Dual-specificity phosphatase 8 (DUSP8) induces drug resistance in breast cancer by regulating MAPK pathways

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ABSTRACT

The aim of the study was to explore the role and molecular mechanism of dual-specificity phosphatase 8 (DUSP8) in the drug resistance of trastuzumab in breast cancer. Real-time PCR and western blot detected the difference in expression of DUSP8 between breast cancer tissue/cells and trastuzumab-resistant tissues/cells. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of DUSP8 in breast cancer. si-DUSP8 or dusp8 overexpression vector was transiently transfected, and the effects of si-DUSP8 on apoptosis, cell viability and cell migration of drug-resistant cell lines were investigated by flow cytometry, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and Transwell assays, and its regulation mechanism finally explored. The results showed that the expression of DUSP8 in breast cancer tissues and cells was significantly higher than in matched non-tumor tissues and cells. DUSP8 was significantly upregulated in non-responsive patients compared with patients who responded to trastuzumab. ROC analysis showed that the area under the curve was 0.732, and the diagnostic sensitivity and specificity were 64.86% and 75.76%. DUSP8 knockdown promotes apoptosis and reduces trastuzumab resistance in BT474/TR and SKBR3/TR cells by inhibiting cell migration and cell viability. Knockdown of DUSP8 increased the expression of p-p38 and p-ERK, and the regulation of DUSP8 in chemotherapy resistance of breast cancer cells may be realized by mediating mitogen-activated protein kinase (MAPK)-related signaling pathways. In conclusion, knockdown of DUSP8 expression in trastuzumab-resistant cells can inhibit cell migration and proliferation, and leads to decreased drug resistance by activating MAPK signaling pathway in trastuzumab-resistant cells.

Significance of this study

What is already known about this subject?
⇒ Loss of dual-specificity phosphatase (DUSP) expression is related to progression of several cancers.
⇒ DUSP1 is overexpressed in breast cancer and increased the resistance of tamoxifen.
⇒ High expression of DUSP8 is associated with poor prognosis in patients with breast cancer receiving radiotherapy.

What are the new findings?
⇒ Knocking down DUSP8 reversed the proliferation and migration of trastuzumab-resistant cells.
⇒ Silencing DUSP8 activates the mitogen-activated protein kinase pathway in drug-resistant breast cancer cells.

How might these results change the focus of research or clinical practice?
⇒ The study revealed the resistance mechanism of trastuzumab in breast cancer cells and identified potential molecular markers and therapeutic targets for patients with breast cancer.

Trastuzumab can target HER2 and interfere with its function, mainly in patients with early HER2-positive mutations or metastatic breast cancer.[2 3] Trastuzumab may be effective in the initial treatment stage, but drug resistance also increases significantly after long-term treatment. Therefore, it is an urgent problem to reveal the drug resistance mechanism of trastuzumab and identify potential molecular targets for diagnosis and treatment of patients with breast cancer.

As the main negative regulator of mitogen-activated protein kinases (MAPKs), change in the expression of dual-specificity phosphatase (DUSP) may affect the results of cancer chemotherapy. DUSPs play various roles in cancer. Some studies have shown that loss of DUSP expression is related to progression of several cancers, while others have shown that increase of DUSP expression is related to cancer progression, drug resistance and poor prognosis. For example, DUSP1 is overexpressed

INTRODUCTION

Breast cancer is one of the most common cancers seriously affecting women's physical and mental health. Human epidermal growth factor receptor 2 (HER2) is overexpressed in about 20% of breast cancer, resulting in poor prognosis and survival rate.[1] Currently, anti-HER2 monoclonal antibodies, including trastuzumab, have been used in the treatment of HER2-positive patients with breast cancer.

in breast cancer and increased the resistance of tamoxifen.[4] High expression of DUSP16 enhanced resistance to cisplatin, carboplatin, oxaliplatin, fluorouracil and epirubicin. Previous studies have shown that high expression of DUSP8 is associated with poor prognosis in patients with breast cancer receiving radiotherapy.[5] However, the specific mechanism of action has not been explored. The aim of this study was to investigate the regulatory effect and mechanism of trastuzumab in DUSP8 breast cancer.

MATERIALS AND METHODS
Patient samples
The study enrolled 70 HER2-positive patients and 35 HER2-negative patients treated with trastuzumab. According to the Immune-Related Response Evaluation Criteria In Solid Tumors, 70 HER2-positive patients were divided into trastuzumab treatment response group (33 cases) and non-response group (37 cases). All patients signed written informed consent.

