

Alpelisib Exhibits a Synergistic Effect with Pyrotinib and Reverses Acquired Pyrotinib Resistance in HER2+ Breast Cancer

Hao Chen

First Affiliated Hospital of Wenzhou Medical University

Yuhao Si

First Affiliated Hospital of Wenzhou Medical University

Jialiang Wen

First Affiliated Hospital of Wenzhou Medical University

Chunlei Hu

First Affiliated Hospital of Wenzhou Medical University

Erjie Xia

First Affiliated Hospital of Wenzhou Medical University

Yinghao Wang

First Affiliated Hospital of Wenzhou Medical University

Jizhao Niu

University of Bristol

Ouchen Wang (✉ woc@wmu.edu.cn)

First Affiliated Hospital of Wenzhou Medical University

Research Article

Keywords: pyrotinib, HER2 positive breast cancers, alpelisib, PI3K p110 α , pyrotinib-resistance

Posted Date: January 5th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1221004/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Human epidermal growth factor receptor 2 (HER2) plays a vital role in breast cancer progression in patients who overexpress HER2, thus promoting the rapid progress of targeted drugs development and therapy strategies advancement targeting this gene. Pyrotinib, approved in clinical by the Chinese State Drug Administration, is a novel pan-ErbB kinase inhibitor and exhibits better efficacy than lapatinib. Alpelisib is a selective PI3K p110 α inhibitor approved for application in HR+, HER2-, PIK3CA mutated breast cancers. We assumed that combining pyrotinib with alpelisib worked better than single-drug treatment.

Methods: We performed the drug combination assay to evaluate the combination index (CI) of pyrotinib and alpelisib in HER2+ breast cancer cell lines. Cell functional assays, RT-qPCR (Real Time-Quantitative Polymerase Chain Reaction) and western blotting were performed to elucidate the combined efficacy of two drugs and explore the underlying mechanism. Then we established the acquired pyrotinib resistant HER2+ breast cancer cell lines and evaluate the combined efficacy of two drugs in pyrotinib resistant cells and explore the potential mechanisms.

Results: Our data exhibited that a combination of alpelisib and pyrotinib showed a synergistic effect in HER2+ breast cancer by enhancing cell proliferation and migration decrease, G0-G1 cell cycle arrest, and apoptosis rate increase. Additionally, alpelisib combined with pyrotinib showed a tremendous synergistic effect in acquired pyrotinib resistant cells.

Conclusions: Our results provided the preclinical evidence that a combination of pyrotinib and alpelisib as an effective therapeutic strategy in treating HER2+ breast cancer, whether patients were sensitive or resistant to pyrotinib treatment.

1. Background

Breast cancer is the most diagnosed cancer and causes significant mortality in women ^{1,2}. Approximately 15-20% of patients with breast cancer overexpress HER2 (human epidermal growth factor receptor 2) ³⁻⁶, which is relevant to more aggressive progression and worse prognosis. HER2 plays an essential role in regulating cancer cells' proliferation, differentiation, and apoptosis ⁷. The relationship between HER2 expression and the aggressive phenotype of HER2+ breast cancer makes this gene appealing for targeted therapies, with an increasing number of drugs being developed to target HER2. Several anti-HER2 drugs are currently available to treat HER2+ breast cancer in clinical. Five targeted drugs, including trastuzumab, pertuzumab, TDM-1, lapatinib, and neratinib, have been approved by the U.S. Food and Drug Administration (FDA) to treat HER2+ breast cancer ⁸⁻¹². Pyrotinib, a novel drug that targets the pan-ErbB receptors, exhibits an excellent anti-tumor activity against HER2+ breast cancer and has been approved by the Chinese State Drug Administration for clinical use recently ¹³⁻¹⁵.

Pyrotinib is a novel oral, irreversible pan-ErbB receptor tyrosine kinase inhibitor (TKI), which shows inhibitory activity against epidermal growth factor receptor (EGFR)/HER1, HER2, and HER4¹³. Clinical trial data revealed the excellent anti-tumor activity of pyrotinib in treating HER2+ breast cancer. In a randomized, phase II trial, the overall response rate in tested patients was 78.5% when treated with pyrotinib compared to 57.1% treated with lapatinib. The median progression-free survival (PFS) was 18.1 months with pyrotinib compared to 7.0 months with lapatinib. Besides, patients showed better tolerance to adverse reactions when treated with pyrotinib¹⁶.

Alpelisib (BYL-719) is a phosphatidylinositol 3-kinase (PI3K) inhibitor that primarily inhibits PI3K p110 α (IC₅₀=5nM), also with lower effects in inhibiting other PI3K catalytic subunit: p110 β (IC₅₀=1200nM), p110 δ (IC₅₀=290nM), p110 γ (IC₅₀=250nM)¹⁷⁻¹⁹. Amplification or mutation of the PIK3CA gene, which encodes the catalytic subunit PI3K p110 α , is observed in approximately 40% of breast cancers^{20,21}. Alpelisib, combined with fulvestrant, is approved to treat HR+, HER2-, PIK3CA mutated breast cancer patients by FDA. Besides, alpelisib is also under development in HER2+ breast cancer in several countries worldwide.

It is vital to develop drug combination strategies to delay drug resistance and lower the dose toxicity of pyrotinib. PI3K/AKT signaling pathway is one of the most crucial activated pathways downstream the HER2^{22,23}. Alpelisib, a PI3K p110 α inhibitor, effective in wild-type and mutated PIK3CA, can effectively inhibit the PI3K/AKT pathway. We inferred that alpelisib would show significant anti-tumor activity in HER2+ breast cancer and could be combined with pyrotinib for a better therapeutic activity. Hence, we hypothesized that the combination of pyrotinib and alpelisib would show a synergistic effect against HER2+ breast cancer. Additionally, drug resistance is the main reason that limits the targeted therapies. There are increasing pyrotinib resistant cases appearing in clinical, thus understanding the potential mechanism underlying the acquired pyrotinib resistance and exploring the substitute treatments are essential. In the present study, we investigated the combined effect of pyrotinib and alpelisib in HER2+ breast cancer. Furthermore, we established a pyrotinib resistant breast cancer cell line to explore the mechanism of drug resistance and investigate the combination effect of pyrotinib and alpelisib in acquired pyrotinib resistant HER2+ breast cancer. The results showed that a combination of pyrotinib and alpelisib be a practical choice for clinical applications to treat HER2+ breast cancer patients, whether they are sensitive or resistant to pyrotinib treatment.

2. Materials And Methods

2.1 Cell lines and cell cultures

Two human HER2+ breast cancer cell lines SK-BR-3 and BT-474 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Two human HR+, HER2-, PIK3CA mutated breast cancer cell lines T47D and MCF-7 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SKBR-3-PR was a pyrotinib resistant cell line obtained by exposing SKBR-3 cells to

increasing concentration of pyrotinib continuously. SK-BR-3 and SKBR-3-PR were cultured in Mcoy'5A (Gibco, US) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), BT-474 and T47D were cultured in RMPI-1640 (Gibco, US) with 10% FBS. MCF-7 was cultured in DMEM (Gibco, US) supplemented with 10% FBS.

All cell lines have recently been tested and confirmed with no mycoplasma contamination.

2.2 Chemicals

Pyrotinib dimaleate (SHR-1258 dimaleate) was obtained from Hengrui Medicine Co. Ltd (China). Alpelisib (BYL-719) and PI-3065 (PI3K p110 δ inhibitor) were purchased from MCE (MedChemExpress, US). Agents were dissolved in dimethylsulfoxide (DMSO) and were kept at -20 °C for future use.

