

**Aim of the study:** To discuss the activation of the signal transduction pathway of phosphatidylinositol 3'-kinase/serine-threonine kinase (PI3K/Akt), one of the important targets of drug resistance of trastuzumab, which provides a theoretical basis for the targeted therapy of drug resistance of trastuzumab in breast cancer.

**Material and methods:** Establish the drug-resistance sub-strain BT-HerR of trastuzumab for the continuous treatment of human breast cancer cell strain BT474, conduct Her-2 phenotype analysis on the drug-resistance cell strain BT-HerR with the FISH method, detect the proliferation inhibition *in vitro* of trastuzumab to BT474 and BT-HerR cells with the MTT method, detect the apoptosis variation after interference of trastuzumab with a flow cytometer and detect p-Akt and apoptosis-related protein expression with Western blot after PI3K/Akt inhibitor LY294002 interferes with the cells.

**Results:** The gene expression of drug-resistance cell strain BT-HerR Her-2 is strongly positive; 72 hours after interference of trastuzumab, the proliferation *in vitro* of the BT474 and BT-HerR cells is inhibited, which is strengthened with the increase of concentration, showing a significant difference ( $p < 0.01$ ); after treatment of trastuzumab, comparison of the cell apoptosis rate of BT474 and BT-HerR shows a significant difference ( $p < 0.01$ ); trastuzumab can only inhibit the Akt protein phosphorylation of BT474, while LY294002 can inhibit the BT-HerR and BT474 Akt protein phosphorylation simultaneously.

**Conclusions:** Akt protein phosphorylation of trastuzumab drug-resistance cells is activated; LY294002, a PI3K/Akt inhibitor, can obviously inhibit Akt protein phosphorylation of trastuzumab drug-resistance cells and there is a clear association between the PI3K/Akt signal transduction pathway and trastuzumab resistance.

**Key words:** trastuzumab, LY294002, PI3K/Akt, breast cancer cell, HER2/neu.

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# Research on drug resistance mechanism of trastuzumab caused by activation of the PI3K/Akt signaling pathway

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Breast cancer is one of the most common female malignant tumors, with morbidity increasing year by year and age of onset tending to be younger, so its harm cannot be neglected. The HER2/neu gene (also called c-erbB2) coding transmembrane receptor tyrosine protein kinase has an overexpression rate of up to 30% in breast cancer and is closely related to adverse prognosis and low sensitivity to radiotherapy and chemotherapy [1, 2]; therefore targeted therapy against HER-2/neu has become a research hotspot in recent years. Trastuzumab (trade name: Herceptin) is a kind of humanized monoclonal antibody resisting the HER-2/neu gene. In 1998, upon the approval of the Food and Drug Administration (FDA), it was used for the first-line treatment of Her-2 positive advanced breast cancer. Trastuzumab has a good treatment effect on Her-2 positive breast cancer and obviously extends the disease-free survival of patients; however, nearly 50% of Her-2 positive patients are not sensitive to trastuzumab treatment [3]. After 1 year's trastuzumab treatment, most Her-2 positive patients will generate drug resistance to trastuzumab. The drug-resistance mechanism of trastuzumab is relatively complex [4]. The activation of the phosphatidylinositol 3'-kinase/serine-threonine kinase (PI3K/Akt) signal transduction pathway is deemed as one of the important mechanisms of trastuzumab drug resistance [5, 6]. With the important gene p-Akt expression that drug-resistance mechanism might occur to trastuzumab in the PI3K signaling pathway as the entry point and through the inhibition of PI3K/Akt activity of trastuzumab drug-resistance breast cancer cells by its inhibitor LY294002. This research aims to discuss the drug-resistance mechanism of trastuzumab, thus providing a theoretical basis for further improving the treatment effect of breast cancer trastuzumab targeted treatment.

