Pyrotinib sensitizes 5-fluorouracil-resistant HER2-positive breast cancer cells to 5-fluorouracil

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Abstract

BACKGROUND: Clinical trials have shown that pyrotinib+ capecitabine significantly improved efficacy of patients with human epidermal growth factor receptor 2(HER2) +breast cancer. However, whether pyrotinib sensitizes 5-Fluorouracil(5-FU)-resistant breast cancer cells to 5-FU is unknown. This study aimed to investigate the effects of pyrotinib on HER2+breast cancer cells with resistance to 5-FU and provide new clues for the pyrotinib treatment in 5-FU-resistant breast cancer.

METHODS: the 5-FU-resistant breast cancer cell lines SK-BR-3/FU and MAD-MB-453/FU were established by continuous exposure of the parental cells to 5-FU.The effects of pyrotinib on these cell lines were examined by growth inhibitory activity assay, reverse transcription-quantitative polymerase chain reaction, Western blot analysis, high-performance liquid chromatography and animal experiments.

RESULTS: Pyrotinib inhibited the proliferation of 5-FU-resistant and parental HER2-positive breast cancer cells and re-sensitized resistant cells to 5-FU by decreasing the expression of thymidylate synthase(TS) and ABC transporter subfamily G member 2(ABCG2). In a xenograft model, combination treatment with 5-FU and pyrotinib showed greater antitumor activity than either agent alone.

CONCLUSIONS: Our results offer a preclinical rationale for clinical investigations of combination treatment with pyrotinib and 5-FU for 5-FU-resistant HER2-positive breast cancer.

1. Background

Breast cancer is the common female tumor[1] and sensitive to chemotherapeutic agent. Among the agents, 5-FU was widely used for the treatment of breast cancer. In cells, It turns into FdUMP which blocks thymidylate synthase (TS) and thus inhibits DNA synthesis[2]. However, acquired chemoresistance to 5-FU frequently leads to a loss of effect on breast cancer. The mechanisms of chemoresistance to 5-FU not fully known. Some reports revealed that chemoresistance to 5-FU was associated with overexpression of TS and increasing efflux of 5FU from cancer cells[3,4]. Thus, it is necessary to overcome chemoresistance to 5-FU and improve outcome of chemotherapy.

HER consists of HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4[5]. Approximately 15–30% of breast carcinomas overexpress HER2[6], Targeting HER2 is an important treatment method for HER2 + breast cancer, and some anti-HER2 drugs, including trastuzumab, pertuzumab, trastuzumab-emtansine and lapatinib[6–8] have significantly improved the outcomes of patients with HER2 + breast cancer. Recently, a novel drug, pyrotinib for targeting HER1, HER2 and HER4, was developed by Jiangsu Hengrui Pharmaceutical (Lianyungang, People's Republic of China), this drug is a small irreversible inhibitor of receptor tyrosine kinase and 3-cyanoquinoline derivative. Clinical trials have indicated that pyrotinib improved the efficacy of patients with HER2 + breast cancer[9, 10]. Developing combination treatment of targeted therapies with cytotoxic agents is critical strategy for HER2 + breast cancer. Some studies showed that combination pyrotinib with capecitabine significantly improved PFS and ORR in patients with HER2 + metastatic breast cancer[11, 12]. These results revealed the synergistic anticancer activities

of pyrotinib plus capecitabine against HER2 + breast cancers. However, there is not study whether pyrotinib can sensitize 5FUresistant breast cells to 5FU. A previous report showed that lapatinib can sensitize 5-FU-resistant breast cancer to 5-FU by downregulating TS activity[13]. In this study, we established HER2 + breast cancer cell lines with resistance to 5-FU and investigated synergistic anti-tumor effect of pyrotinib plus 5-FU on HER2 + breast cancer with resistance to 5-FU. Our results showed that pyrotinib could sensitize 5FUresistant breast cancer cells to 5FU, which provided a new clue for combination treatment with pyrotinib and 5-FU in HER2 + breast cancer with resistance to 5-FU.

2. Materials And Methods

2.1. Chemicals and antibodies

Pyrotinib (PYR) was obtained from Hengrui Medicine Co. (Jiangsu, China); Signal silence TS siRNA, ABCG2 siRNA, the corresponding control siRNAs, fetal bovine serum (FBS), RNAzol® RT kit, Reverse Transcription System kit, cell lysis buffer, BCA Protein Assay Kit, enhanced chemiluminescence (ECL), Lipofectamine 3000 and 5FU were purchased from Sangon Biotech (Shanghai, China). Antibodies against p-AKT (Ser473), AKT, breast cancer resistance protein (BCRP)/ABCG2, pHER2 (Tyr1221/1222), HER2, pHER4 (Tyr1284), HER4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from BD Biosciences (USA).

2.2. Cell lines and cell cultures

The human HER2-negative breast cancer cell lines MDA-MB-468, the human HER2 + breast cancer cell lines SK-BR-3 and MAD-MB-453 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were maintained in DMEM medium containing 10% FBS and 100 units/mL penicillin/streptomycin.

2.3. Establishment of 5FUresistant SK-BR-3 and MAD-MB-453 sublines

SK-BR-3 cells and MAD-MB-453 cells were cultured in DMEM medium with gradually increasing amounts of 5FU for about 3 months. The final concentration of 5FU was 10 μM. The 5-FU-resistant subclone cells were then cloned using the limiting dilution method. Two 5FUresistant SK-BR-3 and MAD-MB-453 subclones, named SK-BR-3/FU and MAD-MB-453/FU, respectively, showed the same growth ability as the parental cells.

2.4. siRNA transfection

Targeted siRNA or negative control siRNA was transfected into the SK-BR-3/FU and MAD-MB-453/FU cell lines using Lipofectamine 3000.The suppression efficiencies of each siRNA were determined by reverse transcriptionquantitative polymerase chain reaction (RTqPCR) and western blot.

2.5. Growth inhibitory activity assay

The CellTiter-Glo® nonradioactive cell proliferation kit (Promega Beijing Biotch Co., Beijing, China) was used to determine cell viability. Briefly,3×10^4 cells were cultured in 96well plates for 24 hours. These cells were then treated with different concentrations of pyrotinib and/or 5-FU for different times. The plates were at room temperature for 30 min. We then added 100 μl of CellTiter-Glo® (Promega) Reagent to each well, shaked the plates for 2 min, and placed the plates at room temperature for 10 min to stabilize the luminescence signal, which was recorded with a GloMax® luminometer (Promega). The rate of viable cells was calculated using the formula: ratio (%) = [luminescence (Treatment) – luminescence (Blank)]/[luminescence (Control) – luminescence (Blank)] × 100%. The drug concentration that inhibited cell proliferation by 50% was IC_{50}.

Interactions between pyrotinib and 5-FU were determined by combination index (CI). The fraction affected (FA) was evaluated by the formula: FA = (100 – growth inhibition)/100. Where CI < 1, = 1, and > 1 indicated synergism, additivity, and antagonism, respectively[14, 15].

