

Circulating Methylated Septin 9 Nucleic Acid in the Plasma of Patients with Gastrointestinal Cancer in the Stomach and Colon^{1,2}

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Abstract

BACKGROUND: Methylated *Septin 9* (mSEPT9) in plasma has recently been suggested as a screening marker for colorectal cancer (CRC) with variable sensitivity. We aimed to determine the usefulness of plasma mSEPT9 for screening CRC and gastric cancer (GC) and its diagnostic role in postoperative CRC patients. **METHODS:** A total of 350 peripheral blood samples from 101 CRC patients, 153 GC patients, and 96 healthy persons were collected. In addition, we obtained 35 follow-up blood samples from 27 CRC patients after curative radical surgery. Plasma mSEPT9, serum carcinoembryonic antigen (CEA), and serum CA19-9 were evaluated with clinicopathologic features. **RESULTS:** The sensitivity of plasma mSEPT9 was 36.6% for detecting CRC and 17.7% for detecting GC, and the specificity was 90.6%. During follow-up periods, mSEPT9 showed negative conversion in eight of nine CRC patients (88.9%) whose plasma mSEPT9 had been positive before radical surgery. The patients with plasma mSEPT9 had a tendency of presence of distant metastasis and lower disease-free survival in both CRC and GC. In GC patients, plasma mSEPT9 was more frequently observed in intestinal (23.5%) and mixed type (40.0%) than diffuse type (7.3%; $P = .009$). Combined analysis of mSEPT9, CEA, and CA19-9 increased the sensitivity for diagnosing GC to 32.7% ($P = .002$). **CONCLUSION:** Considering the high incidence of plasma mSEPT9 in intestinal or mixed type GCs similar to CRCs, GC should be examined through the plasma mSEPT9 screening test. In addition, plasma mSEPT9 is proposed as a follow-up marker in CRC patients, but further validation is required.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers and is a leading cause of cancer-related deaths worldwide [1]. In spite of the advances in the treatment, an advanced stage of CRC at the time of diagnosis is still associated with a very unfavorable prognosis. Screening tests decrease CRC-related mortalities by detecting tumors at earlier stages, thereby improving patient prognosis [2,3]. Details of CRC screening policies vary in different countries, but it is generally agreed that there is convincing evidence for the usefulness of testing fecal occult blood or performing colonoscopy to detect early-stage cancer and adenomatous polyps in patients aged ≥ 50 years [4–6]. Fecal occult

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blood testing and fecal immunochemical tests have a few advantages including easier methods and inexpensiveness [7] but with low sensitivity and specificity. Flexible sigmoidoscopy and colonoscopy demonstrate high sensitivity and specificity in preventive and therapeutic procedures to remove adenomatous polyps or early cancers [6]. However, it is expensive and presents risks such as perforation or bleeding. Therefore, the recommendations for incorporating invasive procedures into screening policies for CRC vary between countries [4,5,8], and moreover, not all adults receive screening tests despite their recommendation by national guidelines [9,10]. Only 64.2% of adults were reported to be recommended for screening in the United States [10], and the rate was lower in Korea at 35.5% [9]. To maximize the number of individuals who participate in screening and reduce colon cancer deaths and the cost of health care [11,12], studies targeted on more sensitive and noninvasive methods such as blood testing to screen CRC are being investigated.

Because CRC is known to develop from the accumulation of genetic and epigenetic changes in colonic epithelial cells, molecular markers targeting genetic and epigenetic alterations have been evaluated in tumor tissues and peripheral blood. Differences in the methylation status of tumor and nontumor DNA were evaluated for various genes [13], and methylated *Septin 9* (mSEPT9) DNA has been proposed as a screening marker for detecting CRC in peripheral blood and tumor tissue [12,14–16]. SEPT9 is a member of the septin family involved in cytokinesis and cytoskeletal organization [17]. Alterations in SEPT9 have been linked to multiple cancers, but the role of SEPT9 in tumorigenesis is still elusive [18]. Instead, several studies have evaluated mSEPT9 in human plasma as a screening marker for CRC [12,14,15]. The sensitivity and specificity of plasma mSEPT9 for detecting CRC varied from 50% to 90% and from 88% to 91%, respectively, depending on the detection methods. Plasma mSEPT9 is suggested to be a screening tool in standard-risk individuals unable or unwilling to receive standard CRC screening tests. However, only limited data regarding CRC screening with mSEPT9 have been analyzed, especially in Asians [19], although some studies have reported differences in tumor DNA methylation between different ethnicities [18,20,21].

