CtIP Silencing as a Novel Mechanism of Tamoxifen Resistance in Breast Cancer

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Abstract

Acquired resistance to the antiestrogen tamoxifen constitutes a major clinical challenge in breast cancer therapy. However, the mechanisms involved are still poorly understood. Using serial analysis of gene expression, we identified CtIP, a BRCA1- and CtBP-interacting protein, as one of the most significantly down-regulated transcripts in estrogen receptor α-positive (ER+) MCF-7 tamoxifen-resistant breast cancer cells. We further confirmed the association of CtIP down-regulation with tamoxifen resistance in an additional ER+ breast cancer line (T47D), strengthening the relevance of the phenomenon observed. In additional studies, we found CtIP protein expression in a majority of ER+ breast cancer cell lines that we tested, but no or very little CtIP expression in ER-negative lines. Furthermore, CtIP protein expression status correlates with clinical response to neoadjuvant endocrine therapy, and patients with progressive disease express significantly lower CtIP protein in their primary breast carcinomas than those who respond. Meta-analysis of seven publicly available gene expression microarray data sets showed that CtIP expression is significantly associated with ER, disease-free survival, and breast cancer metastasis status. Importantly, we found that silencing endogenous CtIP in tamoxifen-sensitive breast cancer cells confers tamoxifen resistance. On the other hand, reexpression of CtIP in tamoxifen-resistant breast cancer cells restores sensitivity to the inhibitory growth effects of tamoxifen. Together, our findings indicate that CtIP silencing might be a novel mechanism for the development of tamoxifen resistance in breast cancer, suggesting that CtIP is likely associated with ER function, and that CtIP gene and protein expression may be useful biomarkers for breast cancer prognosis and clinical management. (Mol Cancer Res 2007;5(12):1285–95)

Introduction

Estrogen plays a pivotal role in the etiology and progression of human breast cancer. Therefore, for a long time, treatment of breast cancer has been directed toward inhibiting the tumor-promoting effects of estrogen. Tamoxifen, a nonsteroidal antiestrogen, has been the gold standard for endocrine treatment of all stages of estrogen receptor α (ER) positive breast cancer for >25 years and was the first approved drug by the Food and Drug Administration as a cancer chemopreventive agent for reducing breast cancer incidence in both premenopausal and postmenopausal women at high risk of breast cancer development (1). As adjuvant therapy, tamoxifen reduces the risk of recurrence and improves overall survival in early breast cancer (2). It was also shown to be effective for patients with untreated metastatic breast cancer (2). Despite the benefits of tamoxifen in treating breast cancer, unfortunately, many tumors that initially respond to tamoxifen therapy develop resistance. This phenomenon has become a serious obstacle in breast cancer treatment. In the clinic, almost all patients with advanced metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen eventually relapse and die from their disease (3). The mechanisms involved in the development of tamoxifen resistance are still poorly understood. Numerous mechanisms have been proposed to contribute to the development of tamoxifen resistance, including altered drug metabolism, loss of expression or mutation of ER, lack of expression of progesterone receptor, increased expression of ERβ, posttranslational modifications of ER, altered expression of coregulators (e.g., increased expression of the coactivator AIB1 or decreased expression of the corepressor NCoR), and increased growth factor signaling (e.g., HER2 signaling pathways; reviewed in refs. 3-9). Interestingly, a recent study showed that DIBA, an ER zinc finger inhibitor, restores the antagonistic action of tamoxifen in tamoxifen-resistant breast cancer cells through targeted disruption of the ER DNA-binding domain and its interaction with the proximal NH2-terminal domain to suppress ligand-dependent and ligand-independent ER transcription and...
influence the recruitment of cofactor to the ER (10). These findings strengthen the important role of ER in the development of tamoxifen resistance and suggest a possible new approach in modifying tamoxifen resistance (10). However, much work is still needed to learn whether some of the postulated mechanisms thus far can explain resistance to tamoxifen therapy in a majority of patients, or simply each of the enumerated possibilities account for minor portions of resistant cases. Thus, studies geared at better understanding the most common mechanisms involved in tamoxifen resistance are of considerable clinical significance.

With the aim of identifying key genes involved in the development of tamoxifen resistance, we defined the global gene expression profiles of recently developed tamoxifen-resistant MCF-7 breast cancer cell lines and compared them with their tamoxifen-sensitive parental MCF-7 counterpart by using serial analysis of gene expression (SAGE). We determined that the mRNA expression of CtIP, a BRCA1- and CtBP-interacting protein, was found to be 15-fold down-regulated in the tamoxifen-resistant cells when compared with their tamoxifen-sensitive counterparts. In this report, we describe these observations and show the functional involvement of CtIP in the development of tamoxifen resistance.

