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PRECLINICAL STUDY

Erythropoietin receptor expression and its relationship with trastuzumab response and resistance in HER2-positive breast cancer cells

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Abstract Resistance to trastuzumab is a major issue in the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer. Several potential resistance mechanisms have been investigated, but the results are controversial and no conclusion has been reached. Erythropoietin receptor (EPOR) may function in cell growth, and expressed in various cancer cells. Because the downstream signaling pathways for EPOR and HER2 partially overlapped, we hypothesized that EPOR may play a role in the inhibition effect of trastuzumab and resistance to trastuzumab. Here, we detected the expression of EPOR mRNA and protein in HER2-positive breast cancer cell lines and tissues. EPOR expressed in SKBR3, MDA-MB-453, and UACC-812 cell lines, but not in BT474. Of the 55 HER2-positive cancer tissues, EPOR was positive in 42 samples and highly expressed (H-score $\geq$ 25) in 24 by immunohistochemistry. The difference between EPOR expression and Ki67 index was significant ($P = 0.033$), and EPOR expression also positively correlated with higher pathological stage (Spearman correlation coefficient = 0.359; $P = 0.007$). Exogenous EPO antagonized trastuzumab-induced inhibition of cell proliferation in HER2/EPOR dual-positive breast cancer cells. We then exposed SKBR3 cells to trastuzumab for 4 months to obtain trastuzumab-resistant SKBR3 cell line, which demonstrated higher phosphorylated EPOR level, higher EPO expression and more extracellular secretion than non-resistant parental SKBR3 cells. Downregulation EPOR expression using short hairpin RNA resensitized trastuzumab-resistant cells to this drug, and SKBR3 cells with EPOR downregulation demonstrated attenuated trastuzumab resistance after the same resistance induction. EPOR downregulation plus trastuzumab produced a synergetic action in the inhibition of cell proliferation and invasion in SKBR3 and MDA-MB-453 cell lines. Therefore, EPOR expression may be involved in tumor progression and proliferation in HER2-positive breast cancer. EPO/EPOR contributes to the mechanism of trastuzumab resistance in SKBR3 cell lines, and EPOR downregulation can reverse the resistance to trastuzumab and increase the inhibition effect of this drug.

Keywords Breast cancer · Erythropoietin receptor · HER2 · Resistance · Trastuzumab

Introduction

Trastuzumab is a recombinant-humanized monoclonal antibody directed against the extracellular domain of human epidermal growth factor receptor 2 (HER2). Studies have shown that a combination of trastuzumab and conventional chemotherapy is significantly more effective in treating HER2-positive metastatic breast cancers than chemotherapy alone [1, 2]. However, the overall response rate to trastuzumab was only 26 % when used as a single therapy and 40–60 % when used in combination with systemic chemotherapy [3, 4]. The majority of patients who achieve an initial response to trastuzumab-based therapy developed resistance within 1 year of the treatment [5, 6]. Elucidating the mechanisms by which tumors escape the cytotoxic properties of trastuzumab is critical to improve the survival of HER2-positive breast cancer patients.

Several potential resistance mechanisms have been described in model systems. These include hyperactivation

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of the PI3K/AKT pathway resulting from mutation or diminished expression of PTEN and mutational activation of the p110 subunit of PI3K, upregulation of other receptor tyrosine kinases such as epidermal growth factor receptor, c-MET or IGF-1R, accumulation of truncated forms of HER2, increased SRC activation, and overexpression of MUC-4 [7–10]. However, the mechanisms of trastuzumab resistance have not been completely elucidated, and none of the markers associated with trastuzumab sensitivity/resistance is sufficiently reliable for clinical use [11].

Erythropoietin (EPO) is a glycoprotein hormone that serves as the primary regulator of erythropoiesis by stimulating growth, preventing apoptosis, and inducing differentiation of red blood cell precursors [12]. Recombinant human EPO (rHuEPO) has been frequently used in the treatment of cancer-related and chemotherapy-induced anemia or fatigue since the 1990s [13]. However, recent studies have suggested that EPO once thought to act solely on erythroid compartment is actually a pleiotropic cytokine [14]. The erythropoietin receptor (EPOR) is also present in multiple types of normal and cancerous tissues [15], suggesting that the EPO/EPOR system governs additional cellular processes besides erythropoiesis. Several clinical trials have concluded that rHuEPO is useful for ameliorating anemia in cancer patients, but fails to improve or even impairs cancer control and survival, including the breast cancer patients treated with trastuzumab [16–18]. Because cancer cells are exposed to endogenous autocrine/paracrine EPO, and endocrine EPO secreted by kidney, exogenous EPO treatment avoidance may not sufficient, therefore EPOR is attracting attention in cancer research. Considering the fact that the downstream signaling pathways for EPO/EPOR and HER2 may overlap or interact each other, we hypothesized that EPOR may have antagonistic effects on trastuzumab-induced antitumor activity in HER2-positive breast cancer cells, and play a role in trastuzumab resistance.

Materials and methods

Cell culture and reagents

The human HER2-positive breast cancer cell lines SKBR3, BT474, MDA-MB-453, and UACC-812 were purchased from the Chinese Academy of Medical Sciences Cell Resource Center (CAMS/PUMC, Beijing). SKBR3, BT474, and UACC-812 cells were maintained in RPMI 1640 (HyClone, Logan, UT, USA) at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2 and MDA-MB-453 cells were maintained in DPMI L15 (Gibco, Grand Island, NY, USA) at 37 °C in a humidified atmosphere of 100 % air. All medium were supplemented with 10 % fetal bovine serum (FBS, Gibco). Trastuzumab was purchased from Genentech (South San Francisco, CA, USA), and EPO from Amgen (Thousand Oaks, CA, USA). Trastuzumab-resistant SKBR3 cell line (SKBR3/R) was developed by continuous exposure to 10 μg/ml trastuzumab for 4 months, during which the medium was replaced every 5 days and cells were passaged when they reached 50 % confluence. Trastuzumab resistance was confirmed by dose–response studies as described below.

