Regulation of Survivin by ErbB2 Signaling: Therapeutic Implications for ErbB2-Overexpressing Breast Cancers

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Abstract

In breast cancer, overexpression of ErbB2 or aberrant regulation of survivin, a member of the inhibitor of apoptosis family, is associated with resistance to chemo/hormone therapy and predicts for a poor clinical outcome. A functional link between the two predictive factors has not been previously shown. Here, using genetic and pharmacologic approaches to block ErbB2 signaling, we show that ErbB2 regulates survivin protein expression in ErbB2-overexpressing breast cancer cells. Selective knockdown of ErbB2 using small interfering RNA markedly reduced survivin protein, resulting in apoptosis of ErbB2-overexpressing breast cancer cell lines such as BT474. Alternatively, inhibition of ErbB2 signaling using lapatinib (GW572016), a reversible small-molecule inhibitor of ErbB1/ErbB2 tyrosine kinases, at pharmacologically relevant concentrations, leads to marked inhibition of survivin protein with subsequent apoptosis. The effect of lapatinib on survivin seems to be predominantly posttranslational, mediated by ubiquitin-proteosome degradation as lactacystin, a proteosome inhibitor, reverses these effects. Furthermore, lapatinib down-regulated the expression of His-tagged survivin, which was under the transcriptional control of a heterologous promoter, providing additional evidence supporting a posttranslational mechanism of regulation. In contrast, trastuzumab and gefitinib failed to down-regulate survivin in ErbB2-overexpressing breast cancer cells. Importantly, the clinical relevance of these findings was illustrated in patients with ErbB2-overexpressing breast cancer whose clinical response to lapatinib was associated with marked inhibition of survivin in their tumors. These findings shed new light on the mechanism by which ErbB2 overexpression protects against apoptotic stimuli in breast cancer and identifies therapeutic interventions to improve clinical outcomes in these aggressive tumors. (Cancer Res 2006; 66(3): 1640-7)

Introduction

Aberrant expression of survival factors protects tumors from cell death following activation of intrinsic or extrinsic apoptotic pathways. Among the major gene families regulating cell survival are the inhibitor of apoptosis proteins (IAP), which in humans consists of eight family members (1). Members of the IAP family protect against apoptosis by either directly or indirectly inhibiting activation of effector caspases (1–4). Included among the most common transcripts selectively expressed in tumors, but not in normal tissue, is survivin, the smallest IAP family member (5, 6). Expression of survivin in tumors correlates with a poor clinical outcome in a variety of malignancies including breast cancer (5, 7, 8).

In nonmalignant proliferating cells, expression of survivin protein is regulated in a cell cycle–dependent manner, transiently up-regulated during G_2-M, followed by its rapid down-regulation on entry into G_1 phase (9–11). Survivin regulates two critical activities during G_2-M. First, it enables cell cycle progression by associating with and stabilizing components of the mitotic spindle apparatus (12–15). And second, by maintaining the integrity of the mitotic spindle, survivin protects against apoptosis triggered by activation of the mitotic spindle checkpoint (16). Induction of survivin during G_2-M is primarily transcriptionally regulated (9) whereas its down-regulation on entry into G_1 is both transcriptional and posttranslational, the latter mediated by the ubiquitin-proteosome pathway (17). Increased survivin protein in tumors does not seem to be solely cell cycle dependent as it occurs in tumor cells that are not actively cycling (18).

Members of the ErbB family of transmembrane tyrosine kinase receptors promote tumor cell growth and survival. Overexpression or gene amplification of ErbB2 (Her-2/neu), which occurs in 20% to 30% of breast cancers, predicts for a poor clinical outcome and resistance to chemo- and hormonal therapies (19, 20). On binding a cognate ligand, ErbB receptors undergo homodimerization or heterodimerization and autophosphorylate cytoplasmic tyrosine residues that serve as binding sites for proteins containing Src homology 2 and phosphotyrosine-binding domains, which in turn link activated ErbB receptors to downstream proliferation [e.g., mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (Erk) 1/2] and survival [e.g., phosphatidylinositol 3-kinase (PI3K)-Akt] pathways (21). ErbB2, which lacks an exogenous ligand, is the preferred partner for other ErbB receptors amplifying the biological signal emanating from ErbB2-containing heterodimers (22, 23). Although the exact mechanism(s) is unknown by which ErbB2 overexpression protects tumors against chemotherapy, it has largely been attributed to activation of the PI3K-Akt survival pathway, which is concomitantly up-regulated in ErbB2-overexpressing breast cancer cells (24–27). Furthermore, ErbB2 heterodimerizes with ErbB3, the latter lacking intrinsic autokinase activity and requiring transactivation through interactions with its ErbB partner (28). ErbB3 contains six PI3K binding sites, making ErbB2/ErbB3 heterodimers among the most potent activators of the PI3K-Akt pathway (29–32).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Here we show that survivin is regulated by ErbB2 and ErbB3, but not by ErbB1. Interrupting ErbB2/ErbB3 heterodimer signaling using RNA interference or lapatinib (GW572016; ref. 33), a potent small-molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, down-regulates survivin and induces apoptosis in ErbB2-overexpressing breast cancer cell lines and in primary tumors. Moreover, down-regulation of survivin by lapatinib is largely mediated by proteosome-dependent degradation. Our results provide insight into the mechanism(s) by which ErbB2 overexpression protects breast cancers from apoptosis and identifies therapeutic strategies to improve clinical outcomes in these aggressive tumors.