2.3 Cell viability assay and drug combination study

CCK-8 cell viability assay was performed to identify the 50% inhibitory concentrations (IC₅₀) and combination index (CI). Cells were seeded in a 96-well plate at a density of 6000 - 12000 cells/well and cultured overnight, then cells were added with drugs at different concentrations. After 72h of treatment, Cell Counting Kit (CCK)-8 reagent (MCE, USA) was added at a concentration of 10%, and cells were incubated in a CO₂ incubator for 3h, OD₄₅₀ was measured. Cell inhibitory rate was calculated as $(OD_{450}^{\text{negative control}} - OD_{450}^{\text{treatment}}) / (OD_{450}^{\text{negative control}} - OD_{450}^{\text{black control}})$, and the 50% inhibitory concentration was determined as IC₅₀ of the drug. The combination index was calculated by using CompuSyn (ComboSyn Inc.) according to the theory of professor Ting-Chao Chou^{24,25}. CI values could indicate antagonistic (> 1.2), additive (1-1.2), and synergistic (< 1) effects of two or more drugs combination.

2.4 Cell proliferation assay and colony formation assay

Cells were seeded in a 96-well plate and cultured overnight. Then cells were added with DMSO, pyrotinib, alpelisib, or combinations. Every 24h, cells were added with CCK-8 and incubated for 3h, then OD₄₅₀ was measured. The experiment was performed three times, and the cell proliferation curves were drawn.

Cells were cultured in a 6-well plate at a density of 3000 cells/well, then cells were added with different treatments the other day. The culture medium containing corresponding drugs was renewed every three days, and cells were cultured for three weeks. Finally, cells were fixed with 4% paraformaldehyde and stained with 0.1% Crystal Violet. The colony (>50 cells/colony) was counted and compared to the controlled group. The experiment was performed in triplicate, and the data were representative of three separate experiments.

2.5 Transwell assay

Cells (10^5) suspended in 300 μ L serum-free medium containing different drug treatments were transferred into the upper chamber of 24-well transwell chambers (#3422, Corning, NY, USA), and 600 μ L medium containing 20% FBS was added to the lower chamber. After incubation for 24 h, cells crossed the membrane were fixed with 4% PFA and stained with 0.1% Crystal violet. For each sample, 5 fields of view were obtained, and cell numbers were counted. The experiment was performed in triplicate, and the data were representative of three separate experiments.

2.6 Cell cycle assay

Cells were starved for 24h and treated with the corresponding treatments for 72h, and then cells were harvested and fixed with 75% ethanal at -20°C overnight. Fixed cells were washed with PBS, resuspended in 100 μ L PBS, stained with 20 μ L 7-AAD in darkness for 15min at room temperature, and finally measured using a flow cytometer (BD Accuri C6 Plus, USA) according to the manufacturer's instruction. The data were analyzed using Flowjo software (v.10.5; TreeStar, CA, USA). The final data were obtained from three independent experiments.

2.7 cell apoptosis assay

Cells were obtained after 72h of treatment and washed twice with PBS. Then cells were re-suspended in 100 μ L $1\times$ binding buffer (BD Biosciences, NJ, USA), added with 5 μ L of Annexin V-FITC and 7-AAD and incubated in darkness for 15 minutes at room temperature. Samples were added with another 400 μ L $1\times$ binding buffer before being measured with a flow cytometer (BD Accuri C6 Plus, USA). The data were analyzed using Flowjo software (v.10.5; TreeStar, CA, USA).

Tunel assays were performed according to the manufacturer's instructions (Beyotime, Shanghai, China). Cells were plated in a 24-well plate and treated with different treatments for 72 hours. Then cells were fixed with 4% PFA for 30 minutes and permeabilized with 0.3% Triton-X-100 for 5 minutes. Cells were incubated with terminal deoxyribonucleotidyl transferase (TdT) at 37°C in darkness for an hour and added with fluorescent mounting media (Beyotime, Shanghai, China). Pictures were taken by using DMi8 (Leica, German).

All assays were performed in triplicate, and the data were obtained from three separate experiments.

2.8 NA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted with Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA was reverse-transcribed with ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real-time PCR analysis was performed on Applied Biosystems Quantstudio5

(Thermo Fisher Scientific, USA) with PowerUp SYBR Green Master Mix (#A25742, Thermo Fisher Scientific, USA) following the manufacturer's protocol. The relative expression of mRNA to GAPDH was calculated. The data were obtained from three separate experiments. The sequences of primers are enlisted in Supplementary Table 1.

2.9 Protein extraction and western blot analysis

Total protein was extracted using Cell lysis buffer for Western and IP (Beyotime, Shanghai, China) supplemented with proteinase and phosphatase inhibitors cocktail tablet (Pierce, Thermo, USA). Protein quantification was performed using the BCA reagent (Pierce, Thermo, USA) according to the manufacturer's instructions, and then samples were denatured with 5× loading buffer at 100°C for 5min. The extracted protein samples were loaded into 8% - 15% SDS-PAGE gel for separation and transferred to 0.45µM polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, USA). The membrane was blocked with 5% skim milk or BSA for an hour. Primary antibodies were added, and the membrane was incubated at 4°C overnight. Subsequently, the membrane was added with fluorescent secondary antibodies (LI-COR Biosciences, USA) and incubated in darkness for an hour at room temperature. The immunoreactive bands were obtained and analyzed using the LI-COR Odyssey CLx (LI-COR Biosciences, USA). The primary and secondary antibodies used are enlisted in Supplementary Table 2.

2.10 Statistical analysis

Student's t-tests and Analyses of variance models were used to compare mean values between the tested and controlled groups. Statistical analyses were performed with IBM SPSS version 22 (SPSS, NY, USA) and GraphPad Prism version 7 (GraphPad Software Inc., CA, USA). Results were presented as mean ± SD of three independent tests. The statistical significance of the difference between tested and controlled groups was assessed at significance thresholds of * (P < 0.05), ** (P < 0.01), and *** (P < 0.001).

3. Results

3.1 Alpelisib showed a synergistic effect when combined with pyrotinib in treating HER2+ breast cancer cells

We first verified the ErbB, HR expression, and PIK3CA mutation situations in four breast cancer cell lines (BT-474, SKBR-3, MCF-7, and T47D). We found that BT-474 and SKBR-3 overexpressed HER2, while BT-474, T47D, and MCF-7 had mutations in PIK3CA (Table 1). We also examined the protein and mRNA expression of EGFR, HER2, and PI3K p110α (Fig. 1A-1C). Then we evaluate the inhibitory effects of pyrotinib and alpelisib on four cell lines. The result indicated that three cell lines were sensitive to alpelisib except for MCF-7 (Fig. 1E). Only BT-474 and SKBR-3 were sensitive to pyrotinib (Fig. 1D). The IC50 values of four breast cancer cells to different treatments were presented in Table 1. To examine the

combined effect of pyrotinib and alpelisib in HER2+ breast cancer cells, we exposed BT-474 and SKBR-3 cells to two drugs individually or in combination with different concentrations and calculated the combination index. The result indicated that a combination of pyrotinib and alpelisib showed a synergistic effect in treating HER2+ breast cancer cells, whether cells had mutations in PIK3CA (Fig 1F, 1G).

Table 1

The molecular and gene signature of 4 breast cancer cell lines and their IC50 to pyrotinib or alpelisib treatment

Cell Line	HER2 Protein Expression ^[1]	PIK3CA mutation situation ^[3]	HR Protein Expression		IC50 (72h) ^[2]	
			ER	PR	PYR	ALP
BT-474	3+	PIK3CA; Simple; p.Lys111Asn (c.333G>C)	+	+	5.78 ± 0.75 nM	0.82 ± 0.03 µM
SKBR-3	3+	-	-	-	4.83 ± 0.88 nM	0.71 ± 0.05 µM
T47D	0-1+	PIK3CA; Simple; p.Leu194Phe (c.580C>T)	+	+	0.8 ± 0.14 µM	0.34 ± 0.03 µM
MCF-7	0-1+	PIK3CA; Simple; p.Glu545Lys (c.1633F>A)	+	+	42.4 ± 55.4 µM	38.3 ± 31 µM

¹ Previously reported in western blotting (Fig. 1).

² IC50 value (drug concentration required to inhibit growth by 50%) compared with vehicle-treated controls and calculated using CompuSyn (ComboSyn Inc.). Values shown are the means of 3 independent experiments ± SD.

