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 extracellular domain (4ED) 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. BCI-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 TamR 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 10 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 cytosstatic 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 cytosstatic effects alone, suggesting a role for apoptosis in these models as well (7). In multiple experimental systems, tamoxifen induces cytochrome 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 mammmary 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 untethered 4ICD BH3-only protein.

Materials and Methods

Plasmids. The HER4 constructs used in this study were all derived from the HER4 Jm-a-Cyt1 isoform. The HER4 expression plasmids pH4E4-EGFP and pH4E4muBH3-EGFP have been described previously (17, 24). The plasmid pRex-flag expressing a flag-tagged version of human ERα was provided by Dr. Brian Rowan (Tulane University, New Orleans, LA). The BCL-2 expression plasmid pcDNA-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 pLSXN-HER2 (26) harboring wild-type human HER2 to generate the clone pLSXN-HER2Δ16 using standard procedures. The exon 16 deletion was confirmed by sequencing. The plasmids pcDNA3-HER2 and pcDNA3-HER2Δ16 were generated by subcloning the 4.5 kb Hind III fragment containing the HER2 open reading frame from pLSXN-HER2 or pLSXN-HER2Δ16, respectively, into the same sites of pcDNA3 (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 (27, 28). 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/TamR2Δ16 cell line was obtained by stably transfecting MCF-7 cells with pcDNA3-HER2Δ16 (Invitrogen) using FuGene 6 (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 pEGFPN3 (Clontech), pH4E4-EGFP (24), or pH4E4muBH3-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 siRNA 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 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).

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 abo-
lished 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 refrac-
tory breast tumors.

Tamoxifen disrupts the ERα/4ICD transcriptional coactiva-
tor 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 ERαs and
coactivators. We therefore determined if tamoxifen disrupts the
interaction between ERαs and the 4ICD coactivator. Consistent with
our published results (19), 4ICD and ERαs were coimmunoprecipi-
tated 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 coacti-
vator whose interaction with ERαs 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 serendipitous activation of the
untethered 4ICD BH3-only protein. In response to a specific apop-
totic signal, BH3-only proteins initiate cell killing by translocating
to the mitochondria where they promote activation of the apop-
totic gateway proteins BAX and BAK (33).

Figure 1. HER4 expression regulates tamoxifen-induced apoptosis of breast
tumor cells. A, suppression of HER4 expression by RNAi abolishes tamoxifen-
induced (5 μmol/L) apoptosis of T47D and MCF-7 ERα(+) breast tumor cell lines.
Cell lines were cultured in phenol red–free medium containing 5% charcoal-
stripped FBS 24 h before treatment with RNAi. Twelve hours after RNAi
expression, cells were left untreated or treated with 100 pmol/L 17-β-estradiol (E2)
alone or in combination with 5 μmol/L 4-hydroxytamoxifen (TAM) for 24 and 48 h.
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). *, significant differences in
each data set as determined by Student’s t test. B, HER4 expression is
suppressed in tamoxifen-resistant MCF-7 cell variants. Western blot analysis of
HER4 expression in cell lysates prepared from the parental MCF-7 cell line and
two tamoxifen-resistant variants TamR, LCC2, and HER2Δ16 each developed in
an independent laboratory. Analysis of α-tubulin expression is included as a
loading control. C, MCF-7 variants lacking HER4 expression are resistant to
tamoxifen-induced apoptosis. Each indicated cell line was cultured for 48 h in
phenol red–free MEM containing 5% charcoal-stripped FBS and then treated for
24 h with 100 pmol/L 17-β-estradiol (Estrogen) alone or in combination with
5 μmol/L 4-hydroxytamoxifen (Tamoxifen). Apoptosis was determined visually
in a Nikon fluorescent microscope by staining the cells with DAPI and
determining the percentage of cells with condensed nuclei.

