Evaluation of Circulating Tumor DNA for Methylated *BCAT1* and *IKZF1* to Detect Recurrence of Stage II/ Stage III Colorectal Cancer (CRC)

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ABSTRACT

Background: Most recurrences of early-stage colorectal cancer detected with current surveillance measures are widespread and incurable. Circulating tumor DNA (ctDNA) may facilitate earlier diagnosis of recurrent colorectal cancer and improve cancer-related outcomes.

Methods: Plasma from patients undergoing standard surveillance after definitive treatment for stage II/III colorectal cancer was assayed with COLVERA and carcinoembryonic antigen (CEA) at a single time point. Results were correlated with radiographic imaging. Assay performance, including sensitivity and specificity for recurrence, were compared. Impact of potentially confounding variables was also explored.

Results: 322 patients were included in the final analysis, and 27 recurrences were documented over a median follow-up period of 15 months. Sensitivity for recurrence was 63% [confidence interval (CI), 42.4–80.6] and 48% (CI, 28.7–68.1) for COLVERA and CEA (≥5 ng/mL), respectively ($P = 0.046$), while specificity was 91.5% (CI, 87.7–94.4) and 96.3% (CI, 93.4–98.1), respectively ($P = 0.016$). Smoking and age were independent predictors of CEA but not COLVERA positivity.

Conclusions: COLVERA was more sensitive but less specific than CEA in detecting recurrent colorectal cancer. Short median follow-up may have been responsible for apparent false positives in COLVERA. Studies with serial sampling and longer follow-up are needed to assess whether earlier detection of colorectal cancer recurrence translates into clinical benefit.

Impact: This prospective study showed that COLVERA (a two-gene ctDNA assay) was more sensitive for detection of recurrence in a cohort of patients undergoing surveillance after definitive therapy for stages II and III colorectal cancer.

Introduction

Colorectal cancer remains a leading cause of cancer-related mortality, accounting for over 53,000 deaths in the United States (1) and 850,000 deaths globally each year (2). Although the overall incidence of colorectal cancer has declined in the United States due to widespread screening, the incidence of colorectal cancer has increased among younger individuals, in particular those between the ages of 20 and 39 (3). Advances in therapeutic approaches for early-stage colorectal cancer, including surgical resection, adjuvant chemotherapy, and (in the case of rectal cancer) neoadjuvant chemoradiation, have improved cure rates to 90% with stage I disease and 75% with stage III disease (1). Although an ever-expanding armamentarium of systemic therapy has improved the prognosis of stage IV colorectal cancer, with 5-year survival approaching 20%, most cases of colorectal cancer recurring after definitive treatment of early-stage disease are incurable (4). The primary motivation for aggressive surveillance after treatment of early-stage colorectal cancer is therefore the detection of limited metastatic disease amenable to potentially curative surgical resection (5, 6).

Surveillance after treatment of nonmetastatic colorectal cancer typically includes serial serum carcinoembryonic antigen (CEA) monitoring and radiographic imaging (most commonly, CT) to detect recurrence in distant sites, as well as colonoscopy to detect intraluminal recurrences, new tumors, or premalignant polyps (7–11). Unfortunately, CEA is not sensitive enough to detect most recurrences when they are potentially curable and is subject to false positivity in nonneoplastic conditions, such as smoking and chronic inflammatory diseases (12, 13). Furthermore, because it is reported as a continuous variable with cutoffs for positivity that vary among laboratories, CEA can cause enough confusion and anxiety among physicians and patients to warrant repeated testing (14, 15). CT scans have limited ability to detect metastases smaller than one centimeter, are prone to false-positive ("incidental") findings, and require radiation exposure (16, 17). Colonoscopy, while an effective tool to detect intraluminal recurrences, is unable to detect extraluminal recurrences (15). Finally, even when the current surveillance approach detects resectable recurrences, improved outcomes reported in the medical literature are prone to lead-time bias (18–20). As a result, a novel noninvasive approach to detecting colorectal cancer recurrence early enough to change its natural history is greatly needed.

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A growing body of evidence suggests that detecting cancer-specific genetic and epigenetic alterations in circulating cell-free DNA (cfDNA) may be a highly sensitive and specific method of diagnosing cancer recurrence (21). Studies have shown the cfDNA technology can detect the presence of primary colorectal cancer (22–27) as well as disease recurring after primary tumor resection (28–30). Multiple cfDNA assays have been developed and tested, including customized gene mutation panels derived from resected tumor samples and assays for specific methylation changes common to most colorectal tumors (24, 29, 31). Methylation-based assays, in particular, show promise because they do not require gene sequencing of the primary tumor to formulate a unique biomarker panel for each patient.