## Material and methods

### Main experiment reagents

Human breast cancer cell strain BT474 was purchased from Chinese Academy of Science Shanghai Cell Bank, trastuzumab from Roche, RPMI 1640 culture medium and fetal calf serum from American HyClone, EDTA-trypsin from American Gibco, penicillin streptomycin solution from Shanghai Beyotime Biotechnology Co., Ltd., 25 cm<sup>2</sup> cell culture bottle, 96-hole and 6-hole culture plate from American Corning, dimethyl sulfoxide (DMSO) and MTT from American Sigma, AnnexinV – FITC/PI apoptosis kit from American BioVision, RIPA lysate, PMSF, prestained protein marker, horseradish peroxidase marker goat anti-rabbit IgG, ECL chemiluminescence kit from Shanghai Beyotime Biotechnology Co., Ltd., and anti-rabbit human phosphorylation protein kinase B (p-AKT) monoclonal antibody, PI3K inhibitor LY294002 and anti-rabbit human  $\beta$ -actin monoclonal antibody all from American Cell Signaling. *In situ* hybridization reagents (including HER-2/CSP17DNA probe kit, 2 × SSC buffer

solution, protease K, 10% NP-40 and DAPI after stain) were purchased from Beijing GP Medical Technologies, Ltd.

### Main experiment equipment

Scientific Model 1205A-type clean bench, Scientific Model 3111-type CO<sub>2</sub> incubator (American FORMA), Model 680-type micro plate reader (Raito Life and Analytical Sciences Co., Ltd.), CKX31-type inverted phase contrast microscope (Japanese OLYMPUS), Epics XL-type flow cytometer (American Beckman Coulter), Power PAC 300-type vertical electrophoresis apparatus, Power PAC200 electro orator and Fluor-S gel imaging analysis system (American Bio-Rad).

### Cell culture

Apply conventional anchorage-dependent cell culture method to culture the cells in RPMI 1640 culture solution containing 10% fetal calf serum and then place them in an incubator containing 5% CO<sub>2</sub> at 37°C. Use 0.25% trypsin for digestion and passage.

### Method of induction to create drug-resistance cell strain [7]

Through 6 months' continuous treatment of BT474, BT-HerR, a drug-resistance sub-strain resisting trastuzumab, is created. From 1 µg/ml, the sub-strain is cultured continuously to its stable growth in low-concentration trastuzumab and its morphology is basically consistent with that of the parental generation. Then the concentration of trastuzumab in culture solution is increased gradually to 10 µg/ml. Maintain the concentration during culture, and change the culture solution once every 4 days. Conduct passage when the density reaches 70–80%. Identify the drug resistance of the sub-strain by using the MTT method.

### Her-2 phenotype analysis of drug-resistance strain in FISH method [8]

**Pretreatment before FISH:** dissociate cells to prepare  $1 \times 10^5$ /ml cell suspension, take 5–10 ml for centrifugation at 1000 RPM for 10 min and abandon the supernatant. Then, add 5 ml 0.56% KCL solution at 37°C, blow and beat the re-suspended cells again and incubate the cells in a water bath at 37°C for 20 min. Add 2 ml of stationary liquid to the test tube, centrifuge it at 1500 RPM for 10 min, suck and abandon the supernatant to 1 ml, add 5 ml of stationary liquid cell re-suspension and place it still at room temperature for 10 min; after that, centrifuge it at 1500 RPM for 10 min again, suck and abandon the supernatant to 1 ml, then add 5 ml of stationary liquid cell re-suspension and place it still at room temperature for 10 min. Centrifuge at 1000 RPM for 10 min, suck and abandon the supernatant to 1 ml, drop the cells on a slide after blow, beat and re-suspension. Age the slide at 56°C for 30 min.

**Hybridization:** When the slide has been air dried, drip 10 µl from the probe to the area for hybridization, then cover it with a cover glass and seal the edge with rubber glue. Place it into an automatic hybridization oven to treat at a denaturalization temperature of 75°C for 5 min and keep at 42°C overnight. Washing after hybridization: remove the cover glass and wash

the slide in 2 × SSC solution twice, each time for 10 min, and then wash in 2 × SSC/0.1% NP-40 solution for 5 min. Then, soak in 70% ethanol solution for 3 min and place the slide in a dark place for air drying. Add 10–20 µl of 4',6-diamidino-2-phenylindole (DAPI) to all sections, cover them with a cover glass before placing them in a dark place for 10 min, and then observe through a special single-channel filter by a fluorescence microscope.