Results

CtIP Expression Is Significantly Down-Regulated in Tamoxifen-Resistant Breast Cancer Cells

To identify genes implicated in the development of tamoxifen resistance, we determined global gene expression profiles of two independently derived isogenic MCF-7 breast cancer cell line variants (TAMR1 and TAMR2) that are resistant to tamoxifen and their parental tamoxifen-sensitive MCF-7 line by using SAGE. As shown in Fig. 1A, in contrast to their parental MCF-7 cells, both TAMR1 and TAMR2 cells cultured in estrogen-depleted medium proliferated in the presence of tamoxifen. Additionally, despite continuous exposure to trans-4-hydroxytamoxifen (4-OH-TAM), both tamoxifen-resistant variants are still estrogen responsive (Fig. 1B) and express equivalent levels of ER protein as their parental MCF-7 cells (Fig. 1D). Therefore, both TAMR1 and TAMR2 cells express functional ER, as do their parental MCF-7 cells. The tamoxifen-resistant phenotypes of these cells seem not to be a consequence of changes in ER expression or function. These data are also consistent with the clinical findings that the majority of patients with acquired resistance to tamoxifen still express functional ERs (11, 12).

By SAGE comparative analyses, we identified that the transcript encoding for CtIP (also known as retinoblastoma binding protein 8, RBBP8) was significantly down-regulated (15-fold) in both tamoxifen-resistant cell lines when compared with their tamoxifen-sensitive parental MCF-7 counterpart (Fig. 1C). Real-time reverse transcription-PCR on the same RNA samples used for SAGE analysis also confirmed significantly lower CtIP mRNA expression in the tamoxifen-resistant cells than in the tamoxifen-sensitive parental MCF-7 cells (data not shown). Next, we determined whether the differential expression of CtIP detected at the mRNA level could also be observed at the protein level. By Western blot analysis, we detected CtIP protein expression in the tamoxifen-sensitive parental MCF-7 line, but not in the two tamoxifen-resistant lines (Fig. 1D). In fact, both TAMR1 and TAMR2 cells seem to express little or no CtIP protein.

To determine whether this was a phenomenon exclusive to MCF-7 cells, we selected another estrogen-responsive and tamoxifen-sensitive breast cancer cell line, T-47-D, and developed a T-47-D/TR variant that is resistant to tamoxifen. To this end, we followed the same procedure as with MCF-7 cells, growing parental tamoxifen-sensitive T-47-D cells under chronic exposure to 4-OH-TAM (1 μmol/L). After an initial growth arrest by tamoxifen, T-47-D cells regained exponential growth ability even in the presence of tamoxifen. As seen in Fig. 1E, after being cultured in tamoxifen for over 3 months, T-47-D/TR cells proliferated in fetal bovine serum–supplemented growth medium even in the presence of tamoxifen, whereas the growth of parental cells was significantly inhibited by tamoxifen.
Furthermore, we determined CtIP protein levels in these T-47-D cells cultured in tamoxifen for 1, 2, and 3 months and compared them with parental T-47-D cells. We observed that consistent with the findings obtained from MCF-7 cells, T-47-D cells also have significantly decreased CtIP protein expression as they became tamoxifen resistant (Fig. 1F).

Together, the above data confirm that the expression of CtIP is significantly decreased in tamoxifen-resistant cells at both mRNA and protein levels and raise the possibility that CtIP silencing could be a novel mechanism for the development of tamoxifen resistance.

CtIP Expression in Various Human Breast Cancer Cell Lines

To perform a comparative analysis of CtIP expression levels, we next determined CtIP protein expression by Western blot analysis in 10 human breast cancer lines. As can be observed in Fig. 2, three of six ER-positive (ER+) breast cancer lines express abundant CtIP (MCF-7, T-47-D, and ZR-75-1) and one of these lines (BT-483) expresses some CtIP; that is, in total, four of six (67%) ER+ cell lines express detectable CtIP. In contrast, four of four (100%) ER-negative (ER–) breast cancer lines either do not express CtIP at all or very little (UAC 812, MDA-MB-231, MDA-MB-435, SKBR3).