Considering that mSEPT9 have been reported to present in other organ cancers [18,22], mSEPT9 in blood sample is suggested not to be specific for CRC. Among various organs, gastric cancer (GC) and small intestinal cancer are morphologically similar to CRC. However, the incidence of small intestinal cancer is very low and we cannot easily meet it at daily practice. GC is the fourth most common malignancy and more common in developing countries and in Asian countries [1]. Intestinal type of GC has very similar histopathology to CRC, and in addition, they share some molecular characteristics including microsatellite instability, hypermethylation of tumor suppressor genes, and some mutations [23,24]. However, mSEPT9 in blood samples of GC patients has not been reported yet.

The present study aimed to investigate the usefulness of mSEPT9 as a diagnostic marker in Korean gastrointestinal cancer patients and to validate the results in healthy controls by performing real-time polymerase chain reaction (PCR) assays with plasma samples from CRC and GC patients and healthy controls. Subsequently, we analyzed the correlation between clinicopathologic characteristics and plasma mSEPT9 in CRC and GC patients, compared the clinical implication of plasma mSEPT9 with serum carcinoembryonic antigen (CEA) and CA19-9 concentration, and evaluated plasma mSEPT9 as a follow-up marker of CRC.

Materials and Methods

Plasma Specimens

To investigate mSEPT9, a total of 254 gastrointestinal cancer patients and 96 anonymous healthy volunteers were enrolled in this study. The cancer patients (101 CRC patients and 153 GC patients) whose diagnosis of CRC or GC was confirmed by endoscopic and histopathologic examination were enrolled. The plasma specimens were collected from the CRC and GC patients before surgical treatment, chemotherapy, or radiation therapy at Seoul National University Bundang Hospital between March 2011 and May 2012. We obtained the pretreatment cell-free plasma samples by collecting remnants of routine blood samples. Using this technique, 35 follow-up specimens were obtained from 27 CRC patients who were treated with curative radical surgery. Clinical and pathologic data for the CRC and GC patients were recorded from the electronic medical record system and histopathologic review. Cancer staging was based on the seventh edition of the Cancer Staging Manual of the American Joint Committee on Cancer [25]. Histologic differentiation was graded on the basis of the World Health Organization (WHO) classification [26]. Follow-up information, including cancer recurrence and the time interval between the date of surgical resection and recurrence, was collected. The patients lost to follow-up and deaths from any cause other than CRC or GC were regarded as censored data for the survival analysis. The mean follow-up period was 518 days (range, 492–543 days) in CRC patients and 413 days (range, 397–460 days) in GC patients. For the disease-free controls, 96 anonymous healthy volunteers were enrolled from the same institute from November 2011 to May 2012. The control subjects did not have any neoplasms in their colorectum or stomach or other organs. This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital. Written consent was obtained from the participants.

Specimen Preparation

Blood samples were collected from remnants of routine blood samples of two 9-ml K₂-EDTA bottles. The specimens were processed and stored within 24 hours of collection. Whole-blood specimens were kept at 2 to 8°C before centrifugation and held at 15 to 30°C for approximately 30 minutes immediately before centrifugation. The blood samples were centrifuged in the blood tubes at room temperature for 10 minutes at 1500g and recentrifuged at 1500g for 10 minutes in 15-ml tubes. The buffy coat layer was not to be disturbed while transferring the plasma. The plasma specimens were stored at -70°C before the analysis. At least 4 ml of plasma was collected for testing.

DNA Extraction

DNA was extracted by using the Abbott m2000sp Sample Preparation System (Abbott Molecular, Abbott Park, IL). The patient specimens and one each of the positive and negative controls included in the Abbott RealTime mS9 Colorectal Cancer Assay Kit (Abbott Molecular) were processed with the Abbott m2000sp. The nucleic acids were eluted by magnetic particle technology to capture the nucleic acids, and unbound sample components were removed by washing.

Bisulfite Conversion and Bisulfite Purification

Bisulfite modification was performed by Abbott Bisulfite Modification Kit according to the manufacturer's protocol. Briefly, 210 µl of bisulfite mix and 38 µl of DNA protect buffer provided in the bisulfite modification kit were mixed with 100 µl of DNA specimen. The tubes

were transferred to a thermal cycler and amplified with the following conditions: 99°C for 5 minutes, 60°C for 25 minutes, 99°C for 5 minutes, 60°C for 85 minutes, 99°C for 5 minutes, and 60°C for 2 hours and 55 minutes, followed by holding at 20°C. Bisulfite purification was performed using an Abbott m2000sp System with Abbott Low Residual Volume Tubes.