**Result assessment:** count 30 cells and perform statistics of the ratio value (Ratio value = total amount of red signal in 30 cell nuclei/total amount of green signal in 30 cell nuclei). A result with a ratio < 1.8 is negative, which indicates no gene amplification of HER-2 in the sample; a result with a ratio > 2.2 is positive, which indicates a gene amplification of HER 2 in the sample; where the ratio is between 1.8 and 2.2, increase the number of counted cells to 100 or repeat the FISH test to determine the final result; in case of aggregation of abnormal cells or appearance of multiple cells with a red signal of each cell nucleus > 10–15, it should be treated as a positive result. If the green signal in each nucleus is > 2.25, it should be considered as a polysomic chromosome.

### Cell proliferation inhibition detection in MTT method [7]

Collect cells at exponential phase, adjust the cell density to  $3 \times 10^4$ /ml and inoculate 100 µl to each hole on a 96-hole culture plate. After 24 hours, add drugs of different concentrations, while setting the zero-setting hole (do not inoculate the cells but add corresponding cell culture fluid) and negative control groups with 6 complex holes set for each group. Incubate in atmosphere of 5% CO<sub>2</sub> at 37°C for 5 days with the solution changed once during this period. After a 5-day intervention of trastuzumab, add 10 µl of MTT solution to each hole of the 96-hole culture plate (5 mg/ml) and continue to culture for 4 hours before stopping to carefully absorb the culture fluid in the holes. Add 100 µl of DMSO to each hole and put the plate on a shaker to vibrate for 10 min so as to fully dissolve the crystals. Measure the absorbance of each hole at OD490 nm of the ELISA Reader. Meanwhile, set zero-setting holes (added with medium, MTT and DMSO), and control holes (added with cells, drug dissolution medium with the same concentration, culture fluid, MTT and DMSO). The formula adopted for the calculation of the inhibition ratio of cell proliferation is:

$$\text{inhibition ratio} = 100\% \times \frac{(\text{OD value of control hole} - \text{OD value of zero-setting hole}) - (\text{OD value of dosing hole} - \text{OD value of zero-setting hole})}{(\text{OD value of control hole} - \text{OD value of zero-setting hole})}$$

Repeat the experiment 3 times.

### Detection of cell apoptosis by flow cytometer

After the cells are treated, collect all anchorage-dependent cells and floating cells to the flow cytometer for centrifugation at 1000 RPM for 5 min. Wash the cells with PBS twice and record the amount. Use 1 × Binding Buffer to re-suspend the cells, adjust the cell concentration to  $1 \times 10^6$ /ml, drip 100 µl of cell suspension to a 5 ml flow tube, and then add Annexin V 5 µl and PI 10 µl to gently blend it and keep in a dark place for 10 min at room temperature. Immediately

detect and analyze the result to calculate the percentage of the viable apoptotic cell.

**Detection of p-Akt of cells and apoptosis related protein expression by Western blot [9]**

Collect cells and add cell lysate before placing on ice for dissociation for 30 min, and then centrifuge it at 12 000 RPM/min for 20 min at 4°C. Retain the supernatant, take protein with the equal amount to conduct SDS-PAGE denatured gel electrophoresis, and then transfer protein to PVDF membrane. Seal the 5% skim milk at room temperature for 2 h and combine with the first antibody overnight. After the membrane is washed, combine it with the secondary antibody for 2 h. Then detect in chemiluminescence method to expose the film, wash down and dry up. Analyze the grey level on the stripe in the picture by Quantity One software through scanning of the gel imaging system. Repeat the procedure 3 times.

**Statistical analysis**

The measured data are presented as mean ± standard deviation ( $\bar{x} \pm s$ ). We used the *t*-test to compare the means of two samples and analyzed the dose-effect relationship using Spearman rank correlation; the variance analysis was conducted using the statistical software SPSS 13.0. All the results indicating  $p < 0.05$  show statistical significance.

