Response prediction and risk stratification of patients with rectal cancer after neoadjuvant therapy through an analysis of circulating tumour DNA

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Summary

Background Multiple approaches based on cell-free DNA (cfDNA) have been applied to detect minimal residual disease (MRD) and to predict prognosis or recurrence. However, a comparison of the approaches used in different cohorts and studies is difficult. We aimed to compare multiple approaches for MRD analysis after neoadjuvant therapy (NAT) in patients with locally advanced rectal cancer (LARC).

Methods Sixty patients with LARC from a multicentre, phase II/III randomized trial were included, with tissue and blood samples collected. For each cfDNA sample, we profiled MRD using 3 approaches: personalized assay targeting tumour-informed mutations, universal panel of genes frequently mutated in colorectal cancer (CRC), and low depth sequencing for copy number alterations (CNAs).

Findings Positive MRD based on post-NAT personalized assay was significantly associated with an increased risk of recurrence (HR = 27.38; log-rank $P < 0.0001$). MRD analysis based on universal panel (HR = 5.18; log-rank $P = 0.00086$) and CNAs analysis (HR = 9.24; log-rank $P = 0.00017$) showed a compromised performance in predicting recurrence. Both the personalized assay and universal panel showed complementary pattern to CNAs analysis in detecting cases with recurrence and the combination of the two types of biomarkers may lead to better performance.

Interpretation The combination of mutation profiling and CNA profiling can improve the detection of MRD, which may help optimize the treatment strategies for patients with LARC.

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Keywords: Circulating tumour DNA; Locally advanced rectal cancer; Minimal residual disease; Neoadjuvant therapy; Next-generation sequencing

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Research in context

Evidence before this study

Despite two large randomized trials (RAPIDO and PRODIGE 23) demonstrated that intensified neoadjuvant chemotherapy before surgery improved both disease-free survival (DFS) and pathological complete response (pCR), the impact is limited and overall survival (OS) benefit is lack. More efficient predictive tool with high accuracy of patient selection for risk-adapted therapy before surgery may optimize the therapeutic decision making. However, a limited number of studies on colorectal cancer are based on preoperative ctDNA to predict the risk of recurrence and pCR.

Added value of this study

In this study, we report the minimal residual disease surveillance based on ctDNA mutation profiling, including a tumour-informed personalized assay and a universal panel. We also profile CNAs in the same samples. This study provides a head-to-head comparison among different approaches for MRD detection in the same samples and may help to determine the best solution for the precise management of patients with LARC.

Implications of all the available evidence

This study explores three approaches for post-NAT MRD detection in predicting disease recurrence for patients receiving two neoadjuvant modalities. Post-NAT provides an opportunity to predict prognosis before surgery and thus neoadjuvant chemotherapy may be selectively applied to those at high risk of recurrence in a timely manner. When combined with a personalized assay, CNA profiling may improve the sensitivity of MRD detection and the recurrence prediction. Furthermore, the combination of a universal panel and CNA profiling provides the possibility of a fully tumour-naive and non-invasive solution for MRD detection.

Introduction

Colorectal cancer is the third most frequently diagnosed cancer globally and has the fourth highest mortality rate[1]; rectal cancer accounts for approximately 28% of these cases.[2] Neoadjuvant chemoradiotherapy followed by total mesorectal excision (TME) is one of the standard treatments for patients with locally advanced (cT3-T4 and/or cN+, AJCC Cancer Staging 7th edition) rectal cancer (LARC).[3,4]

However, two main clinical dilemmas remain. The first problem is the high metastasis rate of 25–40%, which is the main cause of treatment failure in patients with LARC. The second is the low complete response (CR) rates of 10-30%.[3] Therefore, an ongoing paradigm shift in the approach to the administration of chemoradiation has been noted based on the results of several recent pivotal trials.[5] Total neoadjuvant therapy (TNT), a strategy of intensified induction chemotherapy combined with neoadjuvant chemoradiotherapy or short-course radiotherapy, appears to improve compliance, increase disease-free survival (DFS), and facilitate a better short-term response.[6,7] Meanwhile, overall survival benefit was not observed and DFS was improved less than 10%. Nevertheless, the potential to increase the response rate and reduce the incidence rate of metastasis still exist with the discovery of other effective biomarkers in addition to standard clinical features that are considered during the exploration of treatment strategies.

Circulating tumour DNA (ctDNA) has become a promising noninvasive biomarker for the dynamic monitoring of the tumour burden, with proven utility in predicting the prognosis[8–11] and monitoring the treatment response of patients with multiple tumour types. Multiple types of cfDNA-based biomarkers and multiple strategies have been studied in the field.[12–14] Due to the limited yield of cfDNA from one blood draw, a single approach was used to profile a single type of biomarker in most previous studies, and the performance of different biomarkers or strategies was difficult to compare.[15–17] Researchers have not determined whether different cfDNA-based biomarkers can be combined to obtain a better detection of MRD, either.

In this study, we applied a technology supporting multiple tests of one cfDNA sample with different approaches, and we profiled multiple sets of biomarkers to obtain a head-to-head comparison of the performance of MRD detection and prognostic prediction.

Methods

Ethics

This multicentre, open-label, prospective phase II/III randomized trial was approved by the Ethics Committee of National Cancer Center, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (Approval Number: 16-020/1099) and was conducted in the tertiary medical centres according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Patient enrolment and sample collection

This study was registered and the inclusion criteria for patients were presented on ClinicalTrials.gov with the number NCT02533271, STELLAR. The primary endpoint was 3-year relapse-free survival, defined as the time from the date of randomization to the first occurrence of local-regional failure or distant metastasis. The secondary objectives were 3-year local relapse-free survival, distant metastasis-free survival, and overall survival.

The tumour tissues were collected at the diagnostic stage by biopsy sampling, and peripheral blood was

collected in EDTA Vacutainer tubes (BD Diagnostics; Franklin Lakes, NJ, USA) and centrifuged within 2 h of collection at 4000 × g for 10 min to separate plasma and blood cells. Plasma was centrifuged a second time at 12,000 × g for 10 min at 4°C to remove any remaining cellular debris and stored at -80°C.

Neoadjuvant therapy

Patients enrolled were randomly assigned in a 1:1 ratio to short-course preoperative radiotherapy (SCPRT, 5 Gy x 5 alone) with neoadjuvant chemotherapy (NCT) (4 cycles of capecitabine plus oxaliplatin regimen) and preoperative long-course chemoradiotherapy (2 Gy x 25 with capecitabine). The treatment strategies in these two groups were described in detail in STELLAR registration file. Clinical serum levels of the biomarkers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) were monitored at baseline, before surgery and after surgery. CEA and CA19-9 levels were measured with immunoelectrochemiluminescence, with CEA concentrations of < 5.0 ng/mL and CA19-9 concentrations of < 27.0 U/mL considered within the reference range. Chest/abdominal/pelvic CT scans were performed every 3 months during the first two years and then every 6 months for a total of 5 years. Clinicians were blinded to the ctDNA results during the courses of neoadjuvant therapy.

DNA extraction and library preparation

Genomic DNA (gDNA) was extracted from fresh frozen tumour biopsies and WBCs with the QIAamp DNA Mini Kit (Qiagen; Germantown, MD, USA), and cfDNA was extracted from 1.5-4.5 mL of plasma with the Apostle MiniMax cfDNA isolation kit (C40605, Apostle; San Jose, CA, USA). Targeted sequencing of a panel of 509 genes or exomes was performed using genomic DNA obtained from tumour tissue and WBCs as previously described.[18]

Identification of somatic mutations

Briefly, the raw data (FASTQ file) were aligned to the UCSC human reference genome hg19 using Burrows–Wheeler aligner software (BWA, v0.7.15). Basic processing, marking duplicates, local realignments and score recalibration were performed using The Genome Analysis Toolkit (GATK, v3.6), Picard (v2.7.1) and Samtools (v1.3.1). Candidate somatic mutations were detected by comparing sequencing data from tumour tissue samples with MuTect1 and Strelka. All selected mutations were further validated by performing a manual inspection using Integrated Genome Viewer (IGV).

Personalized assay to profile multiple mutations

For each patient, we selected up to 22 somatic mutations from the tumour tissue. We designed customized primers targeting the mutations and used the primers to profile the matched cfDNA with Mutation Capsule technology as previously described.[18] Briefly, the cfDNA was ligated to a customized adaptor and amplified to produce a whole genome library that was subsequently used as a template and amplified with customized primers. Multiplex PCR primer pairs for the two rounds of nested amplification were designed using Oligo software (v7.53) and their uniqueness were verified in the human genome (http://genome.ucsc.edu/) to ensure amplification efficiency. In the first round of amplification, the whole genome library was amplified in 9 cycles of PCR using a target-specific primer and a primer matching the adapter sequence. A second round of 14 cycles of amplification was performed with one pair of nested primers matching the adapter and the target region to further enrich the target region and add the Illumina adapter sequences to the construct. The final libraries were sequenced using the Illumina NovaSeq 6000 platform at a median depth of 6835× after removing duplicate molecules. The median on-target ratio of reads mapped to the target region was 80%.

