



OSMR and SEPT9: promising biomarkers for detection of colorectal cancer based on blood-based tests

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Background: Early screening and monitoring of the stage of colorectal cancer (CRC) is crucial for successful treatment and patient survival. Our purpose was to identify that evaluating methylated oncostatin M receptor (mOSMR) and Septin 9 (mSEPT9) in plasma DNA could be a feasible method for CRC diagnosis.

Methods: Plasma samples (40 samples have matched cancer and adjacent non-cancer tissues) were collected from 187 patients with CRC, 25 patients with polyps and 109 healthy controls. qRT-PCR assay was used to analyze SEPT9 and OSMR mRNA levels. DNA bisulfite treatment, the levels of mSEPT9 and mOSMR in CRC tissues and plasma samples were evaluated by using fluorescence-based real-time PCR assay (MethyLight). mSEPT9 and mOSMR levels in plasma were compared before and after curative resection. Serum CEA, CA19-9, CA72-4 and CA242 were also measured and analyzed in parallel.

Results: SEPT9 and OSMR mRNA levels were significantly lower in CRC tissues compared with those in their adjacent noncancerous tissues ($P=0.006$ and 0.002 , respectively). mSEPT9 and mOSMR were significantly higher in CRC tissues compared with those in their adjacent non-cancerous tissues ($P<0.0001$). Plasma mSEPT9 (cut-off value: 0.777) and mOSMR (0.796) had a sensitivity of 62.6% and 74.9% for CRC, and a specificity of 91.7% and 86.2%, respectively. mSEPT9 and mOSMR were positive in 12.0% and 20.0% in polyps, respectively. Moreover, combined mSEPT9 and mOSMR (77.0%) showed a higher sensitivity than CEA (32.9%), CA72.4 (20.4%), CA199 (13.9%) and CA242 (23.9%), and these blood biomarkers together had a higher sensitivity (83.0%) compared with these individual blood biomarkers.

Conclusions: OSMR methylation (mOSMR) in plasma is useful for diagnosis of CRC as a new biomarker, and the panel of tests to analyze mSEPT9, mOSMR, CEA, CA19-9, CA72-4 and CA242 appears to be feasible and convenient for the diagnosis of CRC.

Keywords: Septin 9 (SEPT9); Oncostatin M Receptor (OSMR); colorectal cancer (CRC); plasma; serum biomarkers

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Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, and its incidence rates are still rapidly increasing in several areas with an aging population and increasing changes in lifestyle habits (1). Taking precaution through CRC screening and the removal of precancerous polyps could dramatically decrease incidence rates (2,3). Several screening options such as colonoscopy, fecal occult blood tests (FOBTs), air contrast barium enemas and CT virtual endoscopy could help to alleviate the incidence of CRC (2,4-6). Colonoscopy is an effective method for early detection, but it has a limited application due to its expensive cost, painful colonic procedure and risk of intestinal perforation (7). FOBT is still a commonly used auxiliary diagnostic method worldwide. However, it has low sensitivity and poor specificity and cannot be used for screening high-risk groups (2). Air contrast barium enema and CT virtual endoscopy are vulnerable to the influence of the intestinal cleaning state. The former has the advantages of the inspection of the whole colon, lacking biopsy or polyps; the latter is costly and requires specialized equipment and techniques. Both methods are not widely accepted by patients (4). Thus, a non-damaging, successful and simple method for screening is needed.

Cancer-specific DNA methylation is common and has been shown to be an early event in several cancer types (8,9). DNA methylation, mRNA and microRNA can be analyzed in stool samples (10-13). However, the transportation and processing of samples can be very inconvenient. The detection and monitoring of circulating tumor-derived DNA in the cancer patient plasma offer exciting opportunities for tumor treatment (14,15). Changes in DNA methylation levels, variations in cancer-associated copy number, and gene mutations have been found in the plasma of patients with different cancer types (16,17). Most of these studies on plasma DNA as a cancer marker have focused on the detection of specific molecular targets by PCR-based methods, such as shotgun massively parallel sequencing (MPS), fluorescence-based real-time PCR assay (MethyLight) and digital PCR (18-21).

