Published in final edited form as:

Clin Gastroenterol Hepatol. 2012 March ; 10(3): 272–7.e1. doi:10.1016/j.cgh.2011.10.008.

The Stool DNA Test is More Accurate than the Plasma Septin 9 Test in Detecting Colorectal Neoplasia

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Abstract

Background & Aims—Several noninvasive tests have been developed for colorectal cancer (CRC) screening. We compared the sensitivities of a multi-marker test for stool DNA (sDNA) and a plasma test for methylated *Septin 9* (SEPT9) in identifying patients with large adenomas or CRC.

Methods—We analyzed paired stool and plasma samples from 30 patients with CRC and 22 with large adenomas from Mayo Clinic archives. Stool (n=46) and plasma (n=49) samples from age- and sex-matched patients with normal colonoscopy results were used as controls. The sDNA test is an assay for methylated *BMP3*, *NDRG4*, *vimentin*, and *TFPI2*; mutant *KRAS*; the β -actin gene, and quantity of hemoglobin (by the porphyrin method). It was performed blindly at Exact Sciences (Madison WI); the test for SEPT9 was performed at ARUP Laboratories (Salt Lake City UT). Results were considered positive based on the manufacturer's specificity cutoff values of 90% and 89%, respectively.

Results—The sDNA test detected adenomas (median 2 cm, range 1–5 cm) with 82% sensitivity (95% confidence interval [CI], 60%–95%); SEPT9 had 14% sensitivity (95% CI, 3%–35%; $P=$.0001). The sDNA test identified patients with CRC with 87% sensitivity (95% CI, 69%–96%); SEPT9 had 60% sensitivity (95% CI, 41%–77%; $P=.046$). The sDNA test identified patients with stage I–III CRC with 91% sensitivity (95% CI, 71%–99%); SEPT9 had 50% sensitivity (95% CI, 28%–72%; $P=.013$); for stage IV CRC, sensitivity values were 75% (95% CI, 35%–97%) and 88% (95% CI, 47%–100%), respectively ($P=.56$). False-positive rates were 7% for the sDNA test and 27% for SEPT9.

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Disclosures: Mayo Clinic has licensed intellectual property to and is a minor equity investor in Exact Sciences; Dr. Ahlquist and Mr. Taylor were inventors of licensed technology. Dr. Ahlquist is a scientific advisor to Exact Sciences. Drs. Zou, Domanico, Berger, and Lidgard are employees of Exact Sciences.

Conclusions—Based on analyses of paired samples, the sDNA test detects non-metastatic CRC and large adenomas with significantly greater levels of sensitivity than the SEPT9 test. These findings might be used to modify approaches for CRC prevention and early detection.

Keywords

colon cancer; early detection; marker; genetics; Plasma Septin 9; Stool DNA; colorectal cancer; adenoma detection

INTRODUCTION

Molecular detection of colorectal cancer (CRC) offers the patient-friendly appeal of noninvasiveness, but there are fundamental biological differences between the different test approaches that influence test performance. A host of compounds from the basic marker classes (e.g. protein, RNA, and DNA) have been assayed from blood, stool, and urine with widely varying results, as reviewed^{1, 2}. Neoplasm detection rates depend on the nature of the marker(s), the mechanism of marker release into the target medium, and marker levels within that medium. Ideally, molecular markers would highly discriminate colorectal neoplasia from normal colon at the tissue level, be continuously released into the target medium from both CRC and advanced precancers, and be present at concentrations sufficient for assay detection. Two emergent molecular approaches for CRC screening—the commercially available plasma assay of methylated *Septin 9* (SEPT9) and a prototype next generation stool DNA (sDNA) test—are based on different biologies and have not been directly compared.

Aberrant methylation of the *Septin 9* gene highly discriminates colorectal neoplasia from normal mucosa at the tissue level³. While assay of naked methylated *Septin 9* in plasma has been shown to detect CRC, it remains unclear whether or not DNA markers from precancerous lesions access the circulation given that precancers by histological definition do not invade the basement membrane or underlying blood vessels. In the four peer-reviewed case-control studies, all from the referral setting³⁻⁶, SEPT9 yielded sensitivities for CRC ranging from 52-73% at specificities ranging from 84-91%; CRC detection rates were higher with late than with early stages. Using an analytically modified assay, SEPT9 detected 90% of referred CRC at 89% specificity in a preliminary report⁷. And, preliminary data from a large study on average-risk persons in the screening setting suggest a CRC detection rate of 50% by SEPT9; post-hoc analyses using a more sensitive assay algorithm suggest a CRC sensitivity of 67% and specificity of 88%⁸. Detection rates of advanced adenomas by SEPT9 have been reported in just two studies^{4, 6}, and sensitivities were only 17% (3 positive out of 18) and 18% (3 of 17). The SEPT9 assay is now commercially available as a laboratory developed test; it has not yet been approved by the Food and Drug Administration.