Cell culture and treatment
Human HER2-positive breast cancer cell lines SKBR-3 and BT474 were purchased from the American Type Culture Collection (Manassas, USA). BT474 and SKBR-3 were cultured in Dulbecco's Modified Eagle Medium (HyClone, Logan, Utah) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, Missouri, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Grand Island, New York, USA) in an incubator with 5% CO_2 at 37°C. Cell growth was observed under light microscope and the culture medium was changed every 3–4 days. The cell lines were authenticated by short tandem repeat profiling to avoid contamination of the required cells.

Construction of SKBR-3/TR and BT474/TR cell lines
Trastuzumab (Herceptin, Roche Diagnostics, Basel, Switzerland) was dissolved in sterile water. In vitro indirect gradient method was used to induce drug-resistant strains. SKBR-3 and BT474 cell lines at logarithmic growth stage were selected and trastuzumab was added after the cell density grew to 50%–60%. The concentration of trastuzumab in the cell culture medium was 2 μg/mL. During the induction period, the concentration of trastuzumab was 5 μg/mL when the cells were stable for 1 week. The cells were treated with a drug concentration of 10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL, 60 μg/mL and 80 μg/mL successively. When the cells could grow stably at the drug concentration of 80 μg/mL, trastuzumab-resistant strain (BT474/TR, SKBR-3/TR) was identified as a successful construction and used for subsequent experiments.

Cell transfection
DUSP8 gene-specific overexpression plasmid (FulenGen, Guangzhou, China) or siRNA-DUSP8 (GenePharma) was transfected into SKBR3/TR and BT474/TR cells. The cells were cultured in antibiotic-free medium 1 day before transfection and transiently transfected when the convergence was about 70% after 18–24 hours. Transient transfection was performed by Lipofectamine 3000 (CAT L3000008; Invitrogen, Carlsbad, California, USA) with a final concentration of 100 nM. After 5-hour incubation at 37°C in 5%

CO_2 constant temperature incubator, a new medium of 10% fetal bovine serum (FBS) was replaced and the overexpression or interference efficiency of DUSP8 was detected by real-time PCR (RT-PCR). The final transfection efficiency could be applied to subsequent experiments.

RNA extraction and quantitative RT-PCR
Total RNA of serum was extracted with TRIzol reagent (Invitrogen). The concentration and purity of RNA were determined by agarose gel electrophoresis or ultraviolet spectrophotometer. When the purity is 1.7–2.1, follow-up experiments were carried out. Reverse transcription of 1 μg total RNA into cDNA (SuperScript III, Invitrogen) was performed. Expression of the gene DUSP8 was detected by RT-PCR (Takara Bio Company, Dalian, China) and assayed using the Bio-Rad CFX96 sequence assay system (Bio-Rad, Berkeley, California, USA). The primer sequences for RT-PCR were as follows: DUSP8, forward: 5′-TGAC-CCAAAACGGAATAAGC-3′, reverse: 5′-AGAGATG-CCAGCCAGACAG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5′-GGTGAAGGTC-GGAGTCAACG-3′, reverse: 5′-CAAAGTTGTCATGGAT-GACC-3′. GAPDH gene is an internal reference gene. The amplification procedures were as follows: predenaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 60°C for 40 s, extension at 72°C for 30 s, 40 cycles, and three multiple holes were set in each experiment. After the reaction, the relative expression levels of each gene were calculated by $2^{-\Delta\Delta Ct}$.

Western blot analysis
The cells were cultured for 48 hours and the cell proteins were extracted with RIPA protein lysis solution (KeyGEN, Nanjing, China). The total protein concentration was quantified by BCA method and the standard curve was drawn. Total proteins were isolated with 10% sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to polyvinylidene fluoride (PVDF) membrane (soaked in anhydrous methanol for 1–2 min) (Millipore, Bedford, Massachusetts, USA). The voltage was adjusted to 90 V and the membrane was transferred for 2 hours. After the film transfer, the membrane was sealed with 5% skimmed milk powder for 1 hour. After sealing, TBST was used to wash the membrane twice and the primary antibody was incubated overnight at 4°C. After TBST was washed, the secondary antibody was incubated for 1 hour at room temperature. The prepared efficient chemiluminescence kit (ECL) luminescent droplets (Thermo Fisher Scientific, USA) were added to the PVDF film and then imaged on the automatic exposure instrument of GENE company. GAPDH was used as control.