Poor Clinical Response to Endocrine Therapy Is Associated with CtIP Deficiency in Breast Cancer Patients

To determine whether there is a relationship between CtIP status and endocrine therapy response in vivo, we evaluated CtIP expression by immunohistochemistry in 59 ER+, non-operable primary breast carcinomas from patients who received endocrine therapy as single neoadjuvant therapy. Immunohistochemistry was done of samples from tumors before initiation of antiestrogen therapy. Based on the clinical response to the therapy after 4 months of follow-up, patients were classified into four groups: complete response, 4 cases (7%); partial response, 32 cases (54%); stable disease, 17 cases (29%), and progressive disease, 6 cases (10%). These cases are representative of a larger cohort previously reported in which complete response was reported to be between 4% and 10% (13). Immunoreactive scores (IRS) for CtIP were used to semiquantify immunohistochemical staining intensity and percentage of positive cells (14, 15). IRS ranging from 0 to 12 represents CtIP protein staining from undetectable to the highest expression level, respectively (Fig. 3A). One-way ANOVA analysis of CtIP IRS revealed significantly different CtIP expression in the four response groups (P = 0.01). Remarkably, we observed that patients who had the worst response to endocrine therapy (defined as progressive disease) had significantly lower CtIP expression than those who had the best response to endocrine therapy (defined as complete response; P = 0.006; Fig. 3B). Moreover, Pearson’s correlation analysis showed a significant correlation between CtIP status and clinical response to endocrine therapy (P = 0.004). In this limited small study, data seem to indicate that poor response to endocrine therapy is associated with CtIP deficiency in breast cancer patients. However, these observations have to be confirmed in larger cohorts.
CtIP Expression Is Associated with ER, Disease-Free Survival, and Breast Cancer Metastasis Status

To further explore the clinical relevance of CtIP expression in breast cancer, we evaluated information of seven publicly available breast cancer gene expression microarray data sets (16-22) through the use of the web-based Oncomine cancer microarray database (23).6 Clinicopathologic and gene expression data from a total of 828 breast carcinomas was obtained using this publicly available resource. Because ER plays a critical role in the clinical management of breast cancer patients, we first analyzed levels of CtIP mRNA expression in the mentioned microarray sets according to ER status of the tumors (Fig. 4A). By using a meta-analysis approach, we directly compared CtIP expression profiles between 590 ER+ and 238 ER− breast carcinomas by combining all seven microarray data sets. We found a significant association between high CtIP expression and ER (+) status in breast carcinomas (P < 0.0001; Fig. 4A). Next, we analyzed CtIP expression profiles versus disease-free survival in two microarray data sets that had at least 5 years of follow-up clinical information available. Analysis from the study of van de Vijver et al. (19) showed a statistically significant association between low expression of CtIP and disease relapse (P = 0.019; Fig. 4B, left). A trend was also found in the Sorlie et al. study (20) but did not reach statistical significance (P = 0.069), possibly due to the low number of samples (Fig. 4B, right). However, by using the meta-analysis approach and pooling both studies together, we found a highly significant association between decreased CtIP expression and disease relapse (P = 0.004; Fig. 4B). Furthermore, CtIP mRNA expression levels were significantly lower in invasive breast carcinomas that had distant metastasis when compared with breast cancer counterparts that did not metastasize (P = 0.029; Fig. 4C).

Silencing Endogenous CtIP in Tamoxifen-Sensitive MCF-7 Cells Confers Tamoxifen Resistance In vitro

To further explore the putative role of CtIP in the development of tamoxifen resistance, we first examined whether silencing the expression of endogenous CtIP in tamoxifen-sensitive parental MCF-7 cells can induce a tamoxifen-resistant phenotype. Knockdown of CtIP protein levels in tamoxifen-sensitive parental MCF-7 cells was achieved using RNA interference techniques. As shown in Fig. 5A, in the resulting clone [MCF-7/CtIP; small interfering (siRNA)] stably transfected with a vector expressing siRNA targeting CtIP mRNA, the silencing of CtIP protein levels reproduces quite closely the expression difference observed between the tamoxifen-sensitive (parental MCF-7) and tamoxifen-resistant (TAMR1 and TAMR2) cells. The negative control siRNA clone [MCF-7/(−) control siRNA] showed unchanged CtIP protein levels when compared with parental MCF-7 cells (Fig. 5A). In addition, we observed an equal level of ER expression in all three clones, indicating that ER protein expression was not affected by the siRNA intervention (Fig. 5A).

