MRI Tumor Regression Grade and Circulating Tumor DNA as Complementary Tools to Assess Response and Guide Therapy Adaptation in Rectal Cancer

Shelize Khakoo[1], Paul David Carter[2], Gina Brown[3], Nicola Valeri[4], Simona Picchia[3], Maria Antonietta Bali[3], Ridwan Shaikh[2], Thomas Jones[2], Ruwaida Begum[1], Isma Rana[1], Andrew Wotherspoon[5], Monica Terlizzo[5], Katharina von Loga[5], Eleftheria Kalaitzaki[6], Claire Saffery[1], David Watkins[1], Diana Tait[1], Ian Chau[1], Naureen Starling[1], Michael Hubank[2], and David Cunningham[1]

ABSTRACT

Purpose: Response to preoperative chemo-radiotherapy (CRT) varies. We assessed whether circulating tumor DNA (ctDNA) might be an early indicator of tumor response or progression to guide therapy adaptation in rectal cancer.

Experimental Design: A total of 243 serial plasma samples were analyzed from 47 patients with localized rectal cancer undergoing CRT. Up to three somatic variants were tracked in plasma using droplet digital PCR. RECIST and MRI tumor regression grade (mrTRG) evaluated response. Survival analyses applied Kaplan–Meier method and Cox regression.

Results: ctDNA detection rates were: 74% ($n = 35/47$) pretreatment, 21% ($n = 10/47$) mid CRT, 21% ($n = 10/47$) after completing CRT, and 13% ($n = 3/23$) after surgery. ctDNA status after CRT was associated with primary tumor response by mrTRG ($P = 0.03$). With a median follow-up of 26.4 months, metastases-free survival was shorter in patients with detectable ctDNA after completing CRT [HR 7.1; 95% confidence interval (CI), 2.4–21.5; $P < 0.001$], persistently detectable ctDNA pre and mid CRT (HR 3.8; 95% CI, 1.2–11.7; $P = 0.02$), and pre, mid, and after CRT (HR 11.5; 95% CI, 3.3–40.4; $P < 0.001$) compared with patients with undetectable or nonpersistent ctDNA. In patients with detectable ctDNA, a fractional abundance threshold of $\geq$0.07% mid CRT or $\geq$0.13% after completing CRT predicted for metastases with 100% sensitivity and 83.3% specificity for mid CRT and 66.7% for CRT completion. All 3 patients with detectable ctDNA post-surgery relapsed compared with none of the 20 patients with undetectable ctDNA ($P = 0.001$).

Conclusions: ctDNA identified patients at risk of developing metastases during the neoadjuvant period and post-surgery, and could be used to tailor treatment.

Introduction

Circulating tumor DNA (ctDNA) could help individualize disease management in locally advanced rectal cancer (LARC) if its full potential is clinically validated (1, 2).

A total of 15%–20% of cases have a complete pathologic response (pCR) to standard-of-care neoadjuvant chemo-radiation (CRT) and such patients have better survival rates (3, 4). Recognition of this and the need to avoid the functional morbidity associated with surgery has led to increased interest in organ preservation (5). The main challenge is appropriate patient selection. Studies have included heterogeneous populations and this has led to difficulty with standardization and data interpretation (6). As a noninvasive, surrogate for the tumor genome, incorporation of ctDNA analysis may be able to improve accuracy by providing information at the molecular level.

In patients that show a poor response to CRT, a trial of neoadjuvant chemotherapy may be offered in an attempt to downsize the tumor and enable less invasive surgery. Early identification of patients with radioresistance or those at risk of developing systemic disease is essential, as these patients may benefit from treatment intensification or alternative treatment strategies.

MRI tumor regression grade (mrTRG) provides an accurate means to assess response to CRT preoperatively and as such, has the potential to allow therapy adaptation while also providing prognostic information (7). Although mrTRG is a measure of local response, it may not be fully representative of systemic disease status. Therefore, integration of longitudinal monitoring with a blood-based biomarker such as ctDNA, with response assessment by imaging, is attractive. Currently, the relationship between detection of ctDNA and response according to mrTRG is unknown.

We investigated whether ctDNA could be an early indicator of response or disease progression to guide therapy adaptation.

Materials and Methods

Study design and participants

This was a single center study (NCT00825110) where consecutive patients meeting the eligibility criteria were prospectively recruited. All patients' aged 18 years or older, with a diagnosis of LARC (cT3–4 and/or node positive) confirmed on histology and absence of metastases on imaging, scheduled to undergo long course CRT at our institution

[1]Department of Medicine, The Royal Marsden Hospital NHS Foundation Trust, London and Surrey, United Kingdom. [2]Clinical Genomics, The Royal Marsden Hospital NHS Foundation Trust, Sutton, United Kingdom. [3]Department of Radiology, The Royal Marsden Hospital NHS Foundation Trust, London and Surrey, United Kingdom. [4]Molecular Pathology, The Institute of Cancer Research/The Royal Marsden NHS Foundation Trust, Sutton, United Kingdom. [5]Department of Histopathology, The Royal Marsden Hospital NHS Foundation Trust, London and Surrey, United Kingdom. [6]Clinical Research and Development, The Royal Marsden Hospital NHS Foundation Trust, London and Surrey, United Kingdom.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: David Cunningham, Royal Marsden NHS Foundation Trust, Sutton, Surrey SM2 5PT, United Kingdom. Phone: +44 (0)20 8661 3156; Fax: +44 (0)20 8643 9414; E-mail: david.cunningham@rmh.nhs.uk

Clin Cancer Res 2020;26:183–92

doi: 10.1158/1078-0432.CCR-19-1996

Translational Relevance

Circulating tumor DNA (ctDNA) has gained recognition as a noninvasive surrogate for the tumor genome. Here we investigate its potential to predict tumor response or disease progression in patients with localized rectal cancer receiving preoperative chemoradiotherapy (CRT). For the first time we show that ctDNA status after CRT is associated with MRI tumor regression grade (mrTRG) response. We confirm that detection of ctDNA after completing CRT is a poor prognostic marker. In addition, we provide novel data that longitudinal ctDNA monitoring during CRT provides information regarding systemic disease status and may therefore be complimentary to mrTRG, which is a measure of local response. ctDNA identified patients at risk of developing metastases during CRT or after surgery and could be used to tailor treatment in patients with rectal cancer from as early as mid CRT where treatment could be intensified or altered in an attempt to prevent the development of metastases.

between February 2015 and November 2016, were eligible. During this time period, patients that did not meet the criteria for LARC but met all other eligibility criteria and had a low rectal tumor and/or an adverse risk feature where the multi-disciplinary team (MDT) recommended long course CRT, were also eligible. CRT consisted of capecitabine 1,650 mg/m^2/day for 6 weeks alongside 50.4–54 Gy radiotherapy. Treatment decisions following CRT were made following MDT discussion and took into account tumor response and patient-related factors. In the absence of disease progression with metastatic disease, treatment options included: surgery, organ preservation, or neoadjuvant chemotherapy (if the risk of R1 resection was high due to poor response to CRT). The study was carried out in accordance with the declaration of Helsinki and approved by a human research ethics committee. All patients provided written informed consent prior to their participation.