Hypermethylation of *BCAT1* and *IKZF1* occurs in 95% of colorectal cancer tumors and can be detected in the blood of patients with colorectal cancer (31, 32). This high level of aberrant methylation in colorectal cancer makes these biomarkers an attractive choice for colorectal cancer recurrence monitoring regardless of underlying colorectal cancer molecular genotype. Retrospective observational studies conducted in Australia have already shown that COLVERA, a qualitative blood test that detects methylated *BCAT1* and *IKZF1* in cfDNA, is more sensitive than and as specific as CEA for predicting recurrence in patients undergoing surveillance after resection of primary colorectal cancer (30, 33). Because reliability of cancer-specific methylation assays may vary based on age, race, and ethnicity, validation of epigenetic biomarkers in a diverse population of patients is warranted to ensure generalizability (34).

The primary aim of this study conducted in the United States was to investigate the sensitivity and specificity of COLVERA, an assay detecting methylation of *BCAT1* and *IKZF1* in cfDNA, to standard CEA, both measured at the same single time point in a cohort of patients undergoing surveillance after definitive treatment of stage II or III colorectal cancer.

Methods

Study population

Adults (≥18 years old) who were undergoing surveillance after definitive therapy for stage II or III colorectal cancer (35) at 24 participating centers throughout the United States were invited to participate in this prospective, cross-sectional observational study. Stage for colon cancer was determined by pathologic evaluation of the resection specimen, whereas stage of rectal cancer was determined by clinical staging prior to neoadjuvant therapy. To be eligible, patients must have completed all components of their definitive treatment without any evidence of residual cancer on standard radiographic imaging. Exclusion criteria included an active synchronous cancer in any organ or any comorbidity that would make standard surveillance medically inappropriate.

The study was approved by the Institutional Review Boards of the 24 participating U.S. centers. Written informed consent was obtained from all subjects prior to any procedures. Patients were recruited between February 2018 to August 2019.

Blood collection and handling

Blood samples were obtained at a single time point within 6 months of scheduled radiologic imaging for surveillance. Blood samples collected after imaging were collected prior to any treatment for confirmed or suspected recurrence. Venous blood was collected in two 9 mL K2-EDTA vacutainers and two 10 mL PAXgene tubes (PAXgene Blood cfDNA Tube, product # 768115, PreAnalytiX). K2EDTA tubes were processed to plasma and stored as described previously (30), and shipped frozen to a central laboratory (Clinical Genomics Pathology Inc). PAXgene blood tubes were shipped at ambient temperature to the central laboratory and processed into plasma within 5 days of collection. All plasma samples were stored at −80°C until assayed. Laboratory personnel were blinded to clinical status at all times.

Recurrence status determination

False-positive rates were determined using radiologic and pathologic data as described previously (30). Local recurrence was defined as recurrence at the site of anastomosis and distant recurrence was defined as recurrence in another visceral organ, nonregional lymph nodes, or the peritoneal cavity. When both local and distant recurrence were documented, recurrence was documented as distant.

Blood testing: *BCAT1/IKZF1* methylation

The two-gene methylation-specific PCR assay COLVERA (Clinical Genomics Pathology Inc.) was performed in accordance with previously published methods (36), with minor modifications for PAXgene plasma using QIAsymphony PAXgene Blood ccfDNA kit (Qiagen; catalog no. 768536) as per manufacturer's instructions. Briefly, cfDNA was isolated from plasma, bisulfite-converted, and assayed in triplicate by real-time PCR for appearance of methylated *BCAT1* and *IKZF1*. COLVERA is reported as a qualitative test for methylated ctDNA reflecting any detectable methylated target for either gene (POSITIVE) or no detected methylated target (NEGATIVE). For comparison to CEA, a quantitative test, the total mass of methylated DNA product was also calculated by measuring PCR results against a standard curve of known target mass and adding results from each of BCAT1 and IKZF1 to yield total methylated DNA.

Blood testing: CEA

The concentration of CEA for each plasma specimen was determined using a commercially available CEA assay as recommended by the manufacturer (Architect, Abbott Diagnostics). Quantitative CEA values were reported for each specimen. A CEA value equal to or greater than 5 ng/mL was considered positive, although CEA positivity at cutoff levels of 7.5, 10, and 15 ng/mL were also reported.

Results of COLVERA and study CEA were not disclosed to patients or their clinicians.

Quality controls

Recurrence status was determined by the treating physicians with supporting documentation provided in the study database. To maintain standardized and unbiased reporting, radiology and pathology reports were reviewed by two reviewers (B.L. Musher and G.P. Young) who were blinded to each site's prior clinical assessment and to the COLVERA and CEA results.