³ The PIK3CA mutation situations were obtained in Website Cellosaurus (<https://web.expasy.org/cellosaurus/>)

3.2 Alpelisib combined with pyrotinib enhanced the inhibitory effect on cancer-associated phenotypes significantly

We first exposed BT-474 and SKBR-3 cells to increasing concentration of pyrotinib (DMSO, 3nM, 6nM, 9nM) and alpelisib (DMSO, 0.1uM, 0.2uM, 0.3uM) for 24h and detected the protein expression of ErbB/PI3K/AKT pathway through western blotting (Fig. 1H, 1I). We determined 6nM of pyrotinib and

0.2 μ M of alpelisib for further experiments, then we exposed BT-474 and SKBR-3 cells to two inhibitors, individually or in combination and detected cancer-associated phenotypes. The combination therapy significantly decreased the cell proliferation, cell clone formation and invasion, increased cell apoptosis rate, and arrested the cell cycle to the G0-G1 stage (Fig 1J, 1K, Fig 2A – 2J).

3.3 The combination of pyrotinib and alpelisib could enhance the downregulation of the PI3K/AKT pathway activation and strengthen the activation of the apoptosis pathway

We detected the protein levels of ErbB and downstream PI3K/AKT signaling pathways. We observed that combined pyrotinib with alpelisib significantly reduced PI3K/AKT signaling pathway activation (Fig 3A, 3C, 3D). Besides, we detected the protein expression levels related to cell apoptosis. The result showed that the apoptosis-related proteins were significantly activated when treating cells with pyrotinib and alpelisib (Fig 3B, 3E, 3F).

3.4 HER2+ breast cancer cells up-regulated ErbB ligand expression to counter alpelisib treatment

To investigate the potential mechanism behind the synergistic effect between pyrotinib and alpelisib, we treated BT-474 and SKBR-3 cells with elevated concentrations of alpelisib (DMSO, 0.05 μ M, 0.5 μ M, 5 μ M). We detected the expression of total EGFR, HER2, and their phosphorylation forms through western blotting. We observed that cancer cells expressed higher activated HER2 and EGFR when treated with alpelisib than controlled groups (Fig 4A-4D). Some underlying mechanisms exist to activate the ErbB receptors to counter alpelisib treatment and cells are more sensitive to pyrotinib. Receptors are activated when combined with their ligands. We hypothesized that the expression of ErbB ligands or proteins that activate these ligands might be up-regulated when cells were treated with alpelisib. Therefore, we detected the mRNA expression levels of two Metallopeptidase (ADAM10 and ADAM17) and seven ErbB ligands (REG, AREG, EPGN, TGF- α , BTC, EGF, HB-EGF) expression levels through qPCR. When treated with alpelisib, BT-474 cells expressed significantly higher levels of HB-EGF, AREG, EPGN, and BTC (REG, TGF- α , and BTC in SKBR-3 cells) (Fig 4E, 4F). The result could explain the higher levels of activated HER2 and EGFR when cells were treated with alpelisib. The schema chart of potential mechanism was shown in Fig 6A.

3.5 HER2+ breast cancer cells acquired pyrotinib resistance by down-regulating phosphorylation of ErbB proteins and escaped target by pyrotinib

To investigate the potential resistance mechanism of pyrotinib in HER2+ breast cancer cells, we constructed a pyrotinib-resistant cell line, SKBR-3-PR. The IC₅₀ of SKBR-3-PR to pyrotinib was 104.97 ± 24.20 nM compared with 4.83 ± 0.88 nM in SKBR-3 cells (Fig. 5a). Then we detected the mRNA and protein expression of HER2, EGFR. At the mRNA level, the expression of ErbB2 was not significantly changed, and the expression of EGFR was up-regulated in SKBR-3-PR cells compared to SKBR-3 cells (Fig 5D). The protein expression of HER2 and EGFR were not changed; however, their phosphorylated forms were significantly down-regulated in SKBR-3-PR cells relative to SKBR-3 cells (Fig 5E, 5F). The results meant the acquired pyrotinib resistant cells were less dependent on ErbB receptors activation and counted more on other pathways and induced cells resistant to pyrotinib treatment.

3.6 Alpelisib showed synergistic effect with pyrotinib in treating acquired pyrotinib resistant HER2+ breast cancer cells by inhibiting the up-regulated of p110δ

We further measured the IC₅₀ values of SKBR-3 and SKBR-3-PR cells for alpelisib treatment and found that SKBR-3-PR cells were less sensitive to alpelisib compared to SKBR-3 cells (Fig 5B, IC₅₀=0.71±0.06μM in SKBR-3, IC₅₀=1.57±0.17μM in SKBR-3-PR), which is consistent with less p110α protein expression in SKBR-3-PR cells (Fig. 5E, 5F). However, we found no significant decrease in AKT activation in SKBR-3-PR cells compared to SKBR-3 cells (Fig 5H, 5I). There might be some other mechanism that existed to compensate for p110α down-regulation. The AKT was mainly activated by PIP3, which was converted from PIP2 by PI3K, so we detected the mRNA expression of other Class I PI3K catalytic subunits (PIK3CB, PIK3CG, PIK3CD) and the PI3K regulatory subunit (PIK3R1) in SKBR-3-PR and SKBR-3 cells. We observed the PIK3CD was up-regulated and the PIK3R1 was down-regulated in SKBR-3-PR cells compared to SKBR-3 cells (Fig 5G). We then detected the expression of proteins they encoded (PIK3CD encoded p110δ, PIK3R1 encoded p85α). P110δ was up-regulated, consistent with its RNA level, but the protein expression of p85α was not changed (Fig. 5E, 5F). We then treated SKBR-3-PR cells and SKBR-3 cells to selective p110δ inhibitor (PI-3065) and found the IC₅₀ value of SKBR-3-PR cells was higher than SKBR-3 cells (Fig. 5J, IC₅₀=1.21±0.13μM in SKBR-3, 0.66±0.08μM in SKBR-3-PR), which meant SKBR-3-PR cells counted more on p110δ. The results could indicate that p110δ was up-regulated and compensated for the p110α. More interestingly, we found that the mTOR was significantly activated in SKBR-3-PR cells (Fig. 5H, 5I), and there might be other mechanisms existing to activate downstream mTOR.

Though less effective than inhibiting p110α, alpelisib showed an inhibitory effect on p110δ. We combined pyrotinib with alpelisib to treat SKBR-3-PR cells and observed that these two drugs exhibited a synergistic effect in pyrotinib resistant cancer cells and reduced cell proliferation, enhanced cell apoptosis, and arrested the cell cycle to the G₀-G₁ stage (Fig. 5C, K-Q).

In summary, SKBR-3-PR cells up-regulated the expression of p110δ to compensate for p110α down-regulation and activate the downstream AKT signaling pathway. Alpelisib could inhibit p110δ in high

concentrations. Thus, alpelisib and pyrotinib showed a synergistic effect in treating acquired pyrotinib resistant breast cancer cells. The schema chart of potential mechanism was shown in Fig 6B.

Discussion

Adverse reactions are the main reasons for drug withdrawal, which is often relevant to high doses. To lower the dose patients needed to reduce the possibility of adverse reaction but with no efficacy reduction, patients are often treated with several drug combinations. Besides, drug combinations are used to delay or reverse the acquired drug resistance. It is crucial to combine pyrotinib with other anti-tumor drugs to extend the validity of pyrotinib when treating patients.

It has been reported that the PI3K-AKT pathway is one of the most crucial downstream signaling pathways of the ErbB receptor. In theory, simultaneously inhibition of upstream signaling molecular ErbB and the downstream signaling molecular p110 α overlap their anti-tumor activities and may not show a synergistic effect when treating HER2+ breast cancer. ADAM (a disintegrin and metalloprotease) proteins are a class of membrane-anchored metalloproteases that process the ectodomains of membrane-anchored growth

factors, cytokines and receptors. Among many ADAM proteins, ADAM10 and ADAM17 were reported to show proteolysis function and release ErbB ligands anchored to the membrane^{26,27}. EGF, HB-EGF, AREG, EREG, EPGN, TFG- α , BTC are the main ErbB ligands that activate HER2 and EGFR²⁸⁻³¹. Our data showed that HER2+ breast cancer cells exposed to alpelisib treatment alone up-regulated the expression of ErbB ligands, which had a feedback activation of the ErbB receptors. Thus HER2+ breast cancer cells treated with alpelisib were more sensitive to ErbB inhibitor, which can explain the synergistic effect of alpelisib and pyrotinib.

