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Growth Factor-Induced Resistance to Tamoxifen is Associated with a Mutation of Estrogen Receptor α and its Phosphorylation at Serine 305

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Abstract

Estrogens play a crucial role in breast tumor growth, which is the rationale for the use of antiestrogens, such as tamoxifen, in women with estrogen receptor (ER)- α -positive breast cancer. However hormone resistance is a major clinical problem. Altered growth factor signaling to the ER α pathway has been shown to be associated with the development of clinical resistance. We previously have identified a mutation that replaces arginine for lysine at residue 303 (K303R) of ER α , which confers hypersensitive growth in low levels of estrogen. To determine if the K303R mutation could participate in the evolution of hormone resistance, we generated MCF-7 breast cancer cells stably transfected with either wild-type (WT) or K303R ER α . We found that the mutation confers decreased sensitivity to tamoxifen in the presence of the growth factor heregulin, using anchorage-independent growth assays. K303R ER α -expressing cells were hypersensitive to growth factor signals. Our data suggest that phosphorylation of serine 305 within the hinge domain of ER α might play a key role in increasing ligand-independent activity of the mutant receptor. We hypothesize that the mutation adapts the receptor for enhanced bidirectional cross-talk with the HER2 growth factor receptor pathway, which then impacts on responsiveness to tamoxifen.

Keywords

breast cancer; estrogen receptor; K303R mutant ER α ; HER2

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Introduction

Estrogens play a crucial role in regulating the growth and differentiation of normal breast epithelium and also of breast cancers, with approximately two-thirds of all breast cancers dependent for their growth on a functional estrogen receptor α (ER α). ER α is a member of the nuclear hormone receptor superfamily that regulates transcription of ER target genes by binding with specific estrogen response elements [1]. However, ER α also regulates the expression of many genes without direct binding to DNA. This occurs via protein-protein interactions with other transcription factors, such as activator protein-1, and with extranuclear signaling complexes that, in turn, modulate downstream gene expression [2, 3]. Therapeutic strategies directed at inhibiting the action of ER α using antiestrogens, such as tamoxifen (Tam), or reducing estrogen levels using aromatase inhibitors, are the standard therapies offered to women with ER α -positive cancer. However, not all patients who have ER positive tumors respond to endocrine therapies (termed *de novo* resistance), and a large number of patients who do respond will eventually develop disease progression or recurrence while on therapy (acquired resistance).

In previous work we identified an A to G somatic mutation at ER α nucleotide 908 (A908G) in early premalignant breast lesions [4]. This transition introduces a lysine to arginine substitution at residue 303 (K303R ER α) within exon 4, at the border between the hinge and the hormone-binding domain of the receptor; the mutation confers increased sensitivity to subphysiological levels of estrogen [4]. Others have reported that the K303R ER α mutation was not present in invasive tumors [5, 6], but we have demonstrated that the detection method used by these other investigators, standard dye-terminator sequencing, was not sensitive for detection of this specific mutation [7]. In addition, Conway *et al* have identified this mutation in 6% of breast cancers using a different detection method [8]. Therefore, the exact frequency of this mutation in breast tumors remains to be established. The mutation resides at major post-translational modifications sites (acetylation, ubiquitination, methylation) adjacent to a protein kinase A (PKA) phosphorylation site at serine residue 305 (S305). Cui *et al.* have demonstrated that this naturally-occurring mutation is a more efficient substrate for phosphorylation by PKA, and is hypoacetylated which subsequently alters estrogen sensitivity [9]. Michalides *et al.* have suggested that phosphorylation of ER α S305 by PKA induces a switch from antagonistic to agonistic effects of Tam, which induces resistance to this antiestrogen [10]. It has also been shown that the ER α S305 site can be an *in vivo* substrate for p21-activated kinase 1 (PAK 1)-mediated phosphorylation, and that activation of ER α S305 might confer a conformational change which allows for a better interaction with ligands such as Tam [11, 12].

We know that estrogen regulation of breast cancer cell growth can also be modulated by complex interactions with a variety of peptide growth factors. A large body of evidence supports the idea that rapid membrane effects of ER α may activate various components of growth factor tyrosine kinase signaling, such as that from insulin-like growth factor-IR (IGF-IR), epidermal growth factor receptor (EGFR), and c-erbB2/HER2 [13–15].. Furthermore, the kinase cascade signaling initiated by growth factor receptors can activate ER α (termed ligand-independent effects) and its coregulatory proteins, causing an interdependent loop of cross-talk that leads to enhanced tumor cell survival and proliferation

[16–19]. Moreover, several preclinical and clinical studies suggest that overexpression of EGFR or HER2, and/or high levels of phosphorylated Akt and extracellular signal-regulated kinases (ERKs) in breast cancers contribute to Tam resistance [20–25]. We therefore hypothesized that the K303R mutation adapts ER α for enhanced reception of intracellular signal transduction, which leads to antiestrogen resistance. To test this hypothesis we used as an experimental model MCF-7 breast cancer cells stably transfected with either wild-type (WT) or the K303R mutant ER α . We found that cells expressing the estrogen hypersensitive K303R ER α mutant showed elevated levels of growth factor signaling, and enhanced cross-talk between the mutant and the HER2 growth factor receptor. These results suggest that the presence of the A908G ER α somatic mutation may be useful as a predictive marker of hormonal response in patients whose tumors exploit ER α and/or growth factor cross-talk to evade treatment.

Materials and methods

Reagents, hormones and antibodies

17 β -Estradiol (E₂), 4-Hydroxytamoxifen (4-OH), Epidermal growth factor (EGF), Insulin like growth factor –1 (IGF-1), and Heregulin (H) were from Sigma (St. Louis, MO). Herceptin was from Genentech (San Francisco, CA). Antibodies used for immunoblotting were: ER α (6F11) from (Novocastra, Newcastle, United Kingdom), progesterone receptor (PR) from DAKO (Carpinteria, CA), Rho GDI α from Santa Cruz Biotechnology (Santa Cruz, CA), total MAPK, total Akt, total c-Src, phosphorylated p42/44 MAPK (Thr²⁰²/Tyr²⁰⁴), Akt (Ser⁴³⁷), c-Src (Tyr⁴¹⁶) from Cell Signaling Technology (Beverly, MA), total HER2 from NeoMarker (Fremont, CA), phosphorylated HER2 (Tyr¹²⁴⁸), phosphor-ER-S305 from UPSTATE (Temecula, CA), and Living colors™ Full Length polyclonal antibody (Clontech, Mountain View, CA).

