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Original Research

A novel treatment strategy of HER2-targeted therapy in combination with Everolimus for HR+/HER2- advanced breast cancer patients with HER2 mutations

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ABSTRACT

The incidence of HER2 somatic mutations in breast cancer is about 2–4%, mainly occurring in the HR+/HER2- subtype. Preclinical studies suggest that HER2 mutations can lead to constitutive HER2 activation, but effective treatment options for the clinical management of patients with HER2 mutations remain obscure. Our study analyzed HER2 mutation status by performing next-generation sequencing using tumor tissues and over 300 plasma samples from 72 metastatic breast cancer patients. We observed that two patients bearing HER2 mutations (Patient #1 bearing S310F and V777L mutations, Patient #2 bearing 778insGSP mutation) achieved a durable partial response to Trastuzumab combined with Everolimus. *In vitro* experiments showed that T47D and MCF7 cells overexpressing these HER2 mutants (S310F, V777L, 778insGSP and L755S) were sensitive to HER2-targeted therapies combined with the mTOR inhibitor Everolimus. These findings provide a treatment option for patients with HER2 mutations by combining HER2-targeted therapies with Everolimus.

Background

Erb-b2 receptor tyrosine kinase 2 gene (*ErbB2*) encodes a receptor tyrosine kinase, also known as human epidermal growth factor receptor 2 gene (*HER2*), which often appears in an amplified form in many cancer types [1,2]. HER2 mutations occurred most commonly in the absence of HER2 amplification and have been reported in several solid tumors, including breast cancer, lung cancer, colorectal cancer, etc. [3–6]. Previous studies have shown that HER2 mutations play a crucial role in the oncogenic activation of HER2 and intracellular signaling, especially leading to the activation of phosphoinositide 3-kinase (PI3K) pathway [4,7,8]. In breast cancer, HER2 mutations occur in approximately 2–4% of patients and are responsive to EGFR/HER2 tyrosine kinase inhibitors (TKIs) such as neratinib [4]. Clinical trial of neratinib (SUMMIT trial; NCT01953926) in patients with HER2-mutant cancers is underway, and an effective therapeutic approach for this subtype of breast cancer patients is still lacking.

Next-generation sequencing (NGS) continues to reveal novel genetic alterations and novel driver events in different cancers. In the current study, we collected more than 300 plasma samples from 72 metastatic breast cancer patients and performed sequencing analysis to identify cancer-associated gene mutations. Two of these HER2-mutant patients received Trastuzumab combined with Everolimus therapy and showed a sustained partial response (approximately 1 year). *In vitro* experiments also showed that T47D and MCF7 cells overexpressing these HER2 mutants (S310F, V777L, 778insGSP and L755S) are sensitive to HER2-targeted therapies combined with the mTOR inhibitor Everolimus. Based on these data, we believe that dual targeting of HER2 and downstream mTOR is a rational therapeutic approach for HER2-mutant breast cancer patients.

Material and methods

Study cohorts and bioinformatics analysis

Seventy-two metastatic breast cancer patients were enrolled from the Second Hospital of Dalian Medical University. The patient follow-up was started on January 1, 2017 and was completed on April 30, 2020. Both

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primary tissue and plasma circulating tumor DNA (ctDNA) samples were collected during the initial sampling. In the subsequent monitoring, plasma ctDNA was collected monthly for NGS testing, and blood testing was also performed at the same time to check for changes of tumor markers. Sixty of enrolled patients were with matched tissue and blood samples. This study was approved by the Ethical Committee of Dalian Medical University (Dalian, China). Gene status (*e.g.* HER2 mutation status) was evaluated as a part of routine clinical care using targeted sequencing of 425 clinically relevant genes. Scans were taken every 3 weeks during treatment and retrospectively evaluated according to RECIST, version 1.1 to determine patient response rate. The quality of life of cancer patients is assessed by the Quality of life (QOL) rating for Chinese cancer patients, which includes 12 items such as appetite and mental condition (Fig. S1A). All patients signed written informed consent documents. Sequencing data of tumor tissues and plasma were analyzed by R language (Version 3.5.2) and illustrated as Oncoprint.

Antibodies and inhibitors

The following antibodies were purchased from Cell Signaling Technology: AKT, phospho-AKT (S473) and phospho-S6 Ribosomal Protein (S235/236). HER2 was purchased from Abcam and phospho-HER2 (Tyr1248) antibody was purchased from Millipore. GAPDH was purchased from Wanleibio. Trastuzumab was obtained from Roche. Lapatinib and Everolimus were purchased from MedChemExpress (MCE).

Cell culture and stable cell lines with mutant and WT HER2

The breast cancer cell line T47D and MCF7 cells were purchased from The American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 media (Gibco, USA) supplemented with 10% fetal bovine serum (Biological Industries, Israel) and penicillin/streptomycin (Hyclone, Austria) in a 5% CO_2 humidified incubator at 37°C. Constructs bearing HER2 somatic mutations were built and verified as Zuo's protocol [8]. Cell lines were subjected to puromycin selection and transfection efficacy was analyzed by Western blotting and immunohistochemistry to confirm HER2 expression.

Immunohistochemistry

15 μL of complete medium with 7×10^3 of T47D or MCF7 cells were mixed with 25 μL of Matrigel (BD Biosciences) and then seeded in pre-warmed 24-well low adhesion plates (Corning; 3473). After incubation in the incubator at 37 ℃ for 20 min, 400 μL of media were added to the wells and replaced every two days. Five days later, medium-Matrigel-cells were centrifuged, and cell pellets were embedded in histogel (Thermo Scientific; Cat#HG-4000-012). The mixture was fixed in 4% paraformaldehyde overnight followed by dehydration, paraffin embedding, sectioning, and immunohistochemistry was performed as previously described [9]. Rabbit anti-HER2 (1: 500) antibody was purchased from Abcam (ab134182).

Colony formation assay

Cells were seeded in triplicate at a low density in 12-well plates and culture medium containing the corresponding drugs was replaced every three days. Trastuzumab, Lapatinib, Everolimus, or Dimethyl Sulfoxide (DMSO) was added at the next day of seeding. After 7 days, the cells were fixed and stained with 0.5% crystal violet solution containing cold methanol. Colonies were dissolved with 50% glacial acetic acid and the optical density (OD) value was measured at 590 nm.

