Estrogen Receptor-α Variant, ER-α36, is Involved in Tamoxifen Resistance and Estrogen Hypersensitivity

XianTian Zhang and Zhao-Yi Wang

Departments of Medical Microbiology and Immunology, Creighton University Medical School, Omaha, Nebraska 68178

Antiestrogens such as tamoxifen (TAM) provided a successful treatment for estrogen receptor (ER)-positive breast cancer for the past four decades. However, most breast tumors are eventually resistant to TAM therapy. The molecular mechanisms underlying TAM resistance have not been well established. Recently, we reported that breast cancer patients with tumors expressing high concentrations of ER-α36, a variant of ER-α, benefited less from TAM therapy than those with low concentrations of ER-α36, suggesting that increased ER-α36 concentration is one of the underlying mechanisms of TAM resistance. Here, we investigated the function and underlying mechanism of ER-α36 in TAM resistance. We found that TAM increased ER-α36 concentrations, and TAM-resistant MCF7 cells expressed high concentrations of ER-α36. In addition, MCF7 cells with forced expression of recombinant ER-α36 and H3396 cells expressing high concentrations of endogenous ER-α36 were resistant to TAM. ER-α36 down-regulation in TAM-resistant cells with the short hairpinRNA method restored TAM sensitivity. We also found that TAM acted as a potent agonist by activating phosphorylation of the AKT kinase in ER-α36-expressing cells. Finally, we found that cells with high concentration of ER-α36 protein were hypersensitive to estrogen, activating ERK phosphorylation at picomolar range. Our results thus demonstrated that elevated ER-α36 concentration is one of the mechanisms by which ER-positive breast cancer cells escape TAM therapy and provided a rational to develop novel therapeutic approaches for TAM-resistant patients by targeting ER-α36. (Endocrinology 154: 1990–1998, 2013)
has been described in breast cancer xenografts that exhibit a switch from a TAM-inhibitory phenotype to a TAM-stimulated one (5, 6). The agonist activity of TAM in this model may be due to the enhanced growth factor signaling that is often associated with acquired TAM resistance (reviewed in Ref. 7). However, the molecular mechanism underlying this type of acquired TAM resistance has not been well established.

During development of acquired antiestrogen resistance, breast cancer cells usually undergo adaptive changes in response to inhibitory effects of antiestrogens (8). Adaptive changes also occur in response to aromatase inhibitor therapy in postmenopausal patients or from oophorectomy in premenopausal patients (9, 10). Using a MCF7 breast cancer model system, Santen’s group demonstrated that deprivation of estrogen for a prolonged period of time confers these cells hypersensitive to low concentrations of estrogen (8–11). In these hypersensitive cells, 17β-estradiol (E2) stimulates cell proliferation at picomolar (pM) range, whereas the wild-type cells require nanomolar (nM) range E2 to induce cell growth (11). However, the exact molecular events in the development of this “adaptive hypersensitivity” have not been elucidated, although up-regulation and membrane localization of ER-α, activation of the nongenomic estrogen signaling, and induction of c-Myc and c-Myb have been proposed to be involved in this process (8, 12).

Previously, our laboratory identified and cloned a variant of ER-α, ER-α36, which has a molecular mass of 36 kDa (13, 14). The transcript of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α gene (15). This ER-α differs from the original 66-kDa ER-α (ER-α66), because it lacks both transcriptional activation function domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains and partial ligand-binding domain (13). ER-α36 is mainly localized near the plasma membrane and mediates membrane-initiated estrogen signaling (14). We also found that the breast cancer patients with tumors expressing high concentrations of ER-α36 benefited less from TAM therapy than those with low concentrations of ER-α36 (16), suggesting that increased ER-α36 concentration is one of the underlying mechanisms of TAM resistance. Recently, we also reported that ER-α36 is able to mediate agonist activity of TAM and ICI 182,780 (17, 18), such as activation of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinases (ERK) and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways, indicating that these antiestrogens may loss their growth-inhibitory activities in cells with increased ER-α36 expression.

Based on these observations, we hypothesized that ER-α36 is involved in TAM resistance. Using ER-positive breast cancer MCF7 cells with different concentrations of ER-α36 as model systems, we investigated ER-α36 function in TAM resistance. Here, we present evidence to demonstrate that ER-α36 plays an important role in TAM resistance presumably through mediating agonist activity of TAM and estrogen hypersensitivity.

