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Circulating Tumor DNA Analyses as Markers of Recurrence Risk and Benefit of Adjuvant Therapy for Stage III Colon Cancer

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IMPORTANCE Adjuvant chemotherapy in patients with stage III colon cancer prevents recurrence by eradicating minimal residual disease. However, which patients remain at high risk of recurrence after completing standard adjuvant treatment cannot currently be determined. Postsurgical circulating tumor DNA (ctDNA) analysis can detect minimal residual disease and is associated with recurrence in colorectal cancers.

OBJECTIVE To determine whether serial postsurgical and postchemotherapy ctDNA analysis could provide a real-time indication of adjuvant therapy efficacy in stage III colon cancer.

DESIGN, SETTING, AND PARTICIPANTS This multicenter, Australian, population-based cohort biomarker study recruited 100 consecutive patients with newly diagnosed stage III colon cancer planned for 24 weeks of adjuvant chemotherapy from November 1, 2014, through May 31, 2017. Patients with another malignant neoplasm diagnosed within the last 3 years were excluded. Median duration of follow-up was 28.9 months (range, 11.6-46.4 months). Physicians were blinded to ctDNA results. Data were analyzed from December 10, 2018, through June 23, 2019.

EXPOSURES Serial plasma samples were collected after surgery and after chemotherapy. Somatic mutations in individual patients' tumors were identified via massively parallel sequencing of 15 genes commonly mutated in colorectal cancer. Personalized assays were designed to quantify ctDNA.

MAIN OUTCOMES AND MEASURES Detection of ctDNA and recurrence-free interval (RFI).

RESULTS After 4 exclusions, 96 eligible patients were eligible; median patient age was 64 years (range, 26-82 years); 49 (51%) were men. At least 1 somatic mutation was identified in the tumor tissue of all 96 evaluable patients. Circulating tumor DNA was detectable in 20 of 96 (21%) postsurgical samples and was associated with inferior recurrence-free survival (hazard ratio [HR], 3.8; 95% CI, 2.4-21.0; $P < .001$). Circulating tumor DNA was detectable in 15 of 88 (17%) postchemotherapy samples. The estimated 3-year RFI was 30% when ctDNA was detectable after chemotherapy and 77% when ctDNA was undetectable (HR, 6.8; 95% CI, 11.0-157.0; $P < .001$). Postsurgical ctDNA status remained independently associated with RFI after adjusting for known clinicopathologic risk factors (HR, 7.5; 95% CI, 3.5-16.1; $P < .001$).

CONCLUSIONS AND RELEVANCE Results suggest that ctDNA analysis after surgery is a promising prognostic marker in stage III colon cancer. Postchemotherapy ctDNA analysis may define a patient subset that remains at high risk of recurrence despite completing standard adjuvant treatment. This high-risk population presents a unique opportunity to explore additional therapeutic approaches.

Editorial

Supplemental content

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Colorectal cancer is a major health burden globally.[1] For patients with stage III colon cancer, the administration of adjuvant chemotherapy improves overall survival.[2-4] The duration of oxaliplatin-based treatment can be adjusted according to pathologic risk category from 3 months for patients with low-risk tumors (≤T3 and N1) to 6 months for patients with high-risk tumors (T4 and/or N2).[5] Oxaliplatin can be omitted in patients older than 70 years based on multiple clinical trials failing to demonstrate a survival benefit in this age group.[6-8] Regardless of the estimated recurrence risk before treatment or the therapy pursued, we cannot yet define a patient subset that remains at high risk of recurrence after completing standard treatment. Also, only when clinical recurrence occurs can treatment failure be acknowledged.

Tumor-specific DNA mutations, also known as circulating tumor DNA (ctDNA), can be detected in the cell-free component of peripheral blood samples in almost all patients with advanced colorectal cancer and many other advanced solid tumors.[9] Several studies, including in colorectal cancer, have shown that detectable ctDNA after surgery for early-stage cancers is associated with a very high risk of recurrence.[10-18] To our knowledge, no published series in any solid tumor type has examined the significance of ctDNA analysis at the completion of adjuvant therapy or the potential of this analysis to inform further treatment.

Herein we report on the results of a correlative biomarker study in patients with stage III colon cancer undergoing standard adjuvant chemotherapy. The primary aim of this study was to demonstrate the association between postsurgical and postchemotherapy ctDNA detection and the risk of recurrence.