Cell viability detected by MTT assay
The transfected breast cancer cells were subcultured and inoculated into 96-well cell culture plates with 200 μL per well and 5×10^3 cells. After 48 hours of culture, cell proliferation was detected by MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and the absorbance value at 490 nm (A490) was detected to draw the cell growth curve. Each group had three multiple holes. The relative survival curve was used to estimate the half

inhibitory concentration (IC50) of the drug, that is, the drug concentration required when the number of viable cells is reduced by half after treatment.

Cell migration detected by Transwell assay

Cells grown to logarithmic stage were evenly inoculated at the top chamber of Transwell (24-well format, 8 µm pore size; BD Biosciences, St Louis, Missouri), 5×10^4 per well. The bottom well containing 10% FBS was used as the growth medium. After 48 hours of cell culture, the cells were taken out and the cells passing through the back of the bottom of the cell were fixed with 4% mostly paraformaldehyde and stained with 1% crystal violet for microscopic examination. After the cells were cultured for 48 hours, the Transwell chambers were removed. The cells that passed through the dorsal surface of the bottom of the chamber were fixed with 4% paraformaldehyde and stained with 1% crystal violet for microscopic examination.

Cell apoptosis

Cell apoptosis was determined using the Annexin V-FITC Apoptosis Assay Kit (Nanjing Jiancheng) according to the instructions. The transfected cells were digested with 0.25% trypsin without EDTA, centrifuged at 1000 rpm for 10 min, and resuspended with phosphate buffer saline and counted. Then 5×10^5 cells were centrifuged at 1000g/min for 5 min. Annexin V-FITC 5 µL were added to gently mix

the cells, and the cells were incubated for 10 min without light. After 5 µL propidium iodide was added to the ice bath, the data were analyzed by flow cytometry using FACSCanto II (BD Biosciences, San Jose, California) and FlowJo V.7.6 software.

Statistical analysis

GraphPad Prism (V.5.01; GraphPad Software, San Diego, California, USA) and SPSS V.23.0 were used for data processing and statistical analysis. Measurement data were expressed as mean±SD ($\bar{x} \pm s$). Differences between two groups were analyzed using two-sided Student's t-test. One-way analysis of variance was used for comparison of multiple groups (>2) and the variance similar between the groups that are being statistically compared. Receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value of DUSP8 in breast cancer. P<0.05 was considered to indicate a statistically significant difference.
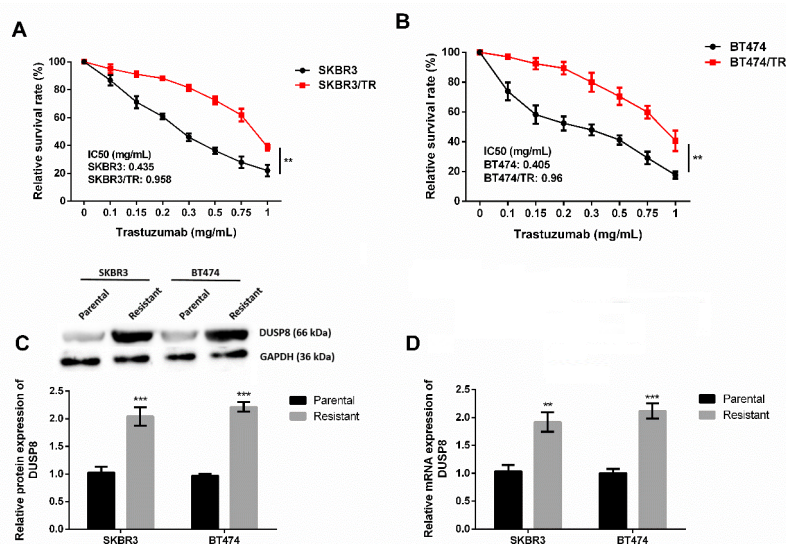
RESULTS