In the present study, we analyzed OSMR, which is methylated frequently in CRC and rarely in cancers of non-digestive organs (22). We also analyzed SEPT9 as a reference to select the best candidates indicative of CRC for early diagnosis and monitoring of markers in plasma samples (23-26). SEPT9 is an existing biomarker for

detecting CRC in blood and is commercially available from the Epigenomics Company. In this study, we aimed to evaluate the effectiveness in CRC diagnosis by analyzing the methylation of SEPT9 and OSMR in plasma, and serum biomarkers (Carcinoembryonic antigen: CEA; carbohydrate antigen: CA19-9, CA72-4 and CA242) were also analyzed in parallel. These serum biomarkers are currently used in routine early diagnosis and for monitoring tumor markers for gastrointestinal neoplasm in our hospital.

Methods

Ethics

The study was approved by the ethics committee of Peking University Cancer Hospital & Institute. Written informed consent was obtained from all participants for obtaining blood or tissue samples.

Human CRC plasma and tissue specimens

Plasma specimens, including 187 patients with CRC (100 males and 87 females, mean age 63.8 years), 25 polyp patients (17 males and 8 females, mean age 50.6 years) and 109 healthy controls (46 males and 63 females, mean age 60.8 years), were gathered from November 2011 to April 2013. About 40 paired tissues matching these plasma were collected immediately after operation and stored in a -80 °C freezer. Inflammatory bowel disease, familial adenomatous polyposis and hereditary non-polyposis CRC were not included in this study. Approximately 109 healthy adults were eligible controls recruited from employee physical examination at Peking University Cancer Hospital & Institute. The control cancer histories were checked in past medical examination records and the subjects provided informed consent for the detection of their methylation state. Plasma samples of polyp patients were obtained from the Sixth Affiliated Hospital, Sun Yat-sen University, and collected before colonoscopy. Plasma samples of CRC patients were collected before surgical resection. None of the patients with CRC received chemotherapy, radiotherapy or any surgical intervention.

Five-milliliter peripheral blood samples were collected using EDTA-containing tubes before any therapeutic intervention at the Department of Gastrointestinal Surgery, Peking University of Cancer Hospital & Institute. The tumor tissues of the CRC patients were obtained during

Table 1 Primer sequences used in the study

Primers	Sequence (5'-3')
mOSMR-MF	TAGGAGTAGCGTTGCGCGC
mOSMR-MR	CCCGAACTTTACGAACGAACG
mSEPT9-MF	AAATAATCCCATCCAACCTA
mSEPT9-MR	GATT-dSp-GTTGTTTATTAGTTATTATGT
mSEPT9-Blocker	GTTATTATGTTGGATTTTGTGGT- TAATGTGTAG-SpC3
mCOL2A1-MF	TCTAACAAATTATAAACTCCAACCACCAA
mCOL2A1-MR	GGGAAGATGGGATAGAAGGGAATAT
OSMR-F	CCTACCTGAAAACCAGAACTGATG
OSMR-R	CAGGAGTCAATGGTAAACGTTTCAG
SEPT9-F	AGTTCAACATCATGGTGGTTCGG
SEPT9-R	ACCCTGGTGTGTCAATCACTGTC
GAPDH-F	GAAGGTGAAGGTCGGAGT
GAPDH-R	GAAGATGGTGATGGGATTTTC
mOSMR-probe	6FAM-CGGGGTACGGAGTTTCGGTC- MGB-1
mSEPT9-probe	6FAM-TTAACCGCGAAATCCGAC-BHQ-1
mCOL2A1-probe	6FAM-CCTTCATTCTAACCCAATACCTATC CCACCTCTAAA-BHQ-1

OSMR, oncostatin M receptor.

their cancer resection surgeries.

RNA isolation, quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, California, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences used are listed in *Table 1*.

