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Chemotherapy Modulates Endocrine Therapy-Related Resistance Mutations in Metastatic Breast Cancer

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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.

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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 microenvironmen-

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tal selective pressure [4]. The constantly varied variation in gene mutations leads to tumor heterogeneity, limiting the effectiveness of targeted therapy.

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 fluorescent *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 2×101-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.

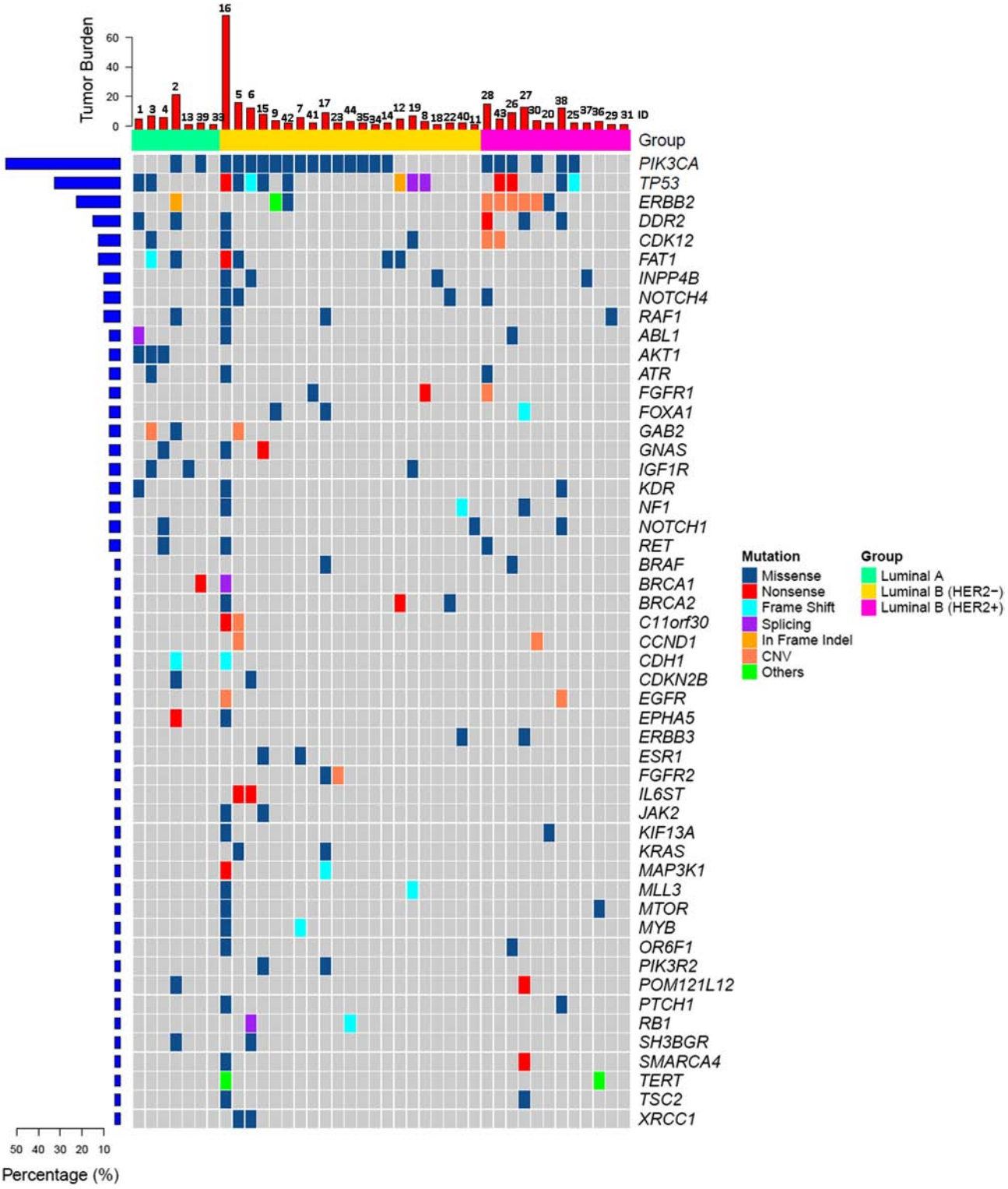


Figure 1. Circulating tumor DNA (ctDNA) gene mutation profiles in 44 ER-positive MBC patients. Each column (labeled with ID number) represents an individual patient.

ctDNA Gene Mutation Frequency

Total cfDNA included ctDNA and other normal cfDNA. 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 cfDNA. For example, *PIK3CA* mutation frequency was 46.6%, indicating 46.6% cfDNA 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 (n<5) 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 the gene mutation trends between progressive disease (PD) and non-PD groups after treatment. Due to the small sample size, Fisher's exact tests (n<5) were performed to evaluate the effect of the mTOR inhibitor. All statistical tests were two-tailed and conducted at a significance level of .05. Statistical analyses were conducted using SAS 9.4 (Cary, NC).