Statistical analysis

The data were analyzed and reported using descriptive statistics, including medians and interquartile ranges (IQR) where appropriate. Demographics and baseline characteristics, stratified by recurrence and nonrecurrence, were evaluated and reported. Stratified populations were compared using two correlated proportions methodologies, and a contingency table was created and analyzed. Assay performance was determined by calculating standard diagnostic characteristics, including sensitivity and specificity, and 95% confidence intervals (CI) were provided for all performance estimates using methods of Clopper Pearson (37). McNemar test for concordance was used to evaluate significance for proportional differences in 2×2 contingency

tables comparing test results in patients with recurrence (sensitivity) and without recurrence (specificity; ref. 38). No continuity correction adjustment was made in the case of zero values in the 2 × 2 contingency table (38). Statistical results were reported in absolute values and relative proportions. All statistical tests were two-sided and a P value of <0.05 determined statistical significance. The study protocol was designed to have an 80% power to detect difference in sensitivity between CEA and ctDNA, assuming a real proportional difference of 30% in 24 paired blood samples (after recruitment, dropout, etc.), based on prior published data (30).

To evaluate the independent predictors of assay positivity, logistic regression was used. Model parameters were selected on the basis of published data demonstrating an effect of age, gender, and race on methylation status and smoking on serum CEA level. Where independence was not observed (e.g., smoking and recurrence) in this study, a linear interaction term was added to the model.

Receiver operator characteristic (ROC) curve analysis is used to explore the sensitivity and specificity of CEA depending on the threshold used to define positivity.

All analyses were carried out in R (version 3.6.2) or Python (version 3.7).

Other

The trial was registered at ClinicalTrials.gov (NCT03706235). This study was conducted in accordance with the Declaration of Helsinki (WMA 2013) and Good Clinical Practice (GCP ICH-E6).

Results

Study population

A total of 537 subjects signed consent and enrolled in this study. Twenty patients ($N = 20$, 3.7%) were excluded from final analysis for failure to satisfy inclusion/exclusion criteria based on additional data that became available after enrollment, and an additional 195 patients were excluded for insufficient clinical or radiographic data, leaving 322 patients for final analysis (**Fig. 1**).

Clinical and demographic characteristics of the evaluable cohort are shown in **Table 1**. The median age of evaluable patients was 63.5 (range 25–89, IQR: 55.25–73.0), and 56% were male. Seventy-two percent identified as White, 13% as Hispanic or Latino, 11% as Black or African American, and 3% as Asian. The majority (72%) had primary colon cancer while the rest had primary rectal cancer. Sixty percent of patients had stage III disease while 40% had stage II disease. Twenty-five percent underwent surgical resection alone, 73% underwent surgery plus additional (neoadjuvant and/or adjuvant) therapy, and 2% underwent definitive chemoradiation (no surgery).

Imaging and specimen collection

By definition, all evaluable patients had at least one imaging study. Those patients in whom recurrence was not detectable had exactly one (negative) study while those whose cancer recurred may have undergone more than one scan to confirm recurrence. As permitted by the protocol, the timing of the blood draw in relation to imaging varied. The majority (66.4%) of patients provided a specimen prior to imaging (median 69 days, IQR: 12.3–112 days), 21.4% provided a blood sample on the day of imaging, and 12.1% provided one after imaging. All blood specimens were, however, collected before treatment for recurrence was initiated. CT imaging alone was used to evaluate recurrence status in 306 of 322 (95%) patients. The other 14 patients had either PET/CT or a combination of CT and MRI. Two patients were diagnosed with local recurrence on endoscopy, and neither had distant metastases on CT imaging.

Recurrence

Recurrence was documented in 27 (8.4%) of the evaluable patients, 25 (93%) of whom had colon cancer and two (7%) of whom had rectal cancer. The recurrence rate for colon cancer (25/233, 10.7%) was significantly higher than that for rectal cancer (2/89, 2.2%; $P = 0.027$). Nineteen (70.4%) of the recurrences occurred in subjects with stage III disease, and the remaining 8 (29.6%) occurred in those with stage II disease. The observed recurrence rates for stage II and stage III patients were 6.2% and 9.8%, respectively ($P = 0.341$). **Table 1** shows the clinical characteristics of the evaluable subjects with recurrence. The proportion of current smokers was higher in patients who exhibited recurrence (38.5%) than in patients who did not recur (13.4%; $P = 0.03$). Twenty-four (89%) recurrences occurred in distant sites, the most common sites being liver ($n = 11$, 46%), peritoneum ($n = 7$, 29%), and lung ($n = 4$, 17%; Supplementary Table S1); the remaining 11% of recurrences were local.

Because patients were eligible to enroll between 1 and 60 months after completion of therapy and because accrual occurred between February 2018 and August 2019, there was considerable variability in time from completion of treatment to enrollment. Median duration of follow-up after completion of therapy until imaging used for this study was 15 months, ranging from 1 to 60 months (IQR: 9.3–22.0 months). Median time to recurrence following completion of therapy for early-stage disease was 12.2 months, ranging from 3.6 to 51.4 months (IQR: 7.4–17.1 months). The median duration of follow-up for evaluable subjects without cancer recurrence was 15 months (IQR: 9.6–22.3 months; Supplementary Fig. S1).

