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Mitotic deregulation by survivin in ErbB2 overexpressing breast cancer cells contributes to Taxol resistance

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Abstract

Purpose—Taxol resistance remains a major obstacle to improve the benefit of breast cancer patients. Here we studied whether overexpression of ErbB2 may lead to mitotic deregulation in breast cancer cells via upregulation of survivin that confers Taxol resistance.

Experimental Design—ErbB2 over-expressing and ErbB2 low-expressing breast cancer cell lines were used to compare their mitotic exit rate, survivin expression level and apoptosis level in response to Taxol. Survivin was then downregulated by antisense oligonucleotides to evaluate its contribution to mitotic exit and Taxol resistance in ErbB2 over-expressing breast cancer cells. At last, specific PI3K/Akt and Src inhibitors were used to investigate the involvement of these two pathways in ErbB2-mediated survivin upregulation and Taxol resistance.

Results—We found that ErbB2-overexpressing cells expressed higher levels of survivin in multiple breast cancer cell lines and patient samples. ErbB2-overexpressing cells exited M phase faster than ErbB2 low-expressing cells, which correlated with the increased resistance to Taxol-induced apoptosis. Down-regulation of survivin by antisense oligonucleotide delayed mitotic exit of ErbB2-overexpressing cells and also sensitized ErbB2 over-expressing cells to Taxol-induced apoptosis. Moreover, ErbB2 upregulated survivin at translational level and both PI3K/Akt and Src activation

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STATEMENT OF TRANSLATIONAL RELEVANCE

Taxol resistance is a major obstacle to improving breast cancer patients' response and survival. A better understanding of the molecular mechanism of Taxol resistance and designing of novel strategies to overcome Taxol resistance are critical for development of more effective therapies. Here, our studies revealed that ErbB2 overexpression led to survivin translational upregulation, which involves activation of both PI3K/Akt and Src pathways. The increased survivin is a key event in ErbB2-mediated mitotic deregulation and subsequent Taxol resistance. Most importantly, combinational treatment using survivin antisense oligos or PI3K/Akt and Src inhibitors with Taxol dramatically sensitized ErbB2-overexpressing breast cancer cells to Taxol treatment. Currently, clinically applicable anti-survivin agents, PI3K/Akt and Src inhibitors are intensively investigated for treating cancer patients. Our data demonstrated an important clinical potential for using combination of survivin inhibitors, and/or PI3K/Akt and Src inhibitors with Taxol to improve Taxol response and the clinical outcome of Taxol-treated patients.

are involved. In addition, combination treatment of Taxol with PI3K/Akt and Src inhibitor led to increased apoptosis in ErbB2-overexpressing breast cancer cells than single treatment.

Conclusions—Survivin upregulation by ErbB2 is a critical event in ErbB2-mediated faster mitotic exit and contributes to Taxol resistance.

Keywords

breast cancer; ErbB2; survivin; mitotic deregulation; Taxol resistance

INTRODUCTION

Taxol (Paclitaxel) is one of the most effective microtubule targeting drugs for breast cancer treatment and it accounts for significant improvements in breast cancer patient's survival in the last two decades. Taxol disrupts microtubule dynamics by promoting tubulin polymerization and stability, which leads to cell cycle arrest at the G₂/M phase, activates mitotic spindle checkpoint, and results in apoptotic cell death (1,2). However, despite a remarkable response of initial treatment with Taxol, progressive diseases eventually develop in most patients. Taxol resistance is a major obstacle that severely limited the improvement of response and survival in Taxol-treated cancer patients (3). Therefore, better understanding of the molecular mechanism of Taxol resistance and strategies to overcome Taxol resistance are urgently needed for effective and individualized chemotherapies.

