

## Retrospective Study

## Clinical value of preoperative methylated septin 9 in Chinese colorectal cancer patients

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**Abstract****BACKGROUND**

The methylated septin 9 (mSEPT9) assay was the first blood-based test approved by the United States Food and Drug Administration as a colorectal screening test. However, the diagnostic and prognostic role of preoperative mSEPT9 for colorectal cancer (CRC) in Chinese patients is still unknown.

**AIM**

To improve the understanding of diagnostic and prognostic factors, serum mSEPT9 was detected in Chinese CRC patients.

**METHODS**

A retrospective analysis of 354 cases, of which 300 had CRC and 54 were normal, was performed in China. Patients' characteristics, treatments, and laboratory data, including age, the date of surgery, Union for International Cancer Control (UICC) stages, distant metastasis (M), and so on, were collected. Methylation levels of SEPT9 were quantified by quantitative, methylation-specific polymerase chain reaction before surgery. In addition, the effects of mSEPT9 on the occurrence and prognosis of 330 CRC cases from The Cancer Genome Atlas (TCGA) database were evaluated using bioinformatics analyses. Potential prognostic factors for overall survival (OS) and progression-free survival (PFS) were evaluated by Kaplan-Meier univariate analysis.

**RESULTS**

Review Procedures" approved by the National Health and Family Planning Committee of China (No. 11, Section 39), informed consent was waived because of the retrospective nature of the study. After the following circumstances have been reviewed and approved by the ethics committee, the informed consent form can be waived if: Research is conducted using human body materials or data that can identify information, and the subjects can't be found, and the research project does not involve personal privacy and commercial interests.

**Conflict-of-interest statement:** The authors have no conflicts of interest to disclose.

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In Chinese CRC patients, positive mSEPT9 was strongly associated with advanced UICC stages, deeper invasion by the primary tumor, and more distant metastasis. Methylation levels of SEPT9 were stage-dependent and showed a stepwise increase in UICC stages (I–IV), primary tumor categories (T1–T4), regional node categories (N0–N2), and distant metastasis categories (M0–M1). The patients with positive mSEPT9 showed a tendency toward lower PFS. After analyzing TCGA clinical data, the high mSEPT9 group was found to be obviously correlated only with more distant metastasis. The patients with high mSEPT9 levels showed a tendency toward lower OS. Besides, nine meaningful mSEPT9 sites were found to provide guidance for the follow-up studies.

## CONCLUSION

MSEPT9 analysis may add valuable information to current tumor staging. Serum mSEPT9 in Chinese CRC patients appears to offer promising novel prognostic markers and might be considered for monitoring CRC recurrence.

**Key words:** Methylated septin 9; Methylated; Colorectal cancer; Diagnosis; Prognosis

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**Core tip:** This study retrospectively explored the value of serum septin 9 methylation (mSEPT9) in the diagnosis and prognosis of colorectal cancer in a Chinese population. Preoperative mSEPT9 levels in 354 enrolled patients were retrospectively analyzed. In addition, the effects of mSEPT9 on the occurrence and prognosis of 330 colorectal cancer cases from The Cancer Genome Atlas database were evaluated using bioinformatics analyses. Besides, nine meaningful mSEPT9 sites were found to provide guidance for the follow-up studies.

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## INTRODUCTION

Colorectal cancer (CRC) remains the third most common cancer expected to occur in men and women<sup>[1]</sup>, accounting for approximately 10% of the global cancer burden. To date, more than 90% of patients with early CRC have survived five years after diagnosis<sup>[2,3]</sup>. However, in the case of regional spread to lymph nodes or adjacent organs, the five-year relative survival rate decreases to 69%, and when there is distant metastasis, it drops sharply to approximately 10%<sup>[3,4]</sup>. Despite significant recent achievements in the diagnosis and treatment of these patients, resulting in partial reductions in overall incidence and mortality, there is no effective diagnostic assay so far for tumor progression or recurrence monitoring, especially *in vitro*.