Assay performance

The results of paired blood testing by recurrence status for all 322 evaluable patients are shown in **Table 2**, and assay results are shown in **Fig. 2**.

On the basis of 27 observed recurrences in this study, the sensitivities of COLVERA and CEA for detecting colorectal cancer recurrence using a single time-point blood test were 63.0% (17/27; 95% CI, 42.4–80.6) and 48.1% (13/27; 95% CI, 28.7–68.1), respectively ($P = 0.046$). COLVERA detected four cancers not detected by CEA. No recurrences associated with a CEA >5 ng/mL had a negative COLVERA assay, meaning COLVERA detected all the recurrences that were detected by CEA.

Among patients whose cancer recurred and in whom COLVERA was negative (i.e., false negatives), there were no defining characteristics of the primary tumor or recurrent disease that met statistical significance in this small sample size. Detailed clinical characteristics for cancer recurrences are provided in Supplementary Table S3.

Because the threshold for CEA positivity can vary among diagnostic laboratories and mild elevations in CEA may be attributed to non-malignant causes (e.g., smoking, intestinal inflammation), sensitivity of COLVERA was compared with sensitivity of CEA across a range of cutoff values. As the threshold for CEA positivity increased from 5 to 15 ng/mL, CEA sensitivity decreased (**Table 3**; **Fig. 2**; additional detail in Supplementary Fig. S2). The specificities of one-time COLVERA and CEA (≥5 ng/mL) were 91.5% (95% CI, 87.7–94.4) and 96.3% (95% CI, 93.4–98.1), respectively ($P = 0.012$). However, since the 297 patients without recurrence underwent imaging only once at a median time of 24 months from end of therapy (Supplementary Fig. S1), it is possible that more recurrences would have been documented with

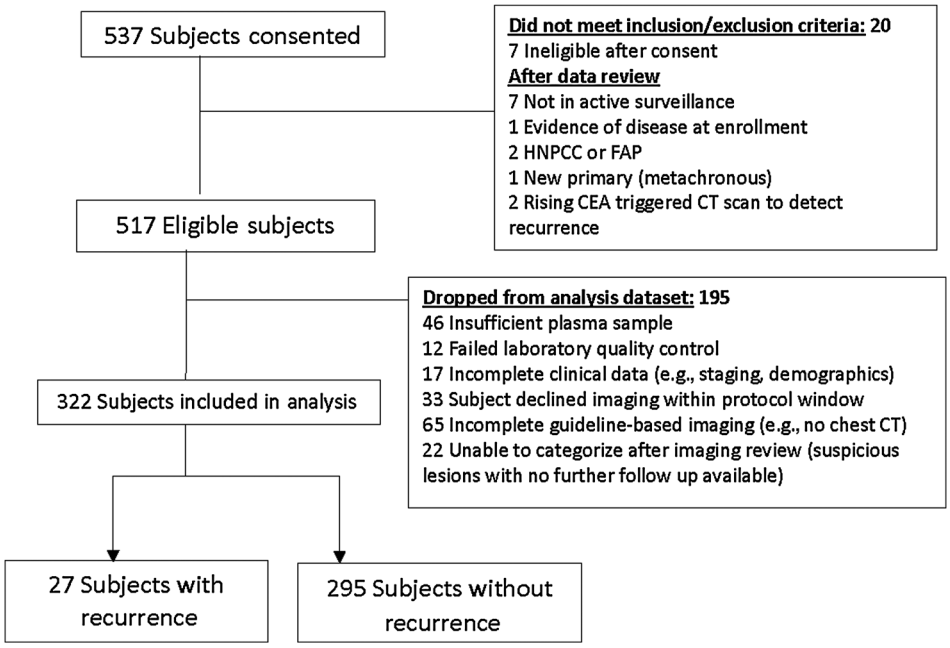

Figure 1.
Disposition of study patients. FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis colorectal cancer.

Table 1. Characteristics of patients included in the primary analysis.

