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Performance of a second-generation methylated SEPT9 test in detecting colorectal neoplasm

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Abstract

Background and Aim: Screening and early detection reduces mortality due to colorectal cancer (CRC). Methylated Septin 9 (SEPT9) is a new blood-based biomarker for CRC. We evaluated the performance of the second-generation SEPT9 assay for the detection of colorectal neoplasm, and compared it with fecal immunochemical test (FIT).

Methods: A total of 135 patients with CRC, 169 with adenomatous polyps, 81 with hyperplastic polyps, and 91 healthy controls were included. The clinical status of all subjects was verified by colonoscopy. In all patients, peripheral blood samples were taken for SEPT9 testing using Epi proColon 2.0 test. For 177 patients, both SEPT9 and FIT were performed.

Results: The sensitivity and specificity of SEPT9 for CRC were 74.8% (95% confidence interval [CI]: 67.0–81.6%) and 87.4% (*vs* non-CRC, 95% CI: 83.5–90.6%), respectively. SEPT9 was positive in 66.7% of stage I, 82.6% of stage II, 84.1% of stage III, and 100% of stage IV CRCs. The sensitivity of SEPT9 for advanced adenomas was 27.4% (95% CI: 18.7–37.6%). The sensitivity and specificity of FIT for CRC was 58.0% (95% CI: 46.1–69.2%) and 82.4% (95% CI: 74.4–88.7%), respectively. SEPT9 showed better performance in CRC detection than FIT, but similar among advanced adenomas.

Conclusions: With improved performance characteristics in detecting CRC, the second-generation SEPT9 assay could play an important role in CRC screening and early detection.

Introduction

The incidence of colorectal cancer (CRC) has increased rapidly in China and continues to be a major public health threat around the world. There is sufficient evidence to support the notion that screening and early detection reduce mortality due to CRC. Fecal occult blood tests (FOBT), especially the guaiac-based test (gFOBT), are the most widely used CRC screening methods. However, the sensitivity of gFOBT is low (35–64% for CRC).[1,2] Although the human hemoglobin-specific fecal immunochemical test (FIT) improves the sensitivity (65–81% for CRC),[2,3] the detection of colorectal neoplasms by FOBT is limited by the fact that some neoplasms may not bleed.[4]

Recently, a new blood-based test, the methylated Septin 9 (SEPT9) assay, was developed. SEPT9 gene is differentially methylated in CRC tissues.[5] Methylated SEPT9 is released from tumor cells into the bloodstream, and can be detected in blood plasma.[6] Several case–control studies demonstrated an overall CRC detection rate of around 65%, with a false positive rate of approximately 10%.[7–9] However, these studies used the earlier versions of the SEPT9 detection kit (Epi proColon 1.0; Epigenomics AG, Berlin, Germany) and were performed in western countries. While a modified next generation protocol may improve the performance of SEPT9 in CRC detection, there are few reports about the commercially available kits. Thus, in the current study, we evaluated the efficacy of the second-generation commercially available SEPT9 assay (Epi proColon 2.0; Epigenomics AG, Berlin, Germany) in a Chinese population, and compared the performance of SEPT9 with FIT.

Methods

Patients. The study was approved by the Beijing Military General Hospital Ethics Committee, and informed consent was obtained from each subject. Based on the primary objective, to estimate sensitivity and specificity of SEPT9 for CRC, and the results of previous studies,[7–9] the planned distribution for the selected subjects was 100 CRC, 150 adenomas, and 150 non-neoplasm (including hyperplastic polyps and healthy controls). Between March 2013 and April 2014, peripheral blood samples were taken from 6298 patients seen in the Beijing Military General

Hospital Endoscopy Center before colonoscopy preparation. Among them, a total of 135 patients with CRC, 169 with adenomatous polyps, 81 with hyperplastic polyps, and 91 healthy controls (no evidence of disease [NED]) were included in the study finally. The clinical status of all subjects was verified by colonoscopy at the Beijing Military General Hospital. None of the patients received chemotherapy, radiotherapy, endoscopic, or surgical intervention before colonoscopy. During colonoscopy, biopsies were taken for histological examination. Patients with known inflammatory bowel disease, Lynch syndrome, familial adenomatous polyposis, Peutz-Jeghers syndrome, or other malignant diseases were excluded. Adenomatous polyps with > 25% villous component, high-grade dysplasia (HGD), or diameter ≥ 10 mm were considered as advanced adenomas.[10] Thus, the cases of adenomatous polyps were subclassified into HGD ($n = 22$), advanced adenomas without HGD ($n = 62$), and non-advanced adenomas ($n = 85$) based on the histopathological characteristics after endoscopic resection. The cases of carcinoma in situ ($n = 5$) were grouped as HGD. In the 135 CRC patients, 90 underwent surgical treatment after colonoscopy and the CRC stages were determined from the resected specimens.[11]