DNA extraction in tissue and plasma samples

Total DNA of frozen tissue was extracted by a TIANamp genomic DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The DNA was eluted in 50 μ L elution buffer, and its concentration was measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher

Scientific, Wilmington, Delaware, USA) and finally stored in a -80°C freezer.

Plasma preparation was performed on all of the peripheral blood samples by centrifugation at 2,000 rpm for 10 min at 4°C , and the plasma portion was recentrifuged at 12,000 rpm for 10 min at 4°C . Plasma samples were stored at -80°C until needed. Cell-free DNA molecules from 2 mL plasma were extracted according to the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, California, USA). During isolation of plasma DNA, carrier RNA (Qiagen, Valencia, California, USA) was added to increase the production.

DNA bisulfite treatment and MethyLight analysis of SEPT9 and OSMR

Sodium bisulfite conversion of tissue and plasma DNA was performed using the EZ DNA methylation-gold kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's protocol. Subsequently, 1 μ L of the eluted tissue and 3 μ L of plasma DNA were used for each respective MethyLight PCR reaction.

Bisulfite-treated DNA was analyzed by a fluorescence-based real-time PCR assay, which was described previously as MethyLight. One pair of primers and a probe for each gene (*OSMR*, *SEPT9* and *COL2A1*) were designed specifically to bind to the bisulfite-converted DNA (*Table 1*). The *COL2A1* gene was analyzed to control DNA amplification and normalized for input DNA. Specificity of the reactions for methylated DNA was confirmed by separately amplifying positively methylated cell line RKO DNA and unmethylated human control DNA with each set of primers and probes. PCRs were carried out in 20 μ L volumes containing 1 \times PCR buffer, 5 mM MgCl_2 , 250 μ M dNTP mixture, 0.5 μ M of each primer, 0.3 μ M of each probe, 1 or 3 μ L bisulfite-treated tissue or plasma DNA, and 0.05 U/ μ L Taq DNA polymerase (HotStar Taq plus, Qiagen, Valencia, California, USA). PCR was carried out in the ABI 7500 fast (Life Technologies, Carlsbad, CA, USA) using the following conditions: 95°C for 5 min, followed by 55 cycles of 94°C for 30 s, 60°C for 60 s. The Ct values of each gene were normalized to the Ct values of *COL2A1*.