Real-time PCR for the Detection of mSEPT9

The Abbott RealTime mS9 Colorectal Cancer Assay System, which consisted of an Abbott RealTime mS9 Colorectal Cancer Amplification Reagent Kit and an Abbott RealTime mS9 Colorectal Cancer Control Kit, was used according to the manufacturer's protocol. A PCR master mix was created with the amplification reagent kit. Activation reagent, oligonucleotide reagent, and AmpliTaq gold enzyme were mixed, and 50 µl of the master mix and 30 µl of the purified product were placed on a 96-well optical reaction plate on the Abbott m2000rt (Abbott Molecular) for real-time amplification. The test was performed in triplicate using the contiguous three wells across the row on the same plate. The endogenous human β -actin gene was used as the internal control for sample extraction, bisulfite modification, and amplification efficiency. Specimens whose cycle number of internal control exceeds the established specification range by mS9 Colorectal Cancer Assay m2000rt software were considered as inhibited and retested starting with sample preparation if possible. The positive and negative controls provided by the manufacturer were processed along with the samples from the DNA extraction stage. The results were reported when both controls were valid. Samples were reported as positive when the internal controls were positive and when at least one of three samples was positive.

Measurement of CEA and CA19-9

Serum CEA and CA19-9 concentrations were analyzed using an electrochemiluminescence immunoassay kit on the Modular Analytics E170 module (Roche Diagnostics Corp, Indianapolis, IN) according to the manufacturer's instructions.

Statistical Analysis

The sensitivity and specificity of plasma mSEPT9 were compared using receiver operating curve with the area under the curve. The correlation between clinicopathologic features and mSEPT9 was analyzed using the Fisher exact method or χ^2 test. The mean values for age, CEA, CA19-9, and tumor size were compared according to mSEPT9 status, using the nonparametric Mann-Whitney test. The Kaplan-Meier method with the log-rank test was performed for the univariate analysis of the effect of mSEPT9 for predicting disease-free survival (DFS). Multivariate analysis was performed using the Cox proportional hazards model to determine hazard ratios. Statistical analysis was performed with SPSS Standard version 17.0 (SPSS Inc, Chicago, IL). All of the *P* values were two-sided, and *P* < .05 was considered to be statistically significant.

Results

Diagnostic Performance of mSEPT9 in Gastrointestinal Cancer Patients

Specimens from 254 gastrointestinal cancer patients and 96 healthy controls were tested for mSEPT9. mSEPT9 was identified in the pre-

treatment plasma of 64 of 254 gastrointestinal cancer patients (25.2%), consisting of 37 CRC and 27 GC patients. Therefore, overall sensitivity was 36.6% [95% confidence interval (CI), 27.8–46.8%] in CRC and overall sensitivity was 17.7% (95% CI, 12.0–24.6%) in GC (*P* = .001). mSEPT9 was detected in 9 (9.4%) of the 96 control specimens collected from the healthy blood donors. The test had an overall specificity of 90.6% (95% CI, 82.9–95.6%). By comparing the results from the cancer patients and healthy controls, the positive detection rate of mSEPT9 was higher in the CRC patients (*P* < .001) and the GC patients (*P* = .05).

Clinicopathologic Characteristics and mSEPT9

The clinical features of the CRC and GC patients according to mSEPT9 were shown in Table 1. The positive rate of mSEPT9 was 30.8% for stage 0/I, 36.7% for stage II, 25.0% for stage III, and 64.7% for stage IV CRC patients, with borderline statistical significance (*P* = .051). The detection of mSEPT9 was significantly higher in the CRC patients with synchronous distant metastasis than in those

Table 1. Clinical Features of a Total of 101 CRC Patients and 153 GC Patients according to mSEPT9 Status.

	mSEPT9			P Value
	Positive	Negative	Total	
101 CRCs				
Age	65.76 ± 11.25	62.34 ± 10.97	63.59 ± 11.14	NS
Gender				NS
Male	17 (32.7%)	35 (67.3%)	52	
Female	20 (40.8%)	29 (59.2%)	49	
TNM stage				
0/I	8 (30.8%)	18 (69.2%)	26	
II	11 (36.7%)	19 (63.3%)	30	.051
III	7 (25.0%)	21 (75.0%)	28	
IV	11 (64.7%)	6 (35.3%)	17	
Distant metastasis				
Absent	26 (31.0%)	58 (69.0%)	84	.008
Present	11 (64.7%)	6 (35.3%)	17	
CEA concentration	44.69 ± 133.65	3.31 ± 6.23	18.21 ± 82.09	.024
Preoperative CCRT				NS
Not done	31 (36.0%)	55 (64.0%)	86	
Done	6 (40.0%)	9 (60.0%)	15	
Location				NS
Right colon	6 (28.6%)	15 (71.4%)	21	
Left colon	31 (38.8%)	49 (61.2%)	80	
Total	37 (36.6%)	64 (63.4%)	101	
153 GCs				
Age	65.30 ± 13.03	60.45 ± 13.34	61.31 ± 13.37	NS
Gender				NS
Male	19 (20.9%)	72 (79.1%)	91	
Female	8 (12.9%)	54 (87.1%)	62	
TNM stage				.016
I	6 (11.8%)	45 (88.2%)	51	
II	7 (15.9%)	37 (84.1%)	44	
III	6 (15.0%)	34 (85.0%)	40	
IV	8 (44.4%)	10 (55.6%)	18	
Distant metastasis				.004
Absent	19 (14.1%)	116 (85.9%)	135	
Present	8 (44.4%)	10 (55.6%)	18	
CEA concentration	5.46 ± 9.89	3.95 ± 10.52	4.21 ± 10.40	NS
CA19-9 concentration	22.22 ± 44.38	28.11 ± 106.47	27.08 ± 98.38	NS
Location of tumor				NS
Lower third	19 (20.0%)	76 (80.0%)	95	
Middle third	4 (10.8%)	33 (89.2%)	37	
Upper third	4 (19.0%)	17 (81.0%)	21	
Total	27 (17.6%)	126 (82.4%)	153	

