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Role of blood mSEPT9 in evaluating tumor burden and disease monitoring in colorectal cancer patients

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Abstract

Purpose: This study aimed to investigate the correlation between mSEPT9 and tumor burden as well as the role of mSEPT9 in monitoring colorectal cancer (CRC) patients.

Methods: A total of 309 patients were recruited and received mSEPT9 detection in this retrospective study. Clinicopathologic characteristics were collected, including age, gender, differentiation, gene mutation, stage, and tumor markers. The correlation between mSEPT9 and clinical tumor burden was analyzed. A relative mSEPT9 value was determined using the $\Delta\Delta C_t$ method.

Results: The overall positivity rate of mSEPT9 was 39.8% in CRC patients. mSEPT9 status was significantly associated with disease status and tumor markers (CEA and CA19-9). The mSEPT9 positivity rates were 15.6%, 50.0%, 64.4%, and 70.0% for P0M0, P1M0, P0M1, and P1M1 patients, respectively ($p < 0.001$). Among 137 CRC patients who received mSEPT9 assay before surgery, the pre-operation mSEPT9 positivity rate increased significantly from stage I to stage IV (Stage I vs. II vs. III vs. IV 25% vs. 59.1% vs. 57.1% vs. 70.0%, respectively). Consecutive blood samples were obtained from 26 patients during therapy. The patients with increased mSEPT9 levels showed a higher progression rate.

Conclusions: mSEPT9 was a biomarker reflecting tumor burden, and serial detections of mSEPT9 could be a promising strategy for disease monitoring in CRC patients.

KEYWORDS
circulating tumor DNA, colorectal cancer, methylated SEPT9, tumor burden

Huiqin Jiang and Qian Yu contributed equally as first authors.

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent tumors worldwide. Despite numerous advances in diagnosis and treatment, CRC is still a leading cause of death.[1] Recurrence and metastasis are the main reasons for the poor prognosis of CRC patients.[2] Carcinoembryonic antigen (CEA) is currently the most commonly used blood marker for therapeutic effect or recurrence monitoring, but its sensitivity and specificity are suboptimal. CEA is less sensitive to early stage CRC than advanced stage CRC.[3] Besides, almost 50% of CRC patients have normal CEA levels at the time of diagnosis. The low sensitivity of CEA hinders its use as a marker for therapeutic effect monitoring, as patients with negative CEA results at baseline cannot be monitored after treatment. Furthermore, CEA is inadequately specific to detect recurrence, since many factors may cause false positivity (e.g., smoking, infections, chronic obstructive pulmonary disease, inflammatory bowel disease, liver disease).[4] Thus, there is a need for more reliable tumor markers reflecting tumor burden in patients with metastatic CRC.

ctDNA is released into the plasma from malignant cells containing tumor-specific mutations or modifications. Thus, it could be utilized for noninvasive tumor detection and tumor burden monitoring.[5] DNA methylation is one of the most common aberrant epigenetic modifications, playing essential roles in tumor initiation and progression.[6] Several methylation markers, including methylated SEPT9, TMEFE2, NGFR, and SHOX2, have been found valuable for blood-based CRC detection.[7,8] The reported sensitivities for blood- and stool-based CRC DNA methylation biomarkers range in between 90 and 95% with a specificity range of 85–94%.[9] DNA methylation markers have also been explored as a potential biomarker for CRC prognosis. Different studies showed that LINE-1, CDKN2A, CHFR, EVL, IGFBP3, KISS1, RET, HTLF, and HPP1 genes hypermethylation was associated with poor survival outcomes in patients with CRC.[10,11] Besides, a number of genes with hypermethylation status have been suggested to clinics as potential predictive biomarkers in CRC patients under chemotherapy, such as SRBC, MGMT, TFAP2A.[10,11] DNA methylation biomarkers could help us improve early detection and management for CRC, having a promising future for CRC diagnosis. Among these DNA methylation biomarkers, SEPT9 has been most rigorously studied, and emerging results have shown that circulating methylated SEPT9 (mSEPT9) is a promising biomarker for CRC detection.[12] The US Food and Drug Administration (FDA) approved Epi procolon 2.0 kit for mSEPT9 detection in 2016. Till now, most studies focus on the early diagnosis of CRC by mSEPT9 assay.[13,14] However, the correlation between clinicopathologic characteristics and plasma mSEPT9 in CRC has been rarely reported. It is also unclear whether mSEPT9 could be used for monitoring tumor burden.