Materials and Methods

Cell culture and reagents. BT474, SKBR3, and Au565 cells were from the American Tissue Culture Collection (Manasus, VA). BT474, SKBR3, and Au565 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and L-glutamine. The LICR-LON-HN5 (HN5) cell line (kindly provided by Helmout Modjtahedi at the Institute of Cancer Research, Surrey, United Kingdom) were maintained in DMEM with high glucose and 10% FCS. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The following antibodies were purchased for Western blot: survivin from R&D Systems (Minneapolis, MN); phosphotyrosine and actin from Sigma-Aldrich (St. Louis, MO); ErbB1 (Ab-12) and ErbB2 (Ab-11) from LabVision (Fremont, CA); p-Akt (Ser437) from Cell Signaling Technology (Beverly, MA); and Akt, p-Erk1/2, Erk1/2, and p-Erk1/2 from Santa Cruz Biotechnology (Santa Cruz, CA). Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay from Roche Diagnostics (Indianapolis, IN) was done according to the instructions of the manufacturer. Lapatinib or *N*-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino}methyl)-2-furyl]-4-quinazolinamine was synthesized as previously described (33). Lapatinib for cell culture work was dissolved in DMSO (34). Lactacystin was purchased from Biosource (Camarillo, CA); LY294002 and PD98059 were purchased from Calbiochem (La Jolla, CA).

siRNA preparation and transfection. Twenty-one-nucleotide small interfering RNAs (siRNA) targeting survivin (accession no. NM_001168.1) and its appropriate nonsilencing control were synthesized by Qiagen (Valencia, CA). Both siRNAs were synthesized using 2′ TOM-phosphoramidite chemistry, deprotected, and high performance purity purified. The survivin targeting sequence (5′-GCAUUCGUCCGGUUGCGCU-3′) corresponds with position 286, relative to the first nucleotide of the start codon. The nonsilencing control (5′UUCUCCGAACGUGUCACGU-3′) is a random sequence with 16-base overlap to *Thermotoga maritimia*, with no other matches. siRNA SMARTpools generated using 2′-ACE chemistry targeting ErbB2 (accession no. AF077350), ErbB3 (accession no. NM_005228), and ErbB1 (accession no. NM_005228), along with the siControl, were purchased from Dharmacon Research (Lafayette, CO). RNAi liposomes were generated using Lipofectamine 2000 from Gibco/Invitrogen (Carlsbad, CA) complexed with siRNA (100 nmol/L final) in Opti-MEM. Complexes were added to exponentially growing cells suspended in Opti-MEM plus 5% FCS and plated in 12-well plates. Eighteen hours after transfection, complexes were aspirated and replaced with normal growth medium. Samples were harvested at various time points thereafter.

Transfection and His-tagged survivin construct. Exponentially growing SKBR3 cells were transfected using Lipofectamine reagent from Invitrogen. Cells (5×10^5) in 35-mm dishes were transfected with 1 mL of mixture containing 1 μg of cytomegalovirus (CMV)-survivin in pcDNA 3.1 with 8-His tag at the NH_2 terminus. After 5 hours, 1.5 mL of fresh medium were added to the dish for 48 hours. Afterwards, G418 (400 μg/mL) was added in the cell culture medium. After 6 weeks of G418 selection, stably transfected cells were used for further experiments. Cells transfected with pcDNA 3.1 vector only served as controls.

SDS-PAGE and Western blot. Transfected cells were lysed in radioimmunoprecipitation assay buffer (20 mmol/L Tris-HCl, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid, 100 mmol/L NaCl) plus protease inhibitors from Roche Diagnostics and the lysates were cleared at 15,000 × *g* for 20 minutes. Protein concentrations were determined using Bicinchoninic Acid Protein Reagent Kit from Pierce (Rockford, IL). Equal amounts of protein were separated on 4% to 12% NuPage Bis-Tris acrylamide gels from Gibco/Invitrogen and transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 hour in 10% milk in PBS-T [PBS-calcium magnesium-free (PBS-CMF)/0.1% Tween 20], then incubated for 16 hours at 4°C in primary antibody in blocking buffer. After four washes in PBS-T, the blots were incubated in horseradish peroxidase–conjugated secondary antimouse or antirabbit from Jackson ImmunoResearch Labs (West Grove, PA) at a 1:2,000 dilution for 1 hour. Blots were again washed four times and signals were detected using enhanced chemiluminescence from Amersham Biosciences (Piscataway, NJ).

