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EphA8 inhibits cell apoptosis via AKT signaling and is associated with poor prognosis in breast cancer

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Abstract. Erythropoietin-producing hepatocellular receptors (Ephs) comprise the largest subfamily of receptor tyrosine kinases and have been reported to be involved in a variety of biological cellular processes, including tumorigenesis and cancer progression. The present study aimed to determine the expression levels and clinicopathological significance of EphA8 in breast cancer (BC) using immunohistochemistry analysis of tissue microarrays. The results of the present study revealed that EphA8 expression levels were upregulated in BC tissue and were associated with tumor size and TNM stage. In addition, upregulated expression levels of EphA8 were identified to be a poor prognostic biomarker for patients with BC. The knockdown of EphA8 expression using short hairpin RNA resulted in increased levels of apoptosis as well as decreased proliferation, migration and invasion of BC cells both *in vivo* and *in vitro*. The knockdown of EphA8 also decreased the phosphorylation of AKT, which was accompanied by downregulation of Bcl-2 expression levels and upregulation of p53, Caspase-3 and Bax expression levels. Moreover, knockdown of EphA8 expression increased the chemosensitivity of BC cells to paclitaxel. In conclusion, the results of the present study indicated that EphA8 may be a useful prognostic marker in BC and that knockdown of EphA8 may represent a novel strategy in adjuvant chemotherapy for the treatment of BC.

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Abbreviations: Eph, Erythropoietin-producing hepatocellular receptor; BC, breast cancer; RTKs, receptor tyrosine kinases; shRNA, short hairpin RNA; CCK-8, Cell Counting Kit-8; OS, overall survival

Key words: EphA8, prognosis, biomarker, AKT

Introduction

Breast cancer (BC) is the most common type of malignant cancer worldwide, with an estimated 2.3 million new cases; mortality for female breast cancer is 12.4 per 100,000 in developing countries and 5.2 per 100,000 in developed countries, respectively (1). Despite developments in the treatment of BC, resistance to conventional cytotoxic drugs is increasing and recurrent BC remains the leading cause of cancer-associated mortality in women worldwide. BC is considered to comprise a heterogeneous group of diseases with distinct clinical, pathological and molecular features (2). Due to the heterogeneity of the tumor and advancements in molecular-targeted drugs in the clinic, the identification of novel prognostic and predictive factors is key for the development of personalized medicine for patients with BC (3). The occurrence, development and metastasis of cancer are associated with cell proliferation and decreased levels of apoptosis (4). Therefore, an improved understanding of the mechanisms underlying the proliferation and apoptosis of BC cells is essential for the identification of diagnostic markers and development of novel effective therapies for patients with BC.

Erythropoietin-producing hepatocellular receptors (Ephs) comprise the largest family of receptor tyrosine kinases (RTKs), which interact with ligands known as ephrins (5). The binding of Ephs and ephrins produces bidirectional signals that affect both Eph- and ligand-expressing cells. Various downstream signaling pathways are linked to Eph/ephrin binding, including the Ras/MAPK, PI3K/AKT/mTOR, FAK/SRC, ABL and RHO/RAC/CDC42 signaling pathways (5,6). Eph/ephrin signaling has been reported to be key for cell positioning and migration during the development of the central nervous system and in the maintenance of long-term potentiation, angiogenesis and stem cell differentiation (7). In humans, 14 members of the Eph family have been characterized to date, which are subdivided into two classes, A (EphA1-8 and EphA10) and B (EphB1-4 and EphB6), on the basis of sequence homology, structure and binding affinity (8). A number of Ephs are required to synergistically maintain sophisticated tissue

organization of the central nervous system (5). Upregulated expression levels of several Eph members, such as EphA2, EphA10 and EphB4, have been detected in various types of tumor, where they are involved in the regulation of cancer proliferation, migration, invasion and angiogenesis (9-11).

EphA8 functions as a receptor for glycosylphosphatidyl-inositol-anchored ephrin-A1-5 (12,13). Upon ligand binding and activation of EphA8 in NIH3T3 and HEK293 cells, p110γ PI3K is recruited in a TK activity-independent manner, which promotes cell adhesion and migration (14). During early brain development, EphA8 serves a role in modulating apoptosis in a caspase-dependent manner in ephrin-A-expressing neuroepithelial cells (15). The expression of EphA8 in neuronal cells induces a sustained increase in MAPK activity, thereby promoting neurite outgrowth (16). In addition, previous studies have indicated that EphA8 expression is dysregulated in several types of cancer (10,17). Our previous study (17) also demonstrated that EphA8 serves as an oncogene and contributes to poor prognosis in gastric cancer by regulating the expression of a disintegrin and metalloproteinase domain 10.

The present study aimed to investigate the expression levels and clinicopathological significance of EphA8 protein in patients with BC. Moreover, the present study determined the functional role of EphA8 in BC cells and the potential of EphA8 expression levels to predict response to adjuvant chemotherapy for BC treatment.

Materials and methods

Patient clinical information and tissue samples. A total of 151 BC, 61 unpaired paracancerous and 30 benign formalin-fixed and paraffin-embedded breast tissue samples were obtained. The median age of the patients was 54.3 years (female, range 27-86 years). Clinical characteristics were obtained from patient medical records. None of the patients had received radiotherapy, neo-adjuvant chemotherapy or immunotherapy before surgery. All samples were obtained from Clinical Biobank in Affiliated Hospital of Nantong University (Jiangsu, China). All patients provided written informed consent. The study was approved by Ethics Committee of Affiliated Hospital of Nantong University.

