Article

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Characterizing the Efficacy and Safety of Chemotherapy Plus Everolimus in HER2-Negative Metastatic Breast Cancer Harboring Altered PI3K/AKT/mTOR

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Background: The clinical outcomes of chemotherapy (CT) for the treatment of metastatic triple-negative (TN) and hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2-) metastatic breast cancer (mBC) have proven to be disappointing. The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway, a tumor-promoting signaling cascade frequently mutated in breast cancer (BC), has been implicated in chemoresistance. In this study, our objective is to investigate the efficacy and safety of combining everolimus with chemotherapy in mBC patients exhibiting mutations in the PI3K/AKT/mTOR pathway.

Methods: We conducted a retrospective analysis to characterize the efficacy, safety, and their association with clinical and molecular characteristics of metastatic lesions in 14 patients with HER2- mBC. These patients harbored at least one altered member of the PI3K/AKT/mTOR signaling pathway and were treated with a combination of a chemotherapy agent and the mTOR inhibitor everolimus (CT+EVE).

Results: The majority of patients belonged to the triple-negative (TN) subtype (9/14, 64.3%), having already undergone 2 lines of chemotherapy (CT) in the metastatic setting (11, 78.6%). These patients carried altered phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and were administered a vinorelbine-containing regimen (10, 71.4%). The objective response rate (ORR) was 42.9%, with a disease control rate of 92.9%. The median progression-free survival (PFS) and overall survival (OS) were 5.9 (95% confidence interval (CI): 4.9–13.6) months and 14.3 (95% CI: 8.5–not reached (NR)) months, respectively. Patients with fewer prior treatment lines tended to exhibit longer PFS. OS, PFS, and ORR were comparable between hormone receptor-positive (HR+) and triple-negative breast cancer (TNBC) patients, but numerical improvements were noted in patients with a single PI3K pathway alteration compared to those with more than one alteration. Genomic alterations that surfaced upon progression on CT+EVE included cyclin dependent kinase 4 (*CDK4*) and epidermal growth factor receptor (*EGFR*) amplification, as well as neurofibromin 1 (*NF1*) mutation, suggesting potential mechanisms of acquired resistance. An analysis of adverse events indicated manageable toxicities.

Conclusions: The findings of this study suggest both activity and safety for the combination of chemotherapy and the mTOR inhibitor everolimus (CT+EVE) in patients with HER2- mBC who have alterations in the PI3K pathway, particularly those who have received fewer prior chemotherapy. However, it is crucial to note that large-scale, randomized control studies are warranted to more comprehensively characterize the efficacy and safety of this combination therapy.

Keywords: metastatic breast cancer; HER2 negative; *PI3KCA*; everolimus; chemotherapy; combination therapy

Introduction

Breast cancer (BC) currently holds the top position among malignancies in terms of incidence and remains the leading cause of cancer-related deaths in women worldwide [1]. Various treatment strategies have been established for this highly heterogeneous disease, with a continuously expanding array of therapeutic options [2,3]. The management of metastatic breast cancer (mBC) involves distinct strategies for different subtypes: hormone receptor-positive (HR+) and human epidermal growth factor receptor 2-negative (HER2-), HER2-positive, and triple-negative (TN). Early treatment for HR+/HER2- mBC primarily relies on endocrine therapy (ET), typically incorporating a cyclin-dependent kinase (CDK) 4/6 inhibitor. In cases where ET resistance develops, the subsequent treatment often involves single-agent chemotherapy (CT), except for the approximately 5% of patients eligible for poly [adenosine diphosphate-ribose] polymerase (PARP) inhibitors or the 1–2% who may benefit from immunotherapy [2]. For metastatic triple-negative breast cancer (TNBC), chemotherapy (CT) currently stands as the primary option for the majority of cases, despite the disappointing outcomes [4]. Combined, these two HER2- subtypes account for approximately 85% of all BC cases.

The phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway stands out as a prominent signaling cascade in cancer. Positioned downstream of RAS and receptor tyrosine kinases at the cell surface, PI3K phosphorylates AKT, a process reversible by phosphatase and tension homolog (PTEN). Subsequently, AKT activates mammalian target of rapamycin complex 1 (mTORC1) by inhibiting tuberous sclerosis complex (TSC) [5]. mTORC1, in turn, promotes tumor aggression through anti-apoptosis, cell proliferation, angiogenesis, and other mechanisms [6]. Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha *(PIK3CA)* encodes the catalytic subunit of PI3K and is frequently mutated in advanced BC, occurring in 30–50% of HR+/HER2- and 5–10% of triple-negative breast cancer (TNBC) cases [7,8]. Genomic aberrations in other members of the pathway, such as mutated *AKT1* or *PTEN* loss, may also contribute to hyperactive signaling. Dysregulated PI3K signaling has been implicated in endocrine therapy (ET) resistance [9], and everolimus, an mTOR inhibitor, is recommended by international guidelines as a later-line systemic option in combination with ET [4]. Conversely, *PIK3CA* mutations have been associated with chemoresistance in HR+/HER2- mBC [10], while the relationship with chemoresistance or chemosensitivity in metastatic TNBC is still under debate. Conflicting reports support either association [10,11].

Given the limited success of CT in the treatment of HER2- mBC, which includes both TN and ET-resistant HR+ patients, numerous studies have explored the effectiveness and safety of combining CT with targeted therapy. These investigations encompass a range of approaches, such as a clinical trial examining paclitaxel plus a pan-PI3K inhibitor [12–15]. Notably, the VicTORia phase II trial specifically compared vinorelbine plus everolimus with vinorelbine monotherapy as a second-line CT for patients who had experienced failure with or were unsuitable for prior anthracycline and/or taxane treatments. Despite being well-tolerated, the combination of everolimus and vinorelbine did not demonstrate superior efficacy compared to vinorelbine alone. Additionally, no correlation was observed between the mutational status of *PIK3CA* and treatment efficacy [15]. In the present study, we conducted a retrospective characterization of the clinical outcomes in 14 HER2- mBC patients with PI3K pathway-altered metastases who received a combination of a chemotherapeutic agent and everolimus (CT+EVE). Our analysis also delved into the molecular characteristics associated with efficacy and potential mechanisms of resistance to CT+EVE.