Analyses of cell cycle and apoptosis. For each sample, the culture medium was collected and the monolayer washed in PBS-CMF. Samples were then trypsinized and collected. Cells were pelleted at 200 × *g*, aspirated, fixed in 70% methanol, and stored at −20°C until ready for analysis. The samples were pelleted at 200 × *g*, aspirated, and then rinsed in 10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 4% FCS, and 0.1% sodium azide, and repelleted. The samples were repelleted, aspirated, and resuspended in PBS-CMF/0.1% Triton X-100, 100 μg/mL RNase A (Sigma-Aldrich), and 40 mg/mL propidium iodide for analysis. DNA content was analyzed using a FACSCalibur from Becton Dickenson (San Jose, CA) flow cytometer emitting a 488-nm beam. Aggregated clumps were omitted from analysis with pulse area versus pulse width gating. Data from 20,000 events were collected and analyzed. For Annexin V staining, cells were treated in six-well plates with lapatinib at the indicated concentrations. After harvesting the cells with trypsin-EDTA, 5,000 cells in 50 μL were sampled on 96-well microplates. The cells were stained directly in the microplate with Annexin V-phycoerythrin and Nexin 7-AAD in 1× Nexin buffer in a 200-μL final reaction volume. After incubating for 20 minutes at room temperature, the reaction samples are ready to be acquired in the Guava PCA-96-system from Guava Technology (Hayward, CA).

Immunohistochemistry. Tumor tissue was prepared and quantitative immunohistochemistry was done as previously described (35). Briefly, quantitative immunohistochemistry was done using antisurvivin antibody from LabVision and protein detection using streptavidin peroxidase.

Results

Modulation of survivin in response to inhibition of ErbB2 signaling in breast cancer cells. Although the effects of ErbB2 on growth and survival pathways have been extensively studied in breast cancer cells, the exact mechanism(s) by which overexpression of ErbB2 protects against apoptosis is not completely understood. Both ErbB2 overexpression and deregulation of survivin predict for a poor clinical outcome in breast cancer, making it tempting to speculate that the two are related. To address this possibility, we used two strategies. First, we genetically depleted survivin using a siRNA construct (Sur286) that reduced levels of survivin protein by 90% in ErbB2-overexpressing BT474 breast cancer cells compared with a control siRNA construct (NSC; Fig. 1*A*). Treatment with Sur286 resulted in a 3-fold increase in apoptosis (<2N DNA content) compared with NSC-treated cells (Fig. 1*B*). Exposure to Sur286 also increased the percentage of cells in G_2-M phase as well as those exhibiting polyploidy (>4N; Fig. 1*B*). The selectivity of Sur286 was shown by its lack of effect on other cellular proteins (e.g., Erk1/2; Fig. 1*A*). In addition, nonspecific induction of IFN, which has been associated with siRNA, was not observed with our siRNA constructs (data not shown). Thus, in contrast to other tumor cell lines that primarily undergo cell cycle arrest (12, 36), depleting survivin in ErbB2-overexpressing breast cancer cells induced apoptosis.

If survivin is regulated by ErbB2, then blocking ErbB2 signaling might modulate expression of survivin and affect cell survival. Selective ErbB2 depletion using siRNA not only down-regulated survivin (Fig. 1*C*) but also increased apoptosis in BT474 cells in a time-dependent manner (Fig. 1*D*), establishing a functional link between ErbB2, survivin, and cell survival. Cells exposed to a control pool of siRNA (siPool) served as controls.

ErbB2 is one of four ErbB receptor family members (ErbB1-4; ref. 21). ErbB3, which lacks intrinsic autokinase activity, heterodimerizes with ErbB2, forming a receptor complex that potently activates the PI3K survival pathway (29–32). Similar to ErbB2, selective depletion of ErbB3 using ErbB3 siRNA (Fig. 1*C*) enhanced apoptosis of BT474 cells in a time-dependent manner (Fig. 1*D*). As a consequence of depleting ErbB3, steady-state survivin and p-Akt protein levels were markedly reduced (Fig. 1*C*), implicating both ErbB2 and ErbB3, possibly through ErbB2/ErbB3 heterodimers, in the regulation of survivin.

ErbB1 (epidermal growth factor receptor) is highly homologous to ErbB2 and frequently expressed in epithelial tumors (21). To determine whether ErbB1 also regulates survivin, we genetically depleted survivin in HN5 cells, a head and neck carcinoma line that predominantly expresses ErbB1 and relatively less ErbB2 and ErbB3 (34). In contrast to BT474, exposing HN5 cells to Sur286 did not increase apoptosis but instead triggered a 6-fold increase in the percentage of cells exhibiting polyploidy and a concomitant increase in the percentage of cells arrested in G_2-M compared with cells exposed to a control siRNA (siPool; Fig. 2*A*). Moreover, siRNA depletion of ErbB1 did not affect survivin or HN5 cell survival (Fig. 2*A* and *B*), suggesting that ErbB1 might not play a significant role in regulating survivin or cell survival in these cells.