One focus of attention in this effort is on ErbB2 (also known as HER2 or neu), a receptor tyrosine kinase that is overexpressed in 20-30% of human breast cancers, and has been shown to correlate with a poor prognosis (4). Numerous studies have shown that ErbB2 overexpression leads to increased cancer cell survival, proliferation, migration, and invasion. Moreover, we and others have found that overexpression of ErbB2 confers on breast cancer cells an increased resistance to Taxol (5-9), although the mechanisms by which ErbB2 overexpression protects cancer cells from Taxol-induced apoptosis are not completely understood. Given that Taxol cytotoxicity is largely due to mitotic arrest followed by mitotic catastrophe and apoptosis, mitosis deregulation by ErbB2 is likely to reduce the efficacy of Taxol. One potential cell cycle regulator that may play a role in ErbB2-mediated Taxol resistance is survivin, which is a member of the inhibitors of apoptosis protein (IAP) family (10). Survivin is preferentially expressed during mitosis and physically associated with the mitotic apparatus to regulate microtubule dynamics during mitosis (11). It also associates with Aurora B kinase and inner centromere protein (INCENP) to form chromosomal passenger complex (12), dictating the localization of the complex, and the activation of Aurora B kinase (13). Therefore, survivin plays an important role in mitotic spindle checkpoint. Indeed, overexpression of survivin may obliterate the surveillance mechanism of the spindle assembly checkpoint, thereby allowing cancer cells to proceed through cell division, leading to tumor-associated cell cycle deregulation (14). Survivin has been indicated as a prognostic marker for poor survival in breast cancer patients (11).

ErbB2 overexpression has recently been linked to survivin upregulation in breast cancer cells and breast cancer patient samples (15-17). Given that ErbB2 overexpression confers Taxol resistance at the G₂/M phase of the cell cycle (2) and that survivin plays critical roles in the regulation of both mitotic exit and cell survival, we tested the hypothesis that mitotic deregulation by survivin upregulation in ErbB2 over-expressing breast cancer cells may confer Taxol resistance.

We found that ErbB2-overexpressing cells exited M phase faster than ErbB2-low-expressing cells, which correlated with increased survivin expression. Down-regulation of survivin by antisense oligonucleotide delays mitotic exit of ErbB2-overexpressing cells, leading to

sensitization of ErbB2 over-expressing cells to Taxol-induced apoptosis. In addition, our data indicated that ErbB2 upregulated survivin at protein translational level that involves both PI3K/Akt and Src downstream of ErbB2.

MATERIALS AND METHODS

Antibodies and reagents

Anti-ErbB2 antibody (Ab3) was from Oncogene Science (Cambridge, MA); anti-survivin and anti-securin antibodies were from Novus Biological (Littleton, CO) and R&D (Minneapolis, MN); anti-PARP antibody was from Santa Cruz Biotech (Santa Cruz, CA); anti β -actin antibody and Cycloheximide were from Sigma (St. Louis, MO); Annexin V-FLUOS staining kit and MTS assay kit were from Roche (Mannheim, Germany) and Promega (Madison, WI), respectively. PI3K/Akt inhibitor LY294002 and Src inhibitor PP2 were from Calbiochem (San Diego, CA). Src inhibitor AZD0530 was provided by AstraZeneca (UK).

Cell lines

MDA-MB-435, BT474, and MCF7 were obtained from ATCC (Rockville, MD). Wild-type *erbB2* transfectants of two of these cell lines, 435.eB and MCF7/HER-2, have been described previously (8,18).

Treatment of cells with survivin antisense oligonucleotide

Survivin antisense (surv-AS, ISIS 23722) and its nonsense control (surv-NS, ISIS 28598) were from ISIS Pharmaceuticals (Carlsbad, CA). Exponential growth MDA-MB-435 cells or BT474 cells were transfected with 2 μ g surv-AS or surv-NS using an Amaxa Nucleofector (Amaxa Biosystems, MD).

Treatment of cells with ErbB2 siRNA

BT474 breast cancer cells were transfected with 100 nM ErbB2 siRNA as previously described (19).

Quantitative-PCR

RNA was extracted with TRIzol, and reverse transcribed to cDNA using Superscript III First Strand Synthesis System (Invitrogen, CA). 1 μ l of cDNA was used as template for quantitative-PCR with FullVelocity SYBR Green QPCR Master Mix (Stratagene, CA). Fold change difference was calculated after normalization to GAPDH. Survivin primers (forward 5' - CCGCATCTCTACATTCAAGAAC-3' Reverse 5' - CTTGGCTCTTCTCTGTCC-3'), GAPDH primers (forward 5' - TGGTATCGTGGAAGGACTCATGAC-3' Reverse 5' - ATGCCAGTGAGCTTCCCGTTCAGC-3')

RT-PCR

RT-PCR was performed by using SuperScriptTMIII one-step RT-PCR kit (Invitrogen, CA).

Western blot analysis

Western blot analysis was performed as previously described (7).