HER2+ breast cancer acquires resistance to anti-HER2 therapy through several different mechanisms. HER2 mutation can induce continuous self-phosphorylation of HER2 or escape distinguished by targeted inhibitors, thus activating the downstream signaling pathway. In some cases, PIK3CA mutation or alternative upstream pathway activation induces continuous activation of the PI3K/AKT signaling pathway. Besides, tumor microenvironment and cell epigenetics change can induce cancer resistance to anti-HER2 therapy³²⁻³⁷. To investigate the potential mechanisms that contribute to pyrotinib resistance and explore the treatment strategies, we compared the differences in acquired pyrotinib resistant cells and their parent cells. In our study, pyrotinib resistance HER2+ breast cancer cells reduced the activation of HER2 and EGFR significantly, which could escape the anti-ErbB therapy and account for the low sensitivity of cells to pyrotinib treatment. P110 δ , the isoforms of P110 α , can also bind to PI3K regulatory subunit P85 α to activate the downstream AKT. P110 δ was reported to be predominantly expressed in white blood cells^{38,39}. However, evidence revealed that p110 δ is also expressed in some cancer cell lines and human tissues such as breast cancer cells and it plays a vital role in breast cancer^{40,41}. In our study, though acquired pyrotinib resistant cells expressed lower levels of p110 α , no significant reduction of the activation of downstream AKT was observed, which meant there might be some alternative molecular

exist to compensate for the down-regulation of p110 α . We observed that p110 δ was significantly up-regulated in pyrotinib resistant cells. Besides, we observed a significant mTOR activation in pyrotinib resistant cells compared to the sensitive ones, which meant there might be other mechanisms upstream of the mTOR that were strengthened to activate mTOR. As a result, activation of AKT/mTOR signaling via the P110 δ up-regulation and other signaling pathways activation enhanced the proliferation and survival of pyrotinib resistant cells.

This study has limitations. Though our findings are compelling, our research is based on in-vitro models. In-vivo assays should be done to verify the synergistic effects observed in cell experiments. Besides, more different pyrotinib resistant breast cancer cell lines need to be established and clinical studies need to be conducted to verify the results and the mechanism observed in this study. Furthermore, clinical trials are required to establish the safety and efficacy of drug combinations in patients.

Conclusions

In conclusion, our study revealed that pyrotinib exhibited synergistic anti-tumor effects with alpelisib in HER2+ breast cancer. Besides, in acquired pyrotinib resistant HER2+ breast cancer, alpelisib also showed significant synergistic antitumor effects with pyrotinib and could reverse drug resistance. These results suggest that the combination of pyrotinib and alpelisib is an effective treatment strategy that can be applied for HER2+ breast cancer patients, whether sensitive or resistant to pyrotinib treatment.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated for this study are included in the article.

Competing interests

No author has financial or other contractual agreements that might cause conflicts of interest.

Funding:

None.

Author contributions

Hao Chen performed study concept, design and writing; Yuhao Si, Jialiang Wen and Chunlei Hu provided acquisition, analysis and interpretation of data, and statistical analysis; Erjie Xia, Yinghao Wang, and Jizhao Niu provided technical and material support; Ouchen Wang performed the development of methodology, review, and revision of the paper.

Acknowledgment

We acknowledge Hengrui for providing pyrotinib compound.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. Nov 2018;68(6):394-424. doi:10.3322/caac.21492
2. Tzahar E, Waterman H, Chen X, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol*. Oct 1996;16(10):5276-87. doi:10.1128/MCB.16.10.5276
3. Wolff AC, Hammond ME, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J Clin Oncol*. Nov 1 2013;31(31):3997-4013. doi:10.1200/JCO.2013.50.9984
4. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. Aug 17 2000;406(6797):747-52. doi:10.1038/35021093
5. Moasser MM, Krop IE. The Evolving Landscape of HER2 Targeting in Breast Cancer. *JAMA Oncol*. Nov 2015;1(8):1154-61. doi:10.1001/jamaoncol.2015.2286
6. Mitri Z, Constantine T, O'Regan R. The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemother Res Pract*. 2012;2012:743193. doi:10.1155/2012/743193
7. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. Feb 2001;2(2):127-37. doi:10.1038/35052073
8. Graziano C. HER-2 breast assay, linked to Herceptin, wins FDA's okay. *CAP Today*. Oct 1998;12(10):1, 14-6.

9. Swain SM, Kim SB, Cortes J, et al. Pertuzumab, trastuzumab, and docetaxel for HER2-positive metastatic breast cancer (CLEOPATRA study): overall survival results from a randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol*. May 2013;14(6):461-71. doi:10.1016/S1470-2045(13)70130-X
10. Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*. Dec 28 2006;355(26):2733-43. doi:10.1056/NEJMoa064320
11. Deeks ED. Neratinib: First Global Approval. *Drugs*. Oct 2017;77(15):1695-1704. doi:10.1007/s40265-017-0811-4
12. Verma S, Miles D, Gianni L, et al. Trastuzumab emtansine for HER2-positive advanced breast cancer. *N Engl J Med*. Nov 8 2012;367(19):1783-91. doi:10.1056/NEJMoa1209124
13. Blair HA. Pyrotinib: First Global Approval. *Drugs*. Nov 2018;78(16):1751-1755. doi:10.1007/s40265-018-0997-0
14. Li X, Yang C, Wan H, et al. Discovery and development of pyrotinib: A novel irreversible EGFR/HER2 dual tyrosine kinase inhibitor with favorable safety profiles for the treatment of breast cancer. *Eur J Pharm Sci*. Dec 15 2017;110:51-61. doi:10.1016/j.ejps.2017.01.021
15. Ma F, Li Q, Chen S, et al. Phase I Study and Biomarker Analysis of Pyrotinib, a Novel Irreversible Pan-ErbB Receptor Tyrosine Kinase Inhibitor, in Patients With Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer. *J Clin Oncol*. Sep 20 2017;35(27):3105-3112. doi:10.1200/JCO.2016.69.6179
16. Ma F, Ouyang Q, Li W, et al. Pyrotinib or Lapatinib Combined With Capecitabine in HER2-Positive Metastatic Breast Cancer With Prior Taxanes, Anthracyclines, and/or Trastuzumab: A Randomized, Phase II Study. *J Clin Oncol*. Oct 10 2019;37(29):2610-2619. doi:10.1200/JCO.19.00108
17. Furet P, Guagnano V, Fairhurst RA, et al. Discovery of NVP-BYL719 a potent and selective phosphatidylinositol-3 kinase alpha inhibitor selected for clinical evaluation. *Bioorg Med Chem Lett*. Jul 1 2013;23(13):3741-8. doi:10.1016/j.bmcl.2013.05.007
18. Gobin B, Huin MB, Lamoureux F, et al. BYL719, a new alpha-specific PI3K inhibitor: single administration and in combination with conventional chemotherapy for the treatment of osteosarcoma. *Int J Cancer*. Feb 15 2015;136(4):784-96. doi:10.1002/ijc.29040
19. Markham A. Alpelisib: First Global Approval. *Drugs*. Jul 2019;79(11):1249-1253. doi:10.1007/s40265-019-01161-6
20. Juric D, Rodon J, Tabernero J, et al. Phosphatidylinositol 3-Kinase alpha-Selective Inhibition With Alpelisib (BYL719) in PIK3CA-Altered Solid Tumors: Results From the First-in-Human Study. *J Clin Oncol*. May 1 2018;36(13):1291-1299. doi:10.1200/JCO.2017.72.7107
21. Pereira B, Chin SF, Rueda OM, et al. The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. *Nat Commun*. May 10 2016;7:11479. doi:10.1038/ncomms11479
22. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer*. Aug 2009;9(8):550-62. doi:10.1038/nrc2664

23. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*. Jul 2002;2(7):489-501. doi:10.1038/nrc839
24. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev*. Sep 2006;58(3):621-81. doi:10.1124/pr.58.3.10
25. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*. Jan 15 2010;70(2):440-6. doi:10.1158/0008-5472.CAN-09-1947
26. Peschon JJ, Slack JL, Reddy P, et al. An essential role for ectodomain shedding in mammalian development. *Science*. Nov 13 1998;282(5392):1281-4. doi:10.1126/science.282.5392.1281
27. Sahin U, Weskamp G, Kelly K, et al. Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J Cell Biol*. Mar 1 2004;164(5):769-79. doi:10.1083/jcb.200307137
28. Esparis-Ogando A, Montero JC, Arribas J, Ocana A, Pandiella A. Targeting the EGF/HER Ligand-Receptor System in Cancer. *Curr Pharm Des*. 2016;22(39):5887-5898. doi:10.2174/1381612822666160715132233
29. Lemmon MA. Ligand-induced ErbB receptor dimerization. *Exp Cell Res*. Feb 15 2009;315(4):638-48. doi:10.1016/j.yexcr.2008.10.024
30. Riese DJ, 2nd. Ligand-based receptor tyrosine kinase partial agonists: New paradigm for cancer drug discovery? *Expert Opin Drug Discov*. Feb 2011;6(2):185-193. doi:10.1517/17460441.2011.547468
31. Trenker R, Jura N. Receptor tyrosine kinase activation: From the ligand perspective. *Curr Opin Cell Biol*. Apr 2020;63:174-185. doi:10.1016/j.ceb.2020.01.016
32. Kong X, Zhang K, Wang X, et al. Mechanism of trastuzumab resistance caused by HER-2 mutation in breast carcinomas. *Cancer Manag Res*. 2019;11:5971-5982. doi:10.2147/CMAR.S194137
33. Hubalek M, Brunner C, Mattha K, Marth C. Resistance to HER2-targeted therapy: mechanisms of trastuzumab resistance and possible strategies to overcome unresponsiveness to treatment. *Wien Med Wochenschr*. Nov 2010;160(19-20):506-12. doi:10.1007/s10354-010-0838-6
34. Mittal D, Vijayan D, Neijssen J, et al. Blockade of ErbB2 and PD-L1 using a bispecific antibody to improve targeted anti-ErbB2 therapy. *Oncoimmunology*. 2019;8(11):e1648171. doi:10.1080/2162402X.2019.1648171
35. Palomeras S, Diaz-Lagares A, Vinas G, et al. Epigenetic silencing of TGFBI confers resistance to trastuzumab in human breast cancer. *Breast Cancer Res*. Jul 5 2019;21(1):79. doi:10.1186/s13058-019-1160-x
36. Dammann R, Schagdarsurengin U, Strunnikova M, et al. Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. *Histol Histopathol*. Apr 2003;18(2):665-77. doi:10.14670/HH-18.665
37. Christodoulou C, Oikonomopoulos G, Koliou GA, et al. Evaluation of the Insulin-like Growth Factor Receptor Pathway in Patients with Advanced Breast Cancer Treated with Trastuzumab. *Cancer Genomics Proteomics*. Nov-Dec 2018;15(6):461-471. doi:10.21873/cgp.20105

38. Chantry D, Vojtek A, Kashishian A, et al. p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. *J Biol Chem.* Aug 1 1997;272(31):19236-41. doi:10.1074/jbc.272.31.19236
39. Vanhaesebroeck B, Welham MJ, Kotani K, et al. P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc Natl Acad Sci U S A.* Apr 29 1997;94(9):4330-5. doi:10.1073/pnas.94.9.4330
40. Sawyer C, Sturge J, Bennett DC, et al. Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Res.* Apr 1 2003;63(7):1667-75.
41. Tzenaki N, Andreou M, Stratigi K, et al. High levels of p110delta PI3K expression in solid tumor cells suppress PTEN activity, generating cellular sensitivity to p110delta inhibitors through PTEN activation. *FASEB J.* Jun 2012;26(6):2498-508. doi:10.1096/fj.11-198192

Figures

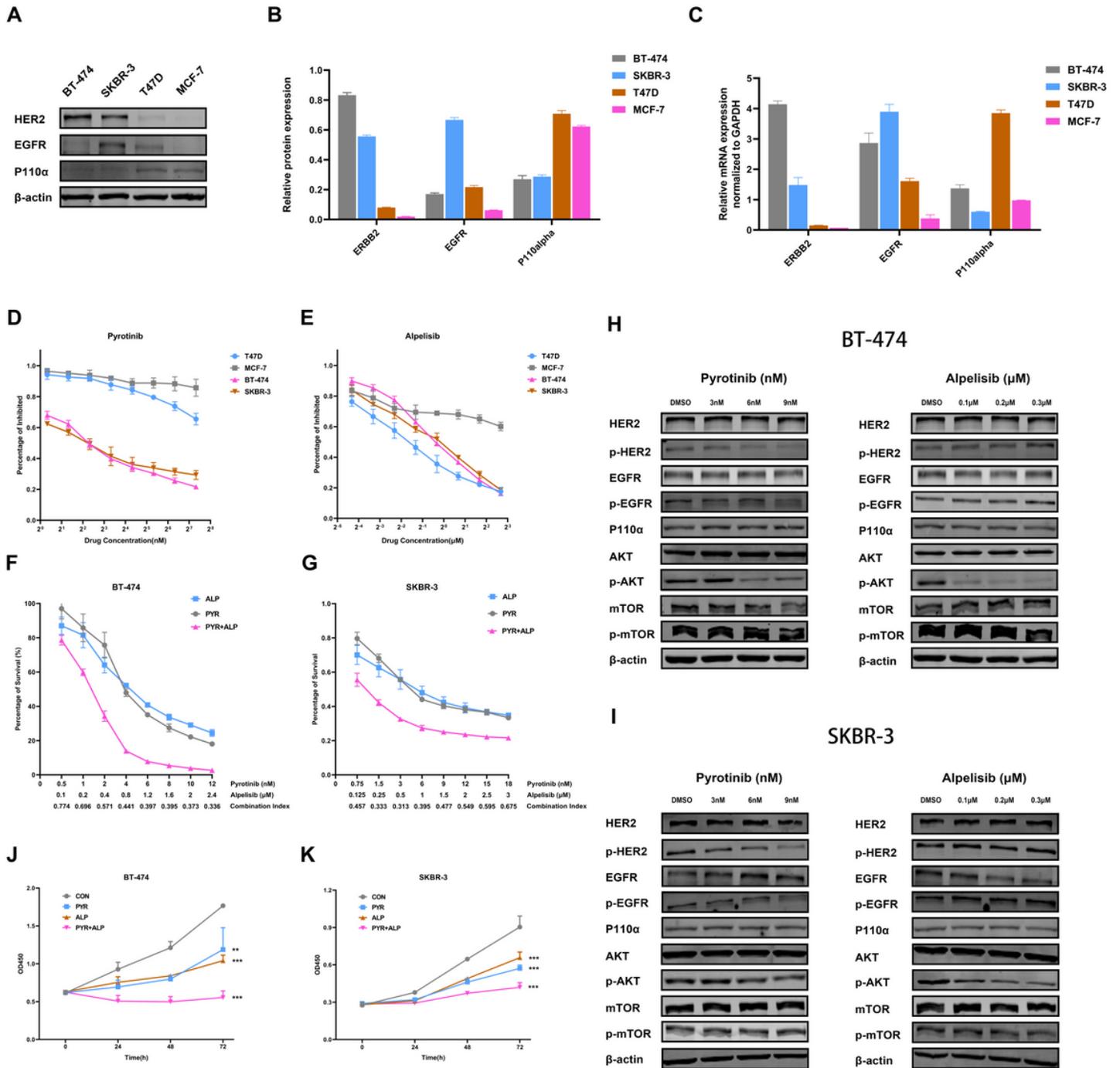


Figure 1

A combination of alpelisib and pyrotinib showed a significantly synergistic treating HER+ breast cancer cells. (A-C) Protein and mRNA expression levels of HER2, EGFR, p110 α in BT-474, SKBR-3, T47D, and MCF-7 cells. (D, E) IC₅₀ values of BT-474, SKBR-3, T47D, and MCF-7 cells treated with pyrotinib and alpelisib for 72h. (F, G) Combination effect curves and combination index (CI) of BT-474 and SKBR-3 cells treated with pyrotinib and alpelisib or combination for 72h. (H, I) Protein expression levels of the ErbB/PI3K/AKT pathway of BT-474 and SKBR-3 cells were treated with increasing alpelisib and pyrotinib

for 24h. (J, K) Cells proliferation curves of BT-474 and SKBR-3 cells treated with control (DMSO), pyrotinib (6nM), alpelisib (0.2μM) or their combinations. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Bars represent group means. n = 3 independent replicates for all experiments.