Procedures

Serial blood samples were collected: pretreatment (within 4 weeks prior to commencing CRT), mid-CRT (3–4 weeks from the start of CRT), after completion of CRT (4–12 weeks from completion), and post-surgery (within 4–12 weeks). For patients pursuing organ preservation, blood samples were collected 3–6 month from the end of CRT until within 3 months of regrowth.

CT to confirm the absence of metastatic disease was carried out at baseline in conjunction with MRI for local staging. MRI was used to assess response 3–6 weeks following completion of CRT and CT evaluated systemic disease status. Radiologists assessing response to CRT were blinded to ctDNA results. All ctDNA analyses were conducted by individuals blinded to the clinical status of patients. Response of the primary tumor was assessed using RECIST version 1.1 and an independent radiologist provided the mrTRG.

Responders were defined as patients achieving a complete response (CR) or partial response (PR) according to RECIST or mrTRG1-2. Patients with stable disease (SD) or progressive disease (PD) by RECIST or mrTRG 3–5 were classed as poor-responders. The mrTRG definitions of response were consistent with that currently being used within the TRIGGER study (NCT02704520), which is evaluating the role of mrTRG as a biomarker to stratify the management of patients with LARC according to mrTRG determined response (8).

For patients proceeding directly to surgery, resected specimens were also assessed by pathologic Mandard TRG, which most closely resembles the mrTRG (Supplementary Table S1). The percentage of Ki-67–positive tumor cells was reported by an experienced pathologist.

Tumor tissue was available for sequencing from 52 patients. DNA was extracted from formalin-fixed, paraffin-embedded tumor tissue. A somatic variant in the genes of interest (*KRAS, NRAS, BRAF, PIK3CA, TP53*, and *APC*) was considered to be present if it had a variant allele frequency (VAF) $\geq$ 5%.

The variant with the highest VAF in tumor tissue was tracked in the corresponding cell-free DNA (cfDNA) by droplet digital PCR (ddPCR), with up to two additional variants tracked per patient where discovered. As defined by others, a plasma timepoint was designated positive if a minimum of two mutant-positive droplets were present for at least one variant (9). The level of ctDNA was reported in copies per mL and fractional abundance (FA) % expressed as the proportion of mutant alleles in the total cfDNA (mutant and wild-type DNA). Further methodologic details are provided in the Supplementary Data.

Statistical analysis

Fisher exact test was used to assess differences in clinical characteristics or radiological response between patients with undetectable and detectable ctDNA at each timepoint. The Mann–Whitney test was used for continuous variables. Spearman rank correlation was used to check for an association between ctDNA level pretreatment and Ki-67 or pretreatment radiological lesion size. If ctDNA from more than one variant was present, the highest detectable value for all analyses was used.

Primary outcome was radiological response by ctDNA detectability per timepoint. Other outcome measures were metastases-free survival (MFS), disease-free survival (DFS), local recurrence-free survival (LRFS), and overall survival (OS). MFS was measured from study entry to development of metastases or death from any cause and was censored at the last follow-up. DFS was measured from date of surgery until relapse or death from any cause and was censored at the last follow-up. LRFS was measured from end of CRT for patients proceeding with an organ preservation approach until local tumor regrowth or death from any cause and was censored at the last follow-up. Regrowth was determined by date of histologic confirmation from biopsy or when unavailable or nonconfirmatory, date of MRI suggestive of regrowth was used if regrowth was subsequently confirmed on histopathology after surgery. OS was measured from study entry to death from any cause or censored by last follow-up if alive. The Kaplan–Meier method was used for the survival estimates while Cox proportional hazards models were used to compare the survival rates between groups and to estimate the HRs. All analyses were performed using Stata software (version 13.1) where $P < 0.05$ (two-sided) were considered significant.

Results

Patient characteristics

A total of 47 patients were evaluable for ctDNA and primary tumor response analysis on MRI by RECIST and mrTRG (**Fig. 1**). Baseline characteristics are summarized in **Table 1**. Median age was 59 years (range 30–83). Forty-six patients (98%) were CDX2 positive and 45 (96%) were proficient for mismatch repair.

On completion of CRT, 8 of 47 (17%) patients had developed metastases with a further 3 developing metastases after surgery. A total of 32% of patients ($n = 15/47$) proceeded with an organ preservation approach following evidence of mrTRG 1 or 2. Regrowth occurred in

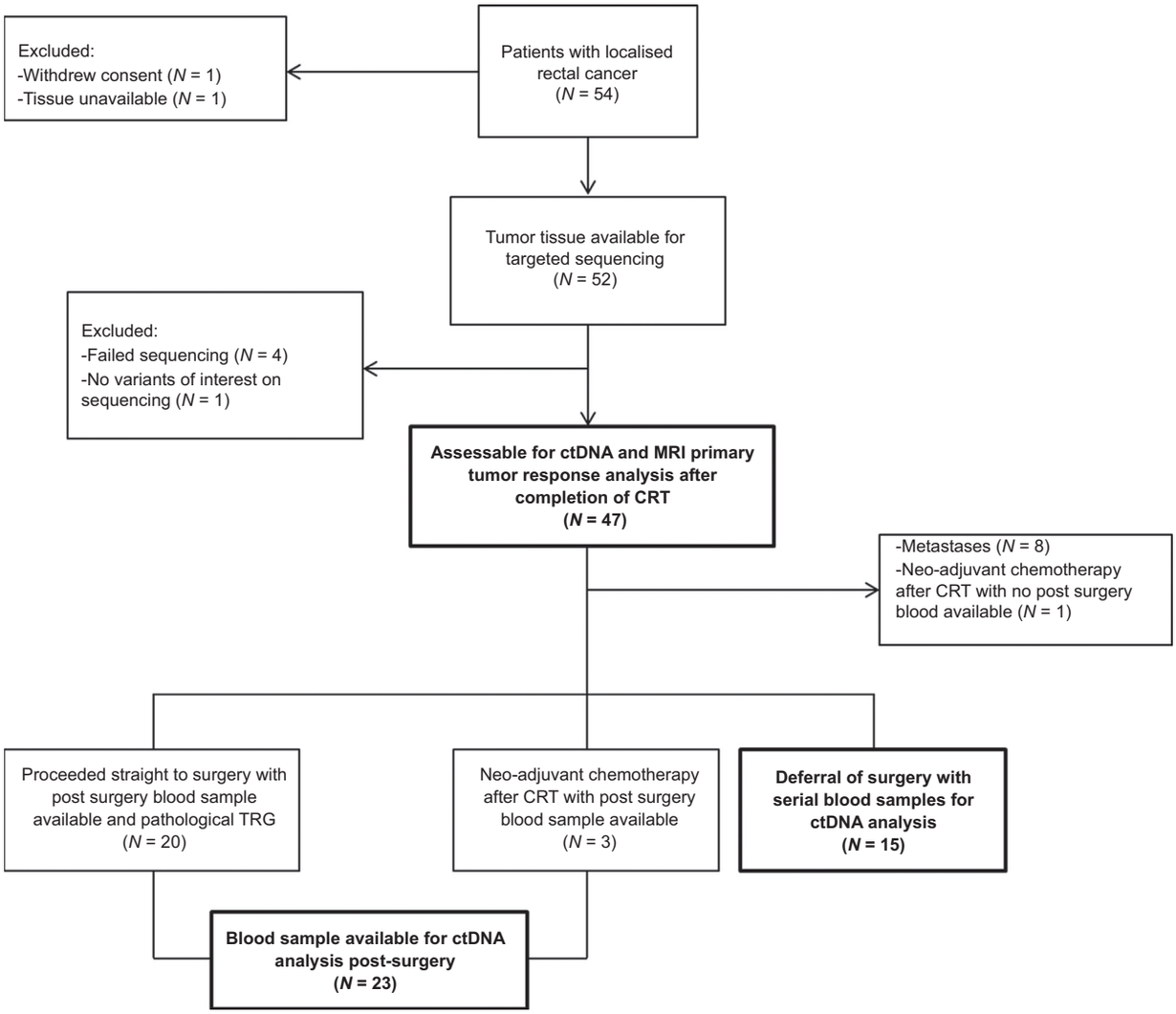

Figure 1.
CONSORT diagram showing patients included in each analysis and reasons for their exclusion.