Expression of DUSP8 in breast cancer and its diagnostic value in breast cancer

Western blot and RT-PCR results showed that DUSP8 expression was significantly upregulated in breast cancer tissues compared with matched non-tumor tissues (p<0.05) (figure 1A). DUSP8 levels were more than two times higher
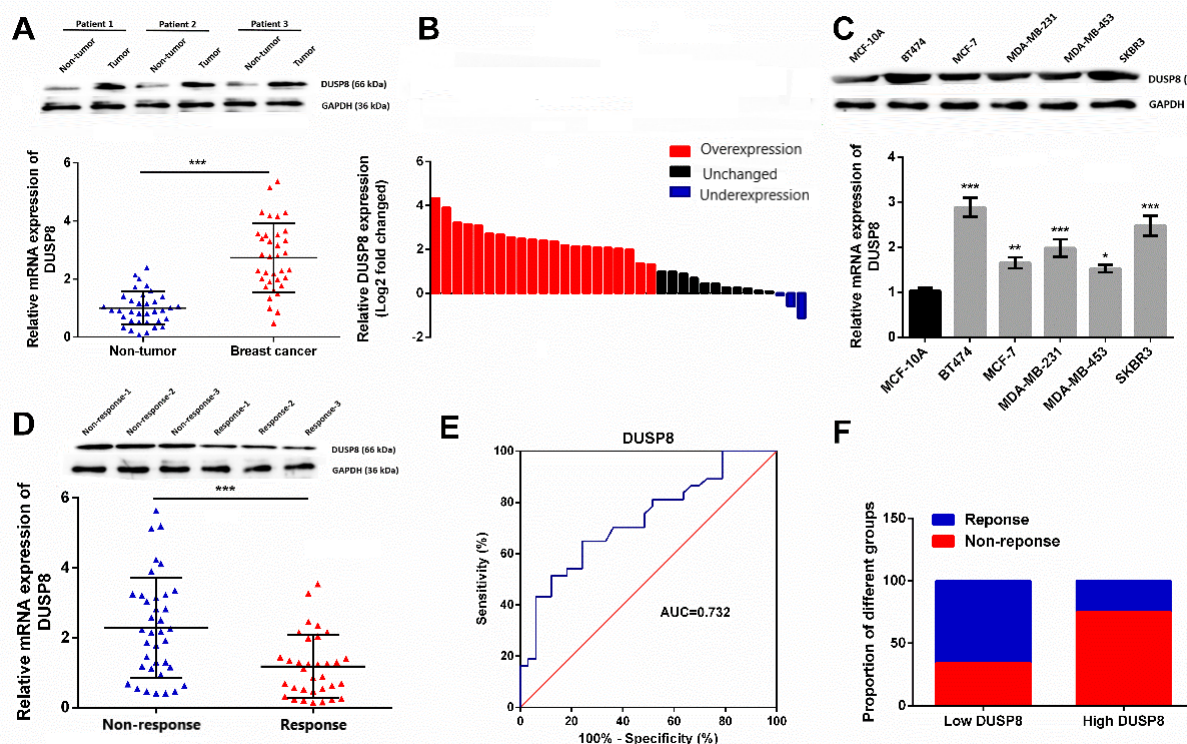
Figure 1 Expression of DUSP8 in breast cancer and its diagnostic value in breast cancer. (A) Expression of DUSP8 protein and mRNA in breast cancer. (B) Differential expression of DUSP8 between paired normal tissues and breast cancer tissues. (C) Expression of DUSP8 protein and mRNA in breast cancer cells. (D) Expression difference of DUSP8 between trastuzumab responders and non-responders. (E–F) ROC curve evaluating the diagnostic value of DUSP8 in breast cancer. *P<0.05, **P<0.01, ***P<0.001. AUC, area under the curve; DUSP8, dual-specificity phosphatase 8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROC, receiver operating characteristics.

Figure 2 Expression of DUSP8 in trastuzumab-resistant strains. (A) IC50 values of trastuzumab on SKBR3/TR and BT474/TR cells. (B) Expression of DUSP8 protein and mRNA in SKBR3/TR and BT474/TR. *P<0.05, **P<0.01, ***P<0.001. DUSP8, dual-specificity phosphatase; IC50, half inhibitory concentration.

in 54.3% (19 of 35) of breast cancer tissues than in matched normal tissues (figure 1B). Similarly, cell assay results showed that DUSP8 expression was significantly elevated in breast cancer cells compared with normal breast epithelial cells (p<0.05) (figure 1C). DUSP8 expression levels were significantly higher in non-responsive patients (n=37) than in trastuzumab-sensitive patients (n=33) (p<0.05). The results are shown in figure 1D. ROC curve was used to evaluate the diagnostic value of DUSP8 in breast cancer, as shown in figure 1E. The area under the curve was 0.732 and the sensitivity and specificity were 64.86% and 75.76%, respectively. The 95% CI was 0.615 to 0.849 and the optimal critical value was 1.445. Using a cut-off value of 1.445, we found a significant increase in the proportion of patients who did not respond to trastuzumab in the group with high expression of DUSP8 compared with the group with low expression (figure 1F).