Clinical samples

Surgical samples of primary HER2-positive breast cancer were collected from 55 female patients treated in Peking University First Hospital Breast Disease Centre during 2011 under a protocol approved by the institutional review board. The mean age of the patients was 57 years (35–86 years); 35 were postmenopausal and 20 were premenopausal. All the patients were recommended to use trastuzumab-based therapy and eleven of them received it. A core biopsy was performed for pathological diagnosis. The HER2 positive was defined as HER2 scored 3+ by immunohistochemistry (IHC) or scored 2+ with *her2* gene amplification by fluorescence in situ hybridization (FISH). None of the patients received preoperative anti-cancer therapy. Fresh sample was fixed in 4 % paraformaldehyde and then paraffin-embedded for IHC. Tumor tissue and paired adjacent normal tissue were frozen immediately after resection and stored in liquid nitrogen for mRNA extraction.

Lentivirus-mediated short hairpin RNA (shRNA) knockdown of EPOR

To generate EPOR stable knockdown cells, SKBR3, SKBR3/R, and MDA-MB-453 cells were transfected with the recombinant lentivirus containing EPOR-knockdown shRNA or a recombinant lentivirus containing scrambled shRNA as negative control, followed by selection with 4 μg/ml puromycin. For construction of the two recombinant lentivirus, the primers of 5′-GATCCCTACAGCTTCTCCTACCAGTTC AAGAGACTGGTAGGAGAAGCTGTAGTTA and 5′-AG CTTAACTACAGCTTCTCCTACCAGTCTCTTGAACTG GTAGGAGAAGCTGTAGG were used to make an EPOR-knockdown shRNA vector targeting the bases 362–382 of EPOR mRNA (NM_000121), and the scrambled shRNA vector for negative control was made to target the sequence of 5′-GACCAGCTTCTCCACAATCAT not complementary to any known human mRNA, as previously described [19]. Successful knockdown of EPOR expression was determined by western blotting and real-time quantitative reverse transcription-PCR (RT-PCR).

Determination of cell viability by MTT assay

Cells were seeded at 6×10^4 cells per well in flat-bottomed 96-well microplates. After 24 h, the cells were treated with appropriate agents for indicated time. The culture medium was replaced with 50 µl MTT-serum-free fresh medium and incubated for 4 h and then 200 µl dimethylsulfoxide were added to dissolve the formazan crystals. Absorbance at 570 nm was measured using a Spectra Max plus microplate reader (Molecular Devices, Sunnyvale, CA, USA). The IC_{50} value was determined from the dose-dependent growth inhibition data with a four-parameter logistic equation by GraphPad Prism software (San Diego, CA, USA). Each experiment was carried out in triplicate and was repeated at least three times.

Western blotting

Cells were resuspended in lysis buffer. After determining the protein concentration, 40 µg of a cell extract sample were run in a SDS-PAGE gel and electroblotted onto polyvinylidene fluoride membrane. After blocking, the blot was exposed to specific antibodies overnight at 4 °C. Antibodies used for immunoblotting were anti-phosphor-AKT (Ser473), anti-AKT, anti-phosphor-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phosphor-STAT5 (Tyr694), anti-STAT5 (all from Cell Signaling, Danvers, MA), anti-phosphor-EPOR (Tyr479), anti-EPOR, and anti-EPO (both from Santa Cruz, Santa Cruz, CA, USA). After washing, the blot was exposed to appropriate secondary antibody at 10,000-fold dilution in blocking solution for 1 h at room temperature. The blot was developed by enhanced chemiluminescence detection (ECL kit; Amersham Pharmacia, Chandler, AZ, USA).

IHC staining of EPOR

IHC was performed on paraffin-embedded sections. After de-paraffin and hydration, the slide was incubated in 0.01 M citrate buffer pH 6.0 heated in a microwave oven for three 5-min, followed by 3 % hydrogen peroxide for 15 min. The slide was then incubated in anti-EpoR polyclonal antibody (Santa Cruz, CA, USA) overnight at 4 °C, washed three times in TBST, incubated in secondary antibody for 30 min at room temperature, then washed three times in TBST, developed with diaminobenzidine for 10 min, and counter-stained with hematoxylin. The H-score was used to express the intensity and percentage of staining. H-score < 25 was considered as low or negative expression, and H-score ≥ 25 as high expression [20].

Quantitative determination of extracellular EPO

SKBR3 and SKBR3/R cells were incubated in serum-free medium for 48 h. Culture conditioned medium was collected for the EPO production assay. EPO production was assessed using an ELISA kit from R&D Systems (Minneapolis, MN, USA) according to the protocol of the manufacturer. The sensitivity limit of the assay is 0.6 m U/ml with a range of 2.5–200 mU/ml. Absorbance at 450 nm was measured using a Spectra Max plus microplate reader.

