated with RFS (all P>0.05).
Further Verification by Propensity Score Matching

The PSM was performed to reduce the potential bias of confounding covariates between each subgroup. Baseline characteristics according to the status of preoperative, postoperative, and longitudinal mSEPT9 after matching are presented in Supplementary Table 1, and those according to CAPEOX/FOLFOX before and after matching are presented in Supplementary Table 3. No significant difference was observed between any 2 groups after matching. The absolute values of standardized differences were all <10%, implying that matched variables were well balanced between the 2 groups. All analyses were performed again after matching, and the results were even more significant than before.

For recurrence detection, there remained no association between preoperative mSEPT9 and RFS after matching (P >0.05, Figure 5A), while postoperative mSEPT9 positivity...
was significantly associated with worse RFS (HR=6.78, 95% CI=3.33-13.97, \( P < 0.001 \), Figure 5B), and longitudinal mSEPT9 performed even better in relapse prediction (HR=7.22, 95% CI=3.57-14.62, \( P < 0.001 \), Figure 5C).

Regarding the role of mSEPT9 in guiding chemotherapy, CAPEOX still had a better RFS than FOLFOX after matching in patients who were postoperatively mSEPT9-positive (HR=7.74, 95% CI=0.95-63.01, \( P < 0.05 \), Figure 5D), as well as in patients who were longitudinally mSEPT9-positive (HR=3.64, 95% CI=1.17-11.32, \( P < 0.05 \), Supplementary Figure 4A). No analysis was performed for PSM in patients who received different durations of therapy because of the limited number of cases.

In addition, postoperative mSEPT9 positivity remained associated with better survival when serum protein biomarkers were not elevated, including CEA (HR=7.63, 95% CI=3.08-18.93, \( P < 0.001 \), Supplementary Figure 4B), CA19-9 (HR=6.05, 95% CI=2.53-14.44, \( P < 0.001 \), Supplementary Figure 4C), and CA242 (HR=7.52, 95% CI=2.85-19.84, \( P < 0.001 \), Supplementary Figure 4D).

**Discussion**

Plasma mSEPT9 detection has emerged as a promising method in CRC screening, diagnosis, and prognosis prediction [23,24]. In this study, we examined the prognostic value of postoperative mSEPT9 in CRC patients and compared it to that of the traditional tumor biomarkers CEA, CA19-9, and CA242. mSEPT9 showed a much better ability to predict recurrence than the 3 tumor biomarkers, with an AUC value of 0.821. Moreover, we continuously monitored mSEPT9 status for 12 months for the first time and defined it as longitudinal mSEPT9. The results
showed that longitudinal mSEPT9 performed best in recurrence monitoring for patients with CRC and even preceded radiological recurrence by a median of 5 months. Furthermore, this study performed pioneering work to reveal the role of mSEPT9 status in ACT selection for CRC patients, in which patients with positive mSEPT9 could benefit more from the CAPEOX regimen than the FOLFOX regimen.

The positivity rate of mSEPT9 was 49.7% before the operation and dramatically declined to 17.7% within 1 month after the operation, which is similar to that reported previously [25]. This conversion of mSEPT9 reflects the tumor burden in real time [13,26], since as a kind of ccfDNA, the half-life of mSEPT9 is approximately 2 h [27]. Therefore, the potential role of mSEPT9 in CRC diagnosis has been extensively investigated and gradually introduced into CRC screening programs [19,22]. Regarding the prognostic value of mSEPT9, several recent studies have...
reported that positive postoperative mSEPT9 was correlated with a worse RFS, and a similar finding also emerged from our study. A study revealed that patients with positive postoperative mSEPT9 had significantly worse cancer-specific survival (CSS) than those with negative mSEPT9, and the correlation was affected by mismatch repair (MMR) status and specific genotype mutation [20]. Huang et al [22] demonstrated that mSEPT9 is a prognostic predictor for stage II-III CRC patients and that its expression is associated with vascular invasion, and other clinical parameters. Nevertheless, few studies have continuously monitored the status of mSEPT9 and evaluated the prognostic value of longitudinal mSEPT9 in CRC patients. Our analysis demonstrated that longitudinal mSEPT9 has a better ability to identify recurrence than postoperative mSEPT9, in which 72.0% patients who had a recurrence had longitudinal mSEPT9 positivity, and 91.7% mSEPT9 positivity was detected early ahead of the radiological evidence with a median lead time of 5 months.

Figure 5. Recurrence-free survival stratified by preoperative (A), postoperative (B) and longitudinal mSEPT9 (C) after propensity matching. (D) Recurrence-free survival stratified by chemotherapy regimen (FOLFOX/CAPEOX) in patients with postoperative positive mSEPT9 after propensity matching. R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.
Serum biomarkers, such as CEA, CA19-9, and CA242, have been widely used clinically for postoperative surveillance in CRC [28-32]. However, the sensitivity and specificity of any single one of them is not sufficient to make it a reliable indicator in CRC monitoring. In our study, the specificity (80.1%) and sensitivity (74.0%) of mSEPT9 for detecting recurrence were remarkably higher than those of any single serum marker, which was similar to that reported by Sun et al, and they also found that mSEPT9 combined with CEA showed a stronger predictive ability [20]. Another recent study revealed that the positive detection rate of the combination of mSEPT9 with CECT was higher than that of single CECT and the combination of tumor markers with CECT [18].

In this study, we mainly focused on the potential value of mSEPT9 in the selection of chemotherapy regimens. Although guidelines have already made recommendations in the selection of chemotherapy regimens for patients with different stages of CRC, when several options exist for first-line treatment, such as FOLFOX and CAPEOX for stage II-III CRC [33-35], choosing the most reasonable regimen for patients remains a challenge due to limited evidence in prognosis studies. Our results revealed that patients with stage II-III CRC who received CAPEOX had a better RFS than those who received FOLFOX, especially those with postoperatively positive mSEPT9. However, the advantage of CAPEOX disappeared in patients with postoperatively negative mSEPT9. When considering the duration of chemotherapy, 3 months of CAPEOX even had a better RFS than 3 months of FOLFOX, which was consistent with several previous studies [36,37].

This could be partly explained by the different frequencies of fluoropyrimidine administration. Patients treated with FOLFOX received fluorouracil administered via intravenous infusion every 2 weeks, while those treated with CAPEOX received capecitabine administered via the oral route twice daily. The latter mode could maintain a stable blood drug concentration and be more effective in terms of MRD clearance than the former [38]. Given the ability of mSEPT9 to detect MRD and the outstanding ability of CAPEOX to remove MRD, CAPEOX would be better than FOLFOX in patients with positive mSEPT9. Correspondingly, patients with negative mSEPT9 had lower MRD levels and risk of recurrence; thus, there was no significant difference between these 2 regimens [39].

Moreover, although FOLFOX and CAPEOX are the first-line ACT regimens, 8.5% of patients were administered second-line FOLFIRI in this study because of the adverse reactions of FOLFOX and CAPEOX, including nausea, vomiting, diarrhea, and hematological toxicities. Meanwhile, stage II patients without high-risk factors who received ACT accounted for 15.5% of all participants due to the comprehensive consideration from clinicians and the willingness of patients, although that was not recommended in the latest guidelines [3].

Septin9 is highly methylated in both colon and rectal cancer tissues [40,41], and it shows few transcriptomic differences in CRC based on tumor location [42]. It has been suggested that CRC detection by mSEPT9 is not influenced by tumor location [23,24,43]. To further identify its validity in predicting recurrence across locations, additional analyses for rectal cancer (Supplementary Figure 5) and colon cancer (Supplementary Figure 6) were performed separately, and these findings remained consistent with our findings from the combined cohort.

This study had some limitations. First, the retrospective nature of this study is still a notable weakness, although PSM was used to reduce bias. Second, with the situations mentioned above and with the aim of ensuring the homogeneity of the population, patients with rectal cancer in our study only received adjuvant therapy, whereas the modern approach to these patients is neoadjuvant therapy. Third, the number of patients was relatively low, and not all patients had mSEPT9 detected before and after surgery and during surveillance; thus, some patients had recurrence but no positive mSEPT9, possibly due to a lack of periodic detection during surveillance.

Conclusions

This study revealed that mSEPT9 detection could identify MRD and dynamically predict the risk of recurrence in resected stage II-III CRC patients. Furthermore, we showed pioneering work that mSEPT9-positive patients could benefit more from CAPEOX than FOLFOX. Thus, postoperative mSEPT9-defined MRD status could be used to guide chemotherapy regimens to improve survival.

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Declaration of Figures’ Authenticity

All Figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.
### Supplementary Table 1. Baseline characteristics of patients with stage II-III CRC after propensity matching.

| Variables                  | Preoperative mSEPT9 after matching at 1: 1 | Postoperative mSEPT9 after matching at 1: 4 | Longitudinal mSETP9 after matching at 1: 2 |
|----------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
|                            | Positive (n=149) | Negative (n=149) | Positive (n=59) | Negative (n=190) | Positive (n=105) | Negative (n=191) | 
| Age, n (%)                 |                            |                            |                            |                            |                            |                            |
| ≤60 years                  | 69 (46.3)                 | 67 (45.0)                 | 21 (35.6)                 | 76 (40.0)                 | 36 (34.3)                 | 73 (38.2)                 |
| >60 years                  | 80 (53.7)                 | 82 (55.0)                 | 38 (64.4)                 | 114 (60)                 | 69 (65.7)                 | 118 (61.8)                |
| Sex, n (%)                 |                            |                            |                            |                            |                            |                            |
| Female                     | 72 (41.6)                 | 60 (40.3)                 | 25 (42.4)                 | 79 (41.6)                 | 38 (36.2)                 | 71 (37.2)                 |
| Male                       | 101 (58.4)                | 89 (59.7)                 | 34 (57.6)                 | 111 (58.4)                | 67 (63.8)                 | 120 (62.8)                |
| Differentiation, n (%)     |                            |                            |                            |                            |                            |                            |
| Well/moderate              | 98 (65.8)                 | 99 (66.4)                 | 31 (52.5)                 | 118 (62.1)                | 61 (58.1)                 | 118 (61.8)                |
| Poor                       | 51 (34.2)                 | 50 (33.6)                 | 28 (47.5)                 | 72 (37.9)                 | 44 (41.9)                 | 73 (38.2)                 |
| Histology, n (%)           |                            |                            |                            |                            |                            |                            |
| Adenocarcinoma             | 140 (94.0)                | 140 (94.0)                | 52 (88.1)                 | 173 (91.1)                | 95 (90.5)                 | 174 (91.1)                |
| Other types                | 9 (6.0)                   | 9 (6.0)                   | 7 (11.9)                  | 17 (8.9)                  | 10 (9.5)                  | 17 (8.9)                  |
| TNM stage, n (%)           |                            |                            |                            |                            |                            |                            |
| II                         | 69 (46.3)                 | 70 (47.0)                 | 18 (30.5)                 | 70 (36.8)                 | 36 (34.3)                 | 63 (33.0)                 |
| III                        | 80 (53.7)                 | 79 (53.0)                 | 41 (69.5)                 | 120 (63.2)                | 69 (65.7)                 | 128 (67.0)                |
| Pathological T stage, n (%)|                            |                            |                            |                            |                            |                            |
| pT1/2                      | 3 (2.0)                   | 3 (2.0)                   | 3 (5.1)                   | 11 (5.8)                  | 5 (4.8)                   | 8 (4.2)                   |
| pT3/4                      | 146 (98.0)                | 146 (98.0)                | 56 (94.9)                 | 179 (94.2)                | 100 (95.2)                | 183 (95.8)                |
| Pathological N stage, n (%)|                            |                            |                            |                            |                            |                            |
| pN0                        | 69 (46.3)                 | 70 (47.0)                 | 18 (30.5)                 | 70 (36.8)                 | 36 (34.3)                 | 63 (33)                   |
| pN1/2                      | 80 (53.7)                 | 79 (53.0)                 | 41 (69.5)                 | 120 (63.2)                | 69 (65.7)                 | 128 (67)                  |
| Location, n (%)            |                            |                            |                            |                            |                            |                            |
| Rectum                     | 86 (49.7)                 | 75 (50.3)                 | 31 (52.5)                 | 93 (48.9)                 | 56 (53.3)                 | 94 (49.2)                 |
| Left colon                 | 54 (31.2)                 | 40 (26.8)                 | 13 (22)                   | 50 (26.3)                 | 29 (27.6)                 | 60 (31.4)                 |
| Right colon                | 33 (19.1)                 | 34 (22.8)                 | 15 (25.4)                 | 47 (24.7)                 | 20 (19)                   | 37 (19.4)                 |
| Recurrence at any site, n (%)|                            |                            |                            |                            |                            |                            |
| Yes                        | 18 (12.1)                 | 15 (10.1)                 | 21 (35.6)                 | 12 (6.3)                  | 34 (32.4)                 | 10 (5.2)                  |
| No                         | 131 (87.9)                | 134 (89.9)                | 38 (64.4)                 | 178 (93.7)                | 71 (67.6)                 | 181 (94.8)                |

CRC – colorectal cancer; mSEPT9 – methylated setpin9; ACT – Adjuvant chemotherapy.
Supplementary Figure 1. (A) Recurrence-free survival stratified by conversion of mSEPT9. (B) Recurrence-free survival stratified by chemotherapy regimens and durations in patients with postoperative positive mSEPT9. R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.

Supplementary Table 2. Relationship between postoperative mSETP9, CEA, CA19-9, and CA242 levels and recurrence status.

|                          | No recurrence | Recurrence |
|--------------------------|---------------|------------|
| **Post-operative mSETP9 positive, n/total (%)** |               |            |
| Postoperative CEA elevated | 7/34 (20.6)   | 5/19 (26.3) |
| Postoperative CEA not elevated | 27/34 (79.4) | 14/19 (73.7) |
| Postoperative CA19-9 elevated | 1/32 (3.1)    | 4/19 (21.1) |
| Postoperative CA19-9 not elevated | 31/32 (96.9) | 15/19 (78.9) |
| Postoperative CA242 elevated | 2/25 (8)      | 4/18 (22.2) |
| Postoperative CA242 not elevated | 23/25 (90.2) | 14/18 (77.8) |
| **Post-operative mSETP9 negative, n/total (%)** |               |            |
| Postoperative CEA elevated | 27/230 (11.7) | 1/13 (7.7) |
| Postoperative CEA not elevated | 203/230 (88.3) | 12/13 (92.3) |
| Postoperative CA19-9 elevated | 15/220 (6.8)  | 0/13 (0.0)  |
| Postoperative CA19-9 not elevated | 205/220 (93.2) | 13/13 (100.0) |
| Postoperative CA242 elevated | 20/168 (11.9) | 1/12 (8.3)  |
| Postoperative CA242 not elevated | 148/168 (88.1) | 11/12 (91.7) |

mSEPT9 – methylated setpin9.
Supplementary Figure 2. Recurrence-free survival stratified by preoperative CEA (A), CA19-9 (B) and CA242 (C), postoperative CEA (D), CA19-9 (E) and CA242 (F), and longitudinal CEA (G), CA19-9 (H) and CA242 (I). R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.

Supplementary Figure 3. Recurrence-free survival stratified by postoperative mSEPT9 in patients with nonelevated postoperative CEA (A), CA19-9 (B) and CA242 (C). R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.
**Supplementary Table 3.** Baseline characteristics of the patients who received CAPEOX and FOLFOX before and after propensity matching.