Methods

Patients and Study Design

As illustrated in Fig. 1, a total of 404 patients diagnosed with HER2-mBC at Zhejiang Cancer Hospital between August 1st, 2017, and November 14th, 2019, underwent screening. The inclusion criteria comprised: (1) detection of $\geq$1 genomic alteration(s) in members of the PI3K pathway through genomic profiling of blood or tumor tissue; and (2) treatment with both everolimus and a chemotherapeutic agent. Sequential exclusions were applied, leading to the exclusion of 314 patients without available genomic profiles obtained through panel-based targeted sequencing, 42 with no alterations in PI3K pathway members, 10 not treated with an everolimus-containing regimen, 21 treated with everolimus plus ET, and 3 patients without available response evaluation. Subsequently, 14 patients met the inclusion criteria for further analyses, with 8 having genomic profiles from tumor tissue and 6 from a blood sample. Alterations in PI3K pathway members were defined as single nucleotide variants and copy number variations in all exons of *AKT1-3*, *MTOR*, *PIK3CA/B/G*, *PIK3R1-2*, and *PTEN*, as well as critical exons, introns, and the promoter region of *PIK3C2B/G*, *PIK3C3*, *PIK3CD*, and *PIK3R3*.

Overall survival (OS) was defined as the duration from the initiation of combination therapy with a CT and everolimus (CT+EVE) to death from any cause. Progression-free survival (PFS) was defined as the duration from the initiation of CT+EVE to disease progression, as assessed by RECIST v1.1, or death from any cause. The objective response rate was defined as the percentage of patients with a best overall response (BOR) of complete response (CR) or partial response (PR) relative to the cohort. The disease control rate was defined as the percentage of pa-

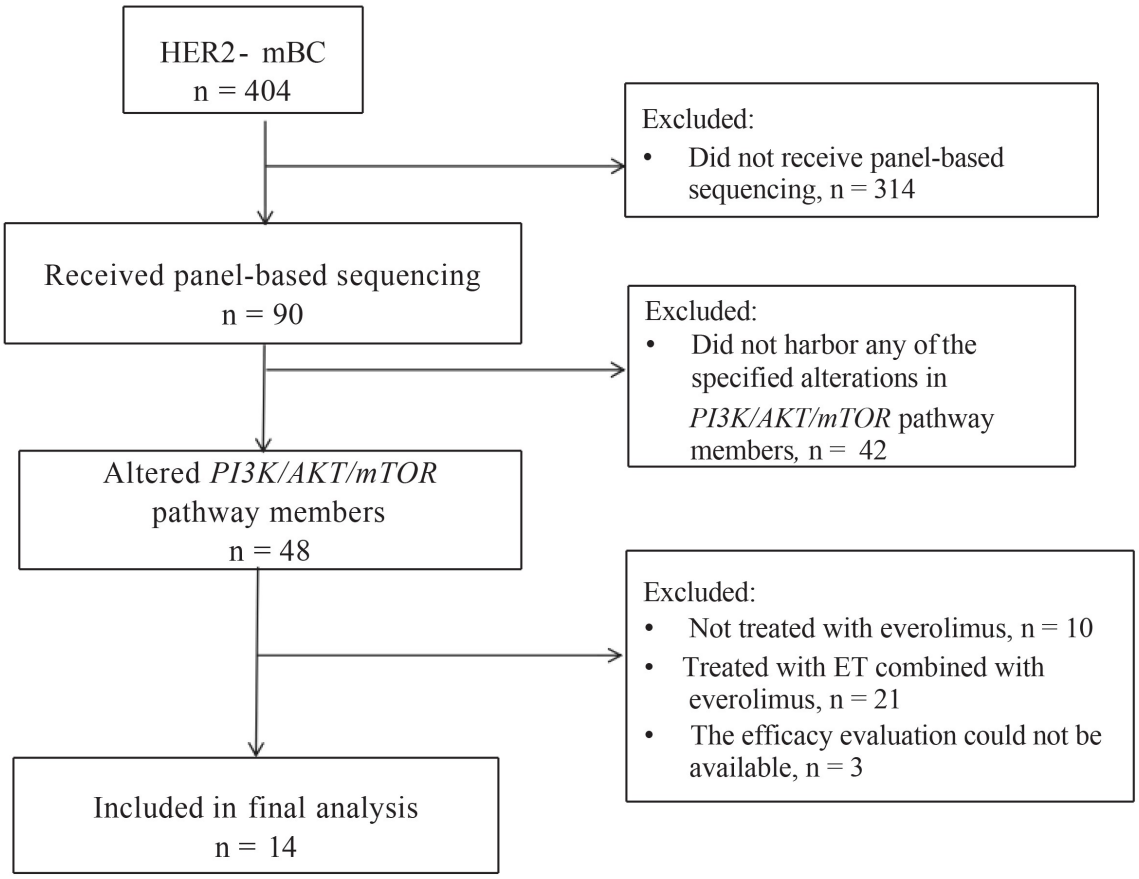

Fig. 1. A schematic illustration of the study design. HER2-, human epidermal growth factor receptor 2-negative; mBC, metastatic breast cancer; PI3K/AKT/mTOR, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin.

tients with a BOR of CR, PR, or stable disease (SD) lasting more than 24 weeks relative to the cohort. Adverse events were graded in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events, version 5.0.

Genomic Profiling with Panel-Based Targeted Sequencing

DNA isolation and targeted sequencing were conducted following established procedures [16]. In brief, DNA extraction from formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples was performed using the QIAamp DNA FFPE tissue kit (56404, Qiagen, Hilden, Germany). Circulating cell-free DNA (cfDNA) was isolated from plasma samples using the QIAamp Circulating Nucleic Acid kit (55114, Qiagen, Hilden, Germany). Subsequent to DNA shearing, fragments ranging from 200 to 400 base pairs were purified with the Agencourt AMPure XP Kit (A63882, Beckman Coulter, Brea, CA, USA). These fragments were then hybridized with capture probe baits on magnetic beads and underwent amplification. The quality and size of the amplicons were evaluated using the high sensitivity DNA kit on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Matched leukocytes served as germline DNA controls for the identification of somatic genomic alterations. Indexed samples were sequenced on the Illumina Nextseq 500 platform with paired-end reads and a target sequencing depth of 1000× for tissue samples and 10,000× for blood samples. Paired white blood cell samples from each patient were utilized to exclude germline mutations.

Statistical Analysis

Fisher's exact test was employed to compare the proportions of values for a nominal variable between two groups. The Wilcoxon signed-rank test was utilized to compare the central tendency of a continuous variable between two groups. The Pearson correlation coefficient was employed to measure the strength of a linear association between two continuous variables. Survival curves were estimated using Kaplan-Meier analysis, and hazard ratios with 95% confidence intervals from the log-rank test were used to compare survival outcomes.