Key Points

Question Can serial analysis of circulating tumor DNA levels provide a real-time indication of adjuvant chemotherapy efficacy in patients with stage III colon cancer?

Findings In this multicenter cohort study of 96 patients with stage III colon cancer, a significant difference in 3-year recurrence-free interval was observed in patients with detectable vs undetectable levels of circulating tumor DNA after surgery (47% vs 76%) and after completion of chemotherapy (30% vs 77%).

Meaning Postsurgical and postchemotherapy circulating tumor DNA analyses may identify patients at high risk of recurrence despite completing standard adjuvant treatment, presenting a unique opportunity to explore additional therapeutic approaches.

Methods

Study Design and Participants

This multicenter cohort study recruited consecutive patients with stage III colon cancer treated at 5 Australian hospitals. Key eligibility criteria included a recent diagnosis of stage III colon cancer with R0 resection; no metastatic disease evident on staging computed tomography (CT) of the chest, abdomen, and pelvis before surgery; an Eastern Cooperative Oncology Group performance status of 0 to 2; and a treatment plan for 24 weeks of adjuvant chemotherapy. Patients with another malignant neoplasm diagnosed within the last 3 years were excluded. The chemotherapy regimen was chosen by the physician, who was blinded to the ctDNA result. We classify the clinical risk group using standard criteria, with high-risk patients defined as those having pT4 and/or pN2 disease according to the pTNM staging system.[5] This study was approved by the human research ethics committees at each contributing hospital, and all participants provided written informed consent. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.

Blood samples for ctDNA and carcinoembryonic antigen (CEA) analysis were collected 4 to 10 weeks after surgery (before commencing any adjuvant chemotherapy) and at the completion of treatment (within 6 weeks of the final cycle of chemotherapy). At each collection point, at least 30 mL of blood was drawn into EDTA tubes, centrifuged twice at 1200*g* and 1800*g*, and stored at −80 °C for retrospective ctDNA analysis.

All patients had a surveillance CT scan 4 to 8 weeks after completion of adjuvant chemotherapy. Thereafter, surveillance included 3-month clinical review and CEA level measurement, with annual CT imaging for 3 years. Serum CEA level was measured by the local diagnostic laboratory at each participating site, with CEA concentrations of less than 5 ng/mL (to convert to micrograms per liter, multiply by 1.0) considered within reference range. Pathology reports from resection specimens were reviewed to assess pathologic prognostic factors.

Tumor Tissue Mutational Analysis and ctDNA Detection

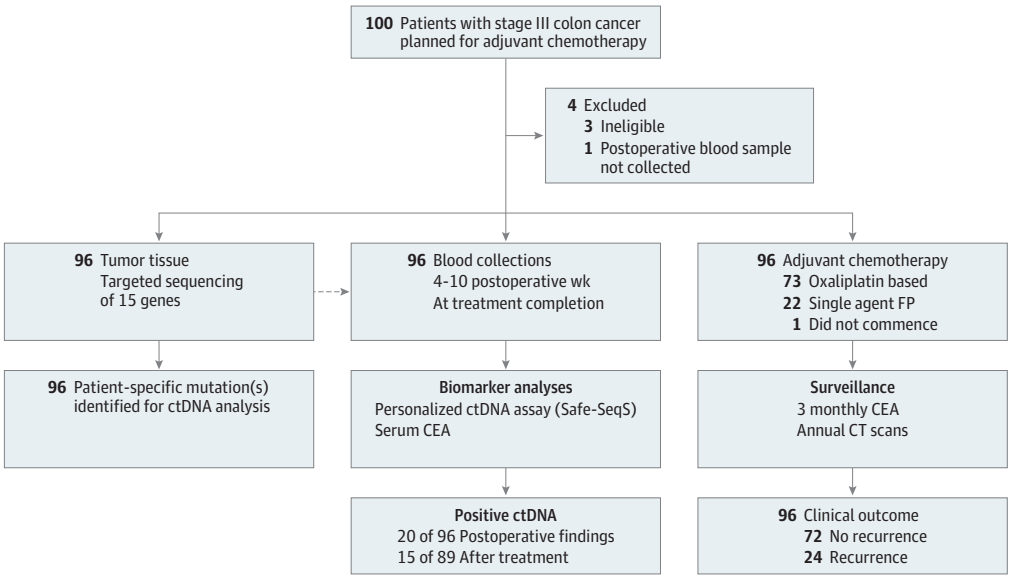
Formalin-fixed, paraffin-embedded tumor tissue from the surgical specimen was analyzed for somatic mutations in 15 genes recurrently mutated in colorectal cancer.[11] Primers were designed and sequencing results were analyzed as previously described.[9,19]