Cell invasion assay

Cells invasion assay was performed using a 24-well Biocoat Matrigel invasion chamber using a 8-µm pore polycarbonate filter, and the surface was coated with 10 % Matrigel. The bottom well was filled with 750 µl medium containing 10 % FBS. Cells were plated in the upper chamber at a density of 6×10^4 cells suspended in 150 µl medium containing 0.5 % FBS supplemented with 2 µg/ml trastuzumab. SKBR3 and MDA-MB-453 cells were allowed to invade at 37 °C in a humidified incubator for 96 and 48 h, respectively. Cells penetrated into the membrane were counted under a microscope (20×) in 10 different fields from triplicate wells.

Real-time quantitative RT-PCR

Total RNA was extracted using a RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 1 µg total RNA was reverse-transcribed to cDNA using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Primers and probes were designed by means of Beacon Designer software (version 3; Premier Biosoft International, Palo Alto, CA, USA), the sequences were EPOR-F: 5′-AGCCCAGAGAGCGAGTTTGA, EPOR-R: 5′-CCACAGGCAGCCATCATTCT, EPOR-probe: 5′-TCACCACCCACAAGGGTAACTTCCAGCT, EPO-F: 5′-GCAGCCTCACCACTCTGCTT, EPO-R: 5′-CGGAAAGTGTCAGCAGTGATTG, EPO-probe: 5′-TCTCCCCTCCAGATGCGGCCTC, GAPDH-F: 5′-CAGTCAGCCGCATCTTCTTTT, GAPDH-R: 5′-GTGACCAGGCGCCCAATAC, and GAPDH-probe: 5′-CGTCGCCAGCCGAGCCACA. The probes were labeled with FAM as reporter dye and TAMRA as quencher dye. Amplification was performed by a TaqMan PCR protocol in an ABI 7300 real-time PCR system (ABI, Foster City, CA, USA). The thermal cycling condition comprised a denaturation step for 5 min at 94 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C.

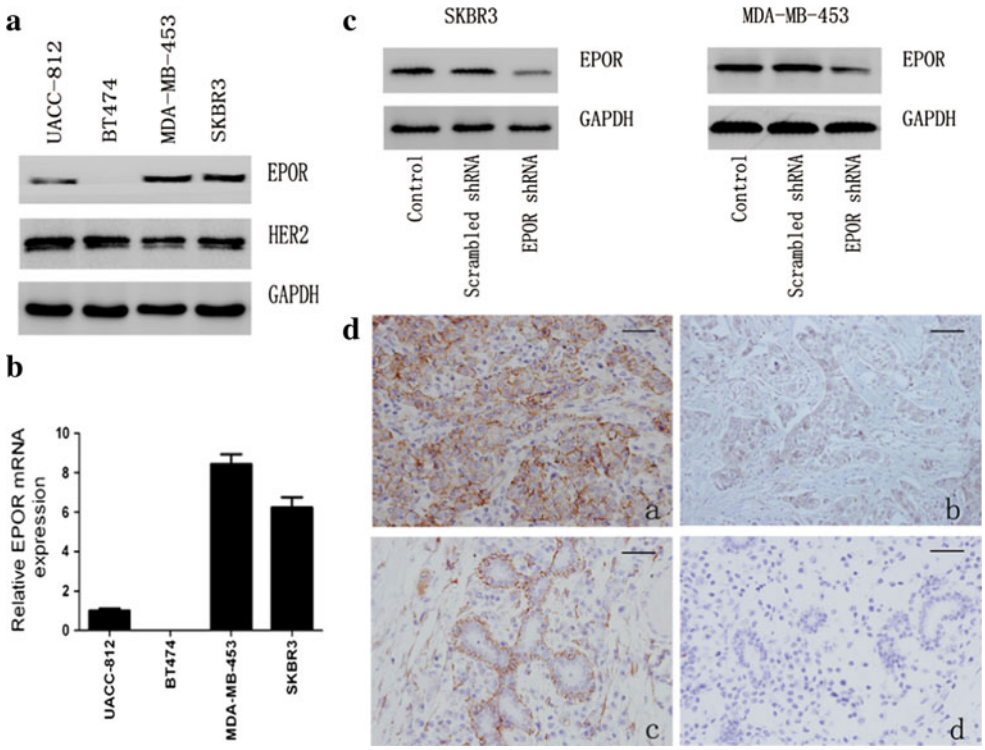

Fig. 1 EPOR expression in HER2-positive breast cancer cell lines and tumor tissues. **a** Among the four HER2-positive breast cancer cell lines, EPOR expression was high in SKBR3 and MDA-MB-453, low in UACC-812, and negative in BT474. **b** Relative EPOR mRNA expression level (mean ± standard deviation) by real-time quantitative RT-PCR in the four cell lines. EPOR mRNA was not detected in BT474 cell line. **c** SKBR3 and MDA-MB-453 cells were transfected with EPOR shRNA or scrambled shRNA as a negative control. Western blotting showed that EPOR expression was decreased in EPOR shRNA transfected cells compared with the scrambled shRNA transfected and parental cells. **d** Representative IHC staining patterns of EPOR in HER2-positive breast cancer samples and adjacent normal tissues. 1 High EPOR expression in cancer; 2 Low EPOR expression in cancer; 3 Positive EPOR expression in adjacent normal tissue; 4 Negative EPOR expression in adjacent normal tissue (*scale bar* = 50 μm)

Statistical analysis

Statistical analyses were carried out in SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Calculation of numerical data (mean ± standard deviation) was performed by two-tailed *t* test and categorical variable was performed by Chi square test or Fisher's exact test. Spearman rank correlation coefficient was determined to correlate EPOR expression and tumor characteristics. A P value <0.05 was considered to be statistically significant.