Table 1. Clinicopathologic characteristics of the 14 included patients in this study.

Patient No.	Age	Molecular Subtypes		Previous line(s) of treatment	Current regimen	Objective response	PFS (m)	PFS of the previous line (m)	OS (m)	Alive	Aberrant PI3K pathway member
		Primary tumor	Metastasis								
P1	54	HR+/HER2+	HR+/HER2-	0	VNR+EVE	PR	9.8	24.9	30.5	NO	PIK3CA p.H1047R
P2	44	TN	TN	2	VNR+EVE	PR	4.9	4.4	8.5	NO	PIK3CA p.H1047R
P3	56	TN	TN	1	GEM+EVE	SD	6.1	13.2	6.3	NO	PIK3CA p.E542K, PTEN p.Q171R, PIK3C2B cn_amp, PIK3CB p.Q637*
P4	51	HR+/HER2-	TN	2	VNR+EVE	PR	13.6	7.4	35.5	YES	PIK3CA p.H1047R
P5	45	HR+/HER2-	TN	2	VNR+EVE	SD	9.7	1.6	14.3	NO	PTEN p.Y16*, PIK3CA p.G1049R
P6	53	TN	TN	2	VNR+EVE	PR	4.9	4	6.6	NO	PIK3CA p.E542K, PIK3CA p.N1044K
P7	60	HR+/HER2-	TN	2	VNR+EVE	SD	8.0	21.7	33.2	YES	PIK3CA p.H1047R, PIK3CA cn_amp
P8	50	HR+/HER2-	HR+/HER2-	2	VNR+EVE	SD	13.7	7.1	44.5	YES	PIK3CA p.H1047R
P9	43	HR+/HER2-	TN	2	VNR+EVE	SD	3.7	9.4	14.2	NO	PIK3CA p.H1047R
P10	51	TN	TN	2	VNR+EVE	PR	3.9	3.2	10.3	NO	PIK3CA cn_amp
P11	55	HR+/HER2-	HR+/HER2-	10	VP-16+EVE	PD	1.7	2.8	2	NO	PIK3CA p.H1047R, PIK3CA p.E726K
P12	49	HR+/HER2-	HR+/HER2-	2	Nab-P+EVE	PD	1.2	1.4	3.5	NO	PIK3CA p.E545Q, PIK3CA p.P539R
P13	29	HR+/HER2-	TN	2	Nab-P+EVE	SD	5.8	6	19.6	YES	PIK3R1 p.L573fs
P14	66	HR+/HER2-	HR+/HER2-	2	VNR+EVE	PR	11.3	11.4	27	YES	PIK3CA p.H1047R

cn_amp, Copy number amplification; EVE, everolimus; GEM, gemcitabine; Nab-P, Nab-paclitaxel; OS, overall survival; PFS, progress-free survival; PR, partial response; SD, stable disease; PD, progressive disease; HR+, hormone receptor-positive; TN, triple negative; VNR, vinorelbine; VP-16, etoposide; PTEN, phosphatase and tension homolog; PI3K, phosphatidylinositol 3-kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha. * represents stop codon.

Patients were classified into two groups based on the number of alterations in PI3K pathway members: 1 vs. >1. Additionally, patients were classified into two groups based on the molecular subtype of hormone receptor-positive (HR+) vs. TNBC. Subgroup survival analysis and analysis of best overall responses were conducted based on these two groups. All tests were two-sided, with a significance level set at $p < 0.05$.

Results

Patient Characteristics

This retrospective study included 14 patients with HER2- mBC who harbored at least one genomic alteration in PI3K pathway members and were treated with a combination of everolimus and a chemotherapeutic agent (CT+EVE). The clinicopathologic characteristics and PI3K pathway status of the patients are summarized in Table 1. All participants were female, with an age range of 29–66 years and a median age of 51 years. Four patients were diagnosed with stage IV disease at initial diagnosis. With the exception of P1, who experienced relapse after tumor removal and adjuvant ET, all patients had received at least one line of therapy in the metastatic setting, with 11 having undergone two prior lines (11/14, 78.6%). The everolimus dosage was 5 mg qd for all patients. The predominant CT agent was vinorelbine (n = 10, 71.4%), administered at 25 mg/m^2, d1, 8, intravenously (iv), every 3 weeks. This was followed by nab-paclitaxel (n = 2, 125–150 mg/m^2, d1, 8, iv, every 3 weeks), gemcitabine (n = 1, 1 g/m^2, d1, 8, iv, every 3 weeks), and etoposide (n = 1, 60 mg/m^2, d1–10, orally, every 3 weeks). The majority of patients presented with TN metastasis (9, 64.3%), while HR+/HER2- (9, 64.3%) was the predominant subtype for the primary lesion. Furthermore, most patients (9, 64.3%) exhibited the same subtype for both primary and metastatic tumors.

A total of 22 alterations were identified in members of the PI3K pathway (Table 1). The majority (17, 77.3%) occurred in *PIK3CA*, with alterations detected in 13 patients. The most frequent *PIK3CA* alteration was p.H1047R (8/17, 47.1%), followed by p.E542K (2/17, 11.8%), and amplification (2/17, 11.8%). Other alterations were observed at a much lower frequency, including *PTEN* in 2 cases (2/22, 9.1%), and *PIK3CB, PIK3C2B*, and *PIK3R1*, each in 1 case (1/22, 4.5%).

