PRECISION MEDICINE

Circulating Tumor DNA Predicts Pathologic and Clinical Outcomes Following Neoadjuvant Chemoradiation and Surgery for Patients With Locally Advanced Rectal Cancer

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PURPOSE This study was designed to assess the ability of perioperative circulating tumor DNA (ctDNA) to predict surgical outcome and recurrence following neoadjuvant chemoradiation for locally advanced rectal cancer (LARC).

MATERIALS AND METHODS Twenty-nine patients with newly diagnosed LARC treated between January 2014 and February 2018 were enrolled. Patients received long-course neoadjuvant chemoradiation prior to surgery. Plasma ctDNA was collected at baseline, preoperatively, and postoperatively. Next-generation sequencing was used to identify mutations in the primary tumor, and mutation-specific droplet digital polymerase chain reaction was used to assess mutation fraction in ctDNA.

RESULTS The median age was 54 years. The overall margin-negative, node-negative resection rate was 73% and was significantly higher among patients with undetectable preoperative ctDNA (n = 17, 88%) versus patients with detectable preoperative ctDNA (n = 9, 44%; $P = .028$). Undetectable ctDNA was also associated with more favorable neoadjuvant rectal scores (univariate linear regression, $P = .029$). Recurrence-free survival (RFS) was calculated for the subset (n = 19) who both underwent surgery and had postoperative ctDNA available. At a median follow-up of 20 months, patients with detectable postoperative ctDNA experienced poorer RFS (hazard ratio, 11.56; $P = .007$). All patients (4 of 4) with detectable postoperative ctDNA recurred (positive predictive value = 100%), whereas only 2 of 15 patients with undetectable ctDNA recurred (negative predictive value = 87%).

CONCLUSION Among patients treated with neoadjuvant chemoradiation for LARC, patients with undetectable preoperative ctDNA were more likely to have a favorable surgical outcome as measured by the rate of margin-negative, node-negative resections and neoadjuvant rectal score. Furthermore, we have confirmed prior reports indicating that detectable postoperative ctDNA is associated with worse RFS. Future prospective study is needed to assess the potential for ctDNA to assist with personalizing treatment for LARC.

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ASSOCIATED CONTENT

Appendix

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INTRODUCTION

Standard treatment for locally advanced rectal cancer (LARC) involves trimodality therapy consisting of neoadjuvant chemoradiation, surgery, and adjuvant chemotherapy. In recent years, total neoadjuvant therapy (TNT) has emerged as a novel paradigm for LARC that aims to address micrometastatic disease earlier in the treatment course.[1,2] However, not all patients require adjuvant chemotherapy,[3] and adopting a uniform TNT approach for LARC carries with it significant risk of overtreatment.[4] Furthermore, after neoadjuvant chemoradiation, 15%-27% of patients are found to have a pathologic complete response (pCR)[5,6] and interest in organ preservation (ie, omission of surgery) in these patients has gained considerable interest in recent years.[7,8] Current practice uses the clinical complete response as the benchmark for identifying patients for whom preservation may be a viable option. However, the clinical response poorly correlates with the pathologic response. In the future, a reliable biomarker of therapeutic efficacy available in real time could be beneficial for assessing the response to treatment and likelihood of benefit from completion of TNT and guiding perioperative management. An National Cancer Institute panel was recently convened and highlighted the need for more studies in this area.[9]

CONTEXT

Key Objective

Patients with locally advanced rectal cancer (LARC) who have minimal residual disease as measured using detectable postoperative circulating tumor DNA (ctDNA) have been shown to have higher risk for recurrence compared with those with undetectable postoperative ctDNA; however, the relationship between preoperative ctDNA and surgical outcome has been less well-studied.

Knowledge Generated

In this study, we analyzed preoperative and postoperative ctDNA in a cohort of patients who received neoadjuvant chemoradiation for LARC. We confirm prior work showing that those who harbor minimal residual disease as measured by postoperative ctDNA following resection are at increased risk for recurrence. Additionally, we show that undetectable preoperative ctDNA was associated with improved surgical outcomes, including lower and more favorable neoadjuvant rectal scores and better margin-negative, node-negative resection rates.

Relevance

Further prospective study is necessary to determine the utility of incorporating ctDNA for clinical decision making for patients with LARC.

