Subtyping of metastatic breast cancer based on plasma circulating tumor DNA alterations: An observational, multicentre platform study

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Summary

Background After multiple lines of therapies, no guideline or consensus is currently available for the treatment of patients with metastatic breast cancer. This study aims to evaluate the efficacy of a novel re-subtyping and treatment strategy based on ctDNA alterations.

Methods This observational, multicentre study recruited 223 patients with metastatic breast cancer intending to receive late-line therapy from Dec 1, 2016, to June 31, 2019. This study took place in Hunan Cancer Hospital, the Forth Hospital of Changsha and Zhuzhou Central Hospital in China. ctDNA alterations were assessed by next-generation sequencing (NGS). Patients with druggable ctDNA alterations were treated with corresponding targeted drugs which are clinically available. Other patients received physician-chosen treatment. This study was registered with ClinicalTrials.gov, NCT05079074.

Findings The progression-free survival (hazard ratio: 0.45, 95% Confidence Interval (CI): 0.33-0.62, $P < 0.0001$) and disease control rate (89.4% *vs.* 65.9%, $P < 0.0001$) were significantly improved in patients who received druggable ctDNA alteration-guided therapy compared with those of patients who received physician-chosen treatment. ctDNA alterations with top rank and high clustering scores were classified into four subtypes based on their functions as follows: 1) extracellular function (ECF), 2) cell proliferation (CP), 3) nuclear function (NF), and 4) cascade signaling pathway (CSP). A significant benefit from ctDNA alteration-guided treatment was observed in patients with NF and CSP ctDNA alterations, with hazard ratios of 0.39 (95% CI: 0.24-0.65, $P = 0.0003$) and 0.14 (95% CI: 0.04-0.46, $P < 0.0001$), respectively.

Interpretation After multiline traditional pathological HR/HER2 subtype-guided therapies, ctDNA testing could identify druggable ctDNA alterations to guide late-line therapy for patients with metastatic breast cancer.

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Keywords: Late-line therapy; Druggable circulating tumor DNA alterations; Genetic alterations of functional pathways; Progression-free survival (PFS); Disease control rate (DCR)

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Introduction

Breast cancer is the most common malignancy in women worldwide. Although the 5-year survival rate increased substantially from 70% in the 1990s to 90%

Research in context

Evidence before this study

Before undertaking this study (December 1st, 2016), we searched PubMed to rapidly review the literature on ctDNA testing in metastatic breast cancer, using the search terms (metastatic breast cancer) AND (circulating tumor DNA). 156 publications were relevant but none focused on ctDNA subtyping.

Added value of this study

In this observational, multicenter clinical study, we found that ctDNA testing could identify druggable ctDNA alterations in the majority of metastatic breast cancer patients who had progressed the early line therapy. Patients who received ctDNA alteration-guided treatment (DDAT) had significantly longer PFS in patients who received physician-chosen treatment (PCT). In addition, ctDNA alterations with top rank and high clustering scores were classified into four subtypes based on their functions as follows: 1) extracellular function (ECF), 2) cell proliferation (CP), 3) nuclear function (NF), and 4) cascade signaling pathway (CSP).

Implications of all the available evidence

This study emphasized that patient had a better response to druggable ctDNA alteration-guided late-line therapy than physician chosen therapy. The implementation of the ctDNA subtype has potential to guide pathway-related drug development for undruggable ctDNA alterations. The increasingly-approved targeting drugs may be able to make the precision treatment feasible for metastatic breast cancer patients.

in the 2010s,[1,2] approximately half million people died from metastatic breast cancer (MBC) worldwide.[3] Currently, the 5-year survival rate of breast cancer (all stage combined) is 90%.[4] The 5-year breast cancer-specific survival rates of stage I, II, III and IV were 98%, 92%, 75% and 27%, respectively, in United States from 2009 to 2015.[5] For patients with HR+/HER2-, HR+/HER2+, HR-/HER2+ and HR-/HER2- breast cancer, the 5-year breast cancer-specific survival rates were 92%, 89%, 83% and 77%, respectively, in United States from 2009 to 2015.[5] Traditionally, treatment for metastatic breast cancer is based on pathological hormone receptor (HR)/human epidermal growth factor receptor 2 (HER2) subtyping. However, the 5-year survival rate is only 27% for stage IV patients.[6] Currently, there is limited knowledge of the mechanisms underlying late-line therapies applied for MBC.[7] The lack of evidence-based guideline complicates clinical decision-making for late-line therapies.

Currently, late-line therapy selection for MBC is based on the pathological HR/HER2 status of the tumor, which dictates the use of endocrine therapy, chemotherapy, or HER2-targeted therapy. However, the HR/HER2 status can be different between the primary tumor and metastases and can also vary among the organs where the disease has metastasized.[8] Another challenge in clinical decision-making is the temporal heterogeneity of breast cancer cells,[9] as an increase in genetic alterations is observed as the disease progresses.

HR/HER2 discordance and heterogeneity are not the only challenges associated with genetic alterations—loss-of-function and oncogenic mutations can also lead to treatment resistance.[10,11] For example, most MBCs that did not respond to multiple lines of therapy are resistant to conventional HR/HER2-based treatment regimens[12]; as results, it is difficult for clinicians to implement effective late-line therapy based on HR/HER2 status alone. Therefore, novel strategies that account for tumor heterogeneity and genetic evolution are needed.

To identify and address the issues caused by heterogeneity, one potential approach is to analyze the circulating tumor DNA, which can be used to monitor clinical prognoses and responses to treatment in MBC patients. In this study, we hypothesized that druggable ctDNA functional aberrations might provide useful information to guide late-line therapy selection.