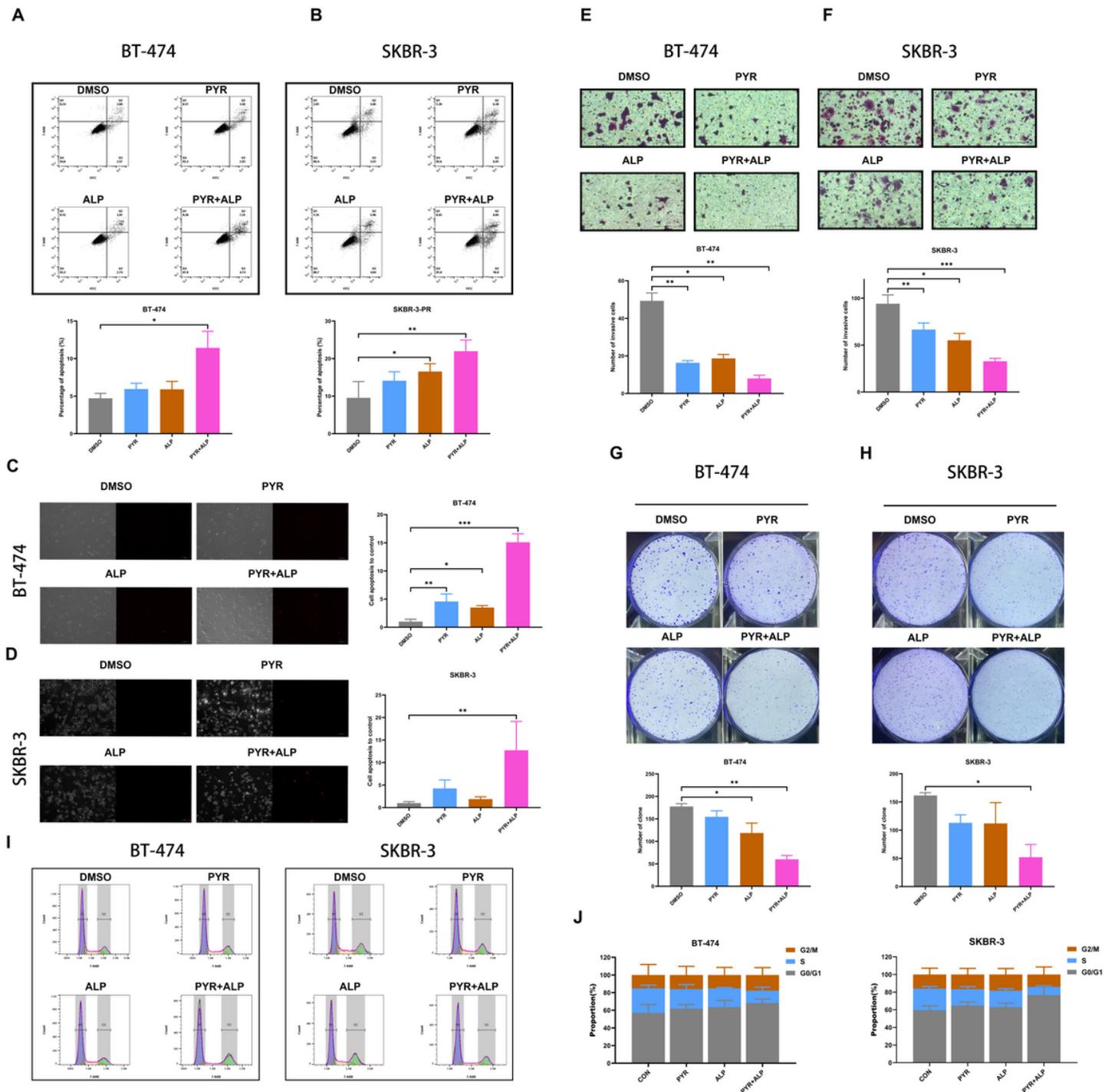


Figure 2

The combination effect of pyrotinib and alpelisib enhancing the inhibitory effect to cancer-associated phenotype in BT-474 and SKBR-3 cells. (A, B) BT-474 and SKBR-3 cells were treated with 6nM pyrotinib,

0.2 μ M alpelisib, or their combinations for 48h and cell apoptosis rates were measured with a flow cytometer. (C, D) BT-474 and SKBR-3 cells were treated with pyrotinib, alpelisib, or their combinations for 48h cell apoptosis were

detected using TUNEL assay. (E, F) BT-474 and SKBR-3 cells were plated in a 24-well transwell plate overnight and treated with pyrotinib, alpelisib, or their combinations for 24h, and cells crossed the membrane were counted. (G, H) BT-474 and SKBR-3 cells were plated in a 6-well plate and treated with pyrotinib, alpelisib, or their combinations for 3 weeks, cell colony numbers were counted. (I, J) BT-474 and SKBR-3 cells were plated in a 6-well plate and treated with pyrotinib, alpelisib, or their combinations for 72h, and cell cycles were analyzed using a flow cytometer. * (P<0.05); ** (P<0.01); *** (P<0.001); **** (P<0.0001). Bars represent group means. n = 3 independent replicates for all experiments.