Subjects ($N = 322$)		All subjects	Recurrence ($N = 27$)	No recurrence ($N = 295$)	*P*
Age	Median (Q1–Q3)	63.5 (55.3–73)	67 (61–72)	63 (55–74)	0.16[a]
Gender	Male (%)	1182 (56%)	18 (66.6%)	164 (55.2%)	0.27[b]
Ethnicity	Hispanic	41 (13%)	2 (7.4%)	39 (13.2%)	0.55[b]
	Not Hispanic	281 (87%)	25 (92.6%)	256 (86.8%)	0.55[b]
Race	Asian	9 (3%)	1 (3.7%)	8 (2.7%)	0.55[b]
	African American	36 (11%)	2 (7.4%)	34 (11.4%)	0.75[b]
	White	232 (72%)	22 (81.5%)	210 (70.7%)	0.37[b]
	Other	3 (1%)	0 (0%)	3 (1.0%)	1.00[b]
Subject ever smoked	Yes	147 (45.6%)	13 (48.1%)	134 (45.4%)	0.79[b]
Subject currently smokes	Yes	23 (15.5%)	5 (38.5%)	18 (13.4%)	0.033[b]
Primary tumor	Colon	233 (72.3%)	25 (92.6%)	208 (70.5%)	0.013[b]
	Rectum	89 (27.7%)	2 (7.4%)	87 (29.5)	0.013[b]
Stage at diagnosis (AJCC 7.0)	IIA	110 (34%)	7 (25.9%)	103 (34.7%)	0.40[b]
	IIB	12 (4%)	1 (3.7%)	11 (3.7%)	1.00[b]
	IIC	7 (2%)	0 (0.0%)	7 (2.4%)	1.00[b]
	IIIA	52 (16%)	5 (18.5%)	47 (15.8%)	0.78[b]
	IIIB	106 (33%)	11 (40.7%)	95 (32.3%)	0.40[b]
	IIIC	35 (11%)	3 (11.1%)	32 (10.8%)	1.00[b]

[a]Two-sided *t* test (normal distribution).
[b]χ^2 test.

Table 2. Performances of the methylated *BCAT1/IKZF1* (COLVERA) and CEA blood tests.

	COLVERA (*BCAT1/IKZF1*)					CEA (≥5 ng/mL)			
	Total	Positive	% Pos	95% CI[a]	OR (95% CI[b])	Positive	% Pos	95% CI[a]	OR (95% CI[b])
Total patients	322	42	13.0%	9.6–17.2	NA	24	7.5%	4.8–10.9	NA
Recurrence	27	17	63.0%	42.4–80.6	18.4 (7.6–44.4)	13	48.1%	28.7–68.1	24.0 (9.1–63.0)
Local	3	1	33.3%	0.8–90.6	5.4 (0.5–61.7)	0	0	0.0–70.8	NA
Distant	24	16	66.7%	44.7–84.4	21.6 (8.4–55.4)	13	54.2%	32.8–74.4	30.5 (11.2–83.3)
No recurrence	295	25	8.5%	5.6–12.3	1	11	3.7%	1.9–6.6	1

Note: Calculation of OR against cases with no recurrence. Local recurrence is defined as in the colon/at anastomosis. Distant recurrence is defined as all other locations.
Abbreviation: NA, not applicable.
[a]Exact Clopper–Pearson confidence limits.
[b]Asymptotic confidence limits.

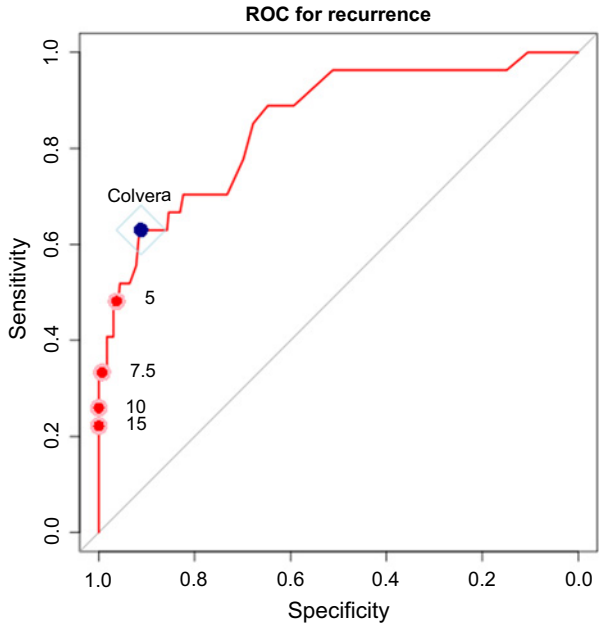

Figure 2.
ROC for recurrences. Observed sensitivity and specificity for the range of CEA values observed (red line) with the cutoff at 5, 7.5, 10, and 15 ng/mL illustrated by the red circles. COLVERA results are reported as positive or negative based on detectability of ctDNA, and the observed sensitivity and specificity are shown (blue diamond).

longer follow-up and further imaging, thus increasing both the sensitivity and specificity of COLVERA.

Gene-level methylation and biomarker quantification

While the methylation levels of *BCAT1* were strongly correlated with methylation levels of *IKZF1* ($R^2 = 0.999$; 95% CI, 0.9986–0.9998; Supplementary Fig. S3) when both biomarkers were detected in a plasma specimen, positivity of only one biomarker was detected in 4 patients with colorectal cancer recurrence: 3 with methylated *BCAT1* and 1 with methylated *IKZF1*.