The clean reads were mapped to the human reference hg19 genome using 'BWA (v0.7.15) mem'[19] with the default parameters. Samtools mpileup[20] was used to identify somatic mutations, including SNVs and INDELs, across the targeted regions of interest. Each uniquely labelled template was amplified, resulting in a certain number of daughter molecules with the same sequence (defined as a UID family). If a mutation is pre-existing in the template molecule (original cfDNA) used for amplification, the mutation should be present in each daughter molecule containing the UID (barring any subsequent replication or sequencing errors). A UID family in which at least 80% of the family members have the same mutation is called the EUID family, indicating that it harbours a mutation that should be true instead of a false-positive mutation due to amplification or sequencing error.[21] The mutant allelic fraction was calculated by dividing the number of alternative EUID families by the sum of alternative and reference families. Tissue-specific mutations with at least one distinct paired duplex EUID family or four distinct EUID families were subsequently manually checked in IGV and verified using a cross-validation method. The candidate mutations were annotated with Ensemble Variant Effect Predictor (VEP).[20]

Development and validation of the ctDNA/cfDNA ratio model

The ctDNA/cfDNA ratio model was developed to estimate the ctDNA fraction based on allelic fraction and sequencing depth of somatic mutations in tumour tissue and matched plasma cfDNA. For each traced somatic mutation, we determined the mutant allelic fraction in the plasma and compared it with the

frequency in the primary tumour. The sample-level estimated ctDNA fraction was determined with maximum likelihood estimation.[22]

CNAs analysis

The raw sequencing data were treated as described above, and the next segmentation analysis was performed using QDNASeq (v1.14.0). The resulting output files were summarized using R software (v4.0.3). Overlap analysis was performed using bedtools (v2.17.0) and plotted with UpSetR (v1.4.0) within the R package (v4.0.3). Chromosome arm-level alterations show cancer-specific patterns. For example, a hierarchical clustering analysis of mean arm-level calls performed across 3,000 TCGA samples revealed that gastrointestinal tumours clustered with gains of chromosomes 8q, 13q, and 20.[23] Some of these CNAs, including gains of chromosomes 1q, 8q, 7,12q, 13q, and 20q and loss of chromosomes 1p, 20p, and 22q,[24–26] were also recurrently identified in our cohort as hot CNAs (34 baseline plasma samples from patients with LARC compared with 70 plasma samples from healthy controls). Therefore, we defined the CNA number as the sum of hot chromosome arms altered ($|Z| > 2$) to represent the level of copy number variation.[23,27]

Statistics

In this clinical cohort-based investigative study, the primary aim was to test the hypothesis that changes in the ctDNA fraction during treatment dynamically reflect minimal residual disease. Correlation analysis between input and estimated ctDNA in ctDNA fraction model and analysis of variance for the assessment of longitudinal plasma samples were the exploratory studies. Method for hypothesis testing and survival analysis was commonly used by previous researchers. Specifically, correlation analysis used Spearman's correlation analysis. For continuous variables, differences in ctDNA fractions between recurrence and non-recurrence groups were assessed with Mann–Whitney (rank sum) test, ctDNA fractions across treatment courses of NAT were assessed by Kruskal-Wallis test and post hoc using Dunn's multiple comparisons test, and the ctDNA fraction was assessed for patients with paired baseline and post-NAT data using Wilcoxon matched-pairs signed rank test. Differences in clinical characteristics between patients with positive and negative ctDNA fractions were evaluated with Fisher's exact test for categorical variables. These statistical analyses were performed with Prism 8 software (v8.4.3). Relapse-free survival (RFS) was measured from the date of randomization to the first occurrence of local-regional failure or distant metastasis. The univariate analysis was conducted using the Kaplan–Meier method with the log-rank test. HR values were calculated using univariate Cox proportional hazard models. The multivariate analysis was based on the Cox proportional hazard model in which the common important factors, such as age, sex, and clinical risk (according to the ESMO guidelines) were included. The survival model was evaluated with the C-index. The Kaplan–Meier curves were verified by performing a time-dependent receiver operating characteristic (ROC) curve analysis, and the area under the curve (AUC) was calculated to evaluate the prognostic performance. These analyses were performed using R software (v4.0.3). P values < 0.05 from a 2-sided test were considered statistically significant in all analyses. A sample of fifty patients was needed to achieve the power of 0.8 in this study as previously described.[28]

Role of the funding source

The sponsors did not have any role in the study design, data collection, data analyses, interpretation, or writing of the manuscript.

Results

Patient characteristics and tissue mutation identification

Patients with locally advanced rectal cancer ($n = 82$; cT3-4N0 or $cT_{any}N1$-2) were enrolled in the trial from December 30, 2016, to October 8, 2018. Twenty-two patients were excluded due to the lack of plasma samples obtained after NAT (Figure 1a). Thirty-one patients were treated with long-course neoadjuvant chemoradiotherapy (LCRT), and 29 patients were treated with short-course neoadjuvant radiotherapy (SCPRT) with neoadjuvant chemotherapy (Table 1). The median follow-up period was 33.25 months (range, 9.63–42.43 months). Seventeen (28.33%) patients were diagnosed with local relapse or metastasis during follow-up, including 5/17 (29.41%) with local relapse, 6/17 (35.29%) with liver metastasis and 6/17 (35.29%) with lung metastasis (Table S1).

One hundred ninety-six blood samples were available during the treatment process, including baseline (collected before NAT, n = 42), in-process (collected during NAT, $n = 35$), post-NAT (collected 2 weeks after SCPRT or LCRT, $n = 60$) and pre-TME (collected before surgery, $n = 59$) samples (Figure 1a). We performed targeted sequencing with a panel of 509 genes or exome sequencing on the genomic DNA isolated from the tumour tissue and matched WBCs, and then identified a median of 51 (range, 3-177) somatic mutations in each tumour (Table S2). The mutational landscape of the top 15 most significantly mutated genes in the cohort was shown in Figure 1b. Customized primers were designed to profile up to 22 somatic mutations in the matched cfDNA with Mutation Capsule technology (Table S3) as previously described.[18]

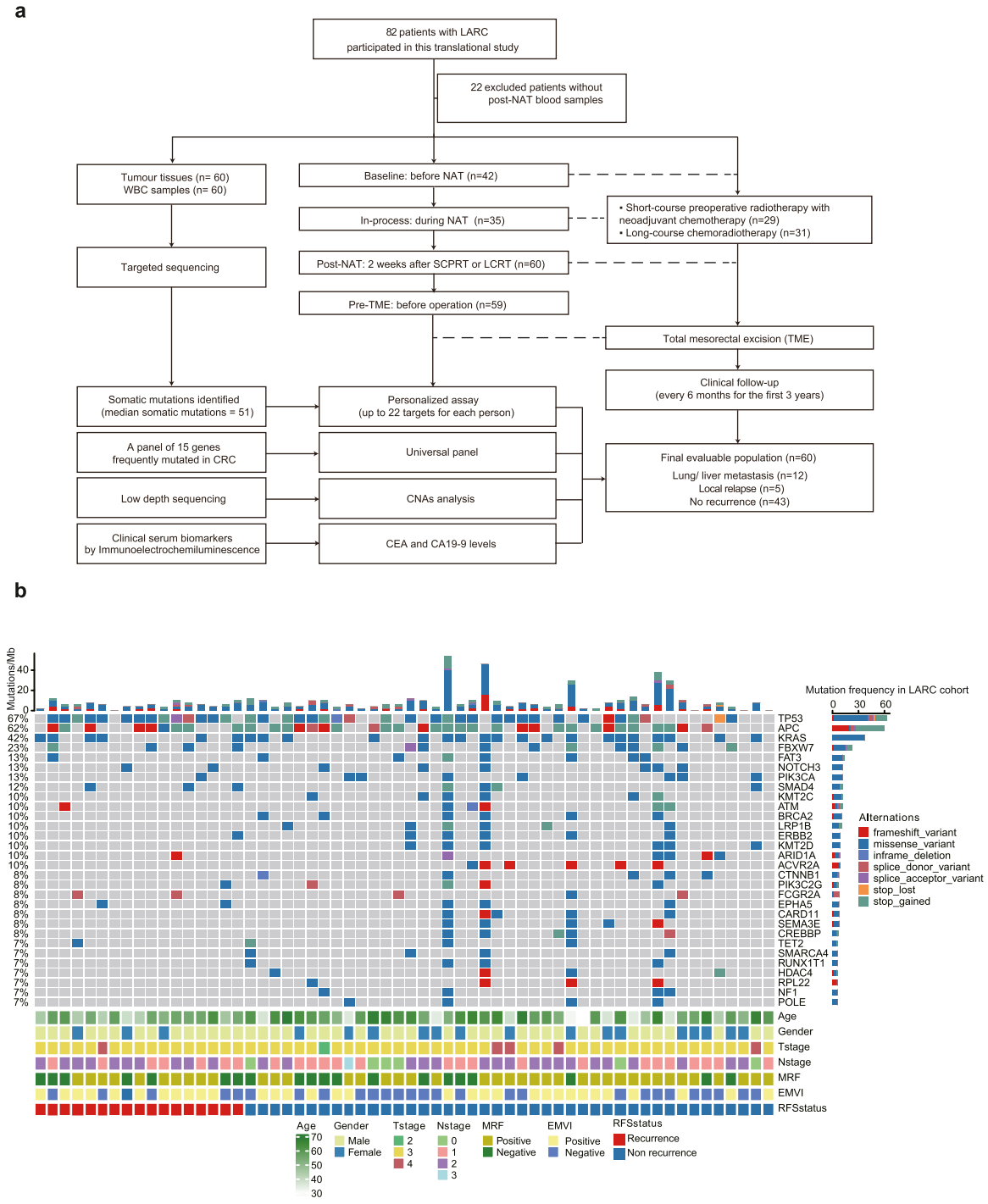

Figure 1. Schematic of the sample collection procedure and mutation landscape. (a) Patient enrolment, sample collections, experimental design and analysis of clinical endpoints. (b) Mutation landscape of tumour tissues and clinical characteristics of the 60 patients with LARC in our cohort. LARC, locally advanced rectal cancer; WBC, white blood cell; NAT, neoadjuvant therapy; SCPRT, short-course preoperative radiotherapy; LCRT, long-course neoadjuvant chemoradiotherapy; TME, total mesorectal excision; CRC, colorectal cancer; CNAs, copy number alterations; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; MRF, mesorectal fascia; EMVI, extramural venous invasion; RFS, relapse-free survival.