2.6. RTqPCR

Total RNA of the cells was extracted using RNAzol® RT and the RNA was reverse transcribed using Reverse Transcription System kit according to the manufacturer's instructions. The sequences of the primers used were as follows:

5′-GATCACAGTCTTCAAGGAGATC-3′ and **5′-CAGTCCCAGTACGACTGTGACA-3′** for ABCG2, **5′CAGATTATTCAGGACAGGGAGTT3′** and **5′CATCAGAGGAAGATCTCTTGGATT3′** for TS, and **5′-CTCATGACCACAGTCCATGCCATC-3′** and **5′-CTGCTTCACCACCTTCTTGATGTC-3′** for GAPDH. The thermocycling conditions for PCR amplification were as follows: 96 °C for 1 min, then 40 cycles of 96 °C for 10 s and 60 °C for 40 s. GAPDH was included as an internal control, and relative gene expression was determined by previously described[15].

2.7. Detection of 5-FU accumulation by HPLC

1×10^8 cells were cultured in 12-well plates for 24 h, and then were induced with or without pyrotinib for 24 h, after washing with PBS, 400 mg/L 5-FU was added, and the cells were cultured for 2 h. Then, the cells were washed with PBS, fresh medium was added, and the cells were cultured with fresh medium for 1 h. After washing with trypsin in PBS, the cells were collected by centrifugation at 3000 × *g* for 5 min. The collected cells were mixed with 200 μL of sterile water, sonicated for 20 s, then centrifugated at 10000 × *g* for 30 min at 4 °C, the 5-FU in supernatant was assessed by HPLC as previously described[16].

2.8. Western blot

Cells were broken with cell lysis buffer. Protein concentrations were measured using BCA Protein Assay Kit according to the manufacturer's instructions. Then, the proteins were separated by 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was incubated with the corresponding primary antibody for 24 h at 4 °C, and then with the secondary antibody

for 2 h at room temperature. Immunoreactive bands were determined with ECL. GAPDH was used as internal standard.

2.9. Animal experiments

Female 6–8-week-old BALB/c null mice were purchased from Sipper-BK Experimental Animal Co. (Shanghai, China) and raised in pathogen-free laboratory. All animal experiments were approved by the Ethics Committee of The First Affiliated Hospital of Hunan Normal University/Hunan Provincial People's Hospital. For xenograft experiments, SK-BR-3/FU cell suspensions (1×10^7cells/100 μl) were injected into subcutaneous tissue the left flank of each mouse. Tumor size was measured with calipers, and the tumor volume was calculated by the formula:1/2 (length × width^2). When the tumor size reached ~ 300 mm^3, the mice were randomly divided into four groups (control, FU, PYR, and FU + PYR, n = 6 mice, each). The control group was given a daily oral gavage and normal saline via tail vein injection three times each week for two weeks. The FU group was given a daily 5-FU (20 mg/kg) via tail vein injection three times each week for two weeks. The PYR group was given a daily 10 mg/kg/d pyrotinib by oral gavage for 24 days and normal saline via tail vein injection three times each week for two weeks. The FU + PYR group was given a daily oral gavage of pyrotinib (10 mg/kg/d) for 24 days and 5-FU (20 mg/kg) via tail injection vein three times each week for two weeks. Tumors were measured at two-day intervals. Twenty-seven days after treatment, all mice were sacrificed by euthanasia and weighed. Tumor samples were collected and analyzed by western blot.

2.10. Statistical analysis

Data are presented as the mean ± SD, statistical significance was determined using Student's t-tests. All statistical analyses were performed using SPSS 21.0 software (SPSS, USA). A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Inhibitory effect of 5FU or pyrotinib on breast cancer cells

We first detected HER2 protein expression in breast cancer cell lines,and the result showed there was high HER2 protein expression in SK-BR-3,MAD-MB-453, SK-BR-3/FU and MAD-MB-453/FU, but not in MDA-MB-468(Fig. 1A). We further examined the individual Inhibitory effect of 5FU and pyrotinib on HER2-positive SK-BR-3,MAD-MB-453, SK-BR-3/FU and MAD-MB-453/FU cell lines. The results showed that pyrotinib similarly decreased the survival of SK-BR-3 and SK-BR-3/FU cells dose-dependently, as well as that of MAD-MB-453 and MAD-MB-453/FU cells (Fig. 1B,1C). The IC_{50} values for pyrotinib in SK-BR-3/FU and MAD-MB-453/FU cell lines were as well as in parental cell lines respectively(P > .05) (Table 1). 5-FU remarkably suppressed the growth of SK-BR-3 and MAD-MB-453 cells dose-dependently, however, the inhibitory effect of 5FU on SK-BR-3/FU cells and MAD-MB-453/FU cells was slight (Fig. 1D,1E). The IC_{50} values for 5-FU in SK-BR-3/FU and MAD-MB-453/FU cell lines were significantly higher than that in

parental cell lines respectively($P < .05$) (Table 1), indicating that SK-BR-3/FU cells and MAD-MB-453/FU cells were considerably less sensitive to 5-FU than the corresponding parental cell lines, with 11-fold and 10-fold increases in resistance, respectively.

3.2. Pyrotinib could sensitize 5FUresistant breast cancer cells to 5FU in vitro

Synergistic effect of pyrotinib in combination with 5-FU on HER2 + breast cancer cells were determined by detecting the efficacy of different concentrations of the combination of pyrotinib and 5-FU to suppress the growth of SK-BR-3, SK-BR-3/FU, MDA-MB-453, and MDA-MB-453/FU cells. The results showed that pyrotinib in combination with 5-FU had synergistic inhibitory effect on not only parental SK-BR-3 and MDA-MB-453 cells, but also 5-FU-resistant SK-BR-3/FU and MDA-MB-453/FU cells (Fig. 2). The IC_{50} values of 5FU in the four cell lines were significantly decreased by cotreatment with pyrotinib (Table 2). These findings indicated that pyrotinib could sensitize 5FUresistant HER2 + breast cancer cells to 5FU.

3.2. Pyrotinib could increase the intracellular concentrations of 5-FU in 5FUresistant breast cancer cells

The intracellular concentration of 5-FU was examined by HPLC. As shown in Fig. 3, the intracellular concentrations of 5-FU were remarkably higher in the SK-BR-3 and MDA-MB-453 cells than in the SK-BR-3/FU and MDA-MB-453/FU cells in without induction with pyrotinib($P < .01$).After induction with pyrotinib, which significantly increased the intracellular concentration of 5-FU in SK-BR-3/FU and MDA-MB-453/FU cells in a dose-dependent manner($P < .05$). However, pyrotinib had no such effect in SK-BR-3 and MDA-MB-453 cells (Fig. 3A,3B).

3.4. Effect of pyrotinib on TS and ABCG2 mRNA expression levels

Because the high expressions of ABCG2 and TS were related to 5FUresistance. Therefore, we investigated TS and ABCG2 mRNA expression levels by RTqPCR. The results showed that TS and ABCG2 mRNA expression levels were higher in SK-BR-3/FU and MDA-MB-453/FU cells than in SK-BR-3 and MDA-MB-453 cells ($P < .05$), respectively (Fig. 4). Consequently, to determine whether pyrotinib had effect on TS and ABCG2 mRNA expression. We observed that pyrotinib decreased TS mRNA expression levels in SK-BR-3, MDA-MB-453, SK-BR-3/FU, and MDA-MB-453/FU cells in a concentration-dependent manner($P < .05$, vs. control group) (Fig. 4A,4B), and pyrotinib also decreased ABCG2 mRNA expression levels in SK-BR-3/FU and MDA-MB-453/FU cells in a concentration-dependent manner($P < .05$, vs. control group), but not in SK-BR-3 and MDA-MB-453 cells ($P > .05$, vs. control group) (Fig. 4C,4D).