Effect of other parameters on assay performance

Age, gender, race, and smoking history may affect epigenetic biomarkers as well as CEA irrespective of disease status and therefore confound detection of colorectal cancer recurrence using these biomarkers. A significantly higher risk for colorectal cancer recurrence was detected among current smokers (OR = 3.82; 95% CI, 1.16–12.0) when compared with never smokers (Supplementary Table S2). To adjust for any confounding effect of this association on performance of COLVERA and CEA, models were adjusted to include an interaction term for smoking and recurrence (**Table 4**). Detection of methylated *BCAT1* and *IKZF1* was not affected by race, gender, age, or smoking history in the recurrence model. Conversely, smoking was a significant independent predictor of CEA positivity. After controlling for recurrence status, age, gender, and race, the OR for having an elevated CEA was 7.7 (95% CI, 1.2–50.0; $P = 0.0313$) in current smokers when compared with nonsmokers. Age was also independently associated with CEA elevation, but with an OR of only 1.07 (95% CI, 1.02–1.14; $P = 0.012$).

Discussion

In this prospective cohort study, COLVERA demonstrated a higher sensitivity than CEA in detecting recurrence of stage II/III colorectal cancer. Smoking and age were independent predictors of CEA positivity, whereas COLVERA positivity was not affected by any clinical or demographic parameter. The sensitivity of CEA declined further as the threshold for positivity increased from 5 to 15. The specificity of COLVERA during this study of relatively short follow-up was lower than that of CEA.

The findings of this study are consistent with prior investigation into the utility of methylation biomarkers (30, 33) for colorectal cancer surveillance. For example, Australian and Chinese studies showed that circulating methylated *BCAT1/IKZF1* and *SEPT9*, respectively, were more sensitive than CEA in detecting colorectal cancer recurrence (33, 39, 40). Because epigenetic biomarkers can be influenced by race and ethnicity (34, 41), the current study—which was conducted in 24 centers, community and academic alike, across the United States—provided the opportunity to validate the findings of prior studies in a diverse population. The final cohort of 322 evaluable individuals included White, Black, Latino, and Asian patients, and race/ethnicity was not an independent predictor of COLVERA positivity. By validating these previously reported data in a diverse U.S. cohort, this study confirms that the methylated *BCAT1* and *IKZF1* assay is reproducible across laboratories and countries, an important consideration for routine use in clinical practice.

Current guidelines (42) for surveillance after definitive colorectal cancer therapy include CEA every 3 to 6 months and CT imaging every 6 to 12 months for 5 years. Because CEA is a continuous variable and subject to mild elevation in nonneoplastic conditions (including smoking and inflammatory diseases), the CEA threshold that triggers additional evaluation varies among clinicians. For example, a clinician may choose not to pursue further evaluation in a smoker unless a CEA exceeds 10. Our study confirmed that smoking was a strong predictor of CEA positivity while the only independent predictor of COLVERA positivity was recurrence status. This study also showed that CEA

Table 3. Performances of the methylated *BCAT1/IKZF1* (COLVERA) and CEA blood tests at various cutoffs for positivity for CEA.

		CEA ≥ 5 ng/mL			CEA ≥ 7.5 ng/mL			CEA ≥ 10 ng/mL			CEA ≥ 15 ng/mL		
Recurrence *N* = 27		**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**	**Positive**	**Negative**	**Total**
COLVERA (BCAT1/IKZF1)	Positive	13	4	17	9	8	17	7	10	17	6	11	17
	Negative	0	10	10	0	10	10	0	10	10	0	10	10
	Total	13	14	27	9	18	27	7	20	27	6	21	27
McNemar test: asymptotic *P* value		$P = 0.046$			$P = 0.005$			$P = 0.002$			$P = <0.001$		

Note: No adjustment is made for zero values in the 2 × 2 Table (1).

Table 4. Independent predictors of COLVERA and CEA positivity.

COLVERA predictors	Log OR	SE	2.5% (CI Log OR)	97.5% (CI Log OR)	*P*
Base[a]	1.00				
(Intercept)	−3.23	1.26	0.0033	0.47	0.01
Age	0.02	0.02	0.98	1.05	0.35
Gender (male)	−0.36	0.39	0.32	1.51	0.36
Previous smoker	−0.45	0.49	0.24	1.68	0.36
Current smoker	0.73	0.70	0.52	8.28	0.30
Recurrence	3.27	0.68	7.00	99.26	1.3E-6
Race (Asian)	−0.29	1.25	0.065	8.67	0.82
Race (Black)	0.31	0.59	0.43	4.32	0.60
Ethnicity (Hispanic)	0.17	0.58	0.38	3.65	0.77
Previous smoker and recurrence	−0.08	1.10	0.11	7.91	0.94
Ever smoker and recurrence	−1.95	1.30	0.01	1.80	0.13
CEA predictors	**Log OR**	**SE**	**2.5% (CI LOR)**	**97.5% (CI LOR)**	***P***
Base[a]	1.00				
(Intercept)	−8.56	2.23	2.42E-6	0.015	0.00125
Age	0.07	0.029	1.01	1.13	0.01
Gender (male)	0.068	0.54	0.69	3.04	0.8491
Previous smoker	0.36	0.71	0.35	5.78	0.61
Current smoker	2.21	0.97	1.36	60.78	0.02
Recurrence	3.49	0.79	6.94	155.10	1.06E-5
Race (Asian)	0.63	1.64	0.075	46.59	0.70
Race (Black)	0.77	0.80	0.45	10.33	0.34
Ethnicity (Hispanic)	−0.73	1.12	0.054	4.33	0.52
Previous smoker and recurrence	0.01	1.24	0.09	11.43	0.99
Ever smoker and recurrence	−1.85	1.45	0.0091	2.70	0.20

