The HER4/4ICD Estrogen Receptor Coactivator and BH3-Only Protein Is an Effector of Tamoxifen-Induced Apoptosis

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Abstract

Greater than 40% of breast cancer patients treated with tamoxifen exhibit de novo or acquired tumor resistance. Recent clinical evidence indicates that loss of expression of HER4 is an independent marker for tamoxifen resistance. In direct corroboration with clinical observations, suppression of HER4 expression in the tamoxifen-sensitive MCF-7 and T47D breast tumor cell lines resulted in resistance to tamoxifen-induced apoptosis. Furthermore, HER4 expression was lost in three independent MCF-7 models of acquired tamoxifen resistance. The HER4 intracellular domain (4ICD) is an independently signaling nuclear protein that functions as a potent ERα coactivator. In addition, mitochondrial 4ICD functions as a proapoptotic BH3-only protein. Tamoxifen disrupts an estrogen-driven interaction between ERα and 4ICD while promoting mitochondrial accumulation of the 4ICD BH3-only protein. BCL-2 inhibition of tamoxifen-induced apoptosis and tamoxifen activation of BAK, independent of BAX, further supports a role for 4ICD during tamoxifen-induced apoptosis. Finally, reintroduction of HER4, but not HER4 with a mutated BH3 domain, restores tamoxifen sensitivity to tamoxifen-resistant T47D cells in a xenograft model. Clinically, breast cancer patients with tumor expression of nuclear 4ICD responded to tamoxifen therapy with no clinical failures reported after 14 years of follow-up, whereas 20% of patients lacking nuclear 4ICD expression succumbed to their disease within 10 years of diagnosis. Our identification of the HER4/4ICD BH3-only protein as a critical mediator of tamoxifen action provides a clinically important role for 4ICD in human cancer and reveals a potential tumor marker to predict patient response to tamoxifen therapy. [Cancer Res 2008;68(15):6387–95]

Introduction

The selective estrogen receptor (ERα) modulator tamoxifen has been used as a single agent for the treatment of ERα(+) breast cancers since 1971. Currently, tamoxifen is the most commonly prescribed antiestrogen therapy and is uniquely effective in the treatment of premenopausal woman with ERα(+) breast tumors. Unfortunately, de novo and acquired tumor resistance to tamoxifen are serious clinical problems. In fact, nearly 30% of patients with ERα(+) breast tumors fail to respond to tamoxifen and up to 40% of initial responders relapse and succumb to their disease (1–3). Because the exact molecular basis for the mechanism of tamoxifen action remains unclear, clinicians lack reliable clinicopathologic indicators to identify patients likely to fail tamoxifen therapy.

Elegant structural studies have shown that tamoxifen binding to ERα not only results in competitive inhibition of estrogen binding but tamoxifen also alters ERα structure, thereby occluding coactivator interactions with ERα essential for estrogen-stimulated tumor cell proliferation (4). As predicted by this mechanism of action, tamoxifen treatment of ERα(+) tumors results in cytostatic effects, reducing breast tumor cell proliferation in part by arresting cell cycle progression (5).

Less clear however is a mechanism to explain the clinical observation that tumors from patients undergoing tamoxifen treatment exhibit increased levels of apoptosis (6, 7). In addition, tamoxifen-induced tumor regression observed in multiple preclinical xenograft models cannot be explained by cytostatic effects alone, suggesting a role for apoptosis in these models as well (7). In multiple experimental systems, tamoxifen induces cytchrome c release from mitochondria (8, 9), the essential and committed step of the mitochondrial or intrinsic apoptotic pathway. Although tamoxifen alters the expression of multiple effectors of the intrinsic apoptotic cascade consistent with increased cell killing, including suppression of antiapoptotic BCL-2 and up-regulation of pro-apoptotic BAX (10–12), the contribution of these alterations to tamoxifen activity in breast tumors requires clinical verification.