Detection of CRC recurrences or metastases in the early stage by constant monitoring may improve long-term outcomes through timely treatment. The American Joint Committee on Cancer (AJCC) Cancer Staging, seventh edition has accepted clinically useful carcinoembryonic antigen (CEA) serum tumor marker as a site-specific prognostic factor in CRC<sup>[5]</sup>. However, the low detection sensitivity of CEA hinders its use for many surgical patients, because patients with negative CEA results before surgery usually cannot be monitored after surgery<sup>[6,7]</sup>. In addition, periodic computed tomography (CT) scanning is another noninvasive method for surgical therapeutic effect assessment<sup>[8]</sup>. However, CT scans have limited sensitivity and high false positive rates, and cannot be used routinely as a monitoring examination due to the danger of long-term radiation<sup>[9]</sup>. Therefore, development of novel, sensitive biomarkers for monitoring recurrences or metastases of CRC is urgently needed.

Hypermethylation of the promoter of septin 9 (SEPT9) has previously been shown to be a sensitive and specific biomarker in various cancers including CRC<sup>[10-13]</sup> and its precursor lesions<sup>[14-16]</sup>. As a result, the methylated SEPT9 (mSEPT9) assay became the first blood-based test approved by the United States Food and Drug Administration

as a CRC screening test<sup>[17]</sup>. A study of Korean CRC patients found that serum mSEPT9 had a tendency to show metastasis and a low disease-free survival rate<sup>[18]</sup>. In a recent study of German CRC patients, mSEPT9 was significantly associated with Union for International Cancer Control (UICC) stages both before and after therapy<sup>[19]</sup>. In addition, quantitative mSEPT9 levels have been successfully applied for the diagnosis of CRC<sup>[19-22]</sup>, and for the screening, diagnosis, monitoring, prognosis, and molecular staging of head and neck squamous cell carcinomas (HNSCC)<sup>[19]</sup>. However, the diagnostic and prognostic role of preoperative mSEPT9 for CRC in Chinese patients is still unknown.

This study assessed the correlation between clinicopathological characteristics and preoperative serum mSEPT9 in Chinese CRC patients and, further, to confirm the correlation between mSEPT9 levels and CRC prognosis by bioinformatics analyses. In addition, we analyzed methylated sites that were co-upregulated or codownregulated in colon and rectum tumors, to provide the theoretical guidance for further research.

## MATERIALS AND METHODS

### *Patients and samples*

This present study was conducted from December 2017 to November 2018 among patients at the Department of Hepatobiliary and Enteric Surgery in Xiangya Hospital. A total of 354 subjects with mSEPT9 serum detection before surgery were recruited from a medicine-pharmacy-nursing integrative parenteral medication rational use and safety early warning platform, the Parenteral Prescription Early Warning and Assessment System, including 300 CRC patients and 54 normal subjects. This study was approved by the Ethical Committee of Xiangya Hospital of Central South University (Approval No. 2018111100).

Three hundred patients presented with histologically confirmed primary CRC. Recurrences or metastases were determined from diagnostic tests (CT scan, magnetic resonance imaging, or colonoscopy) and confirmed through tissue pathology when available<sup>[7]</sup>. Clinical parameters, including mSEPT9 detection results, gender, age, UICC stage, histologic grade, primary tumor (T) categories, regional node (N) categories, distant metastasis categories (M), lymphatic invasion (L), lymph nodal status, vascular invasion (V), and tumor site, were collected. The UICC stage, tumor node metastasis (TNM) categories and histologic differentiation were graded on the basis of the eighth edition of the AJCC<sup>[23]</sup>. Progression-free survival (PFS) time was calculated from the CRC patients' date of surgery to presentation of clinical or pathological evidence of cancer recurrence.

In addition, 330 colorectal adenocarcinomas from The Cancer Genome Atlas (TCGA) Research Network (<http://cancergenome.nih.gov/>) were selected and analyzed retrospectively. Patients whose mSEPT9 levels were less than or equal to median were assigned to the low mSEPT9 group, whereas others were assigned to the high mSEPT9 group. The overall survival (OS) time was calculated from the CRC patients' date of surgery to the date of dead or to the last contact date.