Cell culture

MCF-7 breast cancer cells, originally obtained from Dr. Benita Katzenellenbogen (University of Illinois, Urbana, IL), were maintained on plastic in minimal essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO), 0.1 nmol/L nonessential amino acid, 2 mmol/L L-glutamine, 50 units/ml penicillin/streptomycin, at 37°C with 5% CO₂/95% air. HeLa cells were obtained from American Type Culture Collection (Manassas, VA), and were maintained in the same media. Generation of the yellow-fluorescent protein (YFP)-tagged expression constructs, YFP-WT ER α and YFP-K303R ER α , has been previously described [9]. MCF-7 cells were stably transfected using Eugene 6 according to the manufacturer's instructions (Roche, Indianapolis, IN), and individual clones were isolated and expanded with G418 selection. In some experiments we used a pool of stably transfected cells selected for one week with G418 antibiotic. Stably transfected clones were screened for expression of exogenous and endogenous ER α using immunoblot analysis.

Quantitative image analyses by high throughput microscopy (HTM)

PRL-Hela is a cell line specifically engineered for the single cell study of ER function [26]. PRL-Hela cells contain multiple genomic integrations of a replicated prolactin (PRL)

promoter/enhancer. The multiple integrations (PRL array) are spatially confined and are visualized by the accumulation of fluorescently tagged ER α . PRL-Hela cells transiently expressing YFP-WT ER α or YFP K303R ER α , pretreated with forskolin (10 μ M) for 15 minutes, and then treated with increasing concentration of E₂ or Tam for 30 minutes, were fixed and DAPI-stained as previously reported [26, 27]. The cells were imaged using the Cell Lab IC 100 Image Cytometer (Beckman Coulter, Fullerton, CA) with a Nikon 40X Plan S flour 0.90 NA objective. Two channels were imaged: channel 0 (DAPI stain) was used to find the focus and nuclei, when channel 1 was used to image YFP ER α . A proprietary algorithm (GPRC) developed at Beckman Coulter was used to identify and quantify the YFP-ER α -targeted PRL array. The parameters for the GPRC algorithm were: object scale=30 and minimum peak height=10. Foci identified by the GPRC algorithm were masked. The area of the mask in pixels was the measure of PRL array size. Channel 1 was offset 2 μ m from DAPI focused for cells in all treatment conditions. After image acquisition and application of the GPRC algorithm, the total cell populations for each treatment were progressively filtered (gated) using the same criteria. Nuclei clusters, and mitotic cells were filtered from the total cell population using an intersection of DNA content and DNA clusters gates. In addition, low YFP ER α expression and low aggregate number gates were generated and applied to produce the final cell population to be analyzed. From the final population of cells, the array size was determined using the GPRC mask [28]. The images and masks were visually inspected for accuracy. Unpaired student's t-test assuming equal variance was performed to determine statistical significance (two-tailed, $p < 0.05$). Standard deviations are shown.

Immunoprecipitation and immunoblot analysis

Cells were starved in phenol red free MEM with 5% charcoal-stripped FBS for 48 h and treated as indicated before lysis [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2% NP40, 0.25% deoxycholic acid, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, and 1:100 protease inhibitors cocktail; Calbiochem]. For coimmunoprecipitation experiments, we used 1 mg of total cellular protein and a 1:200 dilution of anti-Living colors Full Length polyclonal antisera (Clontech) that recognizes native and denatured forms of recombinant YFP fusion proteins expressed in mammalian cells, and 2 μ g of HER2 polyclonal antisera overnight, followed by protein A/G precipitation with rotation at 4°C for 2 h. Immunoprecipitated proteins were washed thrice with lysis buffer. Equal amounts of cell extract and immunoprecipitated proteins were resolved under denaturing conditions by electrophoresis in 8% to 10% polyacrylamide gels containing SDS (SDS-PAGE), and transferred to nitrocellulose membranes (Schleicher & Schuell, Keen, NH) by electroblotting. After blocking the transferred nitrocellulose membranes were incubated with primary antibodies overnight at 4°C, with secondary antibodies goat anti-mouse or goat anti-rabbit antisera (1:3000; Amersham Biosciences; Piscataway, NJ) for 1 h at room temperature and developed with enhanced chemi luminescence reagents (Alpha Innotech, San Leandro, CA).

Anchorage-independent soft agar growth assays

Cells (5000 per well) were plated in 4 ml of 0.35% agarose with 5% charcoal-stripped FBS in phenol red-free MEM, in a 0.7% agarose base in six-well plates. Two days after plating, media containing control vehicle or hormonal treatments (E₂ 1 nM, heregulin 2 ng/ml, EGF

10ng/ml, IGF-1 10ng/ml with or without Tam 100 nM) was added to the top layer, and the appropriate media was replaced every two days. In some experiments a pool of stably transfected cells were treated with heregulin with or without herceptin (10 µg/ml). After 14 days, 150 µl of MTT was added to each well and allowed to incubate at 37°C for 4 h. Plates were then placed in 4°C overnight and colonies > 50 µm diameter from triplicate assays were counted. Data are the mean colony number of three plates and representative of two independent experiments analyzed for statistical significance ($p < 0.05$) using a two-tailed student's Test, performed by Graph Pad Prism 5 (GraphPad Software, Inc., San Diego, CA). Standard deviations are shown.

Blocking peptide delivery

A blocking peptide of 13 residues (IKRSKNSLALSC) from the sequence (residues 298–310) surrounding the S305 residue (in bold) of the human ER α was transferred into cells using a cationic amphiphile molecule, PULSinTM delivery reagent (Polyplus transfection, Illkirch, France), as suggested by manufacturer. Briefly, cells were plated in a 6-well plate with regular growth media, and then starved for 48 h in a phenol red free MEM with 5% charcoal stripped FBS. After starvation cells were washed with PBS to remove all traces of serum, and fresh phenol-red free media without serum was added. The mixture containing the S305 blocking peptide (4 µg/well) diluted in 200 µl of 20 nM Hepes, and 16 µl of PULSinTM was incubated for 15 min at room temperature, and then added to the cells. The media was changed after 4 hours of incubation, cells were treated as indicated, and cellular extracts were prepared. Delivery of R-phycoerythrin was used as a positive control and live cells were observed by fluorescence microscopy after 4 h.