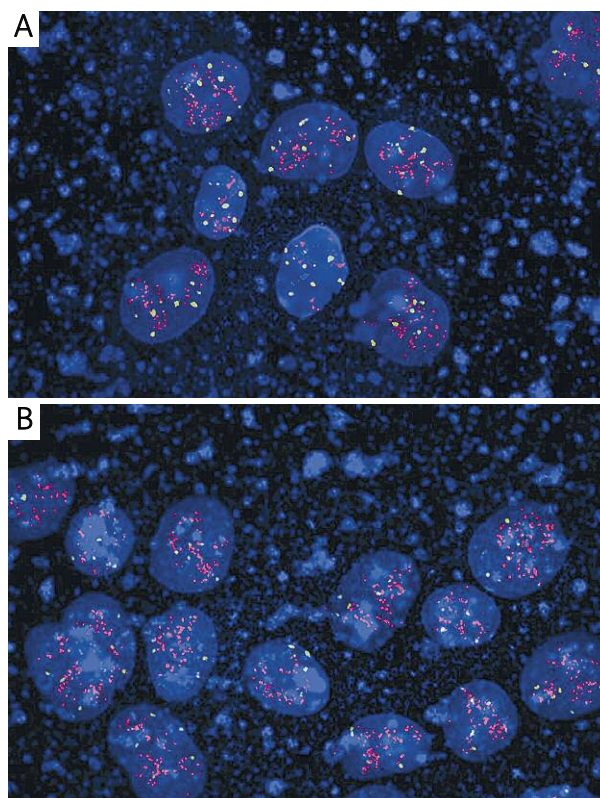
**Results**

**Establishment of cell line of BT-HerR with drug resistance to trastuzumab**

FISH test result of cell line of BT-HerR with drug resistance to trastuzumab See in Fig. 1.

*Inhibition of in vitro proliferation of BT474 and BT-HerR cells by trastuzumab*

It is shown in the result of the MTT method that after trastuzumab with a concentration between 0.1 and 100 µg/ml interferes with cells for 72 hours, the *in vitro* proliferation of the cells is inhibited with the effect on the proliferation increasing with the rising of the concentration. Through calculation, the IC50 of trastuzumab on BT474 cells and drug-resistance cell strain BT-HerR was 1.336 mg/l and 33.994 mg/l, respectively; there was no significant difference ( $p < 0.01$ ). The pairwise comparison difference between experimental groups with different concentrations has statistical significance ( $p < 0.01$ ; Table 1).



**Fig. 1.** Picture **A** is a BT474 cell, Picture **B** is a BT-HerR cell. Her-2 gene amplification detection kit contains 2 DNA probes: GLP HER-2 and CSP 17. The fluorescent signal of the Her-2 DNA probe is jacinth and as a contrast to gene amplification, the fluorescent signal of CSP 17 is green. The ratio of the two signals is used as the basis to judge positive or negative. In this respective, it can be seen that in picture B Her-2 genes amplify in a form of cluster, which indicates strongly positive

*Cell apoptosis of BT474 and BT-HerR under the interference of trastuzumab*

As shown in the test result of flow cytometry, after BT474 cells are interfered with by 10 µg/ml trastuzumab, apoptosis occurs (Fig. 2); the difference between apoptosis rate of BT474 and BT-HerR has statistical significance ( $p < 0.01$ ; Table 2).

