The circulating tumor DNA (ctDNA) alteration level predicts therapeutic response in metastatic breast cancer: Novel prognostic indexes based on ctDNA

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A B S T R A C T

Purpose: Circulating tumor DNA (ctDNA) has good clinical guiding value for metastatic breast cancer (MBC) patients. This study aimed to apply a novel genetic analysis approach for therapeutic prediction based on ctDNA alterations.
Method: This nonrandomized, multicenter study recruited 223 MBC patients (NCT05079074). Plasma samples were collected for target-capture deep sequencing of ctDNA at baseline, after the 2nd cycle of treatment, and when progressive disease (PD) was evaluated. Samples were categorized into four levels according to the number of ctDNA alterations: level 1 (no alterations), level 2 (1–2 alterations), level 3 (3–4 alterations) and level 4 ($\geq$5 alterations). According to ctDNA alteration level and variant allele frequency (VAF), a novel ctDNA-level Response Evaluation Criterion in Solid Tumors (ctle-RECIST) was established to assess treatment response and predict progression-free survival (PFS).
Results: The median PFS in level 1 (6.63 months) patients was significantly longer than that in level 2–4 patients (level 2: 5.70 months; level 3–4: 4.90 months, $p < 0.05$). After 2 cycles of treatment, based on ctle-RECIST, the median PFS of level-based disease control rate (lev-DCR) patients was significantly longer than that of level-based PD (lev-PD) patients [HR 2.42 (1.52–3.85), $p < 0.001$]. In addition, we found that ctDNA level assessment could be a good supplement to radiologic assessment. The median PFS in the dual-DCR group tended to be longer than that in the single-DCR group [HR 1.41 (0.93–2.13), $p = 0.107$].
Conclusion: The ctDNA alteration level and ctle-RECIST could be novel biomarkers of prognosis and could complement radiologic assessment in MBC.

1. Introduction

Although much progress has been made in its treatment, breast cancer remains the most prevalent cancer and a major health burden among females worldwide [1]. Patients with advanced breast cancer, especially those receiving heavily late-line treatments, not only face limited treatment methods but also encounter problems evaluating treatment effects. To assess the treatment response and predict prognosis, appropriate monitoring biomarkers are crucially needed.

Currently, the response evaluation of anticancer therapy is mostly based on radiological assessments according to Response Evaluation Criteria in Solid Tumors (RECIST) [2]. However, repeated radiological assessments are time-consuming, costly, and increase the radiation burden on patients. Compared with radiological assessments, circulating tumor DNA (ctDNA) has unique advantages. ctDNA overcomes tumor heterogeneity, as well as provides molecular information about driver genes, drug resistance genes, and clone structures. It can be used to evaluate the response of lesions that are difficult to assess by imaging (such as bone metastases) [3,4]. Therefore, serial ctDNA is increasingly popular to monitor treatment response and assess tumor burden in many cancers [5–7].

In breast tumors, ctDNA has been indicated to be an effective surveillance tool for monitoring response to treatment and clinical prognosis [4,8]. Especially in advanced or metastatic tumors, ctDNA has

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higher clinical value and development prospects because of its relatively high detection rate [7,9]. However, in clinical practice, the usage of ctDNA remains to be studied. For example, how to make better use of ctDNA to predict the prognosis of patients, and how to confirm the additional clinical utility beyond routine radiological assessment [10].

Herein, we hypothesized that the numbers and variant allele frequency (VAF) of alterations in ctDNA might be able to provide useful information to predict treatment response and monitor disease progression. Four levels (levels 1–4) based on the number of ctDNA alterations were established to explore the differences in the treatment efficacy of breast cancer patients who failed multiple lines (≥2 lines) of chemotherapy/endocrine/targeted therapy. These levels can further be used to assess whether the novel ctDNA-level Response Evaluation Criterion in Solid Tumors (ctle-RECIST) can predict the treatment efficacy independently or in combination with imaging assessment.

2. Methods

2.1. Participants

The study is nonrandomized, observational, and involves multicenter. This study enrolled metastatic TNBC patients who progressed after at least one line of chemotherapy and also enrolled HR-positive or HER2-positive MBC patients who progressed after at least two lines of chemotherapy, antihormone therapy, or anti-HER2 therapy. Therefore, late-line therapy defines the second- or above-line (≥2 lines) in mTNBC patients, and the third- or above-line (≥3 lines) in HR-positive or HER2-positive MBC patients. Totally, 223 patients whom were treated at Hunan Cancer Hospital, the Forth Hospital of Changsha and Zhuzhou Central Hospital in China from January 2016 through June 2019. The patient consent form was approved by the Human Research Ethics Committee at Hunan Cancer Hospital, Central South University (NO2017YS031), and the protocol has been registered on ClinicalTrials. gov with the number NCT05079074. Each participant provided written informed consent to participate in the study.

The inclusion criteria for patients in our study included the following: 1) patients aged >18; 2) recent progression of triple-negative breast cancer after ≥2 lines of chemotherapy or hormone receptor (HR)-positive or human epidermal growth factor receptor 2 (HER2)-positive MBC after multiple lines of endocrine or targeted therapy; 3) no available recommendation for the next treatment regimen; 4) Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; and 5) updated, available pathological HR/HER2 status for metastasis. The exclusion criteria included the following: 1) other malignant neoplasms; 2) immune deficiency or organ transplantation history; 3) heart disease or heart abnormalities, such as cardiac infarction or severe cardiac arrhythmia; and 4) failure of baseline ctDNA detection.

