Abstract

PURPOSE: Accumulation of PIK3CA, ESR1, and GATA3 mutations results in resistance to endocrine therapy in breast cancer patients; however, the response of these genes to chemotherapy is unclear. Therefore, we sought to evaluate the genetic response of circulating tumor DNA (ctDNA) to chemotherapy in metastatic breast cancer patients.

METHODS: The mutation frequency of 1021 genes was examined prior to chemotherapy in ctDNA of 44 estrogen receptor–positive metastatic breast cancer patients. These genes were evaluated again in a subset of patients (n = 24) following chemotherapy. Mutation frequency was defined as the percentage of mutations found in ctDNA compared to total cell-free DNA.

RESULTS: Prior to chemotherapy, PIK3CA was the most commonly mutated gene, with mutation found in 22 of the metastatic breast cancer patients. Following chemotherapy, 16 patients exhibited progressive disease (PD), and 8 patients experienced no progression (non-PD). PIK3CA mutation frequency increased in 56.25% (9/16) of the PD patients but decreased in 62.5% (5/8) of the non-PD patients. As a result, more PD patients exhibited increased PIK3CA mutation frequency than non-PD patients (56.25% vs 0%, P = .002). Further, ESR1 and GATA3 mutations correlated with PIK3CA mutation. Interestingly, patients receiving the mTOR inhibitor everolimus exhibited a lower progression rate (0% vs 62.5%, P = .001), and the combination of everolimus and chemotherapy effectively suppressed PIK3CA, ESR1, and GATA3 gene mutations.

CONCLUSION: Together, these results suggest that mTOR inhibition may be a useful chemotherapy adjuvant to suppress chemotherapy-induced gene mutations that render tumors resistant to endocrine therapy in metastatic breast cancer patients with PD.

Translational Oncology (2019) 12, 764–774

Introduction

Although the 5-year mortality rate of breast cancer has dropped by 34% since 1990, it remains the leading cause of tumor-related death among women [1]. The majority of breast cancer patients benefit from the initial therapy; however, they may eventually develop more aggressive tumor forms, such as metastasized recurrence, that are generally resistant to the treatment [2,3]. In breast cancers, metastasis and drug resistance are often accompanied by genomic instability, alteration of tumor gene subclone changings, and microenvironment-
ESR1 mutation is an independent predictor of poor prognosis for hormone-driven cancers, and low ESR1 expression is a prognostic indicator of aggressive disease and poor survival. In breast cancer patients, about 70% of the tumors express estrogen receptor (ER) and are treated with endocrine therapy [5]. Endocrine therapies include nonsteroidal aromatase inhibitors (anastrozole and letrozole), steroidal aromatase inhibitors ( exemestane), serum ER modulators (tamoxifen, or toremifene), ER downregulators (fulvestrant), etc. However, after 1-5 years of treatment, almost all advanced breast cancer patients eventually become resistant to endocrine therapy [6]. For example, ESR1 mutation plays a key role in the resistance to aromatase inhibitors. Prior to endocrine therapy, ESR1 mutations are rare (<1%) [7], but in advanced patients with previous aromatase inhibitors (AIs) treatment, ESR1 mutations occur more frequently (22%) [8]. Moreover, some studies have reported that ESR1 mutation is an independent predictor of poor prognosis for progression-free survival (PFS) and overall survival [9–11].

GATA3 is another gene which is expressed differentially in ESR1-positive and -negative breast cancers [12]. GATA3 is essential for hormone-driven cancers, and low GATA3 expression is a prognostic indicator of aggressive disease and poor survival [13]. GATA3 mutation occurs approximately 10% for the patients with breast cancer [7]. GATA3 mutation and consequential abnormal expression result in ESR1 ligand activation, leading to endocrine therapy resistance [14].

In addition to the mutation of ESR1 and GATA3, activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signaling pathway also facilitates endocrine therapy resistance in breast cancers [15]. PI3K-AKT-mTOR signaling is one of the most active pathways in breast cancer, and this pathway plays an important role in cell growth, proliferation, survival, and metabolism [16]. Mutations in genes associated with the PI3K-mTOR pathway are common in ER-positive breast cancers. In particular, PIK3CA mutation occurs in approximately 20%-30% of breast cancers [17,18]. Mutations in the PI3K-mTOR pathway can lead to tumor resistance to multiple antitumor agents, including paclitaxel, tamoxifen, trastuzumab, etc. [19]. In addition, the PI3K and ER pathways often play a synergistic role in the tumor progression [20,21].