Results

Ligand-independent signaling to the K303R ER α mutant reduces Tam sensitivity

We have previously shown that K303R ER α mutant-overexpressing cells display enhanced ligand-independent activity when stimulated with cyclic AMP, in part because the mutation generates a more efficient substrate for PKA-mediated signaling [9]. It is well known that ligand-independent signaling can influence cellular responsiveness to Tam [29]. For instance, PKA-mediated phosphorylation of ER α at S305 allows the antagonist Tam to behave as an agonist, which then results in ER α -dependent transactivation [10]. Here we have utilized the stable cell line PRL-Hela containing a multi-copy integrated prolactin enhancer/promoter DNA array that is responsive to ER such that when the receptor is expressed as a YFP-fusion protein, the integration site can be easily visualized (PRL-array) [26]. High-throughput microscopy has been used to identify ligand-independent changes in the size of the PRL-array which is an indicator of the chromatin condensation status at the promoter [28]. Arrays rapidly decondense after E₂ treatment or condense after anti-estrogen treatment (both within minutes). Thus, in PRL-Hela cells, the array size is an indicator of receptor-mediated transcription function in response to different treatments and allows direct and live observation of ER-dependent chromatin remodelling. Therefore using a mammalian-based, stably transfected prolactin promoter array system, we analyzed live cell dynamics on an estrogen-responsive promoter, to visualize chromatin remodelling induced by E₂ and Tam. We transiently transfected PRL-Hela cells with YFP tagged-ER α WT or the

YFP-ER α K303R mutant receptor expression vector, and examined E₂ (Fig 1a) and Tam's (Fig 1b) effect on chromatin remodelling in the presence of forskolin, an activator of PKA signaling. Using high through-put microscopy, the amount of chromatin remodelling was quantified [28]. 24h after transfection cells were pretreated with forskolin (FSK), and then treated with E₂ or Tam at different doses, as indicated, for 30 minutes. Over 60 nuclei in two separate experiments were analyzed to determine the size of the PRL-reporter array. Vehicle control showed similar values for the array size in the presence of the WT receptor and the mutant (Fig. 1b). In PRL-Hela cells expressing the WT receptor, estrogen treatment increased array size in a dose-dependent manner in estrogen concentrations between 10⁻¹² M and 10⁻⁹ M. Mutant-expressing cells showed a linear dose-response in the range of 10⁻¹⁷ M through 10⁻¹¹ M of estrogen. Indeed, mutant ER α expressing cells exhibited a much lower EC₅₀ (3×10^{-13} M) compared with WT ER α -expressing cells (5.1×10^{-11} M). These data demonstrate that K303R mutant-expressing cells are hypersensitive to estrogen and form larger arrays in the presence of low estrogen concentrations. With forskolin treatment, WT and mutant ER α cells stimulated with estrogen both displayed significantly increased array sizes. WT ER α -expressing cells demonstrated a one-log reduction in the EC₅₀ (4.2×10^{-12} M $\pm$ 0.0038 compared to 5.1×10^{-11} M $\pm$ 0.0132 in the absence of forskolin) and a significantly increased average array size (Fig. 1a). Mutant-expressing cells also displayed a significantly increased average size, but without a significant change in the EC₅₀ (8×10^{-13} M $\pm$ 612.5 compared to 3×10^{-13} M $\pm$ 0.0004 in the absence of forskolin). Thus, forskolin increased average array size, and reduced the EC₅₀ of WT ER α cells thus enhancing estrogen sensitivity, whereas forskolin treatment of mutant-expressing cells only increased the array size, suggesting that these cells displayed an inherent hypersensitivity to estrogen. As shown in Fig. 1b, Tam treatment decreased the array size in WT ER α -expressing cells, however the mutant receptor was significantly less responsive to Tam ($p < 0.0001$). Forskolin treatment blocked Tam-induced promoter condensation (reduced size of the array), and the mutant receptor array size was not affected by physiological levels of Tam (100 nM) in the presence of forskolin. All together these results suggest that hormone-independent kinase signaling to the mutant receptor K303R ER α may confer resistance to antiestrogen treatment in breast cancer cells.

MCF-7 K303R-ER α mutant expressing cells exhibit altered growth factor signaling

To further explore ligand-independent activation of signaling in mutant-expressing breast cancer cells, we developed MCF-7 ER α -positive human breast cancer cell lines overexpressing either WT or the K303R ER α mutant. We chose to introduce the mutant receptor into parental WT ER α -expressing cells to simulate the situation in invasive human breast tumors, where WT receptor is most frequently co-expressed along with the mutant [7]. To differentiate the exogenously expressed receptor, we tagged the vector with YFP. Stably transfected clones were screened for expression of ER α using immunoblot analysis (Fig. 2a). Parental MCF-7 cells are shown along with one clone stably expressing YFP-WT, and three clones expressing YFP-K303R ER α (MCF-7 K303R-1–3).

It is well accepted that the response to endocrine therapies in human breast cancer patients correlates with ER α and progesterone receptor (PR) levels. Several studies have shown that patients with ER α /PR-positive breast cancers derive greater benefit from adjuvant hormonal

therapy than those patients whose tumors lack PR [30–32] however it must be noted that other studies have not always found this result [33]. It has been hypothesized that high growth factor signaling activity in breast cancers may be associated with decreased PR levels (for a review see [34]), and elegant studies have shown that up-regulation of pMAPK Erk1/2 leads to a loss of PR via degradation by the 26S proteasome [35]. In clinical samples we have previously reported a borderline significant inverse correlation between the presence of the K303R ER α mutation and the PR-B isoform [7], and we have shown that a reduction in PR-B levels was associated with a poorer response to endocrine therapy [36]. Therefore, we first evaluated the levels of PR-A and B in our parental MCF-7 cells, and two stably transfected clones (Fig. 2b). Parental MCF-7 cells expressed both PR isoforms, with PR-A as the predominant form in these cells. PR is a known estrogen-induced gene, and as expected we saw higher levels of both PR-A and B in MCF-7 cells after estrogen (E₂) treatment. In comparison, MCF-7 WT-overexpressing cells exhibited slightly less PR induction with E₂. In contrast, under control, basal conditions, K303R ER α mutant-overexpressing cells demonstrated much lower levels of both PR isoforms, and less were induced with E₂ treatment. Lower levels of PR mRNA levels were also detected in estrogen-stimulated mutant cells using Real-Time polymerase chain reaction (qPCR), ($p = 0.0008$, data not shown). These results suggest that growth factor signals might be altered in the mutant-overexpressing cells.