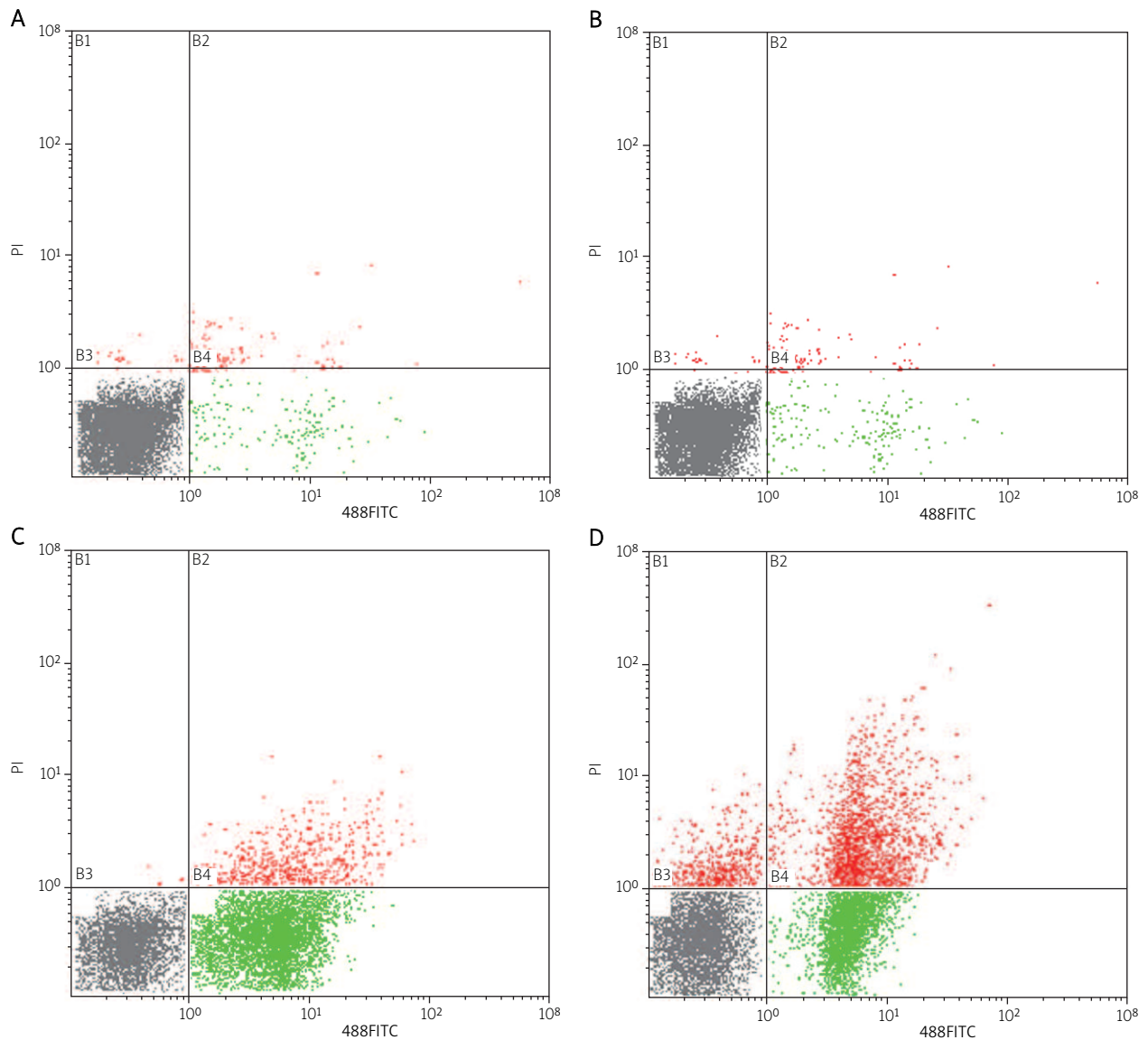
**Influence on BT474 and BT-HerR by PI3K/Akt inhibitor LY294002 and trastuzumab**

In order to observe the influence of PI3K/Akt pathway inhibition on drug resistance of trastuzumab in breast cancer cells, treat the cells with 10 µmol/l PI3K/Akt inhibitor LY294002 for 6 h in advance, then add 10 µg/ml trastuzumab to treat the

**Table 1.** Inhibition ratio on cell proliferation of BT474 and BT-HerR under the interference of trastuzumab with different concentrations for 5 days (% ,  $\bar{x} \pm s$ )

Cell line	Concentration of Trastuzumab (µg/ml)			
	0.1	1	10	100
BT474	10.17 ±1.13	35.95 ±2.56	71.97 ±3.57	79.71 ±1.49
BT-HerR	3.34 ±0.94	9.59 ±2.61	21.34 ±1.60	32.73 ±2.44

Pairwise comparison between experimental groups with different concentrations ( $p < 0.01$ )



**Fig. 2.** **A)** Untreated cells of BT474; **B)** Untreated cells of BT-HerR; **C)** BT474 cells treated with trastuzumab; **D)** BT-HerR cells treated with trastuzumab

**Table 2.** Apoptosis rate of BT474 and BT-HerR under the interference of 10  $\mu\text{g/ml}$  trastuzumab (% ,  $\bar{x} \pm s$ )

