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T47D breast cancer cells switch from ER/HER to HER/c-Src signaling upon acquiring resistance to the antiestrogen fulvestrant

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In this study, T47D cell lines resistant to the antiestrogen fulvestrant were established and analyzed to explore, whether a switch to HER signaling, as seen in fulvestrant resistant MCF-7 cell lines, is a general resistance mechanism. We find that parental T47D cells depend on ER and HER signaling for growth. Fulvestrant resistant T47D cells have lost ER expression and, although HER2 was over expressed, growth was only partially driven by HER receptors. Instead c-Src was important for resistant growth. Thus, the T47D and MCF-7 model system unravel different resistance mechanisms which may be important for fulvestrant resistant breast cancer patients.

1. Introduction

The selective estrogen receptor modulator (SERM) tamoxifen is recommended as first line endocrine therapy for premenopausal women with ER-positive breast cancer [1]. However, some patients are *de novo* resistant to tamoxifen and other patients will, despite an initially responsive disease, acquire resistance and disease progression will occur. Many patients with progression on tamoxifen, will, due to different mechanisms of actions, respond to treatment with the pure antiestrogen fulvestrant (ICI 182,780 or faslodex), which is a selective ER down regulator (SERD) [2]. Binding of fulvestrant to ER prevents ER dimerisation, which results in destabilisation and rapid degradation of the ER-fulvestrant complex and consequently loss of estrogen signaling [3]. Unlike tamoxifen, fulvestrant has no known agonistic effects on cancer cell growth [4] and due to the unique mechanisms of action, fulvestrant delays development of resistance compared to tamoxifen in a xenograft model [5]. The clinical efficacy of fulvestrant in patients with breast cancer progressing on tamoxifen is similar to the effect of the aromatase inhibitor anastrozole [6,7]. However, upon long-term estrogen deprivation by aromatase inhibitors, increased sensitivity to estradiol treatment has been seen in some cell models [8]. Moreover, it is known that cross talk between ER and activated growth factor receptors or their downstream kinases are able to activate ER in a non-genomic manner [9]. Thus, to prevent the action of ER in patients treated with aromatase inhibitors, combination with fulvestrant to degrade ER could be a treatment option. This approach was validated in two recently reported studies [10,11], of which only one showed superiority of the combined treatment [10]. Although combination of different endocrine therapies may be efficient for some breast cancer patients upon relapse, resistance to fulvestrant is, as for tamoxifen, inevitable for patients with advanced breast cancer. Thus, to identify novel treatment for endocrine resistant breast cancer as well as markers for treatment response, it is important to clarify the molecular mechanisms behind acquired resistance to endocrine therapies. *In vitro* cell model systems using human breast cancer cell lines with acquired resistance to different forms of antiestrogens are highly valuable tools to identify changes between antiestrogen sensitive and resistant breast cancer cells [12–22]. Based on the ER-positive human breast cancer cell line, MCF-7, we have established a wide selection of cell lines resistant to different endocrine therapies and explored the molecular mechanisms involved in both tamoxifen and fulvestrant resistance [12–14,21,23–28]. We observed that the expression and activation of proteins from the Human Epidermal growth factor Receptor (HER) family as well as the expression of the HER3/HER4 ligands heregulin2α and 2β were increased in the fulvestrant resistant breast cancer cell lines compared to the expression in the fulvestrant sensitive MCF-7 cell line [24]. Moreover, our antiestrogen resistant breast cancer cell

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lines had increased activation of downstream kinases involved in various signaling pathways including phosphorylation of Akt [23,25,27,28]. In accordance with the increased expression and activation of the HER receptors and their downstream signaling molecules, the cells were growth inhibited by targeting the HER receptors or signaling molecules downstream of the HER receptors [23,24,26]. Thus, based on our studies as well as others it is well recognized that growth of antiestrogen resistant breast cancer cells can switch from being driven by ER to be mediated by the HER receptors upon acquiring antiestrogen resistance [15–21,24,26,28].