This study aimed to answer the following questions: (a) Is there any correlation between mSEPT9 and tumor burden in CRC patients? (b) Do dynamic changes in mSEPT9 levels predict therapeutic efficacy?

2 | METHODS

2.1 | Patients and data collection

A total of 309 CRC patients receiving treatment at Zhongshan Hospital Fudan University between April 2020 and December 2020 were recruited. The inclusion criteria were: confirmed diagnosis of CRC; age >18 years; and signed informed consent. The exclusion criteria were: pregnant woman; diagnosis of any other malignancy in the past. Clinicopathologic characteristics were collected, including age, gender, differentiation, gene mutation, and so on. Patients are divided into groups according to tumor stage and previous treatment: P0M0 (no primary and metastatic lesion; stage I–III CRC patients underwent surgery before plasma collection), P1M0 (with primary lesion, no metastatic lesion; stage I–III patients didn't received surgery before plasma collection), P0M1 (no primary lesion, with metastatic lesion; stage IV patients underwent primary tumor resection before plasma collection), P1M1 (with primary and metastatic lesion; IV patients didn't received surgery before plasma collection). Treatment response was evaluated according to RECIST criteria, version 1.1. Complete Response (CR): Disappearance of all target lesions. Partial Response (PR): At least a 30% decrease in the sum of diameters of target lesions. Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions. Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. Disease control rate (DCR), as well as non-PD rate, was defined as the radio of CR plus PR plus SD to total patients.

The study was approved by the ethics committee of Zhongshan Hospital Fudan University. Informed consent was obtained from each individual participant included in the study.

2.2 | Sample collection and storage

A total of 10 ml of blood was collected from each patient with an EDTA tube and processed immediately by double centrifugation at 1500 g for 12 min. The plasma was stored under −80°C for future analysis.

2.3 | Methylated SEPT9 detection

The detection of methylated SEPT9 was performed using Septin9 assay kit (BioChain Science and Technology, Inc.), following the manufacturer's instructions. Briefly, plasma cell-free DNA was captured, concentrated, and bisulfite-converted, and methylated SEPT9 was detected by real-time polymerase chain reaction (PCR). ACTB (β-actin) served as an internal control. Positive and negative external controls were used in all independent runs. The data from the PCRs of the assay were analyzed using the 1/1 algorithm. A relative methylation value was determined using the $\Delta\Delta C_t$ method as previously described.[15,16] In brief, $\Delta\Delta C_t$ values were calculated as follows:

$\Delta\Delta C_t$ (sample) = ΔC_t (sample) − ΔC_t (calibrator)
ΔC_t (sample) = C_t (ACTB of sample) − C_t (SEPT9 of sample)
ΔC_t (calibrator) = C_t (ACTB of calibrator) − C_t (SEPT9 of calibrator)
Calibrator here referred to the positive control.

2.4 | Statistical analysis

Categorical characteristics were summarized by counts and percentages, and continuous features were described by the mean values. The correlations between clinical factors and SEPT9 status were analyzed using the Chi-square or Fisher exact test. The comparison of the median between two groups was assessed by the Mann–Whitney *U* test. Three or more groups were analyzed by the one-way Kruskal–Wallis test. All the statistical tests were bilateral, and $p < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 18 software (SPSS Inc) and Graphpad Prism 8 (Graphpad Software).

3 | RESULTS

3.1 | Clinical characteristics

In the present study, a total of 309 patients were enrolled. Among them, 167 patients underwent surgery before sample collection, including 122 stage I–III patients and 45 stage IV patients. We divided patients into different groups according to tumor stage and previous treatment; 244 patients were stage I-III, and the other 65 patients were stage IV. 122 of 244 stage I–III CRC patients underwent surgery before plasma collection (P0M0), and 122 of 244 stage I–III patients didn't received surgery before plasma collection (P1M0); 45 of 65 stage IV patients underwent primary tumor resection before plasma collection (P0M1), and 20 of 65 stage IV patients didn't received surgery before plasma collection (P1M1) (Figure 1).

The clinical characteristics are summarized in Table 1. The median age was 63 years (25–88 years). The majority of the CRC patients were male (n = 188, 60.8%). The number of patients with P0M0, P1M0, P0M1, and P1M1 was 122 (39.5%), 122 (39.5%), 45 (14.6%), and 20 (6.4%), respectively. RAS/RAF status was available for 252 patients, and 124 (49.2%) patients presented wild-type RAS/RAF.