Novel high-sensitivity assays have been developed in recent years to detect fragments of circulating tumor DNA (ctDNA) among a background of cell-free DNA in the plasma of individuals with both localized and metastatic cancers.[10-15] Detectable postoperative ctDNA has been shown to accurately identify patients with resected stage II colorectal cancer at risk for recurrence with improved sensitivity compared with standard clinical risk factors or tumor markers.[15] Similarly, detectable postchemoradiotherapy and postoperative ctDNA identify patients with LARC who are likely to subsequently recur.[16] However, studies investigating preoperative ctDNA assessments measured after neoadjuvant therapy are fewer in number and conflicting with respect to whether preoperative ctDNA can be useful for predicting the outcome of surgery.[16,17]

Therefore, in this study, we sought to perform a proof-of-concept analysis evaluating whether ctDNA can identify patients who are likely to have a favorable surgical outcome using composite end points of neoadjuvant rectal (NAR) score[18] and margin-negative, node-negative (R0-NN) resection rates after neoadjuvant chemoradiation. The NAR is a validated surrogate composite end point for early-phase clinical trials involving neoadjuvant therapy for rectal cancer and has been shown to predict overall survival (OS) better than pCR in a previously published independent validation cohort.[18-20] Additionally, we sought to validate prior work[16] showing that those who harbor minimal residual disease (MRD), as defined by ctDNA detection after curative-intent surgery for rectal cancer, are at increased risk for recurrence.

MATERIALS AND METHODS

Patient Population and Treatment

Between January 2014 and February 2018, 29 patients with newly diagnosed locally advanced rectal adenocarcinoma who were being treated at either the Massachusetts General Hospital Center for GI Cancers or the University of Michigan Multidisciplinary Colorectal Cancer Clinic were enrolled onto an institutional-specific institutional review board–approved protocol for biospecimen collection. The present study focused on ctDNA analysis was retrospective. Patients were treated per the recommendations of the treating physician with long-course chemoradiation (conformal external beam radiation therapy [CRT]) consisting of 45 Gy to the pelvis followed by a boost to the mesorectum to 50.4 Gy in 1.8 Gy fractions with concurrent capecitabine or infusional fluorouracil (5FU). Three of the patients who received infusional 5FU also received midostaurin on a separate interventional protocol.

Per the recommendation of the treating physician, some patients received neoadjuvant chemotherapy prior to planned surgical resection and some received adjuvant chemotherapy. Reductions in the dose of chemotherapy prescribed, timing of chemotherapy delivery, and number of cycles received were decided by the treating physician as needed to manage acute chemotherapy–related toxicities.

Tumor Sequencing and ctDNA Analysis

At the time of initial biopsy or surgical resection, patients underwent next-generation sequencing (NGS) of their primary tumor to identify tumor-specific mutations to personalize the ctDNA assay, as described previously.[21,22] Whole blood for ctDNA analysis was collected in two 10 mL Streck tubes generally at baseline prior to neoadjuvant CRT, preoperatively, and postoperatively. Plasma was isolated through two centrifugation steps: the first at room temperature for 10 minutes at 1,600×*g* and the second at room temperature for 10 minutes at 3,000×*g*. Plasma was then stored at –80°C until ctDNA isolation. At the time of analysis, ctDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit according to the manufacturer's instructions, increasing the protein lysis

step from 30 minutes to 60 minutes according to recommendations from the Streck tube manufacturer. ctDNA was analyzed using a highly sensitive and specific droplet digital polymerase chain reaction (ddPCR) method, which allows for detection of one mutant DNA molecule among a background of 10,000 wild-type molecules.[23-25] Methods for ctDNA isolation and analysis have been previously described in detail.[26] Each assay was constructed to detect one or more mutations in peripheral blood specific to individual patients. Specific mutations for ctDNA analysis were chosen after NGS review on the basis of determining that a given mutation was somatic (not germline) and on the basis of probe availability. Probe information is available on request.

Assessment of preoperative clinical response and determination of surgical outcome. As directed by the treating physician, patients also had standard tumor markers (carcinoembryonic antigen [CEA]) drawn before and after neoadjuvant chemotherapy and CRT. The following clinical and treatment factors were noted for each patient: age, sex, tumor location, clinical American Joint Committee on Cancer stage, types and number of cycles of neoadjuvant chemotherapy, radiation technique (three-dimensional conformal, intensity-modulated radiation therapy, etc), total dose received, dose per fraction, and concurrent and adjuvant chemotherapy type.

After neoadjuvant treatment, most patients proceeded to curative-intent surgical resection. However, a subset of patients developed metastatic disease while undergoing neoadjuvant therapy. For patients who underwent surgical resection, the following pathologic outcomes were noted: clinical and pathologic American Joint Committee on Cancer T and N stage, tumor grade, tumor regression grade,[27] R-resection status on the basis of margin status,[28] lymphovascular invasion, and perineural invasion. Surgical specimens from Massachusetts General Hospital were centrally reviewed by a dedicated GI pathologist (J.K.L.) to confirm the tumor regression grade assessment.

Follow-up and determination of recurrence and death. After neoadjuvant treatment and surgery, patients were followed for disease recurrence with serial history and physical examinations, tumor marker assessment (CEA), and computed tomography of the chest, abdomen, and pelvis, or magnetic resonance imaging per treating clinician.[29] Patients were followed from the time of enrollment until the time of death or last follow-up.