MTS assay and Apoptosis analysis

MTS assay and Apoptosis analysis were performed following manufacturer's instructions, respectively.

Mitotic exit measurement

Cells were transfected with or without surv-AS or surv-NS as described above. Forty-eight hours after transfection, the cells were treated for 16 h with culture medium containing 0.4 µg/ml nocodazole (Sigma) to synchronize cells in the M phase. Cells were washed three times and re-cultured to release the cells from M phase arrest. At different time points, cells were collected, cytospinned, and stained with Giemsa. The number of cells in M phase was counted under a light microscope. At least 500 cells were counted for each indicated time point.

Immunohistochemistry analyses for ErbB2 and survivin expression

Tumor samples were collected from patients with primary invasive breast cancer as previously described (20). Immunohistochemistry analysis was performed, as previously described (20). Survivin expression level was scored semi-quantitatively based on staining intensity and distribution using the immunoreactive score (IRS) as following: IRS = SI (staining intensity) × PP (percentage of positive cells). SI was determined as 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. PP was defined as 0, 0-19%; 1, 20%–49%; 2, 50%–69%; 3, 70%–100 positive cells. Final score is defined as: Score 0 for 0; score 1 for (1~3); score 2 for (4~6); score 3 for (7~9). ErbB2 was stained and scored using the DAKO HercepTest (DAKO, CA), and the (++) ErbB2 staining was defined as ErbB2 positive.

Polysomal fractionation

Polysomal fractionation was done as previously described (21). Briefly, cells were lysed with polysome buffer (300mM KCl, 5mM MgCl₂, 10mM HEPES with freshly added 0.5% NP-40, 100µg/ml cycloheximide, 5mM dithiothreitol, 10mM vanadyl adenosine, PH=7.4). Cytoplasm fraction was collected and loaded onto a 15% to 40% linear sucrose gradient, and centrifuged at 38,000g for 90 minutes. 10 fractions were collected to extract RNA for RT-PCR analysis.

Statistical analysis

All *in vitro* experiments were repeated at least three times. The results are presented as means ± SE. Statistical analysis was done using Prism software (GraphPad Software, CA), and two-tailed Student's *t*-test was used for comparisons between groups. *P* < 0.05 was designated as statistically significant.

RESULTS

ErbB2-overexpression in breast cancer cells leads to faster mitosis exit

We and others have demonstrated that ErbB2 overexpression in breast cancer cells confers resistance to Taxol-induced apoptosis (2,8,9,22). Taxol functions by blocking cell cycle at the mitotic exit “checkpoint” and resulting in apoptotic cell death at the M phase (3). Therefore, we test the hypothesis that ErbB2 overexpression in breast cancer cells may cause resistance to Taxol-induced apoptosis by accelerating cell progression through M phase, or a faster mitotic exit. We compared the mitosis exit between ErbB2-high-expressing cells 435.eB (MDA-MB-435 human breast cancer cells stably transfected with wild-type ErbB2) and ErbB2-low-expressing cells 435.neo (vector control) (Fig. 1A) (19). The cells were synchronized at the M phase by nocodazole treatment for 16 hours. Mitotic cells were collected by shake-off and nocodazole was then removed to allow cells to exit from mitosis. Cells were collected at different time points and cells remaining in the M phase were counted under the microscope. We found that about 70% of ErbB2-overexpressing (435.eB) cells, but only about 20% of ErbB2-low-expressing (435.neo) cells, exited the M phase at 90 min after being released from M phase arrest (Fig. 1B). These results indicated that ErbB2-overexpressing cells exit mitosis faster than ErbB2-low-expressing cells.

The degradation of mitosis regulators, such as cyclin B1 and securin, must occur for cells to exit mitosis (23). Indeed, we found that cyclin B1 and securin were degraded faster in 435.eB cells than in 435.neo cells after their release from M phase arrest (Fig. 1C). Additionally, Cdc2-Tyr15 phosphorylation, an inhibitory Cdc2 phosphorylation that signals mitotic exit, increased earlier and more dramatically in 435.eB cells than in 435.neo cells (Fig. 1C). These results are consistent with the observation that ErbB2-over-expressing cells exit the M phase faster than ErbB2-low-expressing cells.