CCRT indicates chemoradiation therapy; NS, not significant.

without metastasis ($P = .008$). There was a tendency for higher CEA concentration in the mSEPT9-positive CRC group (44.69 ng/ml vs 3.31 ng/ml, $P = .024$). Of a total of 101 CRC patients, 15 were treated with preoperative chemoradiation therapy and then radical surgery and 3 were treated with chemotherapy only. Thus, the pathologic characteristics were compared according to mSEPT9 in 83 CRC patients (Table 2). Tumor size was significantly larger in the mSEPT9-positive CRC patients than in the mSEPT9-negative patients ($P = .004$). The CRC patients with lymphatic or perineural invasion did not show higher mSEPT9 positivity than those without invasion ($P > .05$), but the patients with venous invasion showed a higher percentage of mSEPT9 detection than the patients without invasion (63.6% vs 30.6%, $P = .044$). Among the other findings, mSEPT9 positivity was significantly higher in CRCs with peritumoral abscess (75.0%, $P = .004$).

In the GC patients, mSEPT9 was also associated with advanced stage ($P = .016$) and distant metastasis ($P = .004$; Table 1). In contrast to CRC group, CEA concentrations of the mSEPT9-positive GC group were similar to the mSEPT9-negative GC group ($P > .05$). Of a total of 153 GC patients, radical surgery was not performed in 15 patients. Thus, pathologic characteristics were compared according to mSEPT9 in 138 GC patients (Table 2). Interestingly, mSEPT9 detection was more frequently found in intestinal type (23.5%) and mixed type (40.0%) than diffuse type (7.3%, $P = .009$). Among the other findings, mSEPT9 positivity was significantly higher in GCs with expanding border ($P = .001$).

The results of the Kaplan-Meier univariate analysis showed that mSEPT9 positivity was significantly associated with lower DFS in the CRC patients ($P = .034$; Figure 1A). The mSEPT9-positive CRC cases had estimated mean DFS duration of 480 days (95% CI, 426–534) compared with the 539 days (95% CI, 516–563) in the mSEPT9-negative CRC cases. However, mSEPT9 showed borderline prognostic significance in the GC patients ($P = .058$; Figure 1B). mSEPT9 was not an independent prognostic marker in the CRC or GC patients by Cox regression analysis ($P > .05$; data not shown).

mSEPT9 as a Follow-Up Marker

Thirty-five postoperative samples (one to four samples in each patient) from 27 CRC patients treated with curative radical surgery were tested for mSEPT9 (Table 3). Postoperative specimens were obtained 118 days after surgery on average (71–232 days). Among 27 CRC patients with follow-up plasma samples, 24 (88.9%) patients showed negative mSEPT9 after curative radical surgery irrespective of the pre-treatment results. Sixteen patients (61.5%) showed negative results for mSEPT9 before and after surgery. Of the nine patients who were found with mSEPT9 before surgery, negative conversion was observed in eight patients (88.9%).

CRC recurrence was diagnosed in 4 of 27 patients (Table 3), of whom 1 patient was diagnosed as recurrent cancer by plasma mSEPT9. In this patient, two serial plasma samples showed mSEPT9 positivity at 128 and 215 days after the first surgery (Figure W1), but clinical impression was abscess at anastomosis site by computed tomography (CT) imaging. Serum CEA concentration of the patient was 2.9, 4.2, and 12.2 ng/ml at -1, 128, and 215 days, respectively, thus with borderline clinical significance. Recurrence was confirmed by intra-operative frozen tissue examination and radical resection of the recurrent cancer was performed. Negative conversion of mSEPT9 was found after the second surgery for recurrent cancer (at 348 days after

Table 2. Pathologic Characteristics according to mSEPT9 Status in 83 CRC Patients and 138 GC Patients with Pathologic Examination of Radical Surgery Specimens.