Next generation stool DNA (sDNA) testing promises high detection rates of both CRC and precancer buoyed by key technical advances including use of a preservative buffer with stool collection^{9, 10}, highly discriminant marker panels¹¹, and assays with analytical sensitivity 100-1,000 times greater than by earlier approaches¹¹⁻¹³. This approach is biologically based on exfoliation, which occurs continuously from the luminal surface of precancers and all stages of cancer^{1, 14, 15}. A number of small studies using next generation techniques have demonstrated that assay of methylated and mutated gene markers in stool can achieve high clinical sensitivity for both CRC and advanced adenomas^{12, 16, 17}. In a recent large case-control study¹⁸, a prototype multi-marker sDNA test detected 85% of CRCs and 63% of adenomas >1cm at a specificity cutoff of 90%; detection rates rose with increasing neoplasm size but were unaffected by neoplasm site or stage. As part of the Food

and Drug Administration approval process, a multicenter validation study of a refined version of this prototype sDNA test is currently underway.

Luminal exfoliation logically occurs earlier than vascular invasion in the course of colorectal tumorigenesis. Accordingly, we hypothesized higher detection rates of precancers and early stage CRC by sDNA than by SEPT9 testing. To test this hypothesis, we evaluated paired plasma and stool samples from patients with large colorectal adenomas and from those with all stages of CRC.

METHODS

Study Design and Participants

This blinded study, designed and coordinated at the Mayo Clinic, was approved by the Mayo Institutional Review Board and endorsed by the industry collaborators. The primary aim was to compare neoplasm detection rates by sDNA and SEPT9 tests on paired stool and plasma samples from the same case patients. Case patients with CRC or advanced adenomas were selected if they had participated in a recent multicenter evaluation of a prototype next generation sDNA test¹⁸ and if frozen plasma samples obtained prior to neoplasm resection were archived in the Mayo Tumor Registry. The study was not intended to compare specificities between tests. Rather, stool and plasma samples from colonoscopy-normal patients were used as process controls to minimize analytical bias and roughly assess false-positive rates in this outpatient setting. Paired stool and plasma samples were not available on colonoscopy-normal controls; thus, two control groups were selected, each matched on age and sex to cases. Control patients were excluded if they had a personal history of gastrointestinal neoplasia. Results from sDNA testing as determined in the parent multicenter study were used for cases and for stool controls; the database linking sample identification and clinical information has remained blinded to investigators (other than the study statistician) which allowed for the additional comparisons in the present study. Preset historical specificity cutoffs of 90% for sDNA¹⁸ and 89% for the SEPT9⁷ were used, and values exceeding these cutoffs for each test were called positive. Assays for sDNA and SEPT9 were performed in separate laboratories by technicians unaware of the clinical data. Combined data were analyzed independently by a Mayo statistician (DM), and summarized data were simultaneously communicated to all investigators.

Stool DNA Test (sDNA)

Sample collection, storage, processing, assay methods, and primer sequences are described in detail¹⁸ (see also Supplement 1). Whole stools were collected in a preservative buffer and stored at -80°C . The pre-commercial prototype sDNA assay performed at Exact Sciences included the following innovations: direct gene capture from fecal supernatant, an optimized rapid bisulfite treatment process, a panel of broadly informative DNA markers (methylated *BMP3*, *NDRG4*, *vimentin*, and *TFPI2* plus mutant *KRAS*) assayed by the analytically sensitive QuARTS method, quantitative fecal hemoglobin, and use of a logistic regression model for analysis. All sDNA assays were performed in blinded fashion at Exact Sciences Corporation (Madison WI). Test results were considered positive if they exceeded the 90% specificity cutoff, as determined in the parent study¹⁸.