Tissue microarray (TMA) and immunohistochemistry (IHC) staining. IHC was performed to investigate EphA8 protein expression levels in a TMA consisting of 242 4-μm-thick tissue sections. The sections were dewaxed using xylene, followed by rehydration in a descending alcohol series (100, 95, 80 and 70% ethanol). Antigen retrieval was performed by microwave treatment with citrate buffer (pH, 6.0) for 15 min at 750 W. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide solution for 5 min at room temperature. The slides were incubated with EphA8 antibody (1:150; cat. no. 13724-1; ProteinTech Group, Inc.) overnight at 4˚C, followed by secondary antibody (1:500; cat. no. ab7089; Abcam) at 37˚C for 30 min. The labeled antigens were visualized using a DAB Substrate kit (Abcam). EphA8 staining cells were observed under light microscope with a digital camera (Nikon; magnification, x200). EphA8 staining intensity was scored as follows: 0 (-, no staining), 1 (+, mild staining), 2 (++, medium staining) or 3 (+++, intense staining). The percentage of positively stained cells was multiplied by the intensity score to give the final IHC score, which ranged from 0 to 300.

Cell lines and culture. The human BC cell lines HS-578T, LCC, MDA-MB-453 and MCF-7 and the mammary epithelial cell line MCF-10A were purchased from the Chinese Tissue Culture Collection Bioscience (Shanghai, China). MCF-7 cell line was maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) and other cell lines in DMEM (Corning, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

Transfection and reagents. The short hairpin RNA (shRNA) was cloned into pGreenPuro plasmids (Shanghai GenePharma Co., Ltd.) to establish the pGreenPuro/EphA8 shRNA vector, which was then transfected into BC cells with Lipofectamine® 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The shRNA target sequences and negative control (NC) shRNA are listed in Table SI. EphA8 was amplified using the primers 5'-CTGGTGGTGCTTCTG CTCCT-3' and 5'-TGCAGAGGCAGGAAGACAGG-3'. Cells were transfected with50 nM pGreenPuro/EphA8 vector for 6 h, followed by treatment with paclitaxel (0.25 μg/ml; Beijing Solarbio Science & Technology Co., Ltd.) for 48 h separately or in combination.pcDNA3.1 expressing EphA8 was synthesized and obtained from GenePharma. Puromycin was obtained from Beijing Solarbio Science & Technology Co., Ltd. Cells were cultured at 37˚C with 5% CO_2 for 48 h prior to subsequent experiments.

Cell Counting Kit (CCK)-8 assay. Cell proliferation assays *in vitro* were performed using CCK-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Cells were incubated in 96-well plates at a density of $3x10^3$ cells per well for 24 h. Then, after specified time points (24, 48 and 72 h), 10 μl CCK-8 reagent was added and incubated for 1 h at 37˚C. The optical density at 450 nm was measured and the results were expressed the mean ± SEM.

Western blotting. Proteins were extracted from BC cells using RIPA lysis buffer (Beijing Solarbio Life Sciences) and the protein concentration was determined with a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (30 μg/lane) were separated in 4-12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, followed by blocking in 5% non-fat milk (in Tris-buffered saline with 0.1% Tween-20) for 2 h at room temperature. Samples were incubated at 4˚C overnight with the following primary antibodies: EphA8 (1:1,000; cat. no. 13724-1; ProteinTech Group, Inc.), AKT (1:2,000; cat. no. ab179463; Abcam), phosphorylated (p)-AKT (1:500; cat. no. ab38449; Abcam), p53 (1:1,000; cat. no. ab33889; Abcam), Bax (1:1,000; cat. no. ab32503; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam)Caspase-3 (1:1,000; cat. no. ab32351; Abcam), cleaved Caspase-3 (1:200; cat. no. ab2302; Abcam) and GAPDH (1:10,000; cat. no. ab181602; Abcam). Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies from ProteinTech Group, Inc. at room temperature for 2 h. Blots were

detected by enhanced chemiluminescence (Beyotime Institute of Biotechnology). Images were captured using a BioSpectrum Gel Imaging System (Analytik Jena AG) and analyzed using ImageJ 1.51j8 software (National Institutes of Health).

Flow cytometric analysis of apoptosis. Cells were harvested and fixed in 75% ethanol overnight at 4˚C. Cells were centrifuged at 800 x g for 5 min at 37˚C, and resuspended in binding buffer containing Annexin-V APC and PI (both Nanjing KeyGen Biotech Co., Ltd.), which were excited at 633 and 488 nm and emitted fluorescence at 660 and 610 nm, respectively. Following double staining with Annexin V and PI, cells were analyzed by flow cytometry (FACScan; BD Biosicences). Apoptosis analysis was performed using flow cytometric software (CellQuest Pro5.1; BD Biosciences).

Wound healing assay. For the wound healing assay, cells were cultivated in 6-well culture plates ($6x10^5$ cells/well) and grown to 80-90% confluence overnight. The confluent monolayer of cells was scratched with a sterile 200-μl micropipette tip and washed with PBS buffer to clear cell debris. The scratched cells were incubated in serum-free medium for 24 h at 37˚C in a humidified incubator with 5% CO_2. Closed area of the wound was determined under an inverted light microscopy (magnification, x40) at 24 h.

Cell invasion assay. Transwell invasion assays were performed in 24-well (pore size, 8 μm) Transwell plates according to the manufacturer's instructions (Corning, Inc.). The bottom of the Transwell chamber was coated with BD Matrigel Basement Membrane Matrix (BD Biosciences) for 30 min at 37˚C. The upper chamber was filled with $1x10^5$ cells in RPMI-1640. The lower chamber was filled with RPMI-1640 containing 15% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.). Following incubation for 36 h at 37˚C and staining with 0.1% crystal violet for 10 min at room temperature, the number of cells invading through the Matrigel was counted in four randomly selected light microscopic fields of view (magnification, x200).