Results

Demographic and Clinical Features of Patients

A total of 44 ER-positive MBC patients were included in this study. The ER/HER2 subtypes, demographic characteristics, and clinical features of the patients are summarized in Table S1. All patients were female. The average age at the first diagnosis of breast cancer was 44.1 years old. Most patients (95.45%) underwent primary tumor surgery, and half of the total patients received radiotherapy. According to the biopsy IHC results, 31 patients had ER-positive/HER2-negative breast cancer, and 13 patients had ER-positive /HER2-positive breast cancer. Patients progressed to MBC in an average of 4 years and provided samples for the follow-up ctDNA test analysis (Table S1).

At the time of the recruitment, there were no significant differences in lymph nodes, distant metastases, or treatment history observed among patients within each ER/HER2 subtype. Anti-HER2 target therapy was only performed in ER-positive/HER2-positive patients. After MBC was diagnosed, the majority of patients had received one to three rounds (lines) of chemotherapy, endocrine therapy, or targeted therapy. Unfortunately, the response of these patients to treatment was not satisfactory, resulting in continued tumor growth continuation and an unclear treatment plan.

Baseline Mutation Profiling of ER-Positive MBC Patients

ctDNA testing was performed prior to beginning a new treatment regimen. Among the 44 recruited patients, 28/31 (90.32%) ER-positive/HER2-negative patients and 12/13 (92.31%) ER-positive /HER2-positive patients had tumor gene mutations (Figure 1). *PIK3CA* was the most frequently mutated gene in both HER2-negative and HER2-positive MBC patients (Table S2). *TP53* mutation was also common in ER-positive/HER2-negative and ER-positive/HER2-positive patients. More specifically, 3/31 ER-positive/HER2-negative patient had an *ERBB2* mutation (Figure 2*A*), with one frame shift (p.Y772_A775dup, ID=2), one missense mutation (p.S310F, ID=42), and one in-frame indel (p.G776delins, ID=9). *ERBB2* mutations were present in 6/13 (46.15%) ER-positive/HER2-positive patients, with 5 *ERBB2* amplifications and 1 missense mutation (p.S280F, ID=20, Figure 2*B*). In the ER-positive /HER2-positive subtype, one patient had both an *ERBB2* amplification and a missense mutation (p.E844K, ID=27), and one patient had an *ERBB2* amplification and two other missense mutations (p.F279I and p.V670G, ID=3, Figure 2*B*). All *AKT1* mutations were concentrated in the ER-positive/HER-positive subtype (4/31, 12.90%, Figure 2*A*).