Three-dimensional (3D) culture in Matrigel

Cells were grown in 50% precoated Matrigel (BD Biosciences, San Jose, CA, USA) with 50% serum-free medium, and the cells were seeded in 96-well plates and cultured with 2% FBS and 2% Matrigel. Dimethyl sulfoxide (DMSO) vehicle, Trastuzumab, Lapatinib, Everolimus, or Combination was added at the time of seeding. Fresh medium containing the corresponding drugs was replaced every three days. Photographs were taken on the sixth or the eighth day after initial seeding.

Statistical analysis

Unpaired *t*-test was performed to compare the difference between two groups. All statistical analyses were performed using the GraphPad Prism 6.0 software package (GraphPad Software, Inc., San Diego, USA). Statistical significance was identified when $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Genetic alterations in patients with metastatic breast cancer

To examine the genetic alterations existed in metastatic breast cancer patients, tumor and plasma samples from 72 patients with metastatic breast cancer were sequenced to assess the 425 cancer-related genes. The clinicopathological features of the 72 enrolled patients were summarized in Table 1, including ER+/HER2- subtypes ($n = 46$), ER+/HER2+ subtypes ($n = 5$), ER-/HER2+ subtypes ($n = 10$), triple negative subtypes ($n = 10$) and ER+/HER2 unknown ($n = 1$). We analyzed mutation, copy number variation (CNV) and structural variation (SV) of DNA isolated from the samples. The overview of the top 9 high-frequency molecular alterations were detected in the tissue and paired peripheral blood plasma (Fig. 1A). We also compared HER2-mutated patients with non-HER2-mutated patients, and a total of five patients with HER2 mutations were identified (Fig. 1B). We found three out of

Table 1
Clinical characteristics of the 72 enrolled MBC patients.

Variables	Patients
Age at Breast Cancer Diagnosis - no.(%)	
<60Y	65 (90.3)
≥60Y	7 (9.7)
Receptor Subtype - no.(%)	
ER+/HER2-	46 (63.9)
ER+/HER2+	5 (6.9)
ER+/HER2 unknown	1 (1.4)
ER-/HER2+	10 (13.9)
Triple negative	10 (13.9)
T classification - no.(%)	
T1	16 (22.2)
T2	36 (50.0)
T3	5 (6.9)
T4	6 (8.3)
Unknown	9 (12.5)
N classification - no.(%)	
N0	17 (23.6)
N1–3	48 (66.7)
Unknown	7 (9.7)
M classification - no.(%)	
M0	69 (95.8)
M1	3 (4.2)
Primary Clinical Stage - no.(%)	
I	6 (8.3)
II	24 (33.3)
III	30 (41.7)
IV	3 (4.2)
Unknown	9 (12.5)
Histology Subtype (Primary tumor) - no.(%)	
Invasive ductal	38 (52.8)
Invasive lobular	8 (11.1)
Other	21 (29.2)
Unknown	5 (6.9)
Overall Survival - no.(%)	
Median (range) month	69.3 (38.1–121.8)

MBC: metastatic breast cancer.

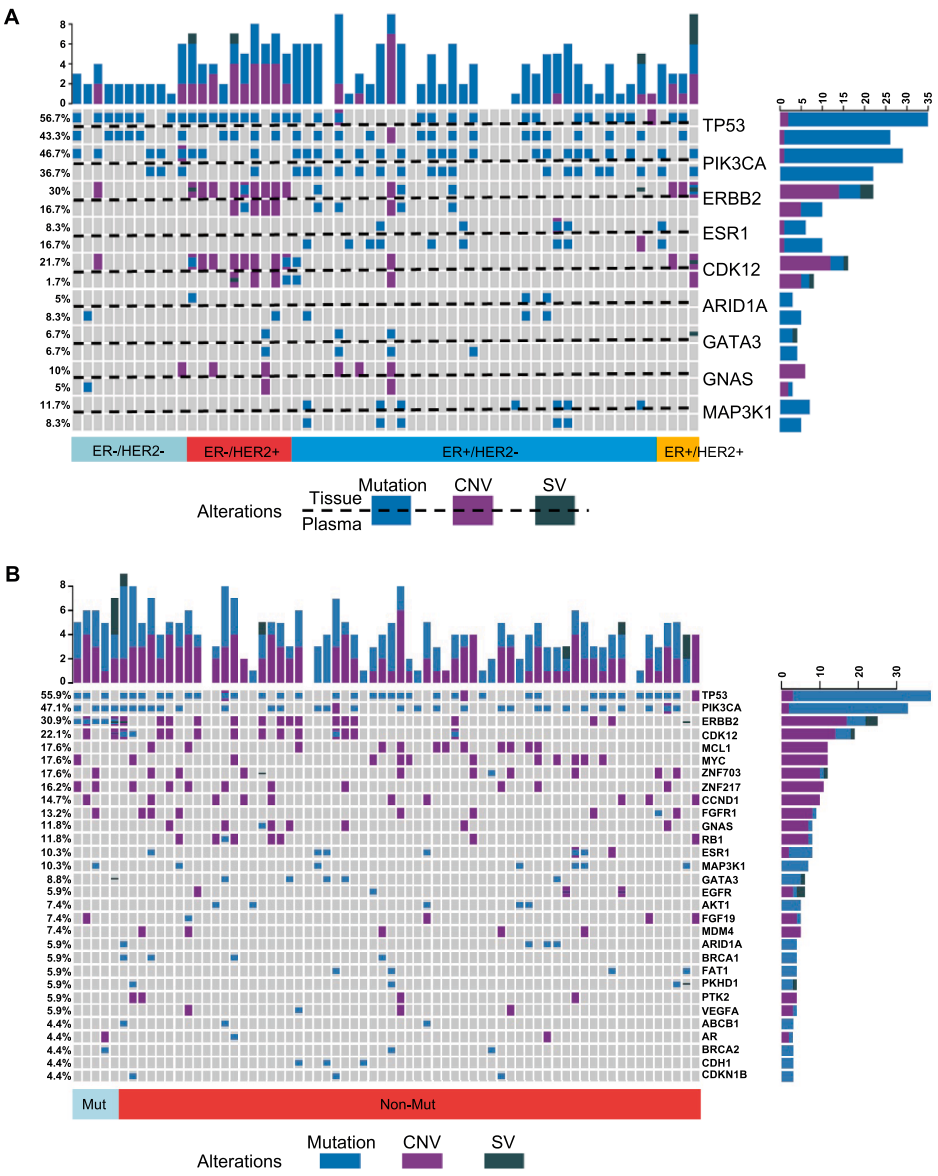
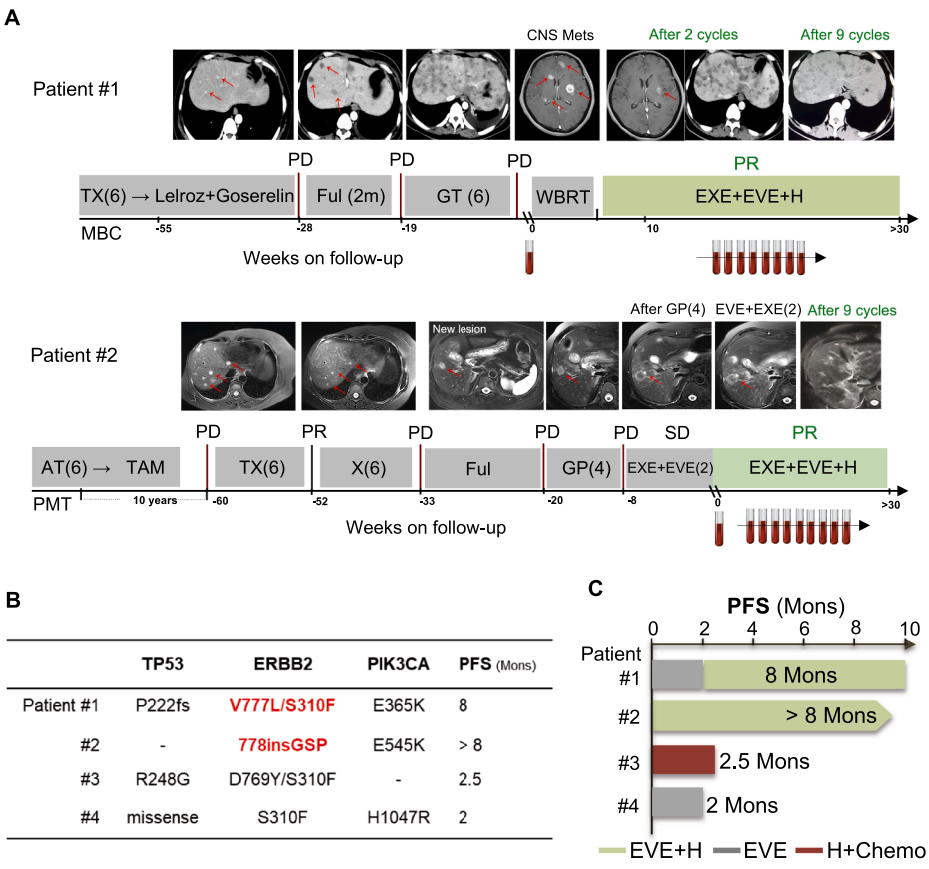