Methods

Study design and participants

We designed an observational, multicentre clinical study to evaluate the circulating tumor DNA (ctDNA) analysis for patients with late-stage breast cancer. Specifically, the study was conducted in Hunan Cancer Hospital, the Forth Hospital of Changsha and Zhuzhou Central Hospital in China. 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. Patients who had druggable ctDNA abnormalities were assigned to the case group. Patients who did not have ctDNA abnormalities or druggable ctDNA abnormalities were assigned to the control group. The control group received physician-chosen treatment. The case group received druggable ctDNA alteration-guided therapy. This study is registered with ClinicalTrials.gov (NCT05079074). The endpoints (PFS and DCR) were confirmed by a retrospective independent radiologic committee. Investigations were performed in accordance with Chinese laws and regulations and the Helsinki declaration after approval by the local ethics committee at each participating hospital. The current

study was approved by the Ethics Committee of Hunan Cancer Hospital, Central South University (approval number 2017YS031) and registered with ClinicalTrials.gov (NCT05079074). The authors declare that they obtained ethics approval and patient consent to participate.

This study recruited 223 consecutive patients with MBC treated at Hunan Cancer Hospital, the Forth Hospital of Changsha and Zhuzhou Hospital Affiliated to Xiangya School of Medicine, Central South University, from December 2016 through June 2019. The patient consent form was approved by the independent ethics committee. Each participant provided written informed consent to participate in the study. The eligibility criteria and exclusion criteria were listed in supplemental protocol file.

Study treatments

This study was conducted according to the flow diagram shown in **Figure S1**. All patients underwent baseline ctDNA testing. The ctDNA analysis revealed that 190 patients had ctDNA alterations. Among these 190 ctDNA alteration-positive patients, clinically relevant drugs targeting the identified alterations were available for 132 patients (Table S1). No drugs were available for the remaining 58 patients, and these patients were treated with physician-chosen therapy. As results, 132 patients received late-line therapy based on druggable ctDNA alterations. Ninety-one patients received physician-chosen late-line therapy.

Baseline ctDNA analyses and imaging (MR/CT) were performed 3−7 days before treatment initiation. Additional imaging was performed every two treatment cycles or whenever progressive disease (PD) was clinically detected. A total of 136 patients underwent the 2nd ctDNA test after completing two treatment cycles; 58 patients underwent the 3rd ctDNA test when PD was confirmed by imaging or if they had clinical symptoms indicating suspected PD. A detailed study protocol is attached in the Supplemental Protocol file.

Endpoints and assessments

All patients received radiology assessments before the initiation of late-line therapy. Radiology assessments of tumor response were conducted every two cycles (6 weeks) of late-line therapy and every two treatment cycles (about 6-8 weeks) thereafter. According to the RECIST 1.1 criterion, PD was defined as 1) a >20% growth of measurable target lesions and an absolute increase in target lesions >5 mm or 2) the presence of new lesions. Partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameter of target metastatic lesions compared to the reference baseline sum of the longest diameter. Stable disease (SD) was defined as shrinkage insufficient to qualify as PR and increases insufficient to qualify as PD.

The primary outcome measure was disease control rate (DCR), which defined as the total rate of CR+PR +SD after the completion of two cycles of late-line therapy. The time frame was from the beginning of the treatment to the end of Cycle 2 (each cycle is 28 days) of treatment. The second outcome measure was the progression-free survival (PFS), which defined as the survival time between the beginning of treatment to death or the progression. The time frame was from the date of recruitment until the date of first documented progression or date of death from any cause, whichever came first, assessed up to 12 months. Due to the security reasons, the death information of the residence was inaccessible. The date of last visit for non-progressive patients was censored for PFS analysis.

Next-generation sequencing for circulating tumor DNA (ctDNA)

Sample processing and DNA extraction. 10ml peripheral blood in Streck tubes was separated by centrifugation at 1600g for 10 min, transferred to microcentrifuge tubes. Then the supernatant was centrifuged again at 16000 g for 10 min to remove cell debris, and then frozen at −80°C. Circulating cell-free DNA (cfDNA) was extracted from 4ml plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen). Germline genomic DNA was isolated from peripheral blood lymphocytes (PBLs) with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The concentration and fragment length of cfDNA was detected by an Agilent 2100 Bioanalyzer.

Library construction. The germline genomic DNA was sheared into fragments at a 200 ∼250 bp peak by Covaris S2 ultrasonicator (Covaris), and indexed NGS libraries were prepared by using NEBNext®Ultra™ NA Library Prep Kit for Illumina®(NEB). cfDNA with median 50ng input was used for library construction, during this process, unique identifiers (UIDs) were tagged on each double-stranded DNA that enables to distinguish authentic somatic mutations from artifacts, improving the ability to precisely track individual plasma molecules.

Target region capture and next-generation sequencing. Customized probes (Integrated DNA Technologies, IDT) covering ∼1.5 Mbp genome and targeting 1021 cancer-related genes were used for hybridization enrichment with germline DNA and cfDNA libraries (Table S2). These libraries were sequenced using a 100bp paired-end configuration on a DNBSEQ-T7RS (MGI Tech, Shenzhen, China) sequencer, producing

5Gb and 15Gb data for germline DNA and cfDNA, respectively.

Raw data processing and tumor somatic variant calling

The sequenced reads were mapped to the reference human genome (hg19) with BWA 0.6.2 at the default parameter, after removing adaptor and low-quality reads. Duplicated reads from cfDNA samples were identified by UID and the position of template fragments to eliminate errors introduced by PCR or sequencing. Duplicated reads were marked and removed using the Picard tools MarkDuplicates (V4.0.4.0) for germline DNA. GATK (V3.4.46) were performed to conduct local realignment around SNVs and indels as well as quality control assessment.