10 of the patients following an organ preservation approach. A post-surgery blood sample (prior to any adjuvant chemotherapy) was available in 23 patients.

Mutation analysis in tissue

The sequencing failure rate (where no results were available from the diagnostic biopsy or the resection) was 4 of 52 (8%).

At least one somatic mutation was identified in the genes of interest of 47 of 48 (98%) of successfully sequenced cases. The median number of mutations in tissue was two (range 0–5). A list of all the mutations in the genes of interest and detection rates is listed in Supplementary Table S2 and Supplementary Fig. S1.

ctDNA detectability in blood

A median of two variants (range 1–3) were tracked in the plasma of each patient (Supplementary Fig. S1). Blood plasma was collected a median of: 6 days prior to commencing CRT [interquartile range (IQR) 4–13], 21 days from the start of CRT (IQR 20–22), and 37 days from completion of CRT (IQR 34–41.5). The post-surgery blood sample was collected a median of 47 days from surgery (IQR 39.5–60.5). The detection rate for ctDNA was: 35 of 47 (74%) pretreatment, 10 of 47 (21%) mid CRT, 10 of 47 (21%) at the end of CRT, and 3 of 23 (13%) post-surgery.

Among the 15 carcinoembryonic antigen (CEA) secretors, pretreatment ctDNA was detectable in all cases compared with 19 of 30 (63%) non-CEA secretors, ($P = 0.008$). No other baseline characteristics were significantly different between patients with detectable or undetectable ctDNA at any timepoint (**Table 1**). However, pathologic node-positive patients on the resection specimen were more likely to have detectable ctDNA post-surgery than node-negative patients ($P = 0.02$).

ctDNA detection and CRT response assessment

Radiological response to CRT was assessed by MRI, a median of 31 days (IQR; 29–33.5) following completion of treatment. RECIST measurement of the primary tumor demonstrated that 37 of 47 (79%)

Table 1. Clinical characteristics and MRI response by ctDNA status.

Variable	Pre CRT ctDNA ($n = 47$)			Mid CRT ctDNA ($n = 47$)			End of CRT ctDNA ($n = 47$)			Post-surgery ctDNA ($n = 23$)		
	Positive ($N = 35$)	Negative ($N = 12$)	*P*	Positive ($N = 10$)	Negative ($N = 37$)	*P*	Positive ($N = 10$)	Negative ($N = 37$)	*P*	Positive ($N = 3$)	Negative ($N = 20$)	*P*
Age, years												
Median	62	57.5	0.37	57	59	0.42	57.5	59	0.69	50	59	0.52
IQR (p25–p75)	50–66	47–60		40–64	51–66		48–64	51–66		37–66	49–65.5	
Gender, *n* (%)												
Male	22 (63)	7 (58)	1.00	8 (80)	21 (57)	0.28	8 (80)	21 (57)	0.28	2 (67)	11 (55)	1.00
Female	13 (37)	5 (42)		2 (20)	16 (43)		2 (20)	16 (43)		1 (33)	9 (45)	
Baseline MRI EMVI status, *n* (%)												
Positive	29 (83)	9 (75)	0.67	10 (100)	28 (76)	0.17	10(100)	28 (76)	0.17	3(100)	17(85)	1.00
Negative	6 (17)	3 (25)		0	9 (24)		0	9 (24)		0	3 (15)	
Baseline MRI CRM status, *n* (%)												
Involved	23 (66)	6 (50)	0.48	7 (70)	22 (59)	0.34	6 (60)	23 (62)	0.76	3 (100)	12 (60)	0.64
Threatened	5 (14)	1 (8)		2 (20)	4 (11)		2 (20)	4 (11)		0	2 (10)	
Safe	7 (20)	5 (42)		1 (10)	11 (30)		2 (20)	10 (27)		0	6 (30)	
Distance from anal verge in cm, *n* (%)												
≤5	9 (26)	3 (25)	1.00	3 (30)	9 (24)	0.70	2 (20)	10 (27)	1.00	1 (33)	4 (20)	0.54
>5	26 (74)	9 (75)		7 (70)	28 (76)		8 (80)	27 (73)		2 (67)	16 (80)	
Stage, *n* (%)												
I–II	3 (9)	3 (25)	0.16	1 (10)	5 (14)	1.00	1 (10)	5 (14)	1.00	0	1 (5)	1.00
III	32 (91)	9 (75)		9 (90)	32 (86)		9 (90)	32 (86)		3 (100)	19 (95)	
cT stage, *n* (%)												
0–2	2 (6)	2 (17)	0.27	0	4 (11)	0.56	0	4 (11)	0.56	0	0	-
3–4	33 (94)	10 (83)		10(100)	33 (89)		10(100)	33 (89)		3(100)	20(100)	
cN stage, *n* (%)												
0	3 (9)	3 (25)	0.16	1 (10)	5 (14)	1.00	1 (10)	5 (14)	1.00	0	1 (5)	1.00
≥1	32 (91)	9 (75)		9 (90)	32 (86)		9 (90)	32 (86)		3 (100)	19 (95)	
pT stage, *n* (%)												
0–2	7 (39)	2 (40)	1.00	2 (33)	7 (41)	1.00	0	9 (45)	0.25	0	9 (45)	0.25
3–4	11 (61)	3 (60)		4 (67)	10 (59)		3 (100)	11 (55)		3 (100)	11(55)	
pN stage, *n* (%)												
0	12 (67)	4 (80)	1.00	5 (83)	11 (65)	0.62	1 (33)	15 (75)	0.21	0	16 (80)	0.02
≥1	6 (33)	1(20)		1 (17)	6 (35)		2 (67)	5 (25)		3 (100)	4 (20)	
Baseline CEA in μg/L, *n* (%)[a]												
<5	19 (56)	11 (100)	0.008	7 (70)	23 (66)	1.00	6 (67)	24 (67)	1.00	2 (67)	14 (70)	1.00
≥5	15 (44)	0		3 (30)	12 (34)		3 (33)	12 (33)		1 (33)	6 (30)	
MRI response by RECIST												
Good responders (CR and PR)	27 (77)	10 (83)	1.00	7 (70)	30 (81)	0.42	8 (80)	29 (78)	1.00	3 (100)	15 (75)	1.00
Poor responders (SD and PD)	8 (23)	2 (17)		3 (30)	7 (19)		2 (20)	8 (22)		0	5 (25)	
MRI TRG response												
Good responders (TRG 1–2)	14 (40)	6 (50)	0.74	3 (30)	17 (46)	0.48	1 (10)	19 (51)	0.03	0	7 (35)	0.53
Poor responders (TRG 3–5)	21 (60)	6 (50)		7 (70)	20 (54)		9 (90)	18 (49)		3 (100)	13 (65)	
Developed metastases, *n* (%)												
No	25 (71)	11 (92)	0.24	6 (60)	30 (81)	0.21	3 (30)	33 (89)	<0.001	0	20 (100)	0.001
Yes	10 (29)	1 (8)		4 (40)	7 (19)		7 (70)	4 (11)		3 (100)	0	

Abbreviation: CRM, circumferential resection margin.
[a]Baseline CEA was unavailable for 2 patients.

of patients had a good response to treatment (CR, $n = 6$ and PR, $n = 31$). The remaining patients had a poor response to treatment (SD, $n = 9$ and PD, $n = 1$; Supplementary Fig. S2A–S2H shows examples of good and poor responders). There was no difference in response determined by RECIST between patients with detectable ctDNA and undetectable ctDNA at any timepoint.