For each patient, 1 mutation identified in the tumor tissue was assessed in the plasma for the presence of ctDNA. Leukocyte germline DNA was used to exclude variants arising from clonal hematopoiesis. If more than 1 somatic mutation was identified in the tumor tissue, the mutation with the highest mutant allele fraction relative to that in healthy control DNA was selected for ctDNA analysis for that patient. We used an error-reduction technology termed *Safe-Sequencing System* (Safe-SeqS) for the detection of low-frequency mutations (eMethods in the Supplement).[19]

Statistical Analysis

Data were analyzed from December 10, 2018, through June 23, 2019. Differences in baseline characteristics between patients with ctDNA-positive and ctDNA-negative findings were assessed using the Fisher exact test for categorical variables and Mann-Whitney (rank sum) test for continuous variables. The primary outcome measure was recurrence-free interval (RFI), measured from the date of surgery to documented first radiological recurrence or death as a result of colorectal cancer, and was censored at last follow-up or noncolorectal cancer-

Figure 1. Patient Enrollment, Sample Collections, and Evaluable Population



CEA indicates carcinoembryonic antigen; CT, computed tomography; ctDNA, circulating tumor DNA; and FP, fluoropyrimidine.

related death. We fitted 2 types of models. For univariate analyses, we used the Kaplan-Meier estimator with the log-rank test. Hazard ratios (HRs) were estimated by univariate Cox proportional hazards regression models. For the multiple variable analysis, a Cox proportional hazards regression model was fitted. Although postoperative ctDNA can be used as a factor in any Cox proportional hazards regression model, ctDNA levels measured after chemotherapy cannot be used to estimate the risk of an event before the chemotherapy is finished. Therefore, to assess serial postsurgical and postchemotherapy ctDNA as a single variable with a single HR estimate, ctDNA was included as a time-varying independent variable. Ties in failure times were handled using the Efron method, and the proportional hazards assumption was tested by a global test of the Schoenfeld residuals. To assess the performance of ctDNA and CEA measurements in estimation of outcomes, we calculated an area under the curve (AUC) with the receiver operating characteristics method of Song and Zhou[20] using the R package survAUC.[21] We used 5-fold cross-validation, repeated 100 times, to estimate the out-of-sample accuracy of estimating cumulative recurrence. The same splits were used for each model. All analyses were performed using a Package for Survival Analysis in S, version 2.38 (https://cran.r-project.org/web/packages/survival/index.html) from the R software, version 3.4.1,[22] where 2-sided $P < .05$ was considered significant.

Results

Clinicopathologic Characteristics and Postsurgical ctDNA Detection

Patient enrollment and the study design are presented in **Figure 1**. We enrolled 100 patients from November 1, 2014, through May 30, 2017. Four patients were excluded from subsequent analysis because postsurgical plasma samples could not be collected (n = 1) or because they were ineligible owing to stage IV disease at diagnosis (n = 3), leaving 96 for the analysis (49 men [51%] and 47 women [49%]; median age, 64 years [range, 26-82 years]). Targeted massively parallel sequencing identified at least 1 somatic mutation in all 96 of the primary tumor tissue samples analyzed. We then designed personalized Safe-SeqS assays for the identified mutations to quantify ctDNA in a total of 174 serial plasma samples.

