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ER-α variant ER-α36 mediates antiestrogen resistance in ER-positive breast cancer stem/progenitor cells

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ABSTRACT

Accumulating evidence indicates that cancer stem cells (CSC) play important roles in breast cancer occurrence, recurrence and metastasis as well as resistance to therapy. However, the roles of breast cancer stem cells in antiestrogen resistance and the underlying molecular mechanisms have not been well established. Previously, we identified and cloned a novel variant of estrogen receptor α, ER-α36, with a molecular weight of 36 kDa. ER-α36 mediates rapid antiestrogen signaling and is highly expressed in ER-positive breast cancer stem/progenitor cells. In this study, we investigated the function and the underlying mechanism of ER-α36-mediated antiestrogen signaling in ER-positive breast cancer stem/progenitor cells. ER-positive breast cancer cells MCF7 and T47D as well as variants with different levels of ER-α36 expression were used. The effects of antiestrogens tamoxifen and ICI 182, 780 on breast CSC's ability of growth, self-renewal, differentiation and tumor seeding were examined using tumorsphere formation, flow cytometry, indirect immunofluorences and *in vivo* xenograft assays. The underlying mechanisms were also analyzed with Western blot analysis. We found that the cancer stem/progenitor cells enriched from ER-positive breast cancer cells were more resistant to antiestrogens than the bulk cells. Antiestrogens increased the percentages of the stem/progenitor cells from ER-positive breast cancer cell through stimulation of luminal epithelial lineage specific ER-positive breast cancer progenitor cells while failed to do so in the cells with knocked-down levels of ER-α36 expression. Our results thus indicated that ER-α36-mediated antiestrogen signaling such as the PI3K/AKT plays an important role in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells.

1. Introduction

Estrogen profoundly influences breast cancer development, which highlights the importance of antiestrogen therapy. However, despite the significant anti-neoplastic activities of antiestrogens, most breast tumors are eventually resistant to antiestrogen therapy. Many initially responsive breast tumors gradually acquire antiestrogen resistance by loss of antiestrogen responsiveness. The mechanisms by which breast tumors loss their antiestrogen responsiveness have not been well established. Several mechanisms have been postulated to be involved in the antiestrogen resistance such as increased growth factor signaling, altered expression of co-regulators, mutations of ER-α [1–3].

Accumulating experimental and clinical evidence indicate that breast cancer arises from mammary stem/progenitor cell populations [4–6]. The major features of cancer stem cells include the ability of self-renewal and generating tumors from very few cells, slow cell division, the ability to produce differentiated cells of different lineages, selective resistance to radio- and chemo-therapy [7,8], constitutive activation of anti-apoptotic pathways and induction of angiogenesis, the ability to migrate and spread in metastasis [9]. Although the possible involvement of breast cancer stem/progenitor cells in antiestrogen resistance has been proposed [10] and demonstrated [11], the exact function and the underlying mechanism of breast cancer stem/progenitor cells in antiestrogen resistance remain largely unknown.

Previously, we identified and cloned a variant of ER-α that has a molecular weight of 36 kDa and was named ER-α36 [12,13].

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ER-α36 is mainly located near the plasma membrane and in the cytoplasm, and mediates rapid estrogen signaling such as activation of the MAPK/ERK [13]. Recently, we reported that the well-known "pure" ER disruptor ICI 182, 780 (Fulvestrant or Faslodex) failed to down-regulate ER-α36 expression [14], and ER-α36 mediated agonist activity of antiestrogens tamoxifen and ICI 186, 780 such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways [15–17]. The breast cancer patients with tumors expressing high levels of ER-α36 less benefited from tamoxifen therapy compared to those with low levels of ER-α36 expression, and ER-α36 expression is significantly associated with HER2 expression [18], suggesting that increased ER-α36 expression is one of the underlying mechanisms of tamoxifen resistance.

Recently, we reported that ER-α36 plays an important role in tamoxifen resistance; tamoxifen resistant cells express high levels of ER-α36 and knockdown of ER-α36 expression in these cells restored tamoxifen sensitivity [17]. More recently, we found that ER-α36 is involved in positive regulation and maintenance of ER-positive breast cancer stem/progenitor cells [19].

In the current study, we investigated the function and the underlying mechanisms of ER-positive breast cancer stem/progenitor cells in antiestrogen resistance and found that ER-α36 plays an important role in antiestrogen resistance of ER-positive breast cancer stem/progenitor cells.