Efficacy and Association with Molecular Characteristics

After a median follow-up of 14 months (range 2–44), 6 patients (42.9%) achieved a partial response (PR) as their best overall response (BOR) on CT+EVE, followed by 6 patients (42.9%) with stable disease (SD), and 2 patients (14.2%) with progressive disease (PD), resulting in an objective response rate (ORR) of 42.9% and a disease control rate (DCR) of 85.7% (Table 1). Fig. 2 provides details on the percentage of tumor reduction/growth relative to baseline since treatment initiation in the 12 patients with measurable target lesions (Fig. 2A) and changes in tumor burden at each follow-up (Fig. 2B). The median progression-free survival (PFS) was 5.9 months with a 95% confidence interval (CI) of 4.9–13.6 months (Fig. 2C), and the median overall survival (OS) was 14.3 months (95% CI: 8.5–not reached; Fig. 2D). One-year PFS and OS rates were 14.3% and 57.1%, respectively. Subgroup analysis showed a trend toward longer PFS in patients with fewer prior lines of treatment (Fig. 2E). Median PFS was 8.0 months for patients with CT+EVE in the 1st- or 2nd-line (n = 2, range 6.1–9.8), 5.8 in the 3rd-line (n = 11, range 1.2–13.7), and 1.7 in later-line settings (n = 1). However, this trend did not reach statistical significance (Wilcoxon signed-rank test $p = 0.876$), likely due to the small subgroup sizes. Furthermore, compared with PFS of the previous line, PFS following CT+EVE was considerably longer (P4, P5, and P8) or comparable (P2, P6, P10, P12, P13, and P14) in a total of 9 (9/14, 64.3%) patients (Fig. 2F). Efficacy also appeared unrelated to hormone receptor status. PFS and OS were not significantly different between HR+ and TNBC patients, although both were numerically longer in HR+ (PFS: 9.8 months, 95% CI: 1.7–not reached; OS: 30.5 months, 95% CI: 3.5–not reached) than in TNBC (PFS: 5.8 months, 95% CI: 4.9–not reached; OS: 14.2 months, 95% CI: 8.9–not reached). When comparing patients with HR+ vs. TNBC metastases, the hazard ratio (HR) was 1.07 (95% CI: 0.26–4.33, $p = 0.930$) for the risk of death and 1.83 (95% CI: 0.53–6.29, $p = 0.334$) for the risk of disease progression or death (Fig. 3A,B). Consistently, no difference was observed in the BOR makeup between the two subgroups ($p = 0.236$, Fig. 3C). Compared with HR+, TNBC were enriched for SD (5/9 in TNBC vs 1/5 in HR+) but lacked PD (0/9 vs 2/5), while the frequencies of PR were comparable (4/9 vs 2/5), resulting in similar ORRs (4/9 vs 2/5, $p = 1.000$) and numerically higher DCR in TNBC (8/9 vs 3/5, $p = 1.000$).

Importantly, survival analysis revealed a more pronounced association between clinical outcomes and genomic aberrations of PI3K pathway members. Patients were categorized into two groups based on the number of alterations in PI3K pathway members: 1 vs. >1. As demonstrated in Fig. 3D,E, patients with >1 alteration exhibited distinctly poorer OS (HR: 3.13, 95% CI: 0.82–11.86, $p = 0.078$) and PFS (HR: 2.75, 95% CI: 0.76–9.87, $p = 0.107$) than those with 1 alteration. Although neither comparison reached statistical significance, the distinctly separate survival curves suggested that this lack may have resulted from insufficient sample sizes. Additionally, patients with 1 alteration were enriched for PR (5/8) compared with those with >1 alteration (1/6), leading to a numerically but not statistically higher ORR (5/8 vs. 1/6, $p = 0.354$) and DCR (7/8 vs. 4/6, $p = 1.000$). Similarly, no significant difference was observed between the BOR makeup of the subgroups ($p = 0.123$), likely due to inadequate statistical power (Fig. 3F).

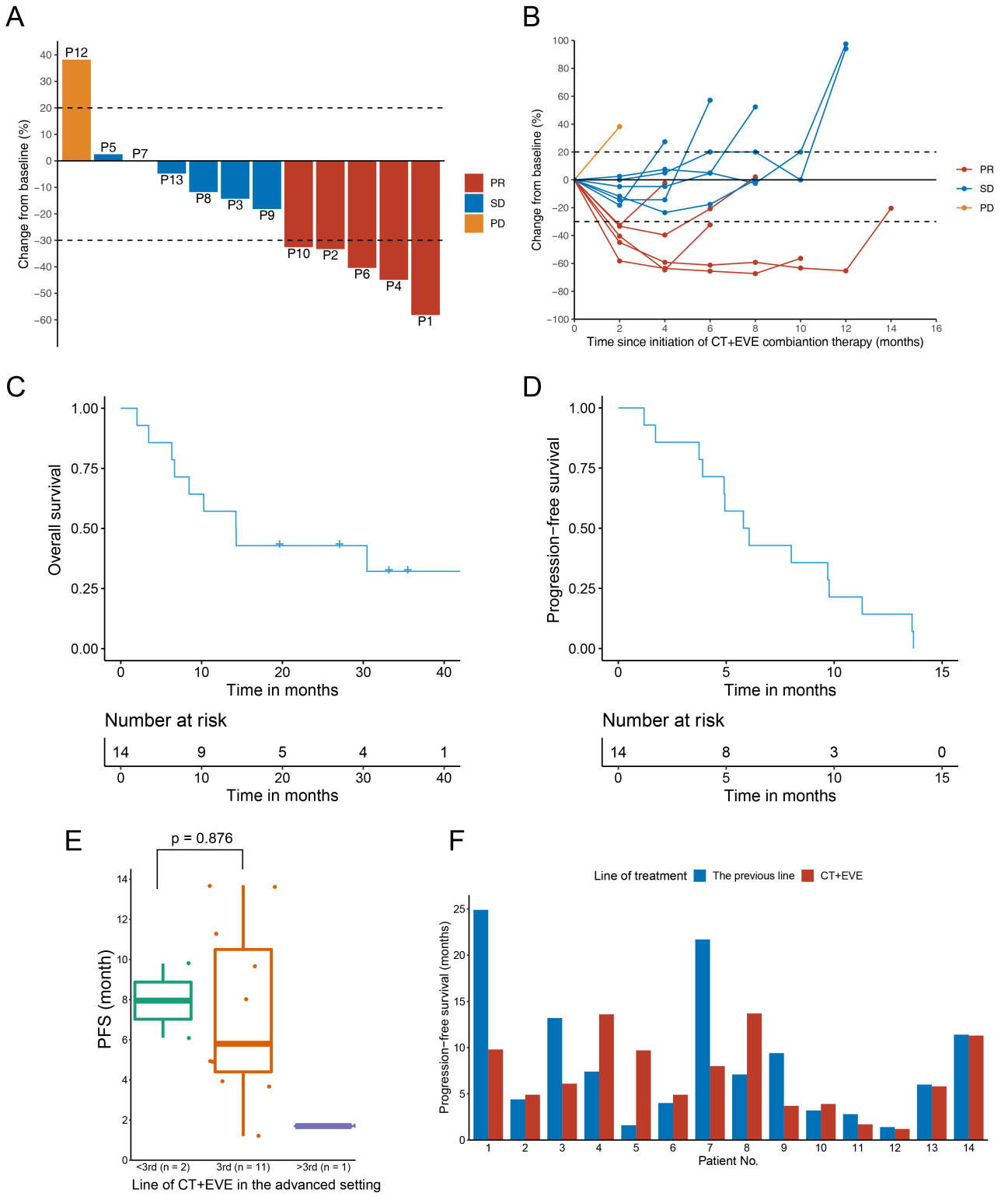

Fig. 2. Response and survival outcomes were evaluated in the 14 patients receiving combination therapy with chemotherapy and everolimus (CT+EVE). (A) By-patient view of tumor response at every 2 cycles after treatment initiation for target lesions. (B) Changes in tumor burden, assessed as the longest linear dimension, over time. (C) Overall survival. (D) Progression-free survival (PFS). (E) Comparison of progression-free survival durations among patients receiving CT+EVE as <3rd, 3rd, or >3rd-line treatment in the advanced setting. (F) PFS of the CT+EVE therapy and the line before by patient.