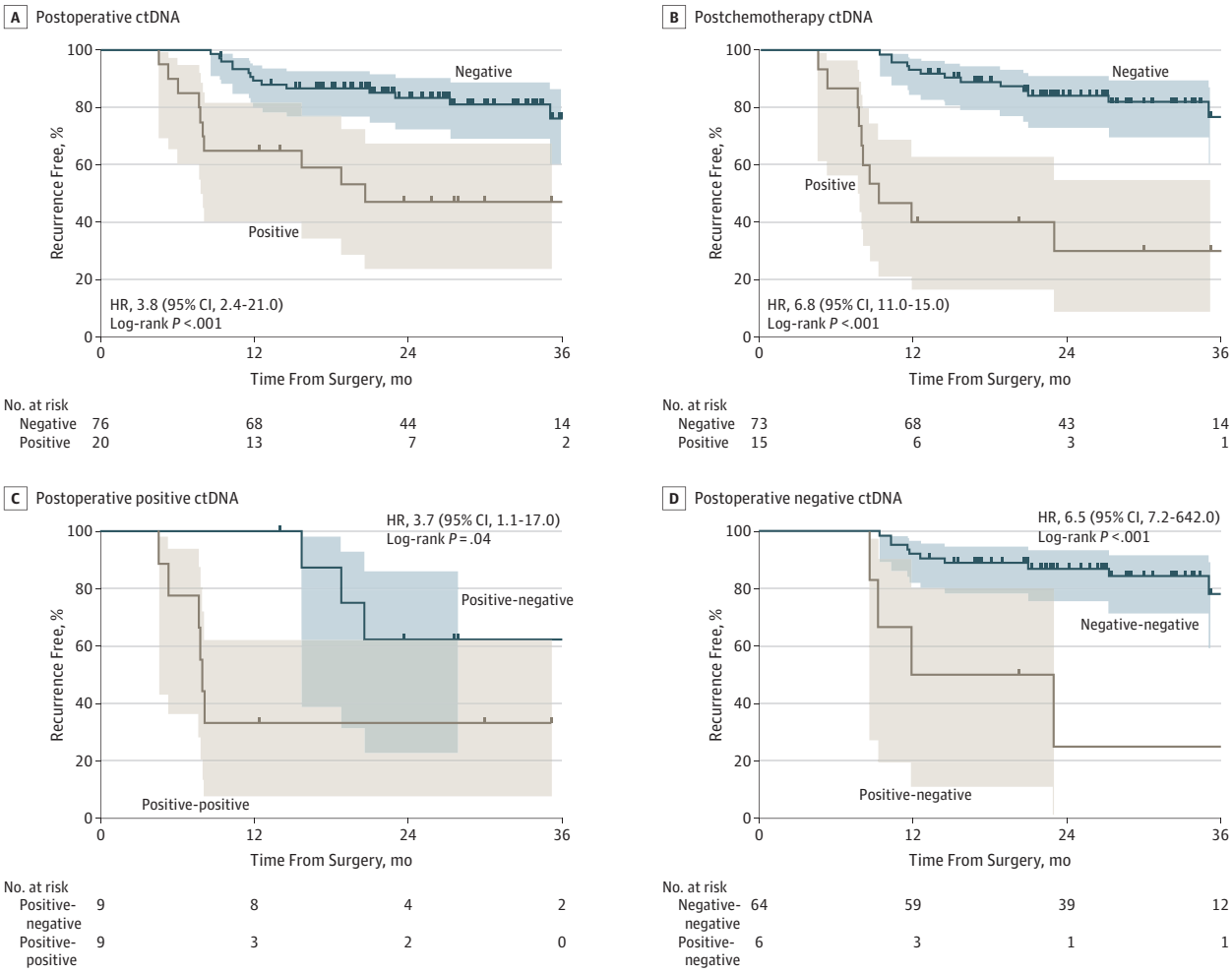
The median time from date of operation to postsurgical blood collection was 42 days (interquartile range, 32-52 days). After the postsurgical blood collection, 73 patients (76%) commenced adjuvant oxaliplatin-based therapy; 22 (23%) received adjuvant fluoropyrimidine alone; and 1 did not commence adjuvant therapy. Twenty-four weeks of adjuvant chemotherapy was planned for all patients. Eighty-five patients (89%) completed at least 12 weeks of adjuvant therapy, and 72 (75%) completed a full course of treatment.

A tumor-specific mutation was detected (ctDNA-positive finding) in the postsurgical plasma sample of 20 of 96 patients (21%). Baseline clinicopathologic characteristics and their association with postsurgical ctDNA status are shown in eTable 1 in the Supplement. Twenty-eight patients had N2 disease. No significant association was found between a positive postsurgical ctDNA finding and any clinical or pathologic factor.

Postsurgical and Postchemotherapy ctDNA

Overall, ctDNA was detectable in 15 of 88 (17%) postchemotherapy samples. Blood samples were collected after chemotherapy (median, 19 days after the last cycle of chemotherapy had been administered [range, 2-47]) in 78 of the 85 patients who completed at least 12 weeks of chemotherapy

Figure 2. Kaplan-Meier Estimates of Recurrence-Free Interval According to Circulating Tumor DNA (ctDNA) Status



HR indicates hazard ratio.