To test whether silenced CtIP expression in parental MCF-7 cells leads to the tamoxifen-sensitive to tamoxifen-resistant transition, we next compared cell proliferation between MCF-7/CtIP siRNA and MCF-7/(−) control siRNA cells under various stimuli. Reduced CtIP expression increased MCF-7/CtIP siRNA cell proliferation under estrogen-deprived conditions compared with control siRNA cells (Fig. 5B). When hormone-starved experimental and control siRNA cells were treated with 4-OH-TAM, growth of the control cells was inhibited by exposure to tamoxifen, whereas growth of MCF-7/CtIP siRNA cells was not inhibited by tamoxifen, indicating acquired resistance to tamoxifen (P < 0.05; Fig. 5C). Furthermore, CtIP-silenced MCF-7/CtIP siRNA cells grew significantly faster than control cells in the presence of both estrogen plus tamoxifen (P < 0.01), indicating that unlike in control cells, tamoxifen indeed fails to inhibit the stimulatory effect of estrogen in CtIP-silenced MCF-7 cells (Fig. 5D). Interestingly, the MCF-7/CtIP siRNA cells still retain response to estrogen-stimulated cell proliferation when exposed to estradiol (Fig. 5E), indicating that ER is still functional and capable of regulating cell growth. In fact, after 10 days in culture in the presence of estrogen, the cell number of both control and MCF-7/CtIP siRNA cells increased ~145-fold (Fig. 5E), which is five to eight times more than that of the MCF-7/CtIP siRNA cells cultured in the presence of tamoxifen (Fig. 5C) or in the absence of estrogen (Fig. 5B), respectively (over the same time period), suggesting that estrogen still seems to stimulate cell proliferation in MCF-7 cells regardless of CtIP status. Taken together, these data indicated that CtIP silencing leads to resistance to the inhibitory growth effects of tamoxifen in vitro.

Reexpression of CtIP in Tamoxifen-Resistant Cells Restores Sensitivity to the Inhibitory Growth Effects of Tamoxifen

Next, we addressed the reciprocal question of whether reexpression of CtIP in tamoxifen-resistant cells abrogates resistance to tamoxifen. Because both tamoxifen-resistant lines have similar proliferation profiles in the presence of tamoxifen, we selected the TAMR1 cell line for further functional studies. To this end, a Tet-off–inducible gene expression system was used to reexpress CtIP in the tamoxifen-resistant TAMR1 cells. TAMR1 cells were transiently cotransfected with Tet-off–inducible vectors containing NH2-terminal Flag-tagged full-length human CtIP cDNA. After transfection, cells were immediately divided equally into two batches. The first batch was treated with 4-OH-TAM or vehicle, and cultured in the presence of doxycycline. The second batch was treated with 4-OH-TAM or vehicle, but cultured in the absence of doxycycline. Expression of FLAG-CtIP was analyzed by immunoblotting after 72 h of transfection. FLAG-CtIP was only detected in TAMR1 cells cultured without doxycycline (Fig. 6A, left), indicating the restoration of CtIP is tightly controlled by doxycycline. Cell proliferation was determined in TAMR1 cells with or without CtIP reexpression. This experiment showed that cells with restored CtIP protein expression displayed a significant growth inhibition by tamoxifen in comparison with control cells having no CtIP restoration (Fig. 6A, middle). Without tamoxifen treatment (vehicle control), transient CtIP restoration had no significant effect on TAMR1 cell proliferation (Fig. 6A, right). Therefore, restoration of CtIP abrogates resistance to tamoxifen in tamoxifen-resistant cells.

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6 http://www.oncomine.org
To further confirm the observations derived from the transient transfection experiments, we developed double stably transfected TAMR1 Tet-off FLAG-CtIP cells. Among the various stably transfected clones obtained, clone 32 was selected for further study. This clone showed no CtIP expression in the presence of doxycycline but similar CtIP protein levels to those produced in parental MCF-7 cells upon doxycycline withdrawal, as determined by Western blot analysis (Fig. 6B).