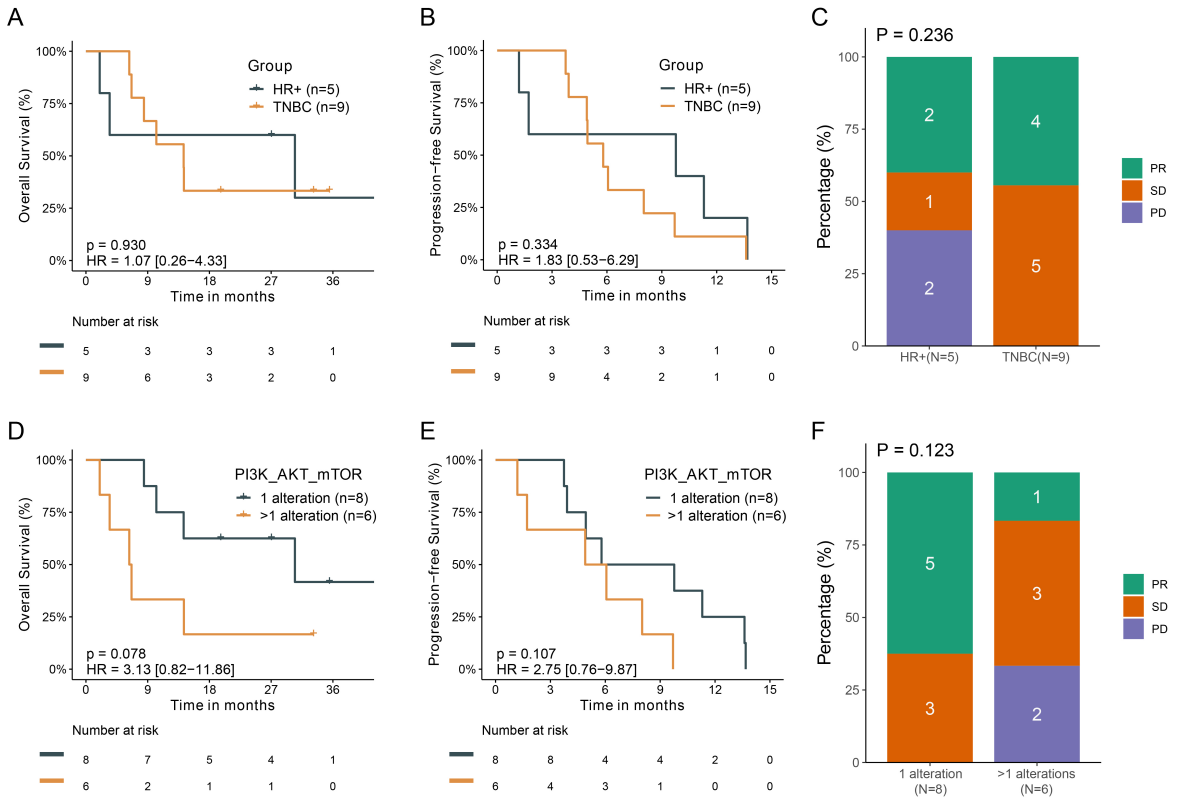

Fig. 3. Survival and response were analyzed according to the number of alterations in PI3K pathway members and molecular subtype respectively. Survival and response outcome comparison between patients with (A–C) HR+ vs TNBC metastases and between those with (D–F) 1 or >1 alterations in PI3K pathway member(s). (A,D) Overall survival. (B,E) Progression-free survival. (C,F) Proportions of best overall responses on chemotherapy plus everolimus. HR+, hormone receptor-positive; TNBC, triple-negative breast cancer.