To examine the potential clinical relevance of the association between survivin and ErbB receptors, expression of survivin was analyzed by immunohistochemistry in 202 breast cancer biopsies with varying degrees of ErbB1, ErbB2, and ErbB3 protein expression. Expressions of survivin, ErbB1, ErbB2, and ErbB3 proteins were scored accorded to the intensity of staining (e.g., 0, 1, 2/3+) and analyzed for statistical correlation. No association was found between survivin and ErbB1 protein expression (P = 0.2678;

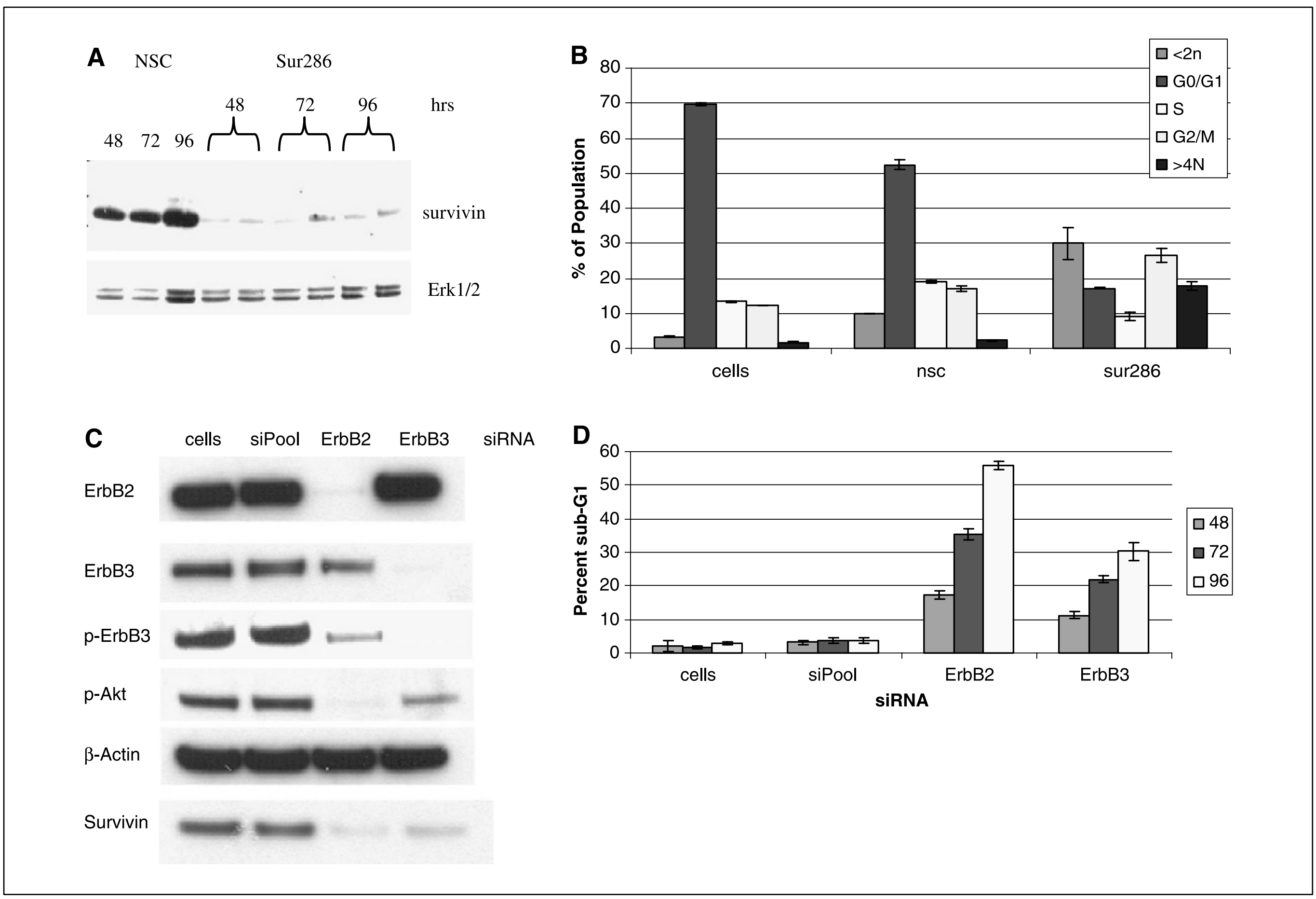

Figure 1. Survivin is regulated by ErbB2 and ErbB3 in ErbB2-overexpressing breast cancer cells where it mediates prosurvival effects. *A,* selective knockdown of survivin protein using siRNA (Sur286). Equal amounts of protein from whole-cell lysates in BT474 cells exposed to Sur286 and a control siRNA construct (NSC) were separated by SDS-PAGE and Western blotted for survivin and Erk1/2. *B,* effects of Sur286 or NSC on cell survival. Cell cycle analysis was done using propidium iodide staining and flow cytometry on BT474 cells treated with Sur286 and NSC siRNA for 48 hours. Untreated cells served as controls. Cells with <2N DNA content (*green*) represent apoptotic cells whereas those with >4N (dark blue) exhibit polyploidy. Representative of three independent experiments. *C,* ErbB2 and ErbB3 knockdown inhibits survivin and p-Akt. Equal amounts of protein from BT474 whole-cell lysates after treatment for 48 hours with the indicated siRNA constructs (siPool/control, ErbB2, and ErbB3) were separated by SDS-PAGE and Western blotted for ErbB2, ErbB3, p-ErbB3, p-Akt, and survivin. Steady-state levels of actin served as a control for equal loading of protein. Untreated cells (*cells*) served as additional controls. *D,* induction of apoptosis following ErbB2 and ErbB3 knockdown. BT474 cells were treated with the indicated siRNA constructs. At 48, 72, and 96 hours following transfection of siRNA constructs, apoptosis (sub-G_1 population) was assessed using propidium iodide staining and flow cytometry. Untreated cells (cells alone) served as additional controls. Representative of three independent experiments.

Supplementary Table S1). In contrast, increased survivin protein expression (e.g., 2/3+) correlated with increased ErbB2 and ErbB3 protein expression with P values of 0.0528 and 0.0039, respectively (Supplementary Figs. S1-3 and Table S1). Thus, the relationship between ErbB2 and ErbB3 overexpression, but not ErbB1, and increased survivin in ErbB2-overexpressing tumor cell lines was also relevant in primary breast cancers.