(92%) and in 66 of the 72 patients who completed 24 weeks of chemotherapy (92%). Summary results of postsurgical and postchemotherapy ctDNA analysis for the 78 patients who completed at least 12 weeks of therapy are shown in eTable 2 in the Supplement. Circulating tumor DNA findings were positive after chemotherapy in 13 (17%) of 78 patients. In the 72 patients who completed all 24 weeks of therapy, ctDNA was detectable after chemotherapy in 10 of 66 (15%).

In the 1 patient who did not receive adjuvant chemotherapy, postsurgical ctDNA finding was positive, and the patient experienced disease recurrence at 6 months. eTable 3 in the Supplement shows the serial ctDNA and CEA results, recurrence outcome, and patterns of failure (where applicable) for all patients. For the 10 patients with a positive postchemotherapy ctDNA finding who experienced recurrence, the median time to recurrence from the postchemotherapy blood draw was 51 days (range, 9-470 days). Of these, recurrence was detected on the planned end-of-treatment imaging in 7 patients; 2 patients underwent earlier-than-planned imaging, one for an elevated postsurgical CEA level that continued to rise during adjuvant chemotherapy and the other for a clinical symptom that was unrelated to the cancer recurrence.

ctDNA Status and RFI

As of October 12, 2018, median follow-up was 28.9 months (range, 11.6-46.4 months). During this period, 24 patients (25%) experienced a recurrence, including 18 of 85 patients (21%) treated with at least 12 weeks of adjuvant chemotherapy, 15 of 72 (21%) who completed 24 weeks of adjuvant chemotherapy, and 1 of 1 (100%) who did not receive chemotherapy. Postsurgical ctDNA was detectable in 10 of 24 patients (42%) with recurrence.

Patients with detectable ctDNA after surgery had an increased risk of recurrence (HR, 3.8; 95% CI, 2.4- 21.0; $P < .001$) (**Figure 2**A). Kaplan-Meier estimates of RFI at 3 years for patients with positive ctDNA findings were 47% (95% CI, 24%-68%) and for those with ctDNA-negative findings were 76% (95% CI, 61%-86%). The ctDNA status of the postchemotherapy sample was strongly associated with RFI (HR, 6.8; 95% CI, 11.0-157.0; $P < .001$) (Figure 2B). Three-year RFI was 30%

Table. RFI Analysis by Clinicopathologic Variables and Postsurgical ctDNA Status

	Analysis			
	Univariate		Multivariable	
Variable	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Age	1.0 (0.97-1.03)	.88	1.0 (1.0-1.0)	.58
Sex, male vs female	1.0 (0.5-2.0)	>.99	0.8 (0.4-1.7)	.60
T stage, T4 vs T1-3	1.9 (0.9-4.0)	.08	NA	NA
N stage, N2 vs N1	2.0 (1.0-4.1)	.07	NA	NA
Lymphovascular invasion, yes vs no	1.1 (0.6-2.3)	.71	1.1 (5.5-2.3)	.77
Clinical risk, high vs low[a]	2.2 (1.1-4.5)	.04	2.5 (1.3-5.0)	.008
Postsurgical ctDNA status, positive vs negative	3.8 (2.2-21.0)	<.001	7.5 (3.5-16.1)	<.001

Abbreviations: ctDNA, circulating tumor DNA; HR, hazard ratio; NA, not available; RFI, recurrence-free interval.

[a] Defined as high (pT4 or pN2) or low (pT1-3 and pN1). Circulating tumor DNA was included as a time-varying independent variable. Because clinical risk is a combination of T and N stage, the latter 2 variables were not included in the multiple variable analysis. An alternative multivariable model including T and N stage but not clinical risk produces similar results.

(95% CI, 9%-55%) for cases with detectable ctDNA and 77% (95% CI, 60%-87%) for those with undetectable ctDNA after chemotherapy.

The RFI outcomes according to the postsurgical and postchemotherapy ctDNA status are shown in Figure 2C-D. In patients with a positive postsurgical ctDNA finding, a positive ctDNA finding after chemotherapy was associated with an inferior RFI compared with patients in whom ctDNA became undetectable after chemotherapy (HR, 3.7; 95% CI, 1.1-17.0; $P = .04$) (Figure 2C). In patients with a negative postsurgical ctDNA finding, a negative ctDNA result after chemotherapy was associated with a superior RFI compared with patients in whom ctDNA became detectable after chemotherapy (HR, 6.5; 95% CI, 7.2-642.0; $P < .001$) (Figure 2D).