Pretreatment, ctDNA was detectable in 3 of 5 (60%), 11 of 15 (73%), 10 of 13 (77%), 8 of 11 (73%), and 3 of 3 (100%) of patients with mrTRG 1, 2, 3, 4, and 5, respectively. Poor responders were more likely to have detectable ctDNA on completion of CRT than good responders (33%, $n = 9/27$ compared with 5%, $n = 1/20$; $P = 0.03$). There was no difference in mrTRG response between patients with detectable and undetectable ctDNA at any other timepoint (**Table 1**).

pCR was reported in 3 of 23 (13%) patients and in all 3 of these cases, ctDNA was detectable pretreatment and became undetectable from mid CRT onwards (Supplementary Fig. S3). All 3 of these cases had mrTRG2 and consistent with the literature, likely had on-going regression during the interval between MRI assessment and surgery which subsequently resulted in pCR (10, 11).

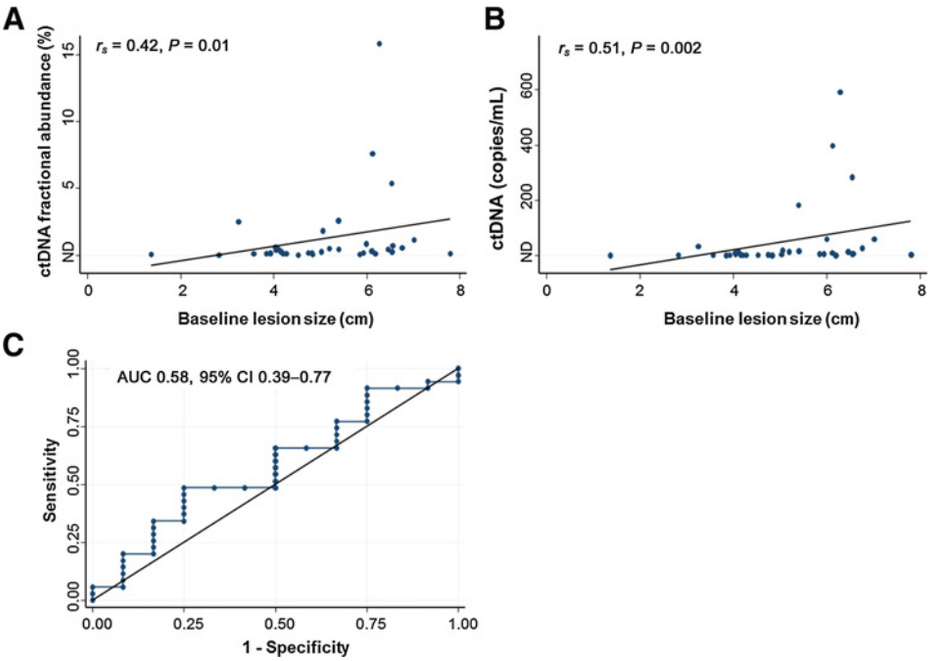

Figure 2.
The relationship between pretreatment lesion size and quantity of pretreatment ctDNA in FA (**A**) and copies/mL (**B**). **C,** ROC analysis to assess whether pretreatment lesion size is a predictor of ctDNA detectability. ND = not detected.

Baseline ctDNA value and primary lesion size

There was no significant difference in the pretreatment lesion size by RECIST between those with detectable and undetectable ctDNA (z = −0.78; $P = 0.45$). ROC analysis was unable to find an optimal pretreatment lesion size threshold to predict ctDNA detectability pretreatment (AUC 0.58; 95% CI, 0.39–0.77). In patients with detectable ctDNA pretreatment ($n = 35$), there was a weak positive correlation between pretreatment lesion size and quantity of ctDNA in copies/mL ($r_s = 0.51$) and FA ($r_s = 0.42$; **Fig. 2**).

ctDNA detectability and survival analyses

At the time of analysis, median follow-up was 26·4 months (IQR 19.7–31.3) and 8 of 47 (17%) patients had died. OS data are immature but preliminary data can be found in Supplementary Fig. S4.

Of the 11 patients that developed metastases, ctDNA detection at the end of CRT was higher ($n = 7$, 64%) compared with those that did not ($n = 3/36$, 8%; $P < 0.001$). Detection of ctDNA pretreatment that persisted at the mid CRT timepoint was also higher in patients that developed metastases ($n = 4/11$, 36%) compared with those that did not ($n = 4/36$, 11%; $P = 0.07$).

In-line with previous observations from our group, there was no difference in MFS by patients with detectable or undetectable ctDNA pretreatment (HR 2.1; 95% CI, 0.5–9.6; $P = 0.33$; **Fig. 3A**; ref. 12). This was also true for the mid CRT timepoint (HR 2.6; 95% CI, 0.9–8.1; $P = 0.09$; **Fig. 3B**). MFS was significantly shorter in patients with detectable ctDNA on completion of CRT compared with patients with undetectable ctDNA (HR 7.1; 95 % CI, 2.4–21.5; $P < 0.001$; **Fig. 3C**). Persistence of detectable ctDNA from pretreatment to the mid CRT timepoint was associated with worse MFS compared with cases where ctDNA was not detected both pretreatment and at the mid CRT timepoint (HR 3.8; 95% CI, 1.2–11.7; $P = 0.02$; **Fig. 3D**). Similarly, persistence of detectable ctDNA from pretreatment to the end of CRT was also associated with worse MFS compared with cases where ctDNA did not persist throughout (HR 11.5; 95% CI, 3.3–40.4; $P < 0.001$; **Fig. 3E**).

For the patients proceeding straight to surgery, the post-surgery sample had detectable ctDNA in all 3 patients that relapsed following surgery and was undetectable in the 20 patients who did not relapse ($P = 0·001$). Of the 20 patients with undetectable ctDNA following surgery, 1 patient died during follow-up but the cause of death was unknown. Patients developed metastases a mean of 128 days from surgery (SD, 82.4). ctDNA was detected a mean of 78 days (SD, 53.0) prior to confirmation of relapse on imaging in the post-surgery sample of these patients. DFS was significantly shorter in the 3 patients with detectable ctDNA post-surgery compared with the 20 patients with undetectable ctDNA (HR 39.9; 95% CI, 4.0–399.5; $P = 0.002$; **Fig. 3F**). Of the 3 patients with detectable ctDNA, 2 patients had an R1 resection compared with 1 patient among the 20 ctDNA-negative patients. Most patients (21/23) received adjuvant chemotherapy following surgery.