Results

EPOR expression in HER2-positive breast cancer cell lines and surgical samples

We analyzed EPOR expression by western blotting and real-time quantitative RT-PCR in four HER2-positive breast cancer cell lines: SKBR3, BT474, MDA-MB-453, and UACC-812. By western blotting, EPOR was expressed at high level in SKBR3 and MDA-MB-453, low level in UACC-812, not expressed in BT474 (Fig. 1a). Similar findings were obtained by real-time quantitative RT-PCR (Fig. 1b). We established EPOR downregulated SKBR3 and MDA-MB-453 cell lines by transfection with lentivirus-mediated EPOR shRNA (Fig. 1c).

We next examined EPOR expression in 55 HER2-positive breast cancer samples and their paired adjacent normal tissues by IHC and real-time quantitative RT-PCR. By IHC, EPOR was expressed in 42 tumor samples, but weakly expressed in only 10 adjacent normal tissues (Fig. 1d); low (including negative) and high expression were found in 31 and 24 tumor samples, respectively. By real-time quantitative RT-PCR, EPOR was expressed in 46 tumor samples and 11 adjacent normal tissues.

Table 1 summarizes the relationship between EPOR expression level and clinicopathological characteristics of the HER2-positive breast cancer patients. Chi square tests or Fisher's exact tests showed the difference was significant between EPOR expression and tumor pathological stage ($P = 0.012$), also EPOR expression and Ki67 index ($P = 0.033$), but not between EPOR expression and hormone receptor status, age, or histologic grade. Spearman

Table 1 EPOR expression level in relation to clinicopathological variables

Variable	Total no.	EPOR, high expression		EPOR, low expression		*P* value*
		No.	%	No.	%	
Age						
<50	18	7	12.7	11	20	0.620
≥50	37	17	30.9	20	36.4	
ER status						
Positive	25	12	21.8	13	23.6	0.551
Negative	30	12	21.8	18	32.7	
PR status						
Positive	22	12	21.8	10	18.2	0.183
Negative	33	12	21.8	21	38.2	
Ki67						
High index	43	22	40	21	38.2	0.033
Low index	12	2	3.6	10	18.2	
Tumor stage						
I	23	5	9.1	18	32.7	0.012
II	24	14	25.5	10	18.2	
III	8	5	9.1	3	5.5	
Tumor grade						
1	8	2	3.6	6	10.9	0.701
2	24	12	21.8	12	21.8	
3	23	10	18.2	13	23.6	

* Chi square test or Fisher's exact test

correlation analysis showed a significant association of EPOR expression and tumor pathological stage ($r = 0.359$; $P = 0.007$).

Effects of exogenous EPO on the cell proliferation inhibition induced by trastuzumab in HER2/EPOR dual-positive breast cancer cell lines

MDA-MB-453, SKBR3, and BT474 cells were grown untreated or treated with 2 μg/ml trastuzumab, 10 U/ml EPO, or both, in medium containing 1 % FBS. After incubation for 5 days, trastuzumab inhibited cell proliferation in the three cell lines, EPO stimulated cell proliferation in EPOR-positive SKBR3 and MDA-MB-453 cell lines, but not in EPOR-negative BT474 cell line, and EPO significantly antagonized trastuzumab-induced cell proliferation inhibition in HER2/EPOR dual-positive SKBR3 and MDA-MB-453 cell lines (Fig. 2a).

To investigate the mechanism by which EPO antagonizes the effect of trastuzumab, we measured the activation of cell signaling pathways by examining the phosphorylation status of Akt, Erk1/2, and STAT5 by western blotting. We found that EPO increased the phosphorylation of Akt, Erk1/2, and STAT5 in EPOR-positive SKBR3 and MDA-MB-453 cell lines (Fig. 2b), the phosphorylation of Akt and Erk1/2 were inhibited by trastuzumab, and EPO counteracted the trastuzumab-induced inhibition. These results suggest that EPOR expressed in breast cancer cells is functional, EPOR and HER2 may partially share the downstream pathways for cell signaling.

The relationship between EPOR and the acquired resistance to trastuzumab

We tried to develop SKBR3 cells resistant to trastuzumab to mimic the acquired resistance in HER2-positive breast cancer patients. SKBR3 cells, EPOR shRNA-transfected SKBR3 cells, and scrambled shRNA-transfected SKBR3 cells were incubated with trastuzumab for 4 months to obtain the trastuzumab-resistant cell lines of SKBR3/R, SKBR3-EPOR-shRNA/R, and SKBR3-scrambled-shRNA/R. The cells were then treated with serial dilutions of trastuzumab for 120 h, and the viability of the cells at various drug concentrations is shown in Fig. 3a. At 2 μg/ml trastuzumab, the mean viability was 83 % for SKBR3-EPOR-shRNA/R cells and was 119 % for SKBR3/R cells; both were remarkably higher than that of the parental SKBR3 cells. The IC_{50} value for trastuzumab was significantly different between parental SKBR3 cells (5.6 μg/ml) and SKBR3/R cells (36.1 μg/ml) (Fig. 3b). SKBR3/R cells became re-sensitized to trastuzumab after transfected with EPOR shRNA, but not after transfected with scrambled shRNA (Fig. 3c).

We also evaluated the phosphorylation of Akt, Erk1/2, and STAT5 possibly involved in HER2 and EPOR cell signaling in SKBR3/R and parental SKBR3 cells. EPOR expression was not changed insignificantly between SKBR3/R and SKBR3 cells; however, we observed a remarkable increase of p-EPOR, p-Akt, and p-STAT5 expression in SKBR3/R cells (Fig. 3d).