### *Methylated SEPT9 detection*

A 10 mL peripheral blood sample was collected with a 10 mL K2EDTA anticoagulant tube for the SEPT9 assay [BioChain (Beijing) Science and Technology, Inc., Beijing, China]. Peripheral blood sample storage and transportation, DNA extraction, and bisulfite conversion were performed manually following the manufacturer's instructions of the Epi proColon 2.0 kit (Epigenomics AG, Berlin, Germany). The mSEPT9 was assayed with the Epi proColon 2.0 kit on an AB7500 Fast Dx Real Time polymerase chain reaction device (Life Technologies) in the Clinical Laboratory of Xiangya Hospital, Central South University. Briefly, a polymerase chain reaction (PCR) test was performed in triplicate with 15  $\mu$ L template DNA per well and run for 45 cycles<sup>[24]</sup>. The instrument software was used to record the PCR results for  $\beta$ -actin (ACTB) and methylated SEPT9 from each of the triplicate reactions. The validity of each sample batch was determined according to methylated SEPT9 and ACTB threshold count (Ct) values for the positive and negative controls. ACTB served as an internal reference to assess the integrity of each sample. According to the instructions, Ct value was less than 41.1 was assigned to the positive mSEPT9 group, whereas those whose Ct value was over 41.1 were assigned to the negative mSEPT9 group.

### *Statistical analyses*

All statistical analyses were performed using SPSS 18 software (SPSS Inc, Chicago, United States). The measurable data was expressed as the mean and standard deviation (SD). Differences of clinicopathological characteristics and Ct values

between groups were compared *via t*-tests and  $\chi^2$  test. The univariate analysis was performed to assess the effect of mSEPT9 to predict PFS and OS by the Kaplan-Meier method. Binary logistic regression was used to analyze the association between each genetic biomarker (*e.g.*, mismatched repair proteins) and mSEPT9. All the statistical tests were bilateral, and  $P < 0.05$  was considered statistically significant.

## RESULTS

### **The mSEPT9 in CRC patients and normal subjects**

Among Chinese CRC patients, the methylated Ct values of 300 primary CRC patients and 54 normal subjects were analyzed. Based on contradictory trends for Ct value and expression, the preoperative serum mSEPT9 levels were significantly higher in CRC patients than in the normal subjects ( $P = 0.008$ ) (Figure 1A). The positive rate of mSEPT9 was 52.3% for CRC patients and 25.9% for normal subjects ( $P = 0.102$ ) (Figure 1B).

Among 351 patients from the TCGA database, the mSEPT9 levels of 330 CRC patients and 21 normal subjects were analyzed. The serum mSEPT9 levels of the CRC patients were higher than those of the normal subjects, but were not statistically significant ( $P = 0.530$ ) (Supplemental Figure 1).

### **Clinicopathological characteristics and mSEPT9 in CRC**

The Chinese CRC patients, clinicopathological features of 300 CRC patients are described in Table 1. As shown in Figure 1C, patients older than 50 years were statistically more numerous than those aged 50 or younger in both positive and negative groups ( $P = 0.016$ ). Through analyzing UICC stages, we found that the positive rate of stage III was observably higher than stage I (46.6% *vs* 31.0%,  $P = 0.012$ ) (Figure 2A); mSEPT9 levels showed a significant increase from UICC stages II to III ( $P = 0.033$ ) and stages III to IV ( $P < 0.0001$ ), but no obvious difference was detected between stages I to II ( $P = 0.898$ , Figure 3A).

In addition, the association of mSEPT9 levels and rate of positive mSEPT9 among primary tumor categories (T1–T4), regional node categories (N0–N2) and distant metastasis categories (M0–M1) were also analyzed. The detection rate of positive T3 was observably higher than that of T1 (51.1% *vs* 40.0%,  $P = 0.019$ ) (Figure 2B). Positive rate and levels of mSEPT9 revealed a significant increase from T3 to T4 ( $P = 0.030$ ,  $P = 0.046$ , respectively) (Figure 2B, Figure 3B). In terms of regional node categories, N0 to N2 showed a gradual increase in mSEPT9 levels ( $P = 0.012$ ) (Figure 3C), but did not show any association with the rate of positive mSEPT9 (Figure 2C). As shown in Figures 2D and 3D, mSEPT9 showed the best ability to discriminate between local and metastatic CRC ( $P = 0.015$ ,  $P < 0.0001$ , respectively). However, higher mSEPT9 levels were not found in CRC patients with lymphatic or vascular invasion than in those without invasion (all  $P > 0.05$ ). We also failed to find association among MLH1, MSH2 (25D12), MSH6, PMS2, and Ki67 and mSEPT9 (all  $P > 0.05$ ) (Supplemental Table 1).

The clinicopathological features of 330 CRC patients from the TCGA database are described in detail in Supplemental Table 2. Similarly, there was a tendency for more distant metastasis ( $P = 0.0001$ ) and more CRC patients older than 50 ( $P < 0.0001$ ) in the high mSEPT9 group, but no significant difference was found in UICC stages, primary tumor categories, or regional node categories (all  $P > 0.05$ ).