To test for altered intracellular signaling, we next examined the effects of short-term treatments with E₂, heregulin (H), EGF (E), and IGF-1 (I) on phosphorylation levels of downstream growth factors signaling components, such as HER2, Akt, and MAPK using immunoblot analysis (Fig. 2c) which is quantitatively represented in Fig. 2d. Cells were maintained under estrogen-depleted conditions for 2 days, and then treated for 10 minutes with E₂ or different growth factors as indicated, and cellular extracts were prepared. MCF-7 K303R mutant cells showed constitutively higher levels of phosphorylated HER2 as well as total HER2, compared to MCF-7 WT ER α -overexpressing cells (Fig. 2c). To begin to understand the mechanism associated with higher levels of total HER2 in the mutant cells we analyzed the expression level of HER2 mRNA in different clones of mutant ER α expressing cells, by qPCR; but we did not find differences in HER2 mRNA levels between WT and K303R ER α expressing cells (data not shown). This result suggests that post-translational modification and increase in protein stability could be involved in the HER2 up-regulation that we found in mutant ER α -expressing cells, and we are currently exploring this possibility. Treatment with E₂, heregulin, EGF, and IGF-1 induced higher levels of pHER2 in mutant-overexpressing cells, but only a small induction was seen in MCF-7 WT cells which contain low endogenous levels of HER2. Treatment with heregulin or EGF led to increased phosphorylation of the downstream signaling molecules Akt and MAPK in mutant-overexpressing cells compared with MCF-7 WT cells. IGF-1 treatment also induced enhanced phosphorylation of Akt, but increased levels of phosphorylated MAPK were only detected in K303R ER α mutant-overexpressing cells. In both cell lines, no enhancement of Akt and MAPK phosphorylation was seen with estrogen treatment.

To investigate if the kinetics of growth factor signaling might be altered in mutant-expressing cells, we performed a time-course study with heregulin treatment in WT ER α and K303R ER α mutant cells (Fig. 2e). After two days of starvation cells were treated for 1,

3, 5, 10, or 20 minutes with heregulin, and then lysed as described in Materials and Methods. As shown in Fig. 2e a rapid response to heregulin was seen within 1' in MCF-7 WT cells with activation of phospho-HER2, and phospho-AKT/phosphoMAPK within 10–20' (numerical quantitation relative to control WT is shown beneath each immunoblot). In contrast, mutant-expressing cells exhibited enhanced activation of pHER2, pAKT, and pMAPK. Compared with WT-expressing cells, these molecules were all stimulated at earlier time points in mutant cells; heregulin induced phosphorylation of MAPK and Akt by 5 minutes, but levels of these phosphorylated kinases were not detectable at this time-point in WT ER α cells. We saw that the non receptor tyrosine kinase c-Src also exhibited an earlier time-point of activation (by 3 min), and higher phosphorylation levels at the activating c-Src tyrosine residue 416 (Tyr⁴¹⁶) was seen in K303R ER α cells. These collective data demonstrate that the mutant-overexpressing cells are hypersensitive to growth factor signal transduction, and suggest that the mutant receptor could impact on ligand-independent signaling pathways commonly known to be employed to evade anti-hormonal therapeutic strategies in breast cancer.

Previous reports have shown that ER α and growth factor pathways can interact at different levels, through a direct association or complex formation of ER α with key signaling molecules such as c-Src, Shc, and the p85 α regulatory subunit of PI3K [37–40]. C-Src family tyrosine kinases are involved in signaling of a number of different growth factor receptors, including EGFR/HER2, in breast cancer cells. In previous work we have reported that MCF-7 cells stably expressing the K303R ER α mutant receptor exhibited increased c-Src kinase activity and c-Src tyrosine phosphorylation, when compared with WT ER α -expressing cells [41]. Therefore we next addressed whether the mutation might alter the ability of the receptor to bind with the HER2 tyrosine kinase receptor, which is expressed at higher levels in mutant cells (Fig. 2c). To evaluate potential protein-protein interactions between WT and mutant receptors with HER2 in our model system, we treated MCF-7 cells overexpressing WT or the mutant for 5' with EGF 100 ng/ml and then lysates were prepared (Fig. 2f). Equal amounts of protein were immunoprecipitated with either anti-YFP antisera or anti-HER2 antisera followed by immunoblot for HER2 and YFP-ER α . As shown in Fig. 2f, similar amounts of input from the whole-cell lysates were used. In the absence of treatment, both WT ER α and HER2 resided in a protein complex; and EGF treatment slightly increased the amount of the protein in the complex. The levels of mutant receptor bound to HER2 under basal conditions were higher, and EGF treatment did not further increase the amount of receptor in the complex. The bottom panel shows that equal amounts of YFP-ER α and HER2 were immunoprecipitated under all conditions tested. These results suggest that the K303R ER α mutant may be constitutively associated with HER2, which could be involved in the enhanced bidirectional crosstalk that we report between ER α and growth factor receptor signaling pathways.