DUSP8 expression in trastuzumab-resistant breast cancer cells

We established two trastuzumab-resistant sublines from HER2-positive parent cell lines SKBR3 and BT474 (SKBR3/TR and BT474/TR, respectively). The concentration–effect curve showed that the IC50 of trastuzumab to SKBR3/TR cells was 0.958 mg/mL and to parental cells was 0.435 mg/mL. The IC50 of trastuzumab against BT474/TR cells was 0.96 mg/mL compared with 0.405 mg/mL for parental cells (figure 2A,B). In addition, DUSP8 expression was significantly increased in BT474/TR and SKBR3/TR cells compared with parental cells (p<0.05) (figure 2C,D).

Effect of DUSP8 on trastuzumab resistance in breast cancer

Si-dusp8 was instantaneously transfected into trastuzumab-resistant breast cancer cells (BT474/TR and SKBR3/TR). The results in figure 3A show that the levels of DUSP8 mRNA and protein were significantly decreased in the

si-DUSP8 group, indicating that si-DUSP8 was successfully transfected. MTT assay showed that the survival rate of BT474/TR and SKBR3/TR cells decreased significantly after DUSP8 knockdown and trastuzumab treatment (p<0.05). This result indicates that knockdown of DUSP8 reduces trastuzumab resistance in BT474/TR and SKBR3/TR cells (figure 3B). In contrast, BT474 and SKBR3 cells were treated with trastuzumab after transient transfection of DUSP8 overexpression vector. The survival rate of SKBR3 and BT474 cells was significantly increased after DUSP8 overexpression (p<0.05). These results suggest that DUSP8 overexpression increases trastuzumab resistance in SKBR3 and BT474 cells (figure 3C,D).

Knocking down DUSP8 reversed the proliferation and migration of trastuzumab-resistant breast cancer cells

First, MTT assay was used to detect the effect of DUSP8 on the viability of trastuzumab-resistant strains BT474/TR and SKBR3/TR cells, as shown in figure 4A. Compared with the negative control group, DUSP8 knockdown significantly inhibited BT474/TR and SKBR3/TR cell viability (p<0.05). In addition, DUSP8 overexpression significantly promoted the cell viability of SKBR3 and BT474 (p<0.05) (figure 4B). Transwell results showed that DUSP8 knockdown inhibited BT474/TR and SKBR3/TR cell migration (figure 4C), and DUSP8 overexpression promoted SKBR3 and BT474 cell migration (figure 4D). Flow cytometry results showed that DUSP8 knockdown promoted apoptosis of SKBR3/TR and BT474/TR cells, while DUSP8 overexpression inhibited apoptosis of SKBR3 and BT474 cells (figure 4E,F).

Silencing DUSP8 activates the MAPK pathway in drug-resistant breast cancer cells

We further explored the regulatory mechanism of DUSP8 in cell biological behavior. The results showed that DUSP8 knockdown increased the expression of p-p38 and p-ERK. We further treated DUSP8-silenced SKBR3/TR and BT474/
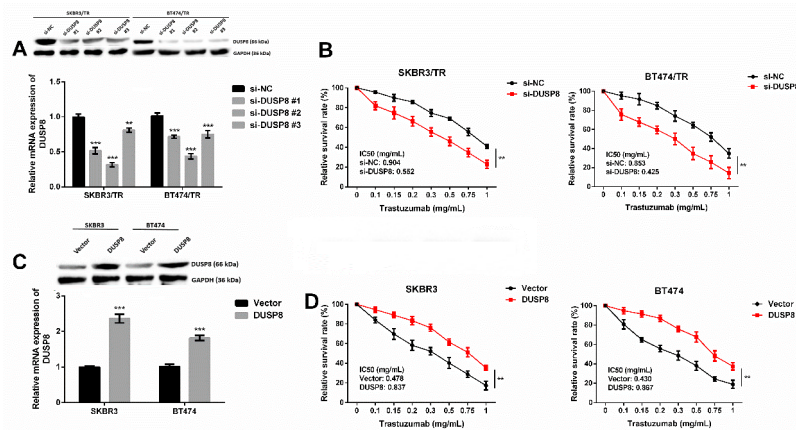
Figure 3 Effect of DUSP8 on drug resistance of trastuzumab in breast cancer. (A) Expression of DUSP8 after transfection of si-DUSP8 in drug-resistant cells. (B) Effect of DUSP8 knockdown on the viability of SKBR3/TR and BT474/TR cells. (C) Expression of DUSP8 after transfection of DUSP8 overexpression vector. (D) Effect of DUSP8 overexpression on drug resistance of SKBR3 and BT474 cells. *P<0.05, **P<0.01, ***P<0.001. DUSP8, dual-specificity phosphatase 8; IC50, half inhibitory concentration.