Characteristics	Baseline ctDNA fraction (*n*=42)			In-process ctDNA fraction (*n*=35)			Post-NAT ctDNA fraction (*n*=60)		
	Positive (*n*=35)	Negative (*n*=7)	*P*	Positive (*n*=17)	Negative (*n*=18)	*P*	Positive (*n*=14)	Negative (*n*=46)	*P*
Age, years									
Mean	53	54		55	56		52	53	
Range	29-68	36-64		36-68	31-67		38-66	29-68	
Sex, *n* (%)									
Male	21	6	0.3898	13	12	0.7112	10	32	>0.9999
Female	14	1		4	6		4	14	
Clinical T stage, *n* (%)									
cT1-2	0	0	>0.9999	0	1	>0.9999	0	1	>0.9999
cT3-4	35	7		17	17		14	45	
Clinical N stage, *n* (%)									
cN0	4	2	0.2574	1	4	0.3377	0	6	0.3201
cN1-3	31	5		16	14		14	40	
Neoadjuvant treatment, *n* (%)									
SCPRT	18	2	0.4143	8	3	0.0750	8	21	0.5474
LCRT	17	5		9	15		6	25	
Pathological T stage, *n* (%)									
ypT0-2	14	2	0.6562	5	7	0.4495	5	6	0.0359(*)
ypT3-4	13	4		9	6		5	32	
Unknown	8	1		3	5		4	8	
Pathological N stage, *n* (%)									
ypN0	21	4	0.6162	11	3	0.0070(**)	6	30	0.2412
ypN1-2	6	2		3	10		4	8	
Unknown	8	1		3	5		4	8	
Pathological complete response, *n* (%)									
Yes	3	0	>0.9999	2	2	>0.9999	1	4	>0.9999
No	24	6		12	11		9	34	
Unknown	8	1		3	5		4	8	
Adjuvant chemotherapy, *n* (%)									
Yes	19	1	0.0961	9	6	0.3145	5	24	0.3652
No	16	6		8	12		9	22	
Pre-op CEA, *n* (%)									
Positive	5	1	>0.9999	2	3	>0.9999	3	6	0.4194
Negative	26	6		15	14		10	37	
Unknown	4	0		0	1		1	3	
Pre-op CA19-9, *n* (%)									
Positive	3	0	>0.9999	1	1	>0.9999	2	2	0.1865
Negative	27	6		14	16		9	40	
Unknown	5	1		2	1		3	4	
RFS events, *n* (%)									
Yes	8	1	>0.9999	6	2	0.1212	13	4	<0.0001(****)
No	27	6		11	16		1	42	
OS events, *n* (%)									
Yes	3	0	>0.9999	3	0	0.1039	4	1	0.0088(**)
No	32	7		14	18		10	45	

Abbreviation: SCPRT, short-course preoperative radiotherapy; LCRT, long-course chemoradiotherapy; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; RFS, relapse-free survival; OS, overall survival.

***Table 1*: Clinicopathological characteristics and clinical endpoint, according to ctDNA fraction status (P-value determined by Fisher's Exact Test).**

Construction of the plasma ctDNA fraction model

Due to the low fraction of residual ctDNA in plasma, the number of cfDNA molecules from one blood draw profiled for a particular mutation site (distinct coverage) might be insufficient to detect the mutation, and the detected allelic fraction of one mutation might not accurately represent the ctDNA fraction. Here, we established a model to estimate the ctDNA fraction based on allelic fraction and sequencing depth of somatic mutations in tumour tissues and paired plasma samples (Figure 2a). For each traced somatic mutation, we determined the mutant allelic fraction in plasma and

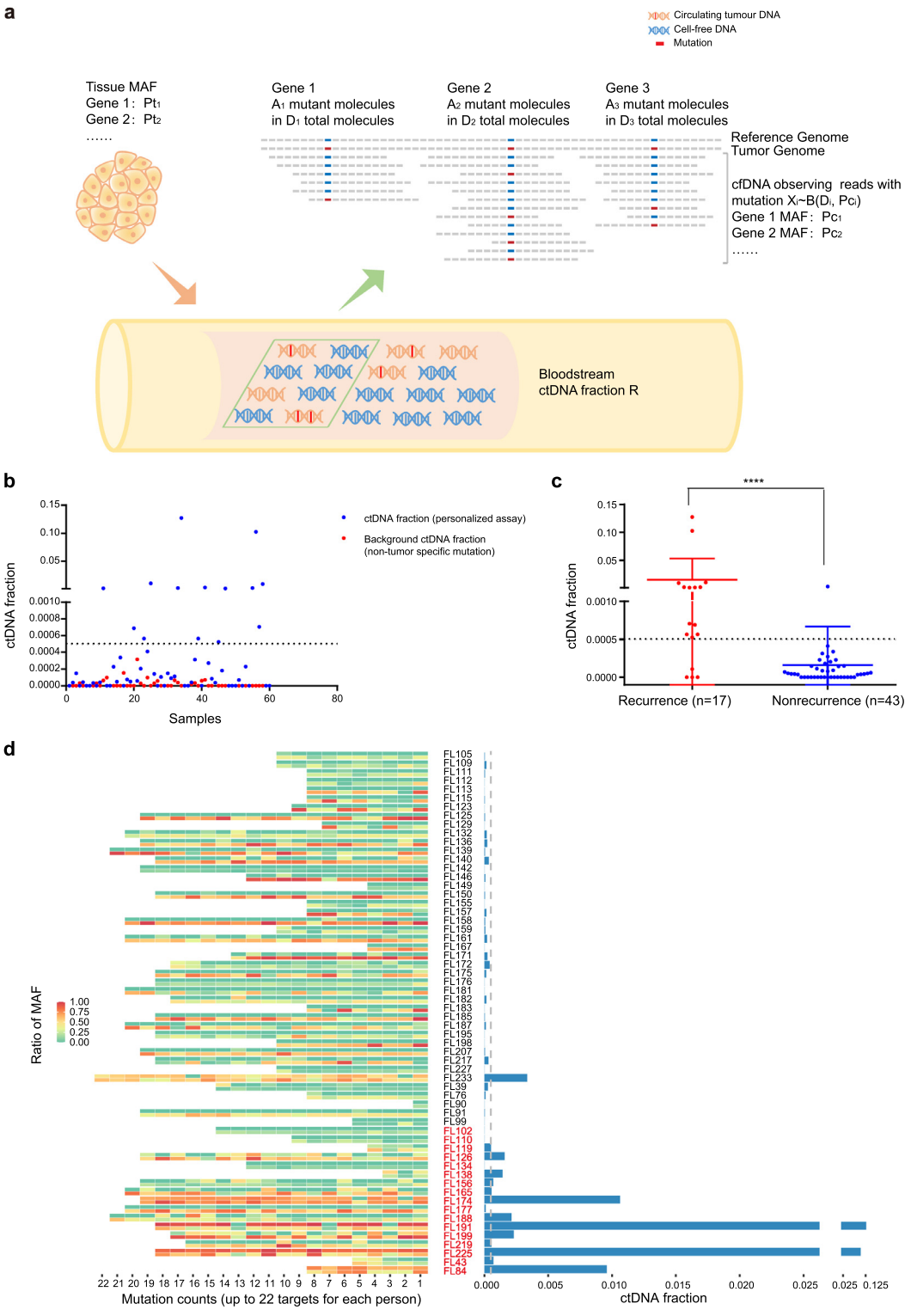

Figure 2. ctDNA/cfDNA ratio model based on personalized assay. (a) Design principle of ctDNA/cfDNA ratio model. A model to estimate the ctDNA fraction based on allelic fraction and sequencing depth of somatic mutations in tumour tissue and paired plasma samples. (b) Biological noise of the 60 plasma samples from patients. The background ctDNA fraction for each sample was calculated based on nontumour-specific mutations. (c) ctDNA fractions are shown as mean ± SD values in patients with recurrence

compared it with the fraction in the primary tumour. The sample-level estimated ctDNA/cfDNA ratio (ctDNA fraction) was determined with maximum likelihood estimation.

We determined the fraction of mutations that were not detected in the matched tumour sample to evaluate the biological noise of random mutations in the cfDNA from the patients in this cohort. We calculated a set of ctDNA fractions based on these non-tumour-specific mutations in the 60 LARC samples and found the background fractions to be lower than 0.05% (Figure 2b, mean = 0.0021%, maximum = 0.032%). The ctDNA fractions of these samples based on tumour-specific mutations were distinct from background fractions (mean = 0.45%, maximum = 12.74%). We classified the ctDNA fraction as positive or negative with a cut-off of 0.05% in further analyses to avoid false-positive MRD detection from background noise mutations (Figure 2b).