Tamoxifen fails to inhibit anchorage-independent growth induced by heregulin in MCF-7 K303R ER α -overexpressing cells

It is already appreciated that altered crosstalk between several receptor tyrosine kinases and ER α may contribute to endocrine resistance [18], and it has been shown that elevated levels of HER2 contribute to resistance in a tamoxifen-resistant subline of MCF-7 cells compared

to unselected parental cells [42]. Since the K303R ER α mutation conferred enhanced heregulin-mediated signaling, we next addressed whether this alteration might confer a decrease in response to antiestrogen therapy. To test this question we next performed anchorage-independent growth assays using our two ER α -overexpressing models. Cells were plated in soft agar and then treated with estrogen (E₂, 1 nM) or heregulin (H, 2 ng/ml), EGF (10ng/ml, E), IGF-1 (10ng/ml, I) in the presence or absence of tamoxifen (Tam, 100 nM). After 14 days of growth colonies >50 μ m in diameter were counted (Fig. 3a). Control (C) basal growth of mutant-expressing cells was higher compared to WT-expressing cells (p=0.001); as expected treatment with estrogen as well as heregulin increased the number of colonies in both cell lines, while EGF and IGF-1 slightly increased proliferation but only in MCF-7 K303R ER α -overexpressing cells. Tam treatment induced a significant reduction in the number of colonies in MCF-7 WT cells under control conditions, and was able to inhibit growth of cells stimulated with estrogen or heregulin (reduction in colonies 78% and 96%, respectively). In contrast Tam treatment was less efficient at inhibiting estrogen or heregulin-stimulated proliferation in mutant-overexpressing cells (reductions were only 54% and 21%, respectively), but effectively reduced EGF and IGF-1 stimulated growth in these cells. Thus, response to Tam was greatly affected by the enhanced growth factor signaling by heregulin in mutant cells; this enhanced sensitivity prevented the major antagonistic activity of tamoxifen on the proliferation of mutant-expressing cells.

Next, we examined whether the HER2 pathway found to be up-regulated in the mutant-expressing cells, was responsible for the higher growth of mutant cells compared to WT ER α cells. We therefore evaluated anchorage-independent growth of either mutant or WT ER α cells after herceptin treatment. Herceptin is a humanized monoclonal antibody directed against the extracellular domain of HER2, and was developed as an agent to inhibit the growth of HER2-overexpressing tumor cells [43, 44]. Cells were plated in soft agar and then treated with heregulin in the presence or absence of herceptin (10 μ g/ml). After 14 days of growth, colonies were counted (Fig 3b). As expected since HER2 levels are low in WT cells, herceptin had no effect on their growth either under basal conditions or with heregulin treatment. In contrast, herceptin significantly inhibited anchorage-independent growth of K303R mutant cells in control untreated conditions, and with heregulin treatment (*p=0.0002 vs control; **p=0.03 vs heregulin treated cells). These data confirm that the HER2 pathway may be responsible for the higher constitutive growth of mutant-overexpressing cells rendering these cells more sensitive to the inhibitory effect of this selective HER2-targeted agent.

Phosphorylation at serine residue 305 (S305) of ER α K303R mutant is involved in growth factor signaling up-regulation

ER α is a known target of several post-translational modifications, such as phosphorylation, sumoylation, and acetylation [9, 45–47]. For instance, receptor phosphorylation, which regulates receptor affinity, coregulator protein binding, and transcriptional activity, can be induced in the absence of ligand via cross-talk with various signal transduction pathways [48]. We previously have reported that the K303R ER α mutant is a more efficient substrate for phosphorylation by PKA at S305 which enhanced hormone sensitivity and stimulated cellular growth [9]. We hypothesize that the phosphorylation status of S305 in the mutant

receptor may control receptor activity, and be a conduit for enhanced downstream cross-talk with growth factor signaling networks. To explore this possibility, we first evaluated the phosphorylation status of the S305 residue in either WT ER α or K303R mutant cells after estrogen treatments between 5 minutes to 2 hours. Cellular extracts were subjected to immunoblot assay using a specific anti-phospho-S305 ER α antibody (Fig. 4a). Estrogen treatment enhanced the phosphorylated levels of S305 within 15' in YFP-WT-expressing cells, and these levels remained elevated at 2 hours. In contrast, K303R ER α mutant-expressing cells exhibited elevated levels of pS305 under basal control conditions, and this elevated phosphorylation remained constant with longer estrogen treatments. Since, it is well established that ER α can be activated in a ligand-independent manner by MAPK [49] at serine 118 (S118), we also evaluated the levels of phospho-S118 in WT ER α or K303R ER α -overexpressing cells under the same conditions described above. We did not detect significant changes in S118 phosphorylation patterns between the two cell lines (data not show). These results suggest that constitutively higher phosphorylation of S305 in the mutant receptor might play a role in the ligand-independent activation of the receptor itself. It is possible that this enhanced S305 phosphorylation within the mutant might play a key role in the observed up-regulation of growth factor signaling cascades seen in these cells as well.

To test this hypothesis we performed immunoblot analysis to evaluate the phosphorylation levels of a number of growth factor signaling components after incubation with an S305 blocking peptide. To block phosphorylation at S305 we delivered a peptide (S305 peptide, residues 298 to 310) to the cells. After peptide delivery, the cells were subjected to short term treatments (10 min) with heregulin and then growth factor signaling molecules were analyzed by immunoblot analysis (Fig. 4b). Heregulin enhanced S305 phosphorylation in K303R mutant-overexpressing cells, but had no effect WT receptor. Phosphorylation of the mutant receptor was abrogated by the S305 peptide. Addition of the S305 blocking peptide also inhibited heregulin-induced phosphorylation of HER2, Akt, and MAPK in both cell lines. Interestingly, reduction in phospho-HER2 and MAPK levels were more pronounced in mutant-expressing cells compared with WT, suggesting that the mutant cells were more sensitive to the inhibitory effect of S305 blocking peptide. These data indicate that phosphorylation of the S305 residue may be crucial in mediating enhanced cross-talk between HER2 and mutant ER α , and suggest that phosphorylation blockade might be a potential therapeutic strategy to block mutant function.

Discussion

Despite the improvements in the efficacy of hormonal therapies for the treatment of breast cancer patients with ER-positive tumors, *de novo* and acquired resistance remain major clinical problems that limit the efficacy of these therapies. In most cases, ER α remains essential to the problem of resistance due to its intimate cross-talk with growth factor signaling pathways [50, 51]. In this study, we show that expression of K303R ER α mutant in ER α -positive MCF-7 breast cancer cells confers a decreased sensitivity to tamoxifen treatment in the presence of growth factor stimulation. Furthermore, this naturally-occurring mutant is constitutively phosphorylated at S305, and shows an enhanced bidirectional cross-talk with the HER2 signaling pathway.