Tumor somatic single nucleotide variants (SNV) and small insertions and deletions (InDel) were profiled by Mutect 2.0. CNVKit was employed to detect copy number alterations (CNV). Structural variations (SV) were analysed by self-developed algorithm NCsv (0.2.3). Variants were filtered to exclude synonymous variants, known germline variants in matched gDNA and dbSNP, and variants that occur at a population frequency of >1% in the Exome Sequencing Project.

cfDNA somatic variant detection performance evaluation

In order to evaluate the accuracy of mutation detection, 100 clinical samples were sequenced in both Illumina and MGI platform. The main method was in accordance with the procedure prescribed in above. The minimum depth of each sample was 1500X. The positive percent agreement (PPA) and positive predictive value (PPV) of non-hot spot mutations with ≥ 1% frequencies and hot spot mutations with ≥ 0.5% were determined with greater than 95% and 99%. The detailed information was presented in Table S3. These results demonstrated the detection method was highly reliable and the detected variants could be applied to assistance in clinic.

Clustering of ctDNA alterations

Hierarchical clustering analysis was performed with the 'hclust' function in the R package (version i386 3.6.2, http://www.r-project.org) by using a set of dissimilarities among the ctDNA alterations being clustered.[13] The 'complete' agglomeration method was used. Next, the cutree function pruned the clustering results of the hclust function. The distribution of ctDNA alterations was pyramidal. To distinguish the frequency of gene alterations, the cutree function was selected, and the pedigree clustering results of 60 categories (scores) were obtained. In the clustering tree, the most recent merge of the left subtree occurred at a lower score than the most recent merge of the right subtree.

Statistical analysis

The R package 'ComplexHeatmap' was used to rank the genetic alterations and ctDNA subtypes at baseline. We used Kaplan–Meier curves and a two-sided log-rank test to ascertain the influence of ctDNA alteration-guided late-line therapy on the PFS of MBC patients. The Cox proportional hazards model was used to estimate the treatment effect and is presented as the hazard ratio and 95% confidence interval (CI). Patients who did not progress were censored at the date of their last follow-up. The subgroup analysis results were plotted by the 'forestplot' package. All statistical analyses were conducted by using SAS 9.4 software (SAS Institute Inc., NC, USA) and R 4.1.1 (https://www.r-project.org/). All hypothesis tests were two sided and conducted at a significance level of 0.05.

Role of the funding source

The fundings support the whole project, including the study design, data acquisition and analysis but authors were responsible for each of these elements. All authors confirmed that they had full access to all the data in the study and accept responsibility to submit for publication.

Results

Patient characteristics

From Dec 1, 2016, to June 31, 2019, 223 eligible patients were recruited. After baseline ctDNA analysis, 132 (59%) patients received druggable ctDNA alteration-guided treatment (DDAT). The remaining 91 (41%) patients, who had no baseline ctDNA alteration (*n* = 33) or who had alterations for which no drugs were clinically available (*n* = 58), received physician-chosen treatment (PCT). The median follow-up time was 6.2 months (interquartile range, 3.1 to 10.4 months).

The patient characteristics were generally well-balanced between the two groups at baseline (**Table S4**). The median age of the study population was 43.5 years (interquartile range, 37 to 52 years). The Eastern Cooperative Oncology Group performance status was 0 in 103 (46.2%) patients and 1 in 120 (53.8%) patients. 138 (49.8%) patients had visceral metastases, and 85 (38.1%) patients had distant lymph node metastases or soft tissue metastases. A total of 115 (51.6%) patients had hormone receptor-positive disease. 57 (25.6%) patients had HER2-positive disease. 190 (85.2%) had invasive ductal carcinoma.

Late-line treatment groups

Recently, multiple molecule-targeting drugs have emerged for the treatment of MBCs, including anti-VEGF drugs, CDK4/6 inhibitors, PARP inhibitors, PI3K inhibitors, EGFR inhibitors, and anti-hormone

drugs. These drugs target the corresponding pathways, and their efficacy against MBCs is promising. Using ctDNA testing, we found that the signaling processes affected by ctDNA alterations in 132 (69.5%) of 190 baseline ctDNA-positive patients involved proteins or pathways for which targeted therapeutics were clinically available. Late-line treatment regimens were applied based on the ctDNA alterations present (Table S1). Patients harboring alterations affecting FGFR/VEGFR pathways were treated with clinically available anti-VEGF/FGFR inhibitors alone or in combination with chemotherapy.[14] Patients harboring alterations affecting cell proliferation pathways were treated with clinically available CDK4/6 inhibitors, which have shown promising anticancer effects.[15] For patients with alterations affecting the HRR pathway, poly ADP-ribose polymerase (PARP) inhibitors were administered.[16] Targeted drugs with good therapeutic effects, such as pyrotinib[17] (with chemotherapy) or T-DM1[18] were administered to patients with alterations affecting the EGFR pathway and PI3K and mTOR inhibitors were administered to patients with alterations affecting the PI3K/AKT/mTOR pathway.[19,20] Overall, we found that the majority of ctDNA-positive patients had druggable ctDNA alterations, and these alterations primarily affected the AKT/mTOR pathway, HRR pathway, EGFR pathway, FGFR/VEGFR pathways, cell cycle pathways, and hormone-related pathways.

Disease control rate and profression-free survival

After 2 cycles of treatment, all 223 patients were monitored for therapeutic responses (disease control) by imaging and were evaluated using the RECIST 1.1 criteria. In total, 45 (20.2%) patients experienced progression, 92 (41.3%) had SD, and 86 (38.6%) achieved PR. The total disease control rate (DCR) (CR, PR and SD) was 79.8% (Table S5). In the DDAT group, 118 (89.4%) patients achieved PR or had SD, while only 60 (65.9%) of 91 patients in the PCT group achieved PR or had SD, indicating a significant benefit from ctDNA alteration-guided treatment (p<0.0001). These findings indicate that MBC responses to druggable ctDNA alteration-guided treatment were superior to the responses to PCT.