3.2 | Correlation between mSEPT9 and tumor burden

Among 309 CRC patients enrolled in the study, the mSEPT9 positive rate was 39.8% (123/309). To assess the clinical utility of mSEPT9 assay in CRC patients, we investigated the association between clinical factors, which could reflect tumor burden, and mSEPT9 status. The mSEPT9 positive rate was 15.6%, 50.0%, 64.4%, and 70.0% for P0M0, P1M0, P0M1, and P1M1 patients, respectively ($p < 0.001$). Besides, mSEPT9 was significantly associated with tumor marker CEA and CA19-9 ($p < 0.0001$ and $p = 0.0001$, respectively) (Table 2).

The level of mSEPT9 in plasma was further examined to study the relationship between mSEPT9 quantity and CRC severity. The mSEPT9 levels showed a significant increment from tumor-free group to tumor-bearing group (Mean $\Delta\Delta C_t$: −6.199 vs. −2.541, $p < 0.0001$). More severe lesions exhibited higher methylation values (Mean $\Delta\Delta C_t$: P0M0 vs. P1M0 vs. P0M1 vs. P1M1 −6.199 vs. −3.272 vs. −1.723 vs. 0.084, $p < 0.0001$). A significant difference of SEPT9 methylation levels was further demonstrated between CEA+ and CEA− group (Mean $\Delta\Delta C_t$: −2.373 vs. −4.827, $p<0.0001$) (Figure 2). This observation suggested that the mSEPT9 level in plasma is correlated with the severity of CRC.

3.3 | Correlation between pre-operation mSEPT9 and pathological characteristics

Pre-operation mSEPT9 status was available for 137 out of 309 patients (44.3%). Detailed clinicopathologic parameters are summarized in Table 3. The mSEPT9 positive rate increased significantly from UICC stage I to stage IV CRC patients. No correlation was found between mSEPT9 and gender, primary tumor site, tumor differentiation, RAS/RAF mutation, or vascular/nerve invasion ($p > 0.05$)

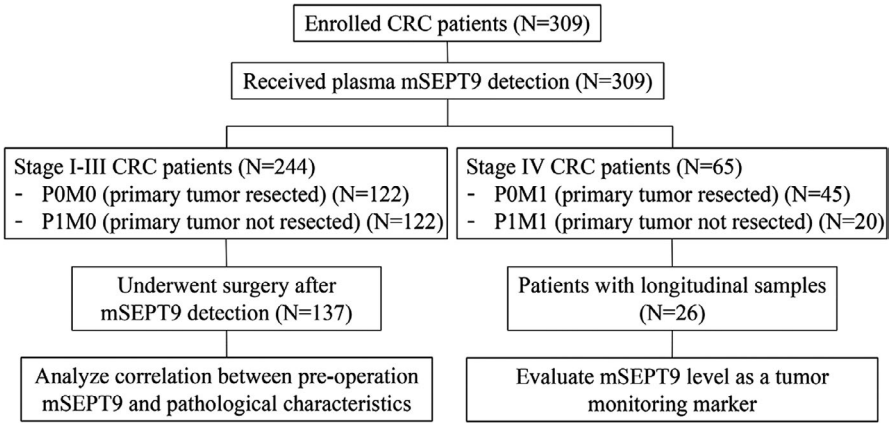

FIGURE 1 Flowchart of this study

(Table 3). These results showed that pre-operation mSEPT9 might be a powerful tool for predicting pathological stage of CRC, regardless of other pathological factors.

TABLE 1 Clinical characteristics

Characteristics	N	%
Median age (years, range)	309	63 (25–88)
Gender		
Male	188	60.8
Female	121	39.2
Primary site		
Right colon	98	31.7
Left colon	95	30.7
Rectal	116	37.6
Status		
P0M0	122	39.5
P1M0	122	39.5
P0M1	45	14.6
P1M1	20	6.4
RAS/RAF mutation		
MT	128	50.8
WT	124	49.2

Abbreviations: MT, mutant type; P0M0, without primary and metastatic tumor; P0M1, without primary tumor and with metastatic tumor; P1M0, with primary tumor and without metastatic tumor; P1M1, with primary and metastatic tumor; WT, wide type.