Fig. 1. Genetic alterations of follow-up population are presented.
(A) Comparison of the consistency of high-frequency molecular alterations detected in plasma with paired tissue samples. **(B)** Mutation map of HER2 mutant and non-HER2 mutant population. CNV: Copy number variation; SV: Structural variation; Mut: mutant; Non-Mut: non-mutant.

five HER2-mutated patients with TP53 mutation or PIK3CA mutation, two with HER2 mutations as well as TP53 mutations and PIK3CA mutations, and two with HER2 mutations as well as CDK12 amplification. By analyzing the cBioPortal database, *ERBB2, PIK3CA, NF1, TP53, PTEN* and *TSC2* were the most frequently altered genes (Fig. S2). For the *ERBB2* gene, the most common mutation forms are L755S, S310F, V777L, and G778_S779insLPS (Fig. S2).

Trastuzumab combined with Everolimus is an effective treatment for ER+/HER2-HER2-mutant breast cancer patients

In our cohort, five patients with HER2 mutations were identified (one ER-/HER2+, three ER+/HER2- and one ER+/HER2+), and the overall HER2 mutation rate was 6.94% (5/72). Two ER+/HER2- patients (Patient #1 and Patient #2) with HER2 mutations (Patient #1 bearing S310F and V777L mutations, Patient #2 bearing 778insGSP mutation) who had developed resistance to multiline endocrine therapies (>3) were subsequently treated with Trastuzumab in combination with Everolimus and achieved a durable partial response (Fig. 2A). After the application of combination therapy for HER2 mutations patients, we monitored the changes of HER2 mutations as well as additional common tumor markers, including carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA125) and carbohydrate antigen 153 (CA153) levels (Fig. S3A and B). The changes of these tumor markers showed similar trends to HER2 mutations. HER2 mutations were lower at the endpoint of combination therapy than at the start of monitoring, indicating the potential efficacy of the combination therapy (Fig. S3A). The progression-free survival (PFS) was more than 8 months compared with Patient #3 and Patient #4 (D769Y/S310F, S310F, respectively), who refused to receive Trastuzumab combined with Everolimus therapy (Fig. 2B and C). From the CT images of Patient #1 and Patient #2, we found that metastases continued to increase despite of receiving multiline regimens including endocrine and chemotherapy (Fig. 2A). After receiving Trastuzumab in combination with Everolimus, the tumor burden was significantly reduced and the quality of life was improved (Figs. 2A and S1B). Among the HER2 mutation cases, there are three cases with PIK3CA mutation simultaneously, Patient #1 carried $HER2^{V777L/S310F}$ and $PIK3CA^{E365K}$, Patient #2 carried $HER2^{778insGSP}$ and



	TP53	ERBB2	PIK3CA	PFS (Mons)
Patient #1	P222fs	V777L/S310F	E365K	8
#2	-	778insGSP	E545K	> 8
#3	R248G	D769Y/S310F	-	2.5
#4	missense	S310F	H1047R	2

Fig. 2. Clinical characteristics of ER+/HER2-mutant breast cancer patients.
(A) Timelines of treatment. Red arrows indicate lesions. Test tube symbol indicated biopsy timepoints. Number in parentheses represents the number of cycles that patients receive this regimen. **(B)** The mutation status of HER2 and other key cancer genes in four patients bearing HER2 mutations. **(C)** Bar chart showing PFS of four patients mentioned above. TX: docetaxel and xeloda; Ful: fulvestrant; GT: gemcitabine and docetaxel; WBRT: whole brain radiotherapy; EXE: exemestane; EVE: Everolimus; H: Trastuzumab; AT: Paclitaxel; TAM: tamoxifen; X: xeloda; GP: gemcitabine and cisplatin; m: month/months; Chemo: chemotherapy; PFS: progression-free survival; PD: progressive disease; PR: partial response; SD: stable disease; CNS Mets: central nervous system metastasis.

