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Original Articles

Nogo-B receptor increases the resistance of estrogen receptor positive breast cancer to paclitaxel

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ABSTRACT

Intrinsic or acquired chemoresistance is a hurdle in oncology. Only 7%–16% of estrogen receptor α (ERα) positive breast cancer cases achieve a pathological complete response (pCR) after neo-adjuvant chemotherapy. Nogo-B receptor (NgBR) is a cell surface receptor that binds farnesylated Ras and promotes Ras translocation to the plasma membrane. Here, we demonstrate NgBR as a potential therapeutic target for ERα positive breast cancer patients to attenuate paclitaxel resistance. NgBR knockdown enhanced paclitaxel-induced cell apoptosis by modulating expression of p53 and survivin in ERα positive breast cancer cells via NgBR-mediated PI3K/Akt and MAPK/ERK signaling pathways. NgBR knockdown attenuated either 17β-estradiol or epidermal growth factor stimulated phosphorylation of ERα at Serine 118 residue. The ChIP-PCR assay further demonstrated that NgBR knockdown decreased ERα binding to the estrogen response element (ERE) of the ERα target gene and increased the binding of p53 to the promoter region of survivin to attenuate survivin transcription. In summary, our data suggest that NgBR expression is essential to promoting ERα positive breast cancer cell resistance to paclitaxel. Findings from this study implicate a novel therapeutic target for treating ERα positive breast cancer in neo-adjuvant/adjuvant chemotherapy.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women [1,2]. Approximately 70%–75% of human breast cancers are estrogen receptor α (ERα) positive, and most breast cancer deaths occur in ERα positive patients [3–5]. Chemotherapy is an important treatment strategy in the management of patients with advanced breast cancer or who desire breast-conserving surgery [5,6]. However, pathological complete response (pCR) rates after neo-adjuvant

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chemotherapy is only achieved in a minority of patients with ERα positive breast cancer [7]. A randomized phase III trial showed that only 7%–16% of ERα positive breast cancer cases achieved their pCR [8]. Many studies indicate that current chemotherapy has much less efficacy in ERα positive breast cancer because estrogen induces chemoresistance by promoting cell proliferation, inhibiting apoptosis, and stimulating both metastasis and angiogenesis [9–13]. Thus, ERα mediated resistance to chemotherapy has become a great challenge to clinical treatment. Taxanes, such as paclitaxel and docetaxel, are among the most commonly used cytotoxic drugs for the treatment of breast cancer [14,15]. Although it is efficacious for many cancers, including ovarian cancer, small cell lung cancer, and many other malignancies [16], the de-novo or acquired resistance to this drug class in ERα positive breast cancer remains a challenge in oncology.

NgBR is a type I receptor with a single transmembrane domain and was identified as a specific receptor for Nogo-B. Our recent findings demonstrated that 1) NgBR binds farnesylated Ras and recruits Ras to the plasma membrane, which is a critical step required for receptor tyrosine kinases (RTKs)-mediated activation of Ras signaling in human breast cancer cells and tumorigenesis [17]; 2) increased NgBR expression is highly associated with survivin (an apoptosis inhibitor) expression in ERα positive breast cancer [18]; 3) NgBR is highly expressed in chemo-resistant human hepatocellular carcinoma and involved in p53-dependnet resistance to 5-fluorouracil [19]. Findings from this study add to the story by elucidating the molecular mechanism by which highly expressed NgBR in ERα positive breast tumor cells enhances the ERα-mediated signaling and resistance to paclitaxel.

2. Material and method

2.1. Antibodies, reagents and siRNA

A peptide (AHHRMRWRADGRSLEK, residues from 81 to 96 of NgBR) was used to immunize rabbits (Epitomics, Burlingame, CA). Antiserum was purified using the same peptide-conjugated SulfoLink Coupling Gel (Pierce, Rockford, IL). Purified NgBR rabbit polyclonal antibody was used for immunostaining. The peptide recognizing epitope 14 to 30 of human Nogo-B was used to immunize rabbits (IMG-5346A, Imgenex, San Diego, CA). Antibodies for NgBR (#ab168351) and Estrogen Receptor alpha (phospho S118) (#ab32396) were purchased from Abcam (Cambridge, MA). Antibodies for phos-Akt (S473 and T308, #9271), phos-p42/44 ERK (#9101), total Akt (#4691), total ERK (#4695) and survivin (#2808) were purchased from Cell Signaling Technology (Beverly, MA). We also used antibodies for p53 (#10442-1-AP) and Hsp90 (13171-1-AP) from Proteintech (Rosement, IL). Estradiol (#E2758), paclitaxel (#T7191), doxorubicin (#44583) and EGF (#E5036) were purchased from Sigma-Aldrich (St. Louis, MO). NgBR siRNA (forward: GGAAAUACAUAGACCUACA, reverse: UGUAGGUCUAUGUAUUUCC) oligonucleotides with 3′ dTdT overhangs were synthesized by QIAGEN (Valencia, CA). The specificity of NgBR siRNA has been validated in our previous publication [20,21]. Control siRNA in experiments refers to a non-silencing siRNA (NSF: UUCUCCGAACGUGUCACGU, NSR: ACGUGACACGUUCGGAGAA) designed and synthesized by QIAGEN. Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) was used for the transfection of siRNA according to the manufacturer's instructions.