In patients deemed suitable for an organ preservation approach ($n = 15$), 10 patients had local regrowth of their tumor a median of 11 months from the end of CRT (IQR 6.25–13.5). LRFS was shorter in patients with detectable ctDNA at the end of CRT compared with patients with undetectable ctDNA (HR 5.8; 95% CI, 0.9–35.3; $P = 0.06$; **Fig. 3G**).

ctDNA quantification to predict development of metastatic disease

In patients with detectable ctDNA, the FA was compared between patients who went on to develop metastases with those who did not using the Mann–Whitney U test (**Fig. 4A**). There was no difference in the distribution of FA pretreatment for patients that developed metastases (median 0.48%; IQR 0.12%–2.5%) from those that did not (median 0.27%; IQR 0.1%–0.5%; z = −1.24; $P = 0.21$). However, the mid CRT FA was significantly higher in patients that developed metastases (median 0.8%; IQR 0.4%–4.6%) compared with those that did not (median 0.03%; IQR 0.02%–0.07%; z = −2.46; $P = 0.01$). Similarly, the FA at the end of CRT was also significantly higher in patients that developed metastases (median 2.3%, IQR 0.2%–7.4%) compared with those that did not (median

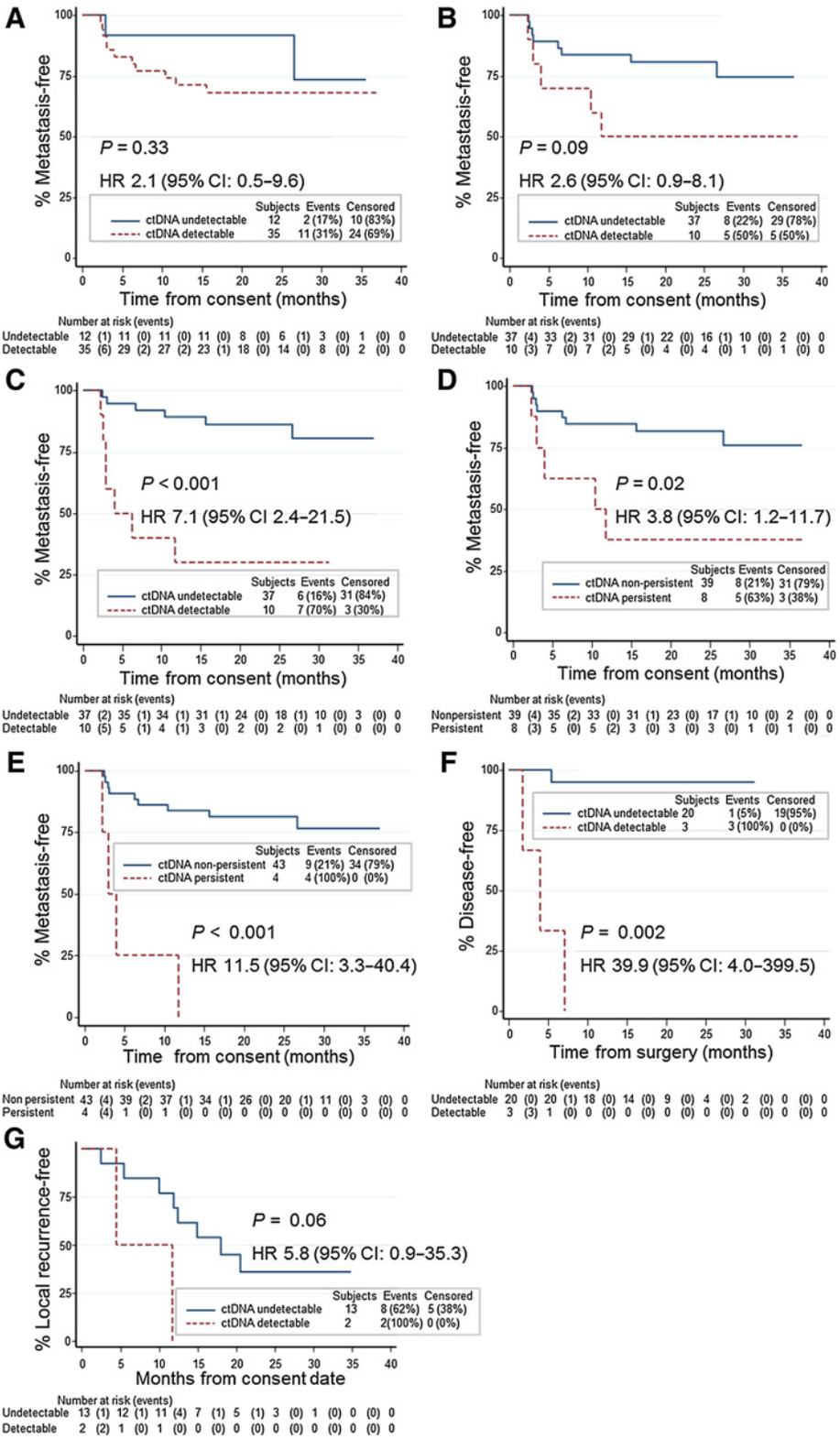

Figure 3.

Kaplan–Meier estimates of MFS by ctDNA status: pretreatment (**A**), mid CRT (**B**), and on completion of CRT (**C**). **D,** Persistence of ctDNA pretreatment and mid CRT compared with not persistent. **E,** Persistence of ctDNA pretreatment, mid CRT, and on completion of CRT compared with not persistent throughout. **F,** DFS from surgery by ctDNA status post-surgery. **G,** LRFS in patients deferring surgery by ctDNA status on completion of CRT.