PIK3CA^{E545K}, and Patient #4 carried HER2^{S310F} and PIK3CA^{H1047R} (Fig. 2B).

HER2 mutant breast cancer cells are sensitive to the combination of HER2-targeted therapies and Everolimus

According to the HER2 pattern diagram, S310F was located in the extracellular domain and the others (V777L, 778insGSP) were clustered in the kinase domain (Fig. 3A). Plasmids harboring HER2 mutations (S310F, V777L, 778insGSP, L755S) were constructed and validated by Sanger sequencing (Fig. 3B). These two cell lines, T47D and MCF7, inherently carry PIK3CA mutations. Therefore, choosing these two cell lines to construct breast cancer cell lines stably expressing HER2 mutations can better simulate the two clinical cases (Patient #1 and Patient #2 carry PIK3CA mutations in addition to HER2 mutations). Mutant or WT HER2 was transduced into T47D and MCF7 cell lines using lentiviral vectors, and immunoblotting was performed to verify this (Fig. 3C). In T47D and MCF7 cell lines, HER2 was activated in the HER2 mutant group compared to the parental control group (Fig. 3C–E).

Then, the cells were treated with DMSO vehicle (0.5%), Trastuzumab (250 μg/mL), Lapatinib (0.5 μmol/L), Everolimus (0.1 nmol/L/0.05 nmol/L), or Combination (Fig. 4A and B). After 7 days of continuous culture, we found that the HER2 mutant-expressing cells stably expressing HER2^{S310F}, HER2^{V777L}, HER2^{778insGSP} and HER2^{L755S} were sensitive to HER2-targeted therapies or Everolimus in contrast to DMSO group. When the HER2-targeted therapies were combined with Everolimus, the number of colonies was the lowest compared to the monotherapy group, indicating that HER2 mutant-expressing cells were sensitive to HER2-targeted therapies combined with Everolimus, and the efficacy of the combination was better than the monotherapy group (Fig. 4A and B). In the T47D cell lines, S310F, V777L and 778insGSP mutations showed more strong sensitivity to the combination drug groups (Trastuzumab + Everolimus, Lapatinib + Everolimus). The inhibitory effects of the combined treatment groups on L775S mutation were slightly worse, but the inhibition was still better than that in the monotherapy groups (Fig. 4C and D). In MCF7 cell lines, Trastuzumab + Everolimus showed a slightly weaker inhibitory effect on V777L and L755S mutations, and a slightly stronger inhibitory effect on S310F and 778insGSP mutations (Fig. 4E and F). Together, these data indicated that HER2 mutant breast cancer cells are highly sensitive to the combination of HER2-targeted therapies and Everolimus.

Lapatinib combined with Everolimus effectively inhibits the 3D growth of HER2 mutant-expressing breast cancer cells

In T47D cell lines, HER2 WT cells expressed higher levels of HER2 protein than other mutant-expressing samples cultured in 3D Matrigel (Fig. 5A and B). While in MCF7 cell lines, HER2 S310F-expressing cells showed higher levels of HER2 expression, and there was little difference in HER2 protein expression between WT cells and other mutant groups in 3D Matrigel (Fig. 5A and C). We also observed that Lapatinib combined with Everolimus markedly reduced the 3D spheroid growth of WT and mutant-expressing cells (Fig. 5D and E). As described above, L755S was not sensitive to Lapatinib, which had been reported in previous studies [4,8]. However, in this study, 3D spheroid growth of HER2 mutant-expressing cells (including L755S) was significantly inhibited by Lapatinib combined with Everolimus, indicating that HER2 mutations were still sensitive to the combination.

Lapatinib combined with Everolimus inhibits HER2 downstream signaling

In the T47D cells stably expressing HER2^{V777L} and HER2^{778insGSP}, we observed that the Lapatinib + Everolimus group showed the stronger inhibition of p-AKT and p-S6K compared with the monotherapy group (Fig. 6A). In the MCF7 cells stably expressing HER2^{V777L} and HER2$^{778-insGSP}$, we observed that the inhibitory effect of the Lapatinib +