Statistical Methods

Prediction of surgical outcomes using ctDNA. Patients were grouped according to whether they had a detectable ($\geq$ 2 mutant ctDNA alleles among a minimum of 2,000 wild-type alleles) versus undetectable (0 mutant ctDNA alleles among a minimum of 2,000 wild-type alleles) *preoperative* ctDNA. Conservatively, if only one mutant ctDNA allele was detected, the patient's ctDNA status was classified as negative.

Composite surgical outcome end points. **R0-NN resection.** Patients were classified as achieving an R0-node negative (R0-NN) resection if surgical margins were negative, and there were no positive lymph nodes on surgical pathology. In addition to pathologic node–positive disease, non-R0-NN resection included patients who had an R1 or R2 resection or those patients who were not resected because of either poor response to neoadjuvant therapy or development of metastatic disease during neoadjuvant therapy.

NAR score. The NAR score[18] was computed for patients who were able to undergo resection of the primary tumor. The NAR score is a composite end point, which reflects the response to neoadjuvant therapy, taking into account downstaging of the primary tumor and pathologic nodal status (Fig 1).[18] As in the work of George et al,[18] patients were grouped into NAR-low or NAR-intermediate and NAR-high categories if their NAR score fell 8-16 and $>$ 16, respectively.

Fisher's exact tests were used to compare the rate of R0-NN resection and the rate of NAR-low or NAR-intermediate score among patients with detectable versus undetectable preoperative ctDNA. Univariate logistic regression with R0-NN status as the dependent variable was conducted separately for the following independent variables: preoperative ctDNA status and preoperative CEA. Univariate linear regression with NAR score as the dependent variable was conducted separately for the following independent variables: preoperative ctDNA status and preoperative CEA.

Predictors of progression-free survival. Patients were grouped according to whether they had a detectable ($\geq$ 2 mutant ctDNA alleles among a minimum of 2,000 wild-type alleles) versus undetectable (0 mutant ctDNA alleles among a minimum of 2,000 wild-type alleles) *postoperative* ctDNA. Conservatively, if only one mutant ctDNA allele was detected, the patient's ctDNA status was classified as negative. Progression-free survival (PFS) was estimated using the Kaplan-Meier method from the date of surgery until the time of radiographic progression (as determined by the interpreting radiologist and confirmed by the treating physician) or date of last follow-up.[30] Patients without radiographic progression at the time of last follow-up were then censored from the PFS estimate. Estimates of PFS

$$NAR = \frac{[5\,pN - 3(cT - pT) + 12]^2}{9.61}$$

FIG 1. Formula used for calculating NAR score as developed by George et al.[18] cT, clinical tumor stage; NAR, neoadjuvant rectal; pN, pathologic nodal stage; pT, pathologic tumor stage.

were calculated separately for patients with detectable versus undetectable postoperative ctDNA, and the Cox proportional hazard model was used to compare the PFS between groups. Positive predictive value (PPV) and negative predictive value (NPV) of postoperative ctDNA for the prediction of recurrence were calculated. PPV was defined as the probability that postoperative ctDNA predicts recurrence (ie, the number of patients with subsequent recurrence and detectable postoperative ctDNA divided by all patients with detectable postoperative ctDNA). NPV was defined as the probability that undetectable postoperative ctDNA predicts subsequent freedom from recurrence (ie, the number of patients without subsequent recurrence and with negative postoperative ctDNA divided by all patients with negative postoperative ctDNA). Statistical analyses were performed using STATA (StataCorp, College Station, TX) and SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

Patient Characteristics

Table 1 illustrates clinical characteristics for the entire cohort. The median age of the cohort was 54 years (range, 45-78 years). All patients received neoadjuvant long-course chemoradiation. Preoperative ctDNA assessments were performed between 0 and 17 weeks preoperatively. Postoperative ctDNA assessments were performed between 1 and 5 months postoperatively. Notably, some patients received neoadjuvant chemotherapy prior to chemoradiation followed by surgery, one patient received neoadjuvant chemoradiation followed by chemotherapy prior to surgery, and some patients received adjuvant chemotherapy after surgery. A patient-level summary table detailing individual treatment course per patient is included in Appendix Table A1.

TABLE 1. Clinical and Treatment Characteristics of the Entire Cohort (N = 29, Except Where Noted)

Clinical Factor	No. of Patients	%
Age, years, median (IQR)	54 (48-66)	
Sex		
Male	15	52
Female	14	48
AJCC 7th edition clinical stage		
II	6	21
III	23	79
Tumor location, median cm from the anal verge (IQR)	7 (3-9.7)	
Neoadjuvant chemotherapy[a]		
Yes	13	45
No	16	55
Adjuvant chemotherapy[a]		
Yes	11	38
No	18	62
Preoperative CEA (n = 27), median ng/mL (IQR)	2.3 (1.1-4.45)	
Postoperative CEA (n = 27), median ng/mL (IQR)	1.80 (1.00-2.5)	
R resection status		
R0	26	90
Non-R0	3	10
Pathologic nodal status (n = 28)		
Positive	8	29
Negative	20	71
Pathologic complete response		
pCR	5	17
Non-pCR	24	83

Abbreviations: AJCC, American Joint Committee on Cancer; CEA, carcinoembryonic antigen; IQR, interquartile range; pCR, pathologic complete response.
[a]Specific chemotherapy regimens for individual patients provided in Appendix Table A1.