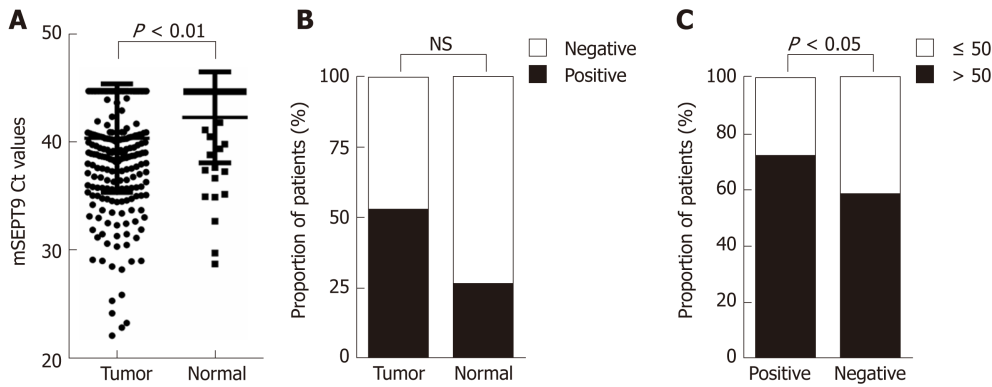
### **Prognostic significance of mSEPT9 in CRC patients**

Kaplan-Meier univariate analysis showed that positive mSEPT9 was obviously associated with shorter PFS among the Chinese CRC patients ( $P = 0.019$ , Figure 4A). The positive mSEPT9 CRC cases were estimated to have an mean PFS duration of 3.7 mo [95% confidence interval (CI): 2.14–5.19] compared with the 6.0 mo (95% CI: 0–13.87) in the negative mSEPT9 CRC cases.

In addition, serum mSEPT9 showed prognostic significance for the CRC patients from the TCGA database ( $P = 0.008$ , Figure 4B). CRC patients with low mSEPT9 levels were found to be correlated with longer OS. The low mSEPT9 CRC cases had an estimated mean OS duration of 8.1 months (95% CI: 6.53–9.27) compared with the 5.1 mo (95% CI: 3.87–6.33) in the high mSEPT9 CRC cases.

### **Significant methylation sites for SEPT9**

In further analyzed TCGA clinical data, 124 mSEPT9 sites were found that showed differential expression among normal subjects and those with colon and rectum adenocarcinoma, respectively (all  $P < 0.05$ ) (Supplemental Figure 2). After analyzing the detailed information of these 124 mSEPT9 sites, 68 co-upregulated and 36 co-downregulated mSEPT9 sites in CRC adenocarcinoma were further observed. We finally confirmed that there were eight co-upregulated mSEPT9 sites (Figure 5) and



**Figure 1** Graphical representations of difference in methylated septin 9 Ct values or proportion of patients between different groups. A: Methylated septin 9 (mSEPT9) Ct values in tumor group and normal group; B: Proportion of patients with positive and negative mSEPT9 in tumor group and normal group; C: Proportion of patients older than 50 and aged 50 or younger in positive group and negative group. The statistical significance for difference of means is shown in P values, *t*-test, or  $\chi^2$  test. mSEPT9: Methylated septin 9.

one co-downregulated mSEPT9 site (cg02975107) through setting a cut-off of a two-fold expression change of mSEPT9.

## DISCUSSION

Most patients with early CRC undergo curatively intended surgery to clear up primary lesions and local lymph node metastasis up. However, 30%-50% of patients would still confront tumor recurrence and might die from metastasis<sup>[25]</sup>. Timely monitoring of recurrence and metastasis is of great significance to the prognosis and survival of patients. In our study, mSEPT9 was proved to be an effective bio-marker for diagnosis, recurrence, and prognosis of CRC in Chinese patients, and nine significant mSEPT9 sites were confirmed for further in-depth consideration.