An ErbB2 tyrosine kinase inhibitor down-regulates survivin, inducing apoptosis. The second strategy to study the relationship between ErbB2 and survivin was a pharmacologic one. We previously showed that inhibition of ErbB receptor signaling using lapatinib (GW572016), a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, induced apoptosis of ErbB2-overexpressing breast cancer cell lines and primary tumors (34, 35, 37). The effects of lapatinib on survivin were shown by Western blot analysis wherein survivin steady-state protein levels were inhibited in a concentration- and time-dependent manner in two ErbB2-overexpressing breast cancer cell lines, BT474 and SKBR3 (Fig. 3*A* and *B*). Inhibition of survivin correlated with a concentration-dependent increase in tumor cell apoptosis (Fig. 3*A*).

To determine whether these observations were generalized to other ErbB-targeted therapies, we next assessed the effects of two ErbB2-targeted therapies on survivin. The first, trastuzumab, is a humanized anti-ErbB2 monoclonal antibody approved for treating patients whose tumors overexpress ErbB2 or exhibit ErbB2 gene amplification (38). Trastuzumab had little effect on cell survival or steady-state survivin protein levels in BT474 cells (Fig. 3*A*). The second, gefitinib, a small-molecule inhibitor of ErbB1 tyrosine kinase activity with reported antiproliferative activity in BT474 cells (39), did not significantly affect survivin in BT474 cells compared with lapatinib (Fig. 3*C*).

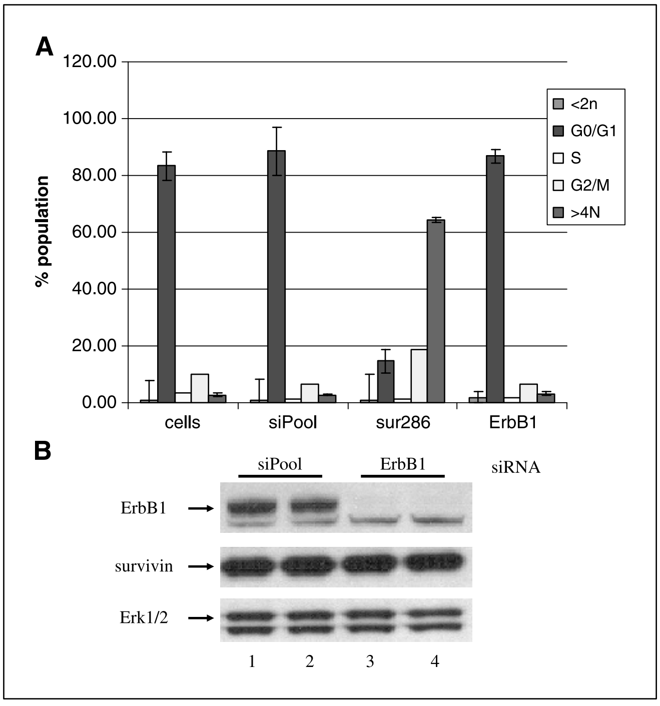

Figure 2. The link between ErbB2, survivin, and cell survival does not seem to be generalized to ErbB1 signaling. *A,* survivin or ErbB1 knockdown does not induce apoptosis in HN5 cells. HN5 cells were treated with the indicated siRNA constructs (siPool/control, Sur286, and ErbB1) for 72 hours; after which, cell cycle analysis was done by propidium iodide staining and flow cytometry. Summary of three independent experiments. *B,* ErbB1 knockdown does not inhibit survivin protein. HN5 cells were treated with the indicated siRNA constructs (*lanes 1* and *2,* siPool/control; *lanes 3* and *4,* ErbB1). After 72 hours, equal amounts of protein were separated by SDS-PAGE and steady-state survivin protein levels were analyzed by Western blot analysis.