Recent clinical studies have revealed that tumor expression of the receptor tyrosine kinase HER4/ERBB4 (referred to here as HER4) improves the overall survival of breast cancer patients with ERα(+) tumors (13–15), raising the possibility that HER4 influences patient response to endocrine therapy. HER4 is a unique member of the epidermal growth factor receptor (EGFR) family and undergoes proteolytic processing at the cell surface to release a HER4 intracellular domain (4ICD; ref. 16) that independently regulates multiple divergent activities in breast tumor cells (17–19). For example, nuclear 4ICD functions as a potent ERα coactivator, directly interacting with ligand-associated ERα at promoters of estrogen response genes and contributing to estrogen-stimulated proliferation of breast tumor cells (19). In nonmalignant breast epithelium, nuclear 4ICD regulates differentiation and lactation in part through transcriptional coactivation of the mammary differentiation factor, STAT5A (20–24).

When excluded from the nucleus, however, cytosolic 4ICD accumulates within mitochondria (17, 18) and triggers breast tumor cell apoptosis through the activity of an intrinsic cell-killing BCL-2 homology 3 (BH3) domain (17). This novel activity for a
receptor tyrosine kinase has been confirmed clinically where we have shown that cytosolic 4ICD but not membrane-localized HER4 is strongly associated with increased breast tumor apoptosis (17). Importantly, HER4 regulation of gene expression and apoptosis are mediated by 4ICD released from the cell surface after proteolytic processing of HER4 (17–19, 21, 24). The molecular mechanisms regulating these divergent 4ICD activities are poorly understood. Here, we show for the first time that 4ICD is an important effector of tamoxifen-induced apoptosis of breast tumor cells. Our results are consistent with a mechanism of tamoxifen action involving tamoxifen disruption of the growth-promoting 4ICD/ERα coactivator complex, thereby unleashing the cell-killing activity of an unthethered 4ICD BH3-only protein.

Materials and Methods

Plasmids. The HER4 constructs used in this study were all derived from the HER4 JM-a/CytI isomorph. The HER4 expression plasmids pHER4-EGFP and pHER4muBH3-EGFP have been described previously (17, 24). The plasmid pErx-flag expressing a flag-tagged version of human ERα was provided by Dr. Brian Rowan (Tulane University, New Orleans, LA). The BCL2 expression plasmid pDNA-BCL2 was provided by the late Dr. Stanley Korsmeyer (Harvard University, Boston, MA).

To generate a plasmid expressing the HER2 oncogenic isoform HER2Δ16 (25), the region of HER2 harboring an exon 16 deletion was amplified by reverse transcription-PCR from BT474 RNA and subcloned into pLXSN-HER2 (26) harboring wild-type human HER2 to generate the clone pLXSN-HER2Δ16 using standard procedures. The exon 16 deletion was confirmed by sequencing. The plasmids pDNA-HER2 and pDNA3-HER2Δ16 were generated by subcloning the 4.5 kb Hind III fragment containing the HER2 open reading frame from pLXSN-HER2 or pLXSN-HER2Δ16, respectively, into the same sites of pDNA3 (Invitrogen).

Cell lines. The T47D and MCF-7 human breast cancer cell lines were purchased from the American Type Cell Culture and maintained according to the manufacturer's recommendations. The tamoxifen-resistant MCF-7 variants TamR and LCC2 have been described previously (17, 31). The LCC2 cell line provided by Dr. Robert Clarke (Georgetown University, Washington, DC) and maintained in Modified Eagle's Medium with 10% fetal bovine serum (FBS). The MCF-7/TAMRΔ16 cell line was obtained by stably transfecting MCF-7 cells with pDNA3-TAMRΔ16 (Invitrogen) using Fugene6 (Roche). Individual transfected colonies were selected in Geneticin (Invitrogen) and isolated using cloning cylinders. The TamR/Vector, TamR/HER4, and TamR/muBH3 cell lines were generated by stably transfecting the TamR cell line with pGFP3N (Clonetech), pHER4-EGFP (24), or pHER4muBH3-EGFP (17), respectively. Individual transfected cell colonies were selected in kanamycin and isolated using cloning cylinders.