Plasma Septin 9 Test (SEPT9)

Archival samples were obtained from the Mayo Clinic Colorectal Cancer Registry. Samples had been prepared as follows: whole blood was centrifuged at 3200rpm/1823g for 10 minutes; plasma was then re-spun at 3200rpm/1823g for 10 minutes and aliquoted into 2 mL cryovials and frozen at -80°C . The time from venipuncture to freezing was <4 hours for all

samples, and none had been previously thawed. This sample preparation was similar to that described by ARUP Laboratories (Salt Lake City UT) ⁷ and endorsed by them prior to study.

Frozen samples (2 vials or approximately 4 mL per patient) were sent to ARUP Laboratories for SEPT9 testing using their commercially available assay⁷. The SEPT9 result was judged as positive if at least 1 out of 3 replicates exceeded a pre-defined marker level. These criteria for positivity yielded a specificity of 89% in a recent preliminary report⁷.

Statistical Methods

Test positivity for both sDNA and SEPT9 tests was defined prior to un-blinding of patient disease status and was based on their respective historical specificity cut-off values. Observed sensitivity and specificity for each test was estimated with corresponding 95% confidence intervals based on the exact binomial distribution. Fischer's exact test for paired proportions was used to compare sensitivity between the two markers overall and within disease subgroups. Plasma samples from patients with normal colonoscopy were used as process controls and for estimating the specificity of SEPT9. Because both plasma and stool were not available on the same normal control patients, a paired assessment of specificity was precluded. To provide an estimate of specificity for sDNA, controls with available stool from the Mayo Clinic archive were randomly matched 1:1 to the plasma control samples on age and gender. These matched stool control samples represented a subset of control stools used to define the cut-off value of the sDNA test in the parent study¹⁸.

RESULTS

Patient and Lesion Characteristics

Patient age and sex distributions for cases and the two groups of process controls are shown (Table 1). The study included a total of 147 patients (52 cases, 49 plasma controls, and 46 stool controls). Cases with paired plasma and stool samples comprised 52 patients with advanced adenoma or CRC. Among the 22 adenomas: median size was 2.0 cm (range 1.0 -5.4) and 55% were located at or proximal to the splenic flexure. Among the 30 CRCs: median size was 4.3 cm (0.8 – 8.3); 50% were proximal; and 7 (23%), 7 (23%), 8 (27%), and 8 (27%) were Stage I, II, III, and IV, respectively.

Comparison of Neoplasm Detection Rates: sDNA vs SEPT9

All Neoplasms—Considering all neoplasm cases (large adenomas + CRC) with paired specimens, sensitivity was 85% (95% CI 72-93%) by sDNA compared to 40% (27-56%) by SEPT9, $p=0.0001$. Because the quantity of plasma received from the archive fell below the desired threshold of 3.6 ml in 8 cases, tests were also compared on only those with plasma volumes ≥ 3.6 ml. In these 44 cases with ≥ 3.6 ml of plasma available for analyses, observed detection rates were essentially the same as in the whole group; sensitivity was 84% (70-93%) by sDNA and 39% (34-55%) by SEPT9, $p=0.0001$.

Adenomas—Detection of large adenomas by sDNA was substantially and significantly higher than by SEPT9 (Figure 1). Sensitivity was 82% (95% CI 60-95%) by sDNA compared to 14% (3-35%) by SEPT9, $p=0.0001$.

Cancers—Detection of CRC was also significantly higher by sDNA than by SEPT9; overall CRC sensitivity was 87% (95% CI 69-96%) by sDNA versus 60% (41-77%) by SEPT9, $p=0.046$ (Figure 2). Differences between tests were most striking for early stage and proximal cancers. Respective detection rates for stages I, II, III, and IV cancers were 86%, 86%, 100%, and 75% by sDNA and 57%, 57%, 38%, and 88% by SEPT9. Among combined stages I-III, sDNA detected 91% (95% CI 71-99%) compared to 50% (28-72%)

by SEPT9, $p=0.013$ (Figure 2); detection rates for stage IV CRC did not differ significantly between tests, $p=0.56$ (Figure 2). Detection of proximal CRC was 92% (64-100%) by sDNA compared to 46% (19-75%) by SEPT9, $p=0.034$; and detection of distal CRC was 81% (54-96%) by sDNA compared to 69% (46-89%) by SEPT9, $p=0.48$ (Figure 3).