TR cells with an MAPK inhibitor (SB203580) and found that p-p38 and p-ERK expressions were reduced. These results suggest that MAPK signaling pathway may be involved in the function of BT474/TR and SKBR3/TR cells (figure 5A,B).

DISCUSSION

Breast cancer is one of the most serious malignant tumors with the highest incidence in women. An epidemiological analysis examining changes in cancer rates among Chinese, South Asian and Vietnamese from 2011 to 2015 found a significant increase in the rates of breast cancer among Chinese and South Asian women.[6] Breast cancer is also the second leading cause of all cancer deaths, accounting for about 90% of all new cancers diagnosed in American women.[7] Trastuzumab is an IgG_1 antibody that binds to HER2 receptor extracellular monoclonal antibody domain IV. The advent of trastuzumab significantly improved progress-free survival and overall survival in patients with HER2-positive breast cancer.[8 9] However, with the increasingly widespread use of trastuzumab, some HER2-positive patients with advanced breast cancer experience disease progression after trastuzumab treatment, and some HER2-positive patients with locally advanced breast cancer experience recurrence and metastasis after trastuzumab treatment.[10]

In recent years, a large number of studies have found that the MAPK pathway, as a highly conserved family of protein kinases, can transmit information from extracellular signals into the nucleus and regulate a variety of biological processes such as cell proliferation, differentiation, migration and apoptosis.[11–13] MAPKs are a family of protein kinases that direct cellular response to a variety of stimuli, including mitogens, osmotic pressures, heat shock and proinflammatory cytokines.[14] MAPKs are activated in the kinase activation circuit by phosphorylation of threonine and tyrosine residues in the conserved label T-X-Y motif. It mainly includes extracellular signal-regulated kinases 1 and 2 (ERK1/2), C-Jun amino-terminal kinase (JNK) and p38.[15] The MAPK/ERK pathway plays an important role

in the development and progression of various tumors. ERK1/2 is a key component of this pathway, and its over-activation has also been shown to promote resistance to chemotherapeutic drugs in many cancer cells.[16] This result was also validated in breast cancer cells, where studies found enhanced ERK pathway activation in doxorubicin-resistant, triple-negative breast cancer cells MDA-MB-231. Further studies on the changes of ERK pathway after epirubicin treatment showed that most positive ERK regulatory genes were upregulated in epirubicin-resistant tumor cells, while most negative regulatory genes were enriched in natural tumor cells.[17] The p38 MAPK signaling pathway is an important part of the MAPK family. Through in vitro and in vivo experiments, researchers claimed that the level of p38 MAPK is closely related to the effectiveness of chemotherapy. This signaling pathway is related to multidrug resistance of some cancers.[18] However, there are also a number of experiments with opposite results, and some studies have shown that the overactivation of p38 MAPK is positively correlated with the sensitivity of tumor chemotherapy.[19]