Suppression of HER4 expression. To suppress expression of endogenous HER4, T47D or MCF-7 cells were transfected with erbB-4/HER4 Antisense SMARTpool using siIMPORTER transfection reagent (Upstate Biotechnology) according to the manufacturer's instructions. Cells similarly transfected with Non-specific Negative Control Pool (Upstate Biotechnology) were used as RNAi controls.

Assays for apoptosis. Apoptosis was quantitated visually using a Nikon fluorescent microscope to determine the percentage of cells with condensed chromatin after 4′,6-diamidino-2-phenylindole (DAPI) staining. All samples were prepared in duplicate, and each experiment was repeated at least thrice. Significant differences between data sets was determined using the paired Student's t test. Apoptosis in histologic sections from paraffin-embedded xenograft tumors was determined using the ApoTag Plus Peroxidase In Situ Apoptosis Detection kit (Chemicon International) exactly as described by the manufacturer. Data are represented as the mean percentage of terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling (TUNEL)-positive cells and SE from at least three individual tumors from each treatment group.

Immunoprecipitation and Western blot analysis. The HEK-293T cells were maintained in phenol red–free DMEM containing 5% charcoal-stripped FBS during the entire immunoprecipitation experiment. Coimmunoprecipitation of ERα and 4ICD was performed by transfecting 5 × 105 HEK 293T cells cultured in 100-mm dishes with pERX-flag and/or pHER4-EGFP using Lipofectamine with Plus reagent (Invitrogen) exactly as described by the manufacturer. At 24 h posttransfection, cells were treated with 100 pmol/L 17β-estradiol alone or in combination with 5 µmol/L tamoxifen for 1 h. Cell lysates were prepared and 1 mg of lysate were immunoprecipitated using α-Flag Agarose (Sigma) or rabbit α-HER4 (Cell Signaling) as described previously (29).

Immunoprecipitates and total cell lysates were analyzed by Western blot as described elsewhere (29) with primary antibodies HER4 (Cell Signaling), Flag M2 (Sigma), α-tubulin (Upstate Biotechnology), TOM40 (Santa Cruz Biotechnology), BAK (Upstate Biotechnology), and BAX (Upstate Biotechnology).

Analysis of isolated mitochondria for 4ICD localization and BAK or BAX dimerization. T47D cells were cultured in phenol red-free RPMI containing 5% charcoal-stripped FBS for 2 d and then stimulated for 1 h with 50 ng/ml of heregulin-β1 (HRG; R&D Systems), 12 h with 1 µmol/L staurosporine (Sigma), or 1 h with 100 pmol/L 17β-estradiol alone or in combination with 5 µmol/L tamoxifen. Mitochondria were isolated and analyzed by Western blot for 4ICD localization or for BAK and BAX dimerization as described elsewhere (17).

Tumor formation in nude mice. Four- to 5-wk-old NU/NU immune-compromised female mice (Charles-River Laboratories) were implanted s.c. in the scapular region with a 60-d release 0.72-mg estradiol pellet (Innovative Research of America) using a precision trochar. Seven days later, the mice were injected s.c. at two rear flank sites with each injection site containing 5 × 106 cells in 100 µL of sterile PBS mixed with 100 µL of Matrigel Basement Membrane Matrix (BD Biosciences). Tumors were allowed to develop for 15 d at which time mice were randomized and implanted with a 60-d release 5 mg tamoxifen pellet (Innovative Research of America) or a 60-d release 5-mg placebo (Innovative Research of America). All mice were anesthetized using isoflurane/Oxigen before surgery. Tumor size was measured every 3 d using a digital caliper, and tumor volume was calculated. Statistical analysis of the data set was performed using the Kruskal-Wallis Test (30).