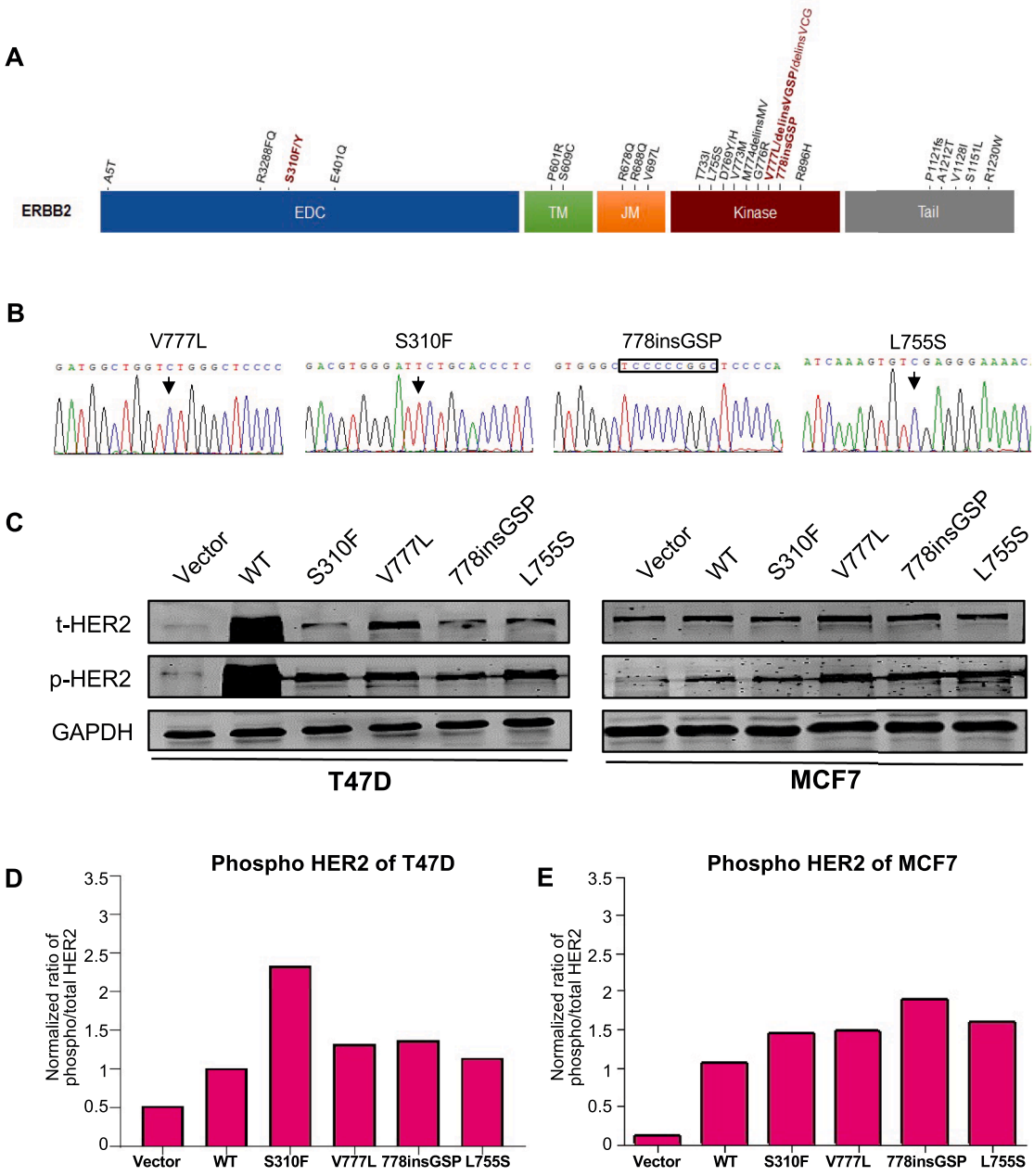


Fig. 3. HER2 mutations could activate HER2.
(A) HER2 somatic mutations in breast cancer. Mutations appearing in patients were highlighted in red. **(B)** Expression of plasmid harboring HER2 mutations detected by Sanger sequencing. Arrows indicated mutant allele. **(C)** T47D cells (left) and MCF7 cells (right) were retrovirally transduced with HER2 WT or the respective mutants. Lysates (50 μg) were prepared and western blotting performed using the indicated antibodies. **(D)** Normalized quantitative histogram of p-HER2 protein expression in T47D cell lines. **(E)** Normalized quantitative histogram of p-HER2 protein expression in MCF7 cell lines.

Everolimus group on p-AKT was similar to that of the monotherapy group, there was a stronger inhibitory effect on p-S6K (Fig. 6B). In the T47D and MCF7 cells stably expressing $HER2^{S310F}$ and $HER2^{L755S}$, p-AKT levels increased reversely after the treatment of Lapatinib + Everolimus (Fig. 6A and B). As reported in the literatures [4,8], these two HER2 mutations are not sensitivity to the HER2 targeted therapies (S310F mutation is insensitive to Trastuzumab, L755S mutation is insensitive to Lapatinib). However, the p-S6K levels were reduced after treatment with Lapatinib + Everolimus, suggesting that the combination was effective for these two HER2 mutations. In conclusion, Lapatinib combined with Everolimus effectively inhibit HER2 downstream signaling. The schematic diagrams were summarized (Fig. 6C).

Discussion

Increasing studies have established the oncogenic roles of activating HER2 mutations and defined them as a distinct molecular subtype [4,5,10]. Many researchers have discovered that HER2 mutations behaved similarly to ERBB2 amplification and served as actionable targets [4,8]. Several HER2 targeted drugs, including Trastuzumab, Lapatinib, Neratinib, Afatinib, and Poziotinib have displayed inhibitory activities toward HER2 mutants in preclinical investigations [4,7,11,12]. Some studies have also shown that HER2 mutations can be acquired in advanced ER+ breast cancer, and may play a significant part in the progression of estrogen independent ER+ tumors [13,14]. HER2 mutations are more than twice as common in endocrine therapy resistant