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

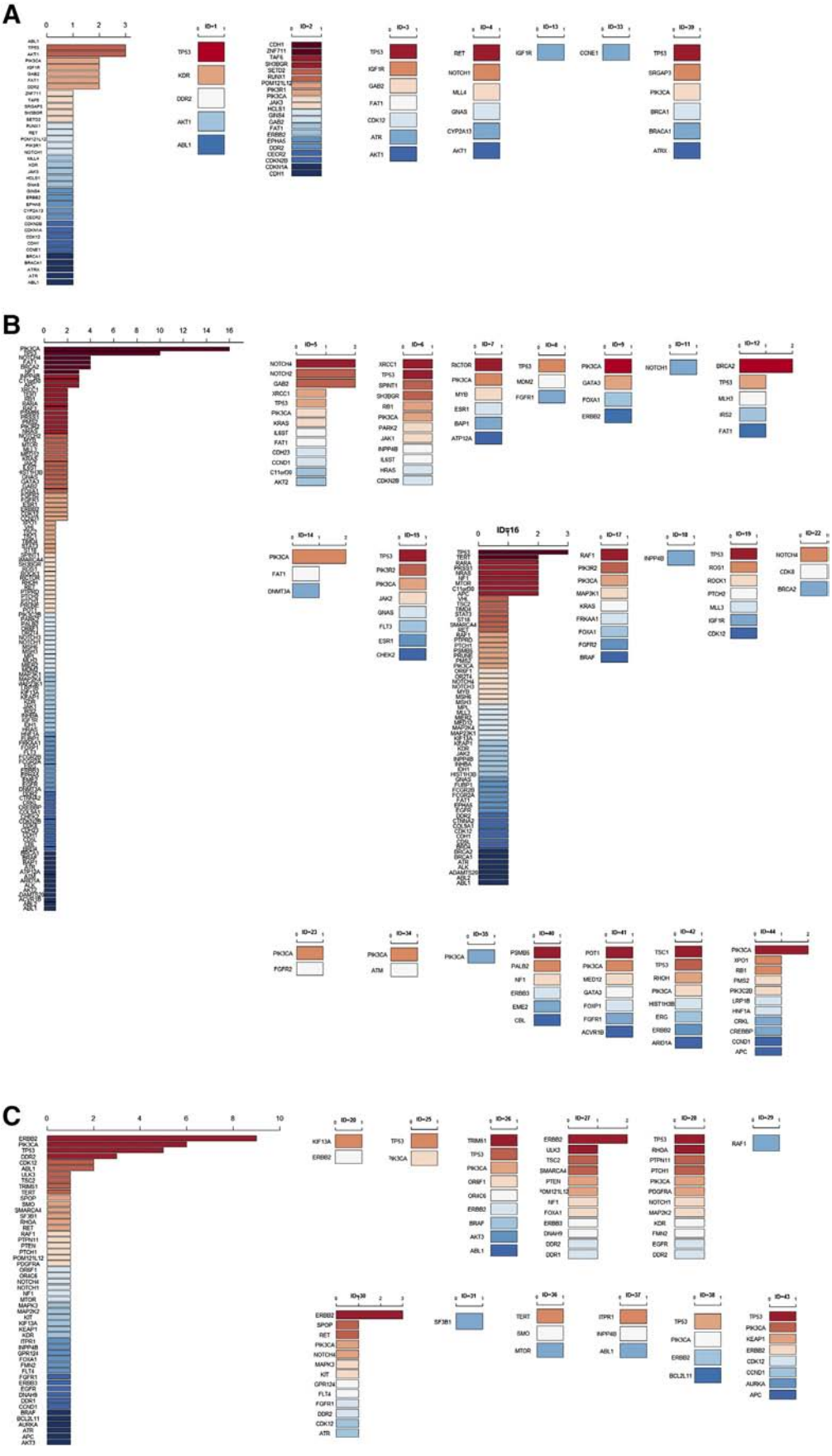
To further investigate the relationship between ctDNA mutation and drug response, we compared the overall trend of ctDNA mutation frequency with disease progression in the 24 patients with ctDNA surveillance results. Within 6 months of chemotherapy completion, 16 had PDs, and 8 patients had non-PD. In the PD group (n=16), the top three genes with increased mutation frequencies were *PIK3CA*, *TP53,* and *ERBB2* (Figure 3*A*). In contrast, the mutation frequencies of *RET*, *FAT1,* and *BRCA1/2* decreased (Figure 3*B*). Significantly more PD patients had increased *PIK3CA* mutation frequency than non-PD patients (56.25% vs 0%, P=.002, Figure 3*A*). 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 3*C*), the frequencies of *PIK3CA*, *ERBB2,* and *TP53* mutations primarily decreased (Figure 3*D*). 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 PI3K/AKT/mTOR pathway [31], and PI3K-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 PI3K-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 4*A*) 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 4*A*).

Furthermore, chemotherapy increased the frequency of *GATA3* mutation in two patient (ID=9, 33, Figure 4*A*) and AKT1 mutation in one patient (ID=33, Figure 4*A*). Interestingly, all of these patients also had PI3K-mTOR–related gene mutations (Figure 4*A*).