Immunohistochemical analysis of xenograft tumors. Two hours before sacrifice, all mice were injected Lp. with 10 µL/g body weight of BrdUrd Cell Labeling Reagent (Amersham). Tumors were excised, fixed overnight in 4% paraformaldehyde, and embedded in paraflin using standard histologic procedures. Immunohistochemical analysis of proliferating cells by BrdUrd incorporation and apoptosis by TUNEL assay have been described in detail elsewhere (17, 31).

Patient population and HER4 immunohistochemistry. A total of 791 archived formalin-fixed paraffin-embedded breast tumors from patients diagnosed at Massachusetts General Hospital between 1976 and 1983 were used for this study. Follow-up for a mean of 15.6 y was used to determine patient outcome after surgery and adjuvant intervention. Additional clinical and pathologic details of this cohort have been described elsewhere (32).

HER4 immunohistochemistry was performed as described previously (17) using HER4 Ab-4 (Neomarkers; Clone HFR-1) directed against the carboxyl terminus of human HER4 at a concentration of 4.0 µg/mL. A tumor was considered positive for nuclear 4ICD if >10% of tumor nuclei exhibited positive immunohistochemical staining. Associations between nuclear 4ICD and patient survival were determined using the Fisher's exact test.

Results

HER4 expression regulates tamoxifen-induced apoptosis of ERα (+) breast tumor cells. Several recent clinical studies indicate that HER4 expression improves the prognosis of patients with ERα (+) breast tumors (13–15). Because the majority of these patients would have received endocrine therapy as part of their therapeutic regimen with tamoxifen being the most commonly prescribed endocrine therapy, we tested the possibility that HER4 regulates tamoxifen response of ERα (+) breast tumor cells. To this
end, we examined the effect of tamoxifen cell killing on the ERα(+), and HER4(+). MCF-7 and T47D breast cancer cell lines after RNA interference (RNAi)-mediated knockdown of HER4 expression. Although tamoxifen has cytostatic effects, consistent with other reports, we observed a dramatic increase in both MCF-7 and T47D cell killing after treatment with physiologic levels of tamoxifen (Fig. 1A). Interestingly, suppression of HER4 expression in each cell line by RNAi pretreatment dramatically inhibited the ability of tamoxifen to induce apoptosis after 24 hours and completely abolished tamoxifen cell-killing activity after 48 hours (Fig. 1A). These results implicate HER4 as an important regulator of tamoxifen-induced apoptosis of ERα(+) breast tumor cells and provide a molecular explanation for the improved prognosis of patients with ERα(+) tumors that also express HER4 (13–15).

Our results coupled with clinical data suggest that loss of HER4 expression may contribute to tamoxifen refractory breast cancer. To test this hypothesis, we examined HER4 expression in three established preclinical cellular models of tamoxifen resistance. The tamoxifen-resistant LCC2 (28) and TamR (27) cell lines were generated in independent laboratories by continuous exposure of MCF-7 cells to tamoxifen. The tamoxifen-resistant HER2Δ16 cell line was derived in our laboratory by overexpressing an oncogenic isoform of HER2 in the MCF-7 cell line. When compared with parental MCF-7 cells, HER4 expression was abolished in each tamoxifen-resistant cell line examined (Fig. 1B), and as predicted, each cell line was resistant to tamoxifen-induced apoptosis (Fig. 1C). Taken together, our results strongly implicate HER4 as an important mediator of tamoxifen-induced cell killing of breast tumor cells. By extension, loss of HER4 expression may represent a clinically important mechanism contributing to tamoxifen refractory breast tumors.