Group	Cell line	Apoptosis rate in early stage (B4)	Apoptosis rate and mortality rate in middle-advanced stage (B2)	Survival rate (B3)
Blank control	BT474	3.38 $\pm$ 0.08	0.58 $\pm$ 0.02	95.34 $\pm$ 1.07
	BT-HerR	3.34 $\pm$ 0.17	1.68 $\pm$ 0.07	94.98 $\pm$ 1.23
Treated with trastuzumab	BT474	24.58 $\pm$ 0.55*	3.60 $\pm$ 0.17	71.76 $\pm$ 2.17
	BT-HerR	6.97 $\pm$ 0.24*	1.84 $\pm$ 0.14	89.07 $\pm$ 1.52

Comparison of apoptosis rate of BT474 and BT-HerR after being treated with trastuzumab, \* $p < 0.01$

cells. Observe whether the apoptosis of drug-resistance cells of BT-HerR changes or not.

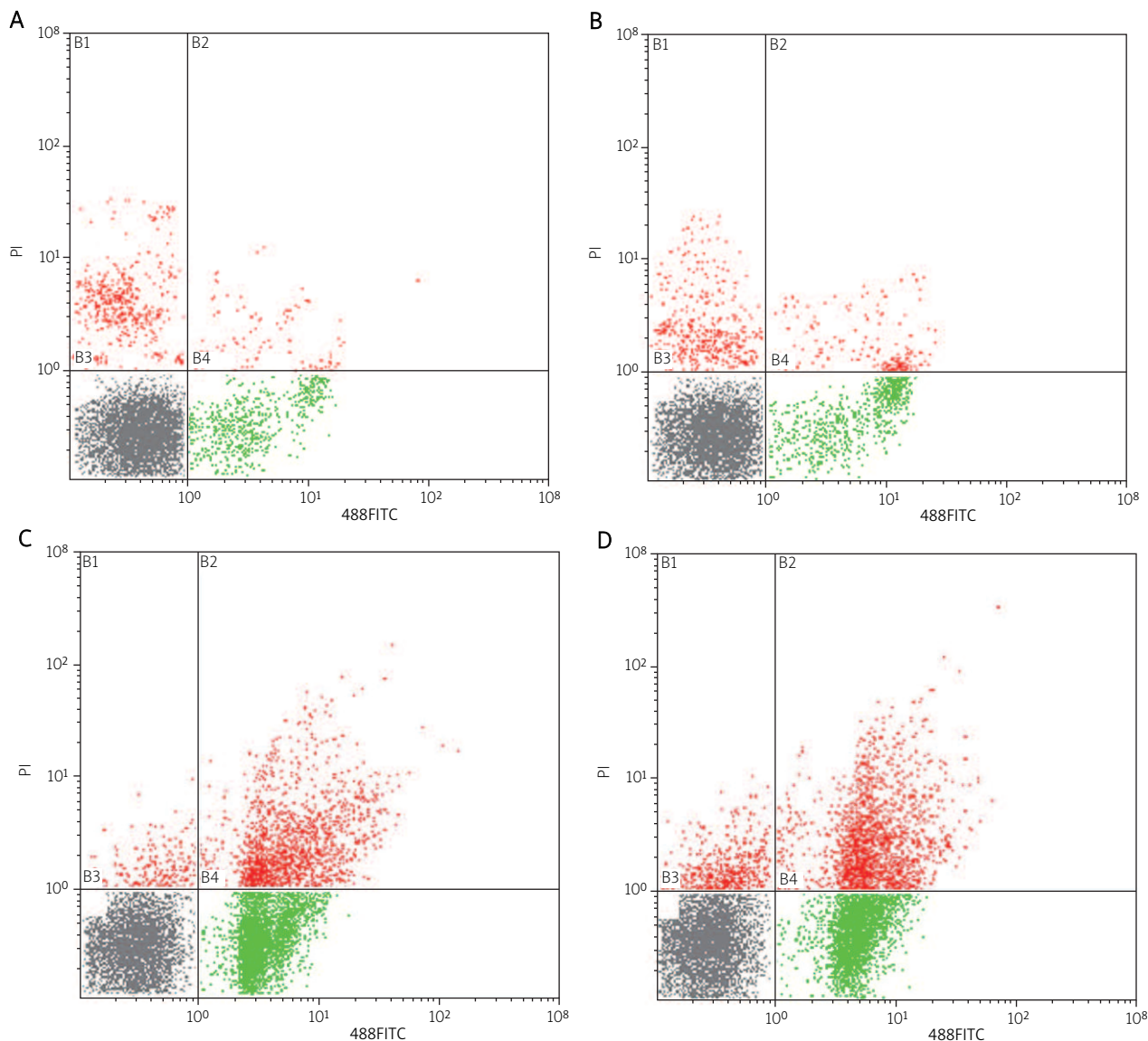
#### *Influence on the apoptosis rate of BT474 and BT-HerR by LY294002 and trastuzumab*