**Materials and Methods**

**Chemicals and antibodies**

E2 was purchased from Sigma Chemical Co (St Louis, Missouri). Anti-phospho-p44/42 ERK (Thr202/Tyr204) (197G2) mouse monoclonal antibody (mAb), anti-p44/42 ERK (137F5) rabbit mAb, anti-phospho-AKT (Ser473) (D9E) rabbit mAb and anti-AKT (pan) (C67E7) rabbit mAb, anti epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) antibodies were purchased from Cell Signaling Technology (Boston, Massachusetts). Antibodies of ER-α66 and β-actin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California). Polyclonal anti-ER-α36 antibody was generated by the custom service provided by the Pacific Immunology Corp (Ramona, California). Polyclonal anti-ER-α36 antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California).

**Cell culture and establishment of stable cell lines**

The MCF7 cell line (American Type Culture Collection, Manassas, Virginia) and its derivatives, as well as H3396 cells (a kind gift from Dr Leia Smith of Seattle Genetics, Bothell, Washington), were maintained at 37°C in a 10% CO2 atmosphere in Improved Minimum Essential Medium (IMEM) without phenol red and 10% fetal calf serum. To establish stable cell lines with knocked down concentrations of ER-α36, we constructed an ER-α36-specific short hairpin RNA (shRNA) expression vector by cloning the DNA oligonucleotides 5’-GATGCCAATAGGTAC TGAATTGATATCCGTTCAGTAC-3’ from the 3’ untranslated region of ER-α36 gene into the pRNAT-U6.1/Neo expression vector from GenScript Corp (Piscataway, New Jersey). Briefly, cells transfected with the empty expression vector and ER-α36 shRNA expression vector were selected with 500-μg/mL G418 for 3 weeks, and more than 20 individual clones from transfected cells were pooled, examined for ER-α36 expression with Western blot analysis, and retained for experiments. For ERK1/2 and AKT activation assays, cells were treated with vehicle (ethanol) and indicated concentrations of TAM or E2.

To examine cell growth in the presence or absence of antiestrogens, cells maintained for 3 days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, Utah) were treated with different concentrations of TAM, E2, or ethanol vehicle as a control. The cells were seeded...
Western blot analysis

For immunoblot analysis, cells washed with PBS were lysed with the lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.25 mM EDTA [pH 8.0], 0.1% sodium dodecyl sulfate, 1% Triton X-100, and 50 mM NaF) plus the protease and phosphatase inhibitors (Sigma Chemical Co). The protein amounts were measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, California). The same amounts of the cell lysates were boiled for 5 minutes in loading buffer and separated on a SDS-PAGE gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were probed with various primary antibodies horseradish peroxidase (HRP)-conjugated secondary antibodies, and visualized with enhanced chemiluminescence detection reagents (GE Healthcare Bio-Sciences Corp, Piscataway, New Jersey). All Western blot experiments were performed at least 3 times. Band densities on developed films were measured and analyzed using Quantity One 1-D Analysis Software version 4.6.7 (Bio-Rad Laboratories).

Statistical analysis

Data were summarized as the mean ± SE using the GraphPad InStat software program (GraphPad, San Diego, California). Tukey-Kramer multiple comparisons test was also used, and the significance was accepted for P < .05.

Results

TAM treatment induces ER-α36 protein concentration in ER-positive breast cancer MCF7 cells

Previously, our laboratory identified and cloned a 36-kDa variant of ER-α, ER-α36, that functions differently from the 66-kDa full-length ER-α, ER-α66 (13, 14). Using an ER-α36-specific antibody, we further found that ER-α36 is highly expressed in established ER-negative breast cancer cells while weakly expressed in ER-positive breast cancer cells such as MCF7 (14). In order to investigate ER-α36 function in the activities of antiestrogens, we first examined whether TAM influences ER-α36 expression in MCF7 cells. The steady state concentration of ER-α36 protein in MCF7 cells treated with 1 μM TAM for different time periods or different concentrations of TAM was examined with Western blot analysis. After TAM treatment, ER-α36 protein concentration was increased in MCF7 cells in a time- and concentration-dependent manner (Figure 1), indicating that TAM is able to increase ER-α36 concentration in ER-positive breast cancer MCF7 cells.