EPO expression and extracellular secretion alternation in trastuzumab-resistant cells

EPO expression increased in trastuzumab-resistant SKBR3 cells compared with parent cells in protein and mRNA levels (Fig. 4a, b). We used ELISA to evaluate the secretion of endogenous EPO in conditioned medium after the cells were incubated in FBS-free medium for 48 h and detected the EPO concentration in SKBR3/R cells cultured medium was remarkable higher than SKBR3 cells (Fig. 4c). EPO concentration in FBS-free medium was below the minimal sensitivity limit of the Epo ELISA kit. These results suggested that EPO expression and extracellular secretion substantially increased after resistance to trastuzumab. As a growth-stimulating factor, these changes might compensate for the inhibition effect of trastuzumab.

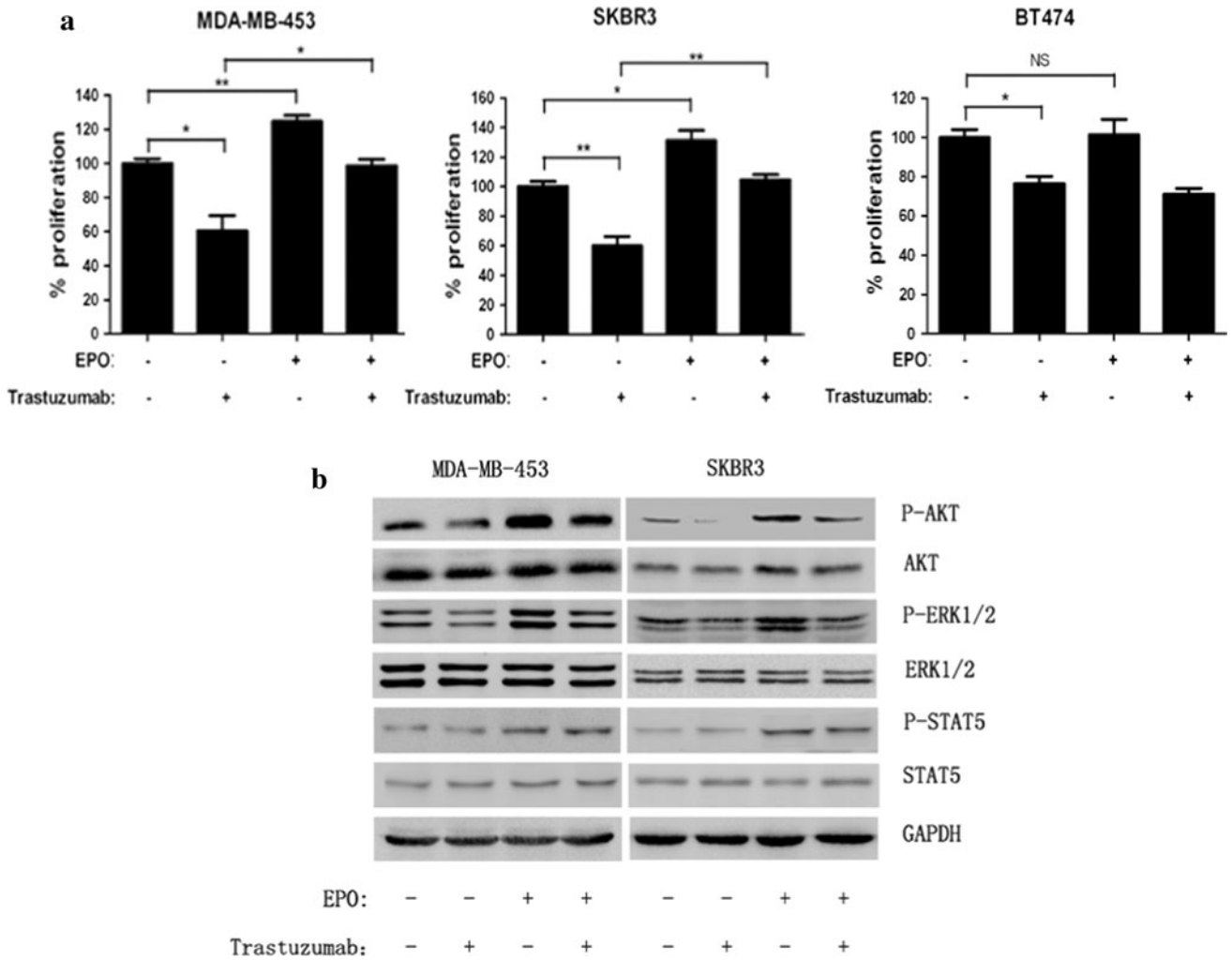

Fig. 2 Exogenous EPO antagonized the effects of trastuzumab in HER2/EPOR dual-positive breast cancer cells. **a** Exogenous EPO on trastuzumab-induced inhibition of cell growth. Cells were grown untreated or treated with 2 μg/ml trastuzumab, 10 U/ml EPO, or both, in 1 % FBS-containing medium for 5 days, then assayed by MTT method to quantify cell proliferation. $*P < 0.05$; $**P < 0.01$; *NS* not significant. **b** Exogenous EPO on trastuzumab-induced changes of cell signaling molecules. The two cell lines were treated as described in **a** for 16 h. Cell lysates were examined by western blotting using indicated antibodies

Synergetic effects of EPOR downregulation plus trastuzumab treatment on the inhibition of cell proliferation and invasion in SKBR3 and MDA-MB-453 cells

EPOR downregulation in SKBR3 and MDA-MB-453 cells resulted in moderate inhibition of cell proliferation and invasion compared with scrambled shRNA-transfected cells and parental cells. 2 μg/ml trastuzumab treatment to SKBR3 and MDA-MB-453 cells also caused moderate inhibition of cell proliferation and invasion compared with scrambled shRNA-transfected cells and parental cells. Notably, EPOR downregulation combined with trastuzumab treatment showed additive effects on the inhibition of cell proliferation and invasion compared with the cells of EPOR downregulation or trastuzumab treatment only (Fig. 5a, b). Together, these data indicated that EPOR interact with those activated by HER2, EPOR downregulation and trastuzumab had a joint action.