3.4 | mSEPT9 level as a tumor monitoring marker

As the mSEPT9 level showed a significant association with tumor burden, we further evaluated the usefulness of mSEPT9 measurement in predicting therapeutic response. Consecutive blood samples were available from 26 patients on therapy. All patients received chemotherapy (XELOX or FOLFOX or FOLFIRI); part of them received additional targeted therapy (cetuximab or bevacizumab). 16/26 (61.5%) patients had decreased mSEPT9 level after treatment; the other 10/26 (38.5%) patients presented increased mSEPT9 level (Table 4). We found that the change of mSEPT9 level correlated with treatment response; the disease control rate (non-PD rate) was much higher in patients with decreased mSEPT9 level after treatment. Besides, the accuracy of mSEPT9 in predicting treatment response was higher compared to CEA (69.2% vs. 53.8%) (Table 5).

4 | DISCUSSION

Accumulated evidences have also verified the value of ctDNA methylation as biomarkers for the screening, early diagnosis, monitoring of therapy response, and prognosis of cancer.[17-21] SEPT9 methylation has been demonstrated as a useful marker for blood-based CRC screening, with a sensitivity of 72–90% and a specificity of 88–90%.[22,23] However, few studies have been conducted to investigate the association between mSEPT9 status and tumor burden, and the role of mSEPT9 level in predicting response is still unclear.

TABLE 2 Correlation between S9 status and clinical tumor burden

Factor	N	S9 positive (%)	S9 negative (%)	*p* Value
Status				
P0M0	309	19 (15.6)	103 (84.4)	<0.0001
P1M0		61 (50.0)	61 (50.0)	
P0M1		29 (64.4)	16 (35.6)	
P1M1		14 (70.0)	6 (30.0)	
Status				
Tumor free	309	19 (15.6)	103 (84.4)	<0.0001
Tumor bearing		104 (55.6)	83 (44.4)	
CEA				
Positive	305	61 (57.6)	45 (42.4)	<0.0001
Negative		61 (30.7)	138 (69.3)	
CA199				
Positive	305	26 (63.4)	15 (36.6)	0.0010
Negative		96 (36.4)	168 (63.6)	
LDH				
Positive	195	6 (66.7)	3 (33.3)	0.1017
Negative		73 (39.2)	113 (60.8)	

Abbreviations: CA19-9, carbohydrate antigen19-9; CEA, carcinoembryonic antigen; LDH, lactate dehydrogenase; P0M0, without primary and metastatic tumor; P0M1, without primary tumor and with metastatic tumor; P1M0, with primary tumor and without metastatic tumor; P1M1, with primary and metastatic tumor.