**FIGURE 4.** CtIP expression is associated with ER status and prognosis in breast cancer. CtIP gene expression profiles and clinicopathologic data of 828 breast carcinomas were obtained from seven published and publicly available breast cancer microarray data sets as described in Materials and Methods. Data were collected and visualized using the Oncomine cancer microarray resource. A. Oncomine database output and meta-analysis showing CtIP expression patterns relative to ER status across seven breast cancer microarray studies. Columns, CtIP transcripts represented as normalized expression units; bars, SE (95% CI). B. Oncomine database output of CtIP expression patterns relative to disease-free survival in two of the seven data sets that have 5 y follow-up information available. The van de Vijver et al. data set (left) shows a significant association between loss of CtIP expression and relapse ($P = 0.019$). The Sorlie et al. study (right) shows a trend that does not reach statistical significance ($P = 0.069$). Meta-analysis (pooling both studies together) shows an excellent statistical inverse correlation between decreased CtIP expression and breast cancer relapse ($P = 0.004$). C. Oncomine database output of CtIP expression patterns related to metastasis status (left, the van de Vijver et al. study; right, the Sorlie et al. study). The analysis shows a statistically significant decrease of CtIP expression in association with metastatic breast carcinomas ($P = 0.029$).
Next, we measured the growth of the double-stably transfected TAMR1 Tet-off FLAG-CtIP cells under conditions in which CtIP restoration was either induced or repressed. Cells from clone 32 were cultured in two different conditions. Half of the cells were grown in hormone-free medium containing doxycycline whereas the other half was cultured in the same medium but devoid of doxycycline. After 3 days of incubation, cells were treated with either 17-β-estradiol (E2), 4-OH-TAM, or vehicle control. As shown in Fig. 6C, the growth of TAMR1 Tet-off FLAG-CtIP cells was significantly inhibited by tamoxifen when doxycycline was removed from the medium compared with cells from the same clone but cultured in the presence of doxycycline (P < 0.05). Cells still responded well to estrogen regardless of doxycycline status (Fig. 6D). Taken together, these results show that sensitivity to the inhibitory growth effects of tamoxifen in previously tamoxifen-resistant cells is restored, at least partially by CtIP reexpression.

**Discussion**

SAGE studies identified CtIP as one of the most significantly down-regulated transcripts in two independently developed tamoxifen-resistant breast cancer cell lines when compared with their tamoxifen-sensitive parental MCF-7 line. Immunoblotting analyses not only validated the SAGE observations but also showed a dramatic difference in CtIP expression between tamoxifen-sensitive and tamoxifen-resistant cells. The down-regulation of CtIP is likely to contribute to the tamoxifen resistance of these cells.

**FIGURE 5.** Silencing of CtIP protein expression in tamoxifen-sensitive MCF-7 cells confers tamoxifen resistance in vitro. A, Western blot analysis of CtIP and ER proteins in parental MCF-7 cells, MCF-7/CtIP siRNA control, and MCF-7/CtIP siRNA stable clones. Growth curves of control and MCF-7/CtIP siRNA cells treated with vehicle control (B) or 4-OH-TAM (C) for 2 wk. D, Growth curves of MCF-7/CtIP siRNA and (-) control cells grown in hormone-free medium and treated with E2 (10 nmol/L) and 4-OH-TAM (1 μmol/L) for 6 d. E, Growth curves of control and MCF-7/CtIP siRNA cells exposed to E2 for 10 d. All experiments were done in triplicate. Points, mean; bars, SE. * and **, P < 0.05 and P < 0.01 by t test, respectively. Note that Y-axis scale for cell number in D is different from that in B, C, and E.
protein expression levels, with high levels of protein expression in parental ER+ MCF-7 breast cancer cells but very little or no CtIP protein product in the ER+ tamoxifen-resistant derivative isogenic cell lines. In addition to the observations in MCF-7 cells, we also found significantly reduced CtIP protein expression in ER+ T-47-D breast cancer cells when they became tamoxifen resistant. Furthermore, consistent with the in vitro observations, we found significant association of CtIP deficiency in breast cancers from patients with poor clinical response to endocrine therapy. By meta-analysis of gene expression data sets, we also found an association of CtIP expression levels with poor outcome. These in vivo data suggest that CtIP gene and protein expression may be useful biomarkers for breast cancer prognosis and clinical management, although it would require further studies in larger patient cohorts. In addition, we found a significant reverse association of CtIP expression with ER status, suggesting that CtIP may function in a pathway or pathways associated with ER signaling and perhaps regulate ER-mediated cell proliferation. All ER− breast cancer cells

![Image](https://example.com/image.png)