2.2. Study design

This multicenter observational study on hospitalized patients was nonrandomized and followed the data design flow shown in Supplementary Fig. 1. Serial blood samples were collected at three defined time points: at baseline (3–7 days before treatment initiation), after 2 cycles of treatment, and when progressive disease (PD) was confirmed by imaging. All patients underwent at least baseline ctDNA testing. Imaging (magnetic resonance imaging/computed tomography) was performed 3–7 days before treatment initiation and after every two treatment cycles until PD was clinically detected. All patients were selected for chemotherapy and/or targeted therapy based on their condition and ctDNA test results. The detailed study protocol is provided in the Supplemental Protocol file.

2.3. ctDNA analysis

The experimental protocol for biospecimen and DNA extraction has

been described in detail in our previous study [11]. For each peripheral blood sample collection, at least 10 mL of blood was drawn into Streck tubes (Streck, Omaha, NE, USA) and centrifuged twice at $1500 \times g$ to separate the plasma from peripheral blood cells. Cell-free DNA (cfDNA) was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), and germline DNA (gDNA) was extracted from peripheral blood cells using the QIAamp DNA Blood Mini Kit (Qiagen); both extractions were performed according to the manufacturer's instructions. Then, cfDNA and gDNA libraries were constructed with the KAPA DNA Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's protocol. The criterion of the sequencing depth was 4000. Our mean sequencing depth was 6000. The amount of cfDNA was 15 ng for library preparation.

Capture probes were designed to cover the coding sequences and exon hotspots of 1021 genes that are frequently mutated in solid tumors [11]. A detailed description of capture experiments has been previously published [12]. Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Integrated DNA Technologies, Coralville, IA, USA), and DNA sequencing was performed using the HiSeq 3000 Sequencing System (Illumina, San Diego, CA) with 2×101-bp paired-end reads. Clonal hematopoietic mutations were filtered as previously described [13]. gDNA from each patient was sequenced as the germline control. Terminal adaptor sequences and low-quality reads were removed from the raw data. The Burrows-Wheeler Aligner tool [14] (version 0.7.12-r1039, http://bio-bwa.sourceforge.net/index.sht ml) was used to align clean reads to the reference human genome (hg19), and Picard (version 1.98) was used to mark PCR duplicates. Realignment and recalibration were performed using GATK (version 3.4–46-gbc02625), and single nucleotide variants (SNVs) were called using MuTect (version 1.1.4) and NChot, software developed in-house to review hotspot variants [12]. Small insertions and deletions (indels) were called using GATK. Somatic copy number alterations were identified with CONTRA (v2.0.8), and significant copy number variations (CNVs) were identified based on the ratio of the standardized depth of coverage between ctDNA and control gDNA. The final candidate variants were all manually verified in the Integrative Genomics Viewer. This sequencing method was previously proven to be credible with simulated cfDNA [12], so we did not validate the mutations found in ctDNA by sequencing tumor biopsies.

Since our previous study suggested that both the tumor mutation load and the clone load of ctDNA alterations would change during treatment [11], both the emerging ctDNA alterations and the change in VAF of the existing ctDNA alterations were under surveillance in this study. Previous study has shown that the sum of the VAFs (%ctDNAsum) was correlated with SUVmax in PET sacn [15]. For every sample, we obtained ctDNA data on two indexes: 1) the number of ctDNA alterations (ctDNA alterations with VAF ≥1% were considered effective alterations and counted in the number) and 2) the sum of the VAF of all ctDNA alterations (VAFsum).

2.4. Tumor mutation burden and ctDNA level

By calculated from big gene panels, tumor mutation burden (TMB) is an important prognostic factor [16–19]. TMB was determined by analyzing the somatic mutations per mega-base (Mb). TMB analysis interrogated SNVs and small indels with the variant allele frequency (VAF) ≥3%. TMB-U (unknown) is defined as the maximum VAF <3%. A cut-off of the top 25% of the TMB of all BC samples from Geneplus database was 9 mutations (Muts)/Mb. In this study, TMB greater than 9 Muts/Mb was defined as TMB-H (high). TMB less than 9 Muts/Mb was defined as TMB-L (low).

In this study, we had time-series information for two-dimensional indexes, the aberrant 0genes and their VAF. Since TMB-high only included the top 25% of the TMB, and two categories TMB-High and TMB-Low were not sufficient enough to evaluate the time-series change of tumor mutation load, so we instead categorized samples into four

levels according to the number of ctDNA aberrations: level 1 (no aberrations), level 2 (1–2 aberrations), level 3 (3–4 aberrations) and level 4 (≥5 aberrations). In hierarchical cluster analysis (hclust, Supplementary Fig. 2), level 1 patients were clustered in the red region; level 2 patients were clustered in the light green region; level 3 patients were clustered in the light blue region and level 4 patients were clustered in the dark blue region. At baseline, 35 (16%), 57 (26%), 57 (26%) and 74 (33%) patients were in level 1, level 2, level3, and level 4, respectively. This four-level category is more evenly distributed than the dichotomy TMB category.