Analysis of ctDNA Trajectory over Time and Treatment

As described above, ddPCR was used to assess one or more tumor-specific mutations (identified by tumor tissue sequencing) in ctDNA at available timepoints for each patient. Figure 2 illustrates examples of ctDNA trajectories for five representative patients, illustrating examples of ctDNA trajectories for favorable and unfavorable surgical outcomes.

ctDNA as a Predictor of Surgical Outcome

Twenty-six patients had preoperative plasma available for analysis. The overall R0-NN resection rate was 73% for the entire cohort. The rate of R0-NN resection was significantly higher among patients with an undetectable preoperative ctDNA (n = 17) compared with those with a detectable (n = 9) preoperative ctDNA (88% R0-NN *v* 44% R0-NN, respectively, P = .028, Table 2). On univariate logistic regression, both ctDNA status and CEA were significantly associated with R0-NN resection (P = .027 and .034, respectively).

The NAR score was able to be computed for 25 patients, and 19 were in the NAR-low or NAR-intermediate category, whereas six were in the NAR-high category. Patients with preoperative undetectable ctDNA had a higher rate of more favorable NAR-low and NAR-intermediate scores (88%) as compared with those with detectable ctDNA (50%, P = .059, Table 2). On univariate linear regression, both ctDNA status and CEA were significantly associated with NAR score (P = .029 and .044, respectively). The pCR rate among patients with detectable versus undetectable preoperative ctDNA was not significantly different, 11% versus 24% (P = .63), respectively (Table 2).

Predictors of PFS

For the subset of patients who underwent curative-intent surgery with postoperative ctDNA available (n = 19), recurrence-free survival was calculated and patients were stratified by detectable versus undetectable postoperative