Xenograft experiments. All animal experiments were performed with 5-week-old BALB/c-Nu female mice (weight, 18-23 g) purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. A total of 24 mice were kept in housing conditions of 40-70% humidity in a 12-h dark/light cycle with free access to food and water at 21-25˚C. Mice were divided into two groups (n=12) and injected subcutaneously with control or shEphA8 stably transfected MCF-7 cells. Tumor growth was monitored externally using a vernier caliper every three days. Tumor volume was calculated using the following formula: V (mm^3)=$\pi ab^2/6$. When BC xenografts reached 100 mm^3 in volume, mice were treated with paclitaxel (30 mg/kg) or PBS (100 μl) via intraperitoneal injection. At 15 days after injection, mice were sacrificed by cervical dislocation and the tumor weight was measured. The present study was approved by the Ethics Committee of Affiliated Hospital of Nantong University (approval no. 2016-070).

Statistical analysis. Data are expressed as the mean ± SD (n≥3). For statistical analysis of the association between EphA8 expression levels and overall survival (OS), the cutoff for low/high expression was determined using X-tile software program (tissuearray.org/rimmlab). Survival curves were generated using the Kaplan-Meier method and analyzed by log-rank test. Multivariate analysis by Cox regression analysis (proportional hazards model) was also performed for the prognostic factors. The significance of differences between groups was tested using χ^2 test for the clinical data of patients. Paired two-sided t-test was used for the comparison between two paired groups for the cell experiments. One-way ANOVA and Dunnett's multiple comparisons test were used for comparisons between more than two groups. The statistical analysis was performed using SPSS version 20.0 (IBM Corp.). The graphs were constructed using GraphPad Prism 7 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

EphA8 expression levels are upregulated in BC compared with paracancerous and benign breast tissue. In order to investigate the significance of EphA8 in the progression of BC, EphA8 protein expression levels were analyzed in 151 tumor, 61 paracancerous and 30 benign breast tissue samples using IHC. Positive EphA8 staining was observed in the cytoplasm and membrane of BC cells (Fig. 1A). The terminal duct lobular unit of the benign breast tissue was negative for EphA8 staining (Fig. 1B). Tissues with EphA8 staining score ≥140 or <140 were defined as high and low expression, respectively. High EphA8 expression was detected in 31.1% of BC but only 13.1% of paracancerous and 16.7% of benign breast tissue samples. The proportion of BC tissue with high EphA8 expression was significantly increased compared with paracancerous and benign breast tissue (χ^2=8.772; P=0.0125; Table I).

The clinicopathological and histopathological characteristics of patients with BC, which were classified according to EphA8 protein expression levels, are summarized in Table SII. High EphA8 expression levels were significantly associated with tumor size and TNM stage (both P<0.001). By contrast, no association was observed between EphA8 expression levels and other clinicopathological parameters, such as age at diagnosis, grade and nodal, estrogen and progesterone receptor (ER and PR, respectively) and Ki-67 status. These findings indicated that upregulated EphA8 expression levels may be associated with tumor growth and poor TNM stage.

Upregulated EphA8 expression levels are associated with poor survival in patients with BC. The association between EphA8 expression levels and the prognosis of patients with BC was subsequently determined. Kaplan-Meier survival curves demonstrated that patients with high EphA8 expression had a significantly shorter OS time compared with patients in the low EphA8 expression group (P<0.001; Fig. 1C). The survival curve with regard to EphA8 expression showed good differentiation between high and low expression with little overlap, indicating the reliability of EphA8 as a prognostic factor. These findings were also validated using univariate Cox regression model (OS: HR, 2.733; 95% CI, 1.421-5.255; P=0.003). In multivariate Cox regression analysis, subsequent to adjustment for grade, tumor size and PR status, high EphA8 expression levels (P=0.022), age at diagnosis (P<0.001), nodal (P=0.030) and ER status

Table I. Erythropoietin-producing hepatocellular receptor A8 expression levels in BC, paracancerous and benign tissue.

Tissue	n	Low expression (%)	High expression (%)	χ^2	P-value
Benign	30	25 (83.3%)	5 (16.7%)		
Paracancerous	61	53 (86.9)	8 (13.1%)		
BC	151	104 (68.9%)	47 (31.1%)		
Total	242			8.772	0.0125[a]

[a]P<0.05. BC, breast cancer.