To determine if tamoxifen promotes 4ICD BH3 domain-
mediated cell killing, we first examined mitochondrial accumula-
tion 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).
HER4/4ICD Regulates Tamoxifen Response

A refractory to tamoxifen-induced apoptosis (Fig. 4) striking contrast, TamR cells expressing HER4-harboring mutations of breast tumor cells. The ability of antiapoptotic BCL-2 to suppress tamoxifen-induced apoptosis in T47D cells. BCL-2 suppresses BH3-only protein-mediated mitochondrial dysfunction by interacting with and sequestering proapoptotic BH3-only proteins (33). Similarly, we have previously shown that BCL-2 interacts with and suppresses the apoptotic activity of 4ICD (17). BCL-2 overexpression completely abolished tamoxifen-induced apoptosis of T47D cells, implicating a role for a BH3-only protein in tamoxifen-mediated breast tumor cell killing (Fig. 3D). Similar results were observed with tamoxifen-treated MCF-7 cells overexpressing BCL-2 (data not shown). BH3-only proteins initiate mitochondrial dysfunction and apoptosis through activation of both BAK and BAX mitochondria pore-forming complexes. Interestingly, 4ICD is unique among BH3-only protein family members in that 4ICD mediates cell killing exclusively through BAK activation, whereas BAX remains unaffected. We therefore examined BAK and BAX-dimerized activation in T47D cells after tamoxifen treatment. Consistent with our previous results, stimulation of T47D cells with the HER4 ligand heregulin, which promotes 4ICD apoptotic activity (17), resulted in dramatic conversion of monomeric BAK to the activated dimer form (Fig. 3C; Supplementary Fig. S2) but had minimal effect on BAX dimerization (Fig. 3D; Supplementary Fig. S2). Similarly, tamoxifen also induced significant levels of BAK dimerization but failed to activate BAX (Fig. 3C and D; Supplementary Fig. S2). Stauroporine, which activates the intrinsic apoptotic pathway in a BH3-only protein–dependent manner, was included as a positive control for BAX dimerization (Fig. 3D). Taken together, these results provide compelling evidence that tamoxifen-induced cell killing of breast tumor cells involves mitochondrial dysfunction mediated by a BAK activating BH3-only protein. Tamoxifen-induced mitochondrial localization of 4ICD coupled with selective activation of BAK further implicates 4ICD as the critical BH3-only protein-regulating tamoxifen-induced apoptosis.

HER4 with an intact 4ICD BH3-domain reverses tamoxifen refractory breast tumor cells to a tamoxifen-sensitive phenotype. We have shown that independently isolated MCF-7 cell line variants with acquired tamoxifen resistance also exhibit suppressed HER4 expression (Fig. 1B). To determine if 4ICD BH3 domain activity can restore tamoxifen sensitivity, wild-type HER4 or HER4 with BH3 domain inactivating mutations (muBH3) were stably reintroduced into the tamoxifen-resistant MCF-7 variant TamR. Significantly, TamR cells with reintroduced HER4 reverted to a tamoxifen-sensitive phenotype with levels of tamoxifen-induced apoptosis equivalent to the parental MCF-7 cells line (Fig. 4A). In striking contrast, TamR cells expressing HER4-harboring mutations inactivating BH3 domain–mediated 4ICD cell killing remained refractory to tamoxifen-induced apoptosis (Fig. 4A). Taken together, these results show that reintroduced HER4 expression can restore tamoxifen sensitivity and strongly implicate the 4ICD BH3-only protein as an essential mediator of tamoxifen-regulated apoptosis of breast tumor cells.

Tamoxifen-induced tumor regression is mediated by the HER4/4ICD BH3-only protein. We extended our in vitro results to a xenograft tumor model and determined if HER4 also mediated tumor regression after tamoxifen treatment. The modified TamR cell lines were injected into the flanks of estrogen-primed nude mice and tumors were allowed to develop to ~100 mm3. After 15 days, established tumors were exposed to tamoxifen or placebo, and tumor volumes were recorded during the 21-day experiment. As expected, the TamR tumors were resistant to tamoxifen treatment with a final tumor volume equivalent to the placebo-treated TamR tumors (Fig. 4B and C). Although TamR tumors expressing HER4 continued to grow for 6 days after tamoxifen exposure, these tumors rapidly regressed by day 9 of tamoxifen treatment (Fig. 4C). Significantly, TamR tumors expressing HER4 with an inactivated BH3 domain continued to grow in the presence of tamoxifen (Fig. 4B and C), providing compelling evidence that the HER4/4ICD BH3-only protein is 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/muBH3 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 expression (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 Erα (+) 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 directly interacts with ERα at selective estrogen-regulated gene promoters in an estrogen-dependent manner. Tamoxifen 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 BCL2 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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References

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