Derived from cell-free DNA (cfDNA) testing, circulating tumor DNA (ctDNA) analysis is a powerful surveillance tool for effective and continuous detection of potential drug-resistant gene mutations [22–25]. Compared with imaging and serum biomarkers, ctDNA testing provides valuable and sensitive information about gene mutations in tumors after the drug-based therapies. For example, in ER-positive breast cancer patients, mutations in PI3K/AKT pathway genes and ESR1 were detected in 15.1% and 2.7% of patients, respectively, and these mutations predicted treatment failure [26].

In this study, 44 ER-positive metastatic breast cancer (MBC) patients were recruited, and their genetic response to chemotherapy was detected using ctDNA testing. The accumulation of PIK3CA, PIK3R2, TP53, NOTCH2, ERBB2/3, ESR1, and GATA3 gene mutations existed after chemotherapy in resistant patients. Among these genes, accumulation of PIK3/AKT, ESR1 and GATA3 mutations may significantly increase the risk of endocrine therapy resistance. Therefore, our findings suggest that drug resistance to endocrine therapy might emerge after chemotherapy in the progressed ER-positive MBC patients via accumulation of the mutations in the specific genes.

### Materials and Methods

#### Patient Cohort and Clinical Data Collection

This study was approved by the Ethics Committee in Hunan Cancer Hospital. A total of 44 ER-positive MBC patients, who were treated from January 2016 to March 2018, were enrolled in this study. Informed consent was obtained from each patient prior to the study onset. All the recruited patients were diagnosed with ER-positive stage IV primary breast malignant tumor or MBC. Patients were aged between 18 and 70 years old, and the heart, liver, and renal functions of the patients were determined to be adequate enough to tolerate chemotherapy. Basic demographic and clinical information, including age, pathology, laterality, stage, metastatic sites, HR/HER2 status, imaging records, and treatment history, were collected from the patients at the beginning of the study.

#### Immunohistochemistry (IHC) Classification

According to the American Society of Clinical Oncology/College of American Pathologists guidelines, ER- and progesterone receptor (PR)-positive tumors were defined as having a minimum of 1% of invasive tumor cells that stained positive for ER and PR. HER2-positive status was defined as “HER2 IHC 3+” or with HER2 copy number or HER2: CEP17 amplification by fluorescence in situ hybridization. ER-positive breast cancer patients were divided into ER-positive/HER2-negative and ER-positive / HER2-positive subtypes.

#### Blood Sample Collection and DNA Extraction

Peripheral blood samples were collected 7 days before the treatment and at the time of the chemotherapy completion (6 months after the initiation of the treatment). Peripheral blood samples were collected in Streck tubes (Streck, Omaha, NE) and centrifuged within 72 hours to separate the plasma from peripheral blood cells. The cfDNA was extracted from plasma based on a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). Genomic DNA (gDNA) was extracted from peripheral blood cells based on a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Both DNA extractions were performed according to the manufacturer’s instructions. The gDNA was sequenced as the control sample.

#### Target Capture and Next-Generation Sequencing

Both cfDNA and gDNA libraries were constructed with the KAPA DNA Library Preparation Kit (Kapa Biosystems, Wilmington, MA) according to the manufacturer’s protocol. Capture probes were designed to cover the coding sequences and the hot exons of 1021 genes that are frequently mutated in the solid tumors. A detailed description of the capture experiments has been reported previously [27]. Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Integrated DNA Technologies, Coralville, IA). DNA sequencing was performed using the HiSeq 3000 Sequencing System (Illumina, San Diego, CA) with 2x101 bp paired-end reads. Clonal hematopoietic mutations, including those in DNMT3A, IDH1, and IDH2, and specific alterations within ATM, GNAS, and JAK2, were filtered as previously described [28]. Passenger mutations were not filtered, as these alterations are somatic.

#### Sequencing Data Analysis

Terminal adaptor sequences and low-quality reads were removed from the raw data. BWA (version 0.7.12-r1039) 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). Single nucleotide variants were identified using MuTect (version 1.1.4) and NChot, a software developed in-house to review hotspot variants [27]. Small insertions and deletions (indels) were also identified using GATK. Somatic copy number alterations were identified with CONTRA (v2.0.8). Significant copy number variation was expressed as the ratio of adjusted depths between ctDNA and control gDNA. The final candidate variants were all manually verified using the Integrative Genomics Viewer. This sequencing method was found to be credible with simulated cfDNA in a previous report [27]. Therefore, we did not validate the mutations found in ctDNA by sequencing tumor biopsies.

Figure 1. Circulating tumor DNA (ctDNA) gene mutation profiles in 44 ER-positive MBC patients. Each column (labeled with ID number) represents an individual patient.
**ctDNA Gene Mutation Frequency**

Total ctDNA included ctDNA and other normal ctDNA. Mutations in ctDNA were identified by comparison to the reference genome (hg18) and gDNA. The ctDNA mutation frequency was defined as the proportion of ctDNA gene mutations in the total ctDNA. For example, **PIK3CA** mutation frequency was 46.6%, indicating 46.6% ctDNA clones contained **PIK3CA** ctDNA mutation.