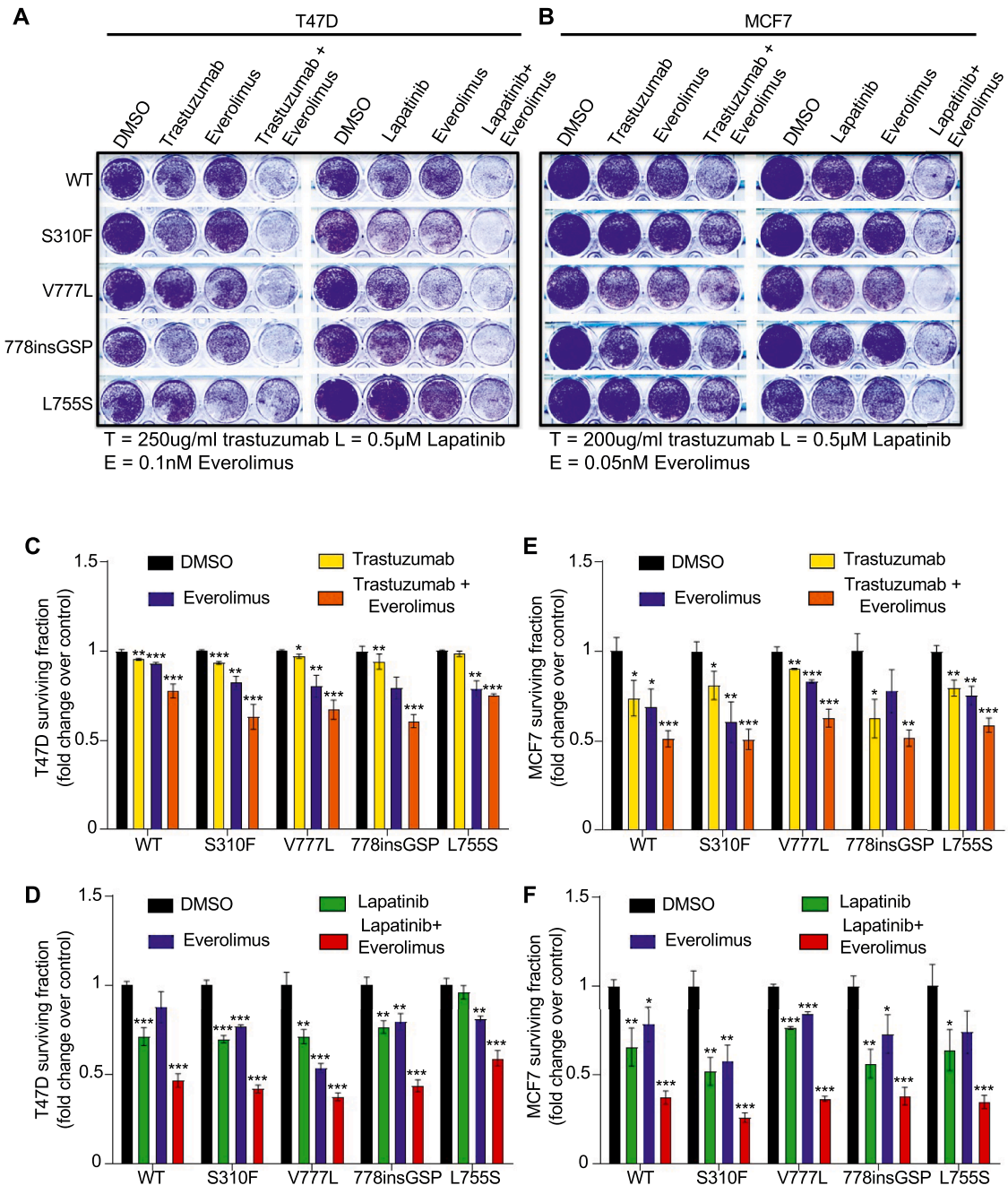


Fig. 4. Colonies of HER2 mutant breast cancer cells are inhibited by HER2-targeted therapies and Everolimus.
(A), (C), (D) Representative images of colony formation assay of T47D cell lines after treatment with DMSO vehicle (0.5%), Trastuzumab (250 μg/mL), lapatinib (0.5 μmol/L), Everolimus (0.1 nmol/L), or combination, and quantified. The data represent the mean ± S.D. values of three independent experiments. *P* values were determined by the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(B), (E), (F)** Representative images of colony formation assay of MCF7 cell lines after treatment with DMSO vehicle (0.5%), Trastuzumab (200 μg/mL), lapatinib (0.5 μmol/L), Everolimus (0.05 nmol/L), or combination, and quantified. The data represent the mean ± S.D. values of three independent experiments. *P* values were determined by the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

tumors [15]. Acquired HER2 mutations are one of the main reasons for endocrine therapy resistance in advanced ER+ breast cancer [13–15]. Dual blockade of HER2 and ER signaling pathways can reverse the endocrine therapy resistance of advanced ER+ breast cancer, such as Neratinib combined with Fulvestrant [13,14]. In an *in vivo* model experiment, to explore an alternative to endocrine therapy resistance caused by acquired HER2 mutations, Croessmann et al. found that the combination of Neratinib and Everolimus showed a better inhibitory effect on tumor growth than the single agent Everolimus. More interestingly, under the three-drug combination therapy of Neratinib, Fulvestrant and Everolimus, the inhibitory effect on tumor growth was much stronger, making the tumor almost completely regressed [14]. In addition, Sudhan et al. found that in HER2-mutant cancers, primary and acquired Neratinib resistance was associated with hyperactivation of TORC1 [16]. The resistance of Neratinib was reversed through the combination of TORC1 inhibitor Everolimus and Neratinib [16]. Recent clinical studies have shown different results for targeted drugs applied in patients with HER2 mutations [17–22]. Ali et al. reported a case of triple-negative breast cancer with $HER2^{V777L}$ and $HER2^{S310F}$ mutations, as well as $PIK3CA^{K111E}$ mutation [23]. The treatment based on Lapatinib and Trastuzumab that lasted for 6 months resulted in rapid improvement of the patient's symptoms. Some clinical trials of Trastuzumab in