Plasma Septin 9 test (SEPT9). In all 476 patients, peripheral blood samples were taken using 10-mL ethylenediamine tetraacetic acid tubes, prior to colonoscopy preparation. Plasma was isolated from whole blood by double centrifugation for 12 min at 1350 rcf and stored at −80°C. Frozen samples were sent to BioChain Medical Laboratory (Beijing, China) for SEPT9 testing using the Epi proColon 2.0 test, according to the manufacturer's instructions and described by Tóth *et al*.[12] The laboratory was blind to the subjects' clinical results. DNA was extracted from the plasma samples and bisulfite conversion was performed. Each bisulfite converted DNA sample was tested in triplicate by real-time polymerase chai reaction (PCR). Following the instruction of manufacturer, the SEPT9 result was considered to be positive if at least two of the three replicates were positive (2/3 rule); conversely, the result was negative if no more than one replicate was positive.

FIT. In the 476 patients, 177 provided a fecal sample for immuno occult blood testing (FIT) before colonoscopy preparation, including 69 cases of CRC, 65 cases of adenomatous polyps, 16 cases of hyperplastic polyps, and 27 cases of NED. Diet and medication were not restricted before providing the fecal sample. The qualitative FIT kits (gold gel stripe) were provided by WanhuaPuman, Inc. (Beijing, China). The tests were performed in the Clinical Laboratory of Beijing Military General Hospital, according to the manufacturers' instructions. The positive threshold of FIT was 0.2 μg/mL.

Statistical analysis. Sensitivity and specificity for each test was calculated with 95% confidence intervals (CI) based on the exact binomial distribution. Chi-squared test was used to compare the detection rates of SEPT9 for CRC between different ages, genders, and location of the tumors (left or right side, depending on the localization of the cancer in relation to the splenic flexure of the colon). McNemar's test was used to compare the detection rates of SEPT9 and FIT for CRC and advanced adenoma. A statistically significant difference was established when $P < 0.05$.

Results

Table 1 showed the demographic data of all 476 patients and the results of SEPT9. The sensitivity and specificity of SEPT9 for CRC was 74.8% (95% CI: 67.0–81.6%) and 87.4% (*vs* non-CRC, 95% CI: 83.5–90.6%), respectively (Table 2). The sensitivity and specificity of SEPT9 for CRC plus HGD was 70.1% (95% CI: 62.6–76.8%) and 89.3% (95% CI: 85.6–92.4%), respectively. The

Table 1 Demographic data of patients and results of the SEPT9 assay ($n = 476$)

	Total	Gender (female/male)	Age (years old) (range, mean ± SD)	SEPT9+ (*n*, %)	SEPT9– (*n*, %)
No evidence of disease	91	49/42	20–80, 50.1 ± 12.8	3 (3.3%)	88 (96.7%)
Hyperplastic polyps	81	37/44	23–76, 52.5 ± 11.2	5 (6.2%)	76 (93.8%)
Adenomatous polyps	169			35 (20.7%)	134 (79.3%)
Non-advanced adenomas	85	24/61	27–81, 53.8 ± 12.5	12 (14.1%)	73 (85.9%)
Advanced adenomas without high-grade dysplasia	62	23/39	30–78, 56.2 ± 11.3	14 (22.6%)	48 (77.4%)
High-grade dysplasia	22	14/8	47–80, 64.4 ± 10.1	9 (40.9%)	13 (59.1%)
Colorectal cancer	135	69/66	28–84, 60.9 ± 12.1	101 (74.8%)	34 (25.2%)

Table 2 Sensitivity and specificity of SEPT9 in identifying colorectal cancer and adenomas