Carcinoembryonic antigen (CEA), CA199, CA72.4 and CA242 test

All blood samples for the CEA, CA19-9, CA72-4 and

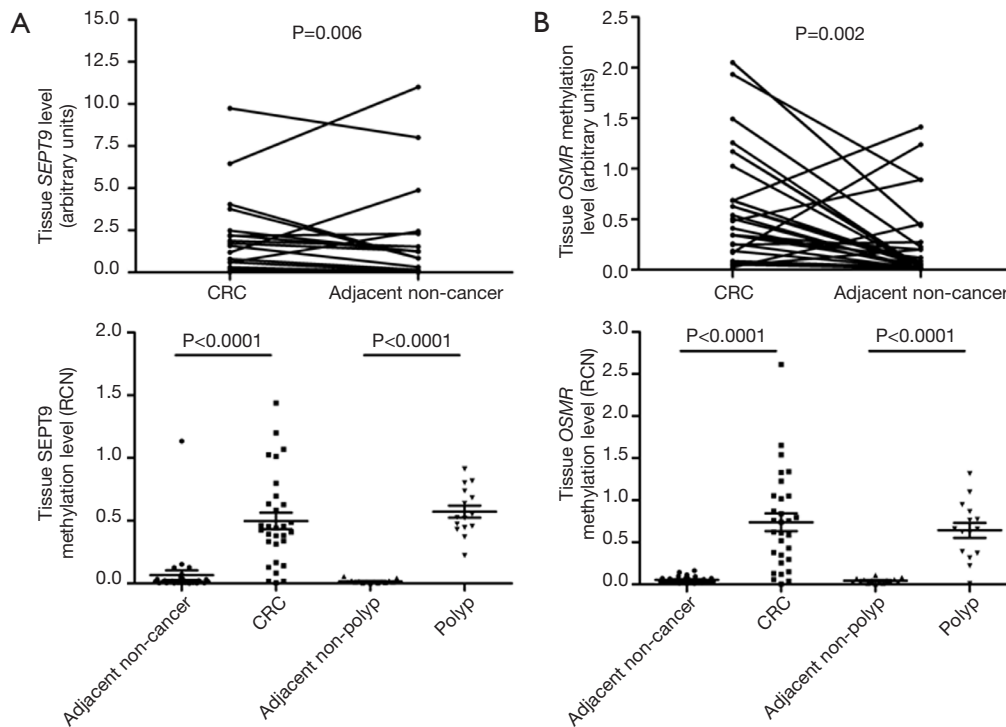


Figure 1 Relative mRNA expression of (A, left upper panel) SEPT9 and (A, right upper panel) OSMR expression in colorectal cancer (CRC) and adjacent non-cancer tissues. (B, left lower panel) SEPT9 and (B, right lower panel) OSMR methylation (mOSMR) in paired CRC tissues and paired polyp tissues. P values were analyzed by the Wilcoxon matched pairs test and the non-parametric t-test.

CA242 tests were analyzed in the clinical laboratory of Peking University Cancer Hospital. Serum levels of CEA, CA199, CA72.4 and CA242 that were higher than 5.0, 37, 6.7 and 20 $\mu\text{g/L}$, respectively, were considered to be in the pathological range.

Statistical analysis

Statistical analysis was carried out by Statistical Analysis System (8.1; SAS Institute, Cary, NC, USA) and Graphpad Prism 5.0 (GraphPad software, San Diego, CA, USA). The non-parametric t test was used to calculate the difference in bisulfite-converted DNA between paired tissue samples. The Wilcoxon matched-pairs test was used to analyze the differences in expression levels between paired tissue samples. The χ^2 test was used to explore associations between methylation and serum biomarker levels and clinicopathological features. The selection of cut-off values was based on the receiver operating characteristics (ROC) curve. $P < 0.05$ was taken as the statistical significance level.

Results

SEPT9 and OSMR mRNA expression in CRC tissues and their methylation levels in CRC and polyp tissues

We compared SEPT9 expression in 30 paired CRC tissue samples and their adjacent non-cancer tissue samples by qPCR. SEPT9 was found to be down-regulated significantly compared with their adjacent non-cancer tissues ($P = 0.006$). Then SEPT9 methylation (mSEPT9) was analyzed in 30 paired CRC tissue samples and 15 paired polyp tissue samples. There was a significantly higher mSEPT9 level in tumors than in adjacent non-cancer tissue samples ($P < 0.0001$), and the methylation levels in polyp tissue samples were also higher than those of the normal counterparts ($P < 0.0001$) (Figure 1A).

We analyzed OSMR expression level in 30 paired CRC tumors and their adjacent non-cancer tissues by quantitative real-time PCR (qPCR). OSMR was found to be down-regulated significantly in primary tumor specimens compared with their adjacent non-cancer tissues ($P = 0.002$). OSMR methylation (mOSMR) was analyzed in