2.5. Therapeutic response evaluation

The therapeutic response evaluation of imaging was based on RECIST 1.1 [2]. The response evaluation was categorized as complete response (CR), partial response (PR), stable disease (SD), or PD. The disease control rate (DCR) refers to the sum of CR, PR and SD. For ctDNA, referring to the RECIST 1.1 criteria, we developed new evaluation criteria based on ctDNA levels and VAF called the ctle-RECIST. Therapeutic responses were classified as level-based PR (lev-PR), level-based SD (lev-SD) and level-based PD (lev-PD). Level-based DCR (lev-DCR) refers to the sum of lev-CR, lev-PR and lev-SD.

The early therapeutic response was determined by the change of ctDNA level from the 1st ctDNA test (at baseline) to the 2nd ctDNA test after 2 cycles of treatment, as follows: 1) lev-PR defined a decrease in the ctDNA level (*eg*, level 4 at baseline to level 1–3 at the 2nd test); 2) lev-PD defined an increase in the ctDNA level (*eg*, level 1–3 at baseline to level 4 at the 2nd test); and 3) if the ctDNA levels were consistent, the sum of the VAFs (VAFsum) of all alterations was compared: a decrease of VAFsum ≥50% was defined as Lev PR; an increase of VAFsum ≥100% was defined as lev-PD; otherwise, it was defined as lev-SD.

The efficacy evaluation of the 3rd ctDNA analysis was compared with the one with the lowest level during treatment (1st ctDNA or 2nd ctDNA analysis). The comparison method was the same as above.

2.6. Statistical analysis

To compare categorical variables, such as associations between patient characteristics and ctDNA alteration levels, Pearson's x^2 test was performed. Progression-free survival (PFS) was calculated from the date of treatment initiation to the date of disease progression or death from any cause. Kaplan-Meier survival plots were generated, and curves were compared using the log-rank test to assess differences in PFS among subgroups. Cox regression analysis was used to identify the significant independent risk factors for PFS. Variables with a p < 0.10 in the univariate analysis were included in the multivariate analysis. These results are presented as hazard ratios (HRs) with 95% confidence intervals (CIs). McNemar and kappa tests were adopted to analyze the agreement between imaging and ctDNA evaluations in terms of treatment response.

Pearson's x^2 test, the log-rank test, and Cox regression analysis were conducted using SPSS software (version 23, SPSS Inc., IBM, NY, USA). Heatmaps of baseline ctDNA alteration profiles stratified by level were plotted by the 'ComplexHeatmap' R package using R software (version 3.6.0, Institute for Statistics and Mathematics, Vienna, Austria; https://www.r-project.org). The Kaplan-Meier plots and the number at risk were determined using MedCalc (version 19.5.3, MedCalc Software Bvba, Ostend, Flanders, Belgium). All statistical tests were two-sided, and p values < 0.05 were considered statistically significant.

3. Result

3.1. Characteristics of the patients and sample information

Of 255 MBC patients who planned to receive late-line treatment, 223 MBC patients were included in this study (Supplementary Fig. 1). The median age of these patients was 44 years, ranging from 25 to 72 years.

The vast majority of patients had ductal carcinoma, accounting for 85.8% (200/233). A total of 55.4% (129/233) of patients were HR positive, and 26.2% (61/233) were HER2 positive. Regarding metastasis, patients with visceral metastasis accounted for 56.7% (132/233), and the most common metastatic sites were the bone, lung and liver, accounting for 46.8% (109/233), 36.9% (86/233) and 30.9% (72/233), respectively. The baseline clinical characteristics of the patients are listed in Table 1.

All 233 patients had baseline ctDNA detection results, of which 190 (85%) were found to have at least one baseline ctDNA alteration (VAF ≥1%), which is presented in heatmaps in Fig. 1 (raw data, Supplementary Table 1). A total of 136 patients underwent 2nd ctDNA detection after completing two treatment cycles, and 58 patients underwent 3rd ctDNA detection when PD was confirmed by imaging or when they presented with clinical symptoms indicating suspected PD (Fig. 2).

3.2. ctDNA alterations and alteration levels at baseline

A total of 452 ctDNA samples were collected from 233 patients. The most common alterations were in *TP53* (164/452, 36.3%), *PIK3CA* (118/452, 26.1%), *ERBB2* (54/452, 11.9%), *BRCA1/2* (43/452, 9.5%), and *ESR1* (26/452, 5.8%). ctDNA alterations differed in different alteration level groups. Following *TP53*, the most commonly affected genes were *BRCA1/2* and *PALB2* in level 2 patients (Fig. 1A), *PIK3CA* in level 3 patients (Fig. 1B), and *PIK3CA* and *ERBB2* in level 4 patients (Fig. 1C).

At baseline, the level 1, 2, 3, and 4 groups contained 33 (14.2%), 58 (24.9%), 57 (24.5%) and 75 (32.2%) patients, respectively. Alteration levels were correlated with the site of metastasis, and higher levels (levels 3–4) were associated with a higher probability of liver metastasis (p = 0.001). Bone metastases were more common in both low- and high-level patients (level 1 and levels 3–4, p = 0.014). CtDNA alteration levels at baseline had no relationship with patient age, breast cancer laterality, histology, HR and HER2 phenotypes, or molecular subtypes.