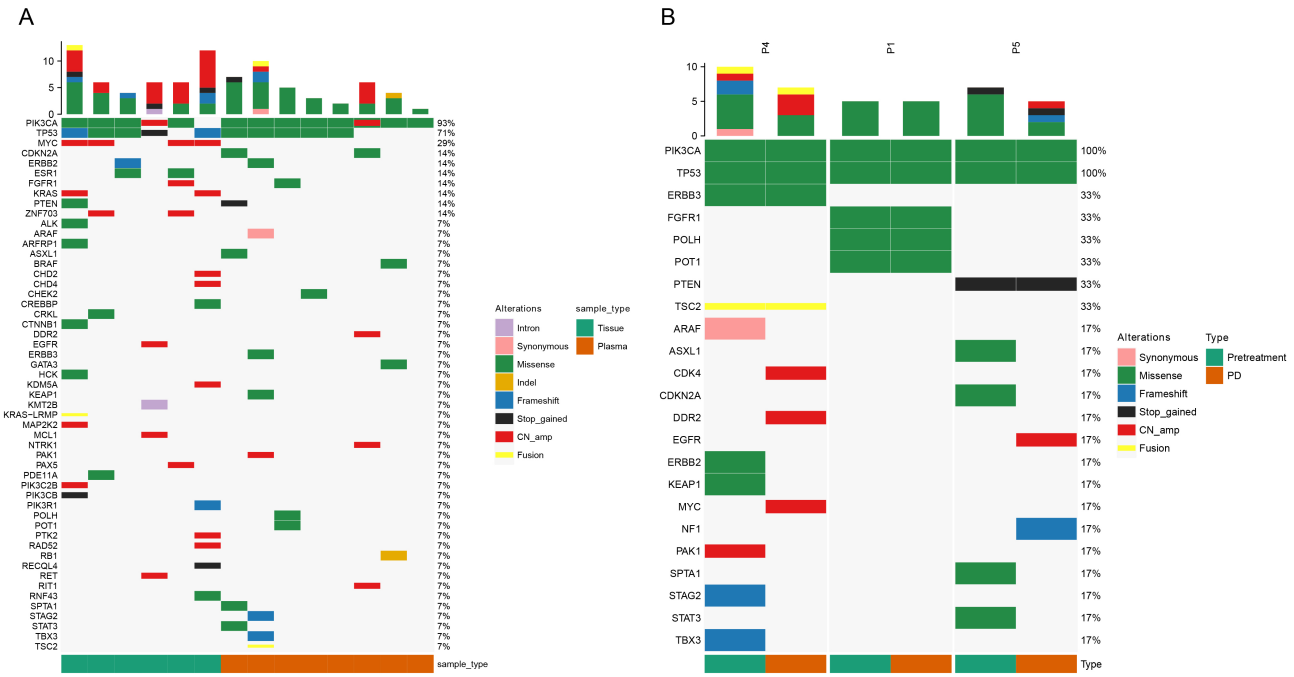

Fig. 4. Genomic profile of the patients. Overview of genomic alterations in (A) all 14 patients and (B) 3 patients with paired samples before and after onset of resistance to the chemotherapy plus everolimus therapy.

Mutational Landscape at Baseline and Potential Mechanisms of Resistance to CT+EVE

Fig. 4A provides an overview of the mutational landscape of all included patients at baseline. *TP53* missense, frameshift, or nonsense mutations were detected in 11 (78.6%) patients. The remaining targeted genes were altered at considerably lower frequencies, including *MYC* (4, 28.5%), *CNKN2A* (2, 14.3%), *ERBB2* point mutations (2, 14.3%), *ESR1* (2, 14.3%), and *FGFR1* (2, 14.3%). Paired samples before and after progression on CT+EVE were available for 3 patients (Fig. 4B). The genomic profile remained the same for P1, suggesting that the possible resistance mechanisms are through the regulation of mRNAs or proteins. P4 and P5 showed distinct sets of newly emerged aberrations. P4 acquired amplifications of *CDK4*, *MYC*, and *DDR2* and lost mutant *KEAP1*, *ERBB2*, *STAG2*, and *TBX3*. P5 harbored acquired epidermal growth factor receptor (EGFR) amplification and neurofibromin 1 (*NF1*) frameshift mutation, while missense mutations in *CDKN2A*, *STAT3*, *ASXL*, and *SPTA1* disappeared. These changes provide candidates for further research to explore their therapeutic relevance and possible countermeasures.

Adverse Events

Safety profiles were available for all 14 patients. The most common adverse events (AEs) of any grade were hematological events, occurring in all cases, followed by alanine and aspartate aminotransferase elevations (7, 50.0%), mucositis (6, 42.9%), fatigue (5, 35.7%), metabolism and nutrition disorders (4, 28.6%), and pneumonia (3, 21.4%). All mucositis and metabolism-related AEs were grade 1 with 2 exceptions, and all 3 pneumonia events were grade 2. Grade $\geq$3 AEs occurred in 7 (50.0%) patients, the 3 most common of which were leukopenia (n = 5), neutropenia (n = 5), and anemia (n = 3). Notably, P6 experienced grade 4 leukopenia and neutropenia, and P9 had grade 4 neutropenia. P1 had five grade 3 AEs: leukopenia, neutropenia, anemia, and the aminotransferase increase. All grade $\geq$3 AEs resolved after symptomatic treatment or regimen suspension, except for dose reduction for P6, for whom vinorelbine dosage was reduced by 20%.

Discussion

The PI3K/AKT/mTOR pathway is frequently altered in HER2- mBC and has been associated with resistance to ET and CT in HR+/HER2- and sensitivity to CT in TNBC [10]. Several regimens combining CT and a kinase inhibitor have been explored but have not shown superior clinical benefit compared with CT alone [12–15]. Everolimus inhibits PI3K signaling by supressing mTOR and is recommended for treating HR+/HER2- advanced BC in the later-line setting in combination with ET [4]. Besides ET, preliminary efforts have been made to examine CT+EVE as a therapeutic option for for HER2- mBC patients indicated for CT [15]. In this study, we retrospectively studied 14 BC patients with HER2- metastases and one or more aberrant PI3K pathway members, all but one of whom had been pretreated with CT in the metastatic setting. CT+EVE regimens elicited a median PFS of 5.9 and OS of 14.3 months, with a trend toward longer PFS in the 1st- or 2nd-line setting, suggesting optimal efficacy as an early treatment. Moreover, distinct changes in genomic profiles were observed in 3 patients after developing resistance to CT+EVE, proposing several potential resistance mechanisms that merit further investigation, including *CDK4* and *EGFR* amplification and NF1 mutation. In addition, CT+EVE also had a manageable toxicity profile with grade $\geq$3 AEs occurring in 50.0% (7/14) of patients, and all resolved after addressing. A phase II umbrella trial (NCT04355858) is currently ongoing to evaluate precision treatment for patients with HR+/HER2-, ET-resistant advanced BC, including nab-paclitaxel plus EVE for a cohort of PI3K pathway-mutated patients. This trial could generate more evidence regarding the efficacy and safety of a CT+EVE regimen for HER2- mBC.

Findings from this study suggested that CT+EVE regimens were effective for HER2-, PI3K pathway-altered mBC patients. The overall median PFS was 5.9 months (95% CI: 4.9–13.6), and the median OS was 14.3 months (95% CI: 8.5–not reached). The ORR was 42.9% (2/5 for HR+ and 4/9 for TNBC), and the DCR was 85.7% (3/5 for HR+ and 8/9 for TNBC). The one-year OS and PFS rates were 57.1% and 14.3%, respectively. The VicTORia phase II trial had several similar settings to this study, as it evaluated everolimus plus vinorelbine (everolimus: 5 mg qd, vinorelbine: 25 mg/m^2, day 1, 8, and 15, q3w) as 2nd-line CT with HER2- locally recurrent or metastatic BC, albeit with no patient selection based on genotype. Median PFS and OS for 17 *PI3K*-mutant patients were 2.3 and 12.4 months, respectively [15]. Acknowledging the weakness of cross-study comparisons, CT+EVE in this study, mostly as 3rd-line CT (11/14), manifested longer PFS and OS than in VicTORia with a similar regimen and dosage scheme.