Tamoxifen disrupts the ERα/4ICD transcriptional coactivator complex. We next determined the molecular mechanism underlying the ability of HER4 to regulate tamoxifen response of breast tumor cells. HER4 is a unique multifunctional member of the EGFR family that undergoes proteolytic processing at the cell surface to release the independently signaling 4ICD. We have shown that 4ICD is a potent ERα coactivator that directly interacts with ERα (19). Tamoxifen functions, in part, by altering ERα structure, thereby occluding interactions between ERα and coactivators. We therefore determined if tamoxifen disrupts the interaction between ERα and the 4ICD coactivator. Consistent with our published results (19), 4ICD and ERα were coimmunoprecipitated from transfected HEK 293T cells and the amount of 4ICD/ERα coimmunoprecipitated complexes was augmented by the addition of estrogen (Fig. 2, compare I0 and I1). Tamoxifen treatment, however, disrupted immunoprecipitable 4ICD/ERα complexes (Fig. 2, compare I1 and I2). These results provide further evidence that 4ICD functions as a canonical ERα coactivator whose interaction with ERα is disrupted by tamoxifen.

Tamoxifen promotes mitochondrial accumulation of the 4ICD BH3-only protein and activation of the intrinsic apoptotic pathway. In addition to the coactivator function of 4ICD, we have also shown that 4ICD translocates to mitochondria where it functions as a proapoptotic BH3-only protein (17, 18). By disengaging 4ICD coactivator activity, tamoxifen may induce cell killing of breast tumor cells by serendipitously activation of the untethered 4ICD BH3-only protein. In response to a specific apoptotic signal, BH3-only proteins initiate cell killing by translocating to the mitochondria where they promote activation of the apoptotic gateway proteins BAX and BAK (33).

To determine if tamoxifen promotes 4ICD BH3 domain-mediated cell killing, we first examined mitochondrial accumulation of 4ICD in response to tamoxifen. Western blot analysis of mitochondrial extracts prepared from T47D cells revealed low
basal levels of mitochondrial 4ICD that was depleted after estrogen treatment (Fig. 3A). The addition of tamoxifen, however, resulted in a dramatic increase in 4ICD mitochondrial accumulation (Fig. 3A).

Tamoxifen treatment of MCF-7 or T47D cells did not, however, alter HER4 expression levels or 4ICD processing when compared with estrogen-treated cells (Supplementary Fig. S1B). Taken together, these observations suggest that tamoxifen disruption of the ERα/4ICD coactivator complex results in mitochondrial localization of the untethered 4ICD BH3-only protein.

To confirm that tamoxifen-induced cell killing involved proteolytic processing of HER4 to generate 4ICD, we blocked HER4 processing by incubating T47D and MCF-7 cells with γ-secretase inhibitors of γ-secretase and BCL-2 expression suppress tamoxifen induced apoptosis of T47D cells. T47D cells were cultured in phenol red–free RPMI containing 5% charcoal-stripped FBS for 48 h and treated with 100 pmol/L 17-β-estradiol alone or in combination with 5 μmol/L 4-hydroxytamoxifen for 48 h. HER4 processing to generate 4ICD was prevented by the addition of 20 nmol/L γ-secretase Inhibitor XXI (Compound E; CE) to the 17-β-estradiol and tamoxifen treatments. Apoptosis was determined visually in a Nikon fluorescent microscope by staining the cells with DAPI and determining the percentage of cells with condensed nuclei (mean ± SE of at least three experiments).

Figure 2. Tamoxifen disrupts the ERα/4ICD coactivator complex. HEK 293T cells were cultured in phenol red–free DMEM containing 5% charcoal-stripped FBS for 24 h then transfected with ERα-flag and/or HER4 expression plasmids. At 24 h posttransfection, cells were left untreated or treated with 100 pmol/L 17-β-estradiol alone or in combination with 5 μmol/L 4-hydroxytamoxifen for 1 h. Coimmunoprecipitation of ERα and 4ICD was analyzed by Western blot. Tamoxifen disrupts both the basal and estrogen stimulated ERα interaction with 4ICD. IP, immunoprecipitation; IB, immunoblotting.

Figure 3. Tamoxifen activates the proapoptotic 4ICD BH3-only protein. A, tamoxifen stimulates mitochondrial accumulation of the 4ICD BH3-only protein. T47D cells were cultured in phenol red–free RPMI containing 5% charcoal-stripped FBS for 48 h, then were left untreated or treated with 100 pmol/L 17-β-estradiol alone or in combination with 5 μmol/L 4-hydroxytamoxifen for 1 h. Mitochondria were isolated and analyzed by Western blot for accumulation of 4ICD. Analysis of TOM40 was included as a loading control. B, inhibitors of γ-secretase and BCL-2 expression suppress tamoxifen induced apoptosis of T47D cells. T47D cells were cultured in phenol red–free RPMI containing 5% charcoal-stripped FBS for 48 h and treated with 100 pmol/L 17-β-estradiol alone or in combination with 5 μmol/L γ-secretase Inhibitor XXI (Compound E; CE) to the 17-β-estradiol and tamoxifen treatments. Apoptosis was determined visually in a Nikon fluorescent microscope by staining the cells with DAPI and determining the percentage of cells with condensed nuclei (mean ± SE of at least three experiments). C, tamoxifen stimulates dimerized activation of mitochondrial BAK. T47D cells were cultured in phenol red–free RPMI containing 5% charcoal-stripped FBS for 48 h then left untreated or treated with 50 ng/mL of HRG, or 100 pmol/L 17-β-estradiol alone or in combination with 5 μmol/L 4-hydroxytamoxifen for 1 h. Mitochondrial extracts were analyzed by Western blot for BAX-dimerized activation.
inactivating BH3 domain–mediated 4ICD cell killing remained striking contrast, TamR cells expressing HER4-harboring mutations in a xenograft tumor model and determined if HER4 also mediated apoptosis of breast tumor cells. Protein as an essential mediator of tamoxifen-regulated apoptosis and strongly implicate the 4ICD BH3-only protein as a critical regulator of tamoxifen activity in vivo. TamR cells expressing HER4 or HER4 with a mutated BH3 domain and treated with placebo both exhibited growth kinetics similar to tamoxifen-treated TamR/HER4 tumors (data not shown).

To determine if tumor regression in HER4-expressing TamR cells was due to increased tumor apoptosis, we examined xenograft tumors histologically for proliferation by BrdUrd incorporation and apoptosis by TUNEL. A significant difference in the levels of tamoxifen-induced apoptosis was observed, with levels of apoptosis in TamR/HER4 tumors >4% compared with <1% observed in TamR and TamR/muBH3 tumors (Fig. 5A and B). The TamR and TamR/muBH3 tumors were however highly proliferative with 14% and 18% of nuclei incorporating BrdUrd, respectively. In contrast, we rarely observed BrdUrd-positive nuclei in the TamR/HER4 tumors (Fig. 5A and B). These results suggest that TamR/HER4 tumor regression in response to tamoxifen is due to a combination of increased apoptosis and impaired proliferation.

**Nuclear 4ICD expression improves patient response to tamoxifen therapy.** To determine the clinical effect of nuclear 4ICD expression on survival of breast cancer patients treated with tamoxifen as a single agent, we analyzed HER4 expression in a cohort of 791 patients. To eliminate complicating and unpredictable factors associated with a combined therapeutic regimen, we focused our analysis on ER/PgR (+) patients that were treated with tamoxifen as a single agent. This is a relatively rare cohort and was limited to 42 patients with 17 patients expressing nuclear 4ICD (Fig. 6A) and 25 patients lacking nuclear 4ICD (Fig. 6A). Despite the small sample size, the results were quite striking with no therapeutic failures observed in the 17 tamoxifen-treated patients with tumor expression of nuclear 4ICD (Fig. 6C). In contrast, 20% (5 of 25) of tamoxifen-treated patients lacking HER4/4ICD tumor expression succumbed to their disease within 10 years of diagnosis (Fig. 6C). These data approached significance with a Fisher’s exact test result of a P value of 0.06. These clinical observations support our preclinical data and further suggest that tamoxifen disruption of the nuclear 4ICD and ERα transcriptional complex results in tumor cell killing mediated by the untethered 4ICD proapoptotic protein. Our data are in concordance with another recent clinical study implicating HER4/4ICD expression as an independent tumor marker for patient response to tamoxifen (34).