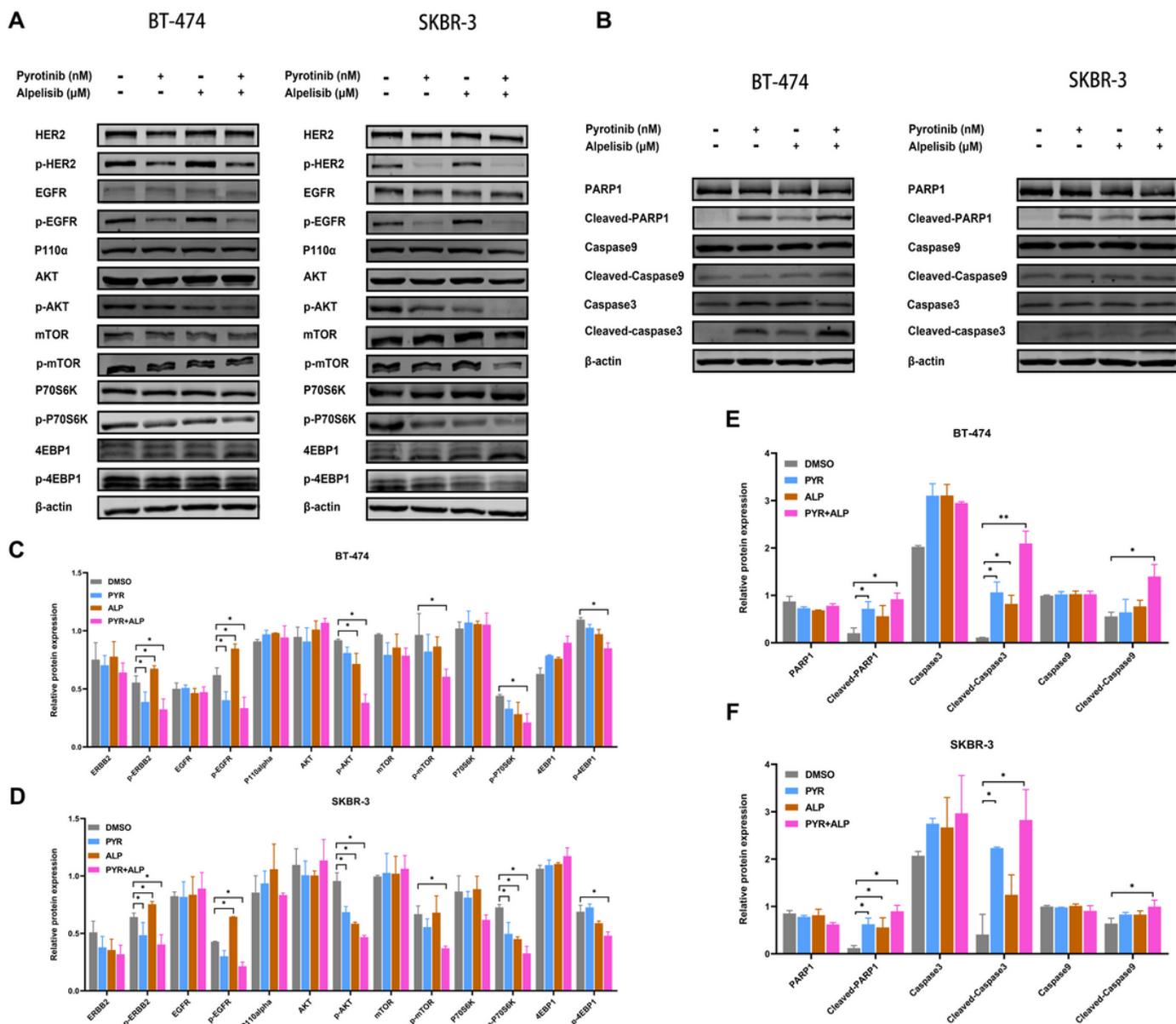


Figure 3

BT-474 and SKBR-3 cells treated with pyrotinib and alpelisib inhibited the ErbB/PI3K/AKT pathway activation and activated the cell apoptosis pathway. (A, C, D) Protein expression levels of the ErbB/PI3K/AKT pathway of BT-474 and SKBR-3 cells treated with pyrotinib, alpelisib, or their combinations for 24h were analyzed. (B, E, F) Protein expression levels of the cell apoptosis pathway of BT-474 and SKBR-3 cells treated with pyrotinib, alpelisib, or their combinations for 24h were analyzed. * (P<0.05); ** (P<0.01); *** (P<0.001); **** (P<0.0001). Bars represent group means. n = 3 independent replicates for all experiments.

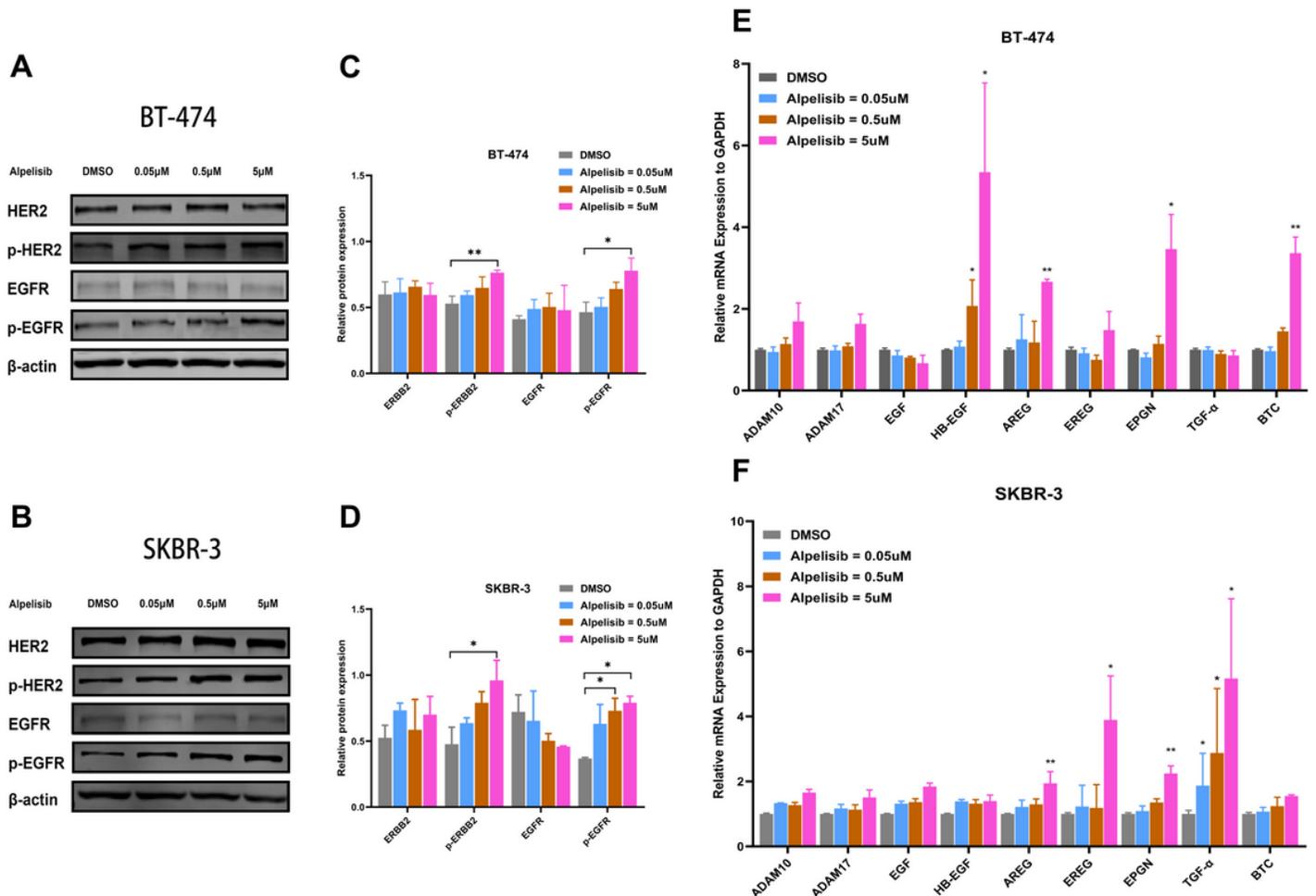


Figure 4

HER2+ breast cancer cells were more sensitive to pyrotinib when treated with alpelisib by up-regulating ErbB ligands and activating ErbB receptors. (A-D) Protein expression of the HER2, EGFR, p-HER2, p-EGFR of BT-474 and SKBR-3 cells treated with increasing concentration of alpelisib (DMSO, 0.05μM, 0.5μM, 5μM) for 24h were analyzed. (E, F) mRNA expression levels of ErbB ligands activation genes (ADAM10 and ADAM17) and ErbB ligands (EGF, HB-EGF, AREG, EREG, EPGN, TGF-α, BTC) for 24h were analyzed. * (P<0.05); ** (P<0.01); *** (P<0.001); **** (P<0.0001). Bars represent group means. n = 3 independent replicates for all experiments. HER2+ breast cancer cells were more sensitive to pyrotinib when treated with alpelisib by up-regulating ErbB ligands and activating ErbB receptors. (A-D) Protein expression of the

HER2, EGFR, p-HER2, p-EGFR of BT-474 and SKBR-3 cells treated with increasing concentration of alpelisib (DMSO, 0.05 μ M, 0.5 μ M, 5 μ M) for 24h were analyzed. (E, F) mRNA expression levels of ErbB ligands activation genes (ADAM10 and ADAM17) and ErbB ligands (EGF, HB-EGF, AREG, EREG, EPGN, TFG- α , BTC) for 24h were analyzed. * (P<0.05); ** (P<0.01); *** (P<0.001); **** (P<0.0001). Bars represent group means. n = 3 independent replicates for all experiments.