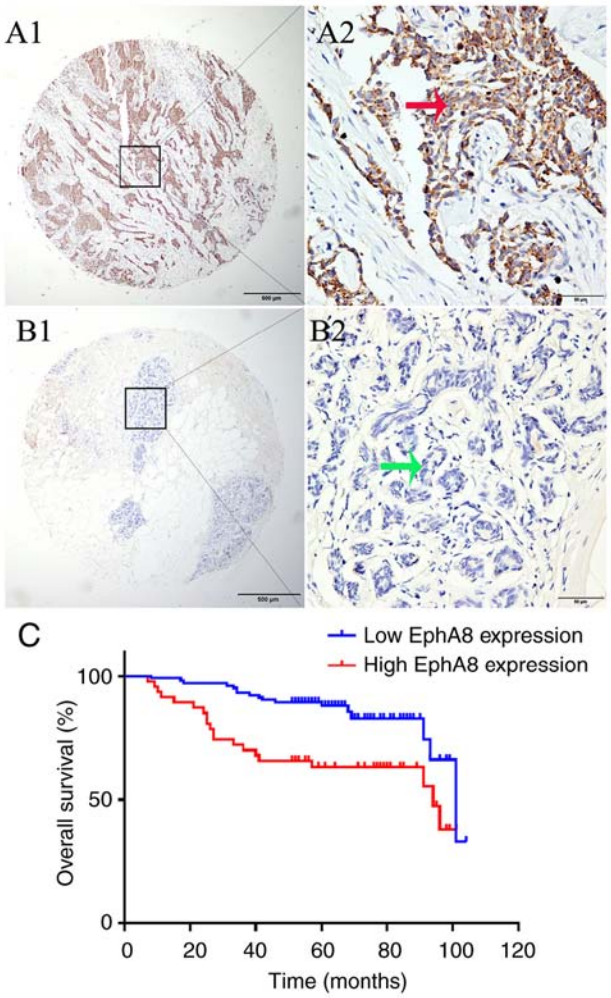

Figure 1. EphA8 expression levels in BC, association with prognosis and immunohistochemistry analysis of tumor microarrays. (A) Positive EphA8 staining in BC tissue. (B) Negative EphA8 staining in benign breast tissue samples. Magnification, x40 (A1 and B1) and x400 (A2 and B2). Positive EphA8 protein expression was indicated by red arrow; negative EphA8 was indicated by green arrow. (C) Kaplan-Meier survival curves of patients with BC with high and low EphA8 expression levels. EphA8, erythropoietin-producing hepatocellular receptor A8; BC, breast cancer.

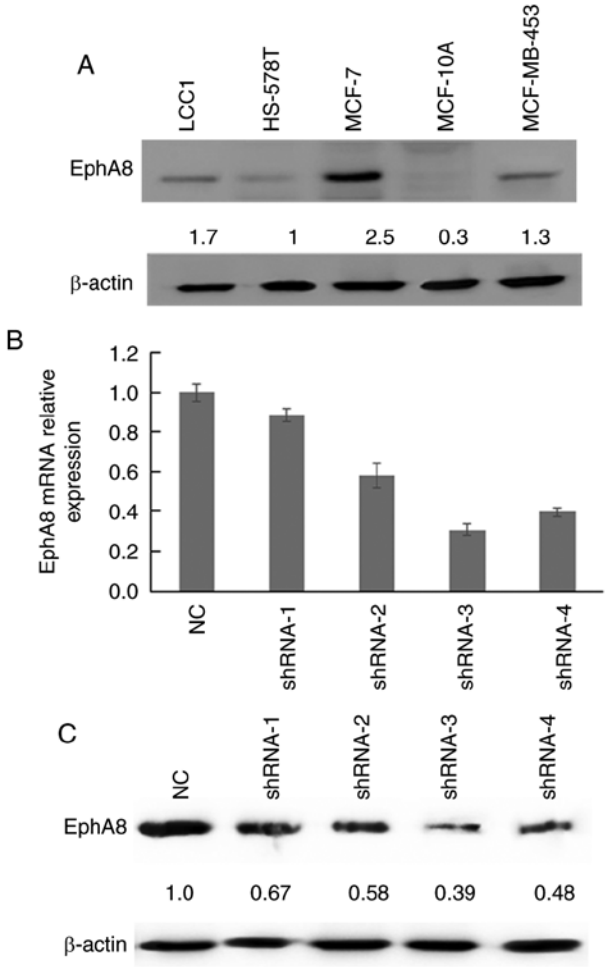

Figure 2. Expression levels of EphA8 in BC cell lines. (A) Protein expression levels of EphA8 in MCF-10A cells and four BC cell lines. (B) mRNA and (C) protein expression levels of EphA8 in MCF-7 cells transfected with shEphA8 (shRNA 1-4) or NC. EphA8, erythropoietin-producing hepatocellular receptor A8; BC, breast cancer; sh, short hairpin; NC, negative control.

(P=0.014) were determined as independent predictive factors of a poor outcome in patients with BC (Table II). Together, these results indicate that EphA8 expression is a negative prognostic factor for survival in patients with BC.

EphA8 expression levels in BC cell lines. EphA8 protein expression levels were subsequently analyzed in four BC cell lines. EphA8 expression levels were significantly upregulated in BC cells compared with the normal MCF-10A cell line. Protein expression levels of EphA8 were highest in MCF-7 cells and lowest in HS-578T cells (Fig. 2A). Therefore, MCF-7 cells were selected for transfection with four types of shEphA8 (shRNA 1-4) and NC. The mRNA expression levels of EphA8 were downregulated in shEphA8-transfected BC cells compared with the NC group, which confirmed the successful transfection of shRNAs targeting EphA8 (Fig. 2B). Western blot analysis also demonstrated that EphA8 protein

Table II. Univariate and multivariate analysis of prognostic factors for overall survival in breast cancer.

Characteristic	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
EphA8 expression, high vs. low	2.733	1.421-5.255	0.003[a]	2.385	1.131-5.031	0.022[a]
Age at diagnosis, years, ≤ median vs. > median	1.965	1.008-3.832	0.047[a]	3.842	1.755-8.410	0.001[a]
NHG, I vs. II vs. III	1.526	0.954-2.440				
Tumor size, mm, ≤20 vs. >20	1.990	0.957-4.138	0.065			
Nodal status, positive vs. negative	5.880	2.835-12.194	<0.001[a]	2.912	1.106-7.665	0.030[a]
TNM stage, 0/I/II vs. III/IV	5.121	2.647-9.908	<0.001[a]	2.339	0.903-6.056	0.080
ER status, negative vs. positive	0.376	0.193-0.734	0.004[a]	0.395	0.188-0.831	0.014[a]
PR status, negative vs. positive	0.596	0.293-1.210	0.152			
Ki-67 status, <14% vs. ≥14%	2.949	1.482-5.866	0.002[a]	1.370	0.652-2.877	0.406

ER/PR expression >10% was considered to be positive. χ^2 was used to test the significance between groups. [a]P<0.05. ER, estrogen receptor; PR, progesterone receptor; NHG, Nottingham histological grade.

expression levels were downregulated in shEphA8-transfected cells compared with the NC group (Fig. 2C). shRNA-3 demonstrated the highest efficiency in silencing EphA8 expression and was used in subsequent loss-of-function experiments.