2. Materials and methods

2.1. Reagents and antibodies

Tamoxifen was purchased from Sigma Chemical (St. Louis, MO, USA) and the ICI 182, 780 was from Tocris Bioscience (Ellisville, MO, USA). The affinity-purified rabbit polyclonal anti-ER-α36 antibody was generated as a custom service from Pacific Immunology Corp. (Ramona, CA, USA). The antibody was raised against a synthetic peptide antigen corresponding to the unique C-terminal 20 amino acids of ER-α36. The specificity of the antibody was tested in ER-α36 expression vector transfected HEK293 cells that do not express endogenous ER-α36. Immunofluorescence assay was also used to demonstrate immunoreactive signals only in transfectants with the ER-α36-expressing vectors but not in transfectants harboring an empty expression vector (data not shown).

The β-actin antibody (1-19), anti-CK18 (DC-10) and anti-CD10 (H-321) antibodies, anti-PCNA antibody (FL-261), the goat anti-mouse IgG–HRP, the goat anti-rabbit IgG–HRP and the donkey anti-goat IgG–HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ER-α antibody (ERAb-16) was purchased from NeoMarkers (Fremont, CA, USA). The antibodies for AKT and p-AKT (Ser473) were purchased from Cell Signaling Technology (Danvers, MA, USA). The ALDH1 antibody was from BD Biosciences (San Jose, CA). PerCP-Cy™5.5 mouse anti-human CD44 (clone C26) and PE mouse anti-human CD24 (clone ML5) were purchased from BD Pharmingen (San Jose, CA, USA). Anti-rabbit Alexa Fluor 488 antibody and anti-mouse Alexa Fluor 555 antibody were from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture, establishment of stable cell lines, and growth assay

MCF7 and T47D cells were purchased from ATCC (Manassas, VA, USA). The cells and their derivatives were cultured in Improved Minimal Essential Medium (IMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino-acids, 1% HEPES buffer, 1% antibiotic-antimycotic from Invitrogen (Carlsbad, CA, USA) and 2 mg/ml bovine insulin (Sigma, St. Louis, MO, USA). All cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

The variants of MCF7 and T47D with different levels of ER-α36 expression were established as described before [17,19]. For antiestrogen treatment, cells grown on attachment dishes were treated with different concentrations of antiestrogens or vehicle (ethanol) as a control for five to seven days as indicated. Treated cells were counted using the ADAM automatic cell counter (Digital Bio, Korea). Three dishes were used for each treatment and experiments were repeated three times.

2.3. Tumorsphere formation and growth assays

To form tumorspheres, cells were seeded into Corning ultra-low attachment 6-well plate (Corning Incorporated, CA, USA) at 10,000 cells/ml and cultured seven days in the tumorsphere medium: phenol-red free DMEM/F12 medium (Invitrogen) supplemented with 1 X B-27 (Invitrogen), 20 ng/ml epidermal growth factor (Sigma–Aldrich) and 20 ng/ml basic fibroblast growth factor (ProSpec, NJ, USA), 0.5 μg/ml hydrocortisone (Sigma). The number of tumorspheres was counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, CA). In addition, tumorspheres were then collected, washed with PBS, and incubated with Trypsin-EDTA (0.25%, 0.5 mM) for two minutes at 37 °C to dissociated cells, and cells were counted using the ADAM automatic cell counter (Digital Bio, Korea).

For antiestrogen treatment, tumorspheres were treated with different concentrations of antiestrogens or vehicle (ethanol) as a control. Three dishes were used for each treatment and experiments were repeated three times.

2.4. Flow cytometry analysis

For $CD44^+/CD24^-$ cell analysis, single cell suspension washed with cold PBS/1% BSA were subsequently incubated with PerCP-Cy™5.5 mouse anti-human CD44 (1:20) and PE mouse anti-human CD24 (1:5) in PBS/1% BSA for 30 min at 4 °C. After incubation, the cells were washed twice with cold PBS/1% BSA and re-suspended in 400 μl cold PBS/1% BSA for flow cytometry analysis.

2.5. Western blot analysis

Cells were washed with cold PBS and lysed with the RIPA buffer containing 1% proteinase inhibitor cocktail solution and 1% phosphatase inhibitor cocktail solution (Sigma). The cell lysates were boiled for 5 min in sodium dodecyl sulfate (SDS) gel-loading buffer and separated on 10% SDS-PAGE gels. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with appropriate primary antibodies and visualized with the corresponding secondary antibodies and the ECL kit (Thermo Scientific, Rockford, IL, USA).

2.6. Indirect immunofluorescence assay

Cells were fixed in 4% paraformaldehyde for 10 min, then permeabilized in 0.1% Triton X-100 for 5 min, blocked in 1% BSA for 30 min, and then incubated with primary antibodies at 4 °C overnight. Secondary antibodies, anti-rabbit Alexa Fluor 488 or anti-mouse Alexa Fluor 555 were then added and incubated for 1 h at room temperature. Cells were washed with PBS and mounted with 10 mg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (Sigma–Aldrich) in aqueous mountant

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