Regulation of survivin by lapatinib is dependent on the proteosome. If the down-regulation of survivin plays a role in lapatinib-induced apoptosis, then overexpressing survivin might protect cells from the antitumor activity of lapatinib. Moreover, if the regulation of survivin by lapatinib is solely transcriptionally mediated, then lapatinib would not be expected to reduce His-tagged survivin protein, which is under the transcription control of a heterologous promoter. To this end, we established stably transfected SKBR3 cell lines expressing His-tagged survivin under the transcriptional control of a heterologous CMV promoter. As shown, induction of apoptosis by lapatinib was unaffected by the expression of His-tagged survivin (Fig. 4*A* and *B*). Protein levels of His-tagged (*dotted line*) and endogenous (*solid line*) survivin were equally down-regulated in response to lapatinib (Fig. 4*B*), implicating a posttranscriptional mechanism by which lapatinib regulates survivin.

The ubiquitin-proteosome pathway plays an important role in regulating apoptosis (40). ErbB2 regulates CXCR4, p53, and the androgen receptor through proteosome-dependent proteolysis (13, 25, 41), making it tempting to speculate that lapatinib regulation of survivin might also be dependent on the ubiquitin-proteosome pathway. Lactacystin, a specific proteosome inhibitor, reversed lapatinib-mediated down-regulation of survivin (Fig. 5, *lane 5*), suggesting that the effects of ErbB2/ErbB3 signaling on survivin are in part dependent on proteosome degradation.

The role of PI3K in mediating the effects of lapatinib on survivin. In hematopoietic and endothelial cells, regulation of survivin has been linked to MAPK-Erk and PI3K-Akt signaling pathways (42, 43). Blocking ErbB2 signaling using either siRNA or lapatinib abrogates downstream MAPK-Erk1/2 and PI3K-Akt signaling (34, 37). To determine the downstream pathways involved in the regulation of survivin by ErbB2, we used specific MAPK/Erk kinase (MEK) and PI3K inhibitors or combinations thereof. Steady-state survivin protein levels were reduced in BT474 cells treated with the PI3K inhibitor LY294002 (10 μmol/L), similar to that achieved using lapatinib alone (Fig. 6). In contrast, the MEK1 inhibitor PD98059 (10 μmol/L) had relatively little effect on survivin (Fig. 6). Based on these results, we propose that lapatinib regulates survivin primarily through its inhibition of PI3K signaling rather than MAPK. However, siRNA knockdown of Akt (simultaneous knockdown of Akt1, Akt2, and Akt3) in BT474 cells did not affect survivin protein levels (Supplementary Fig. S4). These findings suggest that the regulation of survivin by lapatinib seems to be PI3K dependent but primarily Akt independent.

Because ErbB2/ErbB3 heterodimers potently activate the PI3K pathway (29–32), we next examined the effect of ablating ErbB2 kinase activity on the activation state of ErbB3. Steady-state protein levels of activated p-ErbB3 were markedly reduced following ErbB2-targeted siRNA, resulting in the inhibition of PI3K signaling as reflected by a reduction in p-Akt expression (Fig. 1*C*).

Inhibition of survivin in primary tumors correlates with clinical response to lapatinib. To determine if the regulation of survivin by ErbB2 occurs in primary tumor tissue, we did quantitative immunohistochemical analysis on tumor biopsies