3.3. ctDNA alteration levels and treatment efficacy

Thirty-one of 33 patients (93.9%) in level 1 achieved disease control based on radiographic assessment after 2 cycles of treatment, which was significantly higher than patients in level 2 (47/58, 81.0%) and patients in levels 3–4 (95/132, 72.0%), p = 0.020 (Fig. 3A). Kaplan-Meier curves were generated to compare the PFS in different level groups. Baseline ctDNA analyses indicated that the median PFS was 6.63 (5.47–7.79) months in level 1, which was significantly longer than 5.70 (4.54–6.86) months in level 2 (hazard ratio 1.68, 95% CI 1.07–2.62, p = 0.024; log-rank test, p = 0.025) and 4.90 (4.31–5.50) months in levels 3–4 (hazard ratio 1.68, 95% CI 1.13–2.49, p = 0.011; log-rank test, p = 0.011) (Fig. 3B). The results of the univariate and multivariate Cox regression analyses at baseline are summarized in Supplementary Table 2.

Of 136 patients who finished 2nd ctDNA testing after 2 cycles of treatment, there were 28, 33, 22, and 23 patients in the level 1, 2, 3, and 4 groups, accounting for 42.7%, 24.3%, 16.2% and 16.9%, respectively. The number and proportion of level 1 ctDNA in the 2nd ctDNA analysis were significantly higher than those at baseline. For 58 patients who finished 3rd ctDNA testing when imaging evaluation indicated PD, the number of patients in the level 1, 2, 3, and 4 groups was 9, 9, 12 and 28, accounting for 15.5%, 15.5%, 20.7% and 48.3%, respectively. The number and proportion of the level 4 group increased significantly (Table 2).

3.4. Consistency between ctDNA alterations and imaging

We next examined the correlation between ctle-RECIST and RECIST 1.1. The percentage of patients who benefited from treatment based on ctle-RECIST (lev-DCR) and RECIST 1.1 (DCR) after the completion of 2 cycles of treatment was similar (83.8% *vs.* 76.4%), and the overall

Table 1
Patient baseline characteristics.

Characteristics	Total (n = 233)		Alteration level								χ2	P value
			1 (n = 33)		2 (n = 58)		3 (n = 57)		4 (n = 75)			
	n	%	n	%	n	%	n	%	n	%		
Age at diagnosis (years)											3.21	0.360
<45	135	57.94	24	72.73	36	62.07	34	59.65	41	54.67		
≥45	88	37.77	9	27.27	22	37.93	23	40.35	24	32.00		
Laterality											8.946	0.177
Left	118	50.64	22	66.67	29	50.00	24	42.11	43	57.33		
Right	97	41.63	10	30.30	25	43.10	32	56.14	30	40.00		
Bilateral	8	3.43	1	3.03	4	6.90	1	1.75	2	2.67		
Histology											8.831	0.453
Invasive ductal carcinoma	200	85.84	28	84.85	3	5.17	0	0.00	2	2.67		
Invasive lobular carcinoma	10	4.29	3	9.09	1	1.72	1	1.75	5	6.67		
Mixed invasive ductal and invasive lobular	2	0.86	0	0.00	4	6.90	1	1.75	2	2.67		
Others	11	4.72	2	6.06	2	3.45	0	0.00	2	2.67		
HR status											3.363	0.339
Positive	129	55.36	21	63.64	29	50.00	31	54.39	48	64.00		
Negative	94	40.34	12	36.36	29	50.00	26	45.61	27	36.00		
HER2 status											3.996	0.262
Positive	61	26.18	13	39.39	14	24.14	12	21.05	22	29.33		
Negative	162	69.53	20	60.61	44	75.86	45	78.95	53	70.67		
Molecular subtype											13.865	0.127
HR+/HER2-	102	43.78	15	45.45	22	37.93	25	43.86	40	53.33		
HR+/HER2+	27	11.59	6	18.18	7	12.07	6	10.53	8	10.67		
HR-/HER2+	34	14.59	7	21.21	7	12.07	6	10.53	14	18.67		
HR-/HER2-	60	25.75	5	15.15	22	37.93	20	35.09	13	17.33		
Visceral metastasis											6.697	0.082
Yes	132	56.65	15	45.45	30	51.72	36	63.16	51	68.00		
No	91	39.06	18	54.55	20	34.48	21	36.84	24	32.00		
Metastatic site												
Bone	109	46.78	14	42.42	17	29.31	30	52.63	48	64.00	16.623	0.001
Liver	72	30.90	9	27.27	10	17.24	21	36.84	32	42.67	10.622	0.014
Lung	86	36.91	8	24.24	22	37.93	26	45.61	30	40.00	4.128	0.248
Brain	33	14.16	3	9.09	8	13.79	11	19.30	11	14.67	1.816	0.612

Abbreviations: HR, hormone receptor; HER2, human epidermal growth factor receptor 2.

concordance between response evaluations was good (kappa = 0.313, p < 0.001; McNemar's test p = 0.10 > 0.05).

For 58 patients with PD determined by imaging, 77.6% of patients with ctDNA alteration level evaluation had lev-PD, which also reflects the good consistency between the two evaluation methods.