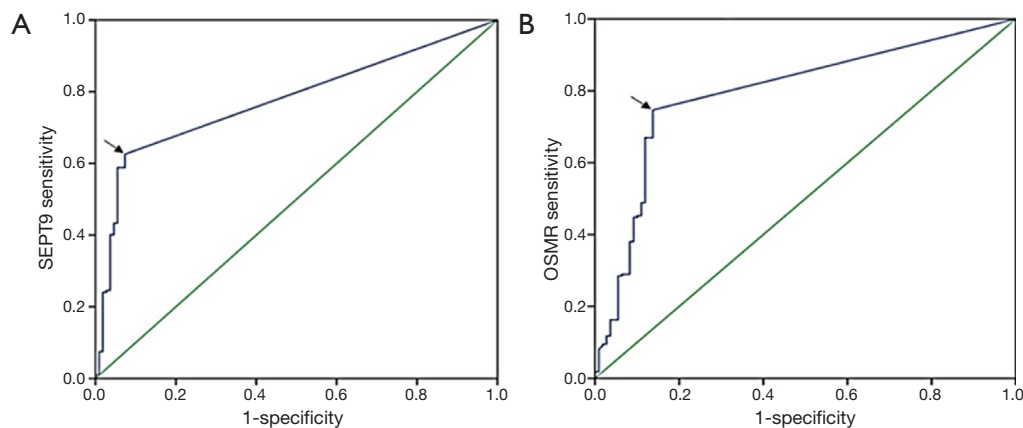


Figure 2 Using the plasma methylation state of (A, left upper panel) SEPT9 and (A, right upper panel) OSMR in normal and CRC patients, receiver operating characteristics (ROC) curves were plotted. SEPT9 and OSMR yielded area under the curve (AUC) values of 0.777 and 0.796 (showed by the black arrow), respectively.

Table 2 Sensitivity of blood-based tumor markers and specificity of blood-based markers in CRC and polyp cases and controls

Tumor marker	Criterion	CRC sensitivity	Polyp sensitivity	Normal specificity
mSEPT9	RCN>0	62.6% (117/187)	12.0% (3/25)	91.7% (100/109)
mOSMR	RCN>0	74.9% (140/187)	20.0% (5/25)	86.2% (94/109)
mSEPT9+mOSMR	RCN>0	77.0% (144/187)	28.0% (7/25)	81.7% (89/109)
CEA	>5.0 µg/L	32.9% (56/170)		
CA72-4	>6.7 µg/L	20.4% (33/162)		
CA19-9	>37 µg/L	13.9% (23/165)		
CA242	>20 µg/L	23.9% (39/163)		
Combination		83.0% (132/159)		

OSMR, oncostatin M receptor; RCN, relative copy number; CRC, colorectal cancer.

30 paired CRC tissue samples and 15 paired polyp tissue samples. mOSMR level was significantly higher in tumors than those in adjacent non-cancer tissue ($P<0.0001$). Methylation levels in polyp tissue samples were also higher than in normal tissues ($P<0.0001$) (Figure 1B).

Sensitivity of blood-based tumor markers for CRC and polyp patient detection

The ROC curve based on the methylation level in plasma of CRC patients and normal controls had an area under the curve value of 0.777 for SEPT9 and 0.796 for OSMR, respectively (Figure 2). As shown in Table 2, mSEPT9 was positive in 117 (62.6%) patients with CRC, 3 (12.0%) patients with polyps, and the specificity in healthy controls was 100 (91.8%) healthy controls using the 1/3 analysis

method. mOSMR was positive in 140 (74.9%) patients with CRC, 5 (20.0%) patients with polyps, and the specificity was 94 (86.2%) healthy controls using the 2/3 analysis method. A combination of mSEPT9 and mOSMR improved sensitivity to 77.0% but decreased true-negatives to 81.7% (89/109) in CRC patients.

Among these 221 CRC patients, CEA, CA72.4, CA199 and CA242 were positive in 56 (32.9%), 33 (20.4%), 23 (13.9%) and 39 (23.9%) patients respectively. The combination of methylation and serum markers dramatically improved sensitivity to 83.0% in CRC patients (Table 2).

Association of biomarkers in blood with clinicopathological factors

The associations of mSEPT9 and mOSMR in plasma, serum

Table 3 Sensitivities of blood biomarkers based on CRC patient clinicopathological variables