	mSEPT9			P Value
	Positive	Negative	Total	
CRC				
Histologic grade				NS
WD	3 (42.9%)	4 (57.1%)	7	
MD	24 (34.8%)	45 (65.2%)	69	
PD	1 (33.3%)	2 (66.7%)	3	
Mucinous	1 (25.0%)	3 (75.0%)	4	
Tumor size	5.75 ± 2.73	3.99 ± 1.92	4.59 ± 2.37	.004
Depth of invasion				NS
pTis/T1	4 (28.6%)	10 (71.4%)	14	
pT2	4 (25.0%)	12 (75.0%)	16	
pT3	14 (35.9%)	25 (64.1%)	39	
pT4	7 (50.0%)	7 (50.0%)	14	
LN metastasis				NS
pN0	19 (35.8%)	34 (64.2%)	53	
pN1	6 (28.6%)	15 (71.4%)	21	
pN2	4 (44.4%)	5 (55.6%)	9	
Lymphatic invasion				NS
Absent	20 (31.7%)	43 (68.3%)	63	
Present	9 (45.0%)	11 (55.0%)	20	
Venous invasion				.044
Absent	22 (30.6%)	50 (69.4%)	72	
Present	7 (63.6%)	4 (36.4%)	11	
Perineural invasion				NS
Absent	19 (30.2%)	44 (69.8%)	63	
Present	10 (50.0%)	10 (50.0%)	20	
Tumor deposits				NS
Absent	22 (34.4%)	42 (65.6%)	64	
Present	7 (36.8%)	12 (63.2%)	19	
Preexisting adenoma				NS
Absent	26 (40.0%)	39 (60.0%)	65	
Present	3 (16.7%)	15 (83.3%)	18	
Other findings				.004
Absent	19 (30.2%)	44 (69.8%)	63	
Mucin production	1 (12.5%)	7 (87.5%)	8	
Peritumoral abscess	9 (75.0%)	3 (25.0%)	12	
Total	29	54	83	
GC				
Lauren classification				.009
Intestinal	12 (23.5%)	39 (76.5%)	51	
Diffuse	6 (7.3%)	76 (92.7%)	82	
Mixed	2 (40.0%)	3 (60.0%)	5	
Tumor size	5.54 ± 3.63	4.37 ± 2.88	4.54 ± 3.02	NS
Depth of invasion				NS
pT1	6 (12.2%)	43 (87.7%)	49	
pT2	1 (4.5%)	21 (95.5%)	22	
pT3	7 (20.0%)	28 (80.0%)	35	
pT4	6 (18.8%)	26 (81.3%)	32	
LN metastasis				NS
pN0	9 (14.1%)	55 (85.9%)	64	
pN1	3 (14.3%)	18 (85.7%)	21	
pN2	1 (4.5%)	21 (95.5%)	22	
pN3	7 (22.6%)	24 (77.4%)	31	
Lymphatic invasion				NS
Absent	10 (14.9%)	57 (85.1%)	67	
Present	10 (14.1%)	61 (85.9%)	71	
Venous invasion				NS
Absent	16 (14.0%)	98 (86.0%)	114	
Present	4 (16.7%)	20 (83.3%)	24	
Perineural invasion				NS
Absent	13 (17.1%)	63 (82.9%)	76	
Present	7 (11.3%)	55 (88.7%)	62	
Tumor border				.001
Expanding	11 (33.3%)	22 (66.7%)	33	
Infiltrative	9 (8.6%)	96 (91.4%)	105	
Preexisting adenoma				NS
Absent	18 (13.8%)	112 (86.2%)	130	
Present	2 (25.0%)	6 (75.0%)	8	
Total	20	118	138	

WD indicates well differentiated; MD, moderately differentiated; PD, poorly differentiated; NS, not significant.