ErbB2 up-regulates survivin expression in breast cancer cells

Survivin plays a critical role in regulating spindle checkpoint during cell cycle (24). To examine whether ErbB2 overexpression in breast cancer cells facilitates M phase exit by modulation of survivin, we examined the survivin expression levels in several ErbB2-low- and ErbB2-over-expressing breast cancer cell lines. Indeed, the ErbB2-over-expressing 435.eB and MCF7.eB stable transfectants expressed higher levels of survivin than did the parental MDA-MB-435 and MCF7 cells (Fig. 2A). Conversely, when we treated BT474 breast cancer cells that overexpress endogenous ErbB2 with ErbB2 siRNA, it downregulated ErbB2 expression, and subsequently reduced survivin expression (Fig. 2A). Importantly, mitotic survivin level was also higher in 435.eB cells than in 435.neo cells (Fig. 2B), which is consistent with the notion that survivin is preferentially expressed during mitosis (25). To determine the relevance of these findings in breast cancer patients, ErbB2-over-expressing and ErbB2-low-expressing breast tumor specimens were stained for ErbB2 and survivin by immunohistochemistry. Five out of the eight ErbB2-overexpressing breast tumors showed very strong survivin staining (+ ++), and two showed strong survivin staining (++). In contrast, survivin was generally expressed at lower levels (+) in tumors that expressed low levels of ErbB2 (supplemental table 1 and Fig. 2C). These data demonstrated survivin upregulation in ErbB2-over-expressing breast cancers, consistent with recent reports (15-17).

Down-regulation of survivin delays mitotic exit of ErbB2-overexpressing cells

As an essential component of mitotic spindle complex, survivin may play a role in ErbB2-mediated faster mitotic exit given that survivin is dramatically upregulated in ErbB2 over-expressing breast cancer cells. To test this hypothesis, we treated 435.neo and 435.eB cells with the survivin antisense oligonucleotide, surv-AS, and with the nonsense oligonucleotide, surv-NS, as control. As shown in Fig. 3A, surv-AS effectively downregulated survivin expression in both 435.neo and 435.eB cells as well as in BT474 cells. Compared to surv-NS treatment, surv-AS did not significantly alter the time course of mitosis exit of 435.neo cells (Fig. 3B). In contrast, surv-AS treatment led to more 435.eB cells remaining in the M phase at 90 min compared to surv-NS-treated cells (32% for surv-NS-treated and 49% for surv-AS-treated cells, $p < 0.05$) after release from M phase arrest (Fig. 3B and C). The data indicated that downregulation of survivin delayed the exit of ErbB2-over-expressing 435.eB cells from mitosis. These findings are further supported by delayed cyclin B1 and securin degradation in surv-AS-treated than in surv-NS-treated 435.eB cells (Fig. 3D). These results indicated that ErbB2-mediated upregulation of survivin contributes to a faster mitotic exit of ErbB2 over-expressing breast cancer cells.

Downregulation of Survivin sensitizes ErbB2 overexpressing cells to Taxol-induced apoptosis

Because survivin contributes to the faster mitotic exit of ErbB2-over-expressing breast cancer cells that are resistant to Taxol-induced mitotic catastrophe/apoptosis, we reasoned that the increased survivin level in ErbB2 over-expressing cells may contribute to ErbB2-mediated Taxol resistance. To test this, we treated 435.neo and 435.eB cells with surv-AS to downregulate survivin expression or with surv-NS as a control. Twenty-four hours later, cells

were treated with Taxol to induce apoptosis. The surv-NS treated 435.neo cells showed increased apoptosis with Taxol treatment, while surv-NS-treated 435.eB cells were resistant to Taxol induced apoptosis as expected (8,9) (Fig. 4A). Interestingly, surv-AS treatment dramatically sensitized the ErbB2-over-expressing 435.eB cells to Taxol-induced apoptosis (Fig. 4A). Consistently, surv-AS-treated 435.eB cells showed increased PARP cleavage than did surv-NS-treated cells (Fig. 4B). These results indicated that survivin plays a critical role in ErbB2-mediated resistance to Taxol-induced apoptosis. Indeed, we found that survivin-high breast cancer patients showed a trend of decreased disease-free survival compared to the survivin-low patients in the ErbB2 positive group following neoadjuvant T/FAC treatment (supplemental Fig.1). We did not observe a significant p value due to the small sample size.