3.5. Effects of pyrotinib on the protein expression of AKT, pAKT, Her-2, pHer-2, Her-4, pHer-4, TS, and ABCG2

Having detected effect of pyrotinib on TS and ABCG2 mRNA expression levels. Next, we examined effects of pyrotinib on the protein expression of protein kinase B(AKT), pAKT, Her-2, pHer-2, Her-4, pHer-4, TS, and ABCG2. The results showed that pAKT, pHer-2, pHer-4, TS, and ABCG2 protein expression levels were higher in SK-BR-3/FU and MDA-MB-453/FU cells than in SK-BR-3 and MDA-MB-453 cells($P < .05$), respectively. Pyrotinib decreased pAKT, pHer-2, and pHer-4 protein expression levels in all four cell lines dose-dependently, without affecting AKT, Her-2, and Her-4 levels (Fig. 5). In addition, pyrotinib decreased ABCG2 protein expression levels in SK-BR-3/FU and MDA-MB-453/FU cells dose-dependently, but not in SK-BR-3 and MDA-MB-453 cells (Fig. 5).

3.6. TS and ABCG2 knockdown restored 5-FU-resistant breast cancer cells to 5-FU

To further confirm the relationship of TS or ABCG2 and 5-FU-resistance in breast cancer cells, we used siRNAs to knock down TS and ABCG2 in 5-FU-resistant breast cancer cells and then treated these cells with various concentrations of 5-FU. The transfection efficiency was determined by RTqPCR, western blot, detection of intracellular concentration of 5-FU and growth inhibitory assay. The results showed TS and ABCG2 mRNA and protein expression levels were significantly lower in SK-BR-3/FU cells and MDA-MB-453/FU cells with TS and ABCG2 knockdown, respectively, than in siRNA-transfected control cells($P < .05$) (Fig. 6A, 6B, 6C, 6D, 6E). The intracellular concentration of 5-FU was remarkably higher in ABCG2-knockdown SK-BR-3/FU and MDA-MB-453/FU cells than in siRNA-transfected control cells($P < .05$). However, TS knockdown had no effect on the intracellular concentration of 5-FU($P > .05$) (Fig. 6F,6G). These findings indicated that the 5-FU is pumped out by BCRP, but not by TS. Next, we measured the changes of 5-FU sensitivity in control cells and TS/ABCG2 siRNA-transfected cells. As shown in Fig. 6H and 6I and Table 3,TS or ABCG2 knockdown increased the sensitivity of SK-BR-3/FU and MDA-MB-453/FU cells to 5-FU compared to that of the control cells, the IC_{50} values for 5-FU in SK-BR-3/FU and MDA-MB-453/FU cells with TS or ABCG2 knockdown were significantly lower than that in siRNA-transfected control cells respectively($P < .05$). In addition, a combination of TS and ABCG2 knockdown made the cells more sensitive to 5-FU than TS or ABCG2 knockdown alone($P < .05$), the IC_{50} values for 5-FU in SK-BR-3/FU and MDA-MB-453/FU cells with a combination of TS and ABCG2 knockdown were noticeably lower than that in TS or ABCG2 knockdown alone respectively ($P < .05$). These results demonstrated that TS and ABCG2 play important roles in 5-FU-resistance in breast cancer cells.

3.7. Pyrotinib restored 5-FU-resistant breast cancer to 5-FU in vivo

After confirming that pyrotinib restored 5-FU-resistant breast cancer cells to 5-FU in vitro. We examined whether pyrotinib could sensitize HER2 + breast cancer with 5-FU-resistance to 5-FU in a xenograft mouse

model. Mice with SK-BR-3/FU xenografts were randomly divided into four groups. The results showed that pyrotinib in combination with 5-FU more effectively inhibited SK-BR-3/FU tumor growth than either pyrotinib or 5-FU alone (Fig. 7A, 7B, 7C). The combination treatment group showed body weights similar to those in the group treated with pyrotinib or 5-FU alone (Fig. 7D), indicating that pyrotinib could restore 5-FU-resistant breast cancer to 5-FU without increased toxicity. Western blot analysis of tumor tissues also showed that TS, ABCG2, pHER2, pHER4, and pAKT were significantly decreased in groups treated with pyrotinib (Fig. 7E, 7F), which were consistent with the results obtained in the cell lines in vitro.

4. Discussion

Breast cancers that overexpress HER2 are generally considered to be poor survival subtypes[17]. Recent advances in anti-HER2 drugs have significantly improved the outcomes of patients with HER2 + breast cancer[18]. However, the curative effects of these targeting drugs alone are limited. Therefore, they are typically administered in combination with chemotherapeutics to maximize the therapeutic effect. 5-FU is commonly used in combination with anti-HER2-targeting therapies for breast cancers[19]. However, some patients with breast cancer treated with 5-FU-based chemotherapy experience recurrence. Vulsteke *et al*. [20] reported that 15.3% of patients with breast cancer experience disease relapse following treatment with 5-FU-based chemotherapy. This recurrence is predominantly due to the development of 5-FU resistance during treatment. About the mechanisms of 5-FU resistance, some studies have highlighted a few, such as overexpression of TS, ABCG2 and dihydropyrimidine dehydrogenase[3, 4]. However, much was still incompletely understood about this process.

In this study, we established two 5FUresistant breast cancer cell lines, SK-BR-3/FU and MDA-MB-453/FU. In vitro, inhibitory effect of 5FU on SK-BR-3/FU cells and MAD-MB-453/FU cells was slight, the IC_{50} values for 5-FU in these cell lines were significantly higher than those in the parental cell lines. In addition, in vivo xenograft experiment confirmed that 5-FU did not suppress SK-BR-3/FU-tumors. These results indicated that SK-BR-3/FU cells and MAD-MB-453/FU cells were highly resistant to 5FU compared to the parental cells. Further We investigated the relationship between 5-FU resistance and TS or ABCG2.

TS is an enzyme that targets 5FU. Our results showed that the mRNA and protein levels of TS were higher in SK-BR-3/FU and MDA-MB-453/FU cells than in the corresponding parental cell lines. Several reports also have shown TS overexpression in some types of 5-FU-resistant cancer cells[21,22]. We used TS siRNAs to knock down TS in SK-BR-3/FU cells and MDA-MB-453/FU cells, the TS mRNA and protein expression levels were found to be significantly lower in TS-knockdown SK-BR-3/FU and MDA-MB-453/FU cells than in the control cells. Moreover, the inhibition rate of 5-FU was also found to be remarkably higher in TS siRNAs-transfected cells than in control siRNAs-transfected cells. Similarly, Kadota et al.[23] also reported that a short hairpin RNA (shRNA) targeting TS effectively downregulated TS expression in 5-FU-resistant tumor cells and the sensitivity to 5-FU were restored in these cells. Therefore, these findings suggested that high TS expression is a mechanism of 5FU resistance.