False-positive Rates

Among the 46 control stools, 3 (7%) were false-positives yielding an observed specificity by sDNA of 93% (95% CI 84-98%). Among the 48 control plasma samples, 13 (27%) were false-positives yielding an observed specificity by SEPT9 of 73% (58-85%).

Because of the unexpectedly high false-positive rate for SEPT9, Tumor Registry data and medical records on all 48 plasma control patients were reviewed. We found one patient with a remote personal history of CRC had not been disqualified and 4 patients with non-gastrointestinal malignancies (including one with multiple myeloma, one with lymphoma, and two with prostate cancer) who presented after the date that stools had been collected and archived; four of these 5 control patients were from the false-positive subset and one from the true-negative subset. If these 5 patients are excluded, then the re-calculated specificity for SEPT9 would be 79% (95% CI 64-90%).

DISCUSSION

In this comparison of test sensitivities using paired stool and plasma samples on cases from the outpatient setting, both curable-stage CRC and large colorectal adenomas were detected at significantly higher rates by sDNA than by SEPT9 testing. These findings lend support to the biological concept that abundant marker release by luminal exfoliation into stool occurs earlier than by vascular invasion into blood during the progression of CRC tumorigenesis. Study results have important implications on the relative effectiveness for CRC prevention and early detection by these two approaches.

The sDNA test detected screen-relevant neoplasms (advanced adenomas and early stage CRC) with high sensitivity. Detection rates observed in the present study generally reflect those in the large multicenter parent study¹⁸. CRC detection by the sDNA test was 87% in the present study compared to 85% in the parent study. The 82% detection rate of large adenomas in the present study was slightly greater than that of similar-sized adenomas in the parent study (e.g. sensitivity for adenomas >2cm was 77%). Size (or luminal surface area) appears to be the lesion characteristic that most critically influences neoplasm detection by sDNA testing. When adjusted for size, adenoma and CRC detection rates by sDNA are comparable¹⁸. In the parent multicenter study¹⁸, 63% of adenomas > 1cm were detected by sDNA testing and detection rates rose further in proportion to enlarging polyp size and increasing risk of progression. Neoplasm site did not affect sDNA results in the present study, which is consistent with earlier findings by our group¹⁸⁻²⁰ and others¹⁶. And, importantly, CRC detection rates were comparably high by sDNA across stages I-III, as was observed in the parent study¹⁸, suggesting that stool marker levels are not affected by depth of tumor invasion.

In the present study, SEPT9 essentially failed to detect large adenomas and detected significantly fewer early stage CRCs than did sDNA. The observed detection rate for large adenomas of 14% did not exceed the background false-positive rate. Other studies have reported comparably low SEPT9 sensitivities for advanced adenoma^{4, 6}. The low adenoma detection rates by SEPT9 cannot be explained by low tissue levels of methylated *septin 9*, as this marker is broadly expressed in adenoma tissue^{3, 7}. The observed CRC detection rate of 60% by SEPT9 is in keeping with sensitivities reported in peer-reviewed publications of 52%³, 58%⁴, 69%⁵, and 73%⁶. Detection rates by SEPT9 were highest for stage IV CRC,

which was the only subset where detection rates for SEPT9 were higher than for sDNA, although the difference was not significant.

Supported by results from this direct comparison study and from the literature, a conceptual model can be derived showing key differences in marker release into blood and stool according to phases of CRC tumorigenesis (Fig. 4). Based on collective observations in patients with colorectal neoplasia, the mechanism of vascular invasion appears to be centrally important for DNA markers to gain entry into blood and plasma. In studies quantifying methylated⁴ or mutated²¹ DNA markers in plasma, marker distributions in patients with advanced adenomas have been essentially no different than in normal controls, levels in stage I CRC overlap substantially with normal controls, and median levels with CRC increase in proportion to stage with 2-4 orders of magnitude differences between stage I and stage IV. Thus, the underlying biology of marker release may represent a fundamental barrier to detection of precursor or pre-invasive lesions by many blood-based tests and limit detection of earliest stage CRC as well. In contrast, evidence from next generation sDNA testing indicate that luminal exfoliation occurs abundantly with large high risk adenomas and across all stages of CRC. It is this difference in the mechanisms of marker release, rather than in marker expression at the tissue level, that likely accounts for the disparate detection rates of large adenomas and earliest stage CRC by sDNA and SEPT9 tests.