**FIGURE 6.** Tamoxifen-resistant cells regain sensitivity to the inhibitory growth effects of tamoxifen upon restoration of CtIP protein expression. **A.** Left, expression of FLAG-CtIP protein in TAMR1 cells transiently cotransfected with inducible FLAG-CtIP expression vectors and cultured in the presence or absence of doxycycline (DOX) for 3 d. Middle and right, transient CtIP restoration partially abrogates resistance to tamoxifen in TAMR1 cells. TAMR1 cells were transiently cotransfected with inducible FLAG-CtIP expression vectors and treated with 4-OH-TAM (middle) or vehicle control (right) in the presence or absence of doxycycline for 3 d. Cell proliferation was determined as shown. Percent cell number (middle) represents cell numbers relative to vehicle control–treated cells. Columns, mean of triplicate determinations; bars, SE. **B.** Total CtIP protein expression in the double-stably transfected TAMR1 Tet-off FLAG-CtIP clone 32 cells in the presence or absence of doxycycline. CtIP expression in parental MCF-7 cells is shown for comparative purposes. **C.** CtIP reexpression upon doxycycline withdrawal restores sensitivity to the inhibitory growth effects of tamoxifen in TAMR1 Tet-off FLAG-CtIP clone 32 cells. The proliferation of clone 32 cells under the treatment of 4-OH-TAM was determined in the presence (black columns) or absence (white columns) of doxycycline. *P < 0.05 by t test. **D.** Effect of E2 on the proliferation of clone 32 cells cultured with or without doxycycline. Note that Y axis scales for cell number in **C** and **D** are different from each other.
tested (intrinsically resistant to tamoxifen) express either none or very little CtIP protein. Interestingly, BT-474, an ER+ but tamoxifen-resistant breast cancer cell line as reported by Wang et al. (10), also expresses nearly undetectable CtIP protein when compared with ER (+) and tamoxifen-sensitive lines such as MCF-7 and T-47-D, which further supports the important role of CtIP in the development of tamoxifen resistance. Finally, based on RNA interference and reciprocal reexpression studies, we were able to reproduce the tamoxifen-resistant phenotype simply by shutting down the expression of one gene (i.e., CtIP). These data show that CtIP silencing is critical for the development of tamoxifen resistance in breast cancer in vitro models and suggest that CtIP silencing may be a novel mechanism by which cells can circumvent the inhibitory effects of tamoxifen to resume proliferation and ultimately acquire resistance to the antiestrogen tamoxifen.

The human CtIP (also known as RBBP8) encodes an 897-amino-acid nuclear protein that is widely expressed in various human tissues (24-27). It was initially identified as a cofactor of transcriptional corepressor CtBP (24). CtIP is also known to interact with tumor suppressors, Rb family proteins (Rb and p130; refs. 25, 28) and BRCA1 (26, 29-31), as well as the transcriptional repressors such as LIM-only protein LMO4 (30) and Ikaros family members (32). Recent studies suggest that CtIP plays an important role in cell cycle regulation and DNA damage response (33-37). Emerging evidence also suggests that CtIP may itself be a tumor susceptibility gene. Analysis of CtIP cDNA from 89 human tumor cell lines revealed 5 missense and 11 silent mutations (26). In a more recent screening study of 109 colon cancers, CtIP was found to be a frequent target for angiogenin, which forms a repressor complex at a recognition site of ZBRK1 in transcriptional repression (41, 42). Significantly, recent studies showed that BRCA1, CtIP, and ZBRK1 form a repressor complex at a recognition site of ZBRK1 in ANGI1 promoter and a defect of this complex formation derepresses ANGI1 transcription, promoting endothelial cell survival and vascular enlargement (43). Interestingly, other studies showed that BRCA1 physically interacts with ER and inhibits transcriptional activity of the receptor (44, 45). In this study, we found that high CtIP expression is significantly associated with ER+ status. Thus, it is possible that CtIP may functionally be linked with ER signaling via its interaction with BRCA1. Moreover, CtIP was also shown to form a complex with BRCA1 and the transcriptional corepressor CtBP, which is important for the repression of p21 promoter activity (29).

Together, it raises the possibility that in physiologic conditions, CtIP could bridge BRCA1 and CtBP to form a transcriptional repressor complex, which in turn may modulate ER signaling pathways through the interaction between BRCA1 and ER. Therefore, we hypothesize that in tamoxifen-sensitive cells, BRCA1, CtIP, and CtBP form a transcriptional repressor complex that leads to inhibition of a full ER+ transcriptional response, accounting for the inhibitory growth effects of tamoxifen. To circumvent the transcriptional inhibitory effects of tamoxifen, tamoxifen-resistant cells silence CtIP expression, which, in turn, disrupts the repressor complex and allows breast cancer cells to resume proliferation. Further characterization of the CtIP signaling pathways may provide insights into how CtIP silencing leads to the development of tamoxifen resistance.