Recent studies have showed that ATP-binding cassette (ABC) transport proteins could pumped cytotoxic drugs out of cells, and was a major mechanism of multidrug resistance (MDR). Breast cancer resistance protein (BCRP), which is also called ABCG2, is a ABC transport protein[24], its overexpression has been observed in some tumors[4, 25]. In addition, it has been reported that there was an effect-dose relationship between resistance to 5-FU and BCRP expression in the cells, and 5-FU resistance could be reversed by knocking down BCRP through siRNA[26]. Our results showed that the intracellular concentration of 5-FU was remarkably lower and the mRNA and protein expression levels of ABCG2 were noticeably higher in SK-BR-3/FU and MDA-MB-453/FU cells than in the parental cell lines. We used ABCG2 siRNAs to knock down ABCG2 in SK-BR-3/FU cells and MDA-MB-453/FU cells, the ABCG2 mRNA and protein expression levels were found to be significantly lower in ABCG2-knockdown SK-BR-3/FU and MDA-MB-453/FU cells than in the control cells. Moreover, the inhibition rate of cell growth and intracellular concentration of 5-FU were also found to be remarkably higher in ABCG2 siRNAs-transfected cells than in control siRNAs-transfected cells. But TS knockdown had no effect on the intracellular accumulation of 5-FU in SK-BR-3/FU and MDA-MB-453/FU cells. The 5-FU resistance of breast cancer cells was reversed by knockdown of ABCG2. These findings indicated that 5-FU is a substrate of BCRP in breast cancer cells and is pumped out by BCRP. In addition, a combination of TS and ABCG2 knockdown made the cells more sensitive to 5-FU than TS or ABCG2 knockdown alone, which suggested that TS and ABCG2 were simultaneously involved in 5-FU-resistance in breast cancer cells.

Some studies showed that a number of targets can restore 5FUresistant breast cancer cells to 5FU[21,22], for example, Minegaki *et al*.[22] reported that valproic acid and suberanilohydroxamic acid, two histone deacetylase inhibitors, re-sensitized 5FUresistant breast cancer cells to 5FU. In this study, our results revealed that pyrotinib not only increased the sensitivity to 5-FU in 5-FU-sensitive breast cancer cells, but also re-sensitized 5-FU-resistant breast cancer cells to 5-FU in vitro and in vivo. In addition, mice that received the combination treatment did not show lower body weights than the mice in the groups treated with 5-FU or pyrotinib alone. Thus, our data show that pyrotinib restored sensitivity to 5-FU, without an increase in toxicity.

We further investigated the mechanisms of pyrotinib re-sensitizing 5-FU-resistant breast cancer cells to 5-FU. Our results showed that pyrotinib markedly decreased TS mRNA and protein expression in both 5FUresistant cell lines as well as the corresponding parental cell lines, thus allowing 5-FU to better suppress the remaining TS activity. Because the apoptosis induced by 5-FU is related to the extent of enzymatic inhibition13. Being consistent with our result, Chefrour *et al*.[13] reported that lapatinib, a reversible inhibitor of HER1 and HER2, decreased TS activity and increased the cytotoxic effect of 5'-dFUR in HER2 + breast cancer cell lines. However, the molecular mechanism by which pyrotinib and lapatinib downregulates TS is unknown.

Moreover, pyrotinib also decreased ABCG2 mRNA and protein expression in both SK-BR-3/FU and MDA-MB-453/FU cells. The intracellular concentration of 5-FU in 5-FU-resistant cells was increased in pyrotinib-treated cells almost to the level of that in the parental cells, and the sensitivity to 5-FU was restored.

However, pyrotinib had no effect on ABCG2 mRNA and protein expression in SK-BR-3 and MDA-MB-453 cells. This might be due to the little ABCG2 expression levels in these cells.

Here, we performed a further investigation of the mechanisms by which ABCG2 expression was inhibited by pyrotinib. Our results showed that the levels of p-AKT, pHer-2, and pHer-4 were higher in SK-BR-3/FU and MDA-MB-453/FU cells than in the parental cell lines, and pyrotinib significantly decreased p-AKT, pHer-2, and pHer-4 levels in SK-BR-3/FU and MDA-MB-453/FU cells, without affecting total AKT, Her-2, or Her-4 levels in virto. pyrotinib-treated SK-BR-3/FU xenograft tissues also had lower pAKT, pHER2 and pHER4 expression, which indicated that BCRP is suppressed by pyrotinib via inhibition of the PI3K/Akt signaling pathway. Similarly, Chefrour *et al*.[13] reported that lapatinib significantly downregulated pHer-2 and p-AKT levels and increased the cytotoxic effect of 5'-dFUR in HER2 + breast cancer cell lines.

Some previous studies showed that HER2 overexpression activated the PI3K/Akt signal transduction pathway and led to increased BCRP expression in breast cancer cells[27, 28]. PI3K inhibition also has been reported to be particularly effective in sensitizing breast cancer to cytotoxic agents by decreasing ACCG2 expression as a consequence of inhibiting p-AKT activity[29, 30]. The present data strongly suggest that the downregulation of ABCG2 by pyrotinib, via inhibition of p-Akt signaling and the pHER2/pHER4 pathway, could be a mechanism of the restoration of sensitivity to 5-FU in 5FUresistant breast cancer cell lines.

5. Conclusions

We showed that the 5FU resistance of breast cancer cells is correlated with the overexpression of TS and ABCG2. This is the first study to demonstrate the role of pyrotinib in restoring the sensitivity to 5-FU in 5-FU-resistant breast cancer cells. Our findings open a new pathway for combination treatment with pyrotinib and 5-FU for the patients with 5-FU-resistant HER2 + breast cancer.

Abbreviations

human epidermal growth factor receptor 2(HER2); 5Fluorouracil(5FU); thymidylate synthase (TS); ABCG2(ABC transporter subfamily G member 2); fetal bovine serum (FBS);glyceraldehyde-3-phosphate dehydrogenase(GAPDH); fetal bovine serum(FBS); enhanced chemiluminescence (ECL); transcriptionquantitative polymerase chain reaction (RTqPCR); protein kinase B(AKT); ATP-binding cassette (ABC); multidrug resistance (MDR); Breast cancer resistance protein (BCRP);

Declarations

ETHICS APPROVAL

The study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Hunan Normal University/Hunan Provincial People's Hospital.

CONSENT FOR PUBLICATION
We have obtained consents to publish this paper from all the participants of this study.

CONFLICT OF INTEREST DISCLOSURES
The authors declare no competing interests.

AVAILABILITY OF DATA AND MATERIALS
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
Experimental design: JNY, PZF, PYY; Experimental execution: JNY, PYY, SC, QL, RZW, YD, LHZ; Computational analysis: JNY, PZF, PYY; Manuscript writing, review and discussion: JNY, PZF, PYY, LHZ.