The duration and magnitude of kinase activation are the main determinants of the biological effects of MAPK signal transduction. This reflects the balance between the activity of upstream activators and the negative regulatory mechanisms of activation of various reaction pathways. Activation of MAPK is accompanied by phosphorylation of threonine and tyrosine residues, and dephosphorylation of either residue is sufficient to inactivate MAPK.[20] This can be done by serine/threonine phosphatase, tyrosine specific phosphatase or by bispectoral phosphatase. Interestingly, to date, the protein phosphatase used to specifically regulate mammalian MAPK phosphorylation and activity is bispecific MAPK phosphatase (MKP).[21] MKP constitutes a unique subgroup of 10 catalytically active enzymes within the large family of cysteine-dependent DUSPs.[22] In other words, most DUSPs negatively regulate MAPK signaling through dephosphorylation, which is usually mediated by dephosphorylation of amino acid residues. This conclusion was also verified in this experiment. We found that knockdown of DUSP8
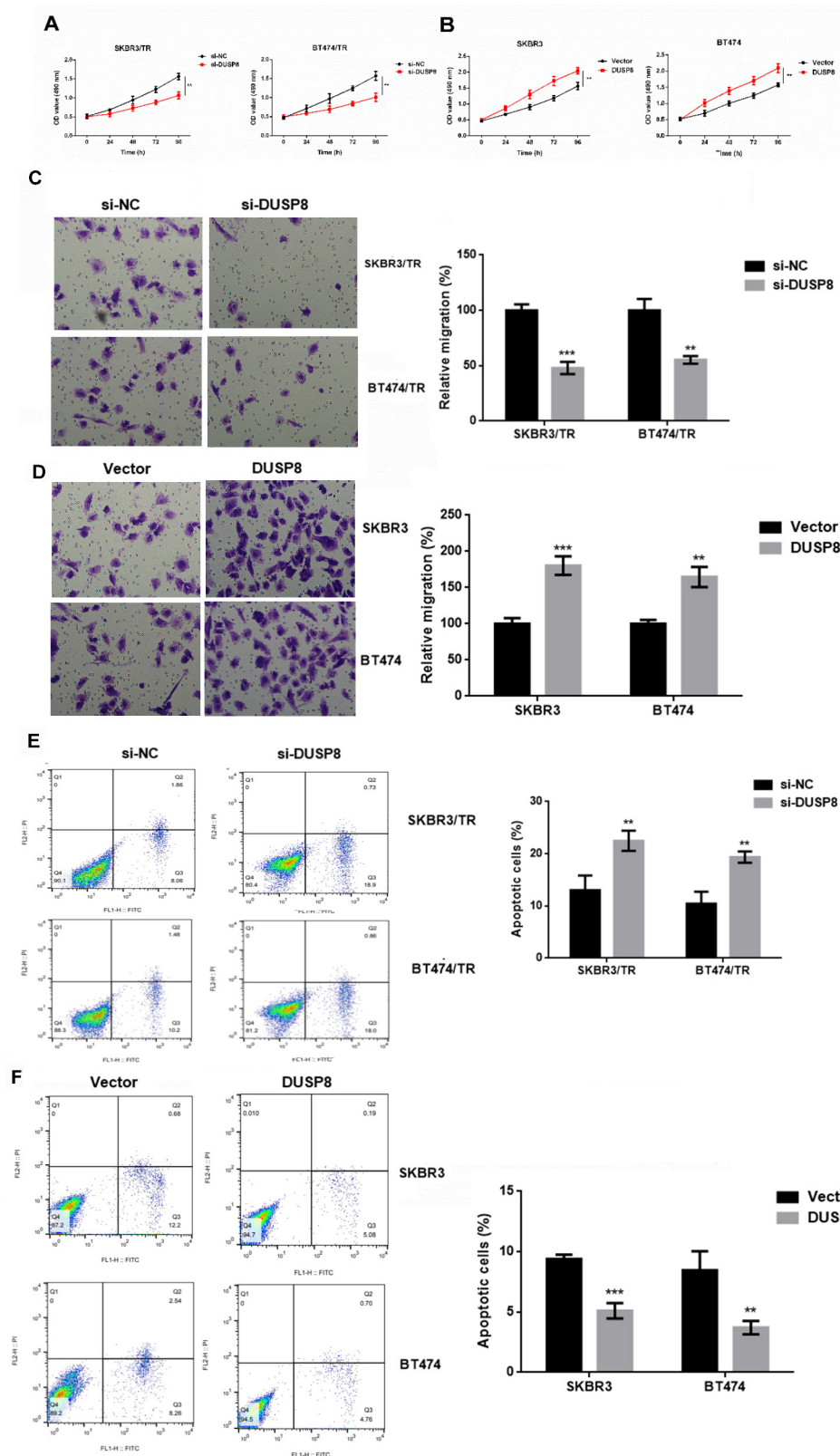
Figure 4 Effect of DUSP8 on proliferation and migration of trastuzumab-resistant cells. (A) Effect of DUSP8 knockdown on cell viability of SKBR3/TR and BT474/TR. (B) Effect of DUSP8 overexpression on cell viability of SKBR3 and BT474. (C) Effect of DUSP8 knockdown on cell migration of SKBR3/TR and BT474/TR. (D) Effect of DUSP8 overexpression on cell migration of SKBR3 and BT474. (E) Effect of DUSP8 knockdown on apoptosis of SKBR3/TR and BT474/TR. (F) Effect of DUSP8 overexpression on apoptosis of SKBR3 and BT474. *P<0.05, **P<0.01, ***P<0.001. DUSP8, dual-specificity phosphatase 8.
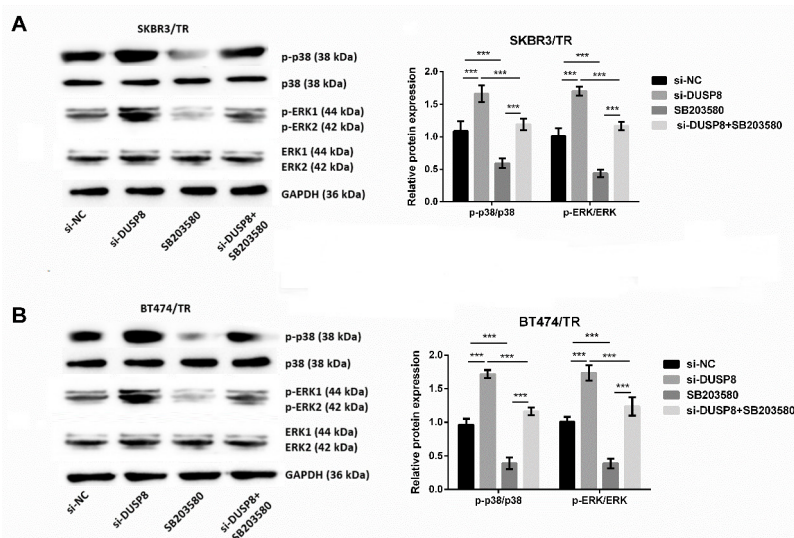
Figure 5 Effect of knocking down DUSP8 on MAPK pathway in drug-resistant breast cancer cells. (A–B) Effects of DUSP8 knockdown and SB203580 on the expression of p-p38 and p-ERK. *P<0.05, **P<0.01, ***P<0.001. DUSP8, dual-specificity phosphatase 8; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase.