EphA8 modulates proliferation, apoptosis, invasion and migration of BC cells in vitro. The pGreenPuro vectors containing shRNA-3 were transfected into the MCF-7 cell line, and puromycin was used to establish the stable EphA8-knockdown MCF-7 cell line. CCK-8 assay was performed to determine the effect of EphA8 on cell proliferation. The proliferation of MCF-7 cells was significantly decreased following knockdown of EphA8 with shEphA8 at each time point (Fig. 3A). In order to investigate whether EphA8 affected BC proliferation by regulating apoptosis or the cell cycle, flow cytometry assay was performed. The cells were treated and harvested 48 h later for analysis of apoptosis and cell cycle distribution. The percentage of apoptotic cells significantly increased (from 2.24 to 13.40%) in the EphA8 knockdown group (Fig. 3B). In order to determine the effect of EphA8 on the migration and invasion of BC cells, Matrigel invasion assay was performed. Following knockdown of EphA8, the number of invasive MCF-7 cells was significantly decreased compared with the control group (P<0.05, Fig. 3C). In order to validate the effect of shEphA8 on cell motility and migration, wound healing assay was performed. Similarly, the knockdown of EphA8 significantly decreased the migratory ability of MCF-7 cells compared with the control group (P<0.05, Fig. 3D). Conversely, overexpression of EphA8 was demonstrated to promote the proliferation, migration, and invasion of HS-578T cells (Fig. S1).

Knockdown of EphA8 inhibits tumorigenicity of BC cells in vivo. In order to investigate the role of EphA8 in BC *in vivo*, nude BALB/c mice were used to determine whether EphA8 influenced the behavior of BC cells. Mice were injected subcutaneously with NC- or shEphA8-transfected MCF-7 cells. Upon BC xenografts reaching 100 mm^3 in volume, mice were treated with intraperitoneal injection of paclitaxel (30 mg/kg) or PBS (100 μl) once a week. Consistent with the *in vitro* findings, the growth of BC cells in the shEphA8 group was notably slower compared with NC (P<0.05, Fig. 4A). Following 2 weeks of intraperitoneal injection, the tumors were harvested (Fig. 4B and C); tumor volume was decreased and tumor growth was notably inhibited following paclitaxel administration (P<0.05, Fig. 4A). Taken together, these findings suggested that EphA8 may serve an oncogenic role in the development of BC.

Knockdown of EphA8 enhances sensitivity of BC cells to paclitaxel chemotherapy. Chemotherapy is an important therapeutic regimen used for the management of patients with advanced BC. Paclitaxel, one of the most effective and widely used drugs, has shown good efficacy against BC; however, long-term use of paclitaxel can promote drug resistance (18). Paclitaxel-based combination therapy increases the survival period of patients with BC; therefore, current clinical treatments often use this strategy (18). The combined knockdown of EphA8 and treatment with paclitaxel significantly decreased tumor cell proliferation, migration and invasion compared with paclitaxel monotherapy *in vitro* (P<0.05; Fig. 3). Similarly, *in vivo*, intraperitoneal injection of paclitaxel in mice in the shEphA8 group significantly impaired tumor growth compared with NC or PBS injection groups (P<0.05; Fig. 4). These *in vitro* and *in vivo* findings suggested that knockdown of EphA8 may enhance the sensitivity of BC cells to paclitaxel.

Knockdown of EphA8 regulates expression levels of apoptosis-associated proteins both in vitro and in vivo. The results of functional experiments revealed that the transfection with shEphA8 significantly induced apoptosis in BC cells. Therefore, changes in the expression levels of apoptotic proteins following EphA8 knockdown were subsequently investigated. Western blotting revealed that the expression levels of cleaved Caspase-3 and Bax were upregulated, whereas Bcl-2 expression levels were downregulated in the shEphA8 group both *in vitro* (Fig. 5A) and *in vivo* (Fig. 5B). The changes in expression levels of apoptosis-associated