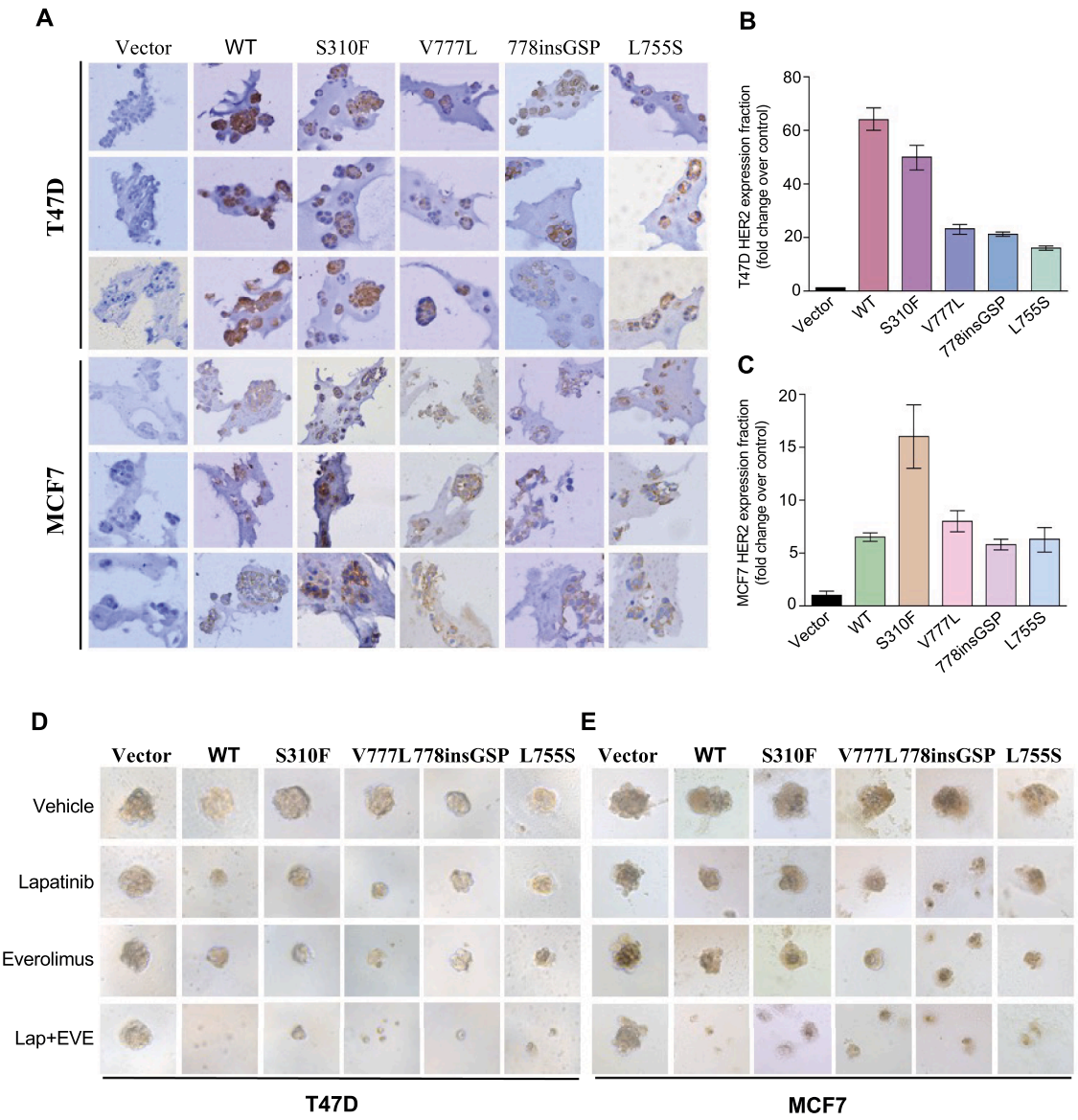


Fig. 5. Lapatinib combined with Everolimus inhibits the 3D growth of HER2 mutant-expressing breast cancer cells.
(A) Immunohistochemical staining of HER2 of T47D cells (upper three rows) and MCF7 cells (lower three rows) stably expressing HER2 WT or mutants. **(B), (C)** Quantification of the T47D **(B)** and MCF7 **(C)** immunohistochemistry staining. The error bars represent the mean ± S.D. values of three independent experiments. **(D)** Parental, HER2 WT or mutants of T47D were seeded on Matrigel in the presence or absence of DMSO vehicle (0.5%), Trastuzumab (100 mg/mL), lapatinib (0.5 mmol/L), or DMSO vehicle (0.5%), lapatinib (0.5 μmol/L), Everolimus (0.1 nmol/L) or combined. Photomicrographs were taken on day 8, magnification × 200. **(E)** Parental, HER2 WT or mutants of MCF7 were seeded on Matrigel in the presence or absence of DMSO vehicle (0.5%), Trastuzumab (100 mg/mL), lapatinib (0.5 mmol/L), or DMSO vehicle (0.5%), lapatinib (0.5 μmol/L), Everolimus (0.05 nmol/L) or combined. Photomicrographs were taken on day 8, magnification × 200.

combination with the HER2 tyrosine kinase inhibitor Neratinib for the treatment of HER2 mutant cancers are ongoing. A clinical trial is investigating the effect of Trastuzumab in combination with Neratinib in HER2-mutant breast cancer (NCT01670877). A phase 2 clinical trial is investigating the efficacy of Neratinib combined with Trastuzumab or Cetuximab in the treatment of HER2-mutant colorectal cancer (NCT03457896). The efficacy of Neratinib was also evaluated in the SUMMIT trial, and favorable responses have been discovered in a subset of HER2-mutant breast cancer patients [17]. However, clinical responses of different HER2 mutations to Neratinib and other HER2 targeted drugs were diverse and often short-lived, suggesting that monotherapy might not be an effective method for treating patients with HER2-mutant cancers [6,24–26].

In this study, 72 patients with metastatic breast cancer were recruited. We identified three patients with HER2 mutations (S310F, V777L, 778insGSP, L841V) from HR+/HER2- breast cancer patients, and two of them (Patient #1 bearing S310F and V777L mutations, Patient #2 bearing 778insGSP mutation) received Trastuzumab combined with Everolimus treatment. HER2 mutations were detected in both plasma and primary tumor tissues of Patient #1 and Patient #2 who received the combination therapy, indicating that the HER2 mutations of these two patients were not acquired under the selective pressure of ER-directed therapy. We herein report their clinical responses to combination of HER2-targeted therapies and the TORC1 inhibitor Everolimus in patients with HER2 mutant breast cancers. Clinical benefits were observed in these two patients who received the combination therapy, HER2 targeted drugs combined with Everolimus.

T47D and MCF7 cells stably expressing $HER2^{S310F}$, $HER2^{V777L}$, $HER2^{778insGSP}$, $HER2^{L755S}$ were successfully constructed, and it was verified that HER2 were activated in these cell lines. The therapeutic activities of the combination approach were also confirmed using *in vitro* assays, showing that HER2 targeted therapies combined with