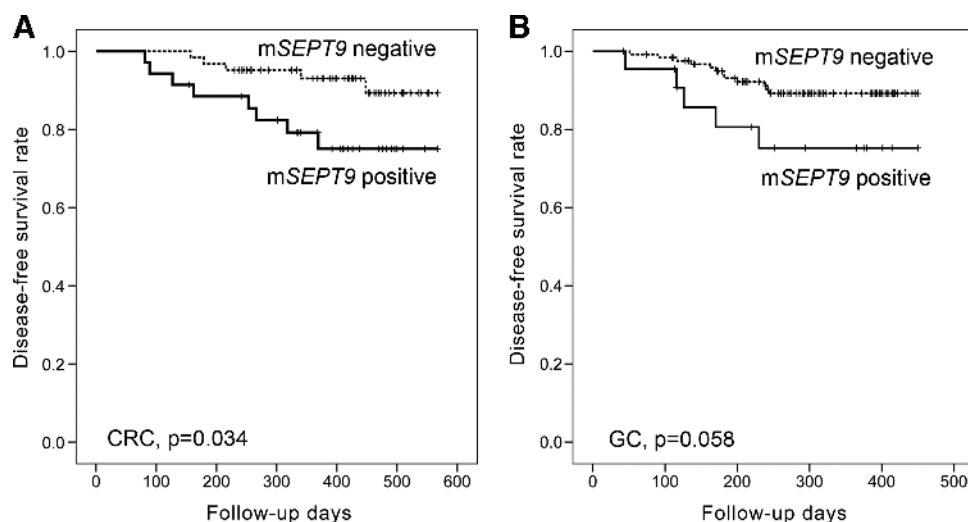


Figure 1. Kaplan-Meier univariate survival curves according to mSEPT9 status in (A) CRC patients and (B) GC patients.

the first surgery). mSEPT9 was also found in the primary and recurrent CRC tissue samples from this patient.

Serum CEA and CA19-9 Concentrations

No serum biomarker currently exists for initial diagnosis of CRC or GC in daily practice, but in 2010, the *AJCC Cancer Staging, Seventh Edition* has accepted serum tumor marker CEA as site-specific prognostic factor in CRC and CEA and CA19-9 as prognostic factors in GC [25]. Thus, concentrations of CEA in CRC patients and CEA and CA19-9 in GC patients were additionally evaluated. A serum concentration higher than 5 ng/ml was defined as CEA positive and a serum concentration higher than 27 U/ml as CA19-9 positive [27]. Positive CEA was found in 21 (20.8%) of the 101 CRC patients, which were correlated with advanced stage and shorter DFS (Table W1). CEA positivity was found in 14 (9.2%) and CA19-9 positivity was found in 22 (14.4%) of 153 GC patients, which were also correlated with advanced stage and shorter DFS (Table W2).

To increase the sensitivity for the diagnosis of CRC or GC, we performed combined analysis of mSEPT9 and serum markers. CEA positivity and/or plasma mSEPT9 was observed in 44 (43.6%) of 101 CRC patients; thus, the sensitivity of the combined analysis was similar to the sensitivity of mSEPT9 for CRC diagnosis ($P > .05$). In contrast to CRC patients, the sensitivity of combined analysis of CEA, CA19-9, and/or mSEPT9 (50 of 153 patients, 32.7%) was

significantly higher than any single marker ($P = .002$ in mSEPT9; $P < .001$ in CEA and CA19-9).

Discussion

In the present study, mSEPT9, which is being evaluated as a non-invasive plasma screening marker of CRC, was tested in Korean CRC patients. Overall sensitivity was 36.6% (95% CI, 27.8–46.8%) and overall specificity was 90.6% (95% CI, 82.9–95.6%). In contrast to our results in Korean CRC patients, sensitivities of plasma mSEPT9 in previous studies ranged from 50.0% to 90.0% and specificities ranged from 73.0% to 94.0% as determined by various methods [12,14,28,29]. Thus, the sensitivity of our results was relatively low, but the specificity was high. The possible causes of low frequency of mSEPT9 in the Korean CRC patients may be ethnic variations in the methylation pattern of SEPT9 or the method of detection. Although ethnic variation in the methylation patterns of SEPT9 has not been studied, differences in methylation status between ethnic groups have been published for other genes in prostate or breast cancer patients [18,20]. The screening of CRC with mSEPT9 has been performed mostly in the United States and Germany and showed sensitivities ranging from 50% to 90% with various methods [12,28,29], whereas a small population study in China showed a sensitivity of 75% using MethyLight assay [19]. Therefore, further investigations are required in various ethnics with various methods. To demonstrate the levels of mSEPT9 in CRC tissue in the Korean population, we evaluated mSEPT9 in 17 CRC tissues and 17 matched normal tissues. mSEPT9 was detected in 16 cancer tissues (94.1%) and 5 normal tissues (29.4%; $P = .001$; data not shown). Therefore, the levels of mSEPT9 in Koreans were higher in cancer tissue than normal tissue, but the detection rate of plasma mSEPT9 was lower than other ethnics. In addition, 25 GC tissues and 25 matched normal tissues were evaluated for mSEPT9. mSEPT9 was detected in 14 cancer tissues (56.0%) and 4 normal tissues (16.0%; $P = .002$; data not shown), which suggested that mSEPT9 was not specific for CRC diagnosis.