The high rate of false-positives with SEPT9 was somewhat surprising, and a post-analysis clinical review revealed that non-gastrointestinal malignancies accounted for 4 (31%) of the 13 false-positives. In other reports^{4, 6}, false-positive rates have been variably high among patients with extra-colonic cancers, inflammatory disorders, or risk factors for CRC. False-positive rates may be lower in patients from the screen-setting, but these observations raise questions about SEPT9 specificity in a general population application and the multiple potential contributors to elevated plasma levels.

Our study has both strengths and limitations. Strengths include the novelty and timeliness of this first direct comparison between sDNA and SEPT9 test sensitivities; availability of paired archival stool and plasma specimens on well-characterized case patients with colorectal neoplasia; and state-of-the-art sDNA and SEPT9 assays performed in separate laboratories under blinded conditions. A limitation of this study involved the use of participants from the referral/outpatient setting, and results may not be representative of those in asymptomatic persons undergoing general screening. Although the sample size in this study was modest, the wide differences in test performances permitted sufficient power to confidently compare detection rates.

Given the findings in the present study, the weight of published evidence, and consideration of the underlying biology of marker release, detection rates by sDNA appear to be higher for early stage CRC and much higher for large adenomas than rates by SEPT9. If such differences hold with application in the general screening setting, then the potential impact on both CRC prevention and early detection would be greater with sDNA than with SEPT9 testing. Further studies are called for to corroborate and extend these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Mary Devens and Julie Simonson for recruitment of patients and maintenance of the clinical database, Tracy Yab for assistance in biospecimen processing and archiving, and Jaci McCormick for assistance

with manuscript submission. We also thank ARUP Laboratories Corporation for provision of plasma *septin 9* assays at no cost.

Roles of Authors: Ahlquist: Principal investigator, study design/oversight, manuscript preparation; Taylor: Marker design, biospecimen preparations, manuscript review; Mahoney: Statistical analyses, manuscript review; Zou: Stool assay design, marker selection, manuscript review; Domanico: Stool assay performance/quality at Exact Sciences, manuscript review; Thibodeau: Laboratory oversight for stool DNA assays at Mayo Medical Laboratories, manuscript review; Berger: Co-management of clinical study, manuscript review; Lidgard: Endorsement of protocol, oversight stool assay development and performance at Exact Sciences, manuscript review

Funding: NIH grant CA 89389, Charles Oswald Foundation, Exact Sciences Corporation, and Mayo Clinic

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Figure 1.

Detection rates of large colorectal adenomas by next generation stool DNA and plasma *septin 9* tests (n = 22). Median adenoma size was 2.0 cm (range 1.0 – 5.4), and 55% were proximal to the splenic flexure.

Figure 2.

Detection rates of colorectal cancer by stool DNA and plasma *septin 9* testing. Rates are compared for the overall group, n = 30; the subset without distant metastases (stages I-III), n = 22; and the subset with distant metastases (stage IV), n = 8.

Figure 3. Comparison of colorectal cancer detection rates by stool DNA and plasma *septin 9* testing according to tumor site. See footnote in Table 1 for definitions of proximal and distal.

Figure 4.

Molecular marker release from colorectal neoplasms into target media. This conceptual model shows proportional differences (illustrated by arrow sizes) expected in rates of marker release into the bloodstream via the mechanism of vascular invasion and into the the stool via the mechanism of exfoliation during progressive phases of tumorigenesis. Marker release into the bloodstream from precursor lesions is negligible but increases progressively with advancing stages of cancer. In contrast, marker release by exfoliation into stool occurs at comparable rates from large precancers and all stages of cancer.

Table 1**Patient and Colorectal Lesion Characteristics**

Patient	Paired Cases (52)	Plasma Controls (49)	Stool Controls (46)
Age, median (25-75%)	69 (61-75)	63 (52-71)	59 (51-66)
Sex, % women	46	47	57
Lesion			
Adenoma (22)			
Size, median cm (range)	2.0 (1.0-5.4)	--	--
Site, % proximal *	55	--	--
Cancer (30)			
Size, median cm (range)	4.3 (0.8-8.3)	--	--
Site, % proximal *	50	--	--
Stage I/II/III/IV **	7/7/8/8	--	--

* The "proximal" colon is defined as the cecum, ascending colon, and transverse colon; and "distal" colorectum as the splenic flexure, descending colon, sigmoid, and rectum.

** Data shown represent numbers of cancer at each stage.