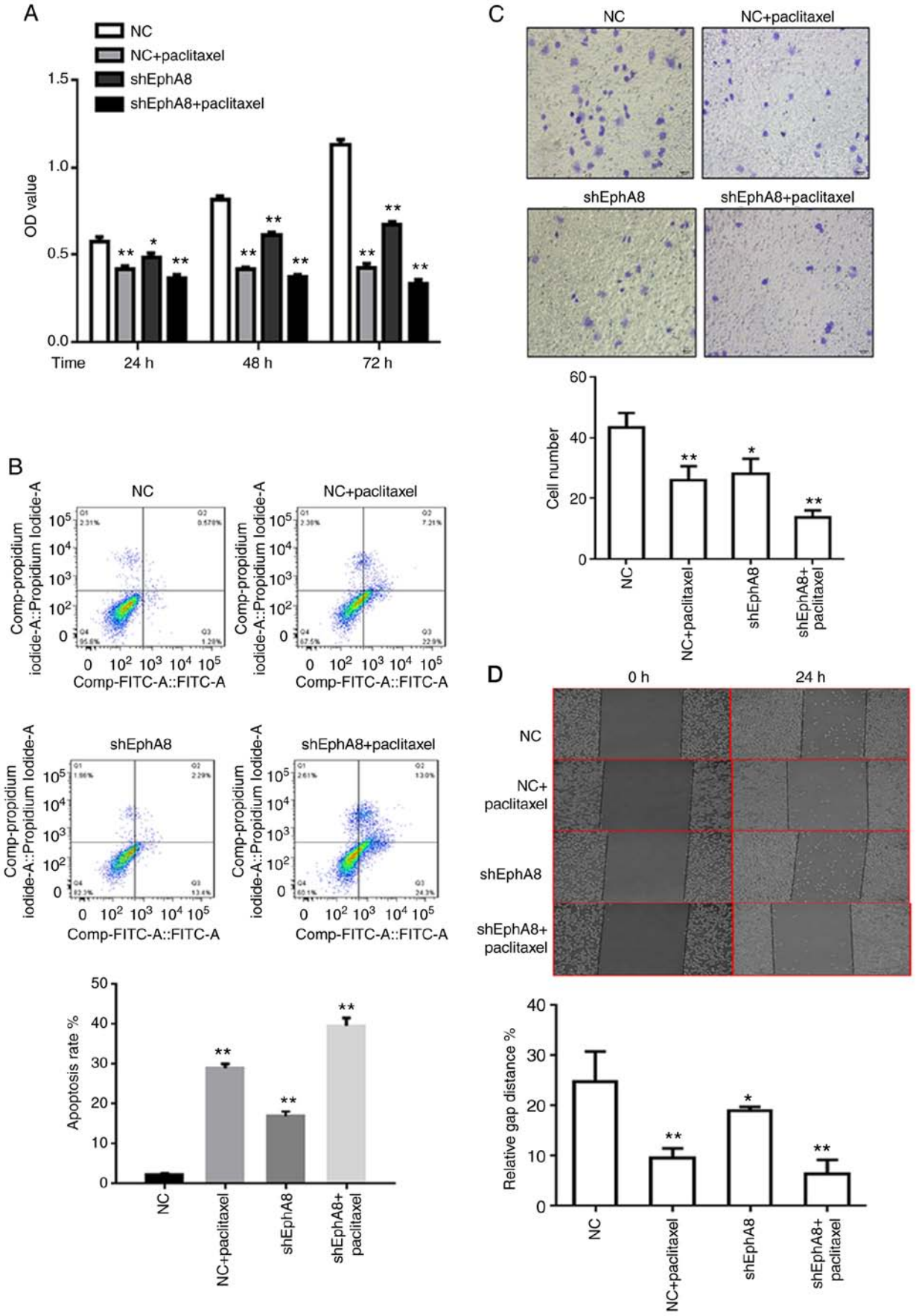

Figure 3. EphA8 knockdown inhibits BC cell proliferation and migration *in vitro* and enhances the sensitivity of BC cells to paclitaxel. Effect of EphA8 knockdown alone or in combination with paclitaxel treatment on BC cell (A) proliferation, analyzed via Cell Counting Kit-8 assay; (B) apoptosis, analyzed using Annexin V-FITC and PI staining followed by flow cytometry; (C) invasion, analyzed via Transwell invasion assay and (D) migration, analyzed via wound healing assay. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01. EphA8, erythropoietin-producing hepatocellular receptor A8; BC, breast cancer; sh, short hairpin; NC, negative control; OD, optical density.