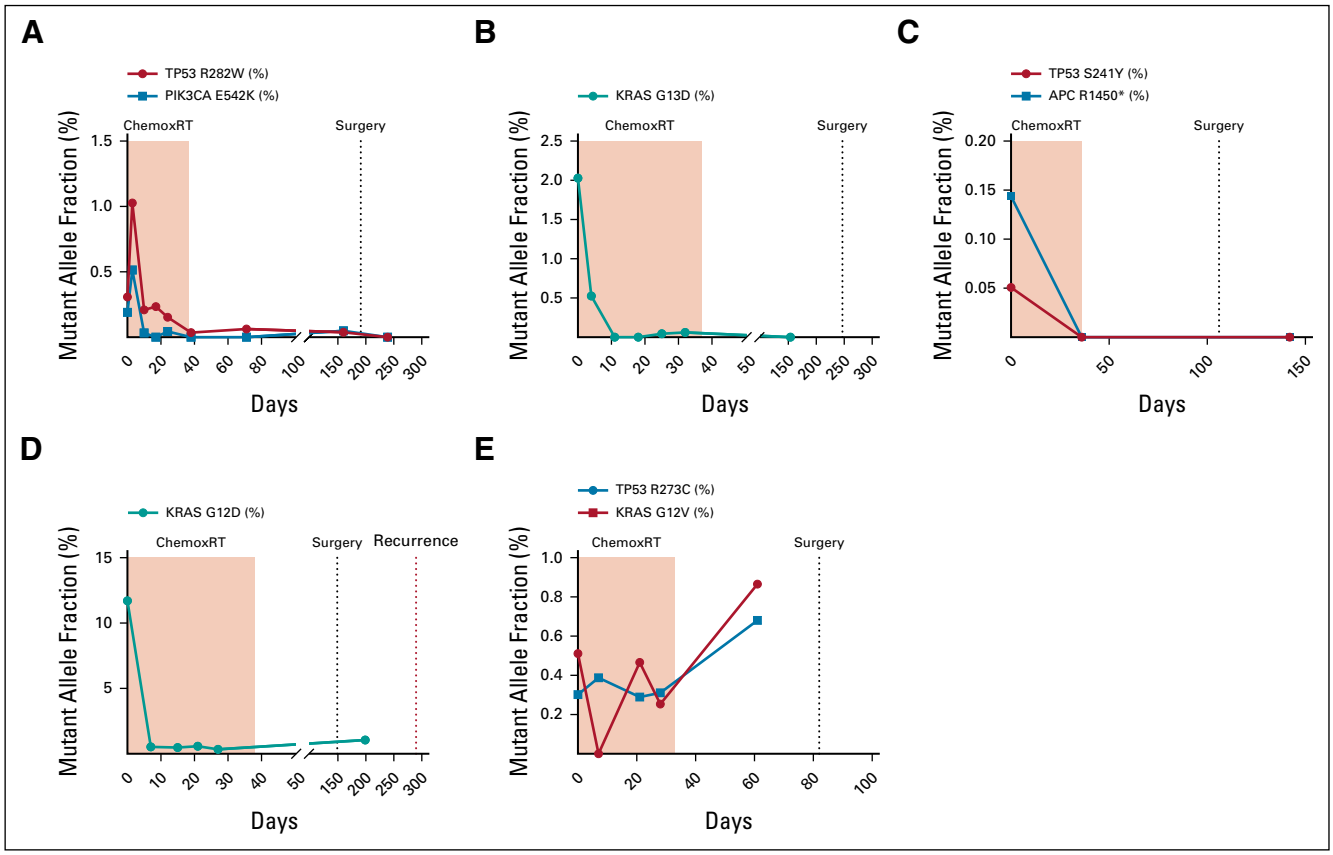

FIG 2. Five representative patient examples. Panel A displays the circulating tumor DNA (ctDNA) trajectory for two mutations (TP53 R282W and PIK3CA E542K) over time for a 53-year-old who received eight cycles of FOLFOX followed by chemoradiotherapy for a TP53 and PIK3CA cT4bN2b locally advanced rectal cancer (LARC). As shown in the panel, his ctDNA decreased during chemoradiation; however, his preoperative computed tomography scan showed a mixed response where the tumor appeared tethered to presacral soft tissue. Surgical pathology revealed significant tumor regression, negative lymph nodes, and negative margins. His postoperative ctDNA assay was negative, and the patient is currently NED 20 months postoperatively. Panel B displays the ctDNA trajectory for a 78-year-old who received neoadjuvant chemoradiation for a T3N1 LARC. Her KRAS G13D ctDNA dropped preoperatively, and she was found to have significant treatment effect with 1-2 mm of residual tumor foci in a 1.5-cm tumor bed. However, her carcinoembryonic antigen (CEA) remained elevated pre- and postoperatively. Panel C displays the ctDNA trajectory for a 50-year-old with a cT2N1 TP53– and APC-mutant LARC, treated with neoadjuvant FOLFOX and conformal external beam radiation therapy (CRT). Her ctDNA decreased during CRT, and she was found to have a pathologic complete response at the time of surgery. Her postoperative ctDNA was negative, and she is currently NED 14 months postoperatively. Panel D displays the ctDNA trajectory for a 48-year-old who received eight cycles of FOLFIRINOX and CRT for KRAS G12D–mutant cT3N2 LARC. His CEA decreased during CRT, but his preoperative ctDNA was positive. Surgical pathology revealed a poor tumor response and six of 14 involved lymph nodes. His postoperative ctDNA was positive, which preceded a radiographic recurrence by 3 months. Panel E displays the ctDNA trajectory for a 45-year-old with TP53- and KRAS-mutant cT4bN2b LARC who received neoadjuvant FOLFIRINOX and chemoradiation prior to surgical resection. Her ctDNA increased during CRT. She underwent resection of rectal primary and partial hepatectomy, which revealed liver metastases (despite a negative liver biopsy prior to chemoradiation).

ctDNA. After a median follow-up of 20 months (interquartile range, 14.0-43 months), 4 of 17 (24%) patients had died and 6 of 19 (32%) had experienced local or distant recurrence. Cumulative incidence estimates of PFS for patients with detectable (n = 4) versus undetectable (n = 15) postoperative ctDNA are shown in Figure 3. Notably, four of four patients with detectable postoperative ctDNA recurred (PPV = 100%), whereas only two of 15 (13%) patients with undetectable postoperative ctDNA recurred (NPV = 87%). Patients with detectable postoperative ctDNA had significantly worse PFS than patients with undetectable postoperative ctDNA (hazard ratio, 11.56; $P = .007$). None of the patients with positive postoperative ctDNA MRD received adjuvant chemotherapy in this cohort.

DISCUSSION

In this proof-of-concept, confirmatory study, undetectable preoperative ctDNA was associated with improved surgical outcomes, including a lower and more favorable NAR score and better R0-NN rates in a cohort of patients treated with neoadjuvant CRT for LARC. We chose to evaluate the relationship between ctDNA and NAR score as this score was developed as a composite short-term end point and predicts OS better than pCR.[18] Additionally, consistent with many

TABLE 2. Results Table Summarizing Relationship Between Preoperative ctDNA, CEA and Surgical Outcomes

Preoperative ctDNA As a Predictor of Surgical Outcome

	Detectable ctDNA	Undetectable ctDNA	*P*, Fisher's Exact Test
R0-NN resection rate	44%	88%	.028
NAR-low or NAR-intermediate score rate	50%	88%	.059
pCR rate	11%	24%	.63

