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Original Article

Essential role of Notch4/STAT3 signaling in epithelial–mesenchymal transition of tamoxifen-resistant human breast cancer

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ABSTRACT

We previously demonstrated that tamoxifen (TAM)-resistant human breast cancer (TAMR-MCF-7) cells showed increased expression of mesenchymal marker proteins compared to the parent MCF-7 cells. Notch is functionally important in the promotion of epithelial–mesenchymal transition (EMT) during both development and tumor progression. Notch1 and Notch4 have been reported as prognostic markers in human breast cancer. Here, we indicated that Notch4, but not Notch1, plays a critical role in the regulation of EMT signaling in TAMR-MCF-7 cells. Notch4 suppression by either Notch inhibitors or Notch4 siRNA attenuated EMT signaling. Tyrosine-phosphorylated STAT3 protein is known as a crucial signaling molecule in the regulation of tumorigenesis and metastasis. We found that TAMR-MCF-7 cells exhibited constitutive STAT3 phosphorylation, and Notch inhibition reduced the level of activated STAT3 in TAMR-MCF-7 cells. An intrasplenic injection model of liver metastases was performed using TAMR-MCF-7 cells. Mice injected with N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, 10 mg/kg) formed smaller splenic tumors and showed a reduced micrometastatic tumor burden in their livers compared with the control group treated with vehicle. To conclude, Notch4 could be a potential target to prevent metastasis in TAM-resistant breast cancer.

Introduction

Despite tamoxifen (TAM) showing clear benefits for the prevention and treatment of estrogen receptor α (ERα)-positive breast cancer, continuous exposure to TAM confers drug resistance [1]. As a consequence, TAM resistance is a considerable limiting factor in the management of advanced breast cancer. Previous studies demonstrated increased cell motility *in vitro* and morphological distinctions between TAM-resistant human breast cancer (TAMR-MCF-7) cells and their parent MCF-7 cells [1,2].

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and gain migratory and invasive properties to become mesenchymal stem cells [3]. As cells undergo EMT, they lose epithelial cell–cell coherence, reorganize their actin cytoskeleton, downregulate the expression of cell adhesion molecules such as E-cadherin and upregulate the expression of mesenchymal markers such as N-cadherin and vimentin [3]. In addition, the activities of matrix metalloproteinases (MMPs) such as MMP2 and MMP9 are elevated during EMT progression [4].

Upregulation of Notch receptors and their ligands has been reported in several types of cancer cells, including breast cancer [5]. The Notch family comprises four receptors, Notch1, 2, 3 and 4 [6]. Notch1 and Notch4 are required for cell proliferation and stimulate matrix invasion in both ERα-negative and -positive breast cancer cells [7]. Moreover, constitutively active forms of Notch1 and Notch4 stimulate the transformation of normal human mammary epithelial cells *in vitro* [8,9]. Consistent with this finding, activation

Abbreviations: AP-1, Activator protein-1; CSCs, cancer stem cells; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EMT, Epithelial-mesenchymal transition; ER, Estrogen receptor; ICD, Intracellular domain; MMP2, Matrix metalloproteinase 2; NF-κB, Nuclear factor-kappa B; PEG 400, Polyethylene glycol 400; STAT3, Signal transducer and activator of transcription 3; TAM, tamoxifen.

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of Notch1 [9] or Notch4 [8] can cause mammary carcinogenesis in mice.

A number of stimuli and transcription factors have emerged as potent EMT drivers during normal development and cancer. Several studies have indicated the Notch signaling pathway as a crucial regulator in the induction of EMT and metastasis [8,10]. Notch ligand binding to an adjacent Notch receptor activates Notch signaling and leads to the morphology and phenotype consistent with mesenchymal transformation [10]. Upon activation, the Notch receptor is cleaved and undergoes conformational changes in which the Notch intracellular domain (ICD) is released through a cascade of proteolytic cleavages by metalloproteases, tumor necrosis factor-α-converting enzyme and the γ-secretase complex [10,11]. Accordingly, γ-secretase inhibition may prevent Notch-induced EMT in cancer cells. Notch4 activation significantly increases the tumorigenic potential of mammary epithelial cells by changing their morphogenetic properties [12]. Yun et al. revealed that PKCα-overexpressing T47D cells showing TAM-resistant phenotype expressed high levels of Notch4, but not of Notch1 [13]. Notch4 is up-regulated in TAM-resistant MCF-7 breast cancer cells which possessed more invasive and migratory phenotype compared to wild type MCF-7 cells [14].

Endogenous activity of the Notch signaling pathway is critical for activation of signal transducer and activator of transcription 3 (STAT3) in neuroepithelial cells [15]. STAT3 is frequently activated in many human cancer types, including breast cancer [16]. Tumorigenic STAT3 activation is often associated with increased malignant cancer behaviors, including uncontrolled growth, EMT, migration, invasion, metastasis and therapeutic resistance [17,18].

Here, we observed that basal expression and activity of Notch4 were amplified in TAMR-MCF-7 cells, and we investigated the role of Notch4/STAT3 signaling in EMT of TAM-resistant human breast cancer, and the potential of Notch inhibitors in the suppression of the metastatic tumor burden *in vivo*.