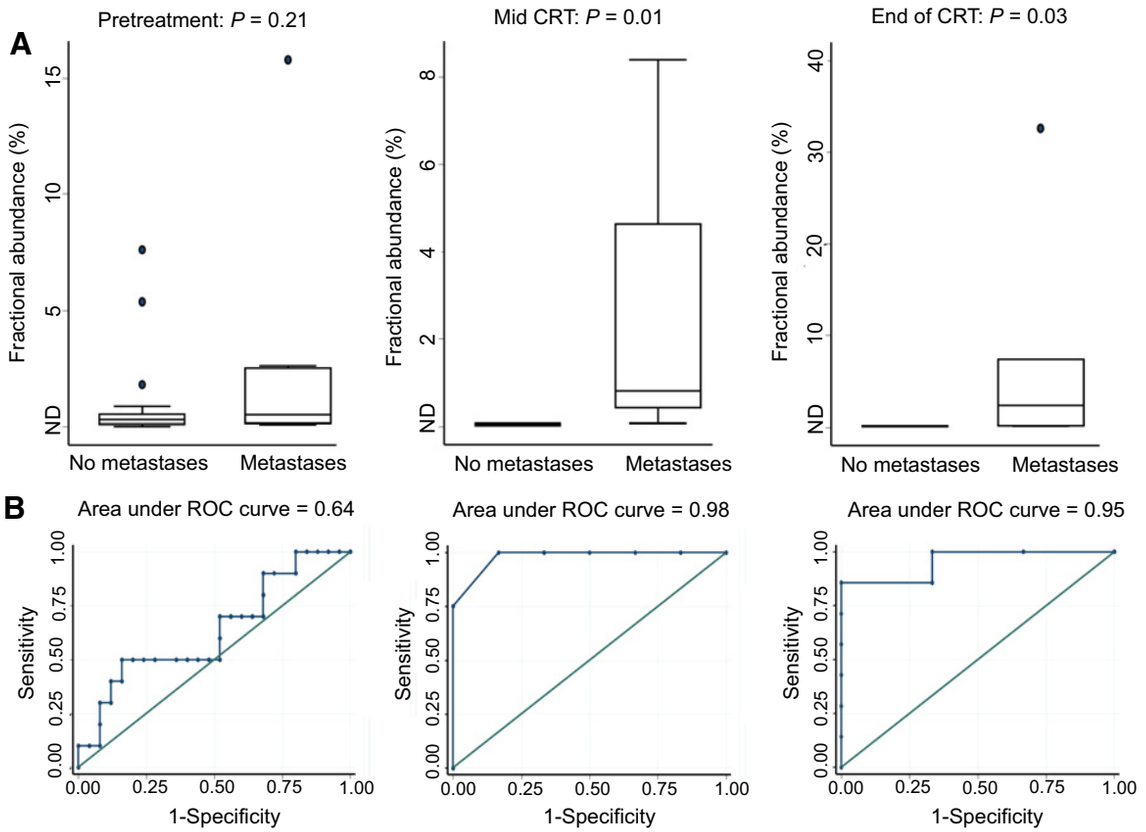

Figure 4.

A, Distribution of FA in patients with detectable ctDNA by whether they developed metastases at each timepoint, ND = not detected. **B,** ROC analysis for ctDNA FA to predict development of metastases at each timepoint.

0.08%, IQR 0.01%–0.2%; z = −2.17; $P = 0.03$). A ROC analysis was performed to assess whether FA is a good predictor for developing metastases (**Fig. 4B**). The pretreatment FA was not a good marker to discriminate between patients that developed metastases from those that did not (AUC = 0.64; 95% CI, 0.42–0.85). However, with a sensitivity of 100% and a specificity of 83.3%, a FA threshold of ≥0.07% at the mid CRT timepoint correctly predicted 90% of the patients that went on to develop metastases (AUC = 0.98; 95% CI, 0.92–1). Similarly, with a sensitivity of 100% and a specificity of 66.7%, a FA threshold of ≥0.13% at the end of CRT correctly predicted 90% of the patients that developed metastases (AUC = 0.95; 95% CI, 0.82–1).

ctDNA and detection of local regrowth in organ preservation patients

Plasma was available a median of 4.5 days (IQR, −15.5–18) from the point of confirmed regrowth ($n = 10$). ctDNA was only detectable in one case of regrowth (Supplementary Table S3).

The tissue from regrowth was therefore sequenced and compared with the diagnostic biopsy to assess whether the mutation profile had changed to account for the low ctDNA detection rate (**Fig. 5**). Eight matching tissue pairs were available. Although some new variants emerged, variants that were originally selected to be tracked in plasma still persisted in the regrowth tissue at a sufficiently high VAF in all the patients.

Discussion

Both mrTRG and ctDNA have attracted a significant amount of research interest although as yet, both are unvalidated biomarkers. Here, we are the first to report that in localized rectal cancer, there is an association between ctDNA status after completing CRT and response as determined by mrTRG ($P = 0.03$). This is primarily driven by the fact that patients with a poor response to CRT were much more likely to have detectable ctDNA following completion of CRT.

In addition, our results confirm that ctDNA is a good surrogate for the emergence of systemic disease with 70% of patients with detectable ctDNA at the end of CRT and all patients with detectable ctDNA post-surgery developing metastatic disease (13–15). We also found that MFS was significantly shorter in patients with persistently detectable ctDNA in the neoadjuvant period or in patients with detectable ctDNA at the end of CRT irrespective of other time points. Our data suggests that in patients with detectable ctDNA at the mid or end of CRT time points, the FA quantity should also be assessed to predict the patients that will go on to develop metastases. This strategy may help select patients that could benefit from treatment intensification or alternative treatment strategies in an attempt to prevent the development of metastases. Most patients with rectal cancer require surgery after CRT, which implies that there is still some tumor activity present, even if at low levels. This may explain why patients with low FA do not have metastases. Our results are consistent with a previous report in patients with

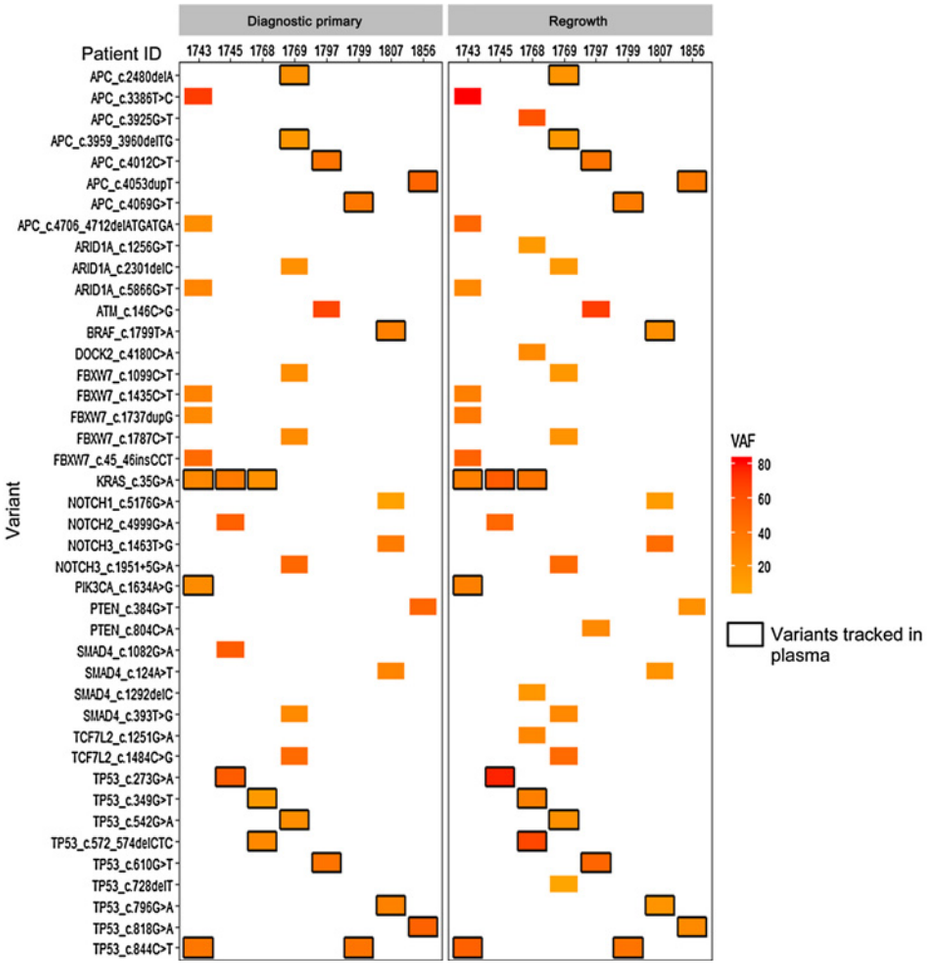

Figure 5.
Full 46 gene panel sequencing tissue results comparing the diagnostic biopsy and regrowth tissue.

localized lung cancer where a threshold of >0.1% during radiotherapy was associated with progression (16).