Discussion

Although trastuzumab significantly improves outcome in HER2-positive breast cancer, primary and acquired resistance severely affects the application of this drug. Elucidating the molecular mechanisms underlying the resistance is essential to trastuzumab and other targeting therapies. Although the mechanism is not fully understood, activation of crosstalk within signaling networks downstream of HER2 is clearly of primary importance. Here, we found that the expression and secretion of EPO, p-EPOR, EPOR

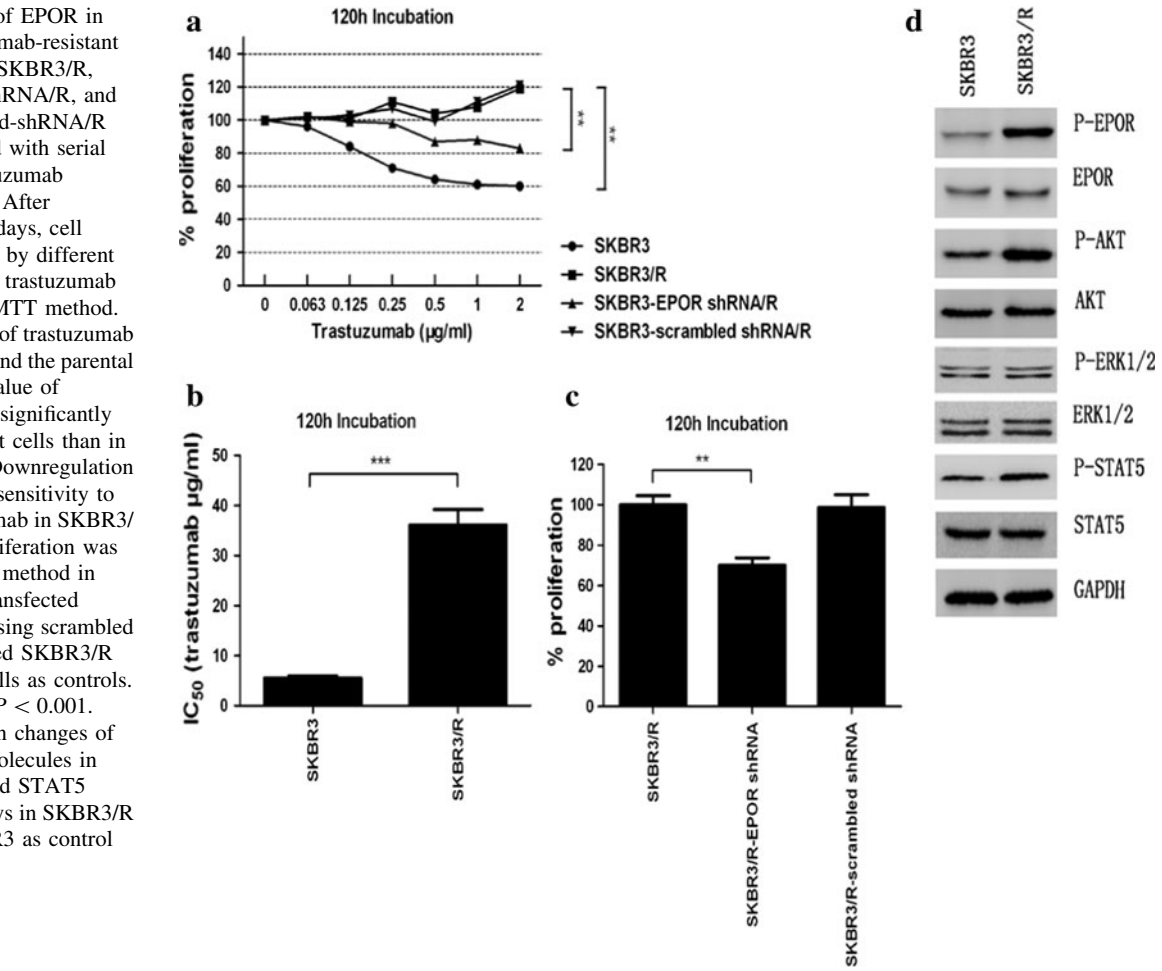

Fig. 3 The role of EPOR in acquired trastuzumab-resistant cells. **a** SKBR3, SKBR3/R, SKBR3-EPOR-shRNA/R, and SKBR3-scrambled-shRNA/R cells were treated with serial dilutions of trastuzumab (0.063–2 μg/ml). After incubation for 5 days, cell growth inhibition by different concentrations of trastuzumab was assayed by MTT method. **b** The IC_{50} value of trastuzumab in resistant cells and the parental cells. The IC_{50} value of trastuzumab was significantly higher in resistant cells than in parental cells. **c** Downregulation of EPOR on the sensitivity to 2 μg/ml trastuzumab in SKBR3/R cells. Cell proliferation was assayed by MTT method in EPOR shRNA-transfected SKBR3/R cells using scrambled shRNA-transfected SKBR3/R and SKBR3/R cells as controls. $^{**}P < 0.01$; $^{***}P < 0.001$. **d** Phosphorylation changes of EPOR and the molecules in PI3K, MAPK, and STAT5 signaling pathways in SKBR3/R cells using SKBR3 as control