As shown in the test result of flow cytometry, there is obvious apoptosis in BT474 and BT-HerR after being treated by LY294002 in advance and added with trastuzumab later. The

difference between the apoptosis rate of BT474 and drug-resistance BT-HerR has no statistical significance.

#### *Influence on p-Akt protein expression in BT474 and BT-HerR cells by LY294002 and trastuzumab*

In order to observe the effect of p-Akt in drug resistance of trastuzumab, respectively treat BT474 and BT-HerR cells with LY294002 and trastuzumab, and collect cell samples to con-



**Fig. 3.** **A)** BT474 cells treated with LY294002; **B)** BT-HerR cells treated with LY294002; **C)** BT474 cells treated with LY294002 with trastuzumab; **D)** BT-HerR cells treated with LY294002 with trastuzumab

**Table 3.** Apoptosis rate of BT474 and BT-HerR cells with or without joint interference of LY294002 and trastuzumab (% ,  $\bar{x} \pm s$ )

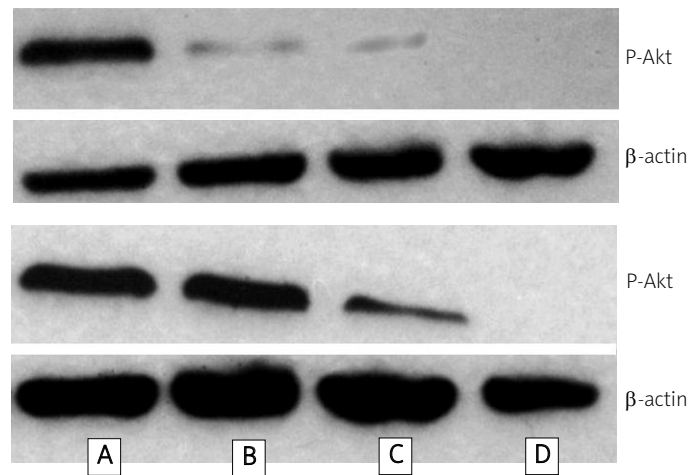
Group	Cell line	Apoptosis rate in early stage (B4)	Apoptosis rate and mortality rate in middle-advanced stage (B2)	Survival rate (B3)
LY294002 group	BT474	13.71 ± 0.67	3.98 ± 0.03	74.38 ± 2.31
	BT-HerR	10.29 ± 1.10	1.51 ± 0.06	79.33 ± 4.02
LY294002 with trastuzumab	BT474	36.42 ± 2.32	17.44 ± 1.32	44.01 ± 3.60
	BT-HerR	32.24 ± 1.74	19.61 ± 1.22	42.65 ± 2.68

duct Western blot test so as to observe the changes in their P-Akt. It can be seen that 10 μg/ml trastuzumab inhibits the Akt protein phosphorylation of BT474, but it cannot inhibit the Akt protein phosphorylation of BT-HerR. However, 10 μmol/l LY294002 can inhibit the Akt protein phosphorylation of both.

**Discussion**

The drug-resistance mechanism of trastuzumab has presently become a hot issue in research, wherein research

on the drug-resistance target spot is considered as the leading priority. Akt is an important downstream target kinase in the PI3K signal transduction pathway. The PI3K/Akt signaling pathway induces the cell to proliferate and differentiate so as to prevent cell apoptosis. This signaling pathway, as an important transduction pathway for cell growth and proliferation, plays an important role in keeping cell malignant biological characteristics [10–12]; therefore, the inhibition of this signaling pathway has become a hot issue for tumor pre-