There were 3 patients with detectable ctDNA on completion of CRT that have not developed metastases within the specified follow-up period. Two of these patients proceeded with an organ preservation approach and both had local regrowth of their tumors. Our data, although limited by numbers, suggests that detection of ctDNA at the end of CRT in patients otherwise presumed to be good candidates for organ preservation, may predict local regrowth. Therefore ctDNA could be a complementary tool to MRI and endoscopic evaluation and detect patients at risk of regrowth among those initially felt to be suitable for an organ preservation strategy. However, undetectable ctDNA at the end of CRT was unhelpful in this regard as 8 of the 13 patients with undetectable ctDNA had evidence of local regrowth (although it should be noted that ctDNA was not detected at any timepoint in three of these cases). Larger prospective studies will be needed to confirm these findings.

We also assessed whether ctDNA was detectable around the time of local regrowth in organ preservation patients. Only 1 patient had detectable ctDNA of the 10 patients experiencing a local regrowth. The low detection rate could not be attributed to a change in the mutation profile from the diagnostic biopsy to the regrowth. It is likely to be related to the sensitivity of our current technique for the presence of very limited disease. In support of this, ctDNA is less likely to be detectable in cases of lower tumor volume or loco-regional recurrence and detection rates appear to decrease with earlier disease stages (14, 17–19). Our data also suggests that ctDNA is more likely to be detectable in node-positive disease on pathology ($P = 0{\cdot}02$) and this association has also been reported by others (14). Interestingly, the one case where ctDNA was detectable at the time of regrowth had the highest nodal burden (N2 disease).

Our pretreatment ctDNA detection rate of 74% was comparable with the published literature (14, 17, 18). However, the incorporation of universal methylation methods as described by others may improve sensitivity (20). Our end of treatment detection rate of 21% was, however, higher and likely reflects the fact that we had several patients that developed systemic progression soon after completing CRT. We speculate that the high progression rate may be due to the fact that a significant proportion of our patients had poor prognostic features at baseline. For example, 81% were MRI detected-EMVI (mrEMVI) positive and this has previously been associated with a higher rate of development of distant metastases compared with mrEMVI-negative cases (21).

It remains unclear why ctDNA consistently remains undetectable in approximately 25% of treatment naïve patients with localized disease. While difficulty detecting pretreatment ctDNA may reflect a less aggressive or smaller tumor, our results do not support this as patients with undetectable ctDNA pretreatment did not have prolonged survival or smaller pretreatment lesions compared with those with detectable ctDNA. Moreover, unlike in early lung cancer,

the level of the proliferation marker Ki-67 did not differentiate between ctDNA detectable and undetectable cases in our patients (Supplementary Fig. S3; ref. 19). Our data is limited in that we did not consider the ratio of proliferation to apoptosis or necrosis and both mechanisms of cell death have been implicated in the pathophysiology for ctDNA release (22, 23). Failure to detect ctDNA might in part be related to technical issues despite using ddPCR, a highly sensitive technique. Another possible explanation may be that ddPCR assays were only based on mutations in 6 genes of interest as they are known to be common in colorectal cancer (24–27). It is possible that the driver mutation may not have been in one of these genes. Our ctDNA analysis was restricted to the known mutation profile of the primary tumor, which could be considered a limitation as clonal evolution cannot be studied. However, in colorectal cancer, primary tumors and metastases exhibit high genomic concordance (28, 29) and focusing on the known mutation profile in the tumor may minimize the risk of false positives particularly in light of recent reports identifying somatic mutations in cfDNA arising from clonal hematopoiesis (30).

To implement the routine analysis of ctDNA into clinical practice, it is essential to establish in which patients it will be a useful marker and ensure adequate turn-around times for reporting results. Our results showed that ctDNA was detectable in all CEA secretors pretreatment, as well as an additional 19 patients who were non-CEA secretors suggesting that it may be a better marker for serial monitoring. Our methodology required the design of multiple assays that were unique to individual patients particularly for variants in large genes such as *APC*. This was time consuming and may limit incorporation of the methodology used here into routine clinical practice. However, improvements in sequencing technology with the ability for ultra-deep sequencing with error correction techniques to reduce false positives might be a viable alternative (31).

Our study has a number of limitations, some of which are inherent to the observational design of the study, as well as the small sample size and the limited number of post-surgery samples which could be collected. However, this is also reflective of the real world setting where the potential treatment options for LARC following CRT can vary depending on response. Our study is strengthened by the fact that: patients were prospectively recruited for the purpose of these analyses and given that this was a single center study, there was consistency in identifying suitable patients for recruitment as the same members of the MDT were involved. In addition, all analyses were conducted in the same laboratory with uniform methodology and laboratory staff were blinded to the clinical status of patients while radiologists were blinded to the ctDNA results. Although the data should be interpreted with caution, the results presented here should be regarded as hypothesis generating and may guide the direction of future study particularly given that risk adapted treatment strategies continue to be a research priority.

In conclusion, our findings support the notion that mrTRG and ctDNA are complementary tools to both assess local response and systemic disease status to guide therapy adaptation in rectal cancer. Prospective trials are in progress and will address the true value of incorporating such strategies into routine clinical practice.

Disclosure of Potential Conflicts of Interest

N. Valeri reports receiving speakers bureau honoraria from Pfizer, Bayer, Eli Lilly, and Merck. D. Tait is an advisory board member/unpaid consultant for Bowel Cancer UK (trustee and consultant medical advisory) and Macmillan Cancer Research (consultant medical advisory). I. Chau is an employee/paid consultant for Eli Lilly, Bristol-Myers Squibb, MSD, Bayer, Oncologie International, Pierre Fabre, Roche, Merck Serono, and AstraZeneca, and reports receiving other commercial research support from Eli Lilly, Sanofi Oncology, Merck Serono, and Janssen Cilag. N. Starling reports receiving commercial research grants from AstraZeneca, Bristol-Myers Squibb, and Pfizer, and reports receiving speakers bureau honoraria from AstraZeneca, Eli Lilly, Merck, Servier, and Pierre Fabre. M. Hubank is an employee/paid consultant for Guardant Health, Bristol-Myers Squibb, and Coleman Research Consulting, and reports receiving speakers bureau honoraria from Roche Diagnostics and Eli Lilly. D. Cunningham reports receiving other commercial research support (to Royal Marsden NHS Foundation Trust) from Amgen (REAL 3 Trial), Sanofi (Sanofi Trial), Merrimack (PLATFORM Trial), AstraZeneca (FRGR Trial), Celgene (Prospect R Trial), MedImmune (PLATFORM Trial), Bayer (PROSPECT R Trial), 4SC (EMERGE Trial), Clovis (Platform Trial), Eli Lilly (PLATFORM Trial), Janssen (IMYC Trial) and Merck (ICONIC/POLEM Trial). No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Khakoo, G. Brown, N. Valeri, I. Chau, N. Starling, D. Cunningham

Development of methodology: S. Khakoo, P.D. Carter, G. Brown, N. Valeri, R. Shaikh, E. Kalaitzaki, N. Starling, M. Hubank, D. Cunningham