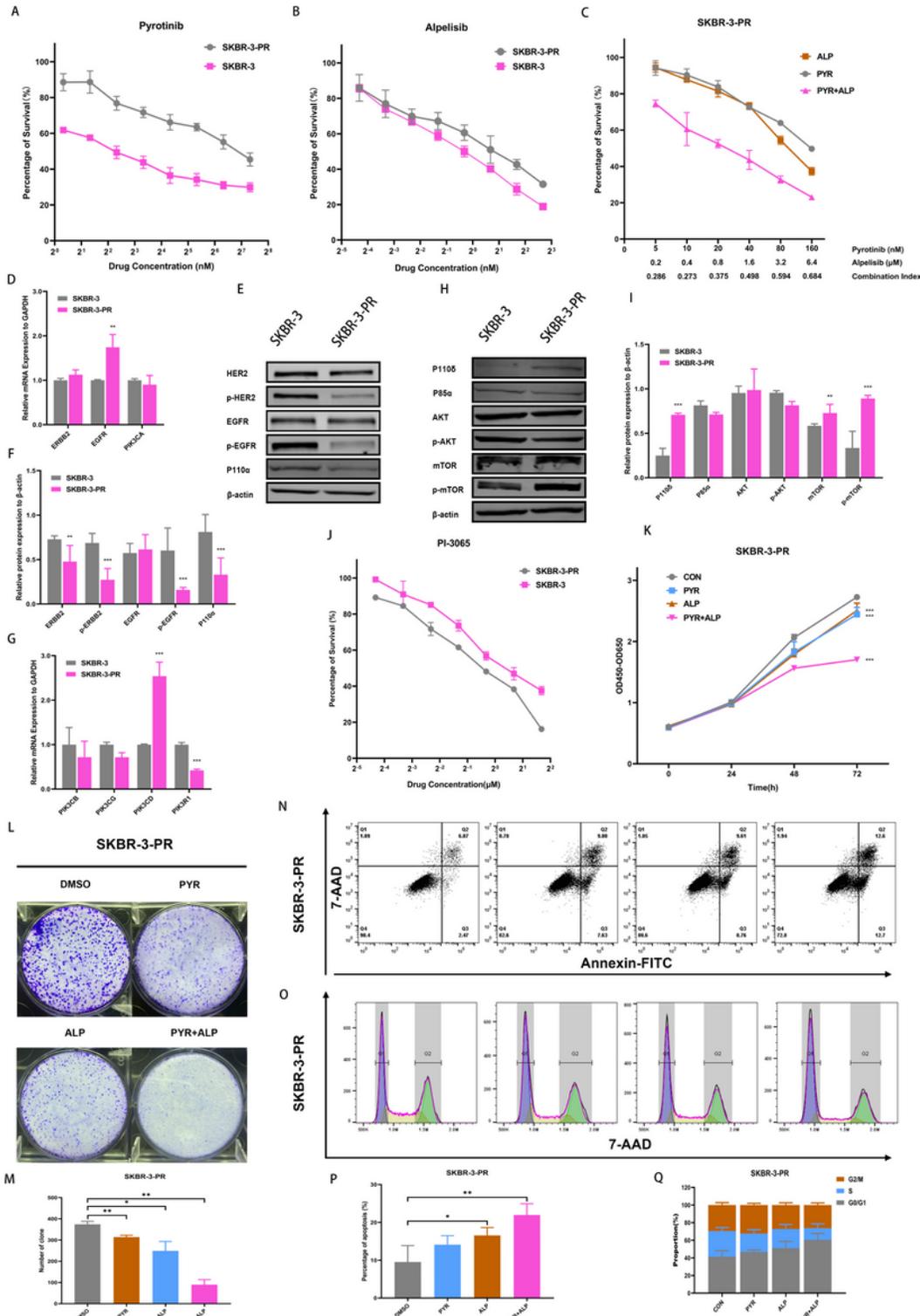


Figure 5

A combination of alpelisib and pyrotinib showed a significantly synergistic when treating acquired pyrotinib resistance cells. (A-B) The IC₅₀ values of SKBR-3 and SKBR-3-PR cells were treated with pyrotinib and alpelisib for 72h. (C) The combination effect and combination index of SKBR-3-PR cells treated with a combination of pyrotinib and alpelisib for 72h. (D-I) mRNA and protein expression of ErbB/PI3K/AKT pathway in SKBR-3 and SKBR-3-PR cells. (J) SKBR-3-PR and SKBR-3 cells were treated with selective p110 δ inhibitor (PI-3065), and the IC₅₀ values were measured. (K-Q) SKBR-3-PR cells were treated with control (DMSO), pyrotinib (30nM), alpelisib (1 μ M) or their combinations and cells proliferation assay, cell colony formation assay, cell apoptosis assay, cell cycle assay were performed. * (P<0.05); ** (P<0.01); *** (P<0.001); **** (P<0.0001). Bars represent group means. n = 3 independent replicates for all experiments.

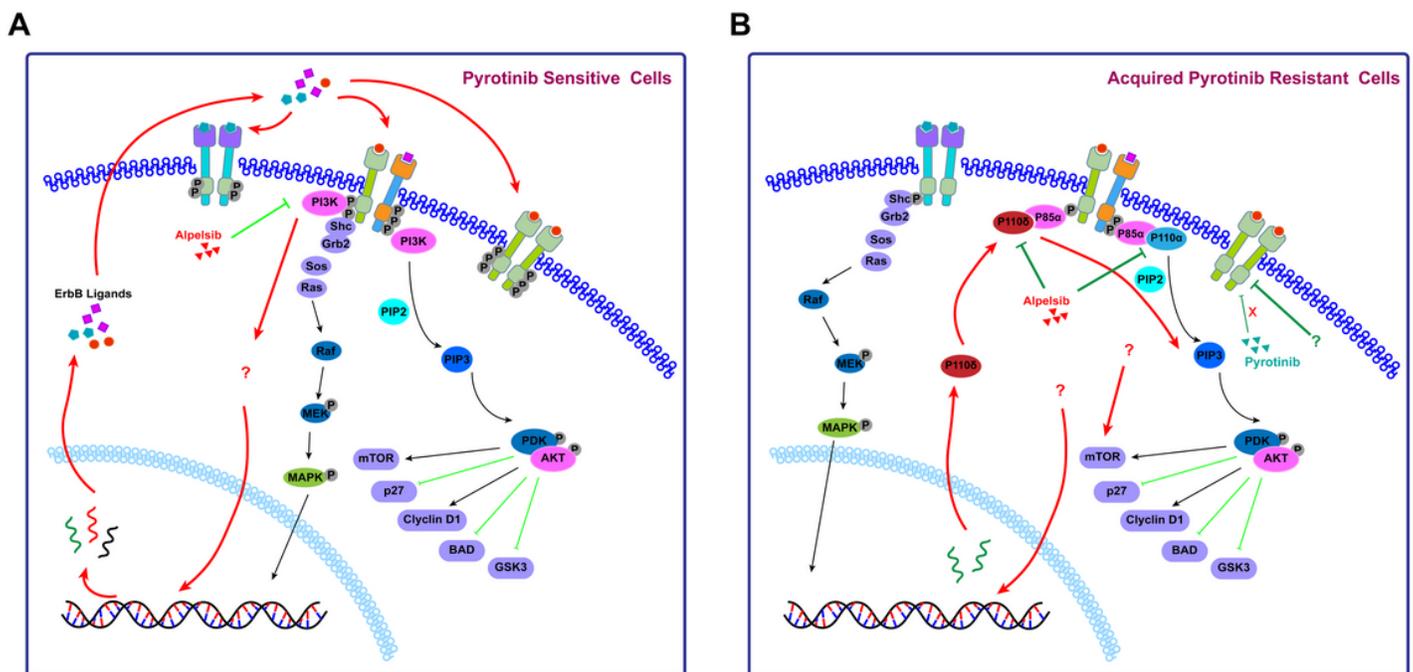


Figure 6

Proposed models illustrating the mechanisms of the combination pyrotinib and alpelisib showed a synergistic effect in treating pyrotinib sensitive or resistant cells. (A) HER+ breast cancer cells up-regulated ErbB ligands expression and activate ErbB, thus were more sensitive to pyrotinib treatment. (B) Acquired pyrotinib resistant cells down-regulated ErbB activation to escape pyrotinib treatment. P110 δ was up-regulated to compensate for p110 α down-regulation. Bars represent group means. n = 3 independent replicates for all experiments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementtable1.xlsx](#)
- [supplementtable2.xlsx](#)