Our study confirmed the value of serum mSEPT9 for CRC diagnosis. Compared with normal tissues in Chinese and TCGA data, serum SEPT9 was found to be hypermethylated in tumor tissues, which was consistent with previous studies<sup>[18,19,26]</sup>. Studies showed that age affected the detection rate of the SEPT9 assay<sup>[27,28]</sup>, and we found that a positive rate of mSEPT9 was strongly associated with CRC patients aged over 50 years both in Chinese and TCGA data. This accords with the definition of an average risk population in National Comprehensive Cancer Network Guidelines for CRC<sup>[29]</sup>. Remarkably, we reported that SEPT9 performs outstandingly as an auxiliary molecular staging parameter in the Chinese population, especially because mSEPT9 levels could distinguish between pathological UICC and TNM stages in an incremental fashion. In addition, our data demonstrated that CRC patients in earlier tumor stages showed lower mSEPT9 levels compared to those with more advanced lesions, which is consistent with studies in German CRC patients<sup>[19,30-32]</sup>. Most importantly, its ability to identify patients with distant metastases emphasizes the potential of mSEPT9 as a bio-marker, which adds valuable information to the classification of tumors<sup>[33-35]</sup>. However, high mSEPT9 group did not show any association with UICC, T, or N stages in patients from the TCGA database, who were from American Indian, Asian, Black, or African American populations. This might be explained by the different study populations. Previous studies found that the incidence of CRC and the sensitivity to the mSEPT9 test assay in different ethnic groups were different<sup>[14,36]</sup>.

In addition, serum mSEPT9 were proved to be an independent predictors of CRC recurrence and unfavorable cancer-specific survival in Chinese and TCGA data, which is consistent with previous studies in Singapore and Germany<sup>[19,37,38]</sup>. The study was performed with a large number of prognostic features and patients; however, much longer prognosis and follow-up time are necessary before final conclusions can be made, and the increasing number of patients with earlier-stage CRC demands a widening of the clinical importance of predictive value for prognosis.

After further analysis of the TCGA clinical data, we obtained nine SEPT9 methylation sites that show two-fold higher or lower mSEPT9 levels in CRC than normal tissues. However, no studies were found that investigate the prognosis of these methylation sites and CRC was found. Surprisingly, cg12783819, which only shows 1.5-fold higher mSEPT9 levels in CRC than in normal tissues, has been proven to be able to assess the diagnosis, prognosis, and molecular staging of

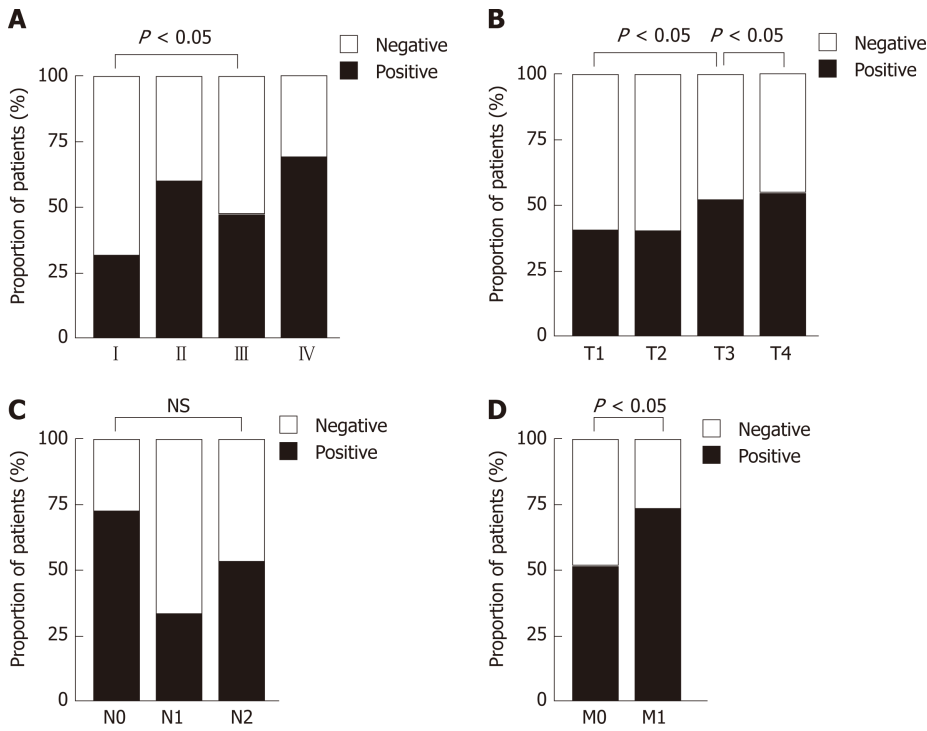
**Table 1 Clinicopathological characteristics based on methylated septin 9 status in 300 colorectal cancer patients**