**Image Evaluation and Definition of Drug Resistance**

MRI/CT image evaluation was performed every two to three treatment cycles according to RECIST 1.1 standards. In targeted therapy-based treatment trials of MBC patients, PFS closely correlates with overall survival [29,30]. Therefore, in this study, PFS was used to evaluate the drug treatment response. Drug resistance was defined as disease progression within 6 months of treatment.

**Statistical Data Analyses**

Continuous variables were summarized as the mean (standard deviation) and median (interquartile range). Categorical variables were reported as counts (percentage). An analysis of variance (ANOVA) was used to compare the continuous variables with symmetrical distributions across subgroups. Chi-square tests or Fisher's exact tests were used to compare differences among subgroups. Kaplan-Meier curves were used to estimate survival distributions against progression, and the log-rank test was used to assess differences in PFS among subgroups. Fisher's exact tests (n<5) were used to compare differences among subgroups. The ER/HER2 subtypes, demographic characteristics, and clinical features of the patients are summarized in Table S1. All patients were treated with chemotherapy, and half of the total patients received radiotherapy. According to the treatment cycles according to RECIST 1.1 standards. In targeted therapy-based treatment trials of MBC patients, PFS closely correlates with overall survival [29,30]. Therefore, in this study, PFS was used to evaluate the response to drug treatment. For both ER-positive/HER2-negative and ER-positive/HER2-positive subtypes, **TP53**, **PIK3CA**, or **ERBB2** mutation was not significantly associated with PFS (Figure S1). However, the small sample size may have contribution to this finding. The **TP53**+/**PIK3CA**- subgroup (**TP53** mutation and **PIK3CA** wild-type) showed a significantly poorer PFS compared to the **TP53**-/**PIK3CA**- subgroup (wild-type **TP53** and **PIK3CA**). These findings suggest that gene mutations may be potential risk factors for poor prognosis.

**PIK3CA Mutation Frequency Increases in Patients with PD and Decreases in Drug-Sensitive Patients**

To further investigate the relationship between ctDNA mutation and drug response, we compared the overall trend of ctDNA mutation frequency with disease progression in the 24 patients with ctDNA surveillance results. Within 6 months of chemotherapy completion, 16 had PDs, and 8 patients had non-PD. In the PD group (n=16), the top three genes with increased mutation frequencies were **PIK3CA**, **TP53**, and **ERBB2** (Figure 3A). In contrast, the mutation frequencies of **RET**, **FAT1**, and **BRCA1/2** decreased (Figure 3B). Significantly more PD patients had increased **PIK3CA** mutation frequency than non-PD patients (56.25% vs 0%, P=0.002, Figure 3A). On the other hand, although rare mutations, such as **TPH2**, **MLL3**, **MED12**, **EGFR**, etc., increased in the non-PD (drug-sensitive) group (n=8) (Figure 3C), the frequencies of **PIK3CA**, **ERBB2**, and **TP53** mutations primarily decreased (Figure 3D). These findings suggest that alteration in **PIK3CA** mutation frequency is most strongly associated with disease progression and drug response; the mutation frequency of **PIK3CA** increased as disease progressed and decreased when treatment was effective.

**Gene Mutations Render Tumors Resistant to Endocrine Therapy**

In breast cancer, an activating mutation of the **PIK3CA** gene is an upstream event in oncogenic activation of the P13K/AKT/mTOR pathway [31], and P13K-mTOR pathway activation further promotes PD and endocrine therapy resistance [15,19]. Therefore, we next examined the mutation frequency of endocrine therapy-related genes, such as **ESR1**, **GATA3**, and P13K-mTOR-related genes, in the 16 PD patients. In this study, 15/16 PD patients received chemotherapy (Table S3). In the ER-positive/HER2-negative patients who received chemotherapy, 6/12 (50%) (ID=5, 6, 7, 9, 14, 16; Figure 4A) exhibited increased **PIK3CA** gene mutation frequency, including a few instances of multiple mutations in the **PIK3CA** gene. In addition to the **PIK3CA**, chemotherapy also increased the frequency of **ESR1** mutation in 3/9 (33.33%) patients (ID=7, 9, 10; Figure 4A).
Furthermore, chemotherapy increased the frequency of GATA3 mutation in two patients (ID=9, 33, Figure 4A) and AKT1 mutation in one patient (ID=33, Figure 4A). Interestingly, all of these patients also had PI3K-mTOR–related gene mutations (Figure 4A).