3.5. ctle-RECIST by ctDNA could be used as an early marker of therapeutic response

Based on ctle-RECIST, there were 72 patients in the lev-PR group, 42 patients in the lev-SD group, and 22 patients in the lev-PD group after the completion of 2 cycles of treatment. Patients in the lev-DCR group had a significantly longer PFS than those in the lev-PD group [median PFS of 6.23 (5.47–6.99) months versus 2.80 (1.08–4.52) months, hazard ratio 2.42, 95% CI 1.52–3.85, p < 0.001; log-rank test, p < 0.001; Fig. 4].

In addition, we found that ctDNA could be used as a good supplement for imaging efficacy evaluation. After the completion of 2 cycles of treatment, according to the two different therapeutic response evaluation criteria of ctDNA and imaging, we divided 136 patients into three types: 94 patients with dual-DCR, 30 patients with single-DCR, and 12 patients with dual-PD. The median PFS in the dual-DCR group was longer than that in the single-DCR group [median PFS was 6.63 (6.03–7.24) months versus 3.93 (2.72–5.14) months, hazard ratio 1.41, 95% CI 0.93–2.13, p = 0.107; log-rank test, p = 0.103; Fig. 5].

4. Discussion

Our study shows that the ctDNA detection success rate in MBC is very high, and the ctDNA alteration level at baseline could predict the DCR and PFS of antitumor treatment well. After 2 cycles of treatment, according to the ctle-RECIST criteria we created, patients with lev-DCR had a longer PFS than those with lev-PD. Moreover, we found that

serial ctDNA measurements could provide greater predictive value on the basis of routine imaging-based assessments because the median PFS in the dual-DCR group tended to be longer than that in the single-DCR group.

The current gold standard for assessing cancer lesions and treatment response is the image-based RECIST 1.1. However, in addition to being time-consuming and expensive, imaging still has some unsolvable disadvantages, such as failing to monitor unmeasured lesions including bone metastases, increasing patients' radiation exposure dose, delaying the discovery time of disease progression, and failing to distinguish the pseudoprogression of immunotherapy [3,10]. Therefore, new biomarkers that can overcome the above shortcomings are needed to assist or even replace radiologic assessment in the future.

CtDNA detection has developed rapidly in recent years. CtDNA can not only overcome intratumoral and intermetastatic heterogeneity and covers DNA fragments from different subclones of tumors but can also dynamically and continuously reflect the progression of disease [9,20]. CtDNA often carries tumor-related genetic abnormalities, such as point mutations, gene amplifications, gene rearrangements, microsatellite instability, allelic heterozygosity deletions, and hypermethylation. ctDNA testing can directly and comprehensively reflect the genetic characteristics of tumors in real time. In particular, serial ctDNA detection and ctDNA dynamics provide an important basis for gene or structural variant analysis, drug use guidance, drug efficacy prediction, drug resistance cause analysis, and recurrence monitoring in both early and advanced breast cancer patients [20–23].