Considering the small number of patients in this study, further investigation is needed to confirm the efficacy of CT+EVE in HER2- mBC. Comparison with other reports of combination therapies led to similar findings of comparable or more favorable survival outcomes. In a phase II trial of biweekly vinorelbine plus oxaliplatin in the 2nd- or 3rd-line setting for mTNBC patients that had progressed on 1 or 2 prior lines of CT in the metastatic setting, median PFS was 4.3 (95% CI: 3.6–5.0) months and median OS was 12.6 (95% CI: 8.1–17.0) months for 38 evaluable patients [17]. The TNBC patients in our study manifested better PFS (median 5.8 months) and OS (median 14.2 months) but with much wider CIs. In a study of ixabepilone and carboplatin in HER2- mBC with $\leq$2 previous CT in the metastatic setting, ORR was 34% and 30.4% for the HR+ and TN patients, respectively. Median OS was 17.9 months

for HR+ and 12.5 months for TNBC. Median PFS was 7.6 months for both subtypes [18]. The HR+ patients in our study had prolonged median OS and PFS of 30.5 and 9.8 months, respectively, although also with sizable CIs. Additionally, a CT plus AKT inhibitor regimen has shown promise in a recent phase II trial [19]. The PAKT study compared paclitaxel plus placebo or plus AKT inhibitor capivasertib in women with untreated metastatic TNBC. PFS was significantly improved with paclitaxel plus capivasertib (9.3 months) than plus placebo (3.7 months) in the 28 *PIK3CA/AKT1/PTEN*-altered patients. For metastatic TNBC with an altered PI3K pathway, determining which one of AKT and mTORC is a more effective target in combination with CT, and whether combining CT and inhibition of both targets would elicit greater clinical benefit while remaining tolerable, is worthy of future investigation.

Although the VicTORia trial suggested no correlation between PIK3CA mutational status and response to paclitaxel plus everolimus [15], in our study, we observed trends indicating that PFS, OS, and ORR tended to improve when comparing carriers of one alteration with those of more than one alteration in the PI3K pathway. However, without functional characterization, it remains unclear whether each of these aberrations would enhance or dampen PI3K signaling. Equally important is understanding how potent everolimus is in inhibiting a PI3K pathway with more than one activating alteration or inducing the death of tumor clones hosting such hyperactive PI3K signaling. Nonetheless, the association between the number of PI3K pathway alterations and survival may facilitate CT+EVE efficacy prediction or prognostication. Interestingly, a recent study of BC transcriptomes suggested that compared with *PIK3CA*-wild type, hotspot *PIK3CA* mutations, when present in one copy, were associated with lower PI3K signaling activity and cancer cell stemness, whereas more than one copy correlated with higher PI3K signaling and stemness scores [20]. It would, therefore, be of interest to clinically validate our finding and to characterize the role of cancer cell stemness in affecting the outcomes of CT plus everolimus or even plus PI3K signaling inhibitors in general.

In addition to potential predictive or prognostic biomarkers, candidate mechanisms of resistance to CT+EVE were identified in three patients with paired samples, all of whom received everolimus plus vinorelbine. Notable candidates included *CDK4* and *EGFR* amplification, *NF1* mutation, and the disappearance of mutated *ERBB2*, *CDKN2A*, and *STAT3*. The amplifications may activate CDK4/6-RB-E2F and EGFR signaling, thereby mediating resistance. In line with this possibility, there is preclinical evidence suggesting combined inhibition of mTOR and CDK4/6 as a prerequisite for long-term growth inhibition of HR+ BC [21]. The therapeutic activity of this strategy, as well as inhibiting EGFR signaling, merits more preclinical and clinical research. NF1 is a tumor suppressor that negatively regulates RAS signaling [22]. On the other hand, NF1 potently stabilizes microtubules [23], and vinorelbine inhibits tumor growth by inhibiting microtubule and mitotic spindle formation, thereby halting cell division. It is, therefore, unclear how the detected mutation affects NF1 activity and vinorelbine efficacy. However, *NF1* loss-of-function mutations were proposed as ET resistance mechanisms in invasive lobular BC [24]. *NF1* may, therefore, be an interesting target for studies of sensitivity to CT and ET. Additionally, STAT3 signaling is implicated in increased cell death in CDK4/6 inhibitor-resistant cells when inhibited together with PARP [25], and is under active development for targeted therapy [26]. Similar to NF1, more research is needed to elucidate the relevance of the disappeared *STAT3* mutation after progression on CT+EVE.

This study is limited by several factors. In addition to the single-center, retrospective nature, the one-arm design limited efficacy and safety comparisons with CT monotherapy. Additionally, the small cohort size also reduced statistical power. Nonetheless, we have provided evidence that CT+EVE was effective and safe for altered HER2- mBC with mostly 2 prior lines of CT and altered PI3K pathway.

Conclusions

Combining CT and PI3K pathway-targeted therapy has been explored as an option for HER2- mBC patients indicated for CT monotherapy, although so far without major breakthroughs. This study suggests promise for CT+EVE as an active option with tolerable toxicities in PI3K pathway-altered HER2- mBC, mostly after 2 lines of prior CT. The study associated fewer lines of previous treatment or fewer PI3K pathway aberrations with survival outcomes and discovered potential mechanisms of acquired resistance. Large-scale, randomized controlled studies are warranted to further examine the therapeutic value of CT plus everolimus for HER2- mBC.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding authors on reasonable request.

Author Contributions

WC, QZ, WY, and XW conceived of and designed the study. RW, QZ, WY, YH, ZC and YZ acquired the data. RW, QZ, WC, XZ, JW, DJ and ZX analyzed and interpreted the data. RW, QZ, and XW wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors approved the final version of the manuscript and are accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of Medical Ethics Committee of Zhejiang Cancer Hospital (No. IRB-2021-159) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients had provided written informed consent for participating in the study.

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Conflict of Interest

XZ is the Publication Manager of Burning Rock Biotech. JW is the Bioinformatics Scientist and DJ is the Medical Science Liaison of Burning Rock Biotech. The other authors declare no conflict of interest.

References

[1] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA: A Cancer Journal for Clinicians. 2020; 70: 7–30.

[2] Waks AG, Winer EP. Breast Cancer Treatment: A Review. JAMA. 2019; 321: 288–300.

[3] Wilcock P, Webster RM. The breast cancer drug market. Nature Reviews. Drug Discovery. 2021; 20: 339–340.

[4] National Comprehensive Cancer Network. NCCN clinical practice guidelines in Oncology:Distress Management. 2023. Available at: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1419 (Accessed: 2 October 2023).