Preoperative CEA (CEA Elevated > 5) As a Predictor of Surgical Outcome

	Elevated CEA	Nonelevated CEA	*P*, Fisher's Exact Test
R0-NN resection rate	33%	89%	.015
NAR-low or NAR-intermediate score rate	40%	89%	.042
pCR rate	0%	30%	.29

Abbreviations: CEA, carcinoembryonic antigen; ctDNA, circulating tumor DNA; NAR, neoadjuvant rectal; pCR, pathologic complete response; R0-NN, margin-negative, node-negative.

studies,[16] we also found that postoperative analysis of ctDNA strongly predicted risk for recurrence following curative-intent surgery for LARC as the recurrence rate among patients with detectable postoperative ctDNA was 100%, but it was only 13% among patients with undetectable ctDNA.

Although further prospective studies are needed, these findings illustrate the potential for ctDNA to predict the treatment response and thus could affect real-time clinical decision making in three main ways. First, as TNT emerges as a paradigm for treatment of LARC,[1,2] widespread adoption of a standard TNT approach may result in overtreatment as some patients may be adequately treated with neoadjuvant chemoradiation and surgical resection alone. This was shown by the ADORE trial, which randomly assigned patients after CRT and surgical resection to adjuvant chemotherapy with FOLFOX versus fluorouracil plus leucovorin.[3,4] Although there was an overall significant improvement in progression-free survival among patients receiving FOLFOX, subgroup analysis revealed that patients with pathologic stage III benefitted from FOLFOX, whereas those with pathologic stage II disease did not,[3] highlighting the need to determine which patients will benefit from intensified adjuvant chemotherapy upfront. The use of ctDNA could potentially help guide which patients are at the highest risk of recurrence and help determine who should receive chemotherapy. Multiple studies have shown that consolidation chemotherapy (after chemoradiation) is safe and effective and the ongoing Organ Preservation of Rectal Adenocarcinoma (OPRA) trial is currently assessing the optimal order of preoperative treatment.[31-35] If TNT is planned with chemoradiation before chemotherapy and ctDNA is predictive of outcome, ctDNA could potentially be used to identify patients who would not necessarily benefit from further chemotherapy with the goal of preventing overtreatment.

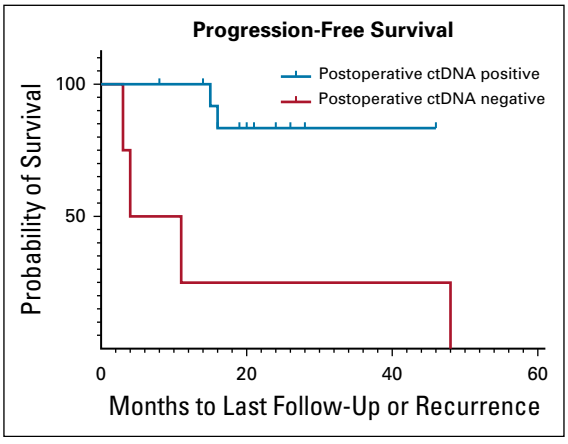

FIG 3. Progression-free survival stratified by postoperative detectable versus undetectable ctDNA status (hazard ratio, 11.56; $P = .007$). ctDNA, circulating tumor DNA.

Second, a subset of patients with a clinical complete response to neoadjuvant therapy may not require surgery to achieve a cure.[7,8] However, it is challenging to identify good candidates for a watch-and-wait approach as standard clinical response assessment does not often correlate with the outcome of surgery. We demonstrated that patients with undetectable preoperative ctDNA following neoadjuvant therapy were more likely to have an R0-NN surgery and favorable NAR score than patients with detectable preoperative ctDNA. Thus, combined with clinical response assessment, ctDNA may represent a promising additional tool to guide future studies for tailoring the TNT approach for individual patients and improving selection for organ preservation.

Third, elevated postoperative ctDNA could possibly help identify a high-risk subset of patients who would potentially benefit from increased surveillance or escalation of adjuvant therapy. The recently published COLOFOL[36] and GILDA[37] randomized trials, which found that intensive surveillance does not prolong survival in patients with nonselected colorectal cancer, have called into question the need for intensive surveillance. Based on prior studies of intensive surveillance, it seems likely that there are high-risk subgroups who will still benefit from more intensive surveillance, which could potentially be selected using postoperative ctDNA. Additionally, it is unknown whether patients with MRD identified with postoperative ctDNA assays would benefit from escalation of adjuvant therapy. In this cohort, none of the patients with positive ctDNA MRD received adjuvant chemotherapy, and it is unknown whether adjuvant chemotherapy would have changed their ultimate disease trajectory. Prospective randomized clinical trials are needed in the MRD space, incorporating ctDNA to determine the utility of ctDNA to monitor the treatment

response and whether escalation of adjuvant therapy for patients with positive ctDNA is beneficial.

