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ORIGINAL ARTICLE

# Non-invasive analysis of tumor mutation profiles and druggable mutations by sequencing of cell free DNA of Chinese metastatic breast cancer patients

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**Keywords**
circulating tumor DNA; druggable mutation; high-throughput DNA sequencing; metastatic breast neoplasm.

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## Abstract

**Background:** Metastatic breast cancer (MBC) remains an incurable disease worldwide. Tumor gene mutations have evolved and led to drug resistance in the treatment course of MBC. However, data on the mutation profiles and druggable genomic alterations of MBC remain limited, particularly among Chinese patients. Our study aimed to depict the mutation profiles and identify druggable mutations in circulating tumor DNA (ctDNA) in Chinese MBC patients.

**Methods:** Targeted deep sequencing of a 1021-gene panel was performed on 17 blood samples and 5 available tissue samples from 17 Chinese MBC patients.

**Results:** We identified 60 somatic mutations in 17 blood samples (sensitivity 100%). Somatic mutations were identified in the blood samples of all patients, and 41.18% (7/17) of patients harbored at least one druggable mutation. A high ctDNA level in plasma is associated with shorter progression-free survival.

**Conclusion:** Targeted deep sequencing of cell free DNA is a highly sensitive, noninvasive method to depict tumor mutation profiles, identify druggable mutations in MBC, and predict patient outcome. Our study shed light on the utility of ctDNA as noninvasive "liquid biopsy" in the management of MBC.

## Introduction

Breast cancer is the most common type of cancer in women across the world, and the incidence is rapidly increasing in China.[1] Metastatic breast cancer (MBC) remains an incurable disease; however, an increasing number of targeted therapies have resulted in ever-improving clinical outcomes. Many studies have shown that clonal evolution of MBC can arise in disease progression or following multiple lines of therapy, leading to treatment failure.[2] Thus, understanding the genomic profile of the tumor is critical for managing MBC, especially with respect to the selection of targeted therapies and when switching regimens. Tumor tissue biopsy in MBC is invasive and often inaccessible (e.g. in bone metastasis). However, as next generation sequencing (NGS) technology has advanced, noninvasive molecular profiling of MBC has become available.

Blood-derived circulating tumor DNA (ctDNA) is reported to be detectable in the plasma of patients with advanced malignancy including BC,[3] acting as a potential noninvasive source to characterize the genomic features of tumors.[4] Many studies on ctDNA have used digital PCR techniques to detect mutations in blood[5,6] these techniques are highly sensitive, but molecular profiling information of tumor tissue is still needed. With the recent development of high-throughput DNA sequencing of targeted regions, we are now able to detect and track tumor-specific somatic mutations in cell free DNA (cfDNA) independently.

To explore whether plasma can be used as "liquid biopsy" in Chinese MBC patients, we conducted a pilot study using a commercially available 1021-gene panel tested on plasma samples, paired peripheral blood mononuclear cell (PBMC) samples, and accessible tumor tissue. Herein, we report the

  

genomic profiles and druggable genomic alterations (GAs) in ctDNA from 17 patients with advanced BC during the course of their standard clinical care.

## Methods

### Patient cohort and sample collection

The study cohort consisted of 17 Chinese MBC patients treated at Sun Yat-Sen Memorial Hospital. This was an observational, non-interventional, retrospective study and was conducted in accordance with recognized ethical guidelines. Written informed consent was obtained from all participants. Patients were treated according to physicians' decisions.

All patients were diagnosed with pathologically confirmed BC. Staging investigations were performed in all patients with breast ultrasound, computed tomography (CT) and/or magnetic resonance (MR) scan and evaluated according to National Comprehensive Cancer Network guidelines. We used tumor markers, breast ultrasound, CT and/or MR to monitor disease every six months and/or when disease progressed.

The clinical characteristics of the study cohort are summarized in Table 1. ER, PR, and HER2 status, as well as Ki67 index were assessed in a single laboratory of the Sun Yat-Sen Memorial Hospital Pathology Department using standard criteria.