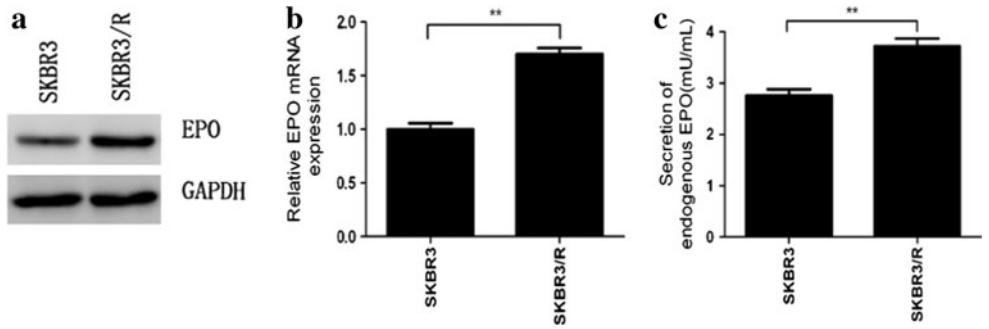

Fig. 4 EPO in acquired trastuzumab-resistant cells. **a** The expression of EPO in SKBR3 and SKBR3/R examined by western blotting. **b** Relative EPO mRNA expression level (mean ± standard deviation) by real-time quantitative RT-PCR in SKBR3 and SKBR3/R cells. **c** Quantitative determination of endogenous EPO in conditioned medium by ELISA, SKBR3 and SKBR3/R cells were incubated in serum-free medium for 48 h. $^{**}P < 0.01$

downstream signaling molecules p-Akt and p-STAT5 dramatically increased after the development of acquired resistance to trastuzumab in SKBR3 cells. Moreover, this resistance could be partially reversed by EPOR downregulation. Subsequently, the activation of EPO/EPOR and its downstream PI3K and STAT5 pathways may take part in the phenotype of trastuzumab resistance.

Cancer cells are heterogeneous in terms of their responses to various drugs. A number of critical proteins must be involved in the development of drug resistance. Cell surface receptors that transduce growth-promoting signals, such as EPOR, are an important group among the critical proteins. EPOR exerts oncogenic effects and therefore it is not surprising that EPOR plays a role in drug