2.2. Obtaining breast tissue samples

Treatment outcomes of breast cancer patients receiving chemotherapy were retrieved from the surgical pathology archives at the Froedtert Hospital & Medical College of Wisconsin and from retrospective chart review. Thirty-eight breast cancer cases were selected for this study. After receiving approval from the Institutional Review Board for the Medical College of Wisconsin, de-identified samples (pre- and post-treatment) were obtained.

2.3. Immunohistochemistry (IHC) staining and scoring

Breast cancer pathological diagnosis was confirmed with hematoxylin and eosin staining of paraffin-embedded tissue sections. Immunohistochemical analysis for Nogo-B and NgBR status was performed on 4-μm sections using respective antibodies from Imgenex (Nogo-B, clone IMG-5346A) and Epitomics (clone 671). The detection system used was 3,3′-diaminobenzidine (DAB) from DAKO (Santa Clara, CA). Slides were counterstained using hematoxylin. Either cytoplasmic or membranous staining for Nogo-B and NgBR was considered positive. Quantitative scoring of NgBR and Nogo-B immunostaining was performed following previously published methods [18,19]. The percentage of positive cancer cells was assigned a score from 0(0%), 1 (1–10%), 2 (11–25%), 3 (26–50%), 4 (51–75%) and 5 (>75%) and was defined as negative (-, IHC score 0 to 1), low (+, IHC score 2 to 3) and, high (++, IHC score 4 to 5). Associations between NgBR, Nogo-B status and clinical parameters were assessed by a chi-square test. All breast cancer cases were histopathologically re-evaluated on hematoxylin and eosin-stained slides by two pathologists (BW and IA).

2.4. Cell culture

T47D breast tumor cells from ATCC were grown in RPMI-1640 (Life Technologies) containing penicillin (100 U/ml), streptomycin (100 mg/ml), 10% (v/v) fetal calf serum (Sigma-Aldrich). MCF-7 breast tumor cells from ATCC were grown in DMEM (Life Technologies) containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% (v/v) fetal calf serum (Sigma-Aldrich). T47D and MCF-7 cells were changed to either 10% or 5% charcoal stripped FBS (Sigma-Aldrich) in phenol red free medium when performing 48 h of 17β-estradiol (E2) treatment. Both cells were changed to 2% charcoal stripped FBS (Sigma-Aldrich) in phenol red free medium when performing 24 h of epidermal growth factor (EGF) treatment. Both cells were changed to serum free, phenol red free medium when performing short time E2 or EGF treatment. All cell lines were cultured in a 37 °C -humidified atmosphere containing 95% air and 5% CO_2.

2.5. Clonogenic survival assay

Cells were seeded in triplicate into a 60 mm culture dish (1000 cells/well). Cells were transfected with non-silencing (NS) control siRNA or NgBR siRNA. At 24 h after transfection, cells were cultured in medium containing E2 or EGF in the absence or presence of paclitaxel at the indicated doses for 48 h. Then, the cells were maintained for 2 weeks. The cell colonies were washed three times with phosphate buffered saline buffer (PBS), fixed in cold methanol for 15 min, and stained with Crystal Violet (Sigma-Aldrich) for 15 min at room temperature.

2.6. Apoptosis assay by AO/EB staining

The cells cultured in 24-well plates were cultured in medium containing E2 or EGF in the absence or presence of the paclitaxel for 48 h. After indicated treatment times, the cells were stained with acridine orange (AO, 100 μg/mL) and ethidium bromide (EB, 100 μg/mL) purchased from Sigma Aldrich, and were observed under a fluorescence microscope (OLYMPUS). The normal cells and early apoptotic cells were stained by AO to display bright green