**Table 1** The clinical characteristics of the study cohort

| Patient characteristics ($n$ = 17) | | |
|---|---|---|
| | Number | Percentage |
| Age at diagnosis (years) | | |
| Mean ± SD | 46 ± 11.20 | |
| Range | 28–62 | |
| Metastatic sites | | |
| 1 | 9 | 52.94 |
| 2 | 5 | 29.41 |
| 3 | 3 | 17.65 |
| ER | | |
| ≥ 10% | 10 | 58.82 |
| < 10% | 7 | 41.18 |
| PR | | |
| ≥ 10% | 3 | 17.65 |
| < 10% | 14 | 82.35 |
| Ki67 | | |
| ≥ 14% | 13 | 76.47 |
| < 14% | 4 | 23.53 |
| *HER2* | | |
| Positive | 2 | 11.76 |
| Negative | 15 | 88.24 |
| Previous therapy | | |
| Yes | 15 | 88.24 |
| No | 2 | 11.76 |

SD, standard deviation.

### Next generation sequencing

A total of 17 blood samples were collected. Blood was processed within one hour of sample collection in ethylenediamine-tetraacetic acid tubes and centrifuged at 3000 rpm for 10 minutes. Plasma was then transferred to new EP tubes and centrifuged at 10 000 rpm to further remove cell debris, and stored at −80°C until DNA extraction. Genomic DNA was extracted from peripheral blood mononuclear cells to generate a reference genome to distinguish germline mutations and single nucleotide polymorphisms (SNPs) for each patient. Archival tumor tissues were also tested if accessible.

Target region capture and enrichment was conducted based on a 1021-gene panel and a customized library provided by Geneplus-Beijing (Beijing, China). All experimental processes were performed following the manufacturer's protocol under strict quality control and assessment. All of the captured DNA fragments were amplified and pooled to obtain multiplex libraries.

All of the samples were sequenced with Illumina 2 × 75 bp paired-end reads on an Illumina HiSeq 3000 instrument according to the manufacturer's recommendations using the TruSeq PE Cluster Generation Kit v3 and the TruSeq SBS Kit v3 (Illumina, San Diego, CA, USA).

### Sequence data analysis

After filtering the adaptor and low-quality sequences from the raw reads, the clean data were mapped to the reference human genome (version hs37d5.fa) aligned with Burrows–Wheeler Aligner (BWA).[7] Somatic small insertions and deletions (indels) and single nucleotide variants (SNVs) were identified using The Genome Analysis Toolkit (https://www.broadinstitute.org/gatk/) and MuTeck,[8] and copy number variations (CNVs) were identified using Contra. PyClone[9] was employed to assess the clonal population structure of ctDNA in each patient. The clonal variant allele frequency (VAF) at each time point was analyzed based on the mean allele fraction (MAF) of mutations contained in the cluster with highest cancer cell fraction (CCF).[10]

### Statistical analysis

A log-rank test was used to assess the association between detection of ctDNA and PFS. Correlations between ctDNA level and clinicopathological markers were assessed using Pearson's chi square test. All statistical analyses and visualizations were performed with GraphPad Prism version 6.0 (La Jolla, CA, USA) or R version 3.4.1 with R package pheatmap, ggplot2 (R Foundation

 

for Statistical Computing, Vienna, Austria). All *P* values are two-sided.

## Results

### Clinical characteristics of the study cohort

Seventeen female patients were enrolled in our study. The average diagnostic age was 46 years. All patients were stage IV. Two patients had primary stage IV BC and were treatment-naive when their blood samples were collected; all other patients had received at least one line of therapy. Of the 17 patients, 10 were ER+/HER2−, 2 were HER2+, and 5 were triple negative BC. The clinical characteristics of the study cohort are summarized in Table 1.

### Somatic mutation profile of circulating tumor DNA (ctDNA) using targeted deep sequencing

Targeted deep sequencing of cfDNA was successfully performed with blood samples collected from the 17 patients. Tumor-specific mutations were identified in cfDNA from the blood samples of all patients (100%), with a median of four somatic mutations per sample (range: 1–9 mutations per sample).

A total of 60 somatic mutations and 1 CNV were detected in the 17 blood samples, with a median MAF of 1.40% (range: 0.06–51.00%). *TP53* (35.29%, 6 patients), and *PIK3CA* (29.41%, 5 patients), were the most frequent mutated genes (Fig 1), which is consistent with the mutation spectrum of primary tumors.[11] *ESR1* (17.65%) and *PTEN* (17.65%) were the third most frequently mutated genes in our study, with mutation frequencies much higher than those reported based on tumor tissue sequencing in the COSMIC database[12] and other studies (*ESR* 7%, *PTEN* 4%).[13]