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Figures

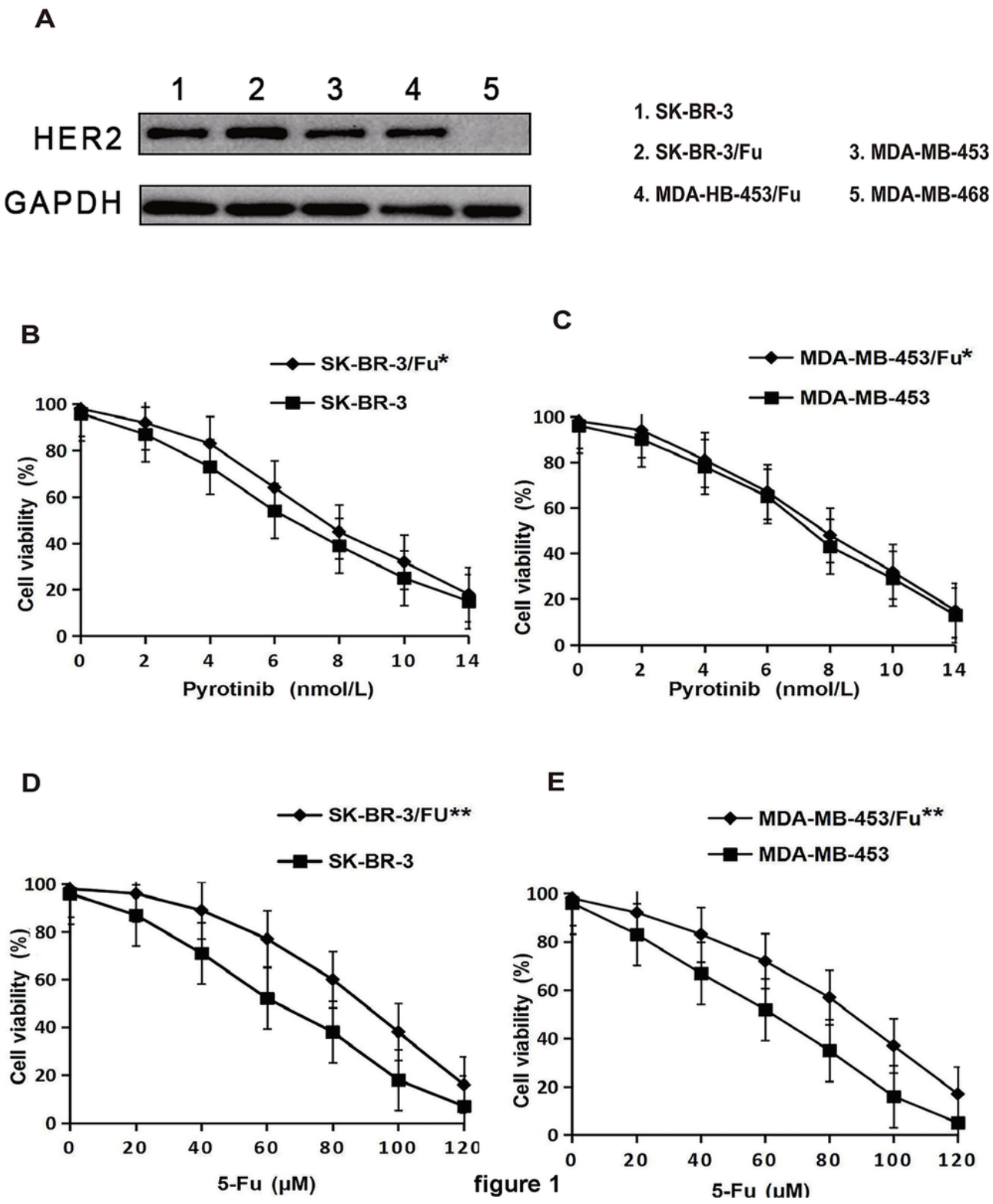

Figure 1

Growth inhibitory effects of 5 FU and pyrotinib in SK-BR-3, SK-BR-3/FU, MDA-MB-453, and MDA-MB-453/FU cells. (A) HER2 protein expression in breast cancer cell lines was detected by Western blot. (B,C,D,E) Cells were treated with various concentrations of 5 FU or pyrotinib for 72 h, and then cell viability was measured. Each point represents the mean ± standard deviation (SD) (n = 3). * $P < 0.05$ vs. the parental cell line and ** $P < 0.01$ vs. the parental cell line. 5 FU, 5 fluorouracil.

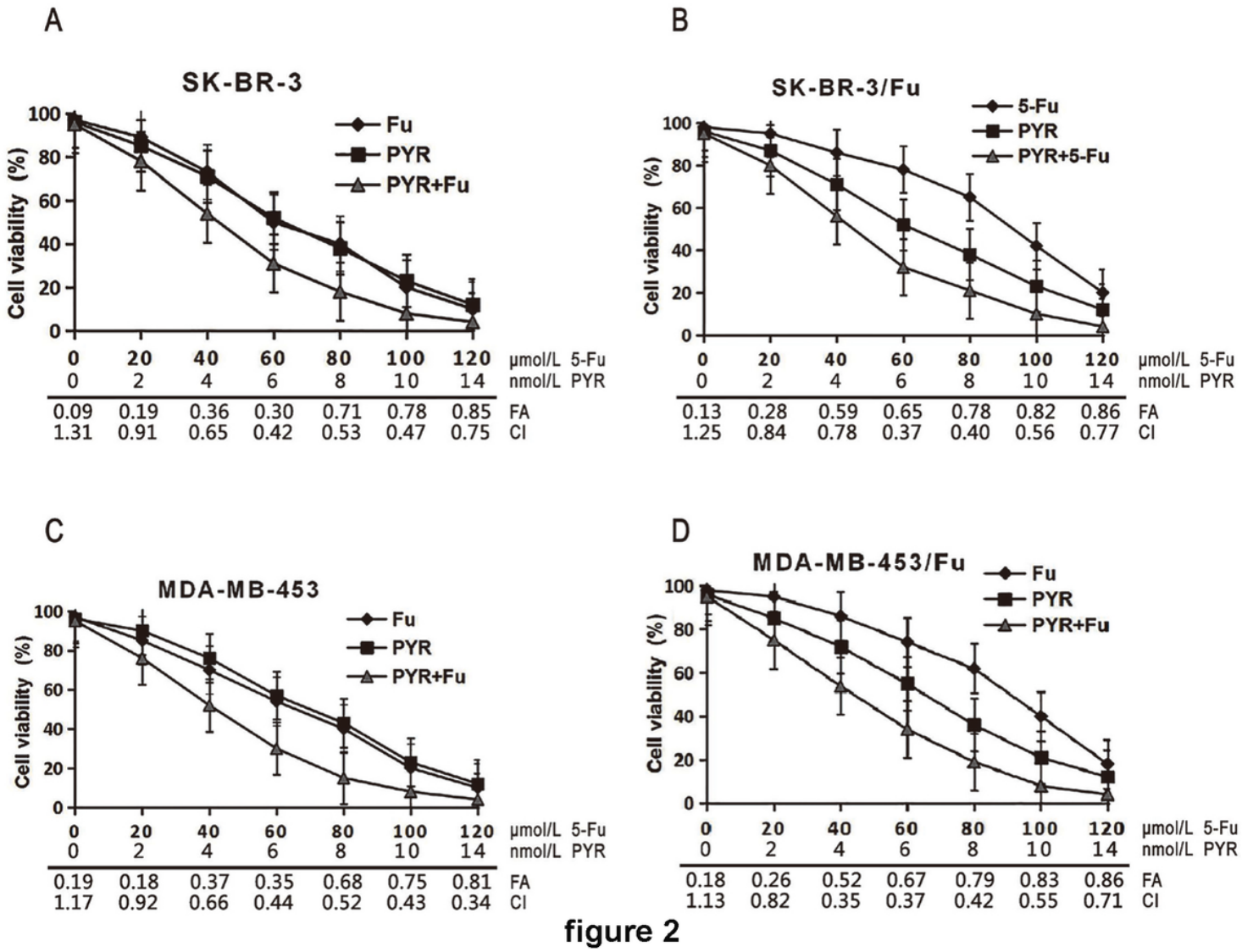

Figure 2

Effect of pyrotinib (PYR) on the growth inhibitory effect of 5-FU in SK-BR-3, SK-BR-3/FU, MDA-MB-453, and MDA-MB-453/FU cells. The cells were treated with various concentrations of 5 FU and/or pyrotinib for 72 h, and then cell viability was measured. The combined drug effect was calculated using the CI equation and the FA of the combinations is presented. Synergy was defined as a CI value <1.0, antagonism was defined as a CI value >1.0, and additivity was defined as a CI value = 1.0. The data points are the mean ± SD percent growth inhibition (from three independent experiments) compared to the controls.