Materials and Methods

Cell Lines and Chemicals

Parental MCF-7 cells used in this study have been described previously (46). The tamoxifen-resistant MCF-7 isogenic cell line variants (termed TAMR1 and TAMR2) were generated in our laboratory by culturing MCF-7 cells under continuous 4-OH-TAM (the active metabolite of tamoxifen; 1 μmol/L) exposure for ~2 years. These cells were maintained in phenol red-free IMEM medium containing 5% fetal bovine serum (TAMR1) or 5% charcoal-stripped fetal bovine serum (TAMR2), glutamine (2 mmol/L), gentamicin (50 μg/mL) and 4-OH-TAM (1 μmol/L). The characterization of parental MCF-7 and TAMR1 cells has been previously described (47, 48). Tamoxifen-resistant T-47-D cells (T-47-D/TR) were generated by growing regular ER+ T-47-D cells in the presence of 4-OH-TAM for over 3 months. These cells were maintained in DMEM (Cambrex Bio Science) medium containing 10% fetal bovine serum and 4-OH-TAM (1 μmol/L). Other breast cancer cell lines used, including SUM-44-PE, ZR-75-1, MDA-MB-231, MDA-MB-435, SKBR3, and BT-474, were maintained in DMEM (Cambrex Bio Science) supplemented with 10% fetal bovine serum. The BT-483 cell line was maintained in RPMI (Cambrex Bio Science) supplemented with 10% fetal bovine serum. The UACC-812 breast cancer cell line was grown in L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum. We purchased 4-OH-TAM and E2 from Sigma-Aldrich.

Serial Analysis of Gene Expression

SAGE was done on tamoxifen-sensitive parental MCF-7 line and tamoxifen-resistant TAMR1 and TAMR2 lines. SAGE library generation, sequencing, and tag extraction were done as previously described (46, 49, 50). SAGE data were analyzed using an ANOVA-based multivariate approach called multiple linear contrast analysis. Two contrasts were defined to identify differentially expressed genes in the tamoxifen-resistant cells, one comparing the average expression level in the two resistant cell lines to that of the parental strain and the second one comparing the expression levels between the two resistant
strains. The null hypothesis for first contrast tests for lack of differential expression between resistant and parental strains and the second tests for consistent expression between the two resistant cell lines. Significance of the null hypotheses for these tests was set at the 95% level after a Bonferroni-type adjustment for the multiplicity of comparisons.

Western Blot Analysis

Cells were washed twice with ice-cold PBS and then lysed with radioimmunoprecipitation assay buffer [10 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, (pH 7.2), 1× protease inhibitor cocktail (Roche)]. Cell lysates were flushed 10 times through a 21-gauge needle and microcentrifuged at 21,000 g for 10 min at 4°C. Supernatants were collected and protein concentration was measured with a Pierce Protein Assay Kit, according to the manufacturer’s instruction. Equal amount (30–50 μg) of protein from each sample was separated on 6% to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes by electroblotting. Blots were first treated with blocking buffer [5% milk in 1× TBS containing 0.1% Tween 20 (TBS-T)] for 1 h and then incubated with primary antibodies for 1 to 2 h. After washing thrice in TBS-T, blots were incubated with horseradish peroxidase–labeled secondary antibodies for 1 h. Labeled proteins were detected using KPL Protein Detector chemiluminescence detection reagents and exposed to X-ray films. All procedures described were carried out at room temperature. Antibodies used were as follows: CtIP (14-1; ref. 27; T-16, Santa Cruz Biotechnology; 19E8, GeneTex), ER (HC-20, Santa Cruz Biotechnology), anti-Flag (M2, Sigma), and β-actin (AC-15, Sigma).

Human Samples

Primary breast cancer formalin-fixed, paraffin-embedded tissue samples were collected retrospectively from 59 postmenopausal patients with stage II to III ER (+) breast carcinomas (median age 78 years; range 60 to 92 years). The 59 patients were treated at a single institution (Instituto Valenciano Oncologia, Valencia, Spain) between 1999 and 2002 with 4 months neoadjuvant endocrine therapy consisting of tamoxifen (23 patients) or letrozole (36 patients) for large nonoperable or locally advanced ER+ breast cancers. These patients were part of a larger study published elsewhere (13). All patients gave written informed consent before the submission of tumor samples for CtIP analyses, and the local ethic committee approved the study protocol and informed consent form. Determination of response to the referred endocrine adjuvant therapy was made after 4 months of patient follow-up. We used one-way ANOVA followed by Tukey’s test post hoc comparisons, and Pearson’s correlation test to assess the association between CtIP protein status (raw IRS scores) and clinical response to endocrine therapy. All statistical analyses were two side, and P < 0.05 was considered as statistically significant. Analyses were conducted using SPSS version 11.5 software (SPSS, Inc.).