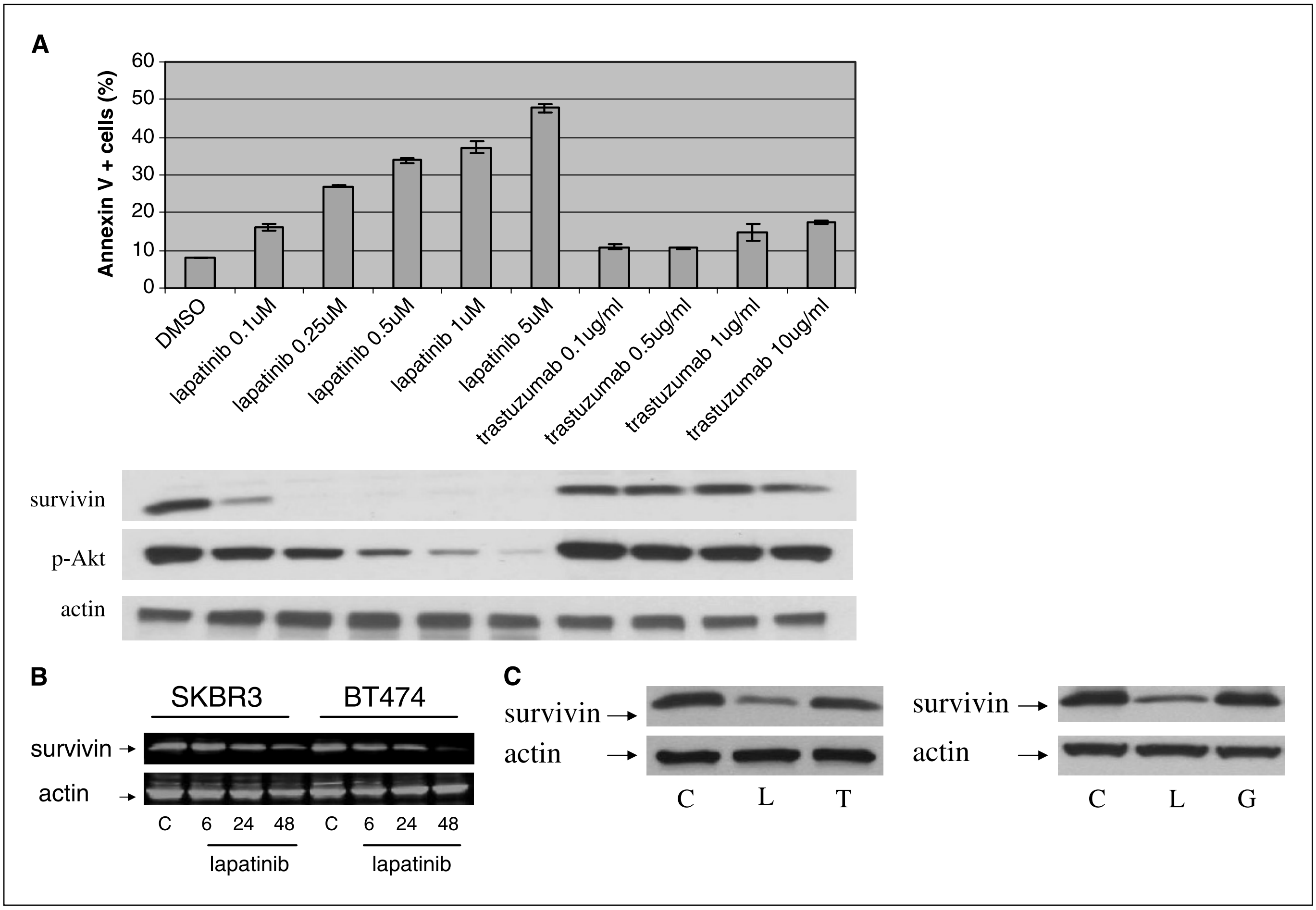

Figure 3. Inhibition of ErbB2 by lapatinib in turn reduces survivin protein in ErbB2-overexpressing breast cancer cells. *A,* concentration-response effect of lapatinib on survivin in BT474 cells. Cells were treated with lapatinib at the indicated concentrations. After 72 hours, apoptosis was analyzed by Annexin V staining and flow cytometry (*top*). Concentration-response effect of trastuzumab is also indicated. Cells treated with vehicle alone (DMSO) served as a control. Samples from the above treatment conditions were also analyzed by Western blot for survivin steady-state protein levels (*bottom*). Actin steady-state protein levels served as a control for equal loading of protein. *B,* lapatinib inhibits survivin in SKBR3 and BT474 cells in a time-dependent manner. Cells were treated with a pharmacologically relevant concentration of lapatinib (500 nmol/L) for 6, 24, and 48 hours. At the indicated times, cells were harvested and equal amounts of protein separated by SDS-PAGE and Western blot analysis were done. Steady-state actin protein levels served as a control for equal loading of lanes. *C,* inhibition of survivin by lapatinib is not shared by other ErbB-targeted agents. BT474 cells were treated with lapatinib (500 nmol/L; *L*), trastuzumab (10 μg/mL; *T*), or gefitinib (500 nmol/L; *G*) for 72 hours; after which, equal amounts of whole-cell lysate were separated by SDS-PAGE and Western blot analysis was done. Cells treated with vehicle alone (DMSO) served as controls (*C*).

obtained from patients with metastatic tumors overexpressing ErbB2 and/or expressing ErbB1, who were treated with lapatinib as part of a phase I clinical trial, the results of which were recently described (35). Fresh tumor biopsies were obtained before and after 21 days of lapatinib therapy. Stained tissue sections were computer analyzed and a mean absorbance score representative of the intensity of staining was assigned to each biopsy. Decreased survivin protein expression at day 21 correlated with tumor regression and clinical response to lapatinib. Figure 7 shows the effects of lapatinib therapy on survivin and TUNEL in one of the patients—all of whom had ErbB2-overexpressing breast cancers—who responded by achieving a partial remission (35). The effects of lapatinib on survivin and TUNEL in this patient are representative of the effects of lapatinib therapy in the other four responders (Supplementary Table S2). As shown, survivin was reduced by 90% compared with pretreated biopsies after only 21 days of lapatinib therapy, with a concomitant increase in tumor cell apoptosis (TUNEL-positive cells; Fig. 7). The effects of lapatinib on survivin were examined in an additional four nonresponders and shown to be essentially unchanged, along with an absence of tumor cell apoptosis (data not shown).