Tumor marker	Sensitivity		P value	Sensitivity		P value
	I+II	III+IV		Rectal	Colon	
mSEPT9	57.1% (60/105)	69.5% (57/82)	0.083	67.6% (73/108)	55.7% (44/79)	0.097
mOSMR	74.4% (78/105)	76.8% (63/82)	0.689	77.8% (84/108)	72.2% (57/79)	0.378
mSEPT9+mOSMR	78.1% (82/105)	76.8% (63/82)	0.837	77.8% (87/108)	73.4% (58/79)	0.248
CEA	24.4% (22/94)	44.7% (34/76)	0.030	36.3% (37/102)	27.9% (19/68)	0.257
CA19-9	4.4% (4/91)	25.7% (19/74)	0.001	12.1% (12/99)	16.7% (11/66)	0.409
CA72-4	14.6% (13/89)	27.4% (20/73)	0.044	19.6% (19/97)	21.5% (14/65)	0.763
CA242	15.9% (14/88)	33.3% (25/75)	0.009	22.4% (22/98)	26.2% (17/65)	0.587
Combination	81.6% (71/87)	84.7% (61/72)	0.603	86.7% (85/98)	77.0% (47/61)	0.114

OSMR, oncostatin M receptor; P values were analyzed by χ^2 test.

Table 4 Methylation levels of mSEPT9 and mOSMR in paired tumor and plasma samples

Case No. in plasma	Case No. in tumors					
	mSEPT9		P value	mOSMR		P value
	Positive	Negative		Positive	Negative	
Positive	28	0	0.001	30	0	0.058
Negative	7	5		8	2	

RCN, relative copy number; OSMR, oncostatin M receptor.

biomarkers or their combination with clinicopathological features are shown in *Table 3*. When we compared mSEPT9 and mOSMR level in colon and rectal cancers, we did not find a significant difference between the two groups (for mSEPT9, $P=0.097$ and for mOSMR, $P=0.378$). There was also no significant difference for serum biomarkers between the colon and rectal cancer groups ($P>0.05$). mSEPT9 was 57.1% and 69.5% positive for stage I+II and III+IV CRC cases ($P=0.083$), respectively. mOSMR was 74.4% and 76.8% positive for stage I+II and III+IV CRC cases ($P=0.689$), respectively. There were significant differences ($P=0.030$, 0.001, 0.044 and 0.009, for CEA, CA199, CA72.4 and CA242, respectively) between the stage I+II and III+IV patients with positive CEA, CA199, CA72.4 and CA242 levels, respectively. A combination of methylation and serum markers improved stage I+II and III+IV detection to 81.6% and 84.7% ($P=0.603$), respectively.

Comparisons of mSEPT9 and mOSMR levels in plasma and their matched tumors

We selected 40 CRC tissue samples that expressed mSEPT9 and mOSMR and collected their matched plasma to

analyze the correlation in the methylation level of these two markers. SEPT9 methylation levels were 87.5% vs. 70.0% in CRC tissues and their matched plasma, and the levels for mOSMR were 95.0% vs. 75.0%. A correlation between the methylation levels in tumor tissues and plasma was present in SEPT9 ($P=0.001$) but not in OSMR ($P=0.058$). Unmethylated SEPT9 and OSMR were observed in 12 and 10 plasma samples, respectively. No false-positive was observed in these two markers in plasma samples (*Table 4*).

Discussion

To reduce the mortality of CRC, CRC screening is needed to identify tumors at earlier stages. However, current screening methods for clinical application are either invasive (i.e., colonoscopy) or have low sensitivity and specificity (i.e., CEA, CA199 and FOBT). With a case-control study, mSEPT9 in plasma was consistently demonstrated to be useful for detecting CRC in several clinical studies in several countries (America, Germany, China and Korea) (25,27,28). The CRC detection rates with mSEPT9 ranged from 36.6% to 90% with different methods (11,16,17,24,25,29). Therefore, we believe that a panel of blood-based

methylation tests will be valuable in screening.