In the four ER-positive/HER2-positive PD patients, 3/4 (75%) exhibited increased PIK3CA mutation frequency, and the remaining
patient had increased PIK3R2 mutation frequency (Figure 4B).

In all, these results indicate that endocrine therapy-related gene mutations (ESR1, GATA3, and PI3K-mTOR–related genes) emerged or mutation frequencies increased in 12/15 (80%) PD ER-positive MBC patients after chemotherapy. The remaining three PD patients had increased mutation frequencies of IGF1R (ID=13, Figure 4A) or

Figure 3. The ctDNA gene mutation frequencies changed in patients with PD and in those sensitive to treatment (non-PD). PD was defined in patients who had disease progression within 6 months. Non-PD was defined in patients who were sensitive to treatment and had no disease progression within 6 months. Dark red represents the most common mutated genes and dark blue represents the rarest mutations. If the mutated genes appeared at the same frequency, they are ranked in alphabetic order.(A) In 16 PD patients, the frequencies of mutations in 102 genes were increased. The most common gene with increased mutations was PIK3CA (asterisk (*)) indicates that significantly more PD patients had increased PIK3CA mutation than non-PD patients (56.25% vs 0%, $P = .002$). (B) In 16 PD patients, the frequencies of mutations in 20 genes were decreased. The most common genes with decreased mutations were RET, FAT1, and BRCA1/2. (C) In eight therapy-sensitive patients, only nine genes had increased frequencies of mutation. No mutation was more common. (D) In 8 therapy-sensitive patients, 32 genes had decreased frequencies of mutations. The most common gene with decreased mutations was PIK3CA.

Figure 2. Baseline circulating tumor DNA (ctDNA) gene mutations in ER-positive/HER2-negative and ER-positive/HER2-positive MBC patients. Dark red represents the most common mutated genes, and dark blue represents the rarest mutations. If the mutated genes appeared at the same frequency, they are ranked in alphabetic order. (A) Rank of the baseline ctDNA gene mutations in all ER-positive/HER2-negative patients (left) and in seven individual patients (ID=1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 33, 34, 35, 39, 40, 41, 42, 44). (B) Rank of the baseline ctDNA gene mutations in all ER-positive/HER2-positive patients (left) and in 12 individual patients (ID=20, 25, 26, 27, 28, 29, 30, 31, 36, 37, 38, 43).
Figure 4. Mutated genes with increased frequencies were ranked in ER-positive/HER2-negative and ER-positive / HER2-positive PD patients. Dark blue represents the most common increased mutations in PD patients. (A) Rank of mutated ctDNA genes with increased frequencies in ER-positive/HER2-negative PD patients (n=3, left) and in individual PD patients (ID=4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16, 33). (B) Rank of increased mutation ctDNA genes in ER-positive / HER2-positive PD patients (n=4, left) and in individual PD patients (ID=25, 26, 27, 36).
TP53/NOTCH2 (ID=8, 12, Figure 4A). These gene mutations were most often found in patients with PD, suggesting that they are likely to affect the drug resistance of MBC.

**Everolimus Decreases PIK3CA Mutation Frequency**

The mTOR inhibitor everolimus is a clinically approved anticancer drug used to treat ER-positive patients [32]. In this study, approximately 36% of the ER-positive/HER2-negative and ER-positive/HER2-positive patients had PIK3CA gene mutations prior to chemotherapy (Table S2). A few of these patients received everolimus and chemotherapy (ID=15, 28, 30, 35, 38). The patients who received everolimus in addition to chemotherapy responded to treatment, and their PIK3CA mutation frequency decreased compared to baseline mutations (Figure 5). In addition, the mutation frequency of ESR1 decreased in patient ID15 (Figure 5A). As shown in the Table 1, patients with everolimus treatment exhibited a lower rate of PD within 6 months (0% vs 62.5%, Fisher’s exact test, \( P=.001 \)). Table S4 listed the treatment regimen for non-PD patients with decreased ctDNA mutation frequencies. No significant differences in rates of PD were observed between PIK3CA mutations and wild-type PIK3CA at baseline; however, significantly more PD patients exhibited increased PIK3CA mutation frequency compared to non-PD patients following chemotherapy (56.25% vs 0%, Fisher’s exact test, \( P=.002 \)). Together, these results suggest that everolimus decreased PIK3CA mutation frequencies.

**Discussion**

In MBC patients with massive tumors, clinicians always recommend chemotherapy at first in order to suppress the tumor burden or relieve symptoms as fast as possible. However, unexpected problems may emerge due to the chemotherapy itself. For example, clinicians have found that patients who were resistant to an initial chemotherapy may also be resistant to the endocrine therapy during the subsequent treatment. Investigation of the underlying mechanism of this resistance suggested that mutation of PI3K-AKT pathway–related genes may be a factor contributing [33,34].