To further evaluate the efficacy of DDAT, Kaplan−Meier curves were generated to compare the PFS of patients who received DDAT with that of those who received PCT. The median PFS of PCT patients was 4.3 months (95% CI: 3.4-4.8); whereas, the median PFS of DDAT patients was 6.3 months (95% CI: 6.0-7.5). The risk for progression among DDAT patients was significantly lower than that among PCT patients (hazard ratio: 0.45, 95% CI: 0.33-0.62, $P < 0.0001$) (Figure 1). Multivariate COX regression analyses also demonstrated that DDAT patients was significantly lower than that among all PCT patients (hazard ratio: 0.49, 95% CI: 0.33-0.73, $P = 0.0004$, Table 1) and PCT patients

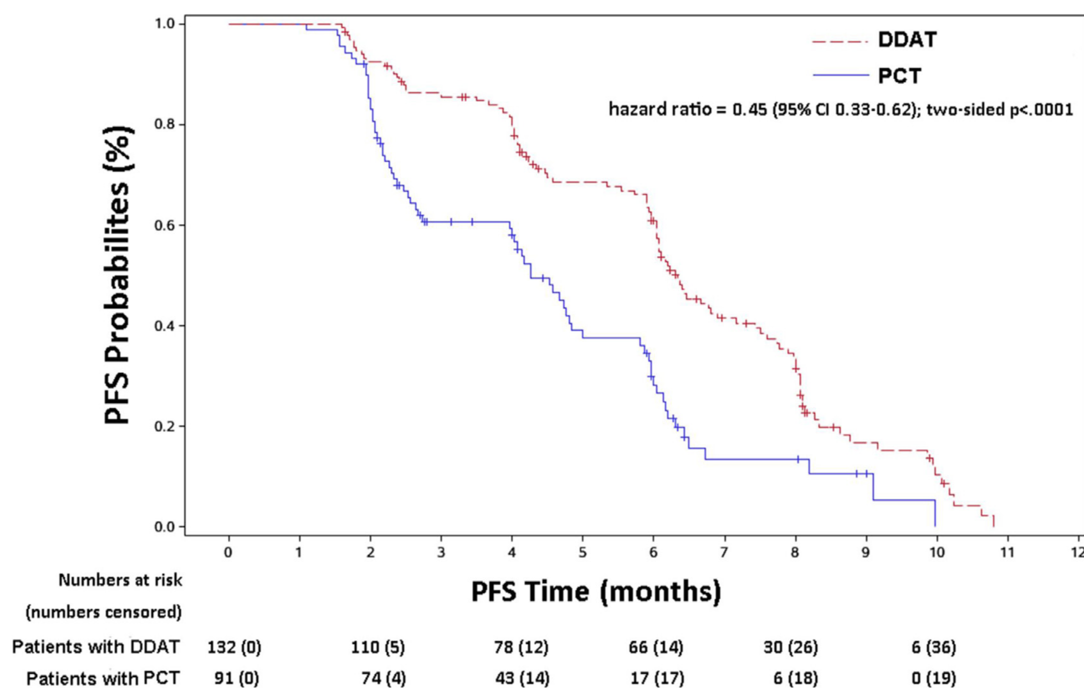

Figure 1. Kaplan−Meier plot of progression-free survival. Dashes represent censored patients. HR=hazard ratio. Univariate Cox regression analysis was performed to calculate the hazard ratio (HR) with 95% confidence interval (CI) of progression in the ctDNA-guided LLT group versus the traditional group.

Variables	Levels	All patients (n = 223) HR	(95% CI)	P-value	Patients with ctDNA alterations (n = 190) HR	(95% CI)	P-value
Age (years)		1.00	(0.98, 1.02)	0.70	1.09	(0.99, 1.03)	0.38
ECOG	0		Ref			Ref	
	1	0.91	(0.64, 1.29)	0.59	0.89	(0.60, 1.32)	0.56
Metastasis sites#	Visceral (with/without bone)	1.73	(1.15, 2.59)	0.008	1.74	(1.12, 2.71)	0.01
	Soft tissue (with/without bone)	0.95	(0.65, 1.38)	0.78	0.92	(0.61, 1.39)	0.69
ER/PR	ER or PR Negative		Ref	0.22		Ref	0.11
	ER and PR Positive	0.80	(0.56, 1.14)		0.72	(0.48, 1.08)	
HER2	Negative		Ref	0.33		Ref	0.03
	Positive	0.82	(0.55, 1.23)		0.58	(0.36, 0.94)	
Pathological type, n (%)	Invasive ductal carcinoma		Ref			Ref	
	Invasive lobular carcinoma	1.63	(0.72, 3.50)	0.24	0.97	(0.34, 2.75)	0.94
	Other	1.06	(0.70, 1.60)	0.79	1.09	(0.70, 1.71)	0.70
Treatment strategies	PCT		Ref			Ref	
	DDAT	0.49	(0.33, 0.73)	0.0004	0.59	(0.39, 0.91)	0.02

***Table 1*: Multivariate COX regression analysis for all patients (n = 223) and patients with ctDNA alterations (n = 190).**
Note: For metastasis sites#, p-value were calculated by comparing the patients with visceral metastases versus non-visceral metastases, and soft tissue metastases versus non-soft tissue metastases. Abbreviation: ER (Estrogen Receptor), PR (Progesterone Receptor), HER2 (Human Epidermal Growth Factor Receptor-2).

who had ctDNA alterations but no available drug (hazard ratio: 0.59, 95% CI: 0.39-0.91, P = 0.02, Table 1). When comparing the PFS between DDAT group and the group of the 58 patients with ctDNA alterations but for whom no drugs were available, the risk for progression among DDAT patients was still significantly lower than that among patients who had undruggable ctDNA alterations and received PCT (hazard ratio: 0.56, 95% CI: 0.38-0.82, P =0.003) (Figure 2).