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



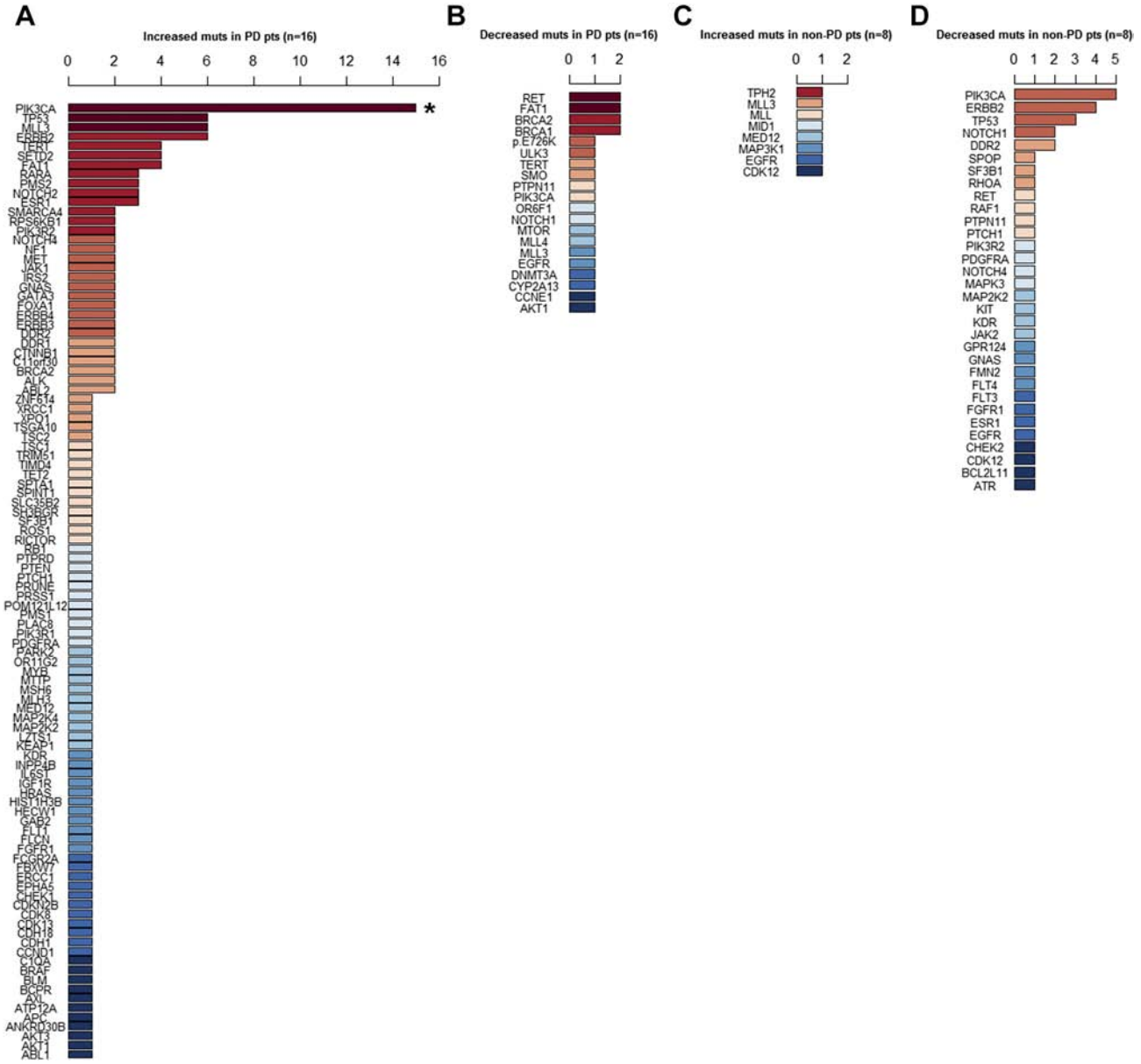


Figure 3. The ctDNA gene mutation frequencies changed in patients with PD and in those sensitive to treatment (non-PD). PD was defined in patients who had disease progression within 6 months. Non-PD was defined in patients who were sensitive to treatment and had no disease progression within 6 months. 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*.(C0 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*.

patient had increased *PIK3R2* mutation frequency (Figure 4*B*). 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 4*A*) or

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).