We analyzed the baseline ctDNA mutations in 44 ER-positive MBC patients prior to chemotherapy. We found that PI3K-AKT pathway–related genes were frequently mutated in these patients. Therefore, the mTOR inhibitor everolimus was recommended in these patients with PI3K-AKT pathway–related gene mutations. A phase III clinical trial (BOLERO-3) has demonstrated that the addition of everolimus to trastuzumab plus vinorelbine significantly improves PFS for patients with trastuzumab-resistant and taxane-pretreated HER2-positive, advanced breast cancer [35]. The underlying mechanism for this effect was reportedly due to the sensitization of tumor cells to chemotherapy by suppression of the functional activation of mutated PIK3CA [20,36–38]. Although some patients complied with the suggested addition of everolimus to their treatment strategy, others declined due to economic reasons. In patients who received everolimus, mTOR pathway–related gene

**Figure 5.** Decreased gene mutations in sensitive MBC patients (\( n=8 \)). Dark blue represents the most common decreased mutations in sensitive patients. (A) In ER-positive /HER2-negative therapy-sensitive patients (\( n=3 \)), the frequencies of mutations in ESR1 (ID=15) and PIK3CA (ID=15, 35) decreased. (B) In ER-positive /HER2-positive non-PD patients (\( n=5 \)), the frequencies of mutations in PIK3CA decreased in three patients (ID=28, 30, 38). Two patients had decreases in the frequencies of RAF1 and SF3B1 gene mutation.
(PIK3R2 and PIK3CA) mutation frequencies decreased (Figure 5), whereas patients who did not receive everolimus had increased PIK3CA, PIK3R2, and AKT1 mutation frequencies (Figure 4). These results support that ER-positive MBC patients might benefit from everolimus in conjunction with chemotherapy.

Apart from the PI3K-AKT pathway genes, ESR1 and GATA3 are endocrine therapy resistance genes in breast cancer [6,14,19]. In ER-positive patients, the presence of ESR1 mutations following endocrine therapy indicates treatment resistance [39–43]. In this study, 31/44 (70.45%) patients had a history of endocrine treatment (Table S1); however, ESR1 mutation was not common, with only two ER-positive/HER2-negative (ID=7, 15) patients presenting with baseline ESR1 mutations. Interestingly, both of these patients also had baseline PIK3CA mutations. One of these patients (ID=7, Table S3) received chemotherapy alone and progressed within 6 months with increased mutation frequencies for both ESR1 and PIK3CA (Figure 4). In contrast, the other non-PD patient (ID=15, Table S4) received everolimus plus chemotherapy and was controlled without progression and with decreased mutation frequencies for both ESR1 and PIK3CA (Figure 5). Moreover, in one patient (ID=9) who had no ESR1 mutation prior to treatment (Figure 2B), ESR1 mutation emerged as PIK3CA and GATA3 mutation frequencies increased and the disease progressed (Figure 4B). These findings suggest that the mutation of PIK3CA and ESR1 varies with progression in MBC patients.

Furthermore, the frequencies of GATA3 gene mutation also increased in two PD patients (ID=33, 9). In one of these patient (ID=33), the GATA3 mutation frequency increased as AKT1 mutation frequency increased. In the other patient (ID=9), the GATA3 mutation frequency increased as both PIK3CA and ESR1 mutation frequencies increased (Figure 4, A and B). Therefore, both GATA3 and ESR1 mutations seem to be coupled with mutation of the PI3K-AKT pathway genes.

Interestingly, in this study we observed chemotherapy-induced selection of preexisting mutations as well as new mutations that arose after chemotherapy. More specifically, some patients had preexisting mutations, and these ctDNA mutation frequencies increased after treatment, indicating the resistance of mutation bearing clones to chemotherapy in patients with PD. In addition, some patients did not have preexisting mutations prior to chemotherapy, and tumor gene mutations emerged after treatment. For example, patient ID33 (Figure 4A) did not have a GATA3 mutation before chemotherapy, but after treatment, the GATA3 mutation frequency was 1.6%. Occasionally, these two phenomena coexisted. For example, patient ID5 did not have a PIK3CA amplification mutation before chemotherapy, but after capcitabine treatment, the PIK3CA amplification mutation frequency was 3.2%. Moreover, this patient also had a PIK3CA H1047R mutation frequency of 42.4% before treatment that increased to 59.6% after chemotherapy.

tDNA mutation is complicated, with both time and space heterogeneity. It is difficult to divide ctDNA mutation into just two or three types. Each mutation may be significant for an individual patient. In this study, we summarized the overall trend of ctDNA mutations following chemotherapy in ER-positive patients. We aimed to provide important and valuable clues for clinicians.