**Fig. 4.** Influence on expression of BT474 and BT-HerR by PI3K/Akt inhibitor LY294002 and trastuzumab. Above are cells of BT474 and below are cells of BT-HerR. The conditions of sample injection are as follows: **A)** Untreated blank control group; **B)** Treat with 10  $\mu\text{g/ml}$  trastuzumab for 24 hours; **C)** Treat the cells for 6 h with 10  $\mu\text{mol/l}$  LY294002; **D)** Add 10  $\mu\text{g/ml}$  trastuzumab to jointly treat for 24 h after treating with 10  $\mu\text{mol/l}$  LY294002 for 6 h

vention and tumor targeted therapy. One of the important functional mechanisms of trastuzumab is to restrain the signal transduction of HER2-PI3K-Akt. The combination of trastuzumab and HER2 can restrain HER3 comprised tyrosine phosphorylation and disturb the formation of HER3 and HER2 heterodimer and HER3 and the PI3Kp85 regulator subunit, so as to restrain the activation of PI3K and reduce Akt phosphorylation [13, 14]. By restraining the PI3K-Akt pathway, trastuzumab can restrain the formation of breast cancer cell clone through HER2 gene amplification and down-regulate cyclin, thereby inducing cancer cell apoptosis. However, at the same time, many cell factors can activate Akt, such as EGF, IGF-1, heregulin and FGF, insulin, interleukin 3 (IL-3), IL-6 as well as VEGF, etc. Clark [15] *et al.* found that Akt of some breast cancer cell strains has high intrinsic activity and the HER-2 positive tumor expresses high-level p-AKT. Furthermore, the continuously activated Akt will lead to a deficiency of cell growth inhibition mediated by trastuzumab, and restrain cell cycle arrest or cell apoptosis mediated by trastuzumab. Therefore, continuous activation of Akt may exist in a trastuzumab drug-resistance cell strain, which may be one of the factors that make trastuzumab resist the drug. Lots of inhibitors, targeted to PI3K-Akt, make PI3K inactivate, restrain the proliferation of trastuzumab drug-resistance cells and accelerate their apoptosis and reverse the resistance of trastuzumab, which indicates that PI3K targeted therapy may overcome trastuzumab resistance [16]. LY294002, transformed from quercetin, is a typical PI3K inhibitor. The inhibitor of targeted PI3K's catalytic subunit p110 can effectively restrain the growth of tumor cells *in vivo* and *in vitro*, block the PI3K/Akt signaling pathway upstream in a non-specific way, and thereby induce tumor cell apoptosis [17, 18]. The present research on the drug-resistance mechanism of trastuzumab is still in exploration. The runaway PI3K/Akt signaling pathway and continuous activation state of p-Akt are just two important target spots for drug resistance of trastuzumab, which need to be further investigated in future experiments.

After the continuous treatment for human breast cancer cell BT474, the drug-resistance sub-strain BT-HerR that resists the trastuzumab is created. After the treatment with trastuzumab, comparison of the cell apoptosis rate of BT474 and BT-HerR shows a significant difference. Also the apoptosis rate of the two cells shows a significant difference. After trastuzumab drug-resistance BT-HerR cells are activated through Akt protein phosphorylation, the activity of trastuzumab drug-resistance breast cancer cell PI3K/Akt is restrained through the PI3K/Akt inhibitor which is LY294002, which proves that trastuzumab can restrain the Akt protein phosphorylation of BT474, but cannot restrain that of the BT-HerR cell. However, LY294002 can restrain the Akt protein phosphorylation of both cells, which further proves that LY294002 restrains the activity of PI3K/Akt, and can also reverse the resistance effect of trastuzumab drug-resistance breast cancer cells to trastuzumab through LY294002. The specific dependence of the existence of the PI3K/Akt pathway and trastuzumab drug resistance further explicates the position of the target spot.

To sum up, this experiment takes the important gene expression p-Akt on the PI3K signaling pathway where a drug-resistance mechanism may occur to trastuzumab as an entry point, adopts the PI3K/Akt inhibitor LY294002 to inhibit the activity of the trastuzumab drug-resistance breast cancer cell PI3K/Akt to prove that activation of the PI3K/Akt signaling pathway is an important mechanism resulting in drug resistance of trastuzumab and identify an important target of trastuzumab drug resistance, which has provided a theoretical basis for individualized clinical therapy of molecule-targeted therapy for breast cancer, a new idea and experimental basis for clinical therapy, and is thus of clinical significance.

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