Discussion

Breast cancers that overexpress ErbB2 or exhibit deregulation of survivin protein have a poor clinical outcome (8, 19, 20). To date, a functional link between these two key prosurvival factors has not been shown. The underlying mechanism(s) responsible for the antiapoptotic effects of ErbB2 overexpression has largely been attributed to the concomitant up-regulation of the PI3K-Akt survival pathway (24–27). We now show that ErbB2 regulates survivin protein in ErbB2-overexpressing breast cancer cells, providing protection against apoptotic stimuli.

Inhibition of ErbB2 kinase using genetic (siRNA) or pharmacologic (lapatinib) interventions triggered apoptosis in ErbB2-overexpressing breast cancer cells (Figs. 1 and 3). Growth and survival signals elicited by activated ErbB2 are largely mediated via PI3K-Akt and Ras-MAPK signaling pathways. Using specific PI3K

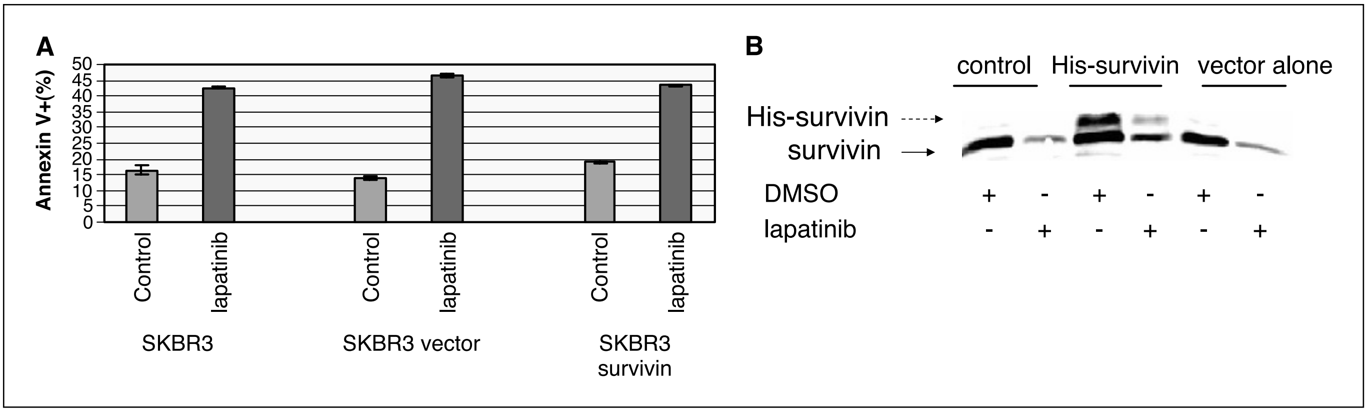

Figure 4. Expression of His-tagged survivin fails to protect ErbB2-overexpressing breast cancer cells from lapatinib-induced apoptosis. *A,* His-tagged survivin under the control of a CMV-heterologous promoter fails to prevent lapatinib-induced apoptosis of SKBR3 cells. SKBR3 cells were transfected with His-tagged survivin under the control of a CMV heterologous promoter. Cells were treated with lapatinib (500 nmol/L) for 72 hours and apoptosis was assessed by Annexin V staining and flow cytometry. Percent Annexin V–positive cells from untransfected SKBR3 cells, SKBR3 cells transfected with vector alone, and SKBR3 cells transfected with His-tagged survivin. Representative of three independent experiments. *B,* His-tagged survivin and endogenous survivin are equally down-regulated by lapatinib. Untransfected SKBR3 cells (*control*), SKBR3 cells expressing His-tagged survivin (*His-survivin*), and SKBR3 cells transfected with vector alone (*vector alone*) were treated with either lapatinib (500 nmol/L) or vehicle (DMSO). After 72 hours, cells were harvested and equal amounts of total cell lysate separated by SDS-PAGE and Western blot analysis was done. Steady-state protein levels of His-tagged (*dotted line*) and endogenous (*solid line*) survivin.

and MEK inhibitors, we were able to show that PI3K signaling, rather than MAPK-MEK, reproduces the effects of lapatinib on survivin, suggesting that the effect of lapatinib on survivin is likely to be mediated in part through PI3K inhibition as a consequence of ErbB2 inactivation. Because ablation of ErbB3 also down-regulated survivin and induced apoptosