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Septin-9 gene methylation and carcinoembryonic antigen as key biomarkers in colorectal cancer

A retrospective study on prognosis and recurrence

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Abstract

This study investigates the clinical value of plasma Septin-9 gene methylation (mSEPT9) and carcinoembryonic antigen (CEA) in colorectal cancer (CRC), and their correlations with clinicopathological features and recurrence. A retrospective study included 81 CRC patients (observation group) and 73 healthy controls (comparison group) from January 2021 to January 2023, with pathological diagnosis as the gold standard. Plasma mSEPT9 (via quantitative PCR) and CEA (via electrochemiluminescence) levels were measured. Associations between mSEPT9 Ct values/CEA status and clinicopathological parameters (tumor location, metastasis, tumor diameter, etc) were analyzed. The observation group was followed for 1 year to assess the relationship between persistent positivity of mSEPT9/CEA before and after surgery and recurrence/metastasis. CEA positivity was significantly associated with tumor location ($P < .0001$), distant metastasis ($P < .0001$), and lymph node metastasis ($P < .0001$). mSEPT9 positivity correlated with distant metastasis ($P = .009$) and tumor diameter ≥ 5 cm ($P = .001$). Compared with negative cases, mSEPT9-positive individuals had a 12.08-fold higher risk of CRC (OR = 12.079; 95% CI = 3.699–39.447, $P < .001$), and CEA-positive individuals had a 5.30-fold higher risk (OR = 5.301; 95% CI = 1.339–20.981, $P = .017$). Postoperative persistent mSEPT9 positivity showed a stronger association with recurrence/metastasis than CEA ($\chi^2 = 7.227$, $P = .007$ vs $\chi^2 = 5.739$, $P = .017$). Combined detection of mSEPT9 and CEA achieved an area under the curve of 0.829, with sensitivity of 78.00% and specificity of 90.00%, outperforming single-marker analysis. Both mSEPT9 and CEA positivity are independent risk factors for CRC. mSEPT9 is closely linked to tumor burden and distant metastasis, demonstrating higher sensitivity in monitoring postoperative recurrence.

Abbreviations: AUC = area under the curve, CEA = carcinoembryonic antigen, CI = confidence interval, CRC = colorectal cancer, Ct = cycle threshold, mSEPT9 = Septin-9 gene methylation, OR = odds ratio, qPCR = quantitative polymerase chain reaction.

Keywords: colorectal cancer, correlation, cycle threshold, mSEPT9, pathological features

1. Introduction

CRC arises through a complex, multistage process influenced by various factors, including dietary habits, lifestyle, and environmental exposures.[1] The disease's pathogenesis involves both irreversible genetic mutations and reversible epigenetic alterations, with DNA methylation being a critical epigenetic mechanism driving its initiation and progression.[2]

Studies have demonstrated that plasma methylation testing of the Septin-9 gene exhibits high sensitivity and specificity for early adjunctive diagnosis of CRC, positioning it as a promising biomarker for detecting this malignancy.[3] Located on chromosome 17q25.3, the SEPT9 gene encodes the Septin-9 protein – a conserved member of the Septin family of GTP-binding proteins. This protein plays a critical role in essential physiological processes, including cytoskeletal remodeling,

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The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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microtubule regulation, vesicular transport, apoptosis, and cell division.[4] Methylation can occur in the nucleotide sequence domain of the promoter of Septin-9 tumor suppressor gene. Catalyzed by DNA methyltransferase, S-adenosylmethionine, as the donor, transfers SAM methyl groups to the 5-position carbon atoms of cytosine and guanine dinucleotide cytosine to form mCpG.[5] The hypermethylation of Septin-9 gene promoter can weaken its original anticancer effect, lead to the disturbance of cell cycle regulation, and promote cell canceration. In general, the increase of mSEPT9 content in human body indicates the occurrence of tumors.[6] These markers are expressed in a variety of malignancies, resulting in low specificity and limited sensitivity and specificity. In the detection of methylation biomarkers for CRC, DNA methylation detection of SEPT9 gene is the only FDA approved methylation biomarker for blood.[7] The sensitivity and specificity of a single biomarker in laboratory diagnosis are low, and the combined detection of multiple biomarkers can improve the diagnostic efficiency.[8]

While many studies emphasize the role of mSEPT9 in early CRC diagnosis, fewer explore its association with clinicopathological features of the disease. Existing research remains inconclusive regarding its utility in identifying lymph node or distant metastases.[9] Moreover, the clinical application value of mSEPT9 in the evaluation of surgical efficacy, tumor burden assessment, recurrence and metastasis monitoring of CRC is still unclear. Therefore, we aimed to explore the value of the combined detection of mSEPT9 and CEA in the early diagnosis of CRC by detecting their levels in peripheral blood.

2. Materials and methods

2.1. Research object

This study has been officially approved by the Ethics Committee (Ethics number: [2020-0131KY]) and was carried out in strict compliance with the relevant guidelines in STROBE's statement. All participants provided written informed consent after receiving a full explanation of the study objectives, procedures, and potential risks and benefits. For patients in the observation group, informed consent was obtained prior to sample collection and data retrieval from medical records. The control group provided consent during their health examination, confirming no history of malignancies or serious chronic diseases. Ethical review waived none of the consent requirements, as all participants met the inclusion criteria and voluntarily agreed to participate. Pathological findings of electronic colonoscopy biopsy specimens or postoperative pathological findings were used as the gold standard. A total of 81 patients diagnosed with CRC were included in the observation group. For comparative analysis, 73 healthy people were included as the comparison group.

2.2. Inclusion and exclusion criteria

The inclusion criteria for patients were as follows: All patients did not receive radiotherapy, chemotherapy, targeted therapy or immunotherapy before enrollment. All patients who participated in the study were fully aware of the study content and volunteered to participate. The age range of the population included in this study was 35 to 75 years. All patients with CRC were diagnosed by pathological examination and their clinical case data were complete. The healthy control group underwent colonoscopy and no obvious abnormality was found. Among the included subjects, there was no history of malignancies other than colorectal malignancies. Patients with CRC who underwent surgery did not receive surgical treatment, radiation therapy, chemotherapy, molecular targeted therapy, or traditional Chinese medicine before surgery. All CRC patients included met the pathological staging criteria stipulated in the CRC Diagnosis and Treatment Guidelines 2018 of the Chinese Society of Clinical Oncology.[10]

The exclusion criteria for both groups were similar, including: We excluded patients with failure of vital organs, including the heart, liver, and kidneys. We excluded women who were pregnant or nursing, as well as those with tumor cachexia. We make sure that patients do not have other malignant tumors or other serious diseases that can cause serious damage to human function. Exclude patients with chronic diseases that affect the levels of tumor markers (such as liver cirrhosis and pancreatitis).

2.3. Experimental methods

In the detection of mSEPT9, we used the mSEPT9 detection kit produced by Bolcheng (Beijing) Technology Co., Ltd. (Beijing, China), which is a PCR fluorescent probe method, each box can perform 30 tests. The kit includes Bolcheng plasma treatment kit and PCR kit. CEA is tested using a CEA assay kit produced by Roche Diagnostics (Basel, Switzerland), which uses electroluminescence (electroluminescence), using the Elecsys CEA system. The detection of mSEPT9 was completed by the 7500 real-time fluorescence quantitative PCR instrument produced by ABI Company in the United States. The detection of CEA relies on the Cobas 8000e 801 automatic chemiluminescence immunoanalyzer manufactured by Roche (Basel, Switzerland). The system's reference interval is set from 0 to 5 ng/L, and a test value >5 ng/L is defined as a positive result.

When performing mSEPT9 detection, we collected 10 mL blood samples using BD Vacutainer K2EDTA (Becton, Dickinson and Company, Franklin Lakes) 10 mL sampling vessels. The collected blood samples were quickly sent to the laboratory of our hospital for the preparation and quality check of plasma samples. If the plasma sample size is <3.5 mL, and there is significant hemolysis, chylblood, or particle precipitation, no testing should be performed and a new blood sample should be collected. If the blood sample cannot be processed immediately,

Figure 1. Collection and storage of blood samples. (A) BD Vacutainer K2EDTA 10mL blood vessels were used to collect 10mL blood samples; (B) preparation and quality check of plasma samples; (C) the blood vessels were centrifuged at 1350 ± 150 relative centrifugal force.

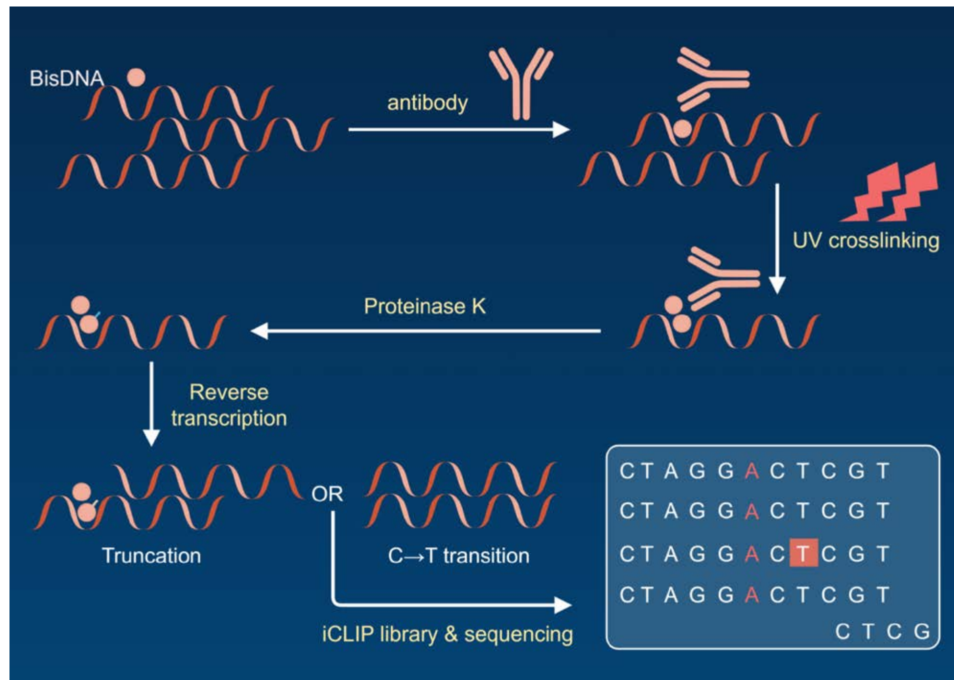

Figure 2. PCR process (by Figdraw.ID:RYYIS6636b). PCR = polymerase chain reaction.

it should be stored at 2 to 8°C for no more than 24 hours, and the blood sample should not be frozen. The blood vessels should be centrifuged at a centrifugal force of 1350 ± 150 relative centrifugal force for 12 minutes. In order to avoid damaging the blood cell layer, the centrifuge brake function is prohibited. After plasma extraction, 3.5mL of plasma was extracted by centrifugation again for 12 minutes at the same centrifugal force. Plasma samples should be stored at –25 to 15°C for no more than 4 weeks; Store at 2 to 8°C for no more than 18 hours. Frozen plasma samples, negative controls and positive controls should be melted at room temperature (15 to 30°C) for 30 minutes, after which the cracking step should be performed within 60 minutes. The plasma samples, positive control products and negative control products were successively added with 3.5mL lysate adsorption solution, and placed in the centrifuge tube at room temperature for 10 minutes. Each batch of testing includes 3 quality control products (high, medium and low methylation levels) to ensure that the inter-batch coefficient of variation (CV) is <10% (Fig. 1).

90 μL of magnetic beads (fresh suspension) and 2.5 mL of absolute ethanol (molecular biology grade, 99.5% purity) were successively added to the centrifuge tube. Close the centrifuge cap and gently shake it 5 to 6 times to mix well. The centrifugal tube is then placed on a wheel rotary mixer and rotated for 45 minutes at room temperature at a moderate speed (approximately 10 to 20 revolutions per minute). Place the 15 mL centrifuge tube on the DynaMagTM-15 magnetic test tube rack for 10 minutes, add 1.5 mL lotion, and use a vortex mixer to ensure that the magnetic beads are completely suspended. Use a disposable pipette to transfer the bead suspension into a labeled 2.0 mL centrifuge tube. Then, the centrifuge tube was placed on the DynaMagTM-2 magnetic test tube rack and adsorbed for 2 to 6 minutes. Use a disposable pipette to remove as much liquid as possible, taking care not to absorb magnetic beads. The centrifuge tube was placed on the nonmagnetic test tube rack, and 100 μL of eluent was added to each centrifuge tube, and the vortex was mixed and the magnetic bead was re-suspended. Put the centrifugal tube into the thermostatic oscillator, set the speed to 1000 RPM, the temperature to 80°C, and shake for 10 minutes. Transfer all eluents to a new 2.0 mL centrifuge tube. If the extracted DNA cannot be used immediately, it can be stored at 2 to 8°C, but not for more than 24 hours.

150 μL of sulfite solution and 25 μL of protective solution are added at one time to a centrifuge tube containing DNA eluent. The centrifuge tube was placed in a thermostatic oscillator and incubated at 80°C for 45 minutes without oscillating. Then, 1000 μL of lotion A and 20 μL of magnetic beads (fresh suspension) were added successively, and the vortex was mixed and placed in a constant temperature oscillator at 23°C, the speed was adjusted to 1000 RPM, and the incubation was 45 minutes. After 3 washes, the centrifuge tube was placed in a thermostatic oscillator for 10 minutes to precipitate and dry, then 60 μL of eluent was added and incubated in a thermostatic oscillator for 10 minutes. The eluent was moved to the 96-well plate, and the 96-well plate was sealed with a viscous film and film sealer. 30 μL of PCR pre-reaction solution was added to the corresponding hole in the selected 96-well plate. Add 30 μL of BisDNA to each hole. If the patient sample and the negative and positive control samples are measured in the same PCR reaction, the PCR reaction can be confirmed to be effective, provided that all the PCR reactions of the control samples meet the requirements of the standard. If the ACTB (actin B) in the control sample shows that the amount of DNA added is sufficient, the PCR result of SEPT9 can be used as the result of this PCR reaction. Conversely, if the Ct value of the ACTB (the cyclic threshold) is greater than a preset critical value, the PCR reaction is considered "invalid" (Fig. 2).

2.4. Statistical analysis

Statistical analysis was performed in IBM Statistic Package for Social Science (SPSS) 26.0 software (IBM, Armonk), while ROC curves were plotted using Medcalc 15.0 software. For counting data, we use the number of cases (N) and the composition ratio (%). When comparing counting data between 2 groups, we use the Chi-square test or Fisher exact probability method. For intragroup comparisons, we use paired Chi-square tests. The selected variables were included in the

binary Logistic regression model to analyze their impact on the study results. The balance of the 2 groups in basic characteristics such as age and gender was verified through baseline data analysis to reduce confounding bias. Subgroup analyses were conducted for key clinical features (including tumor TNM stage, surgical methods, postoperative adjuvant treatment regimens, and baseline CEA levels), and the differences between subgroups were analyzed through interaction tests. We evaluate the performance of the model by calculating the area, sensitivity and specificity under the ROC curve. For counting data, we use the number of column associations to analyze correlations between them. We used a bilateral test with a *P*-value of .05 as the threshold for statistical significance. Only when $P < .05$ were the results considered to be statistically significant. The expression level of the mSEPT9 gene was expressed as the Ct value (cyclic threshold), standardized by the ΔΔCt method, and GAPDH was used as the internal reference gene to correct the differences in RNA extraction efficiency among individuals. The experimental operation was independently completed by 2 certified technicians, and the CV of repeated tests was controlled within 5%. The recurrence endpoint was defined as local recurrence or distant metastasis confirmed by imaging or pathology. The follow-up ended in January 2024, with a median follow-up time of 12 months.

3. Results

3.1. Comparison of clinical data between the 2 groups

There were 46 males (56.8%) and 35 females (43.2%) in the observation group. There were 40 males (54.8%) and 33 females (45.2%) in the comparison group. Chi-square test (X^2=0.062) and $P = .803$ indicated that there was no statistically significant difference in gender distribution between the 2 groups. In the observation group, 36 (44.4%) were < 60 years old and 45 (55.6%) were ≥ 60 years old. In the comparison group, 31 subjects were < 60 years old (42.5%) and 42 subjects were ≥ 60 years old (57.5%). Chi-square test (X^2=0.061), $P = .805$, showed that there was no statistically significant difference in age distribution between the 2 groups. In the observation group, 31 (38.3%) were negative for mSEPT9 and 50 (61.7%) were positive. In the comparison group, 67 (91.8%) were negative for mSEPT9 and 6 (8.2%) were positive. Chi-square test (X^2=47.509), $P < .0001$, indicating that there were extremely significant statistical differences in the detection results of mSEPT9 between the 2 groups. In the observation group, 40 (49.4%) were negative for CEA and 41 (50.6%) were positive. In the comparison group, 66 (90.4%) were negative for CEA and 7 (9.6%) were positive. Chi-square test (X^2=30.126), $P < .0001$, indicating that there were also extremely significant statistical differences in CEA detection results between the 2 groups (Table 1).

3.2. Relationship between pathological features and CEA expression

Although there were slightly more CEA-positive patients in males than females (30 vs 11), the difference was not statistically significant (χ^2=2.909, $P = .088$), indicating that there was no significant association between gender and CEA expression. The proportion of negative and positive CEA expression was similar in patients aged 60 years or not (χ^2=0.106, $P = .745$), indicating that age was not the main factor affecting CEA expression. There was a significant correlation between tumor site and CEA expression (χ^2=16.895, $P < .0001$). This may be related to the physiological and pathological differences of the left and right half colon. The proportion of CEA positive in patients with distant metastasis was significantly higher than that in patients without distant metastasis (χ^2=17.012, $P < .0001$), indicating that CEA positive was closely related to distant metastasis of CRC. There was no significant difference in the proportion of negative and positive CEA expression between tubular adenocarcinoma and mucinous adenocarcinoma (χ^2=0.483, $P = .487$), indicating that pathological classification did not affect the expression of CEA. There was no significant correlation between the expression of CEA and the degree of tumor differentiation (χ^2=0.288, $P = .591$), that is, there was no significant difference in the expression level of CEA in either highly differentiated or moderately poorly differentiated tumors. Tumor diameter was not significantly associated with CEA expression (χ^2=0.358, $P = .550$), indicating that tumor size was not the main factor affecting CEA expression. There was no significant difference in the proportion of negative and positive CEA expression in tumor types (χ^2=1.125, $P = .570$), indicating that tumor types did not affect the expression of CEA. The proportion of CEA positive in patients with lymph node metastasis was significantly higher than that in patients without lymph node metastasis (χ^2=17.201, $P < .0001$), indicating that CEA positive was closely related to lymph node metastasis of CRC. The presence or absence of vascular invasion was close to statistically significant with CEA expression (χ^2=2.223, $P = .136$). Among patients with stage III tumors, the 3-year recurrence-free survival rate of the high-expression group of mSEPT9 (Ct value ≤ 35) was 62.3%, which was significantly lower than that of the low-expression group (78.5%, $P = .012$). However, in patients at stages I and II, there was no statistically significant difference between the 2 groups ($P = .089$), suggesting that the predictive efficacy of mSEPT9 might be more significant in advanced patients. Positive CEA was significantly associated with lymph

Table 1

Comparison of clinical data between the 2 groups [n(%)].

	Observation group (81)	Comparison group (73)	χ^2	*P*-value
Gender				
Male	46 (56.8)	40 (54.8)	0.062	.803
Female	35 (43.2)	33 (45.2)		
Age				
<60 yr old	36 (44.4)	31 (42.5)	0.061	.805
≥60 yr old	45 (55.6)	42 (57.5)		
mSEPT9				
Negative	31 (38.3)	67 (91.8)	47.509	<.0001
Positive	50 (61.7)	6 (8.2)		
CEA				
Negative	40 (49.4)	66 (90.4)	30.126	<.0001
Positive	41 (50.6)	7 (9.6)		

CEA = carcinoembryonic antigen, mSEPT9 = Septin-9 gene methylation.

Table 2

Relationship between pathological features and CEA expression in CRC patients.

	CEA Negative (40)	CEA Positive (41)	*X2*	*P-value*
Gender				
Male	22	30	2.909	.088
Female	18	11		
Age				
<60 yr old	19	18	0.106	.745
≥60 yr old	21	23		
Site of tumor				
Right semicolon	11	30	16.895	<.0001
Left semicolon	29	11		
Distant metastases				
None	27	9	17.012	<.0001
Yes	13	32		
Pathological classification				
Tubular adenocarcinoma	31	29	0.483	.487
Mucinous adenocarcinoma	9	12		
Degree of differentiation				
High differentiation	6	8	0.288	.591
Medium to low differentiation	34	33		
Tumor diameter				
<5 cm	26	24	0.358	.550
≥5 cm	14	17		
Tumor typing				
Lump type	10	9	1.125	.570
Ulcerative	20	25		
Infiltrative	10	7		
Vascular invasion				
None	26	8	17.201	<.0001
Yes	14	33		
Lymph node metastasis				
None	27	21	2.223	.136
Yes	13	20		

CEA = carcinoembryonic antigen, CRC = colorectal cancer.

node metastasis ($P < .0001$) and distant metastasis ($P < .0001$), suggesting that CEA can be used as an auxiliary indicator for evaluating the risk of CRC metastasis, especially for monitoring postoperative lymph node recurrence (Table 2).

3.3. Relationship between pathological features and mSEPT9

The detection results of mSEPT9 were significantly associated with distant metastasis and tumor diameter in CRC patients, but not with gender, age, tumor site, pathological classification, differentiation degree, tumor type, and lymph node metastasis. There was no significant difference in the proportion of males and females in MSEPT9-negative and MSEPT9-positive patients (χ^2=1.704, P = .192), indicating that gender was not significantly associated with mSEPT9 test results. There was no significant difference in the detection results of mSEPT9 regardless of age (χ^2=1.009, P = .315), indicating that age was not the main factor affecting the detection results of mSEPT9. There was no significant difference in the detection results of mSEPT9 for both right and left colon tumors (χ^2=0.030, P = .863), indicating that the tumor location was not correlated with the detection results of mSEPT9. The proportion of mSEPT9 positive in patients with distant metastasis was significantly higher than that in patients without distant metastasis (χ^2=6.739, P = .009), suggesting that mSEPT9 positive may be related to distant metastasis of CRC. No significant difference was found between tubular adenocarcinoma and mucinous adenocarcinoma (χ^2=1.049, P = .306), indicating that the pathological classification did not affect the detection results of mSEPT9. The detection results of mSEPT9 were not correlated with the degree of tumor differentiation (χ^2=0.005, P = .943), that is, there was no significant difference in the detection results of mSEPT9 whether the tumors were highly differentiated or moderately poorly differentiated. The proportion of MSEPT9-positive patients with tumor diameter ≥ 5cm was significantly higher than those with tumor diameter < 5cm (χ^2=10.422, P = .001), suggesting that MSEPT9-positive patients may be associated with larger tumor diameter. There was no significant difference in the detection results of mSEPT9 regardless of tumor type, ulcerative or invasive (χ^2=0.427, P = .808), indicating that the tumor type did not affect the detection results of mSEPT9. There was no significant correlation between the detection results of mSEPT9 and lymph node metastasis (χ^2=0.044, P = .834), that is, the presence of lymph node metastasis did not affect the detection results of mSEPT9. The presence or absence of vascular invasion was close to statistical significance with mSEPT9 (χ^2=2.805, P = .094) (Table 3).

3.4. Relationship between pathological features and positive Ct value of mSEPT9

mSEPT9 positive patients were divided into the high Ct value group (≥35.2) and the low Ct value group (<35.2) according to the median Ct value (35.2). This stratification method was based on the critical value of the kit (38.5) and the distribution characteristics of the samples. There was no significant difference in the distribution of males and females between the 2 groups (P = .242), indicating that gender was not associated with higher or lower mSEPT9 positive Ct values. There was no significant difference in the distribution of age groups (<60 years old and ≥ 60 years old) between the 2 groups (P = .815), indicating that age was not an important factor affecting the positive Ct value of mSEPT9. The distribution of tumors in both the

Table 3

Relationship between pathological features of CRC patients and mSEPT9.

	mSEPT9Negative (31)	mSEPT9Positive (50)	χ^2	*P*-value
Gender				
Male	23	30	1.704	.192
Female	8	20		
Age				
<60 yr old	14	17	1.009	.315
≥60 yr old	17	33		
Site of tumor				
Right semicolon	13	20	0.030	.863
Left semicolon	18	30		
Distant metastases				
None	27	30	6.739	.009
Yes	4	20		
Pathological classification				
Tubular adenocarcinoma	21	39	1.049	.306
Mucinous adenocarcinoma	10	11		
Degree of differentiation				
High differentiation	6	10	0.005	.943
Medium to low differentiation	25	40		
Tumor diameter				
<5 cm	26	24	10.422	.001
≥5 cm	5	26		
Tumor typing				
Lump type	5	8	0.427	.808
Ulcerative	20	35		
Infiltrative	6	7		
Vascular invasion				
None	16	27	0.044	.834
Yes	15	23		
Lymph node metastasis				
None	19	22	2.805	.094
Yes	11	28		

CRC = colorectal cancer, mSEPT9 = Septin-9 gene methylation.

right and the left colon was similar (*P* = .598), indicating that the tumor site was not correlated with the MSEPT9-positive Ct value. There was a significant difference (*P* = .014), and patients with distant metastases were more likely to be present in the low Ct value group. This suggests that a low mSEPT9 positive Ct value may be associated with a more advanced disease state. There was no significant difference in the distribution of tubular adenocarcinoma and mucinous adenocarcinoma between the 2 groups (*P* = .485), indicating that pathological classification was not the key factor affecting the mSEPT9 positive Ct value. The distribution of high differentiation and medium differentiation in the 2 groups was similar (*P* = .724), indicating that the differentiation degree of tumor cells was not related to the level of mSEPT9 positive Ct value. There was a significant difference (*P* = .010), with patients with tumor diameter ≥ 5cm more likely to be present in the low Ct value group. There was no significant difference in the distribution of tumor type, ulcerative tumor or invasive tumor between the 2 groups (*P* = .806), indicating that tumor type is not an important factor affecting the level of mSEPT9 positive Ct value. There was a significant difference (*P* = .035), and patients with lymph node metastases were more likely to be present in the low Ct value group. Association between a positive low Ct value of mSEPT9 and more advanced disease features such as lymph node metastases. There was no significant difference in the distribution of vascular invasion between the 2 groups (*P* = .774), indicating that vascular invasion was not the key factor affecting the positive Ct value of mSEPT9 (Table 4).

3.5. Analysis of influencing factors of CRC

Msept9-positive patients had a significantly increased risk of CRC compared with MSEPT9-negative patients (OR = 12.079; 95% CI = 3.699–39.447), which was highly statistically significant (*P* < .001). Patients with CEA positive also had a significantly increased risk of CRC compared with those with CEA negative (OR = 5.301; 95% CI = 1.339–20.981), an effect that was statistically significant (*P* = .017). Both mSEPT9 and CEA-positive status are important risk factors for CRC. Msept9-positive, with a much higher OR value than CEA positive, suggests a stronger association between MSEPT9-positive status and CRC risk. When assessing CRC risk, clinicians may consider incorporating tests for mSEPT9 and CEA into the routine screening process to more accurately identify people at high risk. Univariate analysis showed that mSEPT9 and CEA positivity were significantly associated with the risk of CRC. After further adjusting for factors such as tumor stage and metastasis status, both remained independent risk factors, suggesting that their predictive value did not depend on clinical stage. The OR value of positive mSEPT9 (12.079) was significantly higher than that of CEA (5.301), indicating that mSEPT9 has a stronger independent predictive ability for CRC and can be used as a core marker for the initial screening of high-risk populations (Table 5).

3.6. Follow-up and auxiliary diagnostic efficacy

CEA expression changed from positive to positive (i.e., positive before and after surgery) in 11 patients. CEA expression changed from positive to negative (that is, positive before surgery and negative after surgery) in 22 patients. CEA expression changed from positive to positive (i.e., positive before and after surgery) in 29 patients. CEA expression changed from positive to negative (that is, positive before surgery and negative after surgery) in 19 patients. X^2=5.739, *P* = .017, indicating a statistically significant association between changes in CEA expression and recurrence or metastasis of CRC. The expression of mSEPT9 changed from

Table 4

Relationship between pathological features of CRC patients and mSEPT9Positive Ct value.

	High Ct value group (29)	Low Ct value group (21)	χ^2	*P*-value
Gender				
Male	16	15	1.366	.242
Female	13	6		
Age				
<60 yr old	12	8	0.055	.815
≥60 yr old	17	13		
Site of tumor				
Right semicolon	13	11	0.278	.598
Left semicolon	16	10		
Distant metastases				
None	17	5	5.990	.014
Yes	12	16		
Pathological classification				
Tubular adenocarcinoma	21	17	0.487	.485
Mucinous adenocarcinoma	8	4		
Degree of differentiation				
High differentiation	7	6	0.124	.724
Medium to low differentiation	22	15		
Tumor diameter				
<5 cm	19	6	6.650	.010
≥5 cm	10	15		
Tumor typing				
Lump type	5	3	0.431	.806
Ulcerative	18	12		
Infiltrative	6	6		
Vascular invasion				
None	17	6	4.428	.035
Yes	12	15		
Lymph node metastasis				
None	14	11	0.082	.774
Yes	15	10		

CRC = colorectal cancer, Ct = cycle threshold, mSEPT9 = Septin-9 gene methylation.

Table 5

Binary logistic regression model of influencing factors of colorectal cancer.

	β	SE	Wald	*P*-value	OR	95% CI
mSEPT9Negative						
mSEPT9Positive	2.491	0.604	17.025	<.001	12.079	3.699–39.447
CEANegative						
CEAPositive	1.668	0.702	5.647	.017	5.301	1.339–20.981

CEA = carcinoembryonic antigen, CI = confidence interval, mSEPT9 = Septin-9 gene methylation, OR = odds ratio, SE = standard error.

positive to positive (that is, positive before and after surgery) in 16 patients. The expression of mSEPT9 changed from positive to negative (that is, positive before surgery and negative after surgery) in 40 patients. The expression of mSEPT9 changed from positive to positive (that is, positive before and after surgery) in 15 patients. The expression of mSEPT9 changed from positive to negative (that is, positive before surgery and negative after surgery) in 10 patients. X^2=7.227, P = .007, indicating a stronger statistical association between changes in mSEPT9 expression and recurrence or metastasis of CRC. When mSEPT9 and CEA were used in combination, the area under the curve (AUC) in the diagnosis of CRC increased to 0.829 (0.756–0.903), the sensitivity and specificity increased to 78.00% and 90.00%, respectively, and the Youden index reached 0.68. This shows that the AUC value and sensitivity are significantly improved by combining the 2 indicators, which is better than the diagnostic efficiency of using either indicator alone. Compared with the traditional noninvasive screening method (FIT), the combined detection of mSEPT9 + CEA maintains a high specificity (90%) while increasing the sensitivity to 78%, showing better comprehensive diagnostic efficacy. The recurrence risk of patients with persistently positive mSEPT9 after surgery (P = .007) was higher than that of CEA (P = .017), suggesting that mSEPT9 may be more sensitive in monitoring minimal residual lesions and early recurrence (Table 6 and Fig. 3).

4. Discussion

The combined application of mSEPT9 and CEA in the adjuvant diagnosis of CRC yielded an AUC of 0.900, demonstrating 80.00% sensitivity, 90.00% specificity, and a Youden index of 0.70. This multi-marker approach improved both the AUC value and diagnostic sensitivity compared to single-marker testing, while retaining comparable specificity. The detection of molecular markers alone is often difficult to achieve the ideal sensitivity and specificity at the same time, and the combined detection of multiple markers is becoming a developing trend of cancer screening and auxiliary diagnosis.[11] Finding a combination of molecular markers that can strike a balance between sensitivity and specificity is a key strategy to improve the detection rate of CRC in the future.[12] During the progression of CRC, the overmethylation of Septin-9 gene promoter will lead to the

Table 6

Relationship between CEA or mSEPT9 expression changes before and after surgery and recurrence or metastasis of colorectal cancer.

	Positive–Positive	Positive–Negative	χ^2	*P*-value
CEA expression				
None relapsed or metastasized	11	22	5.739	.017
Recurrence or metastasis occurs	29	19		
mSEPT9 expression				
None relapsed or metastasized	16	40	7.227	.007
Recurrence or metastasis occurs	15	10		

CEA = carcinoembryonic antigen, mSEPT9 = Septin-9 gene methylation.

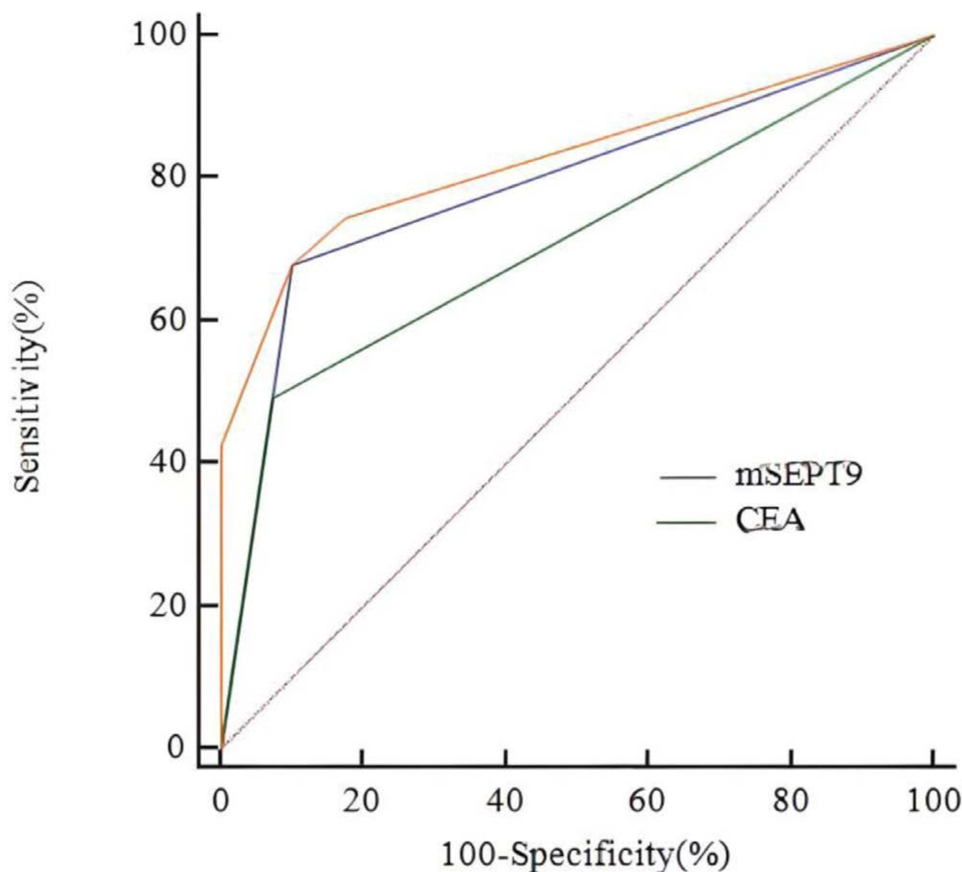

Figure 3. Auxiliary diagnostic effectiveness. As a biomarker for adjuvant diagnosis of colorectal cancer, mSEPT9 achieved an AUC (95% confidence interval) of 0.790 (range 0.705–0.875), demonstrating 68.00% sensitivity and 90.00% specificity. CEA, as another diagnostic index, had an AUC of 0.709 (0.615–0.803), sensitivity of 50.33%, and specificity of 92.50%. When used in combination, the AUC for colorectal cancer diagnosis increased to 0.829 (0.756–0.903), sensitivity and specificity increased to 78.00% and 90.00%, respectively, and the Youden index reached 0.68. AUC = area under the curve, CEA = carcinoembryonic antigen, mSEPT9 = Septin-9 gene methylation.

decline of its cancer-suppressing function, and then promote the abnormal proliferation of cells and eventually cancerous transformation.[13]

In this state, methylated Septin-9 gene fragments may be released from dying cancer cells into the bloodstream, resulting in increased levels of mSEPT9 in the blood.[14] MSEPT9 in plasma is a promising biomarker for CRC, showing high sensitivity and specificity in adjuvant diagnosis.[15] MSEPT9 has shown significant advantages in the early screening and auxiliary diagnosis of CRC. The expression of mSEPT9 in other digestive system tumors, gynecological tumors and esophageal cancer has also been observed, but as an auxiliary tool for the early diagnosis of these malignant tumors, its sensitivity is low.[16] The overall sensitivity of mSEPT9 for adjuvant diagnosis of CRC ranges between 48.2% and 95.6%, while its specificity spans 79.1% to 99.1%. Beyond its utility in screening low- and moderate-risk populations, SEPT9 testing is recommended for opportunistic screening in high-risk individuals. Opportunistic screening involves testing patients who encounter healthcare providers in clinical settings, such as outpatient or inpatient care, particularly those seeking medical attention due to clinical symptoms and thus classified as high risk.[17]

This study demonstrated that Septin-9 DNA methylation Ct values tend to be lower in CRC patients with lymph node metastasis or distant metastasis. For the first time, we performed a stratified analysis of Septin-9 DNA methylation Ct values and observed that CRC patients with mSEPT9-positive expression and low Ct values exhibited significant associations with advanced tumor stage, distant metastasis, lymph node metastasis, and larger tumor diameter. Compared with the mSEPT9 positive expression group with high Ct value, the mSEPT9 positive expression group with low Ct value was more likely to have lymph node and distant metastasis. Real-time fluorescence quantitative polymerase chain reaction (qPCR) showed high

sensitivity in the detection of Septin-9 DNA methylation. Based on a large number of clinical study samples, the mSEPT9 detection kit has determined the optimal critical Ct value, and has been verified by clinical trial samples.[18,19] Studies have mainly focused on the application of mSEPT9 in CRC screening and laboratory diagnosis. The positive expression rate of Septin-9 DNA methylation as determined by Ct values is closely related to the clinicopathological features of CRC, but its application value in the identification of lymph node metastasis is still controversial.[20] Although the quantitative analysis and detection technology of mSEPT9 has high requirements and high cost, which limits its wide application in clinical practice, the semi-quantitative detection and analysis of mSEPT9 is relatively feasible in clinical practice.[21] Further stratified analysis of Ct values can evaluate the severity of CRC to a certain extent and serve as an indicator of risk stratification and early warning.[22] Stratified analysis helps to identify lymph node metastases, considering that lymph node metastases often indicate a later stage of the disease.[23] The detection of mSEPT9 in peripheral blood contributes to the molecular disease staging of CRC, provides valuable information for TNM staging, and is of great significance for clinical treatment guidance.[24]

Although Ct values are limited by differences in tumor burden, DNA release efficiency and the sensitivity of detection instruments among individuals. In this study, the intra-batch CV was controlled within 5% by uniformly using the ABI 7500 fluorescence quantitative PCR instrument and strictly standardizing the plasma processing procedures (such as centrifugal force and storage temperature). However, the instrument differences among different laboratories may still affect the consistency of the results. In the future, a multicenter Ct value calibration system needs to be established, or the percentage of methylation degree (such as β value) can be used as a quantitative indicator to improve comparability.

The expression level of mSEPT9 in peripheral blood of patients with CRC is significantly increased, which makes the detection of mSEPT9 a powerful tool for the early diagnosis of CRC.[25] The detection of mSEPT9 and CEA can improve the detection rate of CRC, and mSEPT9 has shown important application potential in evaluating surgical efficacy, tumor burden and monitoring recurrence and metastasis.[26] The detection rate of mSEPT9 was not related to the gender, age, tumor location and degree of differentiation of patients, while the mSEPT9 positive expression group with low Ct value was significantly associated with distant metastasis, lymph node metastasis and tumor diameter.[27] Compared with the mSEPT9 positive expression group with high Ct value, the low Ct value group was more likely to have lymph node and distant metastasis. Due to the limited sample size of the current study and the fact that the subjects were all from the same hospital, the correlation between mSEPT9 and the pathological characteristics of CRC, as well as its application value in surgical efficacy, tumor burden and recurrence and metastasis, need to be further verified through larger sample size studies.[28] The results of studies on the diagnostic efficacy of mSEPT9 in CRC have great heterogeneity, which may be affected by multiple factors such as gender, race, age, detection methods and environmental factors.[29] Larger prospective studies are necessary to validate the diagnostic potential of mSEPT9.

In the research of CRC (CRC), the detection and analysis of methylation markers is an important research direction. A multicenter prospective cohort study evaluated the role of ctDNA methylation markers in the prediction of CRC recurrence. Studies have shown that the possibility of recurrence in patients with positive ctDNA 1 month after surgery is significantly higher than that in patients with negative ctDNA (hazard ratio 17.5; 95% CI = 8.9–34.4; $P < .001$). Furthermore, the combination of ctDNA and CEA tests can better stratify the recurrence risk (hazard ratio is 19.0; 95% CI = 8.9–40.7; $P < .001$). In postoperative treatment, the recurrence-free survival period of ctDNA-positive patients was significantly shorter than that of ctDNA-negative patients (hazard ratio 13.8; 95% CI = 5.9–32.1; $P < .001$). These results indicate that the longitudinal assessment of ctDNA methylation can achieve the early detection of recurrence, thereby optimizing the risk stratification and postoperative treatment of patients with CRC.[30] Another systematic review and quantitative evaluation study explored the diagnostic value of methylated biomarkers in fecal DNA in CRC and its precursor adenomas. Research has found that the methylation levels of certain genes have a sensitivity of over 70% and a specificity of over 80% in the detection of CRC. These findings indicate that the application of DNA methylation as a minimally invasive biomarker in feces is feasible and can be used for the early screening of CRC.[31] Methylation markers in ctDNA and fecal DNA have significant application potential in the early detection and risk stratification of CRC. These research results provide important scientific basis for the future development of more effective screening and diagnostic tools.

For the first time, we conducted a stratified Ct value analysis of the gradient association between mSEPT9 and metastasis and tumor diameter. We found that the low Ct value group (high methylation level) was significantly associated with lymph node metastasis ($P = .035$), filling the gap in previous studies where "qualitative detection was the main focus and quantitative analysis was lacking." Compared with large-scale studies (such as NCT03214328, n = 2000), the sample size of this study is smaller, and the universality of Ct value stratification needs to be verified in future multicenter studies. This study was a single-center retrospective design. The samples were all from Beijing Tongren Hospital and there might be biases in regions and population characteristics (such as diet and genetic background). The Ct value stratified depends on the single-center critical value. The standardized methylation index was not adopted, which may lead to differences in results among different studies. Subsequently, reference materials need to be introduced for cross-platform calibration.

5. Conclusion

The association between persistent positive mSEPT9 after surgery and recurrence and metastasis showed statistical significance in a single-center retrospective cohort ($P = .007$). However, its clinical value as a recurrence marker needs to be further verified through multicenter and prospective studies (with a planned sample size of ≥ 500 cases and a follow-up period of ≥ 3 years) to reduce selection bias and clarify long-term predictive efficacy.

Author contributions

Data curation: Jing Liu.
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