In addition to CRCs, we investigated plasma mSEPT9 in 153 GC patients because mSEPT9 was reported in other organ cancers [18,22] and intestinal type GC has similar morphologic and molecular features

Table 3. mSEPT9 Status in Follow-Up Plasma Samples of 27 CRC Patients.

mSEPT9		No. of Patients	CRC Recurrence during Follow-Up
Preoperative Samples	Postoperative Samples		
Negative	Negative	16	3
Negative	Positive	2	1
Positive	Negative	8	0
Positive	Positive	1	0
Total		27	4

to CRC. mSEPT9 was infrequently detected in the plasma samples of GCs (17.7%). However, 23.5% of intestinal type and 40.0% of mixed type GCs showed presence of mSEPT9 in the plasma samples, which were similar frequency to CRCs. Furthermore, plasma mSEPT9 was observed in 44.4% (8 of 18) stage IV GCs. Therefore, GCs, especially intestinal/mixed type or stage IV, should be considered in differential diagnosis at the screening test by using plasma mSEPT9. This study is the first to validate a role of plasma mSEPT9 as a noninvasive diagnostic biomarker in large-scaled GC cases.

To validate the specificity of mSEPT9 in CRC patients and to evaluate a possibility of using mSEPT9 as a noninvasive follow-up marker, we investigated plasma mSEPT9 in follow-up samples from 27 CRC patients after curative radical surgery. Twenty-four (88.9%) of 27 patients showed negative mSEPT9 after curative radical surgery and negative conversion was observed in 8 of 9 patients (88.9%), which was similar to the overall specificity (90.6%) of pretreatment plasma mSEPT9. It is suggested that plasma mSEPT9 before and after treatment is specific for CRCs and have potential for a follow-up marker of CRCs. The usefulness of mSEPT9 as a follow-up marker was additionally suggested by the patient in whom recurrent CRC was detected by only plasma mSEPT9 in our cohort. It is the first to demonstrate the negative conversion of plasma mSEPT9 after curative radical surgery and the diagnostic role of mSEPT9 for detecting recurrence. Follow-up results for mSEPT9 in these patients will aid in deciding whether mSEPT9 can be used as a follow-up marker. Further validation is required for the clinical use of plasma mSEPT9 as a follow-up marker of CRCs.

In daily practice, serum CEA and/or CA19-9 concentration is used for screening, prognosis prediction, and follow-up examination in CRC and GC patients. We also evaluated pretreatment serum CEA concentrations in CRC patients and CEA and CA19-9 in GC patients. To increase the sensitivity for the diagnosis of CRC or GC, we performed combined analysis of mSEPT9 and serum markers. The sensitivity of combined analysis was significantly increased in GCs (32.7%; $P = .002$) but not in CRCs. In contrast to CRCs, multiple markers including plasma mSEPT9 and serum CEA and CA19-9 are suggested to be more useful for diagnosing GC than any single marker.

Despite the several reports evaluating mSEPT9 in CRC patients, its clinicopathologic implication has not been confirmed yet. Neither did in GC patients. Thus, we analyzed whether the detection of mSEPT9 showed any relationship with clinicopathologic characteristics and patient outcome. There was no significant difference in mSEPT9 detection according to depth, lymph node metastasis, and lymphatic or perineural invasion in both CRC and GC. However, those patients with distant metastasis had significantly higher positivity for mSEPT9. Furthermore, the mSEPT9-positive group exhibited lower DFS than the mSEPT9-negative group ($P = .034$ in CRC; $P = .058$ in GC). Therefore, mSEPT9 is suggested to be useful for predicting aggressive tumor behavior in CRC and GC patients. In contrast to the present results, septin 9, which is encoded by SEPT9, was identified as a pseudopod-specific protein in various cancer cell lines that is essential for pseudopod protrusion and tumor cell migration and invasion [30]. This discrepancy may be because genetic, epigenetic, and expression status is different according to the various transcripts encoding different isoforms of septin 9, and the relationship between mSEPT9 and septin 9 expression is not clearly elucidated due to isoforms of septin 9 [31]. Further study is needed to clarify it.

In conclusion, mSEPT9 was detected at a relatively low frequency in the Korean CRC patients but highly specific. mSEPT9 detection was correlated with clinicopathologic characteristics such as distant

metastasis and poor DFS. In addition to CRCs, we revealed that plasma mSEPT9 was also observed in GC patients, especially in intestinal/mixed type or stage IV GC patients. Therefore, we suggest that GC should be considered through the screening test using plasma mSEPT9. Follow-up of mSEPT9-positive patients would reveal the usefulness of mSEPT9 as an early detection marker for recurrence.