The evaluation of tumor burden and therapeutic efficacy has been the research focus of the application of liquid biopsy. Blood biomarkers such as CEA or CA-153 are commonly used to assess breast cancer; however, there are problems such as not being timely and lacking sensitivity and specificity [11]. Circulating tumor cells (CTCs) were also widely used as circulating biomarkers that are interrelated biomarkers [20,24]. CTCs are released from the primary tumor or metastatic lesions;
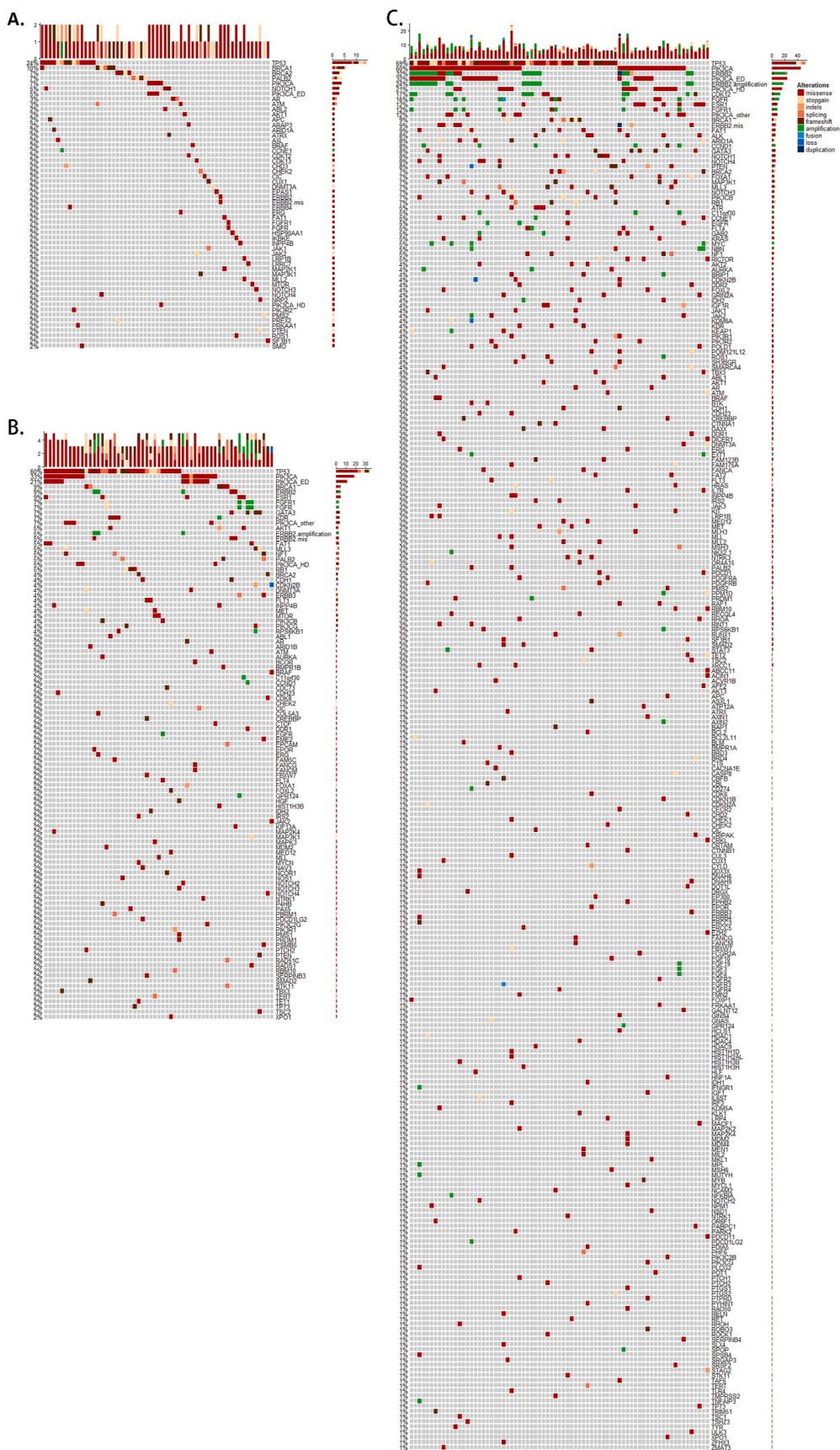
Fig. 1. Heatmaps of baseline ctDNA alteration profiles stratified by level (number of ctDNA alterations). (A) Level 2, n = 58 patients. (B) Level 3, n = 57. (C) Level 4, n = 75.
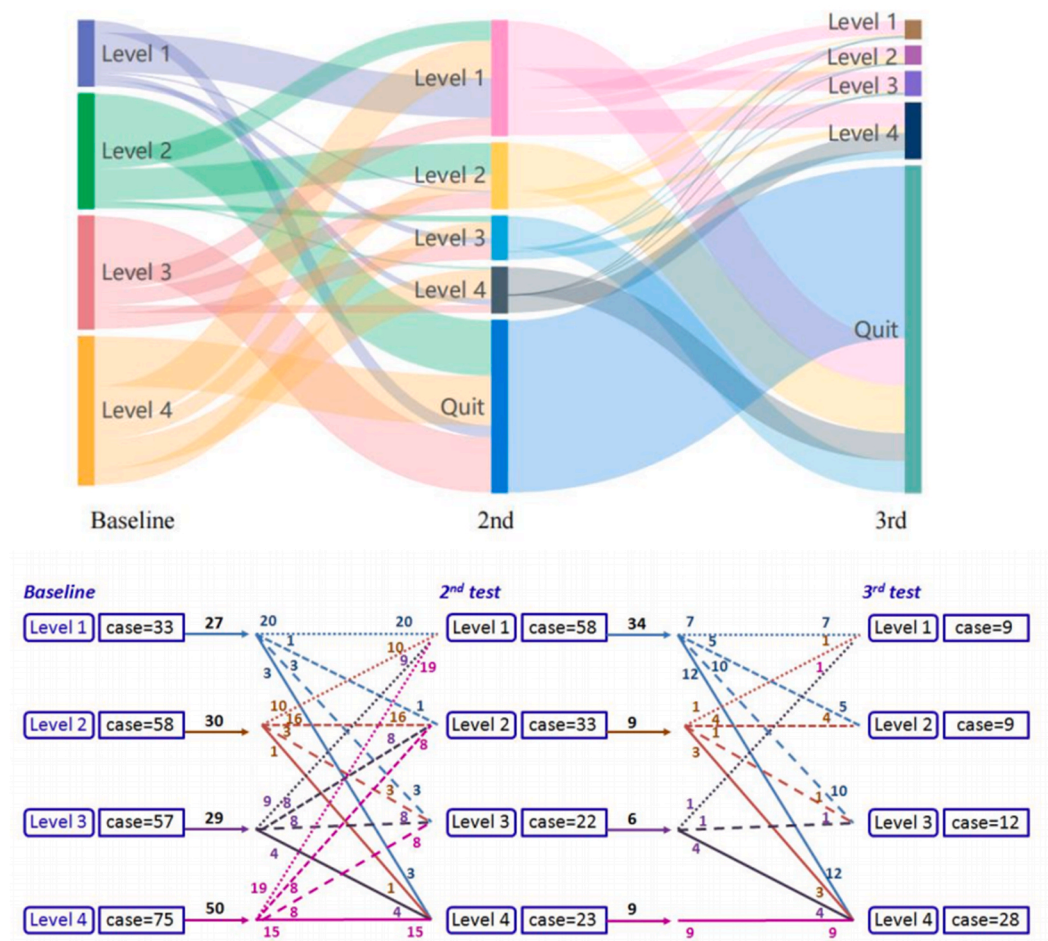
ctDNA alteration level change