The association between the post-NAT ctDNA fraction and survival

For the 60 patients with LARC, a median of 17 somatic mutations (1-22 mutations per person, total = 824) were tracked for each patient in ctDNA. At the post-NAT assessment, 24.64% (203/824) of the tracked mutations were detected with an average allelic fraction of 1.36%, and the ctDNA fraction ranged from 0 to 12.74%. Among the 17 patients with recurrence, 76.47% (13/17) carried a positive (≥ 0.05%) ctDNA fraction in the post-NAT blood sample, while only one (2.33%) of the 43 patients without recurrence exhibited a positive ctDNA fraction (Figure 2c, d), achieving 76.47% sensitivity and 97.67% specificity in recurrence prediction (Table S4). The differences in the ctDNA fraction between the recurrence group (n = 17) and the nonrecurrence group (n = 43) were significant (Mann−Whitney test, $P < 0.0001$) (Figure 2c). Patients with a positive ctDNA fraction (MRD-positive) in post-NAT samples showed a significantly higher risk of recurrence than MRD-negative patients (HR = 27.38; 95% CI, 8.61-87.06; log-rank $P < 0.0001$) (Figure 3a). During the 3-year follow-up, five patients died from metastatic disease, and four of them were MRD-positive. The Kaplan−Meier analysis revealed shorter overall survival (OS) in patients with a positive ctDNA fraction (HR = 17.78; 95% CI, 1.94-162.60; log-rank $P = 0.00054$) (Figure 3b). The post-NAT ctDNA fraction showed a considerable risk classification capability for distant metastasis-free survival (HR = 17.37; 95% CI, 4.62-65.28; log-rank $P < 0.0001$) (Figure S1a) and local relapse-free survival (HR = 20.59; 95% CI, 2.26-187.40; log-rank $P = 0.00016$) (Figure S1b). The post-NAT ctDNA fraction status remained a strong predictor of RFS in both the LCRT group (HR = 31.62, 95% CI: 8.40-118.90, log-rank $P < 0.0001$) and the SCPRT group (HR = 23.61, 95% CI: 6.68-83.44, log-rank $P < 0.0001$).

We further compared the predictive value of the personalized assay (ctDNA fraction) with known risk factors of disease recurrence, including pathological diagnosis and protein biomarkers. The clinical risk was classified as high (ypT3-4 or ypN+) or low (ypT0-2 and ypN0) based on the European Society for Medical Oncology (ESMO) guidelines. The RFS outcomes of risk assessments based on the clinical risk were HR = 2.55; 95% CI, 0.70-9.28; log-rank $P = 0.14$ (Figure 3c). The post-NAT ctDNA fraction remained a strong predictor of recurrence among patients with pathological high risk (HR = 21.27; 95% CI, 5.15-87.92; log-rank $P < 0.0001$) or low risk (HR = 16.39; 95% CI, 1.46-184.30; log-rank $P = 0.0023$) (Figure 3d, e). In addition, post-NAT CEA and CA19-9 concentrations were available for 56 and 53 patients, respectively, with CEA positivity detected in 9 patients and CA 19-9 positivity detected in 4 patients. Among patients with available protein biomarkers, the RFS prediction based on CEA levels was HR = 1.21; 95% CI, 0.34-4.25; log-rank $P = 0.77$ and that for CA 19-9 levels was HR = 1.06; 95% CI, 0.14-8.11; log-rank $P = 0.96$ (Figure S1c, d). Both protein markers exhibited high specificity (85.00% and 92.31%, CEA and CA 19-9, respectively) but low sensitivity (18.75% and 7.14%, CEA and CA 19-9, respectively). On the other hand, the clinical risk showed a high sensitivity (76.92%) but low specificity (48.57%) (Table S4). In the receiver operating characteristic curve analysis of the ctDNA fraction and clinical risk, the ctDNA status had a higher AUC than any traditional risk factor (Personalized assay, AUC = 0.87; CEA level, AUC = 0.52; CA19−9 level, AUC = 0.49; clinical risk, AUC = 0.63) (Figure S2). The combination with the clinical risk did not increase the accuracy of RFS estimation obtained with the ctDNA fraction (Table S1). In the multivariate analysis based on the Cox proportional hazard model of the ctDNA fraction, age, sex, and clinical risk, the post-NAT ctDNA fraction by personalized assay was still the strongest independent risk factor for predicting recurrence

(*n* = 17) and nonrecurrence (*n* = 43) (Mann−Whitney test, ****, $P < 0.0001$). (d) Summary of somatic mutations and post-NAT ctDNA fractions for all patients (*n*=60). The left panel shows the mutations in tumour (bottom bar in each patient) and matched post-NAT plasma samples (top bar in each patient) from 60 patients. Colour gradations represent the ratio of the MAF to the maximum tissue or plasma MAF. The right panel shows the post-NAT ctDNA fractions for each patient. Patients with recurrence are shown in red. ctDNA, circulating tumour DNA; cfDNA, cell-free DNA; MAF: mutant allelic fraction; Pt_i: MAF in solid tumour tissue; Pc_i: MAF in the corresponding plasma sample; D_i: sequencing depth in the plasma sample; A_i: mutation read number in the plasma sample; X_i: observed reads with mutations in the plasma sample; and R: overall ctDNA concentration; SD, standard deviation; NAT, neoadjuvant therapy.

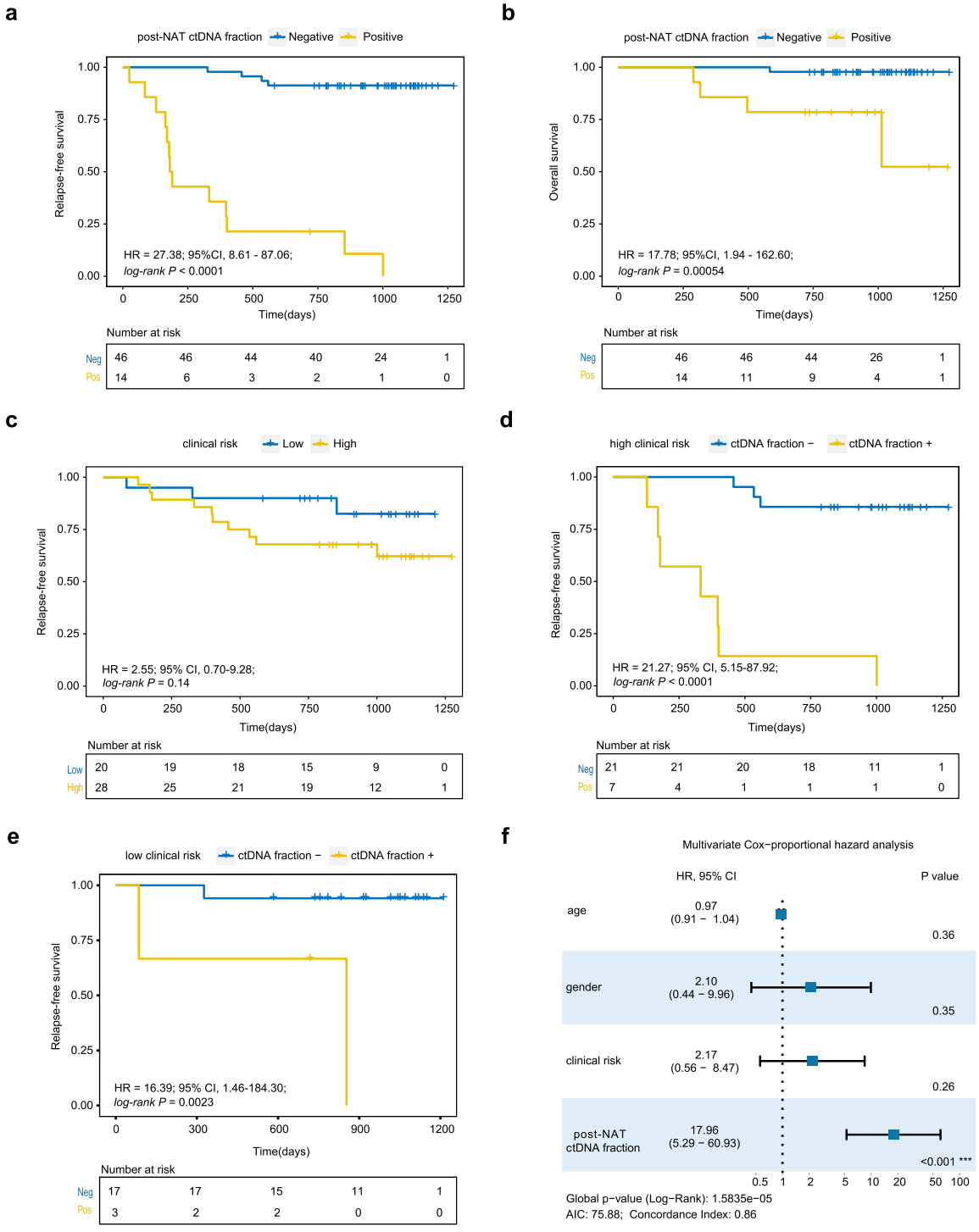

Figure 3. Kaplan–Meier estimates of RFS and OS according to ctDNA fraction. (a) Kaplan–Meier survival analysis showing the probability of RFS as determined by the ctDNA fraction detected in post-NAT plasma samples ($n = 60$). The patient was classified as positive if the post-NAT ctDNA fraction was $\geq 0.05\%$. (b) Kaplan–Meier estimates of OS based on the post-NAT ctDNA fraction ($n = 60$). (c) RFS stratified by clinical risk ($n = 48$): high clinical risk (ypT3-4 or ypN+) and low clinical risk (ypT0-2 and ypN0). (d and e) RFS stratified by post-NAT ctDNA fraction in patients at (d) high clinical risk ($n = 28$) and (e) low clinical risk ($n = 20$). (f) Multivariate Cox proportional hazard analysis including traditional predictors and post-NAT ctDNA fraction ($n = 48$). RFS, relapse-free survival; OS, overall survival; ctDNA, circulating tumour DNA; NAT, neoadjuvant therapy; neg, negative; pos, positive; AIC, akaike information criterion.

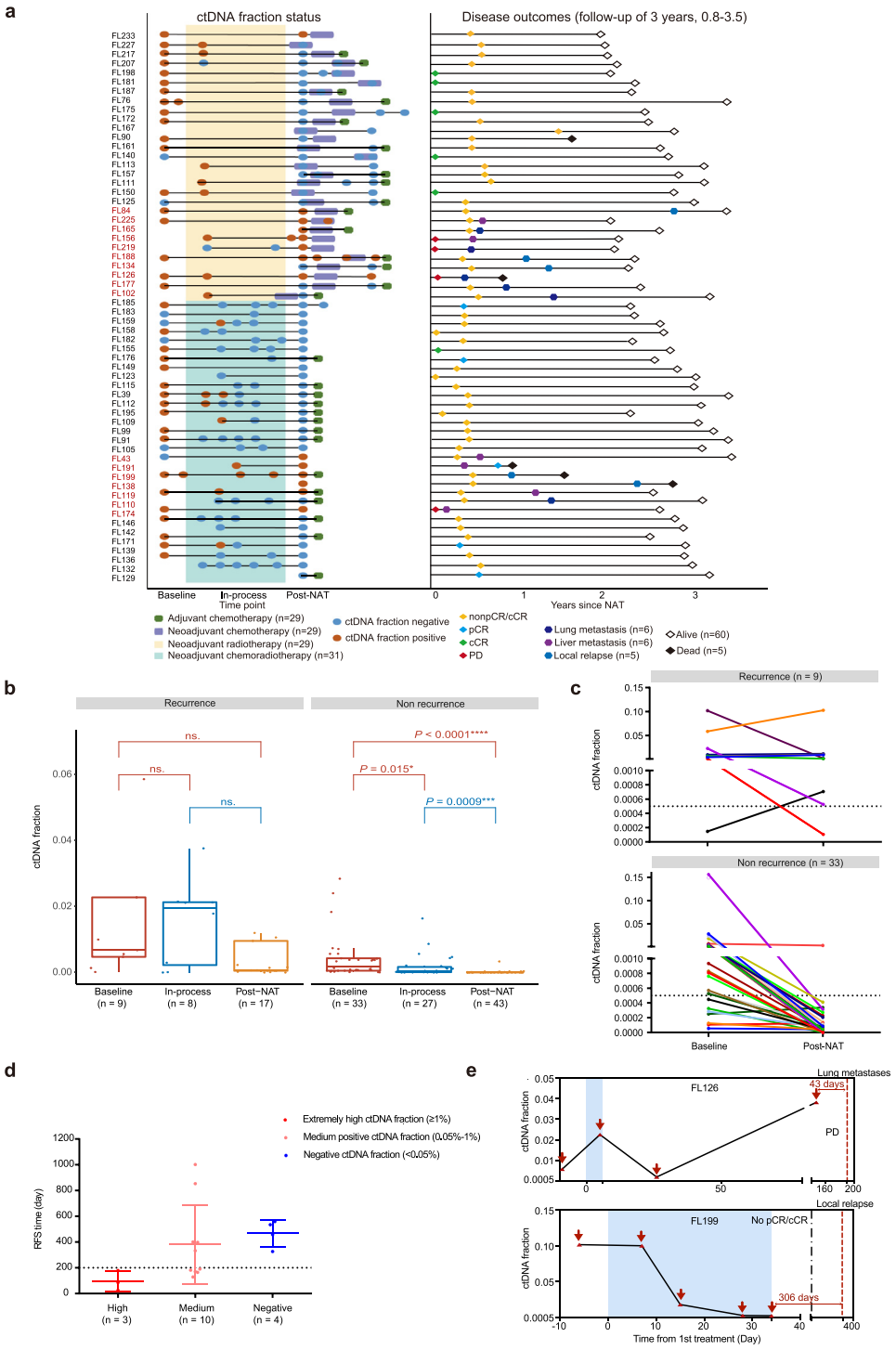

Figure 4. Dynamics of ctDNA fraction during treatment. (a) Longitudinal changes in the ctDNA fraction status, treatment strategies and disease outcomes. (b) ctDNA fractions are shown as median ± IQR values in different courses of NAT (Dunn's multiple comparisons test, ns., $P > 0.05$; *, $P = 0.015$; ***, $P = 0.0009$; ****, $P < 0.0001$). (c) Dynamics of ctDNA fraction in 42 patients with both baseline and post-NAT samples; the dark dotted line indicates the cut-off of 0.05%. (d) Relationships between the RFS time and ctDNA fraction intensity, and RFS time is shown as mean ± SD values. (e) Detailed description of changes in the cfDNA fractions over time for patients FL126 and FL199. ctDNA, circulating tumour DNA; IQR, interquartile range; NAT, neoadjuvant therapy; RFS, relapse-free survival; SD, standard deviation; pCR, pathological complete response; cCR, clinical complete response; PD, progressive disease.

(HR = 17.96; 95% CI, 5.29−60.93; log-rank P < 0.001) (Figure 3f).

Longitudinal status and intensity of ctDNA fraction during neoadjuvant therapy

We next checked longitudinal status of the ctDNA fraction and its possible association with the disease course, therapeutic effect and survival status of all 60 patients (Figure 4a). Compared with baseline and in-process samples, a clear trend of a reduced post-NAT ctDNA fraction was observed in both the recurrence and nonrecurrence groups (Figure 4b), which highlighted the significant therapeutic effect of NAT. We noticed a more substantial reduction in the ctDNA fraction during baseline, in-process and post-NAT stages within the nonrecurrence group (Dunn's multiple comparison test, baseline vs. in-process: P = 0.0130; baseline vs. post-NAT: P < 0.0001; in-process vs. post-NAT: P = 0.0009) compared to the recurrence group (Dunn's multiple comparison test, baseline vs. in-process: P > 0.9999; baseline vs. post-NAT: P = 0.1819; in-process vs. post-NAT: P = 0.4114) (Kruskal−Wallis test, nonrecurrence group, P < 0.0001; recurrence group, P = 0.113) (Figure 4b). Moreover, the post-NAT ctDNA fraction status exhibited the strongest association with RFS, followed by the status at the in-process (HR = 3.61; 95% CI, 0.73-17.91; log-rank P = 0.093) and baseline stages (HR = 1.58; 95% CI, 0.20-12.67; log-rank P = 0.66). For the 17 patients experiencing recurrence, the median lead time between the detection of positive post-NAT ctDNA fraction and finding of radiological recurrence was 10.2 months (range, 0.1-33.2 months) (Wilcoxon matched-pairs signed rank test, P = 0.0001) (Figure S3a). We explored whether ctDNA fraction dynamics were linked to RFS by specifically focusing on the 42 patients with both baseline and post-NAT samples and observed a decreased ctDNA fraction in most patients (85.71%, 36/42). For the 9 patients experiencing recurrence, the ctDNA fraction after NAT increased in 4 (44.44%) patients and decreased but was still positive in 4 (44.44%) patients. In the nonrecurrence group (n = 33), the ctDNA fraction decreased to undetectable levels in 30 patients (90.90%) (Figure 4c). These data showed better predictive value of the post-NAT ctDNA fraction status than the ctDNA fraction dynamics (HR = 7.40; 95% CI: 1.97-27.82; log-rank P = 0.00053; sensitivity of 44.44% and specificity of 93.94%) for RFS estimation.

The ctDNA fraction (post-NAT) in MRD-positive samples varied significantly from 0.05% to 12.74%. We divided the post-NAT samples into two groups to test if the ctDNA fraction values were correlated with the recurrence status: highly positive ctDNA fraction (≥ 1%) and moderately positive ctDNA fraction (0.05%-1%). The RFS of the 3 patients with highly positive post-NAT ctDNA fractions was shorter (< 200 days) than that of the moderately positive group (Figure 4d). In patient FL126 with two post-NAT plasma samples, the ctDNA fraction in plasma was moderately positive (0.16%) at 20 days after NAT and highly positive (3.50%) at 141 days, and lung metastases appeared in this patient only 43 days after the second time point (Figure 4e). In patient FL199 with a moderately positive ctDNA fraction (0.23%), local relapse occurred 306 days later (Figure 4e). The dynamic ctDNA fraction in the remaining samples was shown in Figure S4.