ErbB2 upregulates survivin at the translational level

Our data above demonstrated that survivin upregulation plays a critical role in ErbB2-mediated mitotic deregulation and hence Taxol resistance in breast cancer cells. To gain insight on how to effectively block survivin upregulation, we next investigated how ErbB2 upregulates survivin. The increased survivin in transformed cells has been previously reported as largely due to increased mRNA transcription (26). However, we found that survivin mRNA levels were similar in ErbB2-low 435.neo and ErbB2-high 435.eB cells by quantitative-PCR (Fig. 5A). Survivin is also known to be tightly controlled by ubiquitin/proteasome-mediated degradation (27). Interestingly, we found that survivin protein is degraded at similar rate in 435.eB cells as in 435.neo cell (Fig. 5B). Having ruled out the possibilities of increased mRNA transcription or reduced protein degradation, survivin upregulation in ErbB2-over-expressing cells should largely result from increased protein translation. Since the 16 KDa survivin with only two methionine residues could not be effectively labeled by ³⁵S-methionine for direct measurement of protein synthesis, we examined survivin mRNA distribution on polysomes in 435.neo and 435.eB cells. As shown in Figure 5C, survivin mRNA was distributed to larger polysome fractions in 435.eB cells than in 435.neo cells, indicating increased number of ribosomes associated with survivin transcript in 435.eB cells. Moreover, we also observed a higher level of phosphorylation of 4EBP-1 (P-4EBP-1), an indicator of more active protein translation (Fig. 5D). In addition, when we treated cells with rapamycin, an effective inhibitor of protein translation, survivin protein level was reduced to similar level in 435.eB cells as in 435.neo cells (Fig. 5D). These data suggested that ErbB2-mediated survivin upregulation was largely at the translational level in these breast cancer cells.

Both PI3K/Akt and Src are involved in translational upregulation of survivin and Taxol resistance by ErbB2

ErbB2 has been implicated to upregulate survivin expression via activation of PI3K/Akt pathway (15), although the detailed molecular mechanism remains unclear. Now that we found that ErbB2 overexpression leads to increased 4EBP-1 phosphorylation, allowing for more active translation of survivin, we investigated the possible involvement of PI3K/Akt pathway in ErbB2-mediated 4EBP-1 phosphorylation and survivin upregulation. We treated 435.neo and 435.eB cells with a PI3K inhibitor LY294002. Indeed, 4EBP-1 phosphorylation was reduced, which is accompanied by reduced survivin protein level after LY294002 treatment (Fig. 6A). We have previously shown that ErbB2 overexpression leads to Src activation in 435.eB cells (19). Interestingly, when we treated ErbB2-low 435.neo and ErbB2-high 435.eB cells with Src inhibitor PP2 or AZD0530, survivin protein level was remarkably reduced, which was also accompanied by inhibition of 4EBP1 phosphorylation (Fig. 6A). The treatment with PI3K/Akt inhibitor, LY294002, and Src inhibitors, PP2 and AZD0530, did not reduce survivin mRNA level (Fig. 6B). These data indicated that PI3K/Akt and Src activations downstream of ErbB2 overexpression in breast cancer cells are involved in translational upregulation of survivin via increased 4EBP-1 phosphorylation.

To investigate the contribution of PI3K/Akt and Src pathway activation in the Taxol resistance of ErbB2-overexpressing breast cancer cells, we pre-treated 435.neo and 435.eB cells with either PI3K inhibitor LY294002, or Src inhibitor AZD0530, or control DMSO for 12 hours and then added Taxol. The pre-treatments did not have significant effect on cell cycle profiles of these cells (supplemental fig.2). Remarkably, the combination of LY294002 and Taxol, or AZD0530 and Taxol, greatly increased the sensitivity to Taxol treatment in 435.eB cell as shown in Figure 6C and 6D.