Fig. 2. Sankey diagram and the change in alteration levels in patients. Serial ctDNA surveillance to detect variations in ctDNA-based levels and monitor the treatment response. At baseline and at the 2nd and 3rd tests, patients were divided into four levels according to the number of ctDNA alterations. The numbers on the arrow indicate the number of patients who received the next ctDNA test.
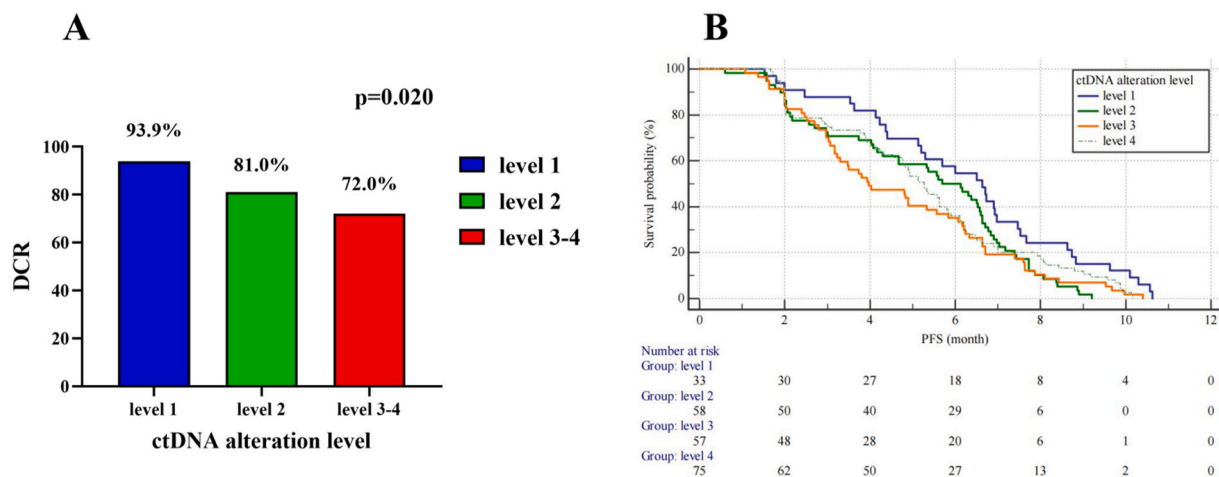

Fig. 3. Kaplan-Meier plot of progression-free survival according to baseline alteration levels. Patients were divided into four levels according to the number of ctDNA alterations: level 1 (no alterations), level 2 (1–2 alterations), level 3 (3–4 alterations) and level 4 (≥5 alterations). (A) DCR was significantly higher in patients with level 1 disease than in patients with level 2 and levels 3–4 disease, p = 0.020. (B) The median PFS of patients in level 1 was significantly longer than that of patients in level 2 (hazard ratio 1.67, 95% CI 1.07–2.62, p = 0.024), level 3 (hazard ratio 1.85, 95% CI 1.19–2.87, p = 0.007) and level 4 (hazard ratio 1.57, 95% CI 1.02–2.34, p = 0.039).

Table 2
Change in ctDNA alteration levels.

Alteration levels	Baseline ctDNA (n = 233)		2nd ctDNA (n = 136)		3rd ctDNA (n = 58)	
	n	%	n	%	n	%
Level 1	33	14.16	58	42.65	9	15.52
Level 2	58	24.89	33	24.26	9	15.52
Level 3	57	24.46	22	16.18	12	20.69
Level 4	75	32.19	23	16.91	28	48.28
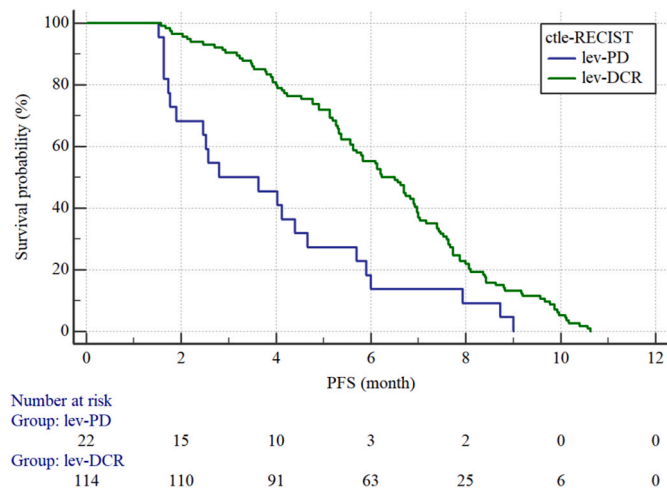
Fig. 4. Kaplan-Meier plot of progression-free survival divided by treatment response. Patients were divided into the lev-DCR and lev-PD groups according to ctle-RECIST. Patients in the lev-DCR group had a significantly longer PFS than those in the lev-PD group (hazard ratio 2.42, 95% CI 1.52–3.85, p < 0.001). DCR, disease control rate. lev-DCR, level-based DCR.
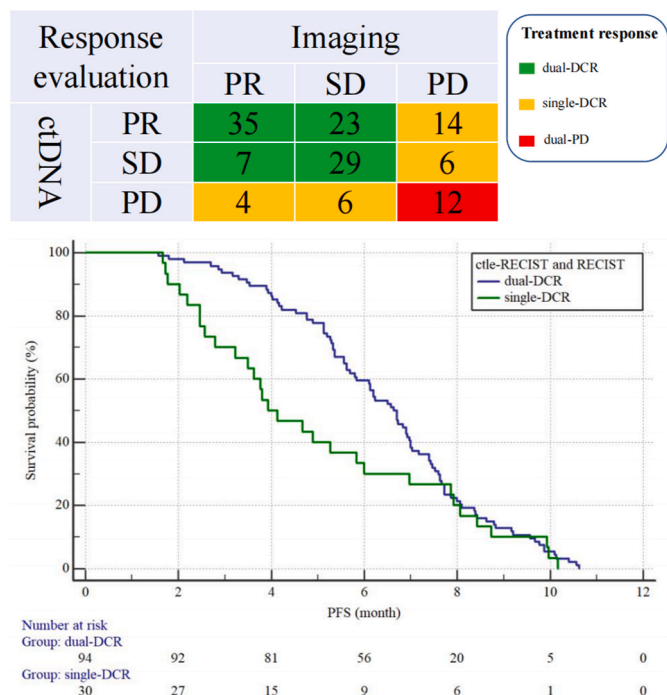