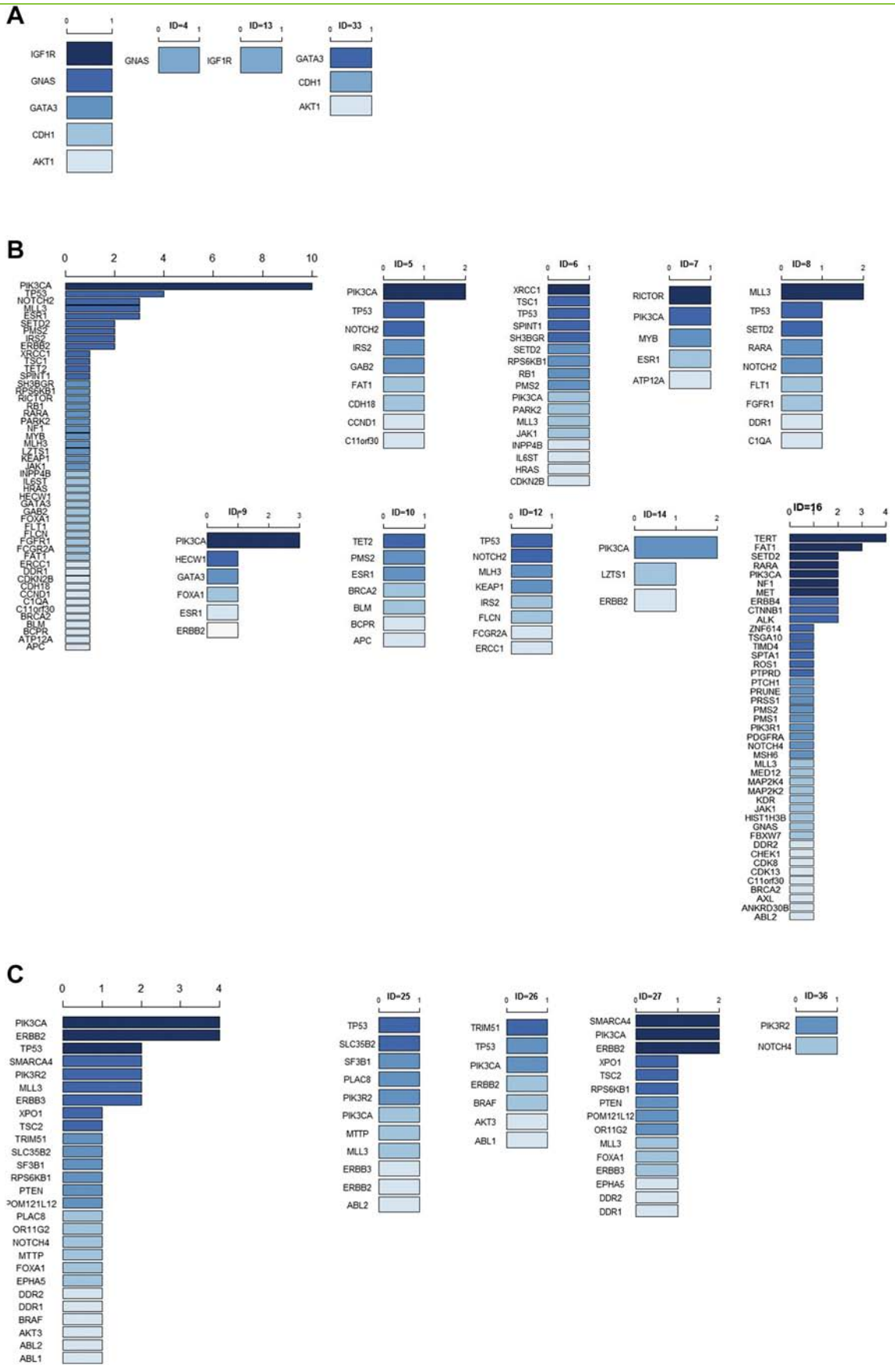


Figure 4. Mutated genes with increased frequencies were ranked in ER-positive/HER2-negative and ER-positive / HER2-positive PD patients. Dark blue represents the most common increased mutations in PD patients.(A) Rank of mutated ctDNA genes with increased frequencies in ER-positive/HER2-negative PD patients (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).