| Variables                  | Postoperative mSEPT9 positive before matching | Longitudinal mSETP9 positive before matching |
|----------------------------|----------------------------------------------|----------------------------------------------|
|                            | CAPEOX (n=20) | FOLFOX (n=30) | p | CAPEOX (n=41) | FOLFOX (n=50) | p |
| Age, n (%)                 |                |                |   |                |                |   |
| ≤60 years                  | 4 (20.0)       | 12 (40.0)      | 0.216 | 11 (26.8)       | 18 (36.0)      | 0.376 |
| >60 years                  | 16 (80.0)      | 18 (60.0)      |   | 30 (73.2)       | 18 (64.0)      |   |
| Sex, n (%)                 |                |                |   |                |                |   |
| Female                     | 6 (30.0)       | 15 (50.0)      | 0.243 | 12 (29.3)       | 18 (36.0)      | 0.274 |
| Male                       | 14 (70.0)      | 15 (50.0)      |   | 29 (70.7)       | 18 (64.0)      |   |
| Differentiation, n (%)     |                |                |   |                |                |   |
| Well/moderate              | 10 (50.0)      | 17 (56.7)      | 0.774 | 25 (61.0)       | 27 (54.0)      | 0.530 |
| Poor                       | 10 (50.0)      | 13 (43.3)      |   | 16 (39.0)       | 23 (46.0)      |   |
| Histology, n (%)           |                |                |   |                |                |   |
| Adenocarcinoma             | 17 (85.0)      | 29 (96.7)      | 0.289 | 37 (90.2)       | 48 (96.0)      | 0.403 |
| Other types                | 3 (15.0)       | 1 (3.3)        |   | 4 (9.8)         | 2 (4.0)        |   |
| TNM stage, n (%)           |                |                |   |                |                |   |
| II                         | 8 (40.0)       | 7 (23.3)       | 0.228 | 15 (36.6)       | 15 (30.0)      | 0.654 |
| III                        | 12 (60.0)      | 23 (76.7)      |   | 26 (63.4)       | 35 (70.0)      |   |
| Pathological N stage, n (%)|                |                |   |                |                |   |
| pN0                        | 8 (40.0)       | 7 (23.3)       | 0.228 | 15 (36.6)       | 15 (30.0)      | 0.654 |
| pN1/2                      | 12 (60.0)      | 23 (76.7)      |   | 26 (63.4)       | 35 (70.0)      |   |
| Pathological T stage, n (%)|                |                |   |                |                |   |
| pT1/2                      | 1 (5.0)        | 2 (6.7)        | 1.000 | 1 (2.4)         | 2 (4.0)        | 1.000 |
| pT3/4                      | 19 (95.0)      | 28 (93.3)      |   | 40 (97.6)       | 48 (96.0)      |   |
| Location, n (%)            |                |                |   |                |                |   |
| Rectum                     | 8 (40.0)       | 16 (53.3)      |   | 19 (46.3)       | 25 (50.0)      |   |
| Left colon                 | 10 (50.0)      | 4 (13.3)       | 0.012 | 18 (43.9)       | 12 (24.0)      | 0.051 |
| Right colon                | 2 (10.0)       | 10 (33.3)      |   | 4 (9.8)         | 13 (26.0)      |   |
| Recurrence at any site, n (%)|            |                |   |                |                |   |
| Yes                        | 3 (15.0)       | 15 (50.0)      | 0.016 | 8 (19.5)        | 28 (56.0)      | 0.015 |
| No                         | 17 (85.0)      | 15 (50.0)      |   | 33 (80.5)       | 22 (44.0)      |   |
Supplementary Table 3 continued. Baseline characteristics of the patients who received CAPEOX and FOLFOX before and after propensity matching.