Clearance of the ctDNA fraction and response to neoadjuvant therapy

Thirty-five patients with a positive ctDNA fraction at baseline were analysed (35/42 patients) to explore the performance of the ctDNA fraction in monitoring the NAT response. With ctDNA clearance defined as ratio of post-NAT ctDNA fraction to baseline ctDNA fraction below 2% (median value of the ratio), 19 (54.29%) patients showed no clearance at the post-NAT time point relative to baseline ctDNA fraction values (Figures 5, S3b). For patients with or without ctDNA clearance, there were 9/16 (56.25%) and 18/19 (94.74%) exhibited nonpCR/cCR (clinical complete response), respectively. The association between ctDNA fraction clearance and response to neoadjuvant therapy was significant (Fisher's exact test, P = 0.013).

MRD detection with a universal panel of CRC driver genes

We profiled the tumour and cfDNA samples with a panel of 15 genes frequently mutated in colorectal cancer[16] to investigate whether a universal panel might achieve similar results for the RFS estimate (Table S5). Among the 57 patients (57/60, 95%) whose tumour tissue harboured somatic mutations in the universal panel, 18 were positive in post-NAT plasma samples, where universal panel positivity was defined as at least one somatic mutation detected in plasma cfDNA. Positive results of the post-NAT universal panel predicted recurrence with 66.67% sensitivity and 78.38% specificity (HR = 5.18; 95% CI, 1.76-15.20; log-rank P = 0.00086) (Table S4 and Figure 6a). The post-NAT universal panel performed well in predicting relapse among patients with a high clinical risk (HR = 7.01; 95% CI, 1.73-28.47; log-rank P = 0.0017) (Figure 6b) but not in patients with a low clinical risk (HR = 4.43; 95% CI, 0.40−49.18 log-rank P = 0.18) (Figure 6c). In the multivariate analysis based on the Cox proportional hazard model, the post-NAT universal panel (HR = 6.01; 95% CI, 1.68-21.50; log-rank P = 0.006) was still the strongest independent risk factor predicting recurrence (Figure 6d). Three patients (3/60, 5%) were unable to be analysed using this approach, as no mutations in the genes in the universal panel were identified in the tumour sample. Compared with the personalized assay, a small and universal panel would provide more

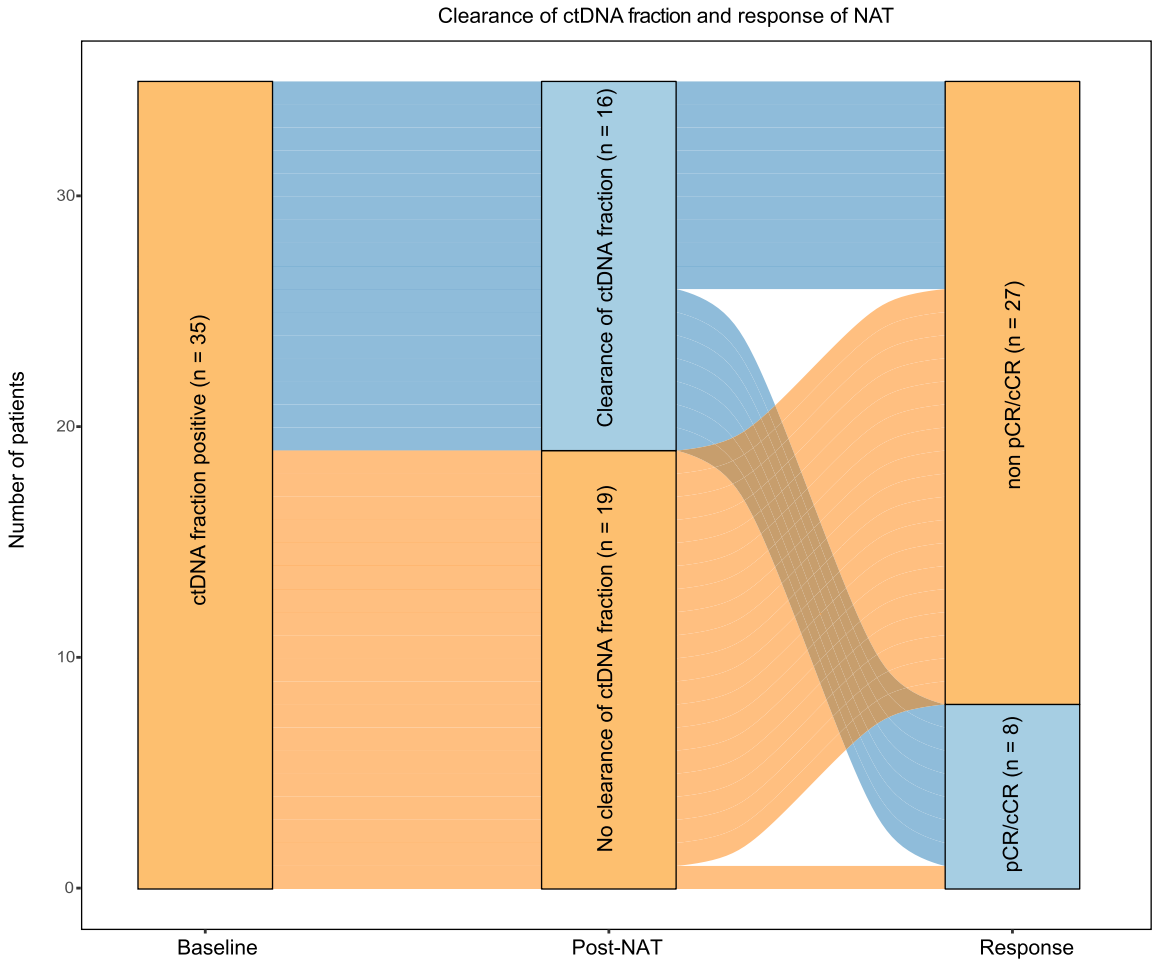

Figure 5. Association of ctDNA fraction clearance with the response to NAT. The Sankey plot shows the association of ctDNA fraction clearance with the treatment response (pCR/cCR, or nonpCR/cCR) in 35 patients with positive ctDNA fraction at baseline. NAT, neoadjuvant therapy; ctDNA, circulating tumour DNA; pCR, pathological complete response; cCR, clinical complete response.

convenience and availability in clinical application for 95% of patients with LARC, but the performance might be compromised.

MRD detection with plasma CNAs

In addition to a mutation profiling, disease progression might also be monitored by profiling copy number alterations (CNAs) with low depth whole genome sequencing of cfDNA.[13] Among the 34 patients with both baseline and post-NAT CNA results, 22 patients with a baseline CNA number ≥ 1 were included in subsequent analyses (Figure S5a). In these samples, we defined baseline-specific CNAs positivity as at least one baseline-specific hot CNA that was identified in post-NAT plasma samples and baseline-specific CNAs negativity as no detection of the baseline-specific hot CNAs in post-NAT plasma samples. Baseline-specific CNAs positivity could predict recurrence with the sensitivity of 66.67% and specificity of 100% (HR = 9.24; 95% CI, 2.28-37.40; log-rank $P = 0.00017$) (Figure 7a). The combination of personalized assay and baseline-specific CNA strategies exhibited the better prediction of RFS (HR = 35.89; 95% CI, 9.93-129.80; log-rank $P < 0.0001$) (sensitivity = 82.35%, specificity = 97.67%). The addition of baseline-specific CNAs also improved the performance of the universal panel in predicting RFS (HR = 6.58; 95% CI, 2.09-20.74; log-rank $P = 0.00022$) (sensitivity = 73.33%, specificity = 78.38%).

In addition, when we used the post-NAT CNA number as an independent biomarker to predict recurrence among all the cases (positive: hot CNA number ≥ 2, negative: hot CNA number < 2) without considering the CNA status in baseline samples, the sensitivity and specificity of the CNA biomarker were reduced to 50.00% and 94.87%, respectively (HR = 7.32; 95% CI,