Fig. 5. Comparison and joint analysis of two response evaluation criteria. The median PFS in the dual-DCR group was longer than that in the single-DCR group [median PFS of 6.63 (6.03–7.24) months versus 3.93 (2.72–5.14) months, hazard ratio 1.408, 95% CI 0.93–2.13, p = 0.107; log-rank test, p = 0.103]. DCR, disease control rate.

they can provide information such as cell morphology and immunocytochemical phenotype, and the count of CTCs is related to the recurrence and prognosis of early breast cancer [25]. At present, CTC research is most appealing for genomic mutational analysis, molecular typing and transcriptome sequencing, which will aid in patient treatment stratification and provide more information for therapeutic choices [20,25].

Currently, no liquid biopsy is approved for response evaluation during treatment, but the results found in this article indicate that this is a promising field [3]. Some previous articles focused on the quantitative level change of ctDNA and the mutation of a single target gene to predict the curative effect, and some newer articles tried to use new analysis algorithms to assess tumor burden or predict treatment efficacy. Darrigues et al. used the quantitative detection of ctDNA levels ratio (Day15/baseline and Day30/baseline) as a biomarker to predict the efficiency of palbociclib and fulvestrant [22]. Yi et al. used PyClone software, which is based on a Bayesian clustering algorithm, to divide ctDNA mutations into trunk mutations and branch mutations, which had significant differences in their effects on drug resistance and PFS and thus had certain curative effect prediction value [26]. Another interesting indicator was the molecular tumor burden index (mTBI), which was established after comprehensive analysis of ctDNA mutations, heterogeneity and dynamic evaluation and has been indicated to be a therapeutic response and prognostic biomarker in MBC patients [10,26]. Tumor mutational burden (TMB) is actively used in clinical decision-making regarding immunotherapy. In the traditional sense, TMB is tumor tissue–based. Although there is controversy, some articles believe that TMB estimated by circulating tumor DNA in blood (bTMB) may serve as a potential biomarker of the response to immunotherapy [27,28].

Our study confirmed for the first time that the baseline ctDNA alteration level is a good predictor of PFS. We established the new prediction method and response evaluation criteria to focus on the whole tumor and abandon the previous practice of focusing on the prediction of PFS by single gene or hotspot mutation analysis (such as *TP53* mutation), which is worthy of further exploration [20,29].

Several articles found that early on-treatment ctDNA dynamics are a surrogate for PFS [4,7,22,26]. However, in previous studies, the quantitative level of ctDNA used to predict the treatment response has certain limitations because sometimes it cannot accurately reflect the tumor burden for tumors with changed clonal structure [10]. Therefore, we proposed ctle-RECIST, which is based on changes in ctDNA alteration levels and the sum of the VAFs of all alterations during treatment compared to baseline, for response evaluation for the first time.

Our study has several limitations. First, not all patients undergo serial ctDNA testing. This not only resulted in a decrease in the sample size of the 2nd and 3rd ctDNA tests but also may cause potential bias. Second, our study lacked a validation cohort to validate the clinical practical value of ctDNA alteration levels and ctle-RECIST for PFS and treatment response prediction.

In summary, the ctDNA alteration level is a good biomarker to predict the efficacy of antitumor therapy at baseline. After two cycles of treatment, the prognosis can be reflected and predicted according to ctle-RECIST. Combining imaging with ctDNA may better achieve disease surveillance in breast cancer patients. Although sufficient clinical validity of ctDNA detection is still needed for its adoption into routine clinical practice, our results prove that our study is a meaningful attempt at the clinical application of ctDNA and provide a new idea for an efficacy evaluation method combined with molecular indicators in the future.

6. Ethics approval and consent to participate

The current study was approved by the Human Research Ethics Committee at Hunan Cancer Hospital, Central South University (NO2017YS031), and the protocol has been registered on ClinicalTrials.gov with the number NCT05079074. The authors declare that they

obtained ethics approval and patient consent to participate.

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Author contribution

Study concepts and design: Q.OY., Z.H., N.X., B.L. All authors participated in the sample collection and data collection. B.L. and Z.H. conducted the data analyses. B.L., Z.H. and J.R. wrote the manuscript and all authors reviewed and approved the final version of the manuscript.

Data availability

All data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2022.07.010.

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