At baseline, PIK3CA gene mutations were common in both HER2-negative and HER2-positive patients. After treatment, the mutation frequency of PIK3CA increased in the majority of ER-positive PD patients. Chemotherapy is a common choice for treatment of MBC; however, baseline TP53 mutation predicts a poor response to chemotherapy in breast cancer patients [44]. Indeed, we found that PIK3CA wild-type patients with baseline TP53 mutation had a significantly poorer PFS than patients without baseline TP53 mutation (P=.04, Figure S1F). PIK3CA mutation has been found to be positive prognostic indicator for overall survival and breast cancer-specific survival in 590 patients (at a single center) and 2587 patients (from 12 independent studies) [45,46]. However, due to the small sample size of this study, this benefit was not significant, and patients with baseline PIK3CA mutation failed to show improved PFS (Figure S1B).

Tumors acquire resistance to systemic treatment as a result of clonal selection [47]. PIK3CA p.E545K mutation is associated with chemoresistance in breast epithelial cells [48], and its mutation frequency increases significantly after paclitaxel treatment [47]. Our study further confirmed this finding, supporting an alternative treatment regimen involving everolimus in ER-positive patients by inhibiting the mTOR pathway.

**Conclusions**

In ER-positive MBC patients with tumor progression, the baseline ctDNA mutation patterns varied across ER/HER2 subgroups. After chemotherapy, ESR1 and GATA3 mutations were coupled with PI3K-AKT1 pathway–related gene mutations. Everolimus treatment in conjunction with chemotherapy suppressed PIK3CA, ESR1, and GATA3 gene mutation. In conclusion, gene mutations that render tumors resistant to endocrine therapy may be suppressed by concomitant mTOR inhibitor treatment. Since our study was limited by a relatively small sample size, future studies should include a larger cohort to compare ctDNA mutation within treatment subgroups and focus on investigation of the underlying effects of everolimus on ctDNA mutations in chemotherapy-resistant MBC patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.02.014.

**Acknowledgements**

We would like to thank the researchers from Genplus Beijing Institute as these individuals sequenced all patients’ ctDNA and gDNA samples for this study.
Author Contributions
Dr. Quchang Ouyang had full access to all data in the study and takes responsibility for the integrity and accuracy of the data analysis.
Study concept and design: Quchang Ouyang and Zheyu Hu
Data acquisition: Yu Tang
Data analysis and interpretation: Zheyu Hu
Drafting of the manuscript: Zheyu Hu
Critical revision of the manuscript for important intellectual content: All authors

Compliance with Ethical Standards
Disclosure of potential conflicts of interest
All authors declared no potential conflicts of interest.

Research Involving Human Participants and/or Animals
This study involved human participants and was approved by the Ethics Committee at Hunan Cancer Hospital. Informed consent was obtained from each patient prior to study onset.