The accuracies of serial ctDNA analysis and clinical risk in estimating radiological recurrence, as assessed by the AUC analysis, is shown in eTable 4 in the Supplement. Circulating tumor DNA has a higher AUC than clinical risk, but the combination of serial ctDNA analysis and clinical risk assessment appears to perform better at estimation than either variable alone.

CEA and ctDNA Levels and RFI

Postsurgical CEA level was elevated in 7 of 96 patients (7%), of whom 6 had detectable ctDNA. Of the 88 patients who had postchemotherapy CEA and ctDNA results available, CEA level was elevated in 12 (14%). An elevated CEA level after surgery or an elevated CEA level after chemotherapy was associated with an inferior RFI (HR after surgery, 3.4 [95% CI, 1.5-50; $P = .02$]); HR after chemotherapy, 3.05 [95% CI, 1.4-21.0; $P = .01$]) (eFigure, A-B in the Supplement). Of the 12 patients with an elevated postchemotherapy CEA level, 6 had detectable ctDNA, and 5 of these (83%) have had recurrence. Of the other 6 patients with an elevated CEA level but negative ctDNA findings after chemotherapy, only 1 (17%) has had a recurrence. Receiver operating characteristics curve analysis suggests that CEA level (AUC = 0.52) did not add accuracy of estimations to any model.

Clinicopathologic Variables and Multivariate Analysis

Clinical risk group (low vs high) was significantly associated with RFI (HR, 2.2; 95% CI, 1.1-4.5; $P = .04$) in univariate analysis and had a trend for association for T stage (HR, 1.9; 95% CI, 0.9-4.0; $P = .08$) and N stage (HR, 2.0; 95% CI, 1.0-4.1; $P = .07$) (**Table**). To adjust for multiple variables in a single model, we used a Cox proportional hazards regression model with ctDNA as a time-varying independent variable. Postsurgical ctDNA status had the strongest independent association with RFI (HR, 7.5; 95% CI, 3.5-16.1; $P < .001$), followed by clinical risk (HR, 2.5; 95% CI, 1.3-5.0; $P = .008$).

Discussion

Several opportunities exist to further personalize the treatment strategy to improve the outcome of patients with stage III colon cancer. One of these opportunities is the discovery and validation of biomarkers that better define an individual patient's risk of recurrence, which is the focus of our study. This information could be used to inform the use of available agents with activity in the adjuvant setting. Alternatively, novel agents could be given in combination with standard chemotherapy or administered after adjuvant therapy completion. The latter approach would be ideally applied to patients who remain at high risk despite completing standard treatment. In patients with stage III colon cancer, we have demonstrated that ctDNA may be a useful prognostic marker after surgery and could guide initial adjuvant treatment. Further, we have demonstrated that ctDNA status after chemotherapy has a stronger association with disease status after surgery and has the potential to inform additional therapy.

We have demonstrated that patients with a positive ctDNA finding after surgery have poor outcomes despite adjuvant chemotherapy, with an estimated 3-year RFI of 47% compared with 76% in those with a negative postsurgical ctDNA finding (HR, 3.8; $P < .001$). This result is consistent with previous studies conducted in patients with resectable liver metastases,[11] stage II colon cancer,[23] and locally advanced rectal cancer.[14] All of these studies have demonstrated that ctDNA detection after surgery is associated with a markedly elevated risk of recurrence. These results have prompted several prospective randomized studies that are currently actively recruiting (DYNAMIC,[24] DYNAMIC-III,[25] and DYNAMIC-Rectal[26]), in which patients are randomized after surgery to standard-of-care or ctDNA-informed adjuvant treatment. In the investigational arms, the duration and intensity of chemotherapy delivered is determined by the postsurgical ctDNA result.

In the present study, we have also demonstrated the potential value of ctDNA as a real-time marker of adjuvant therapy effectiveness. When ctDNA was detectable despite adjuvant chemotherapy, the risk of recurrence was substantially higher than when ctDNA was undetectable after treatment (HR, 6.8; $P < .001$). These data follow from anecdotal observations made in the earlier study of patients with stage II colon cancer,[11] in which 2 of 6 patients with positive ctDNA findings after surgery who then received adjuvant treatment and had no detectable ctDNA at therapy completion remained disease free at a median follow-up of 27 months.