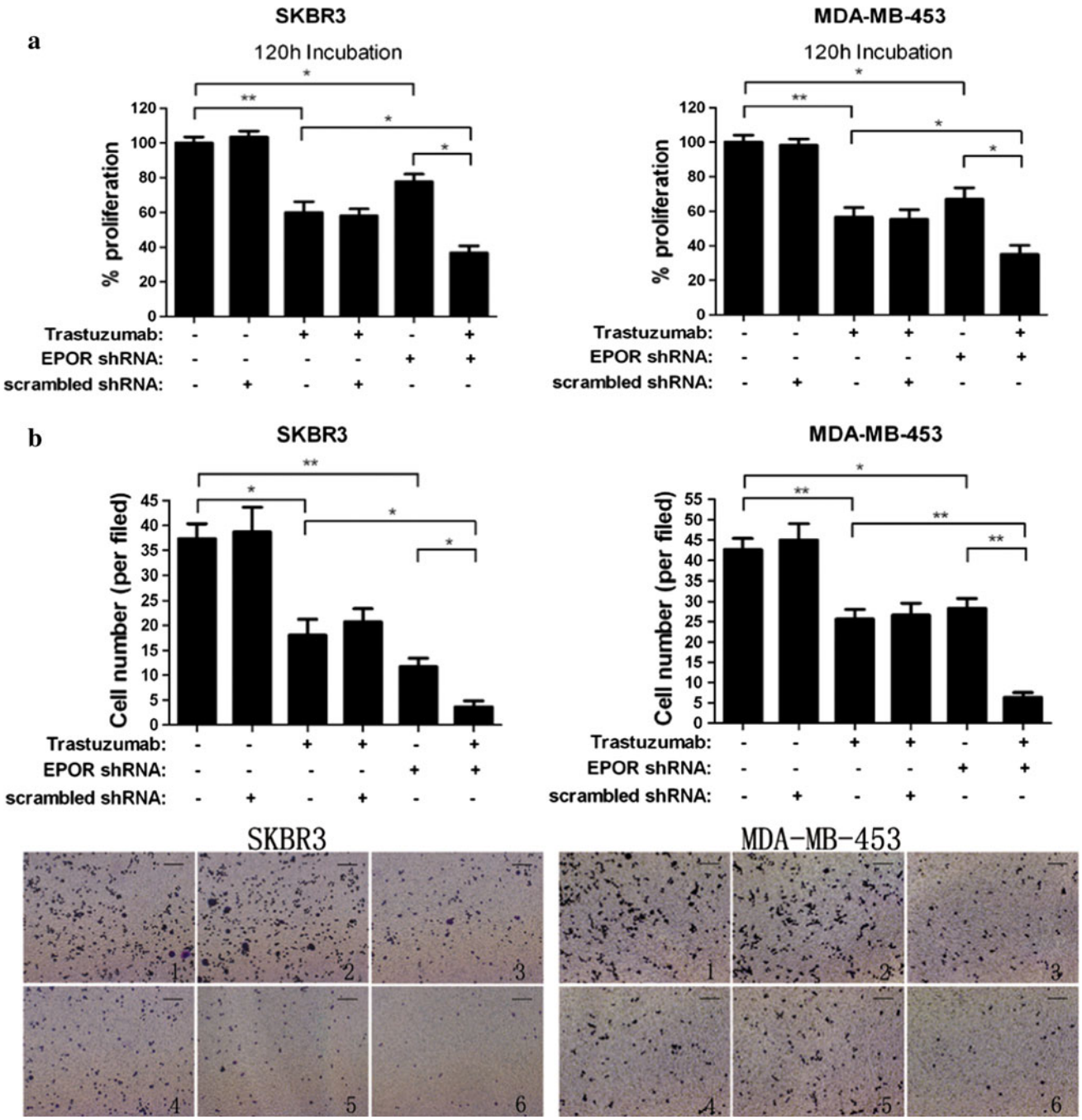

Fig. 5 The synergetic effects of EPOR downregulation plus trastuzumab treatment on cell proliferation and cell invasion in SKBR3 and MDA-MB-453 cells. **a** To evaluate the synergetic effects of EPOR downregulation and trastuzumab treatment, cell proliferation was measured in SKBR3 and MDA-MB-453 cells and corresponding EPOR shRNA-transfected cells incubated with or without 2 μg/ml trastuzumab for 120 h. The lowest cell proliferation was found in EPOR shRNA-transfected cells incubated with 2 μg/ml trastuzumab. **b** The invasion ability was measured using transwell chambers in SKBR3 and MDA-MB-453 cells and corresponding EPOR shRNA-transfected cells incubated with or without 2 μg/ml trastuzumab in the upper chamber. Also, the lowest invasion ability was found in EPOR shRNA-transfected cells incubated with 2 μg/ml trastuzumab. The number of the figures showing invaded cells on filters corresponds to the order of the columns. $*P < 0.05$; $**P < 0.01$ (*scale bar* = 80 μm)

resistance [21, 22]. To our knowledge, this is the first report that EPO/EPOR plays a role in trastuzumab resistance. Downregulation of EPOR expression delayed the resistance and/or re-sensitized cancer cells to this drug.

EPO is widely used to treat cancer-related anemia [23]. However, recent clinical trials have suggested a potential adverse effect of rHuEPO treatment on the outcome of patients. rHuEPO as a supportive therapy in human malignancies should

be prudent [24]. The expression of EPOR in human tumors and tumor cell lines has raised questions regarding the safety of rHuEPO in the treatment of cancer-related anemia in patients with EPOR-positive tumors [25, 26]. Autocrine/paracrine EPO has been shown to affect the tumor microenvironment and involves in cell proliferation, growth, viability, and angiogenesis [15, 27, 28]. On the other hand, Paragh et al. [19] demonstrated that EPOR expressed in A2780 human ovarian carcinoma cells may influence tumor growth and cellular functions even in the absence of EPO. For these reasons, we focused on the role of EPOR in the trastuzumab resistance in breast cancer cells.

We found that EPOR expressed in SKBR3, MDA-MB-453, and UACC-812 cells, but not in BT474 cells, consistent with previous findings [18]. A previous study indicated that EPOR expressed in cancer cells is not functional [29]. However, we demonstrated that EPO activated downstream signaling pathways via EPOR. In addition, high EPOR expression in HER2-positive breast cancer was associated with increased pathological stage and higher Ki67 index, indicating that EPOR expression is related to tumor progression and proliferation. This is in contrast to the findings of Larsson et al., who found that EPOR expression was inversely associated with tumor size [30].

In our study, EPOR expressed in 42 of 55 tumor samples and 10 of the paired adjacent normal tissues by immunohistochemistry. In a previous clinical study, EPOR was identified in clinical breast biopsy specimens containing cancerous tissue, but was not seen in adjacent normal breast tissue, and was also not detected in fibrocystic disease, benign papilloma, or hyperplasia [31]. Thus, EPOR expressed in non-hematopoietic tissues may be associated with oncogenic biology. We also observed that downregulation of EPOR expression combined with trastuzumab treatment have additive effects on the inhibition of proliferation and invasion in SKBR3 and MDA-MB-453 cell lines. HER2 and EPOR may share common signaling pathways for tumor progression and metastasis, and blocking both pathways will produce synergetic inhibition effects.

These data in our study provided the theoretical evidence for developing novel agents targeting EPOR to block this receptor and downstream signaling pathways. The antibody against EPOR might overcome the primary or acquired resistance to trastuzumab, and combined regimens targeting both EPOR and HER2 may be a promising approach to improve the prognosis of EPOR and HER2 dual-positive breast cancer. For EPOR ubiquitously expressed in erythroid cells, the primary side effect of EPOR-targeted therapy might be severe anemia, which can be corrected by blood transfusion. What is more, when using antibodies against EPOR, the structurally similar component could also be blocked. Therefore, EPOR-targeted therapy is promising, but need amount of investigations for safety and tolerability before clinical application.

In conclusion, we have demonstrated an oncogenic role of EPOR in trastuzumab resistance. EPOR downregulation increases the sensitivity of trastuzumab. It is worthwhile to further study whether phosphorylated EPOR or EPO can be used as a marker for trastuzumab resistance in breast cancer. Because of the antagonistic effect of EPO on trastuzumab-induced antitumor activity in HER2/EPOR dual-positive breast cancer cells, EPO may not be safe to treat trastuzumab-induced anemia.

Conflict of interest The authors declare that there are no conflicts of interest.

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