DISCUSSION

We and others have independently demonstrated that ErbB2 overexpression confers Taxol resistance in multiple breast cancer model systems (2,5,6,8,9,22,28). Interestingly, clinical studies of the correlation between ErbB2 status and response to Taxol have led to conflicting results. Some studies suggested that *ErbB2* gene amplification may be a marker of benefit from Taxol (29,30), while other studies showed no association of ErbB2 and Taxol response (31-33), or increased resistance (34). These contradictory results stimulated a healthy debate regarding the role of ErbB2 in Taxol response. One possible reason for the controversy is that ErbB2 is frequently amplified in breast tumors as part of an amplicon on chromosome 17q21. This amplicon contains many genes that are related to breast cancer development (35). It is likely that the companion genes on the ErbB2 amplicon contribute to the reported Taxol sensitivity, while *ErbB2* gene itself actually decreases the sensitivity to Taxol. For example, it has been suggested that Topoisomerase II- α (topoII), co-amplified with ErbB2, plays a role in determining chemosensitivity (36,37). In addition, in clinical studies the comparison was made among different patients with different genetic background (possibly accumulated multiple genetic and epigenetic alterations) and there are plenty of cells within an ErbB2+ tumor that are not overexpressing the receptor. Moreover, the conflicting results are also likely contributed by the unplanned nature of retrospective studies. Nevertheless, there is no clear direct link between ErbB2 overexpression and Taxol sensitivity. In contrast, we compared the Taxol sensitivity between ErbB2-low and — high expressing cells with the same genetic background in our experimental model, which eliminates the multivariate in clinical studies. Taken together, the relationship between ErbB2 and taxol sensitivity/resistance has been conflicting, which deserves further study to reconcile the apparent controversy.

Survivin has attracted considerable attention as a therapeutic target for anti-cancer strategies due to its bifunctional role in regulating cell division and apoptosis, both of which are involved in tumor development. Importantly, its dramatically increased expression in tumor than normal tissue makes it an ideal target for therapy that should specifically inhibit tumor growth or enhance tumor cell apoptosis. Moreover, survivin overexpression also plays a role in chemoresistance, including taxol resistance. However, the mechanisms of survivin mediated Taxol resistance were elusive. Here, our study demonstrated that ErbB2-mediated survivin upregulation contributes to Taxol resistance through survivin-mediated faster Mitosis exit; reducing survivin level in ErbB2-over-expressing cells by antisense oligonucleotides delayed mitotic exit and sensitized cells to Taxol-induced apoptosis. It is also possible that high level of survivin in ErbB2 over-expressing breast cancer cells increased the threshold of spindle checkpoint and even override the mitotic checkpoint, allowing the cells to further progress to G₁ phase after a transient delay of M phase exit caused Taxol treatment. Consistently, expression of survivin correlates with Taxol resistance in ovarian and prostate cancers (38, 39). Moreover, inhibition of survivin enhanced Taxol sensitivity in Hela, and MCF-7 cells (40,41). Our findings here provided further evidence and insight of survivin expression and tumor cell resistance to Taxol. Currently, anti-survivin agents are under extensive study in both research and clinical settings. It will be very important and clinically relevant to design combination therapy targeting survivin plus Taxol treatment for cancer patients who developed

Taxol resistance either due to high ErbB2 expression or due to other molecular mechanisms that upregulated survivin.

Multiple tightly concerted mechanisms are involved in regulating survivin expression at the G₂-M transition of cell cycle, and then degradation upon entry into G₁ phase in non-malignant cells (27). However, survivin expression can be deregulated by various oncogenic pathways in malignant cells and survivin expression may not be solely cell-cycle dependent (42). Here, we found that ErbB2 upregulated survivin expression at the protein translation level, which has not been previously reported. In addition, we found that PI3K/Akt and Src activation, as downstream events of ErbB2 overexpression, contribute to survivin translational level upregulation through activating mTOR/4EBP1 pathway. Indeed, combination of PI3K/Akt inhibitor or Src inhibitor with Taxol dramatically sensitized ErbB2-overexpressing breast cancer cells to Taxol treatment. Since clinically applicable PI3K/Akt inhibitors and Src inhibitors are under intensive investigations for treating cancer patients (43,44), it will be important to investigate whether combination of these pathway inhibitors and Taxol may bring additional therapeutic benefit to patients with ErbB2-over-expressing breast cancers. In summary, our study provided new insights into the mechanisms of ErbB2-mediated Taxol resistance and suggests a clinical potential for survivin inhibitors, and PI3K/Akt and Src inhibitors in sensitizing ErbB2-over-expressing breast cancers to Taxol therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. ErbB2-overexpressing cells exit mitosis faster than ErbB2-low-expressing cells. **A**, ErbB2 levels were detected by immunoblotting in 435.neo and 435.eB cells. **B**, Percentage of cells remained in M phase after nocodazole removal. **C**, Cyclin B1 and securin degradation in 435.neo and 435.eB cells after nocodazole removal. Cyclin B1, Securin, and pY15-Cdc2 were detected by immunoblotting.