The HER receptors are a family of four type-1 transmembrane receptor tyrosine kinases: EGFR/HER1, HER2, HER3 and HER4 [29,30]. In breast tumors, the expression and phosphorylation of HER1, HER2 and HER3 have been associated with a poor prognosis, in contrast to the expression of HER4 which has been linked to a better outcome [31–33]. Upon ligand activation of the HER receptors, downstream signaling pathways are activated, which leads to increased cell proliferation and reduced cell death [29]. Gene amplification or protein over expression of HER2 is seen in 15–20% of early-stage breast cancer [34,35] and is associated with a significantly shorter time to relapse and reduced overall survival [36–39]. Although trastuzumab (Herceptin; Genentech), a humanized monoclonal antibody targeting HER2, has clearly improved the clinical outcome for HER2-positive breast cancer patients in the adjuvant and advanced setting, still many patients exhibit *de novo* or acquired resistance [37,38]. For ER-positive breast cancer patients, amplification or over expression of HER2 reduce the sensitivity to endocrine therapy [39]. This was also demonstrated in a MCF-7 breast cancer cell line ectopically over expressing HER2 [40]. Moreover, combination of endocrine therapy and HER targeted treatment is superior to endocrine therapy alone [41]. Thus, preclinical and clinical data strongly link signaling via the HER receptors to reduced response to endocrine therapy and treatment targeting the HER receptors and their downstream signaling pathways are attractive new therapies.

c-Src belongs to the family of non-receptor tyrosine kinases consisting of 9 members [42]. c-Src is over expressed in human breast cancers [43], and is associated with reduced overall survival of breast cancer patients [44]. *In vitro* studies have shown that dual targeting of c-Src and ER completely prevents development of resistance to tamoxifen [45]. The majority of breast cancers with over expressed or activated c-Src also over express one of the HER receptors [46,47] and in HER2-positive breast cancer, activated c-Src correlates with HER2 positivity and poor prognosis [48]. c-Src can bind to HER1, HER2 and HER3 [47,49–51] and as c-Src activity seems to be required for HER2:HER3 complex formation and subsequent downstream signaling [51], targeting c-Src might be an alternative way of preventing HER-driven cell growth.

So far most model studies investigating antiestrogen resistant breast cancer have been performed using the ER-positive breast cancer cell line, MCF-7. However, in order to determine if the molecular mechanisms driving growth of fulvestrant resistance MCF-7 cells are general for ER-positive breast cancer cell lines and to extend our knowledge of acquired resistance to antiestrogens, we have long-term treated another ER-positive human breast cancer cell line, T47D, with fulvestrant and established two fulvestrant resistant breast cancer cell lines. The work presented here was carried out to characterize the established T47D fulvestrant resistant breast cancer cell lines with respect to cell growth and signaling.

2. Materials and methods

2.1. Cell lines, culture condition and reagents

The T47D cell line was originally obtained from the Human Cell Culture Bank (Mason Research Institute, Rockville, MD, USA). The cells were maintained in growth medium without phenol red (RPMI; Gibco, Invitrogen, CA, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Perbio, Thermo Fisher Scientific, Roskilde, Denmark), 2 mM glutamax (Gibco) and 8 μg/ml insulin (Sigma–Aldrich, Copenhagen, Denmark). The fulvestrant resistant cell lines, T47D/182^R-1 (182^R-1) and T47D/182^R-2 (182^R-2) were established by long term treatment with 100 nM fulvestrant (see Fig. 2) and maintained in the same growth medium as the parental T47D cell lines plus 100 nM fulvestrant (Tocris, Avonmouth, Bristol, UK). For experiments, 2.5×10^5 U penicillin and 31.25 μg/l streptomycin (Gibco) were added to the growth medium. Estradiol (E2) was obtained from Sigma–Aldrich. The HER2 tyrosine kinase inhibitor, AG825 was purchased from Tocris Biosciences (Ellisville, Missouri, USA), whereas gefitinib, CI-1033 and dasatinib were purchased from Selleck Chemicals (Münich, Germany). Stock solutions of 10^{-2} M E2 and 10^{-3} M fulvestrant were dissolved in 96% ethanol, whereas stock solutions of 10 mM gefitinib, 50 mM AG825, 10 mM CI-1033 and 1 mM dasatinib were dissolved in DMSO.