These observations open new opportunities for enriching recruitment to studies of novel therapies in high-risk patients with detectable ctDNA, as was thoroughly explored in a recent commentary by Dasari et al.[27] Such novel therapies could be administered concurrently with standard chemotherapy or alone after standard chemotherapy is completed. Alternatively, high-risk patients with detectable ctDNA could benefit from more intensive follow-up. For example, 3- to 6-month CT scans are currently recommended in National Comprehensive Cancer Network guidelines after resection of liver metastases when the risk of recurrence is also very high. Ideally, the value of more intensive surveillance of patients with detectable ctDNA should be demonstrated in prospective randomized studies.

We also compared the accuracy of ctDNA, a novel but relatively costly blood biomarker, with that of the standard CEA test that is widely used in the clinic to estimate recurrence. The Kaplan-Meier model outputs for these 2 tests demonstrated a slightly higher HR for postsurgical ctDNA (3.8) than CEA (3.4). Notably, the number of patients with a positive postsurgical ctDNA result (20 of 96) is substantially higher than that of patients with an elevated CEA level (7 of 96), suggesting that ctDNA may be more useful clinically to guide adjuvant treatment strategy. Ultimately, the cost-effectiveness of each test depends on several factors other than the cost of the test itself, such as adjuvant treatment received, additional investigations performed, and survival outcome. These factors will be assessed in the context of the ongoing DYNAMIC-III randomized clinical trial.

Limitations

Potential limitations to our study include the modest sample size, low event rate, lack of a validation cohort, and analysis of multiple patient subsets. To fully define the prognostic significance of postsurgical ctDNA as a surrogate marker of recurrence would require analysis in a series of patients with untreated stage III colon cancer; this "perfect" study is clearly not feasible because withholding standard adjuvant treatment would be unethical. The use of adjuvant chemotherapy has likely confounded this postsurgical analysis by preventing recurrence in some patients who would have otherwise had recurrences, meaning the HR would likely be higher in an untreated group. However, adjuvant chemotherapy may also have prevented recurrence in some of the patients with ctDNA-negative findings. In addition, only 42% of recurrences were detected by postsurgical ctDNA analysis. The limited assay sensitivity may have been due to only 1 somatic mutation being assessed in the plasma and potentially could be improved by analyzing multiple mutations in each plasma sample, especially in the setting where the mutation allele frequency is low. In addition, assessing serial ctDNA samples as a single time-varying independent covariate with the Cox proportional hazards regression model is likely to underestimate the HR given that it does not account for measurement errors. Finally, the earlier-than-planned imaging in 2 patients with positive postchemotherapy ctDNA findings due to rising CEA levels or the development of clinical symptoms may have confounded the estimated HR for recurrence.

Conclusions

The results of this study confirm the prognostic significance of postsurgical ctDNA analysis, which has now been demonstrated in multiple series of colorectal cancer and other solid tumors.[11,13,14,18,23,28,29] Our study highlights the potential clinical utility of ctDNA to guide therapeutic decision-making. More specifically, the novel data presented herein suggest that postchemotherapy ctDNA analysis could lead to a more informed selection of patients who could benefit from additional therapeutic approaches, supporting the pursuit of clinical trials of novel agents in this high-risk population. Numerous clinical studies have suggested that the treatment of patients with low burdens of metastatic disease is far more efficacious than the treatment of patients with radiologically detectable disease. Thus, the treatment of patients with detectable ctDNA levels but without radiological evidence of disease after adjuvant chemotherapy could, in theory, eradicate residual disease and increase the chance of cure. This possibility is being further explored in a series of randomized studies that are currently recruiting.

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Author Contributions: Drs Tie, Vogelstein, and Gibbs and Mr Cohen contributed equally to this study. Dr Tie had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Acquisition, analysis, or interpretation of data: Tie, Cohen, Wang, Christie, Simons, M. Lee, Wong, Kosmider, Ananda, McKendrick, B. Lee, Cho, Faragher, Jones, Ptak, Schaefer, Silliman, Dobbyn, Li, Tomasetti, Papadopoulos, Gibbs.
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Supervision: Tie, Wong, Ananda, Tomasetti, Papadopoulos, Gibbs.

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