TAM-resistant ER-positive breast cancer MCF7 cells express high concentration of ER-α36 protein

To examine the possible involvement of ER-α36 in development of acquired TAM resistance, we cultured
MCF7 cells in the presence of TAM (1 μM) for 6 months and pooled all surviving cells to establish a cell line MCF7/TAM. This cell line exhibited resistance to the growth-inhibitory activity of TAM compared with the parental cells and TAM even acted as an agonist in MCF7/TAM cells (Figure 2A). Western blot analysis revealed that MCF7/TAM cells expressed higher concentration of ER-α36 protein compared with the MCF7 parental cells, whereas ER-α66 protein concentration was without significant change (Figure 2B), suggesting that MCF7 cells gained ER-α36 expression during development of acquired TAM resistance. MCF7/TAM cells also expressed increased concentrations of EGFR and HER2 proteins (Figure 2B), indicating that MCF7/TAM cells also gained expression and signaling of the EGFR/HER2 pathway.

High concentration of ER-α36 protein confers TAM resistance

To confirm that elevated concentration of ER-α36 protein is involved in TAM resistance, we sought to downregulate ER-α36 expression in MCF7/TAM cells using the shRNA approach. We established a cell line with knocked down concentration of ER-α36 protein (MCF7/TAM/Si36) from MCF7/TAM cells using the shRNA method as evidenced by Western blot analysis (Figure 3A). We also noticed that the concentrations of EGFR and HER2 were also decreased in MCF7/TAM/Si36 cells (Figure 3A). ER-α36 knockdown restored the sensitivity of MCF7/TAM cells to the growth-inhibitory effects of TAM to a level similar to parental MCF7 cells (Figure 3B). Our data thus suggested that elevated ER-α36 concentration is involved in development of acquired TAM resistance.

To further confirm that elevated ER-α36 expression contributes to TAM resistance, we introduced recombinant ER-α36 into MCF7 cells that express high concentration of ER-α66 protein but lower concentration of ER-α36 to establish a stable cell line, MCF7/ER36. Western blot analysis confirmed that recombinant ER-α36 protein was highly expressed in MCF7/ER36 cells compared with the control MCF7 cells transfected with the empty expression vector (Figure 4A). We also observed that the EGFR expression was strongly increased, whereas the HER2 expression was not significantly increased.
expression was weakly increased in MCF7/ER36 cells (Figure 4A), consistent with our previous report that ER-α36 stabilizes EGFR protein (19). When MCF7/ER36 cells were treated with different concentrations of TAM, these cells are more resistant to the growth-inhibitory effects of TAM compared with the control MCF7 cells (Figure 4B), indicating that increased ER-α36 concentration is one of the underlying mechanism of TAM resistance. We also found a breast cancer cell line H3396 that expressed high concentration of endogenous ER-α36 protein (Figure 4A). Like MCF7/ER36 cells, H3396 cells were more resistant to the growth-inhibitory effects of TAM compared with the control MCF7 cells (Figure 4B).

TAM induces AKT activation in cells expressing ER-α36

Previously, we found that TAM elicited agonist activities, such as activation of the MAPK/ERK and the PI3K/AKT pathways in ER-α36-expressing endometrial cells (15, 16). We sought to determine whether ER-α36 mediates agonist activity of TAM in cells with high concentrations of ER-α36. We first treated MCF7 cells with different concentrations of TAM, and the AKT phosphorylation was measured with Western blot analysis. In control MCF7 cells transfected with the empty expression vector (MCF7/Vector), we found that at lower concentrations from 1 μM to 3 μM, TAM induced the AKT phosphorylation, whereas at 4 μM–5 μM, it failed to do so (Figure 5B). However, TAM at different concentrations failed to induce AKT activation in MCF7 cells with ER-α36 knocked down (Figure 5, A and B), indicating that ER-α36 mediates agonist activity of TAM. However, in MCF7/TAM, H3396, and MCF7/ER36 cells, TAM potently induced the
AKT phosphorylation even at 4 μM–5 μM (Figure 5, C–E). In MCF7/TAM and H3396 cells, TAM induced AKT phosphorylation at 0.4 μM (Figure 5, D and E). To further confirm the role of ER-α36 in the agonist activity of TAM, we also used MCF/TAM and H3396 cells with knocked down concentrations of ER-α36 protein and found that TAM failed to induce AKT phosphorylation in these cells (Figure 5, D and E). Taken together, these results demonstrated that TAM acts as an agonist to induce AKT phosphorylation in cells expressing ER-α36, which provides an explanation to the involvement of ER-α36 in TAM resistance.