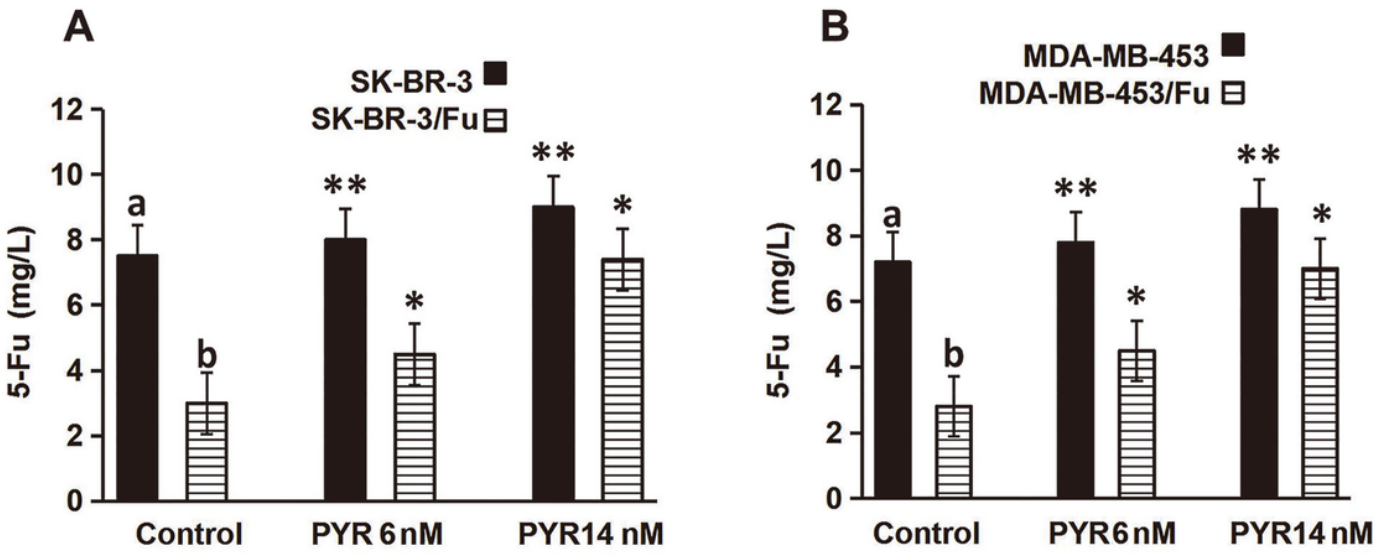

figure 3

Figure 3

Effect of pyrotinib (PYR) on the accumulation of 5-FU in breast cancer cells. After induction with pyrotinib, cells were treated with 5-FU (400 mg/L). The accumulation of 5-FU in these cells were detected by HPLC. The data shown are representative of three independent experiments. (A) Accumulation of 5-FU in SK-BR-3 and SK-BR-3/FU cells. (B) Accumulation of 5-FU in MDA-MB-453 and MDA-MB-453/FU. *P < 0.01 vs. control b, **P < 0.05 vs. control a, and bP < 0.01 vs. control a.

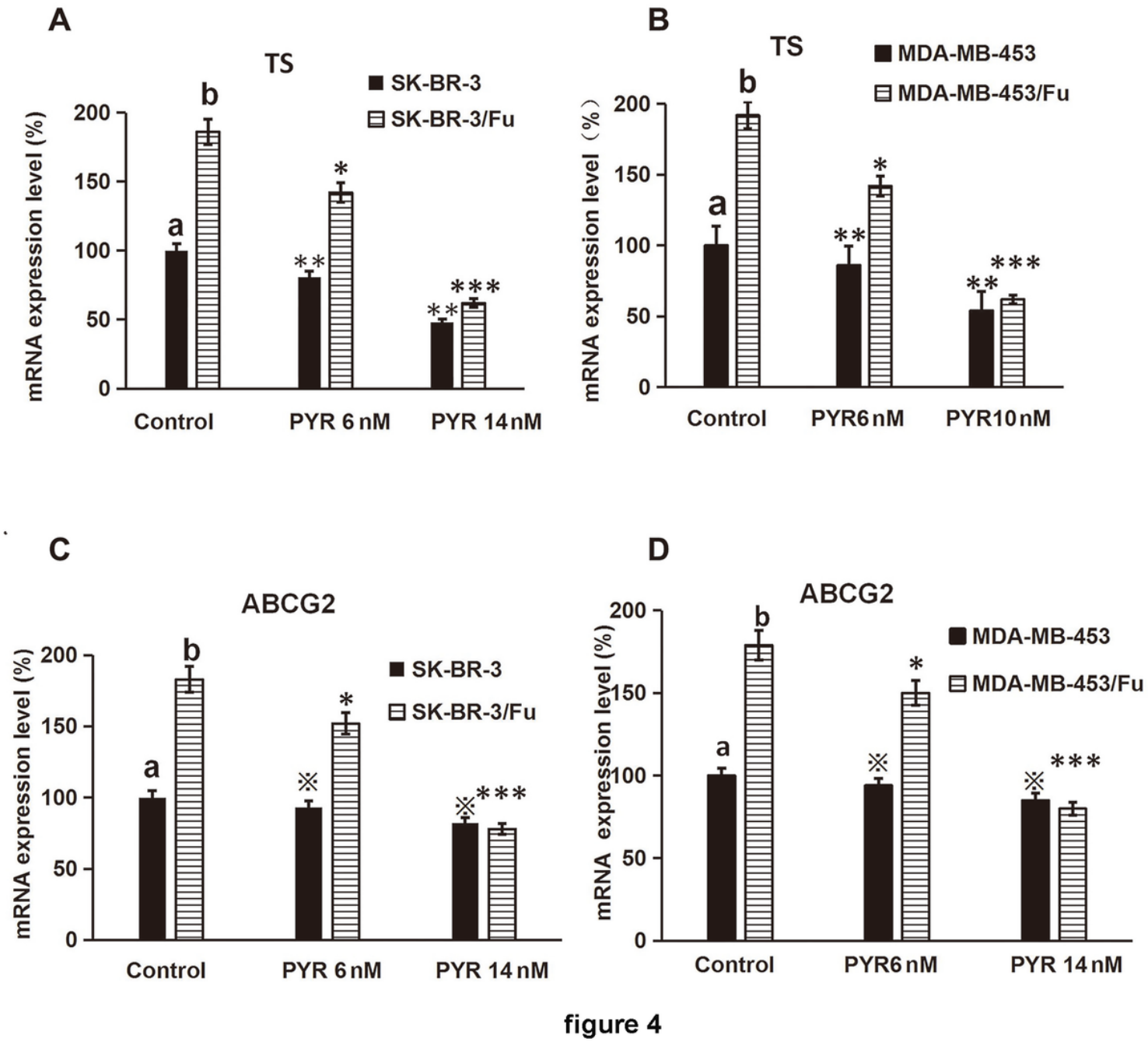

figure 4

Figure 4

Effect of pyrotinib on TS and ABCG2 mRNA expression. mRNA expression was measured by RT qPCR, and GAPDH was used as the internal standard. Each bar represents the mean ± standard deviation (n = 3). *P < 0.05 vs. control b and **P < 0.05 vs. control a, ***P < 0.01 vs. controlb. bP < 0.01 vs. control a, ※P > 0.05 vs. control a. PYR, pyrotinib; TS, thymidylate synthetase; 5 FU, 5 fluorouracil.