### ctDNA profile differs among breast cancer of different hormone receptor status

We also compared the mutation profiles of ER positive and negative patients. *PIK3CA* mutations were frequent across different hormone receptor status (30% in ER-positive and 28.57% in ER-negative patients). However,

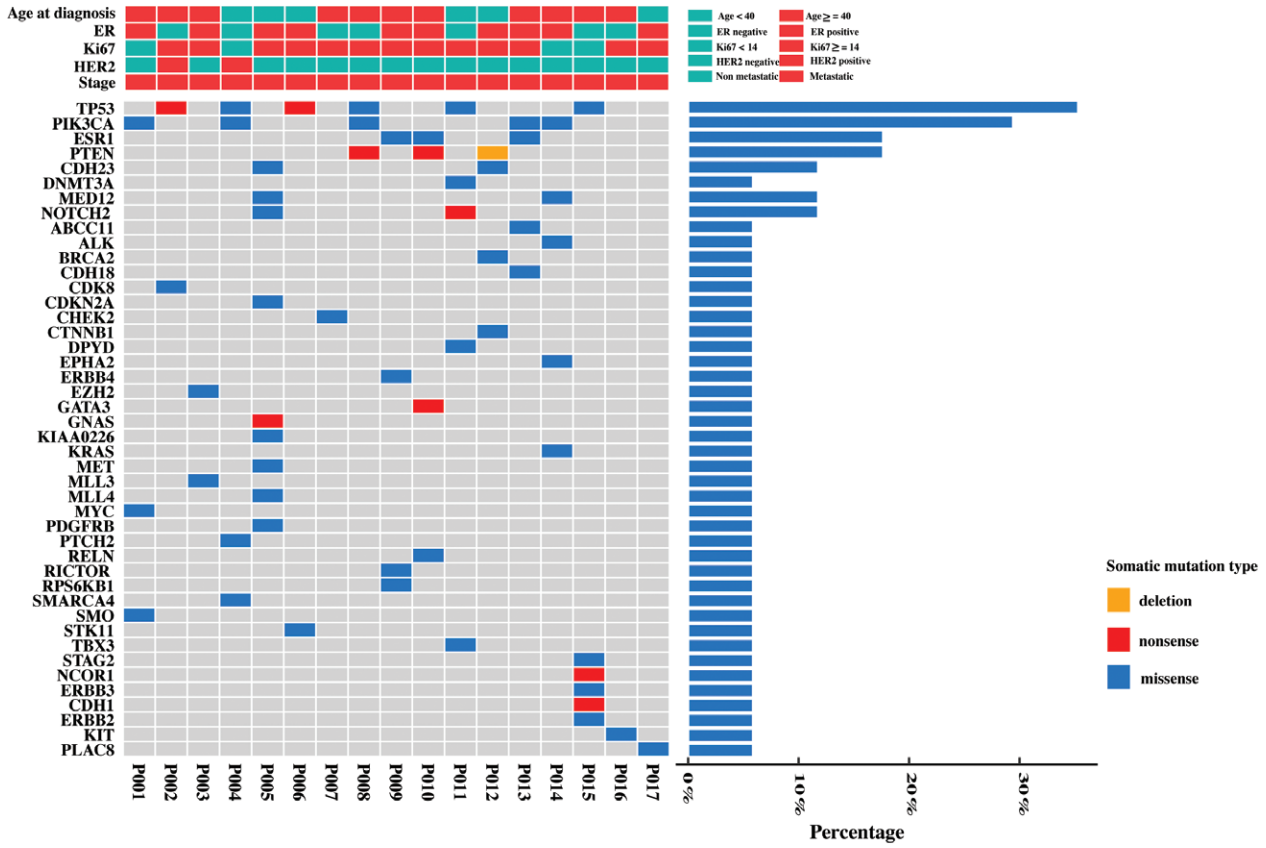


**Figure 1** The frequency and distribution of somatic mutations detected in circulating tumor DNA (ctDNA) of 17 metastatic breast cancer (MBC) patients. The clinical characteristics of 17 MBC patients (upper) and ctDNA profiles among the 17 MBC patients (lower). The mutation frequencies of each gene are shown on the right. Somatic mutation type: (■) deletion, (■) nonsense, (■) missense

  

*TP53* occurred in five out of seven (71.43%) ER-negative patients and only 1 out of 10 (10%) ER-positive patient. All of the *ESR1* mutations were detected in ER-positive patients (3 mutations in 3 patients), which is consistent with the tumor tissue sequencing results of other studies (Fig 2).[11] In addition, we detected *ERBB2* amplification in one patient (P001), whose immunohistochemistry and fluorescence in situ hybridization results were also HER2 positive.

## Concordance of somatic mutations between synchronous and asynchronous tissue and plasma samples

The reliability of ctDNA sequencing has not been fully established and tumor tissue sequencing remains the golden standard. However, invasive procedures are required to procure biopsy samples of MBC and are often difficult to obtain. In our sample, archival tissue samples of five patients were accessible and sequenced (Fig 3). In 80% (4/5) of patients, concordant mutations were found in both tissue and plasma samples. Patient P006 had primary stage IV disease, and paired tumor tissue and blood samples were collected at the same time when the primary tumor was surgically removed. In this case sequencing results of ctDNA and tumor tissue were completely concordant (Fig 3a). Somatic mutations *TP53*$^{W91*}$ and *STK11*$^{L290P}$ were detected in both the patient's tumor tissue and ctDNA. While in the asynchronous samples from P003, P008 and P017, the mutations identified in tissue and plasma were only partly concordant (Fig 3b,e) or completely different (Fig 3c). Only in one patient, P105, who had distant metastases shortly after surgery, the asynchronous tissue and plasma mutations were concordant (Fig 3d).

## Identifications of actionable genomic alterations from ctDNA

We next sought to characterize druggable mutations in the study cohort. We compared somatic mutations detected in the ctDNA of our study cohort with the identified druggable mutations documented in the National Center for Biotechnology Information ClinVar database and previous reports of tissue sequencing results.[14,15] Analysis of ctDNA of the 17 patients revealed that 7 patients harbored a total of 8 druggable somatic mutations and 1 patient had *ERBB2* amplification (Table 2). The most frequent druggable mutations occurred at two hotspots of the *PIK3CA* gene. One was H1047R (4 mutations in 4 patients) at exon 20 encoding the kinase domain, and the other was E542K (detected in 2 two samples of P013) at exon 9 encoding the helical domain. These two hotspot mutations were reported to activate the phosphatidylinositol-3 kinase/protein kinase B/ mammalian target of rapamycin (PI3K/AKT/mTOR) pathway, which diminishes the effects of hormone therapy[16] as well as trastuzumab and lapatinib treatment.[17] However, according to results from BOLERO-2, the beneficial effects of the mTOR inhibitor everolimus are maintained regardless of the *PIK3CA* genotype.[18] The remaining three druggable mutations occurred at the *PTEN* gene, namely, p.K144*, p.Q219*, and p.M134del, which led to the loss of PTEN activity. PTEN can inhibit activity of the PI3K/AKT/mTOR pathway, and *PTEN* gene loss leads to activation of this pathway.[17] Biomarker analyses from BOLERO-1

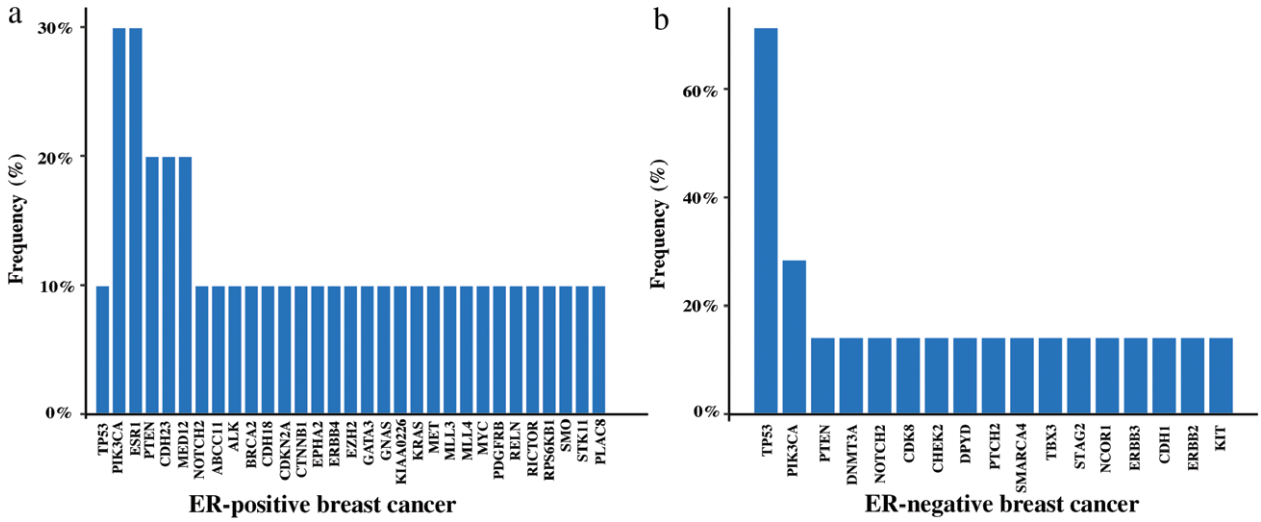


**Figure 2** The distribution of somatic mutations in (**a**) ER-positive and (**b**) ER-negative metastatic breast cancer patients.

 

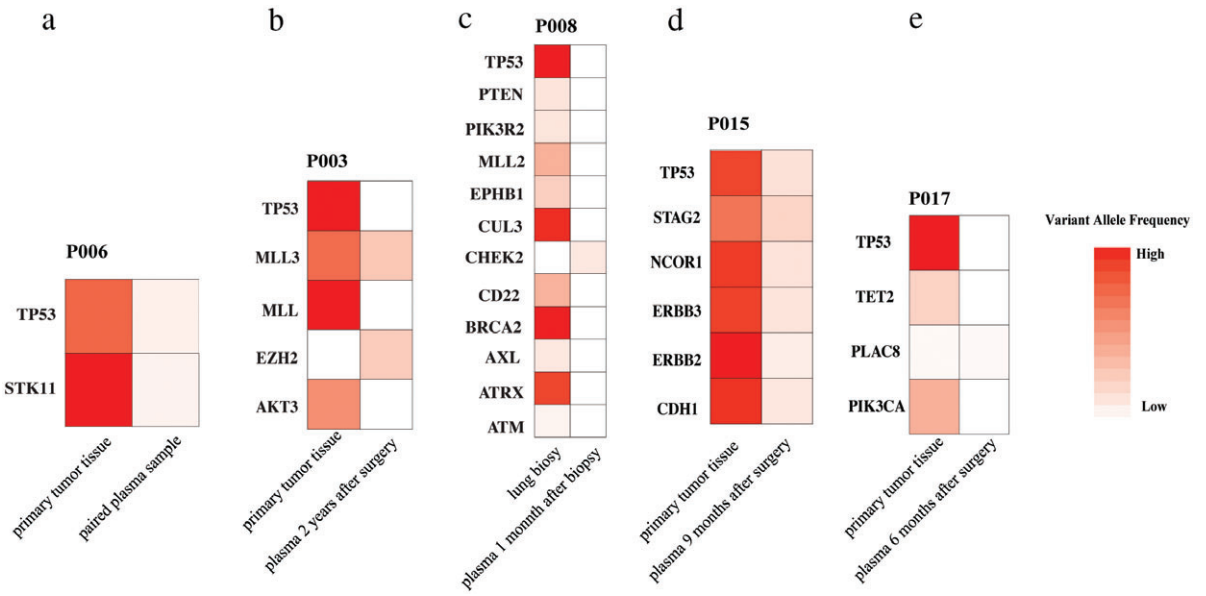


**Figure 3** Somatic mutations detected in synchronous and asynchronous tumor tissue and plasma samples. (**a**) Concordance between tissue and circulating tumor DNA (ctDNA) mutations detected in synchronous tumor tissue and plasma samples. (**b–e**) Tissue mutations and ctDNA mutation assays are not completely concordant in asynchronous tumor tissue and plasma samples.

**Table 2** Druggable mutations detected in cfDNA of the study cohort

| Patient ID | Druggable mutation | Mutation type | cHGVS | pHGVS | Mutant allele frequency (%) | Drug sensitivity not influenced | Resistant drugs |
|---|---|---|---|---|---|---|---|
| P001 | *PIK3CA* | Missense | c.3140A>G | p.H1047R | 1.60 | Everolimus | Trastuzumab/lapatinib |
| P004 | *PIK3CA* | Missense | c.3140A>G | p.H1047R | 1.20 | Everolimus | Trastuzumab/lapatinib |
| P008 | *PIK3CA* | Missense | c.3140A>G | p.H1047R | 7.90 | Everolimus | Trastuzumab/lapatinib |
| P008 | *PTEN* | Nonsense | c.430A>T | p.K144* | 36.50 | Everolimus | Trastuzumab/lapatinib |
| P010 | *PTEN* | Nonsense | c.655C>T | p.Q219* | 39.00 | Everolimus | Trastuzumab/lapatinib |
| P012 | *PTEN* | Deletion | c.402_404delGAT | p.M134del | 3.4 | Everolimus | Trastuzumab/lapatinib |
| P013 | *PIK3CA* | Missense | c.1035T>A | p.N345K | 3.00 | Everolimus | Trastuzumab/lapatinib |
| P014 | *PIK3CA* | Missense | c.3140A>G | p.H1047R | 2.01 | Everolimus | Trastuzumab/lapatinib |

*represents Nonsense mutation (a substitution mutation resulting in a termination codon, foreshortening the translated peptide).
cfDNA, circulating free DNA; cHGVS, complementary DNA human genome variations; pHGVS, protein human genome variations.

and BOLERO-3 trials showed that HER2-positive advanced BC patients with *PTEN* loss in tumors could derive a progression-free survival (PFS) benefit from everolimus.[19,20] Other preclinical studies have suggested that targeting mTOR may restore sensitivity to endocrine therapy in hormone receptor-positive advanced BC patients.[21] Overall, druggable mutations were detected in 50% of our patient cohort. All of the druggable mutations detected in our study cohort were related to the PI3K/AKT/mTOR pathway.

## ctDNA level and clinical outcome in metastatic breast cancer patients

We evaluated the effect of ctDNA mutations on clinical tumor burden and PFS. The VAF of mutations from the major mutated clones (the clone with greatest CCF) was used to access ctDNA levels.[10] Among 17 patients, the ctDNA level varied from 0.06% to 51% (median 2.01%). We found no significant difference between ctDNA level based on the number of metastatic sites or whether there was visceral metastasis (Fig 4a,b), which may be a result of the limited sample size of our study cohort. However, when we further analyzed ctDNA and PFS (16 of 17 patients had available follow-up data), we found patients with higher than median ctDNA levels (> 2.01%) had significantly shorter PFS, and serum tumor marker CA15-3 cannot predict PFS of MBC patients (Fig 4c,d). The median PFS of the high ctDNA level group was less than half the PFS of the low ctDNA level group (138 vs. 386 days; log-rank $P = 0.02$).

  

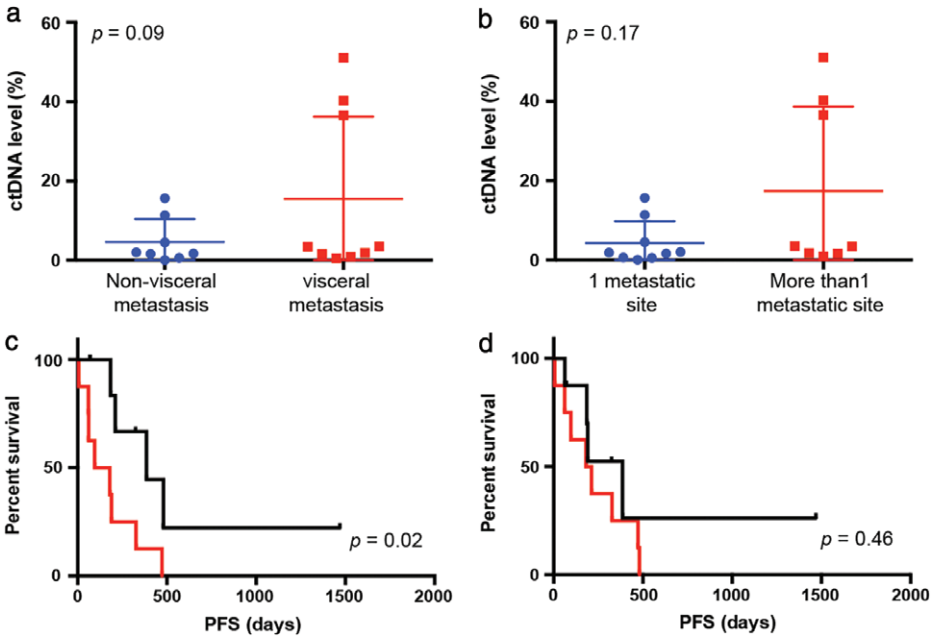


**Figure 4** The association of circulating tumor DNA (ctDNA) level between clinical tumor burden and progression-free survival (PFS). (**a**,**b**) The association between ctDNA level and (**a**) visceral metastasis and (**b**) number of metastatic sites. (**c**) A higher ctDNA level was associated with shorter PFS. (——) low ctDNA level and (——) high ctDNA level. (d) The CA 15-3 level did not predict PFS in metastatic breast cancer patients (——) CA153-low and (——) CA153-high.