Figure S2 shows the treatment trajectories of two DDAT patients with different HR/HER2 statuses. The first patient was a 52-year-old female (ID 61) with HR +/HER2- invasive BC and liver metastases. She experienced progression after receiving a gonadotropic-releasing hormone agonist (GnRHa) for ovarian function suppression (OFS) plus letrozole. Subsequently, she again experienced progression after ten months of fulvestrant treatment. Upon study enrollment, her dominant baseline ctDNA alteration was an *ERBB2* amplification, and she received capecitabine plus pyrotinib. The imaging assessment revealed that the patient had achieved PR at the completion of two cycles of treatment. ctDNA testing showed no aberrations. After twelve months of treatment, an imaging assessment showed diffuse liver metastases, and a ctDNA analysis revealed the reemergence of *ERBB2* amplification. The patient's treatment regimen was then switched to TDM-1. The second patient was a 48-year-old female (ID 35) with TNBC and lung metastases. Despite achieving PR after 6 months of TCb (paclitaxel plus carboplatin), she experienced progression after 5 months without treatment. Her baseline ctDNA alteration was a *BRCA1* mutation; thus, olaparib was administered, which resulted in persistent PR. These results suggest that ctDNA testing could provide important molecular information about MBCs. Druggable ctDNA alteration-guided late-line treatment performed better than PCT.

ctDNA alterations in metastatic breast cancers

To further evaluate the molecular information provided by ctDNA analyses before late-line therapy, we summarized the baseline ctDNA alterations in the analysed MBCs. A total of 223 patients with breast cancer intending to receive late-line therapy underwent baseline ctDNA analysis. A total of 190 (85%) patients were identified to have baseline ctDNA alterations (VAF ≥ 1%), as shown in the Figure 3A heatmaps. ctDNA alterations in *TP53, PIK3CA, ERBB2, BRCA1, ESR1, CDK12*, and *FGFR1* were common in MBC, with alteration rates of 44%, 30%, 13%, 8%, 7%, 6%, and 6%, respectively. Apart from *TP53*, these alterations were all druggable. The top 60 aberrant genes are listed in Figure 3A. The corresponding treatment strategies were listed below the heatmap. The detailed interventional section was listed in the supplemental protocol.

To classify ctDNA alterations and guide late-line therapy in MBC, we used hierarchical clustering analysis (hclust) to cluster all detected ctDNA alterations among 419 samples from 223 patients. As shown in Figure 3B, among 1,021 sequenced genes, aberrations were present in 410. The majority of the aberrations in these 410 genes occurred only once and were distributed throughout the left three-fourths of the pyramid body in the clustering tree. Only a small group of aberrant genes had a score higher than 30 in the 60-score clustering tree. The majority of the genes in this small group were also in the top 60 list of ranked aberrant

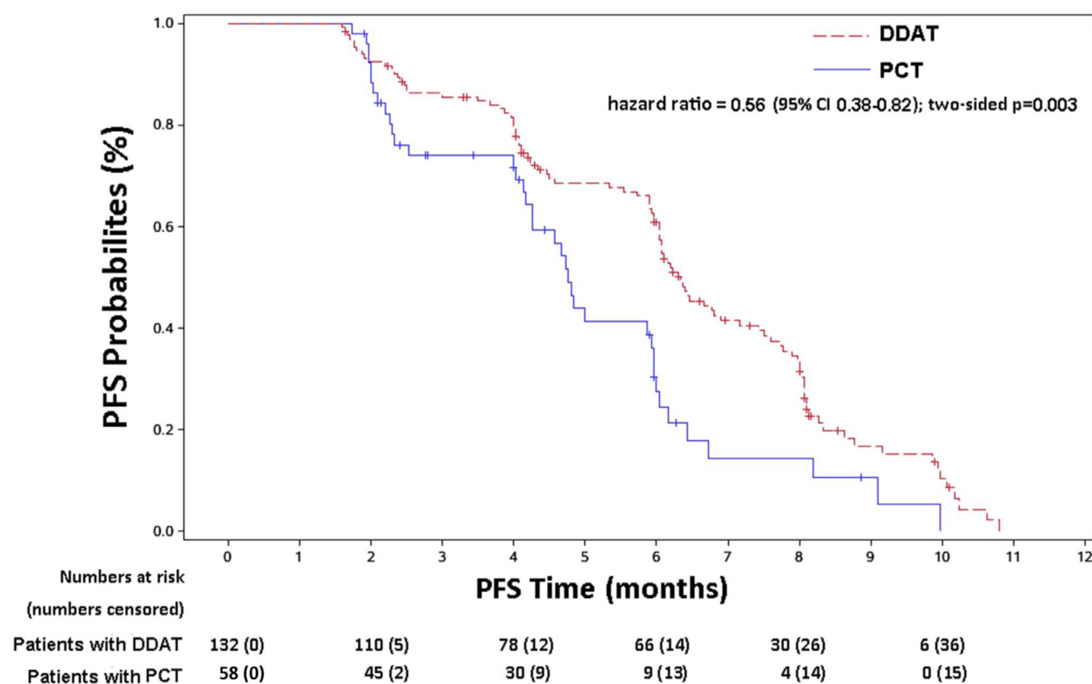

Figure 2. Kaplan–Meier plot of progression-free survival. Dashes represent censored patients. HR=hazard ratio. Univariate Cox regression analysis was performed to calculate the hazard ratio (HR) with 95% confidence interval (CI) of progression in the ctDNA-guided LLT group versus the group of the 58 patients with ctDNA alterations but for whom no drugs were available.

genes (Figure 3A). These genes were categorized based on their function and involvement in pathways as described by GeneCards and validated by GO and KEGG analyses (Table S6-S7), and divided into the following four subtypes (Table 2): subtype 1, extracellular function (ECF) (genes involved in migration, invasion, angiogenesis, hematopoiesis, and immune regulation); subtype 2, cell proliferation (CP) (genes involved in apoptosis, cell cycle, metabolism, and development); subtype 3, nucleus function (NF) (genes involved in DNA damage repair, epigenetics, RNA/protein assembly, and transcriptional regulation); and subtype 4, cascade signaling pathway (CSP) (genes involved in the hormone, PI3K/AKT, MAPK, JAK-STAT and WNT pathways). These findings suggest that a small group of genes are frequently aberrant in MBCs, and most of the genes in this group were druggable.