**Discussion**

Despite widespread clinical use for over 30 years, the molecular regulators of tamoxifen action remain poorly understood. On the one hand, tamoxifen exerts cytostatic effects on some ER (+) breast tumors, which can be explained in part by the ability of tamoxifen to disrupt growth-promoting ERα and coactivator complexes. On
the other hand, a molecular mechanism explaining how tamoxifen disruption of ERα and coactivators contributes to tamoxifen-induced apoptosis of breast tumor cells is less clear. In the current study, we provide multiple lines of experimental evidence, including a preclinical xenograft model, to implicate the 4ICD BH3-only protein as a critical mediator of tamoxifen-induced breast tumor cell killing. For example, in the ER(+)/HER4(+) and tamoxifen-sensitive MCF-7 and T47D breast tumor cell lines, de novo tamoxifen resistance was induced in each cell line when HER4 expression was suppressed using an RNAi strategy. Furthermore, we found that HER4 expression was suppressed in multiple MCF-7 models of acquired tamoxifen resistance developed in independent laboratories with different experimental manipulations including continuous tamoxifen exposure (TamR and LCC2) or HER2 overexpression (HER2Δ16). Indeed, one of the most common models of acquired tamoxifen resistance, the MCF-7/HER18 model developed by the Osborne laboratory, also exhibits suppressed HER4 expression (35). Significantly, when we reintroduced HER4 but not HER4 with a mutated BH3 domain into the TamR MCF-7 variant, tamoxifen sensitivity was restored both in vitro and in a preclinical xenograft model. Taken together, our data establishes an important role for the 4ICD BH3 cell-killing domain in tamoxifen response.

As a cell surface receptor, HER4 must use novel molecular mechanisms to regulate tamoxifen action. HER4 is a unique member of the EGFR family and undergoes proteolytic processing at the cell surface to release the 4ICD transcriptional coactivator. Indeed, in multiple experimental systems, the ability of HER4 to modulate gene expression requires presenilin-dependent γ-secretase cleavage to release the soluble 4ICD transcriptional regulator (18, 19, 21, 24). We have previously shown that similar to other ERα coactivators, 4ICD functions in part by disrupting ERα and coactivator complexes (4). Consistent with the role of 4ICD as an ERα coactivator, we show here that tamoxifen significantly impairs basal and estrogen-stimulated 4ICD complex formation with ERα. It remains unclear however if
tamoxifen disrupts a cytosolic or DNA-bound nuclear ERα/4ICD complex.

Our current results suggest that disruption of the 4ICD/ERα transcriptional complex contributes to tumor regression in response to tamoxifen through activation of the proapoptotic 4ICD BH3 only protein. Indeed, we show that tamoxifen-induced cell killing can be abolished by overexpression of BCL-2. BCL-2 protects tumor cells from apoptosis by sequestering proapoptotic BH3-only protein members, thus implicating a role for a BH3-only protein in tamoxifen cell killing. Importantly, we have previously shown that 4ICD harbors many of the functional characteristics of a proapoptotic BH3-only protein member of the BCL-2 family. For example, when localized to mitochondria, 4ICD induces apoptosis in a BH3 domain–dependent manner through activation of the apoptosis gateway protein BAK. In addition, the cell-killing activity of 4ICD is abolished in cells overexpressing BCL-2 (17). Significantly, we show here that tamoxifen stimulates mitochondrial accumulation of 4ICD, presumably through disruption of the 4ICD/ERα transcriptional complex. Subsequent activation of mitochondrial BAK but not BAX in response to tamoxifen further implicates the 4ICD BH3-only protein as the proapoptotic mediator of tamoxifen-induced cell killing. Indeed, selective activation of BAK is a property unique to the 4ICD BH3-only protein (17). Furthermore, an intact 4ICD BH3 domain was required for ectopic HER4 to reestablish tamoxifen sensitivity in a resistant cell line both in vitro and in a xenograft model. In fact, xenograft tumors of HER4-expressing TamR cells fully regressed with a significant increase in tumor apoptosis when compared with control or tumors expressing HER4 with a mutated 4ICD BH3 domain. Taken together, these results provide important in vitro and preclinical in vivo evidence for 4ICD BH3-domain–mediated cell-killing activity as an important regulator of tamoxifen response in breast cancer.