**ER-α36-expressing breast cancer cells exhibit estrogen hypersensitivity**

Previously, it was reported that cells deprived of estrogen for a long term exhibited hypersensitivity to estrogens (8). We decided to examine whether ER-α36 is involved in development of estrogen hypersensitivity. MCF7/TAM cells were treated with different concentrations of E2 for 7 days. We found that E2 stimulated stronger proliferation in these cells compared with the parental MCF7 cells (Figure 6A). In addition, MCF7/TAM cells exhibited hypersensitivity to E2; at pM range, E2 stimulated proliferation of MCF/TAM cells, whereas E2 stimulated proliferation of the parental MCF7 cells at nM range (Figure 6A). We also found that MCF7/ER36 cells that express recombinant ER-α36 and H3396 cells with high concentrations of endogenous ER-α36 protein also exhibited estrogen hypersensitivity (Figure 6B), suggesting that ER-α36 is involved in estrogen hypersensitivity.

We then examined E2-induced phosphorylation of the MAPK/ERK1/2, a typical nongenomic estrogen-signaling event, in different cell lines. Cells were treated with E2 at different concentrations for 30 minutes, and Western blot analysis with a phospho-specific ERK1/2 antibody was performed. Figure 6C shows that E2 elicited ERK phosphorylation in MCF/TAM cells in a dose-dependent manner starting at a very low concentration, 1 × 10⁻¹⁴ M/L, whereas in the parental MCF7 cells and MCF/
Vector cells, ERK activation requires E2 at $1 \times 10^{-12}$ M/L (Figure 6, C and D). A similar hypersensitivity was also observed in MCF7/ERα36 and H3396 cells (Figure 6, D and E); E2 induced ERK phosphorylation at $1 \times 10^{-14}$ M/L. Our data thus suggested that increased concentration of ER-α36 protein is one of the mechanisms underlying estrogen hypersensitivity.

**Discussion**

TAM therapy is the most effective treatment for advanced ER-positive breast cancer, but its effectiveness is limited by high rate of de novo resistance and resistance acquired during treatment. Many studies were conducted to understand the molecular pathways responsible for the de novo and acquired TAM resistance and have revealed that multiple signaling molecules and pathways are involved in TAM resistance. All of these pathways often bypass the requirement of estrogen signaling pathway for growth of ER-positive breast cancer cells. Previously, we reported that the breast cancer patients with tumors expressing high concentrations of endogenous ER-α36 benefited less from TAM therapy than those with low concentrations of ER-α36 (16), suggesting that elevated concentration of ER-α36 protein may be a novel mechanism underlying both de novo and acquired TAM resistance.

Here, we showed that TAM treatment induced ER-α36 expression and TAM-resistant MCF7/TAM cells selected with long-term cultivation in the presence of TAM expressed elevated concentration of ER-α36 protein. We also showed that MCF7 cells with forced ER-α36 expression and H3396 cells that express high concentration of endogenous ER-α36 protein were relatively more resistant to TAM compared with MCF7 cells. Down-regulation of ER-α36 expression, however, was able to restore TAM sensitivity in MCF/TAM and H3396 cells, indicating that increased ER-α36 concentration is one of the molecular mechanisms by which ER-positive breast cancer develops TAM resistance.

Previously, we found that antiestrogens TAM and ICI 182, 780 failed to block ER-α36-mediated nongenomic estrogen signaling (14). Here, we showed that TAM exhibited a biphasic activation of the AKT kinase in TAM-sensitive MCF7 cells; increasing AKT phosphorylation at low concentrations and failed to do so at higher concentrations. However, in cells with high concentrations of ER-α36 protein, TAM still activates the AKT kinase at higher concentrations, consistent with our recent report that ER-α36 mediates agonist activities of both TAM and ICI 182, 780 (18). Recently, loss of p21 (CDKN1A), a cyclin-dependent kinase inhibitor, was found to be associated with the agonist activity of TAM (20). Likewise, inhibition of p27 (CDKN1B), another cyclin-dependent kinase inhibitor, by Src has been associated with a TAM-resistance phenotype (21). Both p21 and p27 are phosphorylated by the AKT kinase, and this phosphorylation banishes both p21 and p27 from the cell nucleus and keeps them in the cytoplasm (22, 23). Thus, loss of expression and function, and relocalization of either of two G1-checkpoint cyclin-dependent kinase (CDK) inhibitors after AKT phosphorylation, can lead to TAM resistance. We found that in TAM-sensitive MCF7 cells, TAM down-regulated p27 phosphorylation and increased concentration of p27 protein, whereas TAM up-regulated p27 phosphorylation and decreased concentration of p27 protein (our unpublished data). Our results suggested that ER-α36-mediated agonist activity of TAM, such as activation of the PI3K/AKT signaling, is important for ER-α36 function in TAM resistance.