2.2. Quantitative real-time RT-PCR

Cells were grown to 70–80% confluence. Total RNA was purified with the QIAamp RNA blood mini kit (Qiagen, Copenhagen, Denmark) and 0.1 μg total RNA reverse transcribed with AMW reverse transcriptase according to the manufactures instructions (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using a Lightcycler 480 and the Sybr Green I PCR kit both from Roche (Hvidovre, Denmark). Quantification is presented as the ratio between the amount of target gene and β-actin mRNA expression in each sample, as previously described [52,53]. For primer sequences and PCR profiles see [24].

2.3. Cell growth assays

To determine the effect of fulvestrant on growth of the T47D cell line, 4×10^4 -cells/well were seeded in 24-well multidishes in growth medium and allowed to adhere for 2 days. Cell number was determined by manually counting the cells from three wells in a Bürker–Türk chamber (day 0) and growth medium with vehicle or 100 nM fulvestrant was added to the remaining wells. Cell number was determined and medium renewed in the remaining wells every second or third day for a total of 12 days. To determine the doubling time of parental and fulvestrant resistant T47D cell lines, 3×10^4 cells/well were seeded in 24-well multidishes in growth medium and allowed to adhere for 1 day. At selected time points (days 0–12), cells from 3 wells were trypsinized and cell number determined by manually counting the cells in a Bürker–Türk chamber. After each count, growth medium was renewed in the remaining wells. The doubling time was calculated from the exponential part of the growth curve.

All dose response growth experiments were performed in 24-well multidishes except for the experiments with dasatinib, which were performed in 96-well multidishes. To compensate for the uneven plating efficiency, the parental T47D cells were seeded with 4×10^4 cells/well in growth medium and the resistant cells were seeded with 4.5×10^4 cells/well in growth medium with 100 nM fulvestrant in 24-well multidishes. For the growth experiments with dasatinib, the parental cells were seeded with 2300 cells/well in 96-well multidishes in growth medium, whereas the resistant cells were seeded with 2500 cells/well in 96-well multidishes in growth medium with 100 nM fulvestrant.

After 2 days growth (day 0), growth medium was renewed and media containing the compounds of interest were added. The experimental medium was replaced on day 3 and cell number determined on day 5 using a crystal violet colorimetric assay as previously described [54]. Growth experiments with E2 (1 nM) were performed with medium containing 10% newborn calf serum (NCS; Gibco), which contains very low estrogenic activity, 2 mM glutamax and 8 μg/ml insulin. Stability studies were performed with resistant cell lines which have been withdrawn from fulvestrant for 1 and 7 weeks.

2.4. Western blot analysis

Western analyses were performed with cells lysed in RIPA buffer (100 mM NaCl, 20 mM Tris–HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with 1 mM DDT, 1 mM NaF, 10 mM β-glycerol phosphate, 100 μM Na_3VO_4, 150 μM PMSF and 1 tablet/10 ml RIPA complete mini protease inhibitor cocktail (Roche). T47D cells grown for four days in medium with 10% NCS with or without 1 nM E2 were used to explore the expression of ER and estrogen regulated proteins. To investigate the effect of fulvestrant on expression of ER, estrogen regulated proteins, HER receptors and downstream signaling molecules, T47D cells were grown in standard growth medium (with 5% FBS) in the presence or absence of fulvestrant for four days. For expression analysis of HER receptors and their signaling molecules in parental and resistant T47D cells, the cells were grown in standard growth medium (T47D) or in growth medium with 100 nM fulvestrant (resistant cell lines) until 70–80% confluence. To investigate the effect of HER inhibitors on HER receptor signaling, T47D cells and fulvestrant resistant T47D cells, grown in their standard growth medium until 70% confluence, were treated for 1 h with CI-1033 (1 μM), gefitinib (1 and 5 μM) or AG825 (10 and 30 μM).

Protein concentrations in cell lysates were determined with BioRad Protein Assay kit (Bio-Rad Laboratories, Copenhagen, Denmark), 15–20 μg total protein were separated on 4–15% Tris–HCl or 3–8% Tris–Acetate resolving criterion gels (Bio-Rad)