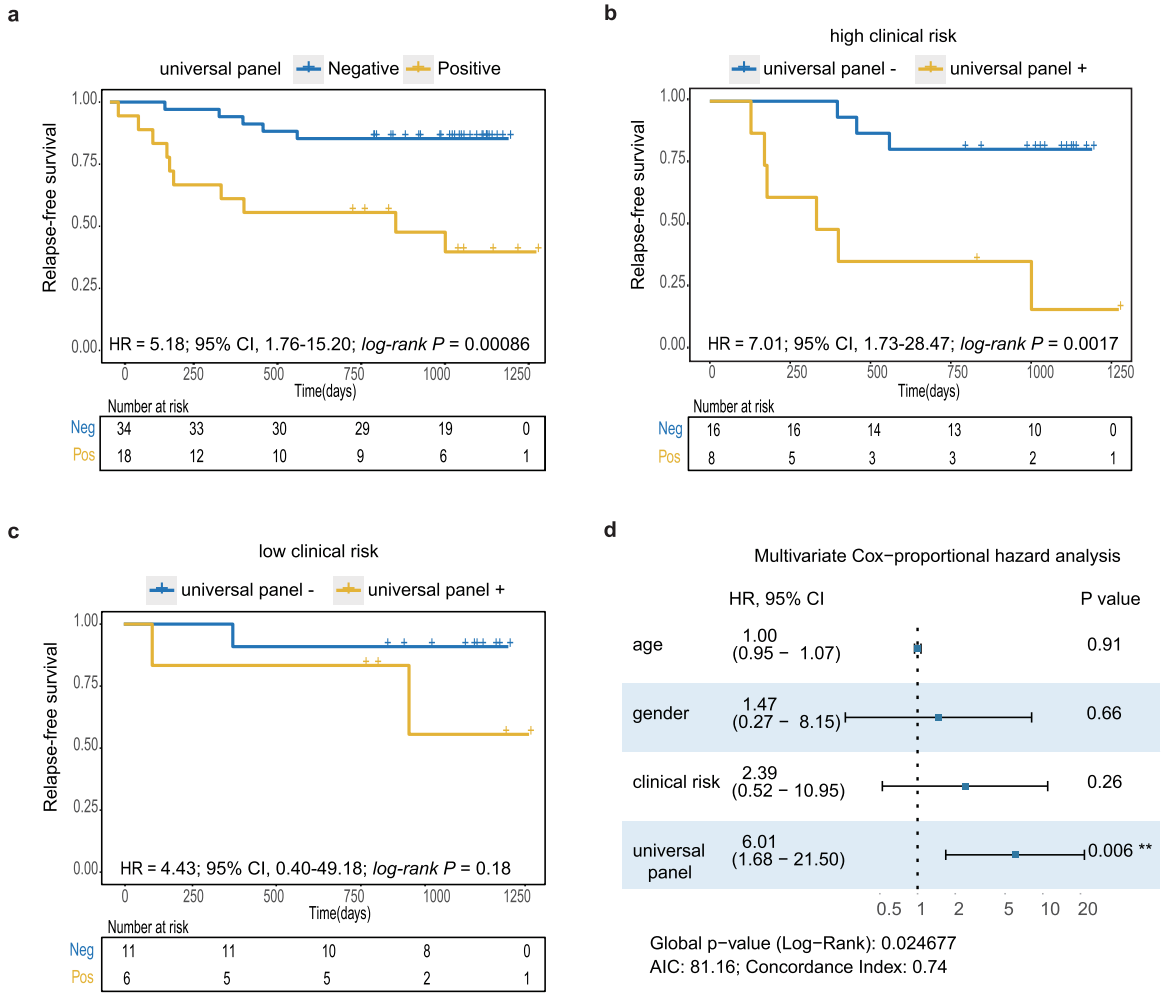

Figure 6. Kaplan−Meier estimates of RFS according to a universal panel. (a) Kaplan−Meier survival analysis showing the probability of RFS as determined by detecting a universal panel in post-NAT plasma samples (n = 52). (b-c) RFS determined by detecting a universal panel in patients at (b) high clinical risk (n = 24) and (c) low clinical risk (n = 17). (d) Multivariate Cox proportional hazard analysis including traditional predictors and a post-NAT universal panel in 41 patients. RFS, relapse-free survival; NAT, neoadjuvant therapy; neg, negative; pos, positive; AIC, akaike information criterion.

2.70-19.85; log-rank $P < 0.0001$) (Figure 7b) (Table S4). The patients with positive post-NAT CNAs were more likely to experience shorter RFS in high clinical risk cases (HR = 5.53; 95% CI, 1.46-20.98; log-rank $P = 0.0049$) (Figure 7c). It showed trend in low clinical risk cases but the sample size was small (Figure 7d). The post-NAT CNAs remained an independent predictor of recurrence when incorporated into a model including traditional predictors (HR = 9.52; 95% CI, 2.14-42.26; log-rank $P = 0.003$) (Figure 7e). The combination of personalized assay and post-NAT CNAs showed 88.24% sensitivity and 93.02% specificity (HR = 38.88; 95% CI, 8.51-177.60; log-rank $P < 0.0001$). The combination of the universal panel and post-NAT CNAs showed 81.25% sensitivity and 73.68% specificity (HR = 8.05; 95% CI, 2.28-28.42; log-rank $P = 0.00012$). The summary of mutation profiling results (personalized assay and universal panel), CNA profiling and the RFS status were shown in Figure 7e.

Discussion

Neoadjuvant chemoradiotherapy is the standard therapy for patients with locally advanced rectal cancer, but treatment strategies to control distant metastases are constantly being updated. The lack of dynamic circulating biomarkers during treatment and short-course substitution of clinical endpoints limits the timely evaluation of curative effects and therefore may be responsible for insufficient treatment or overtreatment.

Several studies show that the presence of ctDNA after surgery is associated with a high risk of recurrence

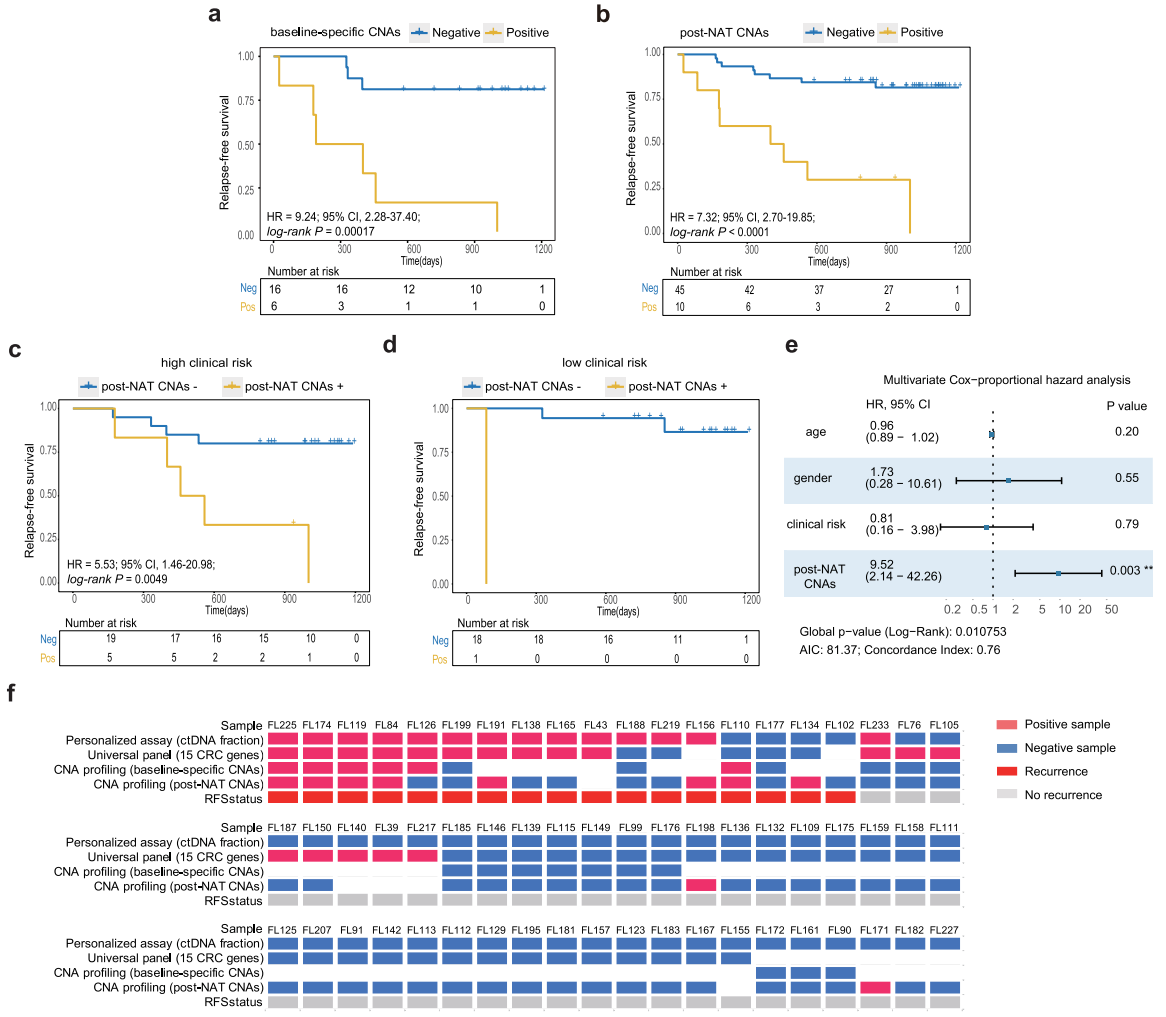

Figure 7. Kaplan−Meier estimates of RFS according to CNA profiling. (a and b) Kaplan−Meier survival analysis showing the probability of RFS as determined by (a) baseline-specific CNAs (n = 22) and (b) post-NAT CNAs (n = 55). (c and d) RFS determined by post-NAT CNAs detected in patients at (c) high clinical risk (n = 24) and (d) low clinical risk (n = 19). (e) Multivariate Cox proportional hazard analysis including traditional predictors and post-NAT CNAs in 45 patients. (f) Summary of mutation profiling results (a personalized assay and a universal panel), CNA profiling results and the RFS status. CNAs, copy number alterations; NAT, neoadjuvant therapy; RFS, relapse-free survival; CRC, colorectal cancer; neg, negative; pos, positive; AIC, akaike information criterion.

in patients with colorectal cancer, where the sensitivity could be 40−50% in predicting relapse.[15−17] In other studies, different types of cfDNA-based biomarkers and different strategies have been applied to detect MRD and to predict the prognosis of patients with CRC.[29−31] Due to the limited yield of cfDNA from one blood draw, only one approach was tested in the cohort of one study, and the performance of the markers and strategies have rarely been compared head-to-head in the same cohort.