Previously, another acquired TAM-resistance phenotype has been described in a human breast cancer xenograft model that exhibits a switch from a TAM-inhibitory phenotype to a TAM-stimulated one. Some breast cancers may be initially inhibited by TAM and later become dependent on TAM for proliferation (5, 6, 24). These xenografts also retain the ability to be stimulated by estrogens (5, 6, 24). In the current study, we found that 1μM TAM stimulated proliferation of MCF7/TAM cells, whereas down-regulation of ER-α36 expression in these cells diminished TAM stimulation. In addition, these TAM-resistant cells retained estrogen responsiveness and even showed estrogen hypersensitivity. Our results thus suggested that elevated ER-α36 concentration is involved in this type of TAM resistance. It also worth noting that the TAM at 1μM failed to stimulate proliferation of MCF7/36 cells that express recombinant ER-α36 and H3396 that express endogenous ER-α36 (Figure 4B). The exact mechanism for this is not known. We observed that TAM-resistant MCF7/TAM cells also gained expression of the growth factor receptors EGFR and HER2, whereas MCF7/ERα36 cells mainly increased expression of the growth factor receptors EGFR and HER2, whereas MCF7/ERα36 cells mainly increased expression of EGFR protein, and H3396 cells only express modest concentration of EGFR. Thus, it is possible that increased expression or signaling of the HER2 receptor in MCF7/TAM cells contributes to the TAM-stimulated proliferation in MCF7/TAM cells. Intriguingly, the expression of both EGFR and HER2 was down-regulated in MCF7/TAM/Si36 cells, consistent with our recent reports, there are positive regulatory loops between ER-α36 and the EGFR/HER2 expression; ER-α36 stabilizes EGFR protein and activates HER2 promoter activity, whereas the signaling of EGFR/HER2 induces ER-α36 expression (19, 25).
Previously, it has been reported that physiological concentrations of E2 exhibit antitumor activity in a TAM-stimulatory MCF7 cell model that was generated by serial transplantation of TAM-resistant tumors in the continuous presence of TAM (26). Based on the laboratory studies, it was recently proposed that physiological concentration of estrogen could be used as a therapeutic approach for these TAM-resistant patients (27, 28). However, the molecular mechanisms underlying this paradoxical phenomenon have not been well elucidated. It is known that estrogen stimulates growth of ER-positive breast cancer cells in a biphasic growth curve, stimulating cell proliferation at low concentrations while failing to stimulate or even inhibiting cell growth at higher concentrations. Our results presented here that elevated ER-α36 concentration rendered cells hypersensitive to E2, shifting the biphasic growth curve to the left. Thus, in cells expressing high concentrations of ER-α36 protein, physiological concentrations of E2 may fail to stimulate proliferation or even inhibit proliferation. Our data thus provided a molecular explanation to the paradoxical phenomenon that some TAM-resistant tumors are simulated by TAM but inhibited by estrogen.

Previously, it was reported that long-term estrogen deprivation with hormonal therapy resulted in “adaptive” changes of breast cancer cells, making these cells hypersensitive to estrogen (8, 11). Recently, we reported that ER-α36 concentration is significantly increased in normal osteoblasts cells from menopausal women (29), suggesting that ER-α36 expression is elevated in response to low concentration of estrogen in menopausal women. Our current data showed that E2 induced ERK phosphorylation and stimulated proliferation at pM range in cells with high concentration of ER-α36 protein while at nM range in cells with low concentration of ER-α36. Thus, our results indicated that gained ER-α36 expression is one of the adaptive changes in breast cancer cells after a long-term estrogen deprivation resulted from antiestrogen treatment.

In summary, here, we provided evidence to demonstrate that ER-α36 is a novel and important player in normal and abnormal estrogen signaling, and ER-α36 is involved in many physiological and pathological processes regulated by estrogen signaling. Our findings that elevated ER-α36 concentration is one of the mechanisms by which ER-positive breast cancer cells escape the antiestrogen therapy provided a rational to develop novel therapeutic approaches for antiestrogen-resistant patients by targeting ER-α36.

Acknowledgments

Address all correspondence and requests for reprints to: Zhao-Yi Wang, Creighton University Medical School, Criss III, Room 352, 2500 California Plaza, Omaha, Nebraska 68178. E-mail: zywang@creighton.edu.

This work was supported by the Department of Defense Grant DAMD 11-1-0497 and the Nebraska Tobacco Settlement Biomedical Research Program Award LB-595 (to Z.-Y.W.).

Disclosure Summary: The authors have nothing to disclose.

References