Note: COLVERA is determined positive if any methylated DNA (either gene) is detected. CEA is evaluated as greater than 5.0 ng/mL. Models are built using logistic regression. ORs are estimated for each covariate independently; SE and 95% CIs are shown for OR.
[a]Never smoker, female, no recurrence, age at zero.

sensitivity progressively decreased as cutoff for positivity increased incrementally from 5 ng/mL to 15 ng/mL, resulting in a more pronounced difference in sensitivity between COLVERA and CEA.

Consistent with prior studies (28), this study showed that the specificity of CEA in the 295 patients without cancer recurrence was higher than that of COLVERA. However, the significance of a false-positive result in this study is unclear due to the relatively short follow-up period. Because CEA and COLVERA results were correlated with only one imaging test, it is possible that some subjects deemed without recurrence were later proven to have recurrent disease after further imaging. Indeed, a recent study showed that adding a second negative CT to confirm recurrence status improves ctDNA specificity (33). More than half of evaluable subjects without recurrence in this study were still within the first two years of surveillance when their blood was sampled and CT report documented. Keeping in mind that the median time to recurrence of stage II/III colorectal cancer is 1.5 to 2 years (43), some of the "false positive" assays may have ultimately been shown to be true positives with longer surveillance. In other words, if COLVERA is sensitive enough to have detected recurrences well before they were radiographically detectable, serial imaging may have yielded higher true-positive and lower false-positive rates, and therefore a higher specificity and sensitivity for COLVERA.

Because earlier detection of colorectal cancer recurrence may facilitate potentially curable metastasectomy, the application of a more sensitive assay, such as one targeting hypermethylated *BCAT1* and *IKZF1* ctDNA, during surveillance might improve outcomes in recurrent colorectal cancer. Consistent with published data showing that methylation alterations to *BCAT1* and *IKZF1* can be identified in plasma well before radiographic recurrence (25, 28, 30, 31, 33), this study demonstrated COLVERA positivity up to 6 months before imaging suggested recurrence. Nevertheless, without directly comparing results of regimented serial CEA and radiographic surveillance with serial ctDNA and radiographic surveillance, it is impossible to know whether application of ctDNA will lead to earlier radiographic detection and, most importantly, whether earlier radiographic detection will translate into improved cancer-related outcomes.

Our study has some limitations. First, because this study included serologic and radiographic data collected at a single point during colorectal cancer, the true sensitivity and specificity of CEA and COLVERA could vary from those reported in this study. Serial radiographic imaging might uncover more cancer recurrences which, in the case of a positive assay, may have converted false positives to true positives (thereby increasing specificity) and converted some true negatives to false negatives (thereby decreasing sensitivity). Second, serial blood collection (for example, every 3–6 months) might convert false negatives to true positives in those patients with previously documented radiographic recurrence, thereby increasing sensitivity. The advantage of serial testing hinges on an assay's ability to detect recurrences (true positives) in previously "missed" cases (false negatives). From a statistical standpoint, it would be important to

investigate whether serial ctDNA biomarker testing would demonstrate an additive effect on programmatic sensitivity when compared with sensitivity of any single assay. Future studies of serial ctDNA surveillance after potentially curative colorectal cancer treatment should therefore investigate whether serial testing improves sensitivity, how frequently testing should be done, and how conversion from a negative result to a positive result correlates with radiographic imaging. Third, in this noninterventional study, a positive ctDNA test did not trigger an earlier imaging study. Given that colorectal cancer surveillance typically includes CEA monitoring (every 3–6 months) at a higher frequency than radiographic monitoring (every 6–12 months), it would have been interesting to see whether the more sensitive COLVERA assay would have triggered more imaging and, in doing so, detected more early recurrences. Fourth, because preoperative CEA and subsequent serial CEA monitoring were not required for enrollment, we could not ascertain how many subjects had CEA-negative tumors ("nonshedders") and may therefore have introduced bias against CEA. In reality, however, CEA is usually not elevated before resection of nonmetastatic colorectal cancer (44), and clinicians monitor with CEA without knowing whether it will be elevated upon colorectal cancer recurrence. The current practice of monitoring patients with CEA, even though many may be "nonshedders," makes searching for alternative assays that much more important. Since *BCAT1* and/or *IKZF1* are methylated in 95% of colorectal cancers (31), COLVERA may instill confidence among clinicians that they are using a biomarker that is biologically more appropriate and also more sensitive than CEA. Finally, this study was not designed to examine the potential impact of earlier detection of colorectal cancer recurrence on survival and, more importantly, mortality. Because the ultimate goal of surveillance is to detect recurrent disease amenable to potentially curative therapy, further investigation will be needed to ensure that earlier detection improves not only median overall survival (18), which is subject to lead-time bias, but also mortality.