Based on our hypothesis that disruption of the nuclear ERα/4ICD transcriptional complex mediates tamoxifen-induced cell killing through release of the 4ICD proapoptotic protein, one would predict that patients with nuclear 4ICD expression will exhibit improved response to tamoxifen. Our clinical data provide compelling support for this hypothesis. After 14 years of follow-up, we have not observed a single failure in our patients whose tumors express nuclear 4ICD and received tamoxifen as their sole therapeutic intervention. Activation of the 4ICD BH3-only protein through disruption of ERα/4ICD transcriptional complexes may emerge as a common mechanism of action for endocrine therapies that target ERα function. For example, HER4 expression was also suppressed in a panel of fulvestrant-resistant breast tumor cell lines (36). Fulvestrant treatment, which leads to ERα degradation, may also disengage ERα/4ICD complexes and promote fortuitous activation of 4ICD cell-killing activity.

There exists a formal possibility that loss of HER4 expression contributes to tamoxifen resistance through disengaged 4ICD coactivation of estrogen-regulated genes. However, mutation of the 4ICD BH3 domain failed to affect HER4 stimulation of estrogen-induced gene expression (Supplementary Fig. S1C), whereas this same functional alteration of HER4 abolished the ability of HER4 to restore tamoxifen sensitivity in a resistant cell line. These results further implicate a role for the 4ICD BH3-only protein in tamoxifen response independent of 4ICD coactivator function.

In summary, we show that suppression of HER4 expression in multiple breast tumor model systems results in tamoxifen resistance, and we further show that the proapoptotic BH3 domain of 4ICD is an important mediator of tamoxifen-induced cell killing and tumor regression in a preclinical xenograft model. In direct corroboration with our preclinical data, a recent analysis of primary breast tumors from patients treated with tamoxifen as a single agent revealed that suppressed HER4 expression was an independent tumor marker for tamoxifen resistance in a multivariate analysis (34). Our clinical results indicating a profound effect of nuclear 4ICD expression on breast cancer patient survival after tamoxifen treatment further implicates 4ICD as a critical mediator of tamoxifen activity and as an important tumor marker for predicting patient response to endocrine therapy. Emerging evidence suggests that the subcellular distribution of cell-surface receptors and their intracellular domains has a profound effect on tumor biology (13, 15, 17, 37, 38). Here, we provide a proof-of-hypothesis to support further studies aimed at deciphering and clinically validating the molecular mechanisms regulating 4ICD subcellular localization and divergent 4ICD activities directly influencing breast cancer progression and therapeutic response.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Figure 6. Nuclear 4ICD expression predicts patient response to tamoxifen. A, example of primary breast tumor staining negative and positive (B) for nuclear 4ICD by immunohistochemistry. C, Kaplan-Meier survival curves for tamoxifen-treated ER/PgR(+) patients by presence or absence of nuclear 4ICD tumor expression. Statistical analysis requires at least one failure in each group. None of the patients with nuclear 4ICD (n = 17) failed tamoxifen therapy with 14 y of follow-up.

References

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