[5] Miricescu D, Totan A, Stanescu-Spinu II, Badoiu SC, Stefani C, Greabu M. PI3K/AKT/mTOR Signaling Pathway in Breast Cancer: From Molecular Landscape to Clinical Aspects. International Journal of Molecular Sciences. 2020; 22: 173.

[6] Szwed A, Kim E, Jacinto E. Regulation and metabolic functions of mTORC1 and mTORC2. Physiological Reviews. 2021; 101: 1371–1426.

[7] Meric-Bernstam F, Zheng X, Shariati M, Damodaran S, Wathoo C, Brusco L, *et al*. Survival Outcomes by *TP53* Mutation Status in Metastatic Breast Cancer. JCO Precision Oncology. 2018; 2018: PO.17.00245.

[8] Loibl S, Poortmans P, Morrow M, Denkert C, Curigliano G. Breast cancer. Lancet (London, England). 2021; 397: 1750–1769.

[9] Araki K, Miyoshi Y. Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor-positive, HER2-negative breast cancer. Breast Cancer (Tokyo, Japan). 2018; 25: 392–401.

[10] Mosele F, Stefanovska B, Lusque A, Tran Dien A, Garberis I, Droin N, *et al*. Outcome and molecular landscape of patients with PIK3CA-mutated metastatic breast cancer. Annals of Oncology: Official Journal of the European Society for Medical Oncology. 2020; 31: 377–386.

[11] Hu H, Zhu J, Zhong Y, Geng R, Ji Y, Guan Q, *et al*. PIK3CA mutation confers resistance to chemotherapy in triple-negative breast cancer by inhibiting apoptosis and activating the PI3K/AKT/mTOR signaling pathway. Annals of Translational Medicine. 2021; 9: 410.

[12] Crown JP, Diéras V, Staroslawska E, Yardley DA, Bachelot T, Davidson N, *et al*. Phase III trial of sunitinib in combination with capecitabine versus capecitabine monotherapy for the treatment of patients with pretreated metastatic breast cancer. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2013; 31: 2870–2878.

[13] Decker T, Overkamp F, Rösel S, Nusch A, Göhler T, Indorf M, *et al*. A randomized phase II study of paclitaxel alone versus paclitaxel plus sorafenib in second- and third-line treatment of patients with HER2-negative metastatic breast cancer (PASO). BMC Cancer. 2017; 17: 499.

[14] Martín M, Chan A, Dirix L, O'Shaughnessy J, Hegg R, Manikhas A, *et al*. A randomized adaptive phase II/III study of buparlisib, a pan-class I PI3K inhibitor, combined with paclitaxel for the treatment of HER2- advanced breast cancer (BELLE-4). Annals of Oncology: Official Journal of the European Society for Medical Oncology. 2017; 28: 313–320.

[15] Decker T, Marschner N, Muendlein A, Welt A, Hagen V, Rauh J, *et al*. VicTORia: a randomised phase II study to compare vinorelbine in combination with the mTOR inhibitor everolimus versus vinorelbine monotherapy for second-line chemotherapy in advanced HER2-negative breast cancer. Breast Cancer Research and Treatment. 2019; 176: 637–647.

[16] Mao X, Zhang Z, Zheng X, Xie F, Duan F, Jiang L, *et al*. Capture-Based Targeted Ultradeep Sequencing in Paired Tissue and Plasma Samples Demonstrates Differential Subclonal ctDNA-Releasing Capability in Advanced Lung Cancer. Journal of Thoracic Oncology: Official Publication of the International Association for the Study of Lung Cancer. 2017; 12: 663–672.

[17] Zhang J, Wang L, Wang Z, Hu X, Wang B, Cao J, *et al*. A phase II trial of biweekly vinorelbine and oxaliplatin in second- or third-line metastatic triple-negative breast cancer. Cancer Biology & Therapy. 2015; 16: 225–232.

[18] Osborne C, Challagalla JD, Eisenbeis CF, Holmes FA, Neubauer MA, Koutrelakos NW, *et al*. Ixabepilone and Carboplatin for Hormone Receptor Positive/HER2-neu Negative and Triple Negative Metastatic Breast Cancer. Clinical Breast Cancer. 2018; 18: e89–e95.

[19] Schmid P, Abraham J, Chan S, Wheatley D, Brunt AM, Nemsadze G, *et al*. Capivasertib Plus Paclitaxel Versus Placebo Plus Paclitaxel As First-Line Therapy for Metastatic Triple-Negative Breast Cancer: The PAKT Trial. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology. 2020; 38: 423–433.

[20] Madsen RR, Erickson EC, Rueda OM, Robin X, Caldas C, Toker A, *et al*. Positive correlation between transcriptomic stemness and PI3K/AKT/mTOR signaling scores in breast cancer, and a counterintuitive relationship with PIK3CA genotype. PLoS Genetics. 2021; 17: e1009876.

[21] Michaloglou C, Crafter C, Siersbaek R, Delpuech O, Curwen JO, Carnevalli LS, *et al*. Combined Inhibition of mTOR and CDK4/6 Is Required for Optimal Blockade of E2F Function and Long-term Growth Inhibition in Estrogen Receptor-positive Breast Cancer. Molecular Cancer Therapeutics. 2018; 17: 908–920.

[22] Tamura R. Current Understanding of Neurofibromatosis Type

1, 2, and Schwannomatosis. International Journal of Molecular Sciences. 2021; 22: 5850.

[23] Smole Z, Thoma CR, Applegate KT, Duda M, Gutbrodt KL, Danuser G, *et al*. Tumor suppressor NF2/Merlin is a microtubule stabilizer. Cancer Research. 2014; 74: 353–362.

[24] Sokol ES, Feng YX, Jin DX, Basudan A, Lee AV, Atkinson JM, *et al*. Loss of function of NF1 is a mechanism of acquired resistance to endocrine therapy in lobular breast cancer. Annals of Oncology: Official Journal of the European Society for Medical Oncology. 2019; 30: 115–123.

[25] Kettner NM, Vijayaraghavan S, Durak MG, Bui T, Kohansal M, Ha MJ, *et al*. Combined Inhibition of STAT3 and DNA Repair in Palbociclib-Resistant ER-Positive Breast Cancer. Clinical Cancer Research: an Official Journal of the American Association for Cancer Research. 2019; 25: 3996–4013.

[26] Yang L, Lin S, Xu L, Lin J, Zhao C, Huang X. Novel activators and small-molecule inhibitors of STAT3 in cancer. Cytokine & Growth Factor Reviews. 2019; 49: 10–22.