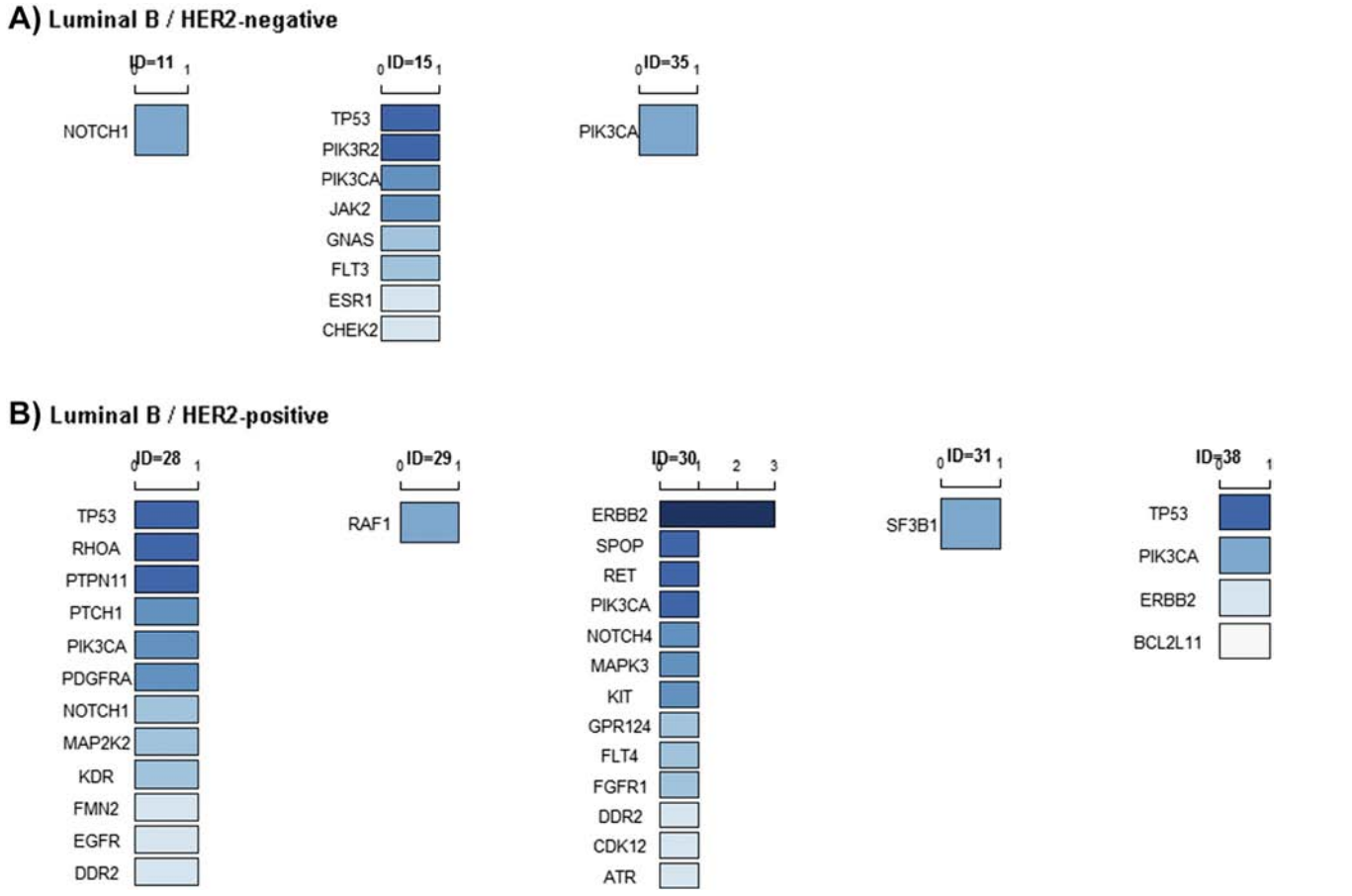


Figure 5. Decreased gene mutations in sensitive MBC patients ($n=8$). Dark blue represents the most common decreased mutations in sensitive patients.(A) In ER-positive /HER2-negative therapy-sensitive patients ($n=3$), the frequencies of mutations in *ESR1* (ID=15) and *PIK3CA* (ID=15, 35) decreased.(B) 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.

TP53/NOTCH2 (ID=8, 12, Figure 4*A*). 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 5*A*). 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 *PI3KCA* 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

Table 1. Effect of Everolimus Treatment on PFS

		Subgroups		
Variable		PD (n=16)	Non-PD (n=8)	P value
Treatment	Everolimus+chemotherapy	0 (0%)	5 (62.5%)	.001
	Chemotherapy	16 (100%)	3 (37.5%)	
Baseline PIK3CA mutation	Yes	8 (50%)	5 (62.5%)	.68
PIK3CA mutation frequency	Increased	9 (56.25%)	0 (0%)	.002
	Decreased	1 (6.25%)	5 (62.5%)	

P value was calculated using Fisher's exact test (n<5) for subgroup comparison between the indicated subgroups.

(*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 2*B*), *ESR1* mutation emerged as *PIK3CA* and *GATA3* mutation frequencies increased and the disease progressed (Figure 4*B*). 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 4*A*) 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 capecitabine 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.

ctDNA 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 S1*F*). *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 S1*B*).

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 everlolimus 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.

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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

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Data analysis and interpretation: Zheyu Hu

Drafting of the manuscript: Zheyu Hu

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest

All authors declared none 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.

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