The A to G somatic mutation of ER α at nucleotide 908 (A908G) was previously identified in about 30% of premalignant breast lesions, but at a higher frequency (50%) in invasive breast tumors [4, 7]. The mutation was found to be associated with biologic measures of poor outcome, including elevated HER2 protein, larger tumor size and axillary lymph node positivity. To date no other somatic ER α mutation has been identified in more than a few invasive breast cancers [52], making this mutation novel. Recently, in a study using a population-based, case-control study design, the A908G mutation was detected, but at a low frequency (7%), in invasive breast tumors [53], confirming our identification of the mutant in cancer. We previously demonstrated that the exogenous expression of the K303R ER α mutant in MCF-7 breast cancer cells conferred a hypersensitive growth in very low physiological levels of estrogen (10^{-12} to 10^{-11} M) [4]. In the present study we focused on the K303R ER α mutation and its potential role in modulating hormonal response in breast cancer cells, and on the molecular pathways that could be involved in its hormone action.

Our studies showed that growth factor signaling pathways were up-regulated in K303R ER α -expressing cells. In particular, MCF-7 mutant ER α expressing cells showed constitutively higher levels of total and phosphorylated HER2, the tyrosine kinase receptor that belongs to the epidermal growth factor receptors family. Preliminary quantitative RT-PCR analysis demonstrated that HER2 mRNA levels were not increased in mutant expressing cells compared to WT ER α -expressing cells. Thus, transcriptional regulation may not be the major mechanism for the observed increased levels of HER2 in mutant cells. In the present study we did not investigate the possible post-transcriptional mechanisms associated with higher levels of HER2, but instead focused on the effects that HER2 overexpression induced on downstream cell signaling and hormone responsiveness in mutant-expressing cells. It is well known that HER2 catalytic activity can amplify the signal of other c-erbB family receptors by the formation of HER2-containing heterodimers, which increases ligand binding affinity and receptor stability [54, 55]. Moreover, it has been shown that c-neu, the mouse homolog of HER2, is able to multimerize, be phosphorylated, and thus activated when present at high density on the cell surfaces [56]. Both mechanisms result in the amplified activation of downstream signaling cascade, such as Akt and MAPK, which are involved in cell survival and proliferation.

We found that the peptide growth factors heregulin and EGF strongly enhanced phosphorylation of the two major downstream signaling cascades Akt and MAPK, in mutant-expressing cells compared to WT ER α -expressing cells. Furthermore, analysis of rapid kinetics showed that many of these downstream molecules, as well as the c-Src non receptor tyrosine kinase, were stimulated at earlier time points in mutant-expressing cells. The rapid responses of these downstream kinase cascades to heregulin suggest that the presence of K303R ER α mutation could modify the responsiveness of the cells to the growth factor signaling possibly through enhanced non-genomic activity of the mutant receptor.

Recent research into the mechanisms associated with Tam resistance suggest that some of the same growth factor receptor pathways implicated in adaptive hypersensitivity, such as Akt and MAPK, or specific oncogenes involved in intracellular signal transduction, become activated and are used to bypass normal hormonal responsiveness. Several reports indicate

that the up-regulation of HER2 tyrosine kinase signaling in breast cancer plays an important role in the development of endocrine resistance [57]. Preclinical studies have demonstrated that HER2 overexpression in ER α positive MCF-7 human breast cancer xenografts rendered them resistant to Tam [58], and markedly increased levels of EGFR and HER2 were found in some sublines of MCF-7 cells with acquired Tam resistance [59, 60]. Tam resistance in these cells was reversed by EGFR/HER2 tyrosine kinase inhibitors, and combined treatment with these inhibitors and Tam was effective in reversing resistance in xenograft models [61], thereby strongly implicating this signaling network in resistance. Although HER2 overexpression occurs only in a minority of ER α -positive patients [62], clinical studies confirm that HER2-overexpressing tumors are less responsive to Tam treatment [63, 64]. In a previous retrospective study we found an association between the K303R ER α mutation and elevated HER2 levels in invasive breast cancer [7]. Here we observed increased HER2 levels in K303R mutant-expressing cells that was concomitant with an altered response to Tam with growth factor stimulation.

We also demonstrated that mutant-expressing cells exhibited a higher level of growth under all conditions tested. Importantly, Tam sensitivity was significantly affected with estrogen and heregulin treatments. Our data indicate that the Tam-resistant phenotype associated with the mutant was most pronounced in the presence of growth factor activation; in the presence of heregulin Tam inhibited soft agar growth only 21% compared with a 96% inhibition in WT ER α -expressing cells. We speculate that bidirectional cross-talk between the HER2 and mutant receptors could play a role in conferring a selective advantage in terms of growth to those patients that express the K303R ER α mutant that are treated with Tam. We are currently examining for the presence of the mutant in a retrospective cohort of patients treated with Tam who have long-term clinical follow-up.

Different therapeutic agents targeting the activity of the c-erbB family of receptors have been recently developed and tested in patients. For instance, herceptin (TrastuzumabTM), a monoclonal antibody against HER2, was approved for therapeutic use in patients with HER2-overexpressing breast cancer [65, 66]. By binding to the juxtamembrane domain of HER2 [67], this agent blocks HER2 homo- and heterodimerization with the other members of the c-erbB family, and thereby interrupts the activation of downstream proliferative signaling. Here we show that herceptin elicited its antiproliferative effects either under basal conditions or with heregulin treatment in K303R ER α mutant cells, but did not affect growth of the WT cells. These findings confirm our hypothesis that the HER2 pathway, which appears to be elevated in mutant cells, could be involved in the regulation of cell growth in breast tumor cells bearing the mutation, probably through increased crosstalk between HER2 and ER α pathways.