Immunohistochemistry Analysis

Immunohistochemical staining was done as previously described (51) with a rabbit polyclonal CtIP antibody (H-300, 1:100 dilution, Santa Cruz Biotechnology). CtIP expression levels was scored blindly by two independent pathologists (D.R.S. and M.I.N.) using the IRS method as previously described (14, 15). In brief, the IRS was calculated by multiplying the percentage of CtIP-positive cells (scored 0 to 4: 0, 0%; 1, 0-25%; 2, 26-50%; 3, 51-75%; 4, >75%) with the CtIP staining intensity (scored 0 to 3: 0, none; 1, weak; 2, moderate; 3, strong).

Meta-analysis of Breast Cancer Microarray Data Sets

CtIP gene expression profiles and clinicopathologic data of 828 breast carcinomas were obtained from seven published and publicly available breast cancer microarray data sets (16-22). The Oncomine cancer microarray database was used for data collection, processing, and visualization (23). CtIP gene expression was log-transformed, median centered for each gene expression data set, and SD was normalized to one per array. The gene module application was used for differential expression analysis (two-sided t test). We used a meta-analysis approach to determine and summarize the CtIP mRNA expression pattern from the seven independent studies. We computed summary estimates (effect sizes) of CtIP expression changes by the standardized mean difference method using the exact t values and sample size for each groups. To calculate the pooled effects of CtIP profile, each study was weighted by the inverse of the individual and between-study variance according to a random-effects model. Meta-analysis was carried out using comprehensive meta-analysis software (Bios tat, Inc.). All effect sizes were presented with 95% confidence interval—based on the estimated variances.

In vitro Cell Proliferation Assays

Cells were cultured in estrogen-free medium for 48 h. On day 0, 1 × 10⁶ cells in estrogen-free medium were plated in triplicate in 12-well plates. E₂ (10 nmol/L), 4-OH-TAM (10 nmol/L), or ethanol (vehicle control, 1 μL/mL) was added directly into the medium at the same time. Fresh medium with the adequate treatment was changed every 2 days. Cell counts were done at various time points as indicated in the figures.

RNA Interference

siRNA expression cassette (SEC) encoding siRNA targeting CtIP mRNA (from 2,492 to 2,512, NM_002894; AATGA- TAGCTTTGGAAGATATG) was generated using the Silencer Express siRNA Expression Cassette Kits (Ambion). A negative control SEC expressing siRNA with no significant homology to human, mouse, or rat genome sequences was also generated by the same method. The SECs were then cloned into the mammalian expression pSEC-puro vector (Ambion). To obtain cell clones that stably expressed siRNAs targeting CtIP, MCF-7 cells were transfected with either pSEC-CtIP-puro or pSEC-Control-puro by electroporation. One day after, puromycin (600 ng/mL) was added into the culture medium. After 3 weeks of selection, puromycin-resistant clones were picked up, expanded, and analyzed for CtIP expression levels by immunoblotting. The stable clone displaying the lowest level of CtIP protein expression was chosen for further studies.
Generation of Double-Stably Transfected Tet-off TAMR1 Cells with Doxycycline-Inducible Restoration of CtIP

The Tet-off gene expression system was purchased from BD Biosciences Clontech. Full-length human CtIP cDNA with three NH2-terminal Flag epitope tags was cloned into pTRE2hyg response vector (pTRE2hyg-FLAG-CtIP). To generate double-stable Tet-off TAMR1 cell clones, cells were cotransfected with pTet-Off and pTRE2hyg-FLAG-CtIP vectors. After electroporation, cells were plated in 10-cm dishes and allowed to grow in regular medium containing 4-OH-TAM (1 μmol/L) plus doxycycline (1 μg/mL) for 48 h. Cells were then selected for resistance to G418 (800 μg/mL) and hygromycin B (200 μg/mL). Fresh doxycycline (1 μg/mL) was added to tamoxifen-containing medium (regular culture medium for TAMR1 cells) every 2 days to maintain a constant suppression of CtIP expression during the selection process. Hygromycin and G418 double-resistant colonies began to appear after 3 to 4 weeks of selection. Thirty-seven large and healthy colonies were isolated using cloning cylinders and transferred to individual wells for expansion. Each clone was healthy and was chosen for further studies.

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