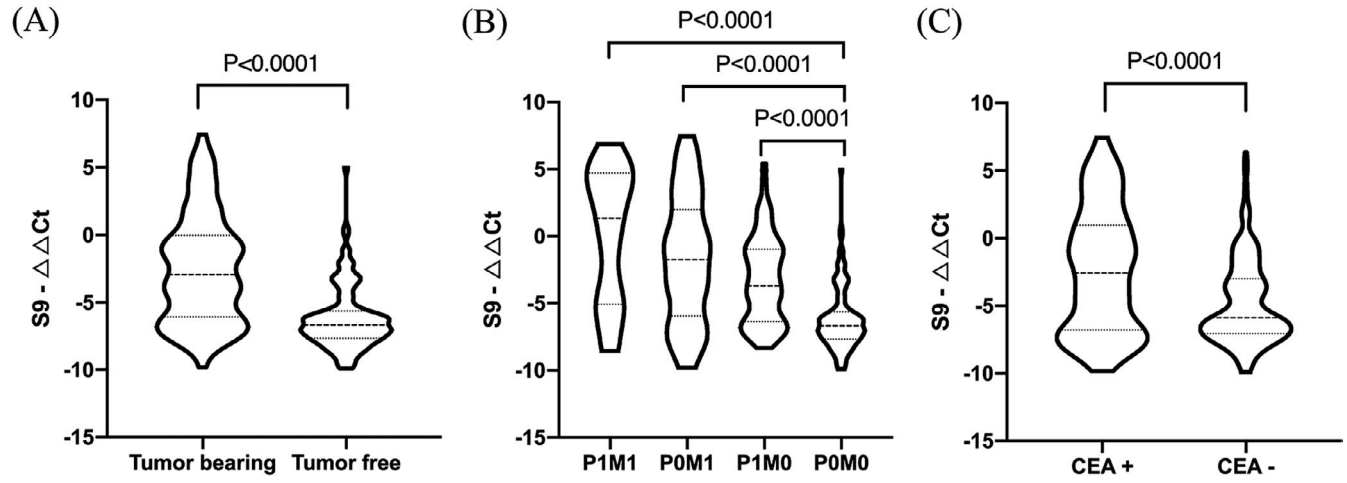

FIGURE 2 Difference in mSEPT9 levels ($\Delta\Delta C_t$) between different groups. (A) mSEPT9 levels in the tumor-bearing group and tumor-free group; (B) mSEPT9 levels in the P1M1, P0M1, P1M0, P0M0 groups; (C) mSEPT9 levels in the CEA+ group and CEA− group

TABLE 3 Correlation between S9 status and clinicopathological factors

		S9 (%)		*p* value	
Factor	**N**	**Positive**	**Negative**	**Univariate**	**Multivariate**
Gender					
Male	137	48 (55.8)	38 (44.2)	0.583	0.396
Female		26 (51.0)	25 (49.0)		
Age					
≥65 years	137	45 (64.3)	25 (35.7)	0.014	0.012
<65 years		29 (43.3)	38 (56.7)		
Vascular invasion					
Yes	121	30 (50.8)	29 (49.2)	0.926	–
No		31 (50.0)	31 (50.0)		
Nerve invasion					
Yes	121	38 (52.8)	34 (47.2)	0.528	–
No		23 (46.9)	26 (53.1)		
Differentiation					
Poor	137	34 (61.8)	21 (38.2)	0.133	0.460
Well		40 (48.8)	42 (51.2)		
RAS/RAF mutation					
MT	121	33 (55.9)	26 (44.1)	0.634	–
WT		32 (51.6)	30 (48.4)		
UICC stage					
I	137	6 (25.0)	18 (75.0)	0.013	0.014
II		26 (59.1)	18 (40.9)		
III		28 (57.1)	21 (42.9)		
IV		14 (70.0)	6 (30.0)		
Primary site					
Right colon	137	23 (57.5)	17 (42.5)	0.249	0.277
Left colon		27 (61.4)	17 (38.6)		
Rectal		24 (45.3)	29 (54.7)		

Abbreviations: MT, mutant type; WT, wide type.

TABLE 4 Characteristics of 26 patients with consecutive mSEPT9 measurement

Characteristics	N (%)
Median age (years, range)	64 (25–77)
Gender	
Male	16 (61.5)
Female	10 (38.5)
Baseline mSEPT9 before treatment	
Negative	8 (30.8)
Positive	18 (69.2)
Baseline CEA before treatment	
Negative	19 (73.1)
Positive	7 (26.9)
mSEPT9 change after treatment	
Decreased	16 (61.5)
Increased	10 (38.5)
CEA change after treatment	
Decreased	18 (69.2)
Increased	8 (30.8)
Primary site	
Right colon	9 (34.6)
Left colon	6 (23.1)
Rectal	11 (42.3)
Systemic treatment	
XELOX	8 (30.8)
FOLFOX	5 (19.2)
FOLFIRI	3 (11.5)
Cetuximab + FOLFIRI	4 (15.4)
Bevacizumab + FOLFIRI	6 (23.1)
Best response (RECIST)	
CR	0 (0)
PR	4 (15.4)
SD	14 (63.8)
PD	8 (30.8)

Abbreviations: CR, complete response; PD, progression of disease; PR, partial response; SD, stable disease

TABLE 5 Accuracy of mSEPT9 and CEA change in predicting treatment response

	Best response (RECIST) (%)		
	Non-PD	PD	Accuracy
Overall	18 (69.2)	8 (30.8)	
mSEPT9 change after treatment			
Decreased	14 (77.8)	4 (22.2)	69.2
Increased	4 (50.0)	4 (50.0)	
CEA change after treatment			
Decreased	12 (66.7)	6 (33.3)	53.8
Increased	6 (75.0)	2 (25.0)	

Previous studies have demonstrated that ctDNA is a highly sensitive biomarker that may reflect tumor burden more accurately than traditional markers for CRC.[24] Our research data also showed that ctDNA mSEPT9 was positively correlated with tumor status and CEA in CRC patients. The mSEPT9 positivity rate was 15.6% in the tumor-free group and 55.6% in the tumor-bearing group ($p < 0.001$). This result suggests that mSEPT9 positivity rate may represent disease severity and possibly be associated with a poor prognosis. Moreover, we found mSEPT9 has a higher positivity rate than CEA in CRC patients, which is consistent with previous reports.[25,26]