In the present study, mSEPT9 and mOSMR were selected for evaluation as noninvasive plasma screening markers for CRC in Chinese CRC patients. mSEPT9 is a familiar marker that was developed by the Epigenomics company (25,30,31). The overall sensitivity in our study was 62.6%, and the overall specificity was 91.7%. The sensitivity and specificity of mSEPT9 were similar to the previous reports. mOSMR has been reported to be frequently methylated in CRC tissues (90%) and less frequently methylated in gastric (33%) and pancreatic (20%) cancers, but rarely methylated in cancers of other organs (13). The overall sensitivity in our study was 74.9%, and the overall specificity was 86.2%. In contrast to our results in Chinese patient plasma, the sensitivity of fecal mOSMR in previous studies was 38% by TaqMan-MSP (32). The evaluation of mOSMR in CRC plasma shows that the sensitivity is higher than that in fecal samples.

To demonstrate the levels of mSEPT9 and mOSMR in Chinese patients, we detected mSEPT9 and mOSMR in CRC tissues and their matched adjacent non-cancer tissues. mSEPT9 and mOSMR were highly detected in cancer tissues compared with their matched adjacent non-cancer tissues (both $P < 0.0001$). Therefore, the levels of mSEPT9 in Chinese patients were higher in cancer tissues than their matched adjacent non-cancer tissues, but the sensitivity of plasma mSEPT9 was higher than that in Koreans (36.6%), likely due to the different detection methods (33). In addition, the level of mOSMR in Chinese patients was the same as in the previous report by Deng *et al.* (22), but the sensitivity of plasma mOSMR was higher in our current investigation due to the method of detection or ethnic variations in the methylation pattern of OSMR.

Notably, false positive methylation of SEPT9 and OSMR was not detected in plasma, and our findings suggested that mSEPT9 and mOSMR in plasma could match their methylation in the corresponding tumor tissues. Although the P value of OSMR failed to reach statistical significance ($P = 0.058$), it may be induced by minute quantity of circular tumor DNA and exhibit less sensitivity with current qPCR (34).

We also analyzed the relationship between the level of mSEPT9 or mOSMR in plasma samples and the level of serum biomarkers and clinicopathologic characteristics. There was not a significant difference in mSEPT9 or mOSMR in CRC tumors and plasma samples of patients with CRC with different locations and TNM stages. In

daily practice, serum CEA, CA72-4, CA19-9 and CA242 concentrations are now used for screening and follow-up examination in CRC patients. We analyzed pretreatment serum CEA, CA72-4, CA19-9 and CA242 concentrations in our study subjects. The sensitivity of these serum biomarkers (CEA: 32.9%, CA72.4: 20.4%, CA199: 13.9% and CA242: 23.9%) were lower compared with mSEPT9 (62.6%) and mOSMR (74.9%). In our study, the sensitivity of the combined analysis of mSEPT9 and mOSMR improved to 77.0% in CRCs, and the specificity of combined analysis did not decrease substantially when compared with the single marker (Table 3). However, the combination of methylation and serum biomarkers dramatically improved CRC detection (83.0%). Thus, multiple markers, including plasma mSEPT9 and mOSMR, and serum CEA, CA72-4, CA19-9 and CA242, might be more useful for CRCs in screening and monitoring.

In conclusion, plasma mSEPT9 and mOSMR were detected at a high frequency in Chinese patients. Both were not correlated with clinicopathologic characteristics. Our investigation assessed the level of mSEPT9 and mOSMR, and clinical serum biomarkers in CRCs. The results indicate that mSEPT9 and mOSMR are sensitive biomarkers for the detection of CRC, with sensitivities of 62.6% and 74.9%, especially for the sensitivity of combination (77.0%). Therefore, the combination of multiple markers might need to be validated in future investigation (in our study, the sensitivity was 83.0% for the combination of methylation and serum biomarkers). Both of these markers are more sensitive than FOBT and serum biomarkers commonly used in clinics. Therefore, the analyses of methylation levels of SEPT9 and OSMR in plasma, especially OSMR as a new biomarker, are useful for the diagnosis of CRC. The panel including SEPT9, OSMR and serum biomarker (CEA, CA19-9, CA72-4 and CA242) analysis appears feasible and convenient for the diagnosis of CRC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2016.03.07>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Peking University Cancer Hospital & Institute. Written informed consent was obtained from all participants for obtaining blood or tissue samples.

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