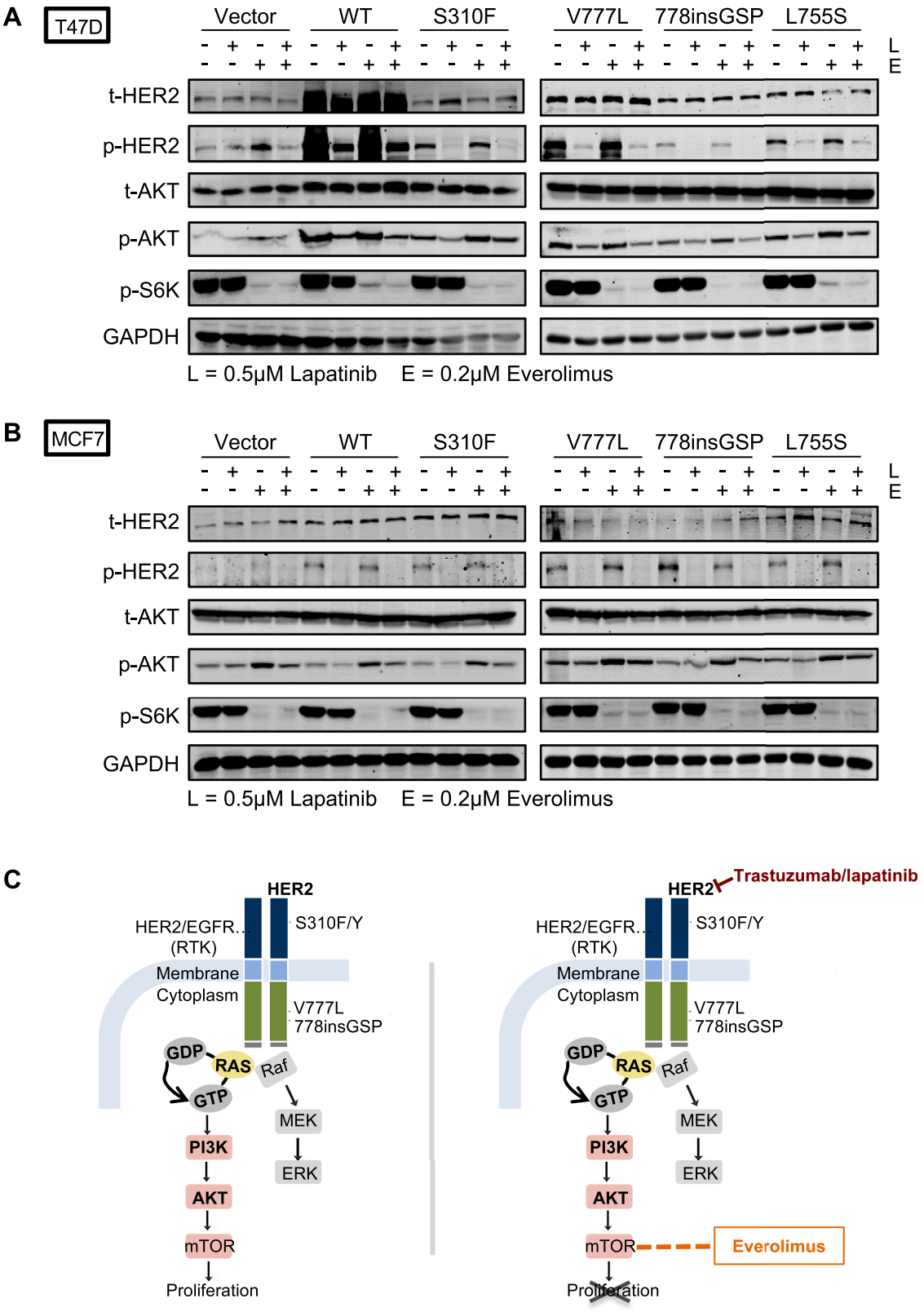


Fig. 6. Lapatinib combined with Everolimus could affect the expression of HER2 downstream proteins.
T47D or MCF7 parental, $HER2^{WT}$, $HER2^{S310F}$, $HER2^{V777L}$, $HER2^{778insGSP}$, or $HER2^{L755S}$ cells were treated with indicated concentration of lapatinib (L), Everolimus (E) or the combination for 4 h. Cell lysates (50 μg) were prepared and blotted with the indicated antibodies. **(A)** Stably transduced T47D cell lines. **(B)** Stably transduced MCF7 cell lines. **(C)** Schematic diagram showing HER2 mutations could activate HER2 (left), and HER2-targeted therapies with Everolimus could inhibited HER2 mutant-expressing cells proliferation (right).

Everolimus inhibit the proliferation and 3D growth of HER2 mutant-expressing cells and the activity of HER2 downstream signaling. Moreover, the Lapatinib combination group showed better efficacy than the Trastuzumab combination group *in vitro* analyses. The above evidence suggests that reversible HER2 inhibitor Lapatinib combined with Everolimus might be a better choice for HER2 mutant cancers. Lapatinib is a potent EGFR and ErbB2 inhibitor, which can inhibit EGFR and ErbB-2 receptor autophosphorylation [27]. Lapatinib could also induce ferroptosis in breast cancer cells, not via targeting EGFR and HER2 [28]. In ER+ breast cancer, PI3K/AKT/mTOR pathway was also activated which is associated with tumorigenesis and resistance to endocrine therapy [29–30]. These also raise the possibility that other mechanisms exist for the blocking effect of lapatinib. In addition, a novel activating HER2 mutation, 778insGSP, was also functionally characterized in our investigation. Together, our findings have shed light on the therapeutic options for patients with HER2 mutations. Longitudinal biopsy of peripheral blood might be a good way to comprehensively and quantitatively assess the patient's pre-treatment status, and be conducive to the development of individualized treatment, providing new treatment methods and ideas for the clinic. In the future, we hope to provide a larger sample of clinical data in follow-up studies.

Ethics approval and consent to participate

This study was reviewed and approved by the Ethics Committee of The Second Hospital of Dalian Medical University. All patients provided informed consents.

CRediT authorship contribution statement

Jing Ma: Visualization, Methodology, Funding acquisition, Data curation, Writing – review & editing, Writing – original draft. **Xuelu Li:** Visualization, Methodology, Funding acquisition, Data curation, Writing – review & editing, Writing – original draft. **Qianran Zhang:** Funding acquisition, Data curation, Writing – review & editing, Writing – original draft. **Ning Li:** Funding acquisition, Data curation, Writing – review & editing, Writing – original draft. **Siwen Sun:** Funding acquisition, Data curation, Writing – review & editing, Writing – original draft. **Shanshan Zhao:** Funding acquisition, Data curation, Writing – review & editing, Writing – original draft. **Zuowei Zhao:** Visualization, Funding acquisition, Data curation, Writing – review & editing, Writing – original draft, Supervision. **Man Li:** Visualization, Funding acquisition, Data curation, Writing – review & editing, Writing – original draft, Supervision.

Declaration of Competing Interest

All authors have stated that they have no conflicts of interest.

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Supplementary materials

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