## Discussion

Our study characterized the mutation profile in a cohort of Chinese MBC patients. We have shown the feasibility of analyzing ctDNA to characterize genomic alterations in MBC. The high frequency (50%) of druggable mutations among the patients suggests that ctDNA is potentially of great clinical utility in the management of MBC. We also showed that a high ctDNA level is associated with poor PFS in MBC patients.

In our study cohort, ctDNA showed high sensitivity (100%) in plasma derived from MBC patients. In previous reports, ctDNA was detectable in > 75% of patients with advanced malignancies.[22] On one hand, the high tumor burden in our MBC patient cohort (47.06% patients had more than one metastatic site) contributed to the high sensitivity. On the other hand, the rapid development of sequencing techniques in recent years has greatly improved the sequencing depth and coverage of cfDNA assays. The commercial panel we applied in our study covered 1021 genes with a target region of 1.1 Mb, which also contributed to the high sensitivity. In 2013, Dawson *et al.* successfully detected ctDNA in 29 out of 30 women (97%) in whom somatic genomic alterations were pre-identified in tumor tissue.[5] However, in our study we achieved high sensitivity without prior knowledge of tissue mutation using a broad-coverage 1021-gene panel. Although only five tissue samples were available because of the difficulties of sampling, we found that tissue mutation and ctDNA mutation is highly concordant in synchronous paired tissue and plasma samples. In contrast, tissue mutation and ctDNA mutation detected in asynchronous tissue and plasma samples were only partly matched or even completely discordant. These findings suggest that ctDNA can reflect real-time tumor mutation profiles and shows potential tumor clonal evolution during disease progression or under the pressure of treatment.

Another important strength of our study is the high frequency of druggable mutations independently detected in plasma samples without the need for biopsy. In our study cohort, 41.18% of patients ($n$ = 7) had druggable mutations; if we include *ERBB2* amplification, actionable genomic alterations were detected in the blood samples of 47.06% of patients ($n$ = 8). Druggable genomic alterations in tumor tissue have been investigated by many studies across different cancer types.[23,24] In 2017, a large-scale study evaluated druggable mutations in 10 000 metastatic cancer tissue samples of different cancer types.[25] The study revealed that BC ranked third in terms of the prevalence of actionable mutations at 63%, which indicates the importance of genomic profiling in MBC. Our study further shows the utility of ctDNA analysis as a noninvasive method to depict genomic alterations in MBC. The high frequency of druggable mutations, mainly located in *PIK3CA* and *PTEN*, suggests that the PI3K/AKT/mTOR pathway plays an important role in MBC. The PI3K/AKT/mTOR pathway can be targeted by the clinically available mTOR inhibitor everolimus, as shown in BOLERO-2. Currently, the PI3K inhibitor buparlisib has shown promising results in penetrating endocrine-resistant HR+/HER2+ MBC in the phase III clinical trial, BELLE-2.[26] In the BELLE-3 trial, buparlisib plus fulvestrant also showed longer PFS compared to a fulvestrant plus placebo group

 

in HR+/HER2− MBC.[27] Thus, monitoring the presence and dynamics of these mutations is of clinical importance. Notably, *ESR1* mutations were also detected at a high frequency in ER-positive MBC patients. *ESR1* mutations are commonly detected after therapy for metastatic disease and the presence of *ESR1* mutations indicates the development of endocrine resistance, especially aromatase inhibitors.[28] Schiavon *et al.* reported that patients with *ESR1* mutations in their ctDNAs had substantially shorter PFS on subsequent aromatase inhibitor-based therapy.[29] Given these results, although there is currently no targeted therapy for *ESR1* mutations, they should be carefully considered in disease management.[29]

The ctDNA level is reported to be associated with PFS in other cancer types, such as lung cancer.[10] The cfDNA tumor fraction is also reported to be associated with survival in MBC.[5,30] Our study confirms that a higher ctDNA level is associated with shorter PFS in a small cohort of MBC Chinese patients. Our results shed light on the potential of ctDNA as liquid biopsy to depict genomic alterations and identify druggable mutations, which can complement and substitute multiple biopsies in the management of MBC.

## Acknowledgment

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## Disclosure

No authors report any conflict of interest.

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