increased the expression of p-p38 and p-ERK, but had no significant effect on the expression of p38 and ERK1/2.

More and more studies are indicating that DUSP family molecules may be involved in the process of tumor development or tumor resistance therapy. Moreover, the expression of DUSP family molecules varies in specific tumors.[23] DUSP8 is an important member of the DUSP family. Its gene is located on chromosome 11 q15.5, and its encoding product is DUSP8 protein, which locates in the cytoplasm and nucleus and negatively regulates JNK and p38 pathways in MAPK signaling pathway through selective dephosphorylation.[24] However, we found that DUSP could also affect the biological behaviors of trastuzumab-resistant breast cancer strains by regulating p-ERK expression. In addition, we found that DUSP8 was upregulated in patients with HER2-positive breast cancer who did not respond to trastuzumab therapy, and that DUSP8 was overexpressed in parental breast cancer cells, which enhanced the proliferation and migration of breast cancer cells and increased trastuzumab resistance. Knockdown of DUSP8 expression in trastuzumab-resistant cells inhibited cell proliferation and migration. In conclusion, DUSP8 is upregulated in trastuzumab-resistant cells and acts as a negative regulator of MAPK signaling; knocking down DUSP8 promotes phosphorylation of p38 and ERK. It further inhibited cell migration and cell viability of drug-resistant strains and promoted cell apoptosis. The sensitivity of breast cancer cells to chemotherapy was then enhanced. The above results also reflect the positive correlation between the activation of p38 and ERK signaling pathways and the chemotherapy sensitivity of breast cancer. This result is consistent with some reported results. For example, Mansouri et al[19] compared the activation of p38 MAPK in cisplatin-sensitive and drug-resistant ovarian cancer cell lines and found that the activation degree of p38 MAPK in sensitive strains was higher than in drug-resistant strains. Drug resistance of ovarian cancer cells was significantly increased after treatment with inhibitors of p38 MAPK. Overexpression of DUSP16 in tumor cells leads to increased resistance to cell death after chemotherapy. Conversely, inhibition of DUSP16 in cancer cells increases their sensitivity to therapy. In addition, further study of the mechanism found that DUSP16 inhibited the activation of JNK and p38, thereby reducing the accumulation of Bax in the mitochondria and thus reducing apoptosis. However, it has also been shown that activated MAPK pathway promoted the resistance of breast cancer tumor cells to tamoxifen, and antagonizing nuclear p-ERK may sensitize breast cancer cells to tamoxifen treatment.[25]

Therefore, knockdown of DUSP8 expression in trastuzumab-resistant cells inhibits cell proliferation and migration and activates MAPK signaling pathway in trastuzumab-resistant cells, leading to decreased drug resistance.

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