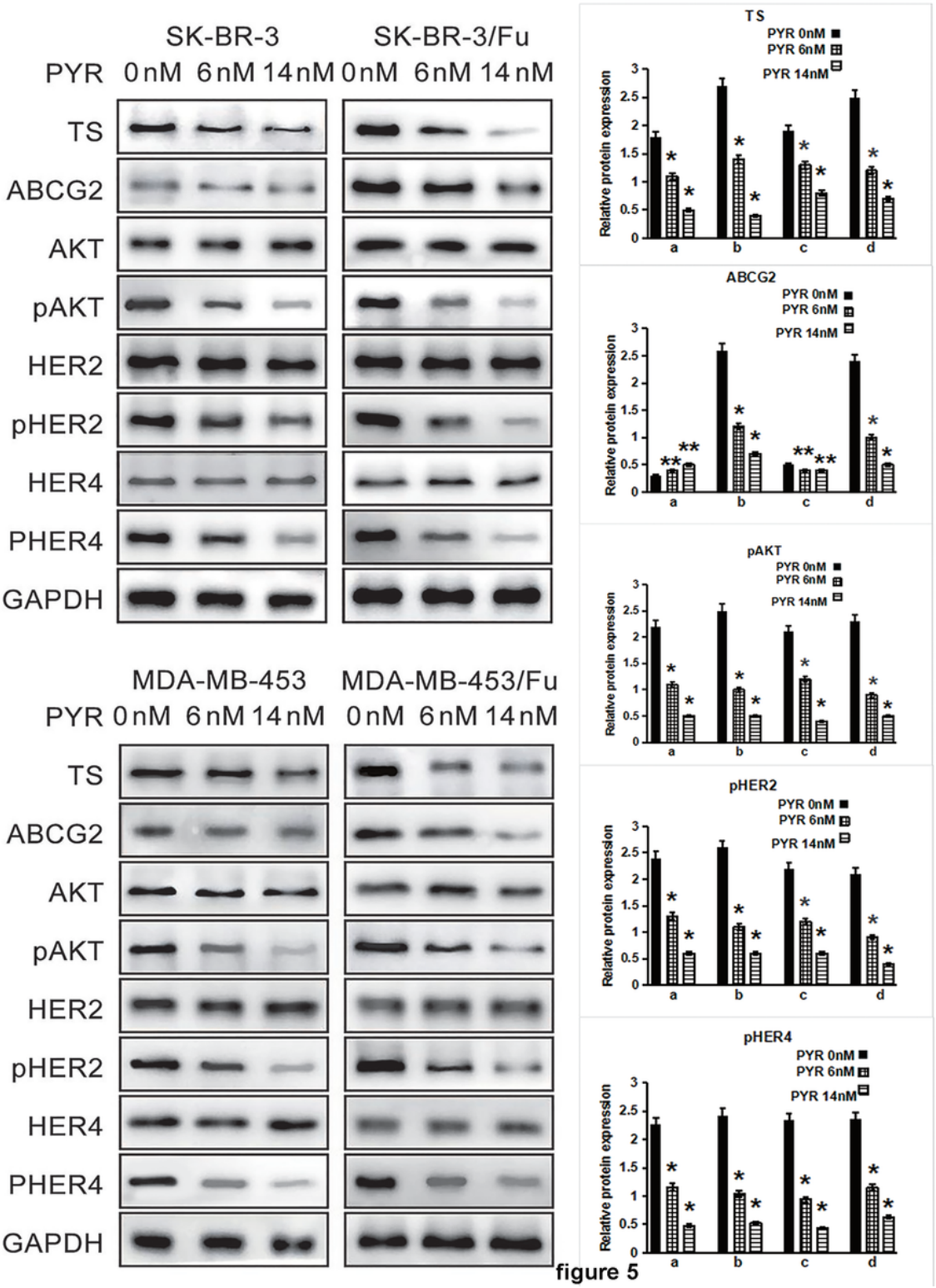

Figure 5

Effects of pyrotinib on the protein expression of AKT, p-AKT, Her-2, pHer-2, Her-4, pHer-4, TS, and ABCG2. Western blot analysis of AKT, pAKT, TS, ABCG2, HER2, pHER2, HER4, and pHER4.*P < 0.05 vs. control , **P > 0.05 vs. Control.a:SK-BR-3,b:SK-BR-3/FU,c:MDA-MB-453,d:MDA-MB-453/FU