References
[1] DeSantis C, Ma J, Bryan L, and Jemal A (2014 Jan-Feb). Breast cancer statistics, 2013. CA Cancer J Clin. 64(1), 52–62.
[2] Hu W, Tan C, He Y, Zhang G, Xu Y, and Tang J (2018). Functional miRNAs in breast cancer drug resistance. Onco Targets Ther. 11, 1529–1541.
[3] O’Driscol L and Clynes M (2006 Aug). Biomarkers and multiple drug resistance in breast cancer. Curr Cancer Drug Targets. 6(5), 365–384.
[4] Aparicio S and Caldas C (2013 Feb 28). The implications of clonal genome evolution for cancer medicine. N Engl J Med. 360(9), 842–851.
[5] Ariazi EA, Ariazi JL, Cordera F, and Jordan VC (2006). Estrogen receptors as therapeutic targets in breast cancer. Curr Top Med Chem. 6(3), 181–202.
[6] Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, and Cao X, et al (2013 Dec). Comprehensive molecular portraits of human breast tumours (2012 Oct 4).
[7] O’Driscoll L and Clynes M (2006 Aug). Biomarkers and multiple drug resistance in breast cancer drug resistance. Curr Cancer Drug Targets. 6(5), 365–384.
[8] Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, and Cao X, et al (2013 Dec). Comprehensive molecular portraits of human breast tumours (2012 Oct 4).
[9] DeSantis C, Ma J, Bryan L, and Jemal A (2014 Jan-Feb). Breast cancer statistics, 2013. CA Cancer J Clin. 64(1), 52–62.
[10] Usary J, Llaca V, Karaca G, Presswala S, Karaca M, and He X, et al (2017 Aug). Kinetics, prognostic and predictive values of ESR1 circulating mutations in metastatic breast cancer patients progressing on aromatase inhibitor. Oncotarget. 8(23), 38056–38060.
[11] Usary J, Llaca V, Karaca G, Presswala S, Karaca M, and He X, et al (2017 Aug). Kinetics, prognostic and predictive values of ESR1 circulating mutations in metastatic breast cancer patients progressing on aromatase inhibitor. Oncotarget. 8(23), 38056–38060.
[12] Tie J, Kinde I, Wang Y, Wong HL, Roeber J, and Christie M, et al (2015 Aug). Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol. 26(8), 1715–1722.
[13] Khan K, Rata M, Cunningham D, Koh DM, Tunariu N, and Hahne JC, et al (2017 Aug). Functional imaging and circulating biomarkers of response to regorafenib in treatment-refractory metastatic colorectal cancer patients in a prospective phase II study. Gut. , 68.
[14] Cheng H, Liu C, Jiang J, Luo G, Lu Y, and Jin K, et al (2017 May 15). Analysis of ctDNA to predict prognosis and monitor treatment responses in metastatic pancreatic cancer patients. Int J Cancer. 140(10), 2344–2350.
[15] Guibert N, Mazieres J, Delaunay M, Casanova A, Farella M, and Keller L, et al (2017 Jun 66). Monitoring of KRAS-mutated ctDNA to discriminate pseudo-progression from true progression during anti-PD-1 treatment of lung adenocarcinoma. Oncotarget. 8(23), 38056–38060.
[16] Zhou et al. (2019). Chemotherapy Modulates Endocrine Therapy-Related Resistance Mutations. Translational Oncology. Vol. 12, No. 5, 2019.
[17] Bachman KE, Argani P, Samuels Y, Silliman N, Prat J, and Szabo S, et al (2004 Aug). The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol Ther. 3(8), 772–775.
[18] Saal LH, Holm K, Maurer M, Memeo I, Su T, and Wang X, et al (2005 Apr 1). PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res. 65(7), 2554–2559.
[19] Brown KK and Toker A (2015). The phosphoinositide 3-kinase pathway and therapy resistance in cancer. F1000Prime Rep. 7, 13.
[20] Basela J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2012 Feb 9;366(6):520-9.
[21] Zardavas D, Fumagalli D, and Loi S (2012 Nov). Phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin pathway inhibition: a breakthrough in the management of luminal (ER+/HER2-) breast cancers? Curr Opin Oncol. 24(6), 623–634.
[22] Cheng H, Liu C, Jiang J, Luo G, Lu Y, and Jin K, et al (2017 May 15). Analysis of ctDNA to predict prognosis and monitor treatment responses in metastatic pancreatic cancer patients. Int J Cancer. 140(10), 2344–2350.
[23] Guibert N, Mazieres J, Delaunay M, Casanova A, Farella M, and Keller L, et al (2017 Jun 66). Monitoring of KRAS-mutated ctDNA to discriminate pseudo-progression from true progression during anti-PD-1 treatment of lung adenocarcinoma. Oncotarget. 8(23), 38056–38060.
[24] Tie J, Kinde I, Wang Y, Wong HL, Roeber J, and Christie M, et al (2017 Aug). Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Ann Oncol. 26(8), 1715–1722.
[25] Khan K, Rata M, Cunningham D, Koh DM, Tunariu N, and Hahne JC, et al (2017 Aug). Functional imaging and circulating biomarkers of response to regorafenib in treatment-refractory metastatic colorectal cancer patients in a prospective phase II study. Gut. , 68.
[26] Takeshita T, Yamamoto Y, Yamamoto-Ibusuki M, Tomiguchi M, Sueta A, and Murakami K, et al (2018 Feb 26). Clinical significance of plasma cell-free DNA mutations in PIK3CA, AKT1, and ESR1 gene according to treatment lines in ER-positive breast cancer. Mol Cancer. 17(1), 67.
[27] Yang X, Chu Y, Zhang R, Han Y, Zhang L, and Fu Y, et al (2017 Jul). Technical validation of a next-generation sequencing assay for detecting clinically relevant levels of breast cancer-related single-nucleotide variants and copy number variants using simulated cell-free DNA. J Mol Diagn. 19(4), 525–536.
[28] Phallen J, Sausen M, Adleff V, Leal A, Hruban C, and White J, et al (2017 Aug). Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl Med. 9(403), 16.
[29] Saad ED and Katz A (2009 Mar). Progression-free survival and time to progression as primary end points in advanced breast cancer: often used, sometimes loosely defined. Ann Oncol. 20(3), 460–464.
[30] Li L and Pan Z (2017 Jul). Progression-free survival and time to progression as real surrogate end points for overall survival in advanced breast cancer: a meta-analysis of 37 trials. Clin Breast Cancer. 25.
[31] Raphael J, Desautes D, Pritchard KL, Petkova E, and Shah PS (2018 Mar). Phosphoinositide 3-kinase inhibitors in advanced breast cancer: a systematic review and meta-analysis. Eur J Cancer. 91, 38–46.
[32] Myonnamah NE, Chen D, He W, Sung P, Samoila A, and You D, et al (2017 Mar 14). Correlation between PIK3CA mutations in cell-free DNA and everolimus efficacy in HR(+) HER2(-) advanced breast cancer: results from BOLORE-2. Br J Cancer. 116(6), 726–730.
[33] Chen IC, Hsiao LP, Huang IW, Yu HC, Yeh LC, and Lin CH, et al (2017 Aug 29). Phosphatidylinositol-3-kinase inhibitors, buparlisib and alpelisib, sensitize estrogen receptor-positive breast cancer cells to tamoxifen. Sci Rep. 7(1), 9842.
[34] Sabine VS, Crozier C, Brookes CL, Drake C, Piper T, and van de Velde CJ, et al (2014 Sep 20). Mutational analysis of PI3K/AKT signaling pathway in tamoxifen exemestane adjuvant multidisciplinary patholog. J Clin Oncol. 32(27), 2951–2958.
[35] Andre F, O’Regan R, Osrugroh M, Toi M, Xu B, and Jerusalem G, et al (2014 May). Everolimus for women with trastuzumab-resistant, HER2-positive, advanced breast cancer (BOLORE-3): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet Oncol. 15(6), 580–591.
[36] Di Nicolantonio F, Arena S, Tabernero J, Grosso S, Molinari F, and Macarulla T, et al (2010 Aug). Deregression of the PI3K and KRAS signaling pathways in human cancer cells determines their response to everolimus. J Clin Invest. 120(8), 2858–2866.
[37] Peng T and Dou QP (2017 Sep). Everolimus inhibits growth of gemcitabine-resistant pancreatic cancer cells via induction of caspase-dependent apoptosis and G2/M arrest. J Cell Biochem. 118(9), 2722–2730.
Martin LA, Pancholi S, Farmer I, Guest S, Ribas R, and Weigel MT, et al (2012 Oct 17). Effectiveness and molecular interactions of the clinically active mTORC1 inhibitor everolimus in combination with tamoxifen or letrozole in vitro and in vivo. Breast Cancer Res. 14(5), R132.

