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 up-regulation of survivin that confers Taxol resistance.

Experimental Design: ErbB2-overexpressing 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 down-regulated by antisense oligonucleotides to evaluate its contribution to mitotic exit and Taxol resistance in ErbB2-overexpressing 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 up-regulation 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-overexpressing cells to Taxol-induced apoptosis. Moreover, ErbB2 up-regulated survivin at translational level and PI3K/Akt and Src activation 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 up-regulation by ErbB2 is a critical event in ErbB2-mediated faster mitotic exit and contributes to Taxol resistance.

Taxol (paclitaxel) is one of the most effective microtubule-targeting drugs for breast cancer treatment, and it accounts for significant improvements in the survival of breast cancer patient 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% to 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 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 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 up-regulation 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 mitotic exit and cell survival, we tested the hypothesis that mitotic deregulation by survivin up-regulation in ErbB2-overexpressing 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-overexpressing cells to Taxol-induced apoptosis. In addition, our data indicated that ErbB2 up-regulated survivin at protein translational level that involved PI3K/Akt and S6c downstream of ErbB2.
fraction was collected and loaded onto a 15% to 40% linear sucrose gradient and centrifuged at 38,000 × g for 90 mins. Ten fractions were collected to extract RNA for reverse transcription-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), 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 shown 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; ref. 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 minutes 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 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 minutes after being released from M phase arrest (Fig. 1B). These results indicated that ErbB2-overexpressing cells exit mitosis faster than ErbB2-low-expressing cells.

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![Fig. 1. ErbB2-overexpressing cells exit mitosis faster than ErbB2-low-expressing cells.](image)

A. ErbB2 levels were detected by immunoblotting in 435.neo and 435.eB cells. B. percentage of cells remaining 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 α-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 μg/mL) for 16 h to synchronize at G2/M phase, and then cell lysates were subjected to immunoblotting with indicated antibodies. C. representative images of immunohistochemical staining for ErbB2 and survivin expression in one ErbB2-overexpressing and one ErbB2-low-expressing tumor samples.
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-expressing and ErbB2-overexpressing breast cancer cell lines. Indeed, the ErbB2-overexpressing 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 small interfering RNA, it down-regulated 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-overexpressing and ErbB2-low-expressing breast tumor specimens were stained for ErbB2 and survivin by immunohistochemistry. Five 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 (Supplementary Table S1 and Fig. 2C). These data showed survivin up-regulation in ErbB2-overexpressing 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 up-regulated in ErbB2-overexpressing breast cancer cells. To test this hypothesis, we treated 435.neo and 435.eB cells with the survivin antisense oligonucleotide and with the nonsense oligonucleotide as control. As shown in Fig. 3A, survivin antisense effectively down-regulated survivin expression in 435.neo and 435.eB cells, as well as in BT474 cells. Compared with survivin nonsense treatment, survivin antisense did not significantly alter the time course of mitosis exit of 435.neo cells (Fig. 3B). In contrast, survivin antisense treatment led to...
more 435.eB cells remaining in the M phase at 90 minutes compared with survivin nonsense–treated cells (32% for survivin nonsense–treated and 49% for survivin antisense–treated cells; P < 0.05) after release from M phase arrest (Fig. 3B and C). The data indicated that down-regulation of survivin delayed the exit of ErbB2-overexpressing 435.eB cells from mitosis. These findings are further supported by delayed cyclin B1 and securin degradation in survivin antisense–treated than in survivin nonsense–treated 435.eB cells (Fig. 3D). These results indicated that ErbB2-mediated up-regulation of survivin contributes to a faster mitotic exit of ErbB2-overexpressing breast cancer cells.