The existence of bidirectional cross-talk between ER α and growth factor receptor pathways, and its involvement in the development of endocrine resistance has been well documented [16, 19]. Several studies have demonstrated direct or indirect activation of growth factor signaling via ER α . For example, ligand-independent activation of serine 118 ER α by EGFR/MAPK-mediated phosphorylation regulates growth of tamoxifen-resistant MCF-7 breast cancer cells [68]. Chung *et al.* have also demonstrated that HER2 and ER α can directly interact at the cell membrane [69], and this interaction protected breast cancer cells from

Tam-induced apoptosis. Moreover, membrane or cytoplasmic ER α can induce phosphorylation of EGFR through activation of G-proteins, c-Src, and matrix metalloproteinases [70], and can directly interact with adaptor proteins such as c-Src, Shc and the p85 α regulatory subunit of PI3K [37–40]. These processes activate downstream kinases that in turn activate ER α and its coregulatory proteins, thus also enhancing genomic activities of the receptor [71, 72]. All together these effects amplify the bidirectional crosstalk which multiplies signals between and downstream of the growth factor receptors and ER α , thus sustaining survival and proliferative signals in breast cancer cells.

The present findings suggest an enhanced hormone-independent physical association/complex between the mutant receptor and HER2 compared to WT receptor. This suggests that the mutation, present in the hinge region of the receptor, may increase the ability of ER α to interact with HER2 or other components of the complex. As yet, the interaction surface between HER and ER α has not been defined. Altered interactions as we describe may imply that Tam may not antagonize the mutant receptor because the HER2 pathway may be dominant and non-genomic action predominates. This is consistent because we have already demonstrated that the mutation can alter coregulator protein binding. The mutation demonstrates enhanced binding ability to bind to the TIF-2 coactivator [4], and the AIB coactivator at very low and physiological levels of estradiol, but decreased binding to the corepressor NCoR1. Mutant receptor binding to BRCA-1 has also been shown to be enhanced. These collective data suggest that altered affinity for ER coregulators, and possibly signaling molecules such as HER2, could be one mechanism by which the K303R mutation confers hypersensitivity to low levels of estrogen, and reduced sensitivity to Tam. The mutant receptor appears to exhibit increased ligand-independent activity which bypasses antiestrogen treatment.

To explore why the K303R ER α mutation has increased cross-talk with the HER2 pathway, we focused on the differences in post-translational modifications between WT and mutant receptor. It is known that ER α activity can be modulated by several post-translational modifications, such as protein phosphorylation [45], acetylation [46], ubiquitination [73], and sumoylation [47]. The majority of studies in this field have focused their attention on the phosphorylation status of the receptor, and its effect on receptor activity. For instance, receptor phosphorylation by different kinases such as c-Src, PKA, MAPK, and Akt can all regulate receptor affinity, coregulator protein binding, and transcriptional activity [45]. We have previously shown that the K303R ER α mutation renders the receptor a more efficient substrate for PKA-induced phosphorylation at residue S305 which has distinct biological results—enhanced hormone sensitivity for growth [9]. Phosphorylation at the S305 residue can also be mediated by both protein kinase A (PKA) and p21-activated kinase-1 (PAK-1) signaling network [9, 12].

Several reports have identified the serine residue at 305 as a physiologically important site that modifies response to Tam. In particular it has been demonstrated, using fluorescence resonance energy transfer (FRET) analysis, that PKA signaling to ER α S305 causes a conformational arrest in the ER α and switches Tam from an antagonist to an agonist [10]. Michalides *et al.* have also demonstrated that PKA phosphorylation at S305 ER α induces Tam resistance through an altered orientation of ER α towards the co-activator SRC-1 [74].

In addition, S305 ER α phosphorylation by PAK-1 up-regulates cyclin D1 expression in breast cancer cells [75]. Here we show that K303R mutant cells have elevated phosphorylation levels of S305 compared with WT-expressing cells suggesting that the mutant have constitutive ligand-independent activity. The contribution of the S305 site in enhanced cross-talk with HER2 was established using a blocking Peptide. We show that heregulin-stimulation enhanced S305 phosphorylation in mutant expressing cells, but it did not significantly influence the phosphorylation status of WT receptor. Interestingly, we found that the S305 peptide affected heregulin-induced ER α phosphorylation, and prevented downstream phosphorylation events, such as activation of MAPK and Akt. These effects were even more prominent in K303R mutant cells possibly due to prominent role of the S305 site in ligand-independent activity of the mutant receptor. A similar experimental approach was used by Varricchio *et al.* [76]. They demonstrated that a six aminoacid peptide surrounding the phosphotyrosine residue 537 was able to block ER/c-Src interactions, cyclin D1 expression, and growth of MCF-7 and LNCaP cells.

We have previously shown that transcriptional activity of the mutant receptor was induced at very low concentrations of estradiol (10^{-12} M), and only the specific lysine to arginine substitution at 303 residue resulted in a receptor with enhanced sensitivity to estrogen. We have also demonstrated that cAMP-dependent signaling can enhance the receptor's intrinsic sensitivity to hormone, and that blocking PKA activity reversed the hypersensitive proliferative phenotype in mutant-expressing cells [9]. The data obtained herein using live cell dynamics agrees with these earlier results. The live cell high through-put analyses allowed us to study mutant receptor/promoter interaction and chromatin remodelling [26, 28]. Our results confirm that the K303R mutant is inherently hypersensitivity to estrogen, and only WT receptor showed increased sensitivity to estrogen after forskolin treatment. We used the same experimental approach to test for altered Tam activity in the presence of forskolin. Tam treatment inhibited transcriptional responses in WT, but at statistically significant lower levels in mutant cells. Forskolin treatment blocked Tam-induced promoter condensation, suggesting that ligand-independent kinase signaling to the mutant receptor decreased tamoxifen sensitivity.

Clinical studies have reported that breast tumors with HER2 amplification show reduced levels of PR [77], and the absence of PR is a marker of a more aggressive phenotype [78]. Patients whose tumors lack PR derive less benefit from adjuvant hormonal therapy [30–32]. *In vitro* studies suggest that amplified growth factor signaling may underlie the reduction of PR levels in breast cancer cells. [79]. In this report we found that PR protein expression was almost undetectable in K303R cells under basal conditions, and estrogen induced only a small increase in PR content, compared to the induction elicited in WT-expressing cells. The lack of PR expression observed in K303R mutant-expressing cells may be the consequence of altered growth factor signaling that contributed to the Tam-resistant phenotype observed in our model system.

We conclude that the K303R ER α hypersensitive phenotype involves an integration of post-translational modification events, such as phosphorylation at S305, with enhanced bidirectional cross-talk between the mutant and growth factor receptors such as HER2, and that genomic and nongenomic mechanisms probably contribute to Tam resistance. Because

our molecular and biological data demonstrate that the ER α mutation may be resistant to Tam, we suggest that the mutation is a potential novel predictive marker of hormonal response in breast cancer tumors. In addition, our molecular studies suggest that use of a specific blocking peptide to prevent S305 phosphorylation of the mutant may reduce ligand-independent activity, and be a new therapeutic approach to treat patients with mutation-positive tumors which are resistant to Tam therapy.

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Fig. 1.

E₂ and Tam effects on large-scale chromatin structure in PRL-Hela cells. Cells transiently expressing YFP-ERα WT (WT) or the YFP-K303R ERα (K303R) mutation were pretreated with forskolin (FSK) for 15' minutes and then were treated with E₂ (**a**) or Tam (**b**) at different doses for 30 minutes. After fixing and counter-staining with DAPI, cells were imaged and array size was quantified using HTM as described in Materials and Methods. Results are expressed as array size normalized to control cells obtained from three independent experiments.

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E

Fig. 2.

Growth factor signaling in MCF-7 WT-ERα and MCF-7 K303R ERα-expressing cells. **(a)** MCF-7 cells stably transfected with a yellow-fluorescent protein (YFP)-tagged expression construct YFP-WT ERα (MCF-7 WT) and different clones transfected with the mutant YFP-K303R ERα construct (MCF-7 K303R-1, -2, -3) were screened for expression of exogenous (96 kDa) and endogenous ERα (66 kDa) by immunoblot analysis using an antibody against human ERα. Parental cells (MCF-7) were used as a control for endogenous ERα expression. Rho GDIα was used as a control for equal loading and transfer. Numbers below the blot represent the ratio between YFP-ERα and endogenous ERα protein expression. **(b)** MCF-7 parental, MCF-7 WT, and MCF-7 K303R-1 cells were serum-starved for 48h, and then treated with or without 1 nM E₂ for 24 hours before lysis. Equal amounts of total cellular extract were analyzed for progesterone receptor (PR-A and PR-B) levels by Western blotting. **(c)** MCF-7 WT and MCF-7 K303R-2 cells were treated with vehicle ethanol (C), 1 nM E₂, 2 ng/ml heregulin (H), 10 ng/ml EGF (E) and 10 ng/ml IGF-1 (I) for 10 min before lysis. Levels of phosphorylated (p) HER2 (Tyr¹²⁴⁸), Akt (Ser⁴⁷³), and MAPK (Thr²⁰²/Tyr²⁰⁴), at the indicated residues, and total non-phosphorylated protein were measured in cellular extracts by immunoblot analysis. **(d)** Quantitative analysis of the blots shown above. **(e)** MCF-7 WT and MCF-7 K303R-2 cells were treated with heregulin (H) at 2 ng/ml for different times before lysis. Cellular extracts were analyzed as in panel **c**, and for p-Src (Tyr⁴¹⁶) at the indicated residues. Numbers below the blot represent fold change in protein expression of MCF-7 K303R-2 cells compared to MCF-7 WT cells. **(f)** MCF-7 WT (WT) and MCF-7 K303R-3 (K303R) cells after 48h of starvation were treated with or without EGF 100 ng/ml for 5 min before lysis. YFP-WT ERα and YFP-K303R ERα proteins were immunoprecipitated using an anti-YFP polyclonal antibody (IP:YFP), or an anti-HER2 polyclonal antibody (IP:HER2), and immunoblotted (IB) with HER2 and anti-ERα antibodies, respectively. Whole-cell lysates (Input) were used as input controls. Rho GDIα was used as a control for equal loading and transfer. Immunoblots are representative of three separate experiments.

A

Fig. 3.

Heregulin treatment reduced the ability of tamoxifen to inhibit anchorage-independent growth of MCF-7 K303R cells. (a) MCF-7 WT and MCF-7 K303R-2 stably transfected cells were seeded (5000/well) in 0.35% agarose and then treated with vehicle (C), E₂ (1 nM), heregulin (2 ng/ml, H), EGF (10ng/ml, E), IGF-1 (10ng/ml, I) with or without Tam (100 nM). Cells were allowed to grow for 14 days and the number of colonies >50 μ m were quantified and the results were graphed. * $p=0.0001$ vs control (C) of MCF-7 WT cells. (b) Immunoblot analysis showing YFP-ER α and endogenous ER α protein expression (upper

panel) in the pool of stable transfectants. Numbers below the blot represent fold change in protein expression of MCF-7 K303R-2 cells compared to MCF-7 WT cells. MCF-7 WT-P and MCF-7 K303R-P pool of transfected and overexpressing cells were plated in soft agar and then untreated or treated with heregulin 2 ng/ml (H) in the presence or absence of Herceptin (10 µg/ml). * $p=0.0002$ vs control (C); ** $p=0.03$ vs heregulin (H) treated cells (bottom panel); standard deviations are shown.

Fig. 4.

Serine residue 305 (S305) in the K303R ER α mutant is involved in growth factor signal up-regulation. **(a)** MCF-7 WT and MCF-7 K303R-1 cells were treated for different times with E₂ (1nM) before lysis. Cellular extracts were analyzed for phosphorylation levels of S305 YFP ER α (pS305) and total non-phosphorylated YFP-ER α . **(b)** Cells were incubated with the S305 peptide (4 μ g/well) for 4 hours in serum-free media and then treated with or without heregulin (2 ng/ml) for 10 min before lysis. Levels of phosphorylated (p) S305 YFP-ER α , HER2 (Tyr¹²⁴⁸), Akt (Ser⁴⁷³), and MAPK (Thr²⁰²/Tyr²⁰⁴), and total non-phosphorylated proteins were measured in cellular extracts by immunoblot analysis. Blots are representative of three separate experiments. Rho GDI α was used as a control for equal loading and transfer