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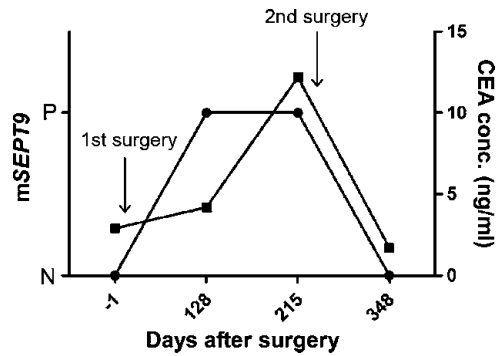


Figure W1. Plasma mSEPT9 and serum CEA concentrations in the patient with recurred CRC. mSEPT9 was negative and serum CEA was 2.9 ng/ml at 1 day before the first operation. At 128 days after the first operation, mSEPT9 was positive, but CEA was 4.2 ng/ml and clinical impression was abscess at anastomosis site by CT imaging. Serum CEA was 12.2 ng/ml at 215 days. Recurrence was diagnosed by the second operation at 217 days. Negative conversion of mSEPT9 was found after second operation (at 348 days; N, negative; P, positive; closed circle, mSEPT9; closed square, CEA concentration).

Table W1. Clinical Features of a Total of 101 CRC Patients according to Serum CEA Status.

	CEA		Total	P Value
	≥5 ng/ml	<5 ng/ml		
Age	63.86 ± 11.45	63.53 ± 11.13	63.59 ± 11.14	NS
Gender				.018
Male	6 (11.5%)	46 (88.5%)	52	
Female	15 (30.6%)	34 (69.4%)	49	
TNM stage				<.001
0/I	0 (0%)	26 (100%)	26	
II	5 (16.7%)	25 (83.3%)	30	
III	6 (21.4%)	22 (78.6%)	28	
IV	10 (58.8%)	7 (41.2%)	17	
Distant metastasis				<.001
Absent	11 (13.1%)	73 (86.9%)	84	
Present	10 (58.8%)	7 (41.2%)	17	
mSEPT9				.001
Negative	7 (10.9%)	57 (89.1%)	64	
Positive	14 (37.8%)	23 (62.2%)	37	
Preoperative CCRT				NS
Not done	18 (20.9%)	68 (79.1%)	86	
Done	3 (20.0%)	12 (80.0%)	15	
Location of tumor				NS
Right colon	5 (23.8%)	16 (76.2%)	21	
Left colon	16 (20.0%)	64 (80.0%)	80	
Mean DFS (days)	400.3 ± 39.5	547.6 ± 9.5	517.5 ± 13.0	<.001*
Total	21 (20.8%)	80 (79.2%)	101	

CCRT indicates chemoradiation; NS, not significant; DFS, disease-free survival.

*By log-rank test of Kaplan-Meier survival curves.

Table W2. Clinical Features of a Total of 153 GC Patients according to Serum CEA and CA19-9 Status.

	Total	CEA		P Value	CA19-9		P Value
		≥5 ng/ml	<5 ng/ml		≥27 U/ml	<27 U/ml	
Age	60.31 ± 13.37	66.00 ± 7.59	59.73 ± 13.71	NS	64.95 ± 13.88	59.53 ± 13.18	NS
Gender							NS
Male	91	11 (12.1%)	80 (87.9%)	NS	13 (14.3%)	78 (85.7%)	
Female	62	3 (4.8%)	59 (95.2%)		9 (14.5%)	53 (85.5%)	
TNM stage				.013			.002
0/I	51	1 (2.0%)	50 (98.0%)		2 (3.9%)	49 (96.1%)	
II	44	4 (9.1%)	40 (90.9%)		5 (11.4%)	39 (88.6%)	
III	40	4 (10.0%)	36 (90.0%)		8 (20.0%)	32 (80.0%)	
IV	18	5 (27.8%)	13 (72.2%)		7 (38.9%)	11 (61.1%)	
Distant metastasis				.013			.006
Absent	135	9 (6.7%)	126 (93.3%)		15 (11.1%)	120 (88.9%)	
Present	18	8 (27.8%)	13 (72.2%)		7 (38.9%)	11 (61.1%)	
mSEPT9				NS			NS
Negative	126	9 (7.1%)	117 (92.9%)		17 (13.5%)	109 (86.5%)	
Positive	27	5 (18.5%)	22 (81.5%)		5 (18.5%)	22 (81.5%)	
Location of tumor				NS			NS
Lower third	95	9 (9.5%)	86 (90.5%)		15 (15.8%)	80 (84.2%)	
Middle third	37	4 (10.8%)	33 (89.2%)		5 (13.5%)	32 (86.5%)	
Upper third	21	1 (4.8%)	20 (95.2%)		2 (9.5%)	19 (90.5%)	
Mean DFS (days)	413.1 ± 8.5	293.2 ± 46.0	424.6 ± 7.4	<.001*	344.4 ± 31.4	423.7 ± 8.1	<.001*
Total	153	14 (9.2%)	139 (90.8%)		22 (14.4%)	131 (85.6%)	

NS indicates not significant; DFS, disease-free survival.

*By log-rank test of Kaplan-Meier survival curves.