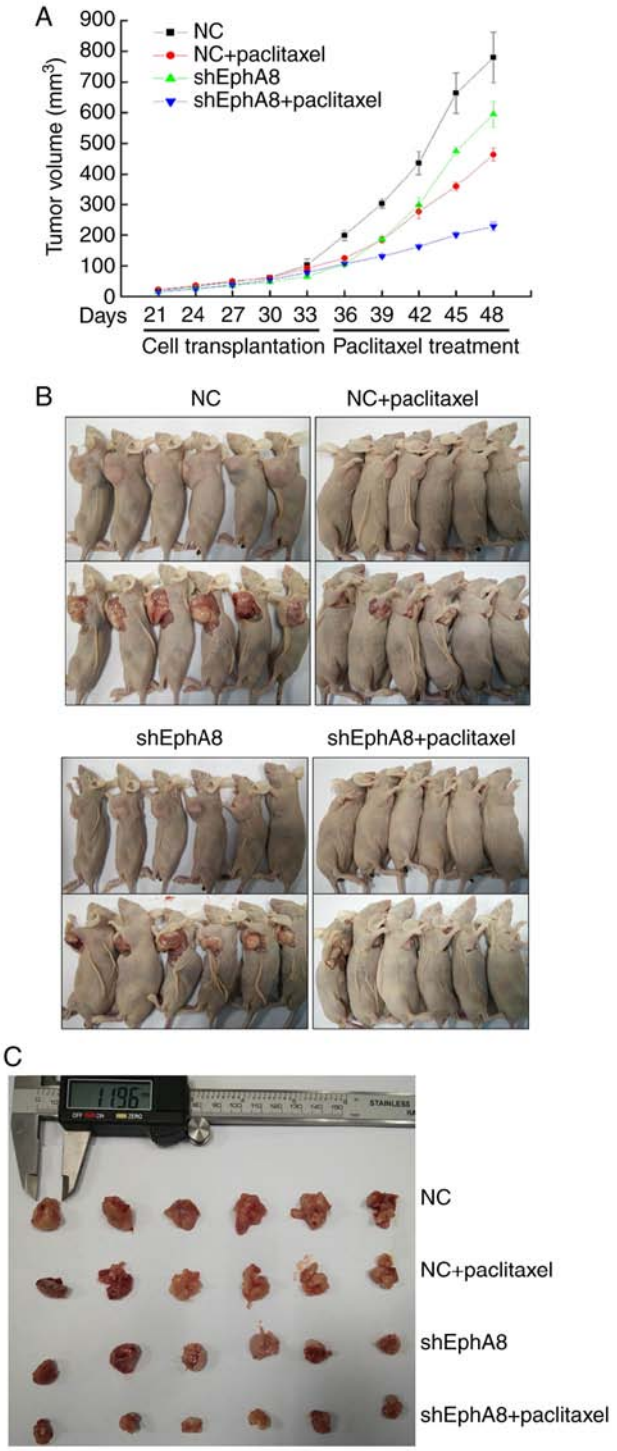

Figure 4. Knockdown of EphA8 inhibits tumorigenicity of BC cells *in vivo*. (A) Tumor growth curves for shEphA8- or NC shRNA-transfected MCF-7 cells treated in the presence or absence of paclitaxel. Images of (B) nude BALB/c mice and (C) tumors from mice subcutaneously injected with shEphA8- or NC shRNA-transfected MCF-7 cells treated in the presence or absence of paclitaxel. EphA8, erythropoietin-producing hepatocellular receptor A8; BC, breast cancer; sh, short hairpin RNA; NC, negative control.

proteins were more significant following combined treatment with paclitaxel and transfection with shEphA8 compared with either paclitaxel treatment or shEphA8 transfection-alone. As alterations in the PI3K/AKT signaling pathway are among the most common genomic abnormalities in BC, and the activation of the PI3K/AKT pathway suppresses the function of

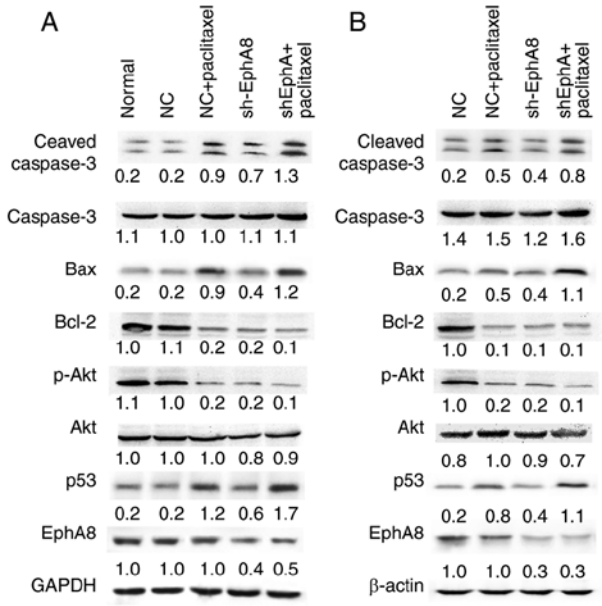

Figure 5. Knockdown of EphA8 in combination with paclitaxel treatment regulates expression levels of apoptosis-associated proteins in breast cancer cells *in vitro* and *in vivo*. (A) Western blot analysis of cleaved Caspase-3, Bax, Bcl-2, p-AKT, p53 and EphA8 in shEphA8 stably transfected MCF-7 cells compared with shNC cells *in vitro*. GAPDH was used as the loading control. (B) Western blot analysis of cleaved Caspase-3, Bax, Bcl-2, p-AKT, p53 and EphA8 in tumors of nude mice from each group. β-actin was used as the loading control. Densitometric values are listed below each blot. EphA8, erythropoietin-producing hepatocellular receptor A8; p-, phosphorylated; NC, negative control; sh, short hairpin.

p53 to inhibit mitochondrial pathway-induced apoptosis (19), the expression levels of p-AKT and p53 expression of in BC cells following the transfection with shEphA8 or paclitaxel treatment were investigated. The protein expression levels of p-AKT were downregulated, whereas those of p53 were upregulated in shEphA8-transfected or paclitaxel-treated cells (Fig. 5). These results indicated that knockdown of EphA8 enhanced activation of Caspase-3 and may be involved in regulating the expression levels of p53 and Bax; this may be a potential mechanism by which EphA8 regulates apoptosis in BC. In addition, transfection of shEphA8 in combination with paclitaxel treatment may exert a more significant effect compared with paclitaxel treatment-alone; this effect may be due to enhanced sensitivity of BC cells to chemotherapy.

Discussion

RTKs are key regulators of signaling transduction pathways and have been discovered to promote the malignant progression of numerous types of solid tumor (20). EphA8 is a member of the Eph RTK subfamily, which transduces signals via ligand-induced activation, and has previously been shown to serve role in tumorigenesis (21). Previous studies have demonstrated that EphA8 promotes cell adhesion and migration via the PI3K and MAPK kinase signaling pathways during normal embryonic development (16,22). However, to the best of our knowledge, the role of EphA8 in BC tumorigenesis and its potential molecular mechanisms have not yet been investigated.

The present study demonstrated that expression levels of EphA8 were upregulated in BC tissue and this was significantly associated with tumor size and TNM stage. Multivariate Cox regression analysis identified that EphA8 was an independent predictive factor of poor outcome in patients with BC. Subsequently, functional studies were performed to determine the effect of knockdown of EphA8 on tumor biology; the results revealed that knockdown of EphA8 inhibited proliferation and induced apoptosis in BC cells. These findings suggested the potential of EphA8 as a target for anticancer therapy in BC.