Several points require further consideration. First, our findings corroborate and complement ctDNA analysis from a larger cohort of patients with LARC treated with neoadjuvant therapy.[16] Tie et al[16] analyzed plasma for ctDNA from 159 patients before and after neoadjuvant therapy and following surgical resection. Although they observed that detectable postoperative ctDNA predicted subsequent recurrence, they did not observe a correlation between post-CRT ctDNA and pCR surgical outcome and concluded that ctDNA measured after CRT cannot differentiate between minimal and no residual disease or be used to select patients for a nonoperative approach.[16] Murahashi et al[17] also investigated ctDNA following neoadjuvant treatment for LARC. They also did not observe a significant association between preoperative ctDNA and pCR; however, they did observe that change in ctDNA was associated with patients who achieved a pCR or those who were managed by the watch-and-wait approach for more than 12 months after achieving a clinical complete response.[17] Although our study was limited by small numbers, we also did not observe a significant relationship between preoperative ctDNA and pCR. However, we did observe a significant relationship between preoperative ctDNA and composite end point markers of surgical outcome, namely, favorable NAR score and R0-NN resection, which have not been previously assessed. We chose to evaluate composite end points (NAR and R0-NN resection) in our analysis instead of individual surgical outcomes to capture more aspects of downstaging. The NAR score is a validated surrogate end point, which has been shown to predict OS better than pCR by considering both tumor and nodal downstaging and was chosen as the primary end point for the ongoing NRG GI002 trial (ClinicalTrials.gov identifier: NCT02921256) evaluating veliparib and pembrolizumab in the neoadjuvant setting for LARC. Further prospective study of multiple assays will be necessary to further understand these differences and further elucidate the utility of incorporating such assays into clinical decision making.

Our study has several limitations. First, the sample size of this cohort is small. As a result, although we were able to show that both ctDNA and CEA are correlated with surgical outcome, the small sample size of our cohort precluded the ability to ascertain the extent to which ctDNA adds over CEA and other clinical factors to predict surgical and oncologic outcomes. This relationship will be important for further study in larger prospective cohorts. Second, although many patients in our cohort received neoadjuvant chemotherapy prior to chemoradiation, not all these individuals had plasma available for ctDNA analysis prior to initiation of any systemic therapy. Thus, for the small subset of patients who had a negative ctDNA assay at all timepoints collected, it is unclear whether the negative assay reflected the treatment response to initial systemic therapy or whether these patients had undetectable ctDNA because of low rates of ctDNA shedding. To mitigate this concern, we assayed ctDNA to a limit of detection of 0.05% in each case, although some patients may have harbored ctDNA levels below this limit. Although our study used custom ddPCR to assess ctDNA in each patient, newer technologies assessing multiple mutations by NGS and/or methylation and epigenomic markers could represent more optimal approaches for assessment of residual disease through ctDNA.[38] A third limitation is that there was variability in the timing of collection of preoperative and postoperative timepoints, but our small sample size precluded assessment of whether this altered our result. Future prospective studies should minimize this heterogeneity with prespecified collection times.

Despite these limitations, our study suggests that ctDNA may be useful for identifying patients who will have a favorable surgical outcome following neoadjuvant chemoradiation for LARC and confirms prior studies indicating that patients with postoperative ctDNA MRD are at higher risk for recurrence. Further study is necessary to examine the utility of incorporating a ctDNA assay for tailoring the TNT approach for individual patients or using ctDNA along with other clinical factors to aid in identification of patients who may be suitable for an organ preservation approach following neoadjuvant treatment. Additionally, identifying patients early following surgery who are at high risk of recurrence could provide an opportunity for alteration of surveillance, potential early intervention, and thus offer a window for a second chance of cure for these individuals, particularly in patients where adjuvant therapy may not otherwise be used.[39] Further prospective study is necessary to determine the utility of ctDNA in personalized therapy for patients with LARC.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