	Sensitivity	95% CI	Specificity	95% CI
Colorectal cancer	74.8% (101/135)	67.0–81.6%	87.4% (298/341)	83.5–90.6%
Colorectal cancer + high-grade dysplasia	70.1% (110/157)	62.6–76.8%	89.3% (285/319)	85.6–92.4%
Colorectal cancer + advanced adenomas	56.6% (124/219)	50.0–63.1%	92.2% (237/257)	88.4–95.1%
Colorectal cancer + adenomas	44.7% (136/304)	39.2–50.4%	95.3% (164/172)	91.4–97.8%
Adenomas	20.7% (35/169)	15.1–27.3%		
Advanced adenomas	27.4% (23/84)	18.7–37.6%		

false positive rate of SEPT9 (positive in NED and hyperplastic polyps) was 4.7% (95% CI: 2.2–8.6%). In the CRC group, there was no significant difference in the positive rates of SEPT9 between different ages, genders, or location of the tumors (Table 3). In the 90 cases of CRC whose stages were identified based on the surgical resected specimens, SEPT9 was positive in 66.7% of stage I (12/18), 82.6% of stage II (19/23), 84.1% of stage III (37/44), and 100% of stage IV (5/5) (Table 4). The sensitivity of SEPT9 for advanced adenomas was 27.4% (95% CI: 18.7–37.6%).

There were 177 patients underwent SEPT9 and FIT simultaneously. Table 5 showed the results of SEPT9 and FIT in these patients. The sensitivity and specificity of FIT for CRC was 58.0% (95% CI: 46.1–69.2%) and 82.4% (95% CI: 74.4–88.7%), respectively. SEPT9 detected 25 cancers that were missed by FIT, whereas FIT detected 12 cancers that were missed by SEPT9 (Table 5). The difference in discordant test results was significant ($P = 0.033$). In a post-hoc analysis among CRC without metastasis (stage I–III), the sensitivity of SEPT9 was statistically superior to that of FIT (76.4% *vs* 56.4%, $P = 0.041$). However, there was no significant difference in sensitivity among advanced adenomas between the two tests ($P = 0.774$).

Table 3 Results of SEPT9 in identifying colorectal cancer between different ages, genders, and tumor locations

	SEPT9+	SEPT9–	*P*-value‡
Age			
< 60 years old (*n* = 57)	39 (68.4%)	18 (31.6%)	0.144
≥ 60 years old (*n* = 78)	62 (79.5%)	16 (20.5%)	
Gender			
Male (*n* = 66)	47 (71.2%)	19 (28.8%)	0.346
Female (*n* = 69)	54 (78.3%)	15 (21.7%)	
Tumor location†			
Left side (*n* = 93)	72 (77.4%)	21 (22.6%)	0.301
Right side (*n* = 42)	29 (69.0%)	13 (31.0%)	

†Depending on the localization of the cancer in relation to the splenic flexure of the colon.
‡Chi-squared test was used.

Table 4 Detection rate of SEPT9 in different stages of colorectal cancer

	Stage I	Stage II	Stage III	Stage IV
SEPT9+	12 (66.7%)	19 (82.6%)	37 (84.1%)	5 (100%)
SEPT9–	6	4	7	0
Total	18	23	44	5

Discussion

The SEPT9 gene codes for a Guanosine triphosphate (GTP)-binding protein associated with filamentous structures and cytoskeleton formation, and plays a role in multiple cancers as either an oncogene or a tumor suppressor gene.[13] Regulation of SEPT9 expression is complex and not well understood; however, hypermethylation of this gene was recently introduced as a biomarker for early detection of CRC.[5] In the current study, we assessed the performance of the second-generation commercially available SEPT9 assay in a case–control setting. Compared with the first generation kit, the new kit improves in DNA extraction and PCR protocol. More importantly, the second-generation SEPT9 assay uses three separate PCR replicates instead of two replicates, which has been confirmed to enhance the sensitivity.[14,15] A sensitivity of 74.8% and specificity of 87.4% for CRC were observed. The sensitivity observed in our study was similar to the reports of Tóth *et al*.[12] which were performed in a Hungarian population using the same kit (79.3% for CRC). In the previous case–control studies which used the first generation SEPT9 detection kit (Epi proColon 1.0), the sensitivity of SEPT9 for CRC ranged from 58% to 69% and the specificity ranged from 86% to 90%.[6–9] Recently, a large prospective study on average-risk population using the first-generation SEPT9 assay showed a CRC sensitivity of 48.2%, while post-hoc analyses using a modified protocol that mimicked the second-generation assay suggested a sensitivity of 63.9% for CRC.[14] These results suggested an improved sensitivity and comparable specificity of the second-generation SEPT9 assay than the first generation kit, though direct comparison was absent yet. Moreover, our study showed that the results of SEPT9 assay were independent of factors such as age, gender, and tumor localization, which were similar to the previous reports.[12,14,15]