Parameter		Positive group (%)	Negative group (%)	P
Gender	Male	84 (28.0)	81 (27.0)	0.585
	Female	73 (24.3)	62 (20.7)	
Age	≤ 50 yr	45 (15.0)	60 (20.0)	0.016
	> 50 yr	112 (37.3)	83 (27.7)	
UICC stage	I	9 (3.0)	20 (6.7)	0.020
	II	46 (15.3)	32 (10.7)	
	III	62 (20.6)	71 (23.7)	
	IV	13 (4.3)	6 (2.0)	
	Unknown	27 (9.0)	14 (4.7)	
Histologic grade	Low level	115 (38.3)	103 (34.3)	0.972
	High level	18 (6.0)	17 (5.7)	
	Not recorded	24 (8.0)	23 (7.7)	
Primary tumor (T) category	T1	2 (0.7)	3 (1.0)	0.002
	T2	15 (5.0)	23 (7.7)	
	T3	70 (23.3)	67 (22.3)	
	T4	40 (13.3)	34 (11.3)	
	Not recorded	30 (10.0)	16 (5.3)	
Regional node (N) category	N0	57 (19.0)	23 (7.7)	0.852
	N1	32 (10.7)	67 (22.3)	
	N2	37 (12.3)	34 (11.3)	
	Not recorded	31 (10.3)	16 (5.3)	
Distant metastasis (M)	Absent	141 (47.0)	137 (45.7)	0.015
	Present	16 (5.3)	6 (2.0)	
Lymphatic invasion (L)	Absent	58 (19.3)	60 (20.0)	0.217
	Present	51 (17.0)	52 (17.3)	
	Not recorded	48 (16.0)	31 (10.3)	
Lymph nodal status	No node involved	51 (17.0)	52 (17.3)	0.048
	1-3 lymph node involved	27 (9.0)	40 (13.3)	
	> 4 lymph node involved	31 (10.3)	20 (6.7)	
	Not recorded	48 (16)	31 (10.3)	
Vascular invasion (V)	Absent	36 (12.0)	31 (10.3)	0.278
	Present	70 (23.3)	76 (25.3)	
	Not recorded	51 (17.0)	36 (12.0)	
Tumor site <sup>a</sup>	Left colon	44 (14.7)	31 (10.3)	0.022
	Right colon	27 (9.0)	11 (3.7)	
	Rectum	74 (24.7)	86 (28.6)	
	Unable to distinguish	12 (4.0)	15 (5.0)	

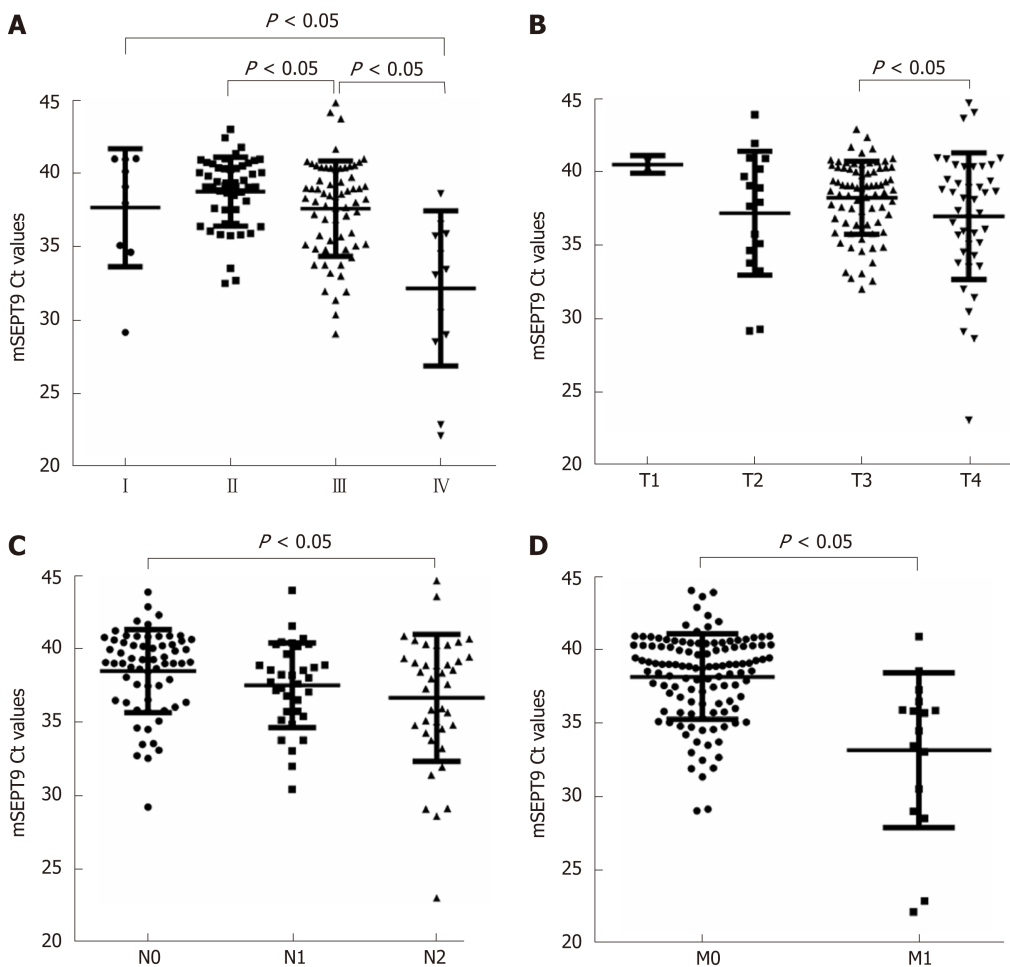
<sup>a</sup>Right colon includes cecum through transverse colon, whereas left colon includes splenic flexure, descending colon, and sigmoid colon. Clinicopathological characteristics between positive group and negative group were analyzed using  $\chi^2$  test. UICC: Union for International Cancer Control.