In summary, this study confirms the findings of prior studies using methylated *BCAT1* and *IKZF1* (30, 33) to detect colorectal cancer recurrence. In both the U.S. and Australian cohorts, head-to-head comparison of COLVERA to CEA at a single time point confirmed that COLVERA detected more radiologically confirmed recurrences. Furthermore, because COLVERA positivity is independently associated with recurrence and was not confounded by race, age, gender, and smoking in a U.S. population, COLVERA may be more applicable in routine clinical practice. Collectively, these findings provide a compelling rationale for a prospective randomized controlled trial investigating whether, when compared with CEA, COLVERA facilitates earlier diagnosis of colorectal cancer recurrence and, in turn, improves cancer-related outcomes.

Disclosure of Potential Conflicts of Interest

B.L Musher reports other from Clinical Genomics (as the sponsor of this study, Clinical Genomics reimbursed institution for the costs of enrolling and following patients; participating investigators did not receive any salary support) during the conduct of the study. J.E. Melson reports personal fees from Clinical Genomics (served prior to the study as a consultant for Clinical Genomics) outside the submitted work and served as a consultant in protocol development for Clinical Genomics (once study started and subjects accrued at institution, no longer served as a consultant for Clinical Genomics). M. Hill reports other from Ipsen (advisory board) outside the submitted work. I. Khan reports other from Clinical Genomics (prorated payments per accrual) during the conduct of the study. S.E. Lyons reports other from Clinical Genomics (Clinical Genomics paid for/sponsored the study at Ascension Providence Hospital) during the conduct of the study. J. Orsini reports other from Clinical Genomics (Clinical Genomics paid for/sponsored the study at institution) during the conduct of the study. S.K. Pedersen reports personal fees from Clinical Genomics (salaried employee) during the conduct of the study, as well as a patent pending and issued and assigned to Clinical Genomics. B. Robb reports grants from Clinical Genomics during the conduct of the study. J. Saltzman reports personal fees from Clinical Genomics outside the submitted work. S. Gaur reports other from Clinical Genomics (full-time employee) during the conduct of the study. M. Tuck reports other from Clinical Genomics (full-time employee) during the conduct of the study. L. C. LaPointe reports personal fees from Clinical Genomics (salaried employee) during the conduct of the study, as well as a patent for biomarkers described in this article pending and issued (inventor; assigned to Clinical Genomics). G.P. Young reports personal fees from Clinical Genomics (consultant) during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

B.L. Musher: Conceptualization, resources, supervision, validation, investigation, methodology, writing–original draft, writing–review and editing. **J.E. Melson:** Conceptualization, resources, investigation, methodology. **G. Amato:** Data curation, formal analysis, validation, writing–review and editing, biostatistician for the study. **D. Chan:** Resources, investigation, writing–review and editing. **M. Hill:** Resources, investigation, writing–review and editing. **I. Khan:** Resources, investigation. **S.T. Kochuparambil:** Resources, investigation, writing–review and editing. **S.E. Lyons:** Resources, investigation, writing–review and editing. **J. Orsini Jr:** Resources, investigation. **S.K. Pedersen:** Conceptualization, validation, methodology, writing–review and editing. **B. Robb:** Resources, investigation. **J. Saltzman:** Resources, methodology, writing–review and editing. **J. Silinsky:** Resources, investigation, writing–review and editing. **S. Gaur:** Resources, validation, investigation, methodology, writing–review and editing. **M.K. Tuck:** Conceptualization, data curation, supervision, validation, methodology, writing–original draft, project administration, writing–review and editing. **L.C. LaPointe:** Conceptualization, data curation, formal analysis, supervision, validation, methodology, writing–original draft, writing–review and editing. **G.P. Young:** Conceptualization, validation, methodology, writing–original draft, writing–review and editing.

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Evaluation of Circulating Tumor DNA for Methylated *BCAT1* and *IKZF1* to Detect Recurrence of Stage II/Stage III Colorectal Cancer (CRC)

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