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Khakoo, P.D. Carter, G. Brown, M.A. Bali, R. Shaikh, R. Begum, I. Rana, A. Wotherspoon, M. Terlizzo, D. Watkins, D. Tait, I. Chau, M. Hubank, D. Cunningham

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Khakoo, P.D. Carter, G. Brown, N. Valeri, S. Picchia, R. Shaikh, T. Jones, A. Wotherspoon, K. von Loga, E. Kalaitzaki, D. Tait, I. Chau, M. Hubank, D. Cunningham

Writing, review, and/or revision of the manuscript: S. Khakoo, P.D. Carter, R. Shaikh, I. Rana, A. Wotherspoon, K. von Loga, E. Kalaitzaki, D. Tait, I. Chau, N. Starling, M. Hubank, D. Cunningham

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Begum, I. Rana, A. Wotherspoon

Study supervision: C. Saffery, D. Watkins, N. Starling, D. Cunningham

Other (reporting pathology samples and assessing tumor regression grade in resection specimens): M. Terlizzo

Other (trial co-ordination): C. Saffery

Acknowledgments

We thank all patients and their families that were involved in the study. The UK National Health Service provided funding to the UK National Institute for Health Research Biomedical Research Centres at The Royal Marsden Hospital/Institute of Cancer Research (London, UK).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 22, 2019; revised August 30, 2019; accepted October 21, 2019; published first December 18, 2019.

References

1. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. N Engl J Med 2018;379:1754–65.
2. Khakoo S, Georgiou A, Gerlinger M, Cunningham D, Starling N. Circulating tumour DNA, a promising biomarker for the management of colorectal cancer. Crit Rev Oncol Hematol 2018;122:72–82.
3. Janjan NA, Khoo VS, Abbruzzese J, Pazdur R, Dubrow R, Cleary KR, et al. Tumor downstaging and sphincter preservation with preoperative chemoradiation in locally advanced rectal cancer: The M. D. Anderson Cancer Center experience. Int J Radiat Oncol Biol Phys 1999;44:1027–38.

4. Maas M, Nelemans PJ, Valentini V, Das P, Rödel C, Kuo LJ, et al. Long-term outcome in patients with a pathological complete response after chemoradiation for rectal cancer: a pooled analysis of individual patient data. Lancet Oncol 2010; 11:835–44.
5. Hendren SK, O'Connor BI, Liu M, Asano T, Cohen Z, Swallow CJ, et al. Prevalence of male and female sexual dysfunction is high following surgery for rectal cancer. Ann Surg 2005;242:212–23.
6. Tekkis P, Tait D, Cunningham D, Brown G. Is organ preservation in rectal cancer ready for prime time? Lancet 2018;391:2480–2.
7. Patel UB, Taylor F, Blomqvist L, George C, Evans H, Tekkis P, et al. Magnetic resonance imaging-detected tumor response for locally advanced rectal cancer predicts survival outcomes: MERCURY experience. J Clin Oncol 2011;29:3753–60.
8. Battersby NJ, Dattani M, Rao S, Cunningham D, Tait D, Adams R, et al. A rectal cancer feasibility study with an embedded phase III trial design assessing magnetic resonance tumour regression grade (mrTRG) as a novel biomarker to stratify management by good and poor response to chemoradiotherapy (TRIGGER): Study protocol for. Trials 2017;18:394.
9. Garcia-Murillas I, Schiavon G, Weigelt B, Ng C, Hrebien S, Cutts RJ, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015;7:302ra133.
10. Sun Z, Adam MA, Kim J, Shenoi M, Migaly J, Mantyh CR. Optimal timing to surgery after neoadjuvant chemoradiotherapy for locally advanced rectal cancer. J Am Coll Surg 2016;222:367–74.
11. Probst CP, Becerra AZ, Aquina CT, Tejani MA, Wexner SD, Garcia-Aguilar J, et al. Extended intervals after neoadjuvant therapy in locally advanced rectal cancer: the key to improved tumor response and potential organ preservation. J Am Coll Surg 2015;221:430–40.
12. Sclafani F, Chau I, Cunningham D, Hahne JC, Vlachogiannis G, Eltahir Z, et al. KRAS and BRAF mutations in circulating tumour DNA from locally advanced rectal cancer. Sci Rep 2018;8:1445.
13. Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016;8:346ra92.
14. Tie J, Cohen JD, Wang Y, Li L, Christie M, Simons K, et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. Gut 2019;68:663–71.
15. Carpinetti P, Donnard E, Bettoni F, Asprino P, Koyama F, Rozanski A, et al. The use of personalized biomarkers and liquid biopsies to monitor treatment response and disease recurrence in locally advanced rectal cancer after neoadjuvant chemoradiation. Oncotarget 2015;6:38360–71.
16. Chaudhuri AA, Lovejoy AF, Chabon JJ, Newman A, Stehr H, Merriott DJ, et al. Circulating tumor DNA analysis during radiation therapy for localized lung cancer predicts treatment outcome. Int J Radiat Oncol 2017;99:S1–2.
17. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.
18. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 2018;359:926–30.
19. Abbosh C, Birkbak NJ, Wilson GA, Jamal-Hanjani M, Constantin T, Salari R, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. Nature 2017;545:446–51.
20. Bachet JB, Bouché O, Taieb J, Dubreuil O, Garcia ML, Meurisse A, et al. RAS mutation analysis in circulating tumor DNA from patients with metastatic colorectal cancer: the AGEO RASANC prospective multicenter study. Ann Oncol 2018;29:1211–9.
21. Bugg WG, Andreou AK, Biswas D, Toms AP, Williams SM. The prognostic significance of MRI-detected extramural venous invasion in rectal carcinoma. Clin Radiol 2014;69:619–23.
22. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65.
23. Mouliere F, El Messaoudi S, Pang D, Dritschilo A, Thierry AR. Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. Mol Oncol 2014;8:927–41.
24. Muzny DM, Bainbridge MN, Chang K, Dinh HH, Drummond JA, Fowler G, et al. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330–7.
25. Roth AD, Tejpar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: Results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 2010;28:466–74.
26. Day FL, Jorissen RN, Lipton L, Mouradov D, Sakthianandeswaren A, Christie M, et al. PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer. Clin Cancer Res 2013;19:3285–96.
27. Domingo E, Ramamoorthy R, Oukrif D, Rosmarin D, Presz M, Wang H, et al. Use of multivariate analysis to suggest a new molecular classification of colorectal cancer. J Pathol 2013;229:441–8.
28. Brannon AR, Vakiani E, Sylvester BE, Scott SN, McDermott G, Shah RH, et al. Comparative sequencing analysis reveals high genomic concordance between matched primary and metastatic colorectal cancer lesions. Genome Biol 2014;15: 454.
29. Moorcraft SY, Jones T, Walker BA, Ladas G, Kalaitzaki E, Yuan L, et al. Molecular profiling of colorectal pulmonary metastases and primary tumours: implications for targeted treatment. Oncotarget 2017;8:64999–5008.
30. Genovese G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 2014;371:2477–87.
31. Mansukhani S, Barber LJ, Kleftogiannis D, Moorcraft SY, Davidson M, Woolston A, et al. Ultra-Sensitive mutation detection and genome-wide DNA copy number reconstruction by error- corrected circulating tumor DNA sequencing. Clin Chem 2018;64:1626–35.