TABLE A1. Patient-Level Summary Table

Patient ID	Age	Sex	Clinical Stage	Pathologic Stage	Neoadjuvant Chemotherapy	Neoadjuvant Chemoradiation	Concurrent Chemotherapy	Adjuvant Chemotherapy	Positive Nodes	Resection Margin	pCR	RONN	Pre-Op ctDNA	Post-Op ctDNA	Pre-Op CEA	Post-Op CEA	Recurrence
1	60	M	cT3N1	ypT3N1b	No	Yes	Capecitabine	No	Yes	R0	No	No	Unavailable	Positive	Unavailable	Unavailable	Yes
2	30	F	cT4N2	ypT2N0	No	Yes	Capecitabine	Yes, FOLFOX	No	R0	No	Yes	Unavailable	Negative	Not elevated	Not elevated	No
3	67	M	cT3N1	pT3N0	No	Yes	Capecitabine	Yes, FOLFOX	No	R0	No	Yes	Positive	Unavailable	Not elevated	Not elevated	No
4	45	F	cT4N1	ypT4N1	No	Yes	Capecitabine	Yes, FOLFOX	Yes	R0	No	No	Negative	Unavailable	Unavailable	Unavailable	No
5	53	F	cT3N1	ypT2N0	No	Yes	Capecitabine	Yes, FOLFOX	No	R0	No	Yes	Positive	Unavailable	Not elevated	Not elevated	No
6	54	F	cT4N0	pT3N0	No	Yes	Capecitabine	Yes, capecitabine	No	R0	No	Yes	Negative	Unavailable	Not elevated	Not elevated	No
7	61	F	cT3N0	ypT0N0	No	Yes	Capecitabine	No	No	R0	Yes	Yes	Negative	Unavailable	Not elevated	Not elevated	No
8	45	M	cT3N2	ypT3N2b	Yes, FOLFOX	Yes	Capecitabine	No	Yes	R0	No	No	Unavailable	Positive	Not elevated	Not elevated	Yes
9	63	M	cT3N1	ypT3N1b	No	Yes	Capecitabine	No	Yes	R0	No	No	Positive	Unavailable	Not elevated	Not elevated	No
10	48	M	cT3N2	ypT3N2a	Yes, FOLFIRINOX	Yes	Capecitabine	No	Yes	R0	No	No	Positive	Positive	Not elevated	Elevated	Yes
11	78	F	cT3N1	ypT2N0	Yes, FOLFOX	Yes	CI-5FU	No	No	R0	No	Yes	Negative	Unavailable	Not elevated	Not elevated	Yes
12	71	M	cT3N2	ypT3N1a	Yes, FOLFOX	Yes	CI-5FU	No	Yes	R1	No	No	Positive	Positive	Elevated	Elevated	Yes
13	48	M	cT4Nx	ypT3N0	Yes, FOLFIRINOX	Yes	CI-5FU	No	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	Yes
14	67	F	cT3N1	ypT3N0	No	Yes	Capecitabine	No	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	No
15	50	F	cT4N2	ypT2N0	Yes, FOLFIRINOX	Yes	CI-5FU	No	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	No
16	43	M	cT4N1	ypT1N0	Yes, FOLFOX	Yes	Capecitabine	No	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	No
17	70	M	cT3N1	YpT0N0	No	Yes	Capecitabine	No	No	R0	Yes	Yes	Negative	Negative	Not elevated	Not elevated	No
18	57	M	cT3N1	ypT0N0	No	Yes	Capecitabine	Yes, FOLFOX	No	R0	Yes	Yes	Positive	Unavailable	Not elevated	Not elevated	No
19	33	F	cT4aN1	YpT0N0	Yes, FOLFOX	Yes	CI-5FU	No	No	R0	Yes	Yes	Negative	Negative	Not elevated	Not elevated	No
20	67	M	cT3N0	ypT3N0	No	Yes	CI-5FU/midostaurin	Yes, FOLFOX	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	Yes
21	64	M	cT3N0	ypT2N0	No	Yes	CI-5FU/midostaurin	Yes, FOLFOX	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	No
22	53	M	cT4bN2b	ypT2N0	Yes, FOLFOX	Yes	CI-5FU	No	No	R0	No	Yes	Positive	Negative	Not elevated	Not elevated	No
23	45	F	cT4bN2b	ypT4N1cM1b	Yes, FOLFIRINOX	Yes	Capecitabine	No	Yes	R0[a]	No	No	Positive	Negative	Elevated	Not elevated	NA (metastatic at surgery)
24	66	F	cT3cN+	ypT2N0	Yes, FOLFIRINOX	Yes	CI-5FU	No	No	R0	No	Yes	Negative	Negative	Not elevated	Not elevated	No
25	51	M	cT3N1	ypT1N0	No	Yes	CI-5FU/midostaurin	Yes, FOLFOX	No	R0	No	Yes	Negative	Negative	Elevated	Elevated	No
26	69	F	cT4N+	ypM1	Yes, FOLFOX	Yes	CI-5FU	No	Unknown	R2[a]	No	No	Positive	Positive	Elevated	Elevated	NA (metastatic at surgery)
27	55	M	cT3N0	ypT2N0	No	Yes	Capecitabine	Yes, CAPEOX	No	R0	No	Yes	Negative	Negative	Elevated	Not elevated	No
28	50	F	cT2N1	ypT0N0	No	Yes	Capecitabine	Yes, FOLFOX	No	R0	Yes	Yes	Negative	Negative	Not elevated	Not elevated	No
29	50	F	cT3N+	ypT3N1c	Yes, FOLFOX	Yes	Capecitabine	No	Yes	R0	No	No	Negative	Negative	Elevated	Not elevated	No

CEA, carcinoembryonic antigen.
[a]Liver metastasis resected.