Effect of ctDNA classification on druggable ctDNA alteration-guided treatment

To assess the effect of ctDNA classification on the outcome of druggable ctDNA alteration-guided therapy, univariate Cox regression analysis was performed to evaluate the risk factors for PFS. Figure 4 summarizes the analysis of patients with different ctDNA subtypes. Compared with patients who received PCT, patients with ctDNA subtype 3 (NF) gained a significant benefit from ctDNA alteration-guided treatment (HR: 0.394, 95% CI: 0.236-0.652, P = 0.0003). The PFS revealed that patients with ctDNA subtype 4 (CSP) obtained an even stronger protective effect from ctDNA alteration-guided treatment (HR: 0.142, 95% CI: 0.044-0.463, P < 0.0001). However, patients with ctDNA subtype 1 (ECF) and subtype 2 (CP) aberrations did not obtain a significant benefit from ctDNA-guided treatment, with HRs of 0.847 (95% CI: 0.33-2.171, P = 0.73) and 0.611 (95% CI: 0.283-1.322, P = 0.211), respectively. These results suggest that druggable ctDNA alteration-guided treatment can significantly improve the PFS of patients with ctDNA alterations that affect cascade signaling pathways and nuclear functions.

Discussion

Currently, the lack of evidence-based guidelines complicates clinical decision-making for MBCs. Even if it is based on HR/HER2 stratification, there is no clear treatment plan. In addition, HR / HER2 status may be different when the disease progresses and new metastases appear. In this study, although we performed stratified analyses by HR/HER2 subtypes, the results were not significant. On the contrary, ctDNA subtypes are more representative. Genetic alterations may underlie resistance to late-line therapy, and researchers have spent considerable amount of efforts on developing strategies to address therapeutic resistance. PlasmaMATCH trial has proven that ctDNA detection can provide accurate

and rapid genotyping for mutation-oriented therapy in advanced breast cancer.[21] In this retrospective, observational, multicentre study, we aimed to evaluate ctDNA-based strategies for decision-making in MBCs. Here, we selected approximately 70 ctDNA alterations with high clustering scores or high aberration rates in MBCs and classified them into four functional subtypes. Compared with physician-chosen treatment, druggable ctDNA alteration-guided treatment could significantly improve the PFS, especially for patients with ctDNA subtype 3 (NF) and subtype 4 (CSP) alterations. In patients with subtype 3 ctDNA alterations who harbor alterations in homologous recombination repair (HRR) pathway, PARP inhibitors provide a significant overall survival benefit compared with standard chemotherapy.[22] For patients with subtype 4 ctDNA alterations, several targeted drugs with good therapeutic effects are available for late-line treatment, such as pyrotinib[17] and T-DM1[18] for patients with EGFR pathway alterations and PI3K and mTOR inhibitors for patients with PI3K/AKT pathway alterations.[19,20]

In this study, seventy ctDNA alterations, commonly existed in MBC, were divided into four types according to their functions. The ctDNA-guided treatment was carried out according to the molecular targets of existing therapeutic drugs or their related targeted pathways. Its curative effect was significantly better than that based on traditional HR/HER2 classification. This study provided more relevant molecular targets for the current late-line treatment and more treatment opportunities for MBC patients, not limited to the treatment opportunities guided by routine HR/HER2. PlasmaMATCH trial recruited four cohorts with ESR1, HER2, AKT1 and PTEN alterations, and the response rate was 8%, 25%, 22% and 11%, respectively.[21] In this study, we did not only focus on several specific alterations. Instead, we summarized the popular genetic alterations into four types and targeted drug were given according to their