Amedos M, Vicier C, Loi S, Lefebvre C, Michiels S, and Bonnfoi H, et al (2015 Dec). Precision medicine for metastatic breast cancer—limitations and solutions. Nat Rev Clin Oncol. 12(12), 693–704.

Spoerke JM, Gendreau S, Walter K, Qiu J, Wilson TR, and Savage H, et al (2016 May 13). Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. Nat Commun. 711579.

Fribbens C, Garcia Murillas I, Beaney M, Hrebien S, O’Leary B, and Kilburn L, et al. Tracking evolution of aromatase inhibitor resistance with circulating tumour DNA analysis in metastatic breast cancer. Ann Oncol. 2018 Jan 1;29(1):145-53.

Schiavon G, Hrebien S, Garcia-Murillas I, Cutts RJ, Pearson A, and Tarazona N, et al (2015 Nov 11). Analysis of ESR1 mutation in circulating tumor DNA demonstrates evolution during therapy for metastatic breast cancer. Sci Transl Med. 7(313), 313ra182.

Fribbens C, Garcia Murillas I, Beaney M, Hrebien S, O’Leary B, and Kilburn L, et al (2017 Oct). Tracking evolution of aromatase inhibitor resistance with circulating tumour DNA analysis in metastatic breast cancer. Ann Oncol. 4.

Andersson J, Larsson L, Klaar S, Holmberg L, Nilsson J, and Inganas M, et al (2005 May). Worse survival for TP53 (p53)-mutated breast cancer patients receiving adjuvant CMF. Ann Oncol. 16(5), 743–748.

Kalinsky K, Jacks LM, Heguy A, Patel S, Drobnjak M, and Bhansot UK, et al (2009 Aug 15). PIK3CA mutation associates with improved outcome in breast cancer. Clin Cancer Res. 15(16), 5049–5059.

Dumont AG, Dumont SN, and Trent JC (2012 Jul). The favorable impact of PIK3CA mutations on survival: an analysis of 2587 patients with breast cancer. Chin J Cancer. 31(7), 327–334.

Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, and Piskorz AM, et al (2013 May 2). Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature. 497(7447), 108–112.

Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, and Pearlman RV, et al (2005 Dec 1). Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res. 65(23), 10992–11000.