Detection rates of advanced adenomas by SEPT9 have been reported in two case–control studies,[7,9] and sensitivities were only 17% (3/18) and 18% (3/17). In the prospective study by Church *et al*.,[14] the sensitivity for advanced adenomas was only 11.2%. In our study, the sensitivity of the second-generation SEPT9 assay for advanced adenomas was 27.4%, which was higher than the previous reports but still limited. As SEPT9 is broadly expressed in adenoma tissues,[6] the lower performance in detecting adenomas could be explained by differences in the release of marker into blood, since vascular invasion is a later event during CRC tumorigenesis.[16] This explanation is consistent with the fact that positive

Table 5 Results of SEPT9 and fecal immunochemical test (FIT) in 177 patients tested with both

	Total	FIT+ (*n*, %)	SEPT9+ (*n*, %)	FIT+ SEPT9+	FIT+ SEPT9–	FIT– SEPT9+	FIT– SEPT9–
No evidence of disease	27	1 (3.7%)	1 (3.7%)	0	1	1	25
Hyperplastic polyps	16	5 (31.3%)	3(18.8%)	1	4	2	9
Adenomatous polyps	65	13(20.0%)	12(18.5%)	5	8	7	45
Non-advanced adenomas	30	3 (10.0%)	3 (10.0%)	1	2	2	25
Advanced adenomas without high-grade dysplasia	26	7 (26.9%)	7 (26.9%)	3	4	4	15
High-grade dysplasia	9	3 (33.3%)	2 (22.2%)	1	2	1	5
Colorectal cancer	69	40(58.0%)	53(76.8%)	28	12	25	4

rates of SEPT9 were increased along with the progression of tumors in our study (i.e. from low-grade dysplasia, HGD, to CRC stage I–IV).

FIT is now increasingly used instead of gFOBT because it may provide improved performance characteristics than gFOBT.[17] In previous reports, FIT yielded sensitivities for CRC ranging from 65% to 81% and specificities ranging from 87% to 97%.[2,3] Thus we compared SEPT9 with FIT in a paired manner. In our study, the sensitivity and specificity of FIT for CRC was 58.0% and 82.4%, respectively. The SEPT9 assay showed better performance in CRC detection than FIT, but similar performance for advanced adenomas. However, the FIT kit we used showed a lower sensitivity than the previous reports. More recently, the study of Johnson *et al*.[18] demonstrated a slightly superior sensitivity for CRC of SEPT9 than FIT but without statistical significance (73.3% *vs* 68.0%).

In this study, we found that 28 out of 69 CRC patients were detected by both SEPT9 and FIT, while 12 were detected by FIT but not SEPT9, and 25 were detected by SEPT9 but not FIT. Since the detected patients by SEPT9 assay and by FIT did not completely overlap, and the two tests detected CRC in entirely different manner, it is possible that the combined use of SEPT9 and FIT assays can increase the detection rate of CRC. The future CRC screening may benefit from this combination.

There were some limitations in our study. First of all, it was a retrospective case–control study, and the subjects were not all asymptomatic average-risk persons. So the performance estimates may differ substantially in prospective screening setting in which the test would finally be applied.[14] Secondly, though we compared tests performance between the SEPT9 assay and FIT, some important parameters such as patient compliance or cost-effectiveness could not be assessed in this study. However, some researches showed that many people who currently avoid CRC screening would be willing to take a simple blood test.[19] Thus the blood-based SEPT9 test may have advantages of patient acceptability compared with existing tests.

In conclusion, our study supports the use of SEPT9 test for CRC screening. The second-generation SEPT9 test showed better performance in CRC detection than the FIT kit we used, and may be superior to the first-generation SEPT9 products. The combination of the SEPT9 assay and FIT may represent a promising direction of future CRC screening, but its effect still needs further investigation. However, the sensitivity of SEPT9 for advanced adenomas was not better than FIT. Finally, the modified SEPT9 assay should be tested further in a prospective screening setting.

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