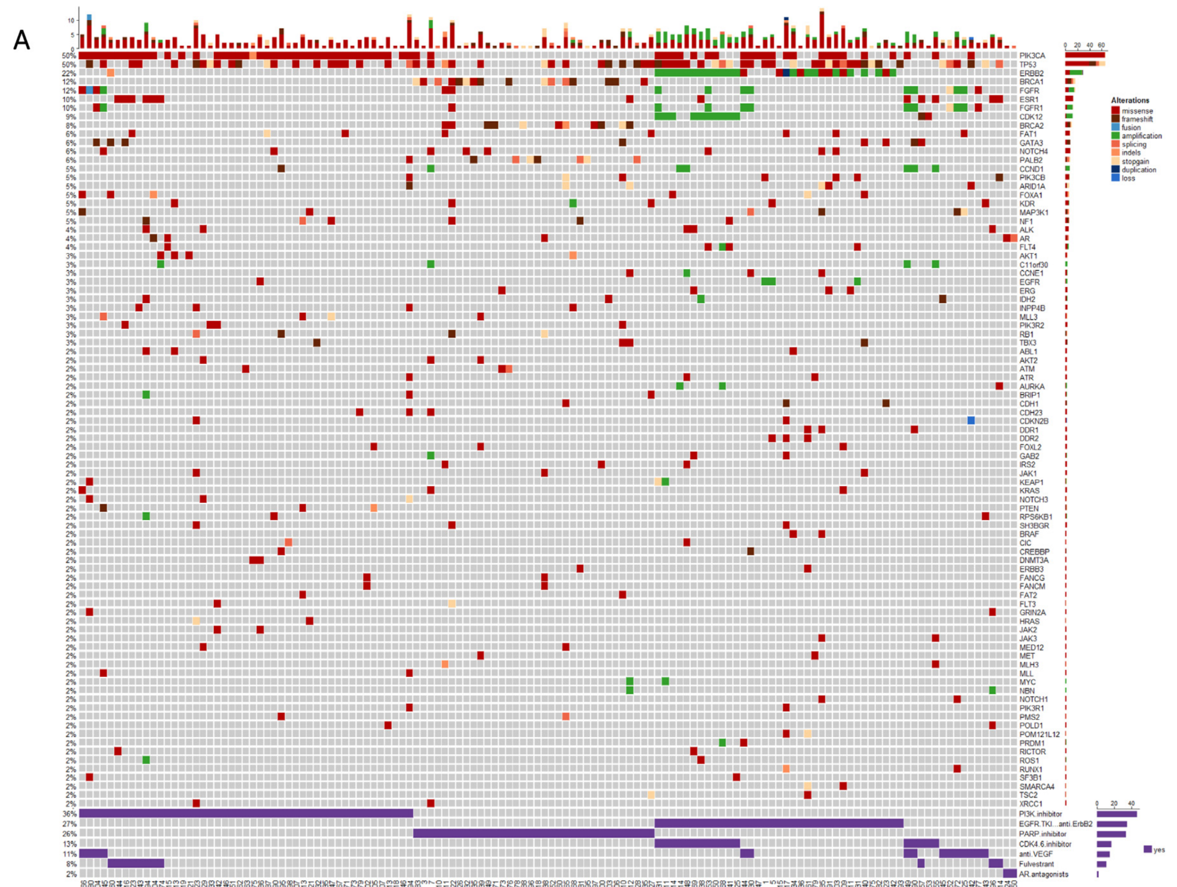

Figure 3. ctDNA alterations.

A. Heatmaps of baseline ctDNA alteration profiles and the corresponding treatment strategies for patients with

B. Circle plot of 442 ctDNA alterations among 420 samples from 223 patients. Genes were clustered by the 'complete' method of the hclust function in R. The colors indicate the cutree score, ranging from 1 (blue) to 60 (red). Nearly three-fourths of all ctDNA alterations had a low score (blue, left and bottom regions of the circle).

Figure 3. Continued

related functional pathways. This ctDNA-guided therapy showed a median PFS was 6.3 months. And the response (PR+CR) rate was 42.4% at the completion of two treatment cycles.

This study started in 2016, when the clinical guidelines recommended 1st line trastuzumab plus chemotherapy and 2nd line lapatinib plus capecitabine or trastuzumab with other chemo-drugs for HER2-positive MBCs, aromatase inhibitors (AIs) for 1st and fulvestrant for 2nd line HR-MBC treatment, and taxane-based chemotherapy (for previous anthracycline-based treatment resistance), single drug chemotherapy and cisplatin/carboplatin plus gemcitabine or cisplatin plus vinorelbine (for previous anthracycline-based and taxane-based treatment resistance) for metastatic TNBC patients. No standard recommendation for late-line therapy was available at that time. Multiple important clinical trials, including trials evaluating tucidinostat, pyrotinib, eribulin and olaparib, were conducted during the study period.[17,23-25] Taking advantage of ctDNA surveillance strategies, we found these drugs to be promising for targeting genetic aberrations and overcoming treatment resistance. Some may argue that most patients received standard treatments according to current guidelines. However, from 2016 to 2021, Chinese guidelines for breast cancer treatment were changed and updated for more than five times. According to NCCN and CSCO guidelines, many drugs evaluated in 2016 are now recommended as early-line therapeutics. The efficacy of these drugs has been confirmed by well-designed clinical trials. Therefore, in this study, we did not aim to emphasize the treatment efficiency of novel treatment drugs; instead, we demonstrated that ctDNA surveillance is a useful tool for providing comprehensive genetic information for accurate treatment.

As a potential genetic marker,[26,27] ctDNA provides important evidence for drug use guidance, drug efficacy prediction, drug resistance mechanism analysis, and recurrence monitoring.[28-30] This study classified the top-ranked genetic aberrations into four subtypes, and supports that the genetic subtype system is better than the scheme selected by doctors. One limitation of the current study is that not all patients undergo serial ctDNA testing in clinical practice. Moreover, data from additional patients are necessary to confirm our findings; in future, we will recruit additional participants in

Subtypes	Aberrant genes	Encoded protein and functions	Therapies[a]
Subtype 1			anti-VEGFR[a], anti-FGFR[a], etc
Angiogenesis & hematopoiesis	FLT1	VEGFR1, Fms-related TK 1, angiogensis and metastasis	anti-VEGF[a]: Bevacizumab
	KDR	VEGFR2, angiogensis and metastasis	
	FLT4	VEGFR3, Fms-related TK 4, angiogenesis and metastasis	
	FLT3	GFR-TK3, Fms-related TK 3, angiogenesis and hematopoiesis	
	FGFR1	Fms-like TK2, FGFR1, angiogenesis and migration	
	FGFR3	FGFR3, angiogenesis and migration	
Subtype 2			
Cell cycle	CCND1	Cyclin D, G1/S checkpoint	CDK4/6 inhibitor[a]: Palbociclib, Abemaciclib, Ribociclib
	CDKN2B	p15/INK4B, control G1 progression	
	CCNE1	Cyclin E1, G1/S checkpoint	
	CHEK2	Checkpoint kinase 2, in response to replication blocks and DNA damages	
Subtype 3			
DNA damage repair	ATM	Response to DNA-damage and regulate BRCA1, CHK2, RAD17, etc;	PARP inhibitor[a]: Olaparib
	ATR	DNA damage sensor, promote DNA repair and recombination	
	C11orf30	EMSY transcriptional repressor, interacting to BRCA2	
	PALB2	Partner and localizer of BRCA2, permits the stable intraneclear localization of BRCA2	
	BRCA2	Breast cancer type 2 susceptibility protein, homologous recombination of DSD repair	
	BRCA1	Breast cancer type 1 susceptibility protein, DNA repair of DSB and recombination	
	NBN	Nibrin, MRE11/RAD50 DSB repair complex member	
	PMS2	DNA mismatch repair endonuclease	
Subtype 4			
Hormone pathway	ESR1	Estrogen receptor 1, ER pathway activation	Endocrine therapy[a]: Fulvestrant
	AR	Androgen receptor, stimulates transcription of androgen responsive genes and downstream pathway	AR antagonists: Bichloramide, Nzalutamide
PI3K/AKT/mTOR pathway	PTEN	Tumor suppressor gene, negatively regulate PI3K pathway	PI3K inhibitor: Alpelisib; mTOR inhibitor[a]: Everolimus
	PIK3CA	PI3K catalytic subunit α, PI3K-AKT-mTOR pathway activation	
	PIK3CB	PI3K catalytic subunit β, PI3K-AKT-mTOR pathway activation	
	PIK3R1	PI3K regulatory subunit 1, negatively regulate PI3K-AKT-mTOR pathway	
	PIK3R2	PI3K regulatory subunit 2	
	AKT1	Serine-threonine protein kinase, regulate cell proliferation through PI3K-AKT pathway	
	AKT2	PI3K-AKT signaling pathway	
	AKT3	PI3K-AKT signaling pathway	
'	MTOR	Mammalian target of rapamycin, modulates cell response to stress	