| Variables                  | Postoperative mSEPT9 positive after matching at 1:1 | CAPEOX (n=13) | FOLFOX (n=13) | P       | Longitudinal mSEPT9 positive after matching at 1:1 | CAPEOX (n=30) | FOLFOX (n=30) | P       |
|----------------------------|-----------------------------------------------------|---------------|--------------|--------|---------------------------------------------------|---------------|--------------|--------|
| Age, n (%)                 |                                                     |               |              |        |                                                   |               |              |        |
| ≤60 years                  |                                                     | 4 (30.8)      | 3 (23.1)     | 1.00   | 7 (23.3)                                          | 8 (26.7)      | 1.00         |        |
| >60 years                  |                                                     | 9 (69.2)      | 10 (76.9)    |        | 23 (76.7)                                         | 22 (73.3)     |              |        |
| Sex, n (%)                 |                                                     |               |              |        |                                                   |               |              |        |
| Female                     |                                                     | 4 (30.8)      | 5 (38.5)     | 1.00   | 9 (30.0)                                          | 10 (33.3)     | 1.00         |        |
| Male                       |                                                     | 9 (69.2)      | 8 (61.5)     |        | 21 (70.0)                                         | 20 (66.7)     |              |        |
| Differentiation, n (%)     |                                                     |               |              |        |                                                   |               |              |        |
| Well/moderate              |                                                     | 8 (61.5)      | 7 (53.8)     | 1.00   | 18 (60.0)                                         | 18 (60.0)     | 1.00         |        |
| Poor                       |                                                     | 5 (38.5)      | 6 (46.2)     |        | 12 (40.0)                                         | 12 (40.0)     |              |        |
| Histology, n (%)           |                                                     |               |              |        |                                                   |               |              |        |
| Adenocarcinoma             |                                                     | 12 (92.3)     | 12 (92.3)    | 1.00   | 28 (93.3)                                         | 28 (93.3)     | 1.00         |        |
| Other types                |                                                     | 1 (7.7)       | 1 (7.7)      |        | 2 (6.7)                                           | 2 (6.7)       |              |        |
| TNM stage, n (%)           |                                                     |               |              |        |                                                   |               |              |        |
| II                         |                                                     | 6 (46.2)      | 4 (30.8)     | 0.688  | 12 (40.0)                                         | 10 (33.3)     | 0.789        |        |
| III                        |                                                     | 7 (53.8)      | 9 (69.2)     |        | 18 (60.0)                                         | 20 (66.7)     |              |        |
| Pathological N stage, n (%)|                                                     |               |              |        |                                                   |               |              |        |
| pN0                        |                                                     | 6 (46.2)      | 4 (30.8)     | 0.688  | 12 (40.0)                                         | 10 (33.3)     | 0.789        |        |
| pN1/2                       |                                                     | 7 (53.8)      | 9 (69.2)     |        | 18 (60.0)                                         | 20 (66.7)     |              |        |
| Pathological T stage, n (%)|                                                     |               |              |        |                                                   |               |              |        |
| pT1/2                       |                                                     | 1 (7.7)       | 0 (0.0)      | 1.00   | 1 (3.3)                                           | 1 (3.3)       | 1.00         |        |
| pT3/4                       |                                                     | 12 (92.3)     | 13 (100)     |        | 29 (96.7)                                         | 29 (96.7)     |              |        |
| Location, n (%)            |                                                     |               |              |        |                                                   |               |              |        |
| Rectum                     |                                                     | 4 (30.8)      | 7 (53.8)     |        | 15 (50.0)                                         | 18 (60.0)     |              |        |
| Left colon                 |                                                     | 7 (53.8)      | 2 (15.4)     | 0.119  | 11 (36.7)                                         | 9 (30.0)      | 0.735        |        |
| Right colon                |                                                     | 2 (15.4)      | 4 (30.8)     |        | 4 (13.3)                                          | 3 (10.0)      |              |        |
| Recurrence at any site, n (%)|                                                   |               |              |        |                                                   |               |              |        |
| Yes                        |                                                     | 1 (7.7)       | 6 (46.2)     | 0.030  | 4 (13.3)                                          | 12 (40.0)     | 0.039        |        |
| No                         |                                                     | 12 (92.3)     | 7 (53.8)     |        | 26 (86.7)                                         | 18 (60.0)     |              |        |

CRC – colorectal cancer; mSEPT9 – methylated setpin9; ACT – adjuvant chemotherapy.
Supplementary Figure 4. (A) Recurrence-free survival stratified by chemotherapy regimen (FOLFOX/CAPEOX) in patients with longitudinal positive mSEPT9 after propensity matching. Recurrence-free survival stratified by postoperative mSEPT9 in patients with nonelevated postoperative CEA (B), CA19-9 (C) and CA242 (D) after propensity matching. R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.
Supplementary Figure 5. Recurrence-free survival of patients with stage II-III rectal cancer stratified by preoperative (A), postoperative (B) and longitudinal mSEPT9 (C). Recurrence-free survival stratified by chemotherapy regimens in patients with postoperative negative (D) and positive (E) mSEPT9. (F) The ROC curve for mSEPT9, CEA, CA19-9 and CA242. RC – rectal cancer; ROC – receiver operating characteristic. R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.
Supplementary Figure 6. Recurrence-free survival of patients with stage II-III colon cancer stratified by preoperative (A), postoperative (B) and longitudinal mSEPT9 (C). Recurrence-free survival stratified by chemotherapy regimens in patients with postoperative negative (D) and positive (E) mSEPT9. (F) The ROC curve for mSEPT9, CEA, CA19-9 and CA242. CC – colon cancer; ROC – receiver operating characteristic. R software 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for Figure creation.

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