Materials and methods

Antibodies and reagents

Anti-E-cadherin and anti-N-cadherin antibodies were purchased from BD Transduction (San Jose, CA). Anti-Snail antibody was supplied from Abcam (Cambridge Science Park, UK). Anti-Notch1, anti-Notch4 and phospho-STAT3, anti-STAT3, anti-c-Jun, anti-c-Fos, Jun B, Jun D antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-P65, anti-Notch1 (for immunohistochemistry), anti-Notch4 (for immunohistochemistry) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated, donkey anti-rabbit IgG, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). DAPT (GSI-IX) was obtained from Selleck Chemicals Biotechnology (Houston, Texas). siGENOME SMARTpools for Notch1 and Notch4 were obtained from Dhamarcon (Thermo Scientific, Waltham, MA). 5-Bromo-4-chloro-3-indoylphosphate/nitro-bluetetrazolium was from Life Technologies (Gaithersburg, MD). Other reagents were obtained from Sigma (St. Louis, MO).

Cell culture and establishment of tamoxifen-resistant MCF-7 cells

MCF-7 cells were cultured at 37 °C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. TAMR-MCF-7 cells were established using the methodology reported previously [19]. Briefly, MCF-7 cells were cultured in phenol-red-free DMEM containing 10% charcoal-stripped, steroid-depleted FBS (Hyclone, Logan, UT) and 4-hydroxytamoxifen. The cells were continuously exposed to 4-hydroxytamoxifen and its concentration was gradually increased from 0.1 μM to 3 μM over a 9-month period.

Preparation of nuclear extracts

Cells were washed with ice-cold PBS. The cells were then scraped, transferred to microtubes, and allowed to swell after adding 100 μl of a hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenyl methyl sulfonyl fluoride. The lysates were incubated for 10 min on ice and centrifuged at 7200 × g for 5 min at 4 °C. Pellets containing the crude nuclei were resuspended in 50 μl of an extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenyl methyl sulfonyl fluoride and incubated for 30 min on ice. The samples were centrifuged at 15,800 × g for 10 min to obtain supernatants containing the nuclear fractions. The nuclear fractions were stored at −70 °C until needed.

siRNA knockdown assay

siGENOME SMARTpools for Notch1 and Notch4 (Dharmacon, Waltham, MA) were used to knock-down Notch1 and Notch4 expression in MCF-7 and TAMR-MCF-7 cells. Briefly, cells were grown in six-well dishes, then Notch1 siRNA or Notch4 siRNA were transfected using DharmaFECT-1 transfection reagent (Dharmacon, Waltham, MA) according to the manufacturer's protocol. As a transfection control, cells were transfected with control siRNA (nontarget) from the Dharmacon (Thermo Scientific, Waltham, MA). Whole-cell lysates were prepared and subjected to Western blot analyses. siRNA oligo sequences (Notch4-ICD #1, GCCCAACCCUGCGAUAAUG; Notch4-ICD #2, CAACGUAACCACUGGGAUC) were synthesized by Bioneer (Korea). Nontargeting control was purchased from Bioneer (Korea). siRNAs were transfected into TAMR-MCF-7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Immunoblot analysis

Cells were lysed in a buffer containing 20 mM TrisHCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10,000 × g for 10 min to remove debris, and the proteins were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted with specific primary and corresponding peroxidase-conjugated secondary antibodies.

Immunoprecipitation

Cells were solubilized in lysis buffer. An equal amount of each protein lysate was incubated with anti-STAT3 antibody overnight at 4 °C, followed by incubation with 20 μl of protein G-Sepharose beads for 2 h. The immune complexes were analyzed by Western blot analyses with anti-Notch4 antibody.

Measurement of cell proliferation

Cells were cultured in 10% FBS-containing medium comprising with or without Notch inhibitor for the indicated times. Then viable adherent cells were stained with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (2 mg/ml) for 4 h. Media were then removed and the formazan crystal-stained cells were dissolved in 200 μl dimethylsulfoxide. Absorbance was measured at 540 nm using a microtiter plate reader (Berthold Tech., Bad Wildbad, Germany).

Reporter gene assay

A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine the promoter activity. Briefly, the cells were transiently transfected with 1 μg of MMP2-Luc, pNF-κB-Luc or pAP-1-Luc reporter plasmid and 20 ng of phRL-SV40 plasmid (Renilla luciferase expression for normalization) (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Both firefly and Renilla luciferase activities in the cell lysates were measured using a luminometer (Tristar LB 941, Berthold Tech., Bad Wildbad, Germany). The relative luciferase activity was calculated by normalizing the promoter-driven firefly luciferase activity to that of *h*Renilla luciferase.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA), and complementary DNA was synthesized by reverse transcription using an oligo (dT) primer. PCR was performed using the selective primers as described in the following table. The band intensities of the amplified DNAs were compared after visualization on a UV transilluminator.

Sequence of primers:

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
MMP2	GTATTTGATGGCATCGCTCA	CATTCCCTGCAAAGAACACA
Notch1	GCAAGAACGCCGGGACAT	CTCGTTACAGGGGTTGCTGA
Notch4	TCGGACTTGGTCCGTAGACT	TCTGCTCTGGTGGGCATACA
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Trans-well migration assay

An *in vitro* cell migration assay was performed using a 24-well Trans-well polystyrene membrane with 8 μm size pores (3422; Corning, Cambridge, MA). The lower side of the upper chamber was covered with type I collagen (Sigma–Aldrich,