We further examined the relationship between pre-operation mSEPT9 status and clinicopathological factors in 137 CRC patients. Our data demonstrated that pre-operation mSEPT9 was an independent predictor of the UICC stage. CRC patients in earlier tumor stages showed lower mSEPT9 levels compared to those with more advanced lesions. The results indicated that mSEPT9 performed outstandingly as an auxiliary molecular staging parameter. Moreover, pre-operation mSEPT9 status did not show any association with gender, primary tumor site, and RAS/RAF status, indicating that ctDNA methylation seems likely to be independent of various molecular subtypes of CRC. As previous studies reported, abnormal DNA methylation is an early and frequent event in cancer development. Different cancer patients may have different methylation patterns, but there are common DNA methylation changes within each cancer type.[27-29] Since DNA methylation is a very consistent feature of cancer compared with highly individualized tumor-specific DNA mutations, assays for detecting aberrant ctDNA methylation are much more widely applicable than mutation-based assays.[30,31]

Interestingly, our present study revealed that the mSEPT9 positivity rate was higher in patients ≥65 years of age than in younger patients. Previous studies reported that aberrant methylation patterns detected in cancer might be present in normal aging cells.[32-35] About 5% of the CpG sites exhibited significant methylation change with the increase in age,[36,37] and approximately half of them are the same genes that are involved in the tumorigenesis of CRC.[38] This may partially explain why elderly patients with CRC showed higher mSEPT9 positivity rate.

Previous studies showed that the dynamics of ctDNA hypermethylation level correlated with treatment response. For example, the blood MGMT hypermethylation levels could predict the response after treatment in patients with metastatic CRC.[39] In another study, the detection of ctDNA methylation markers could predict treatment response and prognosis of HCC.[17] Studies found that the detection sensitivity of the mSEPT9 was positively correlated with the severity of colorectal disease. Patients with higher histological grade and later stage showed a higher positive rate of mSEPT9.[33] Meanwhile, the mSEPT9 level is positively correlated with the tumor size, suggesting that the plasma mSEPT9 level could be an indicator for disease progression or relief.[40] This phenomenon is related to the biological properties of methylation markers, as the amount of ctDNA released from tumor into blood generally correlates with tumor burden. This property is crucial for mSEPT9 to be used for monitoring, as only markers with quantitative relationship with

disease progression or relief can provide meaningful interpretation for assessment. In the present study, the disease control rate was significantly higher in patients with decreased mSEPT9 level after treatment. Our data indicated that plasma mSEPT9 might be a useful biomarker in the evaluation of therapeutic efficacy.

Since the current commercial test only provides qualitative interpretation as it does not plot the standard curve for absolute quantification, we used the $\Delta\Delta C_t$ method adapted for DNA methylation analyses to determine a relative methylation value of SEPT9 as previously described.[15,16] $\Delta\Delta C_t$ value was positively correlated with tumor burden, which means that CRC patients with more severe lesions exhibited higher $\Delta\Delta C_t$ values. Therefore, $\Delta\Delta C_t$ is a relatively good alternative value for continuous disease monitoring. However, to expand the clinical application of mSEPT9 in disease monitoring, quantitative detection is necessary in the future.

There were some limitations in the present study. Firstly, it was a single-center, retrospective study in a heterogeneous patient cohort. More extensive multicenter studies are needed to validate our findings further. Moreover, the number of patients undergoing series mSEPT9 detection was small, which could not provide sufficient power to make sound conclusions.

5 | CONCLUSION

In conclusion, the present study provides evidence that mSEPT9 was a biomarker reflecting tumor burden, and serial detecting of mSEPT9 could be a promising strategy for disease monitoring in CRC patients. Further prospective studies in a larger group of patients are warranted.

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CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

AUTHORS CONTRIBUTIONS

Wei Guo contributed to study concept and design. Huiqin Jiang and Qian Yu performed the experiments, analyzed the data, and wrote the article. Xinning Chen, Chunyan Zhang, Junfei Shen, Minna Shen, Yihui Yang, Beili Wang, and Baishen Pan contributed to the experimental work and the collection of patients' features. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All of the data are available and included in this manuscript.

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