Fig. 2.

ErbB2 regulates survivin expression in breast cancer cells. **A**, ErbB2 overexpression leads to increased survivin protein expression. MDA-MB-435 and MCF7 breast cancer cells were stably transfected with wild type *ErbB2* gene. Cell lysates were subjected to immunoblotting with antibodies against ErbB2, survivin, and β -actin. BT474 cells were transfected with ErbB2 siRNA or control siRNA, and cell lysates were subjected to immunoblotting with indicated antibodies. **B**, 435.neo and 435.eB cells were either untreated or treated with nocodazole (0.4 μ g/ml) for 16 hours to synchronize at G2/M phase, and then cell lysates were subjected to immunoblotting with indicated antibodies. **C**, Representative images of IHC staining for ErbB2

and survivin expression in one ErbB2 over-expressing and one ErbB2 low-expressing tumor samples.

Fig. 3.

Upregulation of survivin by ErbB2 plays a role in the faster mitotic exit of cells. **A**, 435.neo and 435.eB cells were treated with survivin antisense (surv-AS) or nonsense (surv-NS) oligonucleotides. Survivin was detected by immunoblotting. **B**, Effect on mitotic exit after surv-AS transfection. Twenty-four hours after surv-AS transfection, cells were synchronized at G2/M phase by nocodazole (0.4 μ g/ml) treatment for 16 h, and then released from cell cycle arrest by washing out nocodazole. Cells were then collected at different time points, stained with Giemsa, and counted under a microscope. **C**, Representative results from (B). **D**, Cyclin B1 and Securin degradation in surv-AS- or surv-NS-treated 435.neo and 435.eB cells after

nocodazole removal. Cells were treated as in (B). Cell lysates were collected at different time points, and analyzed for Cyclin B1 and Securin levels by immunoblotting.

Fig. 4.

Downregulation of survivin by antisense oligonucleotides sensitizes ErbB2-overexpressing cells to Taxol-induced apoptosis. **A**, Twenty-four hours after surv-AS or surv-NS transfection, cells were treated with 20 nM Taxol for 0, 48, or 72 h. Apoptosis was examined by Annexin-V staining and flow cytometry analysis. **B**, PARP cleavage in surv-AS- or surv-NS-treated 435.neo and 435.eB cells after Taxol treatment (20nM for 72 hours).

Fig. 5.

ErbB2 upregulates survivin at post-transcription level. **A**, Survivin mRNA level is similar in 435.neo and 435.eB cells. The fold difference is a ratio difference of survivin mRNA after normalization to GAPDH. **B**, 435.neo and 435.eB cells were treated with 100ng/ml cycloheximide (CHX) for 1, 2, 4 hours, cell lysates were collected for immunoblotting with antibodies against survivin and β -actin. The relative intensity of survivin band was normalized to its β -actin loading. Degradation curves were drawn based on three independent experiments. **C**, Survivin mRNA distribution in polysome fractions of 435.neo and 435.eB cells, one representative of three independent experiments was shown. **D**, 435.neo and 435.eB cells were treated with rapamycin (25 μ M) for 5 hours, cell lysates were collected for immunoblotting with antibodies against survivin, β -actin, p-4EBP-1 and total 4EBP-1.

Fig. 6. PI3K/Akt and Src are involved in translational upregulation of survivin and Taxol resistance by ErbB2. **A**, 435.neo and 435.eB cells were treated with LY294002 (20 μ M), or rapamycin (25 μ M), or Src inhibitors PP2 (25 μ M) and AZD0530 (25 μ M) for 5 hours, cell lysates were collected for immunoblotting analysis with indicated antibodies. **B**, 435.neo and 435.eB cell were treated as in A, RNA samples were collected, reverse transcribed into cDNA, and followed by Q-PCR with survivin specific primers and GAPDH primers as control. **C**, 435.neo and 435.eB cells were pre-treated with DMSO, LY294002 (5 μ M), or AZD0530 (1 μ M) for 12 hours, then Taxol (50nM) was added for 48 hours. Viable cells were detected by MTS assay.

Bar figure represents the growth inhibition. **D**, Representative images of the effects of various treatments (as in C) in 435.neo and 435.eB cells.