German HNSCC and CRC patients<sup>[19,20]</sup>. The result prompts us to explore the potential association between these nine methylation sites and in Chinese CRC patients in the future.

In conclusions, serum SEPT9 methylation testing is a powerful additional diagnostic tool and promising, novel prognostic markers. Patients with initially high mSEPT9 levels may benefit from intensive therapy and close monitoring of disease development, thereby improving outcomes for CRC patients. These patients may benefit from early systemic treatment.

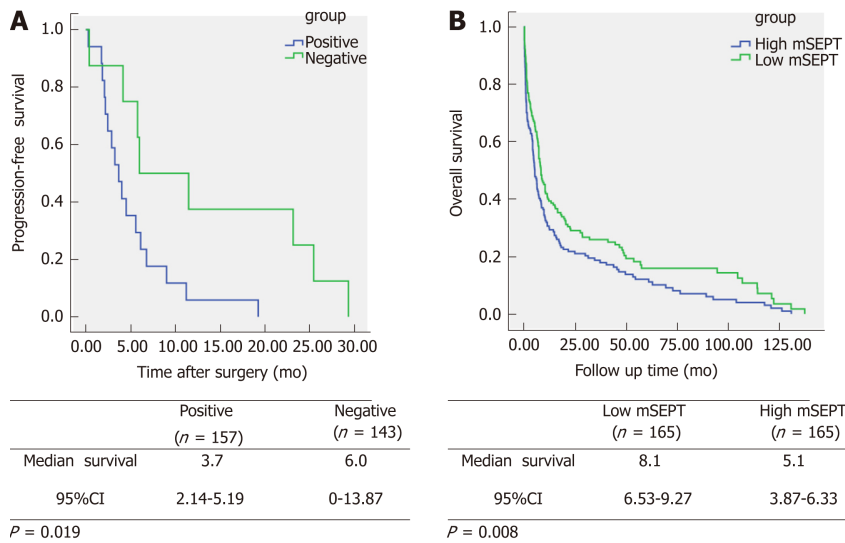


**Figure 2** Graphical representations of the proportion of patients with positive and negative methylated septin 9 with different tumor status. A: Union for International Cancer Control stages; B: Primary tumor categories; C: Regional node categories; D: Distant metastasis categories. The statistical significance for difference of means is shown in *P* values and  $\chi^2$  test).