In the present study, we applied a technology, Mutation Capsule, to profile different sets of biomarkers in the same cfDNA sample. In this case, we can compare the MRD based on different strategies, including tumour-informed personalized assay, universal panel of key driver genes, as well as tumour-naive CNA profiling. Although the tumour-informed personalized assay shows the highest accuracy in the detection of MRD, this solution requires a series of processes, including profiling tumour tissue, designing personalized assays, and profiling cfDNA with the assay. The complicated process takes a longer time and requires more labour. The universal panel avoids the long turnaround time by profiling the tumour and matched cfDNA with the same panel at the same time. However, the performance was compromised and some cases were not

available by this solution. The tumour-naive CNA solution has further waived the requirement for tumour tissue, making it suitable for the scenario where tumour tissues are not available or an additional biopsy must be performed to obtain tissue.[12,32] In addition, mutation profiling and CNAs exhibited a complementary pattern in the detection of recurrence cases, and the combination of these two types of biomarkers enhanced the prediction of RFS. A larger cohort and more patients are needed to further validate the predictive value of this complicated combination of biomarkers.

Our results revealed potential applications of a post-NAT ctDNA analysis in predicting recurrence before surgery. Previous studies have shown the value of a postoperative ctDNA analysis in patients with colorectal cancer.[15,16,33] However, 4–10 weeks may be needed to collect postoperative blood samples and at least 3 weeks for the analysis of cfDNA. A greater than 6-week delay in the administration of adjuvant chemotherapy has been reported to reduce survival.[34] Recently, total neoadjuvant therapy with intensified chemotherapy improved DFS by decreasing metastasis in two randomized trials.[6,7] The post-NAT analysis provides an opportunity to predict the prognosis before surgery, and thus adjuvant chemotherapy, even ideally intensified neoadjuvant chemotherapy, may be precisely administered to those at high risk of recurrence in a timely manner.

This study had several limitations. First, the sample size was modest, and a limited number of patients were included in each subgroup, such as longitudinal plasma samples or patients who accepted LCRT/SCPRT. Second, intervention studies are required to explore the potential clinical utility of ctDNA to guide therapeutic decision-making and to determine whether the administration of neoadjuvant chemotherapy under ctDNA guidance may exert a positive effect on survival.

In conclusion, this study of patients with LARC indicated that the ctDNA fraction at the post-NAT time point was a highly sensitive indicator defining a patient population at high risk of recurrence that was superior to clinicopathological measures currently used to guide adjuvant chemotherapy decisions. Notably, the CNAs analysis improved the performance of MRD detection and the risk classification ability of both the personalized assay and the universal panel. They may have the potential to guide neoadjuvant therapy intensity.

Contributors

YXZ, JJ, YCJ designed the study. WYL, YFL, QQS, YT, NL, SLC, JMS, SLW, and YXL performed data acquisition. WYL, YFL, QQS, and JJW analysed the data. WYL, YFL, and YCJ verified the data. WYL, YFL, and JJW wrote the manuscript. YCJ, YXZ, and JJ revised the manuscript. All authors read and approved the final manuscript.

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Data sharing

All relevant data are within the paper and its supplementary files. The raw data used and/or analysed during the study are available in the Genome Sequence Archive for Human repository [HRA001933 in https://bigd.big.ac.cn/gsa-human/].

Declaration of interests

YCJ is one of the cofounders, has owner interest in Genetron Holdings, and receives royalties from Genetron. The other authors have declared that no competing interest exists.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103945.

References

1 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin*. 2020;70(1):7–30.
2 Siegel RL, Miller KD, Fedewa SA, et al. Colorectal cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(3):177–193.
3 van Gijn W, Marijnen CAM, Nagtegaal ID, et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol*. 2011;12(6):575–582.
4 Glynne-Jones R, Wyrwicz L, Tiret E, et al. Rectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2017;28:iv22–iv40.
5 Fokas E, Glynne-Jones R, Appelt A, et al. Outcome measures in multimodal rectal cancer trials. *Lancet Oncol*. 2020;21(5):e252–ee64.
6 Bahadoer RR, Dijkstra EA, van Etten B, et al. Short-course radiotherapy followed by chemotherapy before total mesorectal excision (TME) versus preoperative chemoradiotherapy, TME, and optional adjuvant chemotherapy in locally advanced rectal cancer

(RAPIDO): a randomised, open-label, phase 3 trial. *Lancet Oncol*. 2021;22(1):29–42.
7 Conroy T, Bosset JF, Etienne PL, et al. Neoadjuvant chemotherapy with FOLFIRINOX and preoperative chemoradiotherapy for patients with locally advanced rectal cancer (UNICANCER-PRODIGE 23): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2021;22(5):702–715.
8 Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008;14(9):985–990.
9 Azad TD, Chaudhuri AA, Fang P, et al. Circulating tumor DNA analysis for detection of minimal residual disease after chemoradiotherapy for localized esophageal cancer. *Gastroenterology*. 2020;158(3):494–505. e6.
10 Bradon R, McDonald TCC, Sammut SJ, Ahuva Odenheimer-Bergman BE, Nieves Perdigones. Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer. *Sci Transl Med*. 2019;11(504):eaax7392.
11 Christensen E. Early detection of metastatic relapse and monitoring of therapeutic efficacy by ultra-deep sequencing of plasma Cell-Free DNA in Patients With Urothelial Bladder Carcinoma. *J Clin Oncol*. 2019;37(18):1547–1557.
12 Cristiano S, Leal A, Phallen J, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature*. 2019;570:385–389.
13 Zviran A, Schulman RC, Shah M, et al. Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nat Med*. 2020;26(7):1114–1124.
14 Dasari A, Morris VK, Allegra CJ, et al. ctDNA applications and integration in colorectal cancer: an NCI Colon and Rectal-Anal Task Forces whitepaper. *Nat Rev Clin Oncol*. 2020;17(12):757–770.
15 Tie J, Cohen JD, Wang Y, et al. Circulating tumor DNA analyses as markers of recurrence risk and benefit of adjuvant therapy for stage III colon cancer. *JAMA Oncol*. 2019;5(12):1710–1717.
16 Tie J, Wang Y, Tomasetti C, 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(346):346ra92.
17 Wang Y, Li L, Cohen JD, et al. Prognostic potential of circulating tumor DNA measurement in postoperative surveillance of nonmetastatic colorectal cancer. *JAMA Oncol*. 2019;5(8):1118–1123.
18 Qu C, Wang Y, Wang P, et al. Detection of early-stage hepatocellular carcinoma in asymptomatic HBsAg-seropositive individuals by liquid biopsy. *Proc Natl Acad Sci U S A*. 2019;116(13):6308–6312.
19 Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2010;26(5):589–595.
20 Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078–2079.
21 Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci U S A*. 2011;108(23):9530–9535.
22 Zhao D, Yue P, Wang T, et al. Personalized analysis of minimal residual cancer cells in peritoneal lavage fluid predicts peritoneal dissemination of gastric cancer. *J Hematol Oncol*. 2021;14(1):164.
23 Taylor AM, Shih J, Ha G, et al. Genomic and functional approaches to understanding cancer aneuploidy. *Cancer Cell*. 2018;33(4):676–689. e3.
24 Douville C, Moinova HR, Thota PN, et al. Massively parallel sequencing of esophageal brushings enables an aneuploidy-based classification of patients with Barrett's esophagus. *Gastroenterology*. 2021;160(6):2043–2054. e2.
25 Douville C, Springer S, Kinde I, et al. Detection of aneuploidy in patients with cancer through amplification of long interspersed nucleotide elements (LINEs). *Proc Natl Acad Sci U S A*. 2018;115(8):1871–1876.
26 Bailey MH, Tokheim C, Porta-Pardo E, et al. Comprehensive Characterization of Cancer Driver Genes and Mutations. *Cell*. 2018;174(4):1034–1035.
27 Oh CR, Kong SY, Im HS, et al. Genome-wide copy number alteration and VEGFA amplification of circulating cell-free DNA as a biomarker in advanced hepatocellular carcinoma patients treated with Sorafenib. *BMC Cancer*. 2019;19(1):292.
28 D AS, Richter JR. Nomograms for calculating the number of patients needed for a clinical trial with survival as an endpoint. *Biometrics*. 1982;38(1):163–170.
29 Parikh AR, Van Seventer EE, Siravegna G, et al. Minimal residual disease detection using a plasma-only circulating tumor DNA assay in colorectal cancer patients. *Clin Cancer Res*. 2021;27:2890–2898.
30 Zhou J, Wang C, Lin G, et al. Serial circulating tumor DNA in predicting and monitoring the effect of neoadjuvant chemoradiotherapy in patients with rectal cancer: a prospective multicenter study. *Clin Cancer Res*. 2020;27(1):301–310.
31 Henriksen TV, Tarazona N, Frydendahl A, et al. Circulating tumor DNA in Stage III colorectal cancer, beyond minimal residual disease detection, toward assessment of adjuvant therapy efficacy and clinical behavior of recurrences. *Clin Cancer Res*. 2022;28(3):507–517.
32 RCS AZ, Shah M, Hill STK, et al. Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nat Med*. 2020;26(7):1114–1124.
33 Tie J, Cohen JD, Wang Y, et al. Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: a prospective biomarker study. *Gut*. 2019;68(4):663–671.
34 Sun Z, Adam MA, Kim J, et al. Determining the optimal timing for initiation of adjuvant chemotherapy after resection for stage II and III colon cancer. *Dis Colon Rectum*. 2016;59(2):87–93.