**Down-regulation of survivin sensitizes ErbB2-overexpressing cells to Taxol-induced apoptosis.** Because survivin contributes to the faster mitotic exit of ErbB2-overexpressing breast cancer cells that are resistant to Taxol-induced mitotic catastrophe/apoptosis, we reasoned that the increased survivin level in ErbB2-overexpressing cells may contribute to ErbB2-mediated Taxol resistance. To test this, we treated 435.neo and 435.eB cells with survivin antisense to down-regulate survivin expression or with survivin nonsense as a control. Twenty-four hours later, cells were treated with Taxol to induce apoptosis. The survivin nonsense–treated 435.neo cells showed increased apoptosis with Taxol treatment, whereas survivin nonsense–treated 435.eB cells were resistant to Taxol-induced apoptosis, as expected (refs. 8, 9; Fig. 4A). Interestingly, survivin antisense treatment dramatically sensitized the ErbB2-overexpressing 435.eB cells to Taxol-induced apoptosis (Fig. 4A). Consistently, survivin antisense–treated 435.eB cells showed increased poly (ADP-ribose) polymerase cleavage than did survivin nonsense–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 with the survivin-low patients in the ErbB2-positive group following neoadjuvant T/FAC treatment (Supplementary Fig. S1). We did not observe a significant P value because of the small sample size.

**ErbB2 up-regulates survivin at the translational level.** Our data above showed that survivin up-regulation 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 up-regulation, we next investigated how ErbB2 up-regulates 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 up-regulation in ErbB2-overexpressing cells should largely result from increased protein translation. Because 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 Fig. 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 up-regulation was largely at the translational level in these breast cancer cells.

**PI3K/Akt and Src are involved in translational up-regulation of survivin and Taxol resistance by ErbB2.** ErbB2 has been implicated to up-regulate 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 up-regulation. 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 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 up-regulation 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 pretreated 435.neo and 435.eB cells with PI3K inhibitor LY294002, Src inhibitor AZD0530, or control DMSO for 12 hours and then added Taxol. The pretreatments did not have significant effect on cell cycle profiles of these cells (Supplementary Fig. S2). Remarkably, the combination of LY294002 and Taxol or that of AZD0530 and Taxol greatly increased the sensitivity to Taxol treatment in 435.eB cell, as shown in Fig. 6C and D.

Discussion

We and others have independently shown that ErbB2 overexpression confers Taxol resistance in multiple breast cancer model systems (2, 5, 6, 8, 9, 22, 28). Interestingly, clinical studies on 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), whereas other studies showed no association of ErbB2 amplification 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-alpha, coamplified 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 anticancer strategies because of its bifunctional role in...
regulating cell division and apoptosis, 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 showed that ErbB2-mediated survivin up-regulation contributes to Taxol resistance through survivin-mediated faster mitosis exit; reducing survivin level in ErbB2-overexpressing cells by antisense oligonucleotides delayed mitotic exit and sensitized cells to Taxol-induced apoptosis. It is

Fig. 6. PI3K/Akt and Src are involved in translational up-regulation of survivin and Taxol resistance by ErbB2. A. 435.neo and 435.eB cells were treated with LY294002 (20 μmol/L), rapamycin (25 μmol/L), or Src inhibitors PP2 (25 μmol/L) and AZD0530 (25 μmol/L) for 5 h; 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 quantitative PCR with survivin-specific primers and glyceraldehyde-3-phosphate dehydrogenase primers as control. C. 435.neo and 435.eB cells were pretreated with DMSO, LY294002 (5 μmol/L), or AZD0530 (1 μmol/L) for 12 h; then, Taxol (50 nmol/L) was added for 48 h. Viable cells were detected by MTS assay. Bar, growth inhibition. D, representative images of the effects of various treatments, as in C, in 435.neo and 435.eB cells.
also possible that high level of survivin in ErbB2-overexpressing 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 by 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, antisurvivin agents are under extensive study in 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 because of high ErbB2 expression or because of other molecular mechanisms that up-regulated survivin.

Multiple tightly concerted mechanisms are involved in regulating survivin expression at the G1-M transition of cell cycle and then degradation upon entry into G1 phase in nonmalignant 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 up-regulated 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 up-regulation 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. Because 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-overexpressing 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-overexpressing breast cancers to Taxol therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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