Table 2 *(Continued)*

Subtypes	Aberrant genes	Encoded protein and functions	Therapies[a]
MAPK pathway	EGFR	Epidermal growth factor receptor, autophophorylation to promote MAPK, PI3K and JNK pathways	EGFR inhibitor[a]
	ERBB2	Erb-B2 receptor TK 2, bind to ligand-bound EGF receptor to activate MAPK and PI3K pathways	Anti-HER2 therapy[a]: Pyrotinib, TDM-1
	ERBB3	Erb-B2 receptor TK 3, bind to ligand-bound EGF receptor to activate MAPK and PI3K pathways	

***Table 2*: Functions and corresponding therapies for aberrant genes in ctDNA subtypes.**
Abbreviations: TK, tyrosine kinase; GFR, growth factor receptor; VEGFR, vascular endothelial growth factor receptor; IGF1R, insulin like growth factor 1 receptor; FGFR, fibroblast growth factor receptor; IDH2, isocitrate dehydrogenase 2; ATM, ataxia-telangiectasia mutated; ATR, ataxia telangiectasia and rad3-related protein; DSD, double-strand DNA; DSB, double-stranded break.

[a] Therapies represent the treatment drugs that target to ctDNA aberrations. Anti-VEGF and anti-FGFR drugs were applied in subtype 1 patients, including Bevacizumab. CDK4/6 inhibitors were used in subtype 2 patients, such as Palbociclib, etc. PARP inhibitors, such as Olaparib, were used in subtype 3 patients. In subtype 4 patients, endocrine therapy was used in patients with ESR1 or AR aberration, including Fulvestrant, Bicalutamide, Enzalutamide; PI3K inhibitor or mTOR inhibitor (Everolimus) were used in patients with PI3K/AKT/mTOR pathway aberrant patients. Anti-ERBB drugs, such as Pyrotinib and T-DM1, were used in ERBB-aberrant subtype 4 patients.

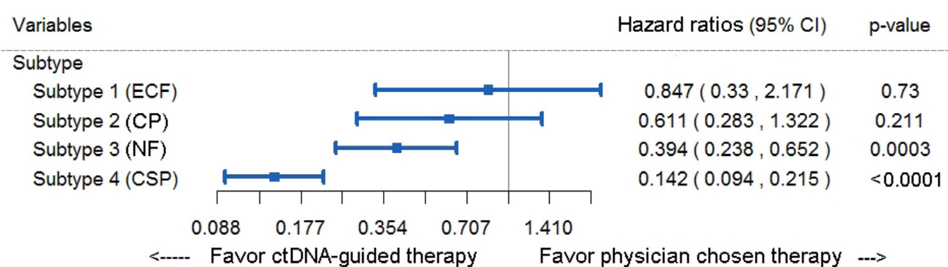

Figure 4. Subgroup analyses of hazard ratios for progression-free survival by ctDNA-based subtype.

the near future and obtain more data on the molecular and genetic profiles of metastatic cancer patients. Another potential limitation is drug availability. Specific targeted drugs are not available for all ctDNA alterations. If all of these aberrations have corresponding targeting drugs, the genetic subtype system might be more sufficient in providing targeting information. ctDNA-based clinical trials are needed in the future. Currently, no guidance is available for genetic analysis-based clinical trials evaluating targeting drugs. Clinical trials evaluating targeting drugs do not examine drug targets. IHC/FISH for HER2 is conducted only when anti-HER2 therapy is administered, and no other molecules involved in resistance are tested. Thus, we lack information regarding drug resistance mechanisms. We hope that this study provides more options for clinical practice.

Although the total sample size of this study was sufficient for the current study, the sample size was not enough for the analysis of each specific ctDNA change and targeted drugs. As a non-randomized observational study, this study may have confounding problems. In order to adjust for potential confounding, multivariate analyses were needed. To verify the generalizability of this study, randomized controlled clinical trial with larger sample size is necessary in the future.

The present study clarifies the late-line treatment strategies according to the ctDNA alterations. Patients with ctDNA alterations that are involved in druggable pathways received ctDNA alteration-guided treatment and showed promising treatment outcomes. Therefore, continuous ctDNA surveillance may not only provide treatment clues for patients with long-term survival, but also promote drug development for the currently-undruggable ctDNA alterations.

Contributors

Quchang Ouyang and Zhe-Yu Hu were responsible for the verification of the underlying data. Quchang Ouyang and Zhe-Yu Hu were responsible for conception, study design, data analysis, interpretation and manuscript writing. Liping Liu, Ning Xie, Can Tian, Bingliang Wang, Lixin Zhou, Wei Zhou and Quchang Ouyang were responsible for the provision of study materials or patients. Yu Tang, Liping Liu, Ning Xie, Can Tian, Bingliang Wang, Lixin Zhou and Wei Zhou were responsible for the collection and assembly of data. Xuefeng Xia and Yikai Wang were responsible for the study protocol and manuscript editing. All authors were responsible for the final approval of the manuscript. All authors confirmed that they had full access to

all the data in the study and accept responsibility to submit for publication.

Data sharing statement

We used NGS sequencing data from the private Geneplus database and from hospitals. Geneplus sequencing data will be made available upon reasonable request.

Declaration of interests

The authors declare that they have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.eclinm.2022.101567.

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