Ephs have previously been reported to be responsible for cytoskeleton activity, cell adhesion, motility, invasion, neo-angiogenesis, cell shape and epithelial-mesenchymal transition (8,23). With such wide-ranging effects, the altered function of Ephs has been implicated in tumorigenesis and cancer progression. For example, the overexpression of Ephs is associated with poor clinical outcome and cancer progression (24,25). EphA2 and EphB4 are the two most extensively studied members of the Eph RTK family in BC (20,26,27). The upregulation of EphA2 expression levels is significantly associated with poor prognosis and resistance to therapeutic agents in HER2-positive patients with BC. The ephrin-A1/EphA2 signaling axis regulates glutamine metabolism in HER2-overexpressing BC and EphA2 promotes BC tumorigenesis in the absence of ephrin-A1 (20). In addition, knockdown of EphA2 in trastuzumab-resistant BC cells restores BC cell sensitivity to trastuzumab treatment *in vivo* (26). Therefore, targeting EphA2 in BC may serve as a novel strategy to inhibit tumorigenesis and reverse trastuzumab resistance. Recent studies have suggested that EphB4 may be associated with tumor angiogenesis, growth and metastasis (27,28). Downregulation of EphB4 expression levels using small interfering RNAs or antisense oligonucleotides has been demonstrated to inhibit malignant cell behavior of BC (27). However, other studies have reported conflicting results on the role of EphB4 in tumor progression. For example, following stimulation of EphB4 with ephrin-B2, EphB4 acts as a tumor suppressor in a xenograft model of BC by activating a tumor suppressive pathway involving Abl family TKs and the Crk adaptor protein (28). These conflicting findings may be due to the bidirectional signaling of EphB4/ephrin and crosstalk with other signaling pathways. Although the role of Eph in BC has been reported in previous studies (24,27), the exact role and signaling pathway of EphA8 remains unclear.

Due to the association between EphA8 expression levels and poor survival in patients with BC in the present study, the function of EphA8 in BC was investigated both *in vivo* and *in vitro*. The findings of the present study revealed that the knockdown of EphA8 significantly induced apoptosis of BC cells. Activation of PI3K/AKT signaling regulates its downstream proteins, including Bad, Bcl-2, Caspase-3 and other effector proteins (29), and suppresses the function of p53 to inhibit apoptosis via the mitochondrial pathway (30,31). In addition, a previous study discovered that EphA8 promotes integrin-mediated cell adhesion and migration during normal embryonic development via the PI3K signaling pathway (12). Thus, the present study investigated the effects of EphA8 knockdown on the activity of the PI3K/AKT signaling pathway. The protein expression levels of p-AKT and Bcl-2 were downregulated in EphA8-knockdown BC cells, whereas the expression levels of caspase-3, p53 and Bax were upregulated. Based on this finding, it was hypothesized that EphA8 may inhibit apoptosis of BC cells, at least partly, by activating the PI3K/AKT signaling pathway. Other members of the Eph family activate the PI3K/AKT signaling pathway (32) and Eph expression patterns vary in different types of tumor cell. For example, in BC stem cells, EphA8 is the most upregulated transcript relative to normal breast epithelial cells (33).

One of the most significant challenges in the treatment of BC is that cancer cells become insensitive or resistant to radio- or chemotherapy-induced inhibition of cell growth and induction of apoptosis. Taxanes are the most widely used chemotherapeutic agents for the treatment of BC (18). Paclitaxel binds to β-tubulin to mechanistically stabilize tubulin polymerization, which results in G_2/M arrest and subsequent apoptosis via the mitochondrial pathway (18,34). Following long-term paclitaxel exposure in BC therapy, abnormal activation of PI3K/AKT, NF-κB and MAPK signaling induces paclitaxel resistance (35). Paclitaxel-based combination therapy increases the survival period of patients with BC; therefore, current clinical treatments often use this strategy (18). Thus, the present study analyzed the effects of the combination of paclitaxel treatment and transfection with shEphA8 on BC cells. Knockdown of EphA8 combined with paclitaxel treatment increased the chemosensitivity of BC cells to paclitaxel and significantly decreased tumor cell proliferation, migration and invasion compared with paclitaxel monotherapy. These results suggested that silencing of EphA8 may inhibit PI3K/AKT signaling, which may increase the chemosensitivity of BC cells to paclitaxel. These data provided an improved understanding of BC progression and the effect of combined chemotherapy for BC treatment. However, further studies are required to determine the association between EphA8 and the PI3K/AKT signaling pathway and to verify the potential application of these findings for BC chemotherapy.

In conclusion, the findings of the present study suggested that the expression levels of EphA8 may be upregulated in BC and are associated with tumor size and TNM stage. Upregulated expression levels of EphA8 were also shown to be a biomarker for a poor prognosis in patients with BC. Moreover, knockdown of EphA8 expression inhibited tumorigenesis and induced apoptosis of BC cells *in vitro* and *in vivo*. In addition, knockdown of EphA8 increased the sensitivity of BC cells to chemotherapeutic reagents by inhibiting PI3K/AKT signaling. These findings may have important implications for understanding BC progression and improving BC treatment.

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Availability of data and materials

All data generated or analyzed in this study are available from the corresponding author on reasonable request.

Authors' contributions

QN conceptualized the study. GHW and KN acquired and analyzed the clinical data. CG and JC performed the *in vitro* experiments. JH and XDW analyzed the data and prepared the figures. GHW and XDW wrote, reviewed and revised the manuscript. GHW and QCN confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Affiliated Hospital of Nantong University (approval no. 2016-070). All experiments complied with the Helsinki declaration and institutional guidelines. All samples were obtained from patients who had provided written informed consent for the use of their tissue for the purposes of research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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