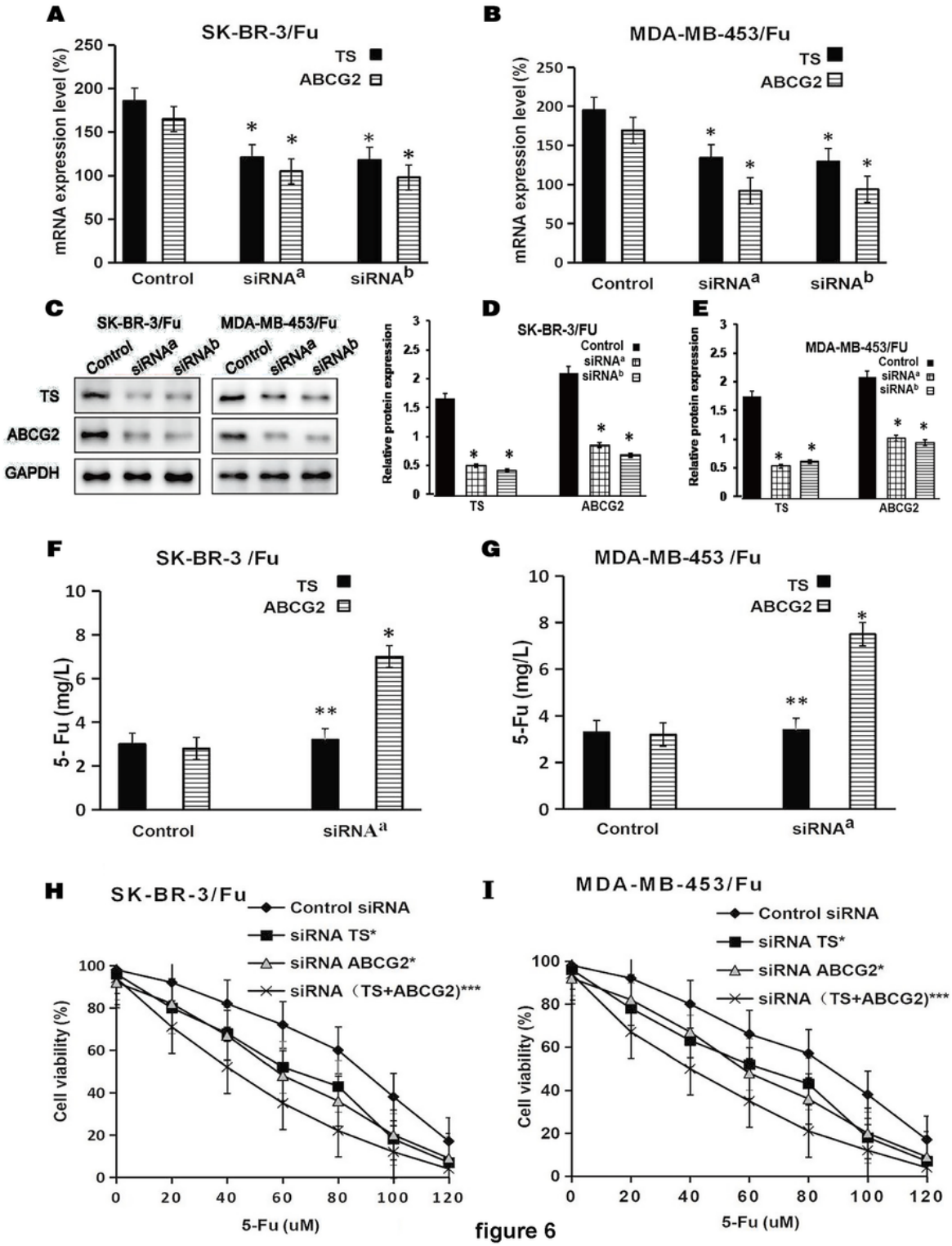

Figure 6

Effect of TS and ABCG2 knockdown on the 5-FU sensitivity of 5-FU-resistant breast cancer cells. (A, B) Transfection efficiency was measured by qRT-PCR. Data are presented as the mean ± SD of triplicate experiments. (C) Transfection efficiency was measured by western blotting. (D, E) The data shown are representative of three independent experiments in detecting TS and ABCG2 expression. (F,G)The accumulation of 5-FU in the cells was detected by HPLC. Shown are representative data from three

independent experiments. (H,I) Cells were treated with 5-FU for 72 h, and the effect of TS and ABCC5 knockdown on 5-FU sensitivity was measured. Data are presented as the mean ± SD of triplicate experiments. *P < 0.05 vs. the Control, **P > 0.05 vs. the Control, ***P < 0.01 vs. Control siRNA-, TS siRNA-, or ABCG2 siRNA-transfected cells. aTS or ABCG2 knockdown, bTS and ABCG2 knockdown.

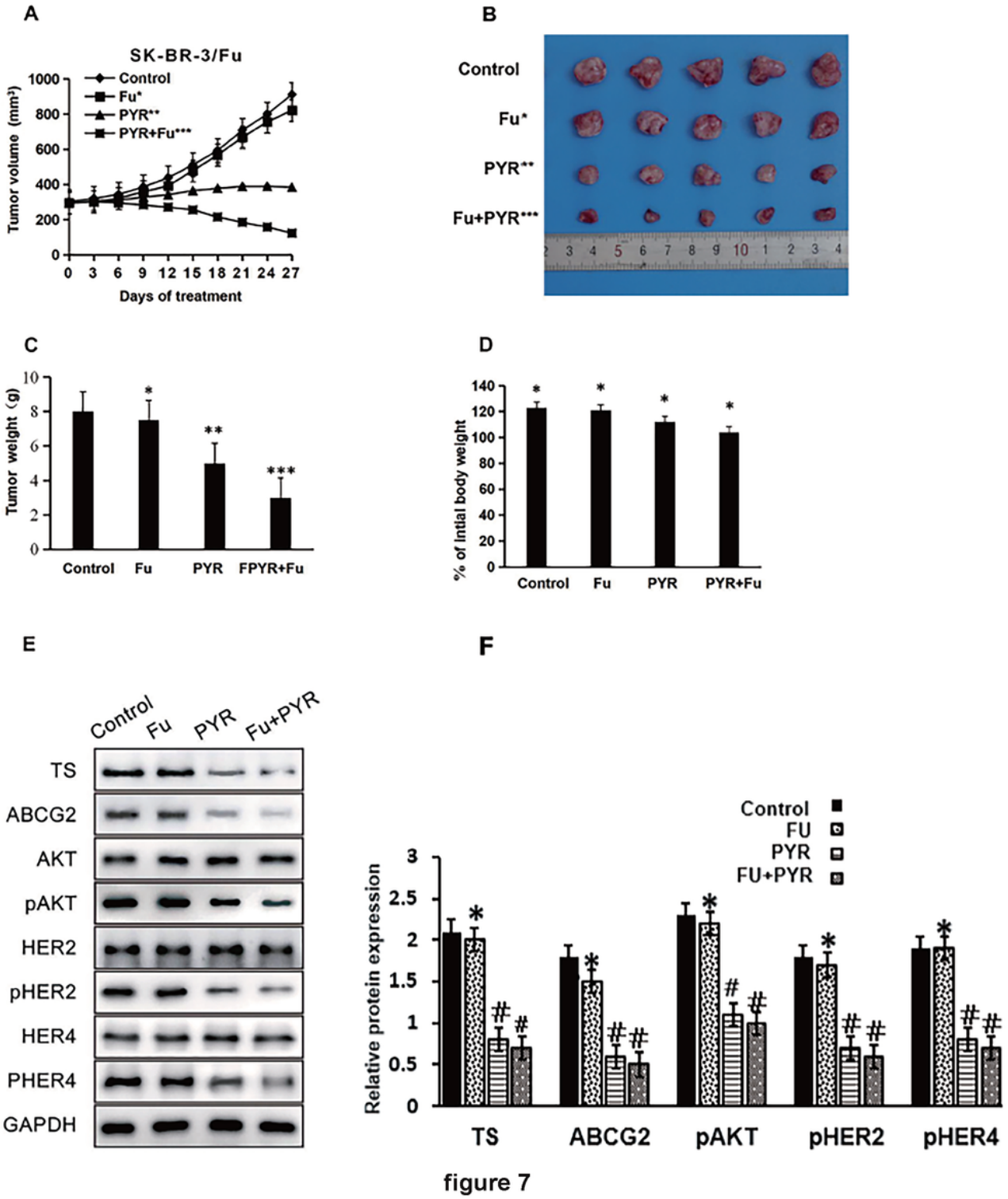

figure 7

Figure 7

Pyrotinib sensitizes 5-FU-resistant breast cancer cells to 5-FU in vivo. Nude mice bearing SK-BR-3/FU tumors were treated with saline (control), FU (20 mg/kg), PYR (10 mg/kg), or FU (20 mg/kg) + PYR (10 mg/kg) for 27 days. (A) Tumor volume, which was assessed every three days after the onset of treatment. (B, C) Representative tumors and tumor weights at the end of the experiment (day 27). (D) Body weights on day 27. (E) Western blot analysis of TS, BCRP, p-AKT, AKT, p-HER2, HER2, p-HER4, HER4, and GAPDH in harvested tumors. (F)The data shown are representative of three independent experiments in detecting TS□ABCG2 p-AKT, p-HER2 and p-HER4 expression. *P >0.05 vs. Control, **P < 0.05 vs. control, ***P < 0.01 vs. Control□□P < 0.01 vs. Control.

Supplementary Files

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