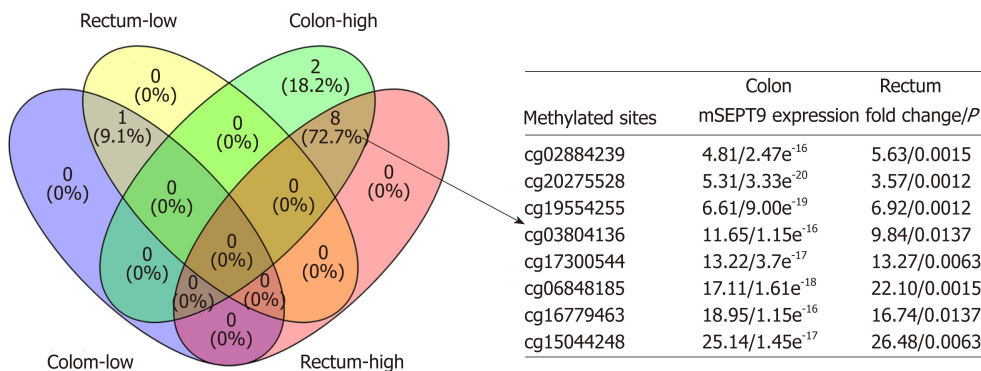


**Figure 3** Graphical representations of methylated septin 9 Ct values in different tumor status. A: Union for International Cancer Control stages; B: Primary

tumor categories; C: Regional node categories; D: Distant metastasis categories. The statistical significance for difference of means is shown in *P* values and *t*-test. mSEPT9: Methylated septin 9.



**Figure 4** Kaplan-Meier univariate survival curves according to methylated septin 9 status. A: Progression-free survival time; B: Overall survival. The statistical significance for difference of means shown in *P* values and Kaplan-Meier univariate analysis. mSEPT: Methylated septin; CI: Confidence interval.



**Figure 5** Venn diagram of eight co-upregulated methylated septin 9 sites and one co-downregulated methylated septin 9 site in colon and rectum adenocarcinoma. "Rectum-low" and "Rectum-high" represented sites that showed low or high expression in rectum adenocarcinoma, and "Colon-low" and "Colon-high" represented sites that showed low or high expression in colon adenocarcinoma. Eight co-upregulated methylated septin 9 (mSEPT9) sites also showed mSEPT9 expression fold of rectum/ colon adenocarcinoma compared to normal subjects and corresponding *P* value. mSEPT: Methylated septin.

## ARTICLE HIGHLIGHTS

### Research background

The methylated septin 9 (mSEPT9) assay was the first blood-based test approved by the United States Food and Drug Administration as a colorectal screening test. Previous researchers found that mSEPT9 was a powerful screening, diagnostic, monitoring, and prognostic tool for German colorectal cancer (CRC) patients. However, the diagnostic and prognostic value of mSEPT9 in Chinese CRC patients is still unknown, and may be affected by differences in ethnicity and socioeconomic status.

### Research motivation

To explore the diagnostic and prognostic value of serum mSEPT9 for Chinese CRC patients.

### Research objectives

This study aimed to explore the diagnostic value of preoperative serum mSEPT9 in the Chinese population, and then assess the value of quantitative mSEPT9 levels for CRC staging. In addition, Chinese population and TCGA database information were combined to determine the prognostic significance of mSEPT9 by bioinformatics analyses.



### Research methods

Three hundred fifty-four subjects (300 CRC, 54 normal) from China and 351 subjects (330 CRC, 21 normal) from the TCGA database including American Indian, Asian, Black, and African American populations were retrospectively analyzed. Preoperative mSEPT9 levels were quantified by quantitative methylation-specific polymerase chain reaction. Kaplan-Meier univariate assay was performed to analyze potential prognostic factors including overall survival (OS) and progression-free survival (PFS).

### Research results

In Chinese CRC patients, positive mSEPT9 and quantitative mSEPT9 levels were strongly associated with clinico-pathological parameters. The patients with positive mSEPT9 showed a tendency toward lower PFS. Higher mSEPT9 levels were correlated with more distant metastasis among the TCGA database patients, and patients with high mSEPT9 levels showed a tendency toward lower OS.

### Research conclusions

Testing for mSEPT9 is a powerful diagnostic and promising prognostic tool for Chinese CRC patients; it may add valuable information to current tumor staging and holds the potential to monitor CRC recurrence.

### Research perspectives

This study assessed the correlation between clinicopathological characteristics and preoperative serum mSEPT9 in Chinese CRC patients and, further, to confirm the correlation between mSEPT9 levels and CRC prognosis by bioinformatics analyses. In addition, we analyzed methylated sites that were co-upregulated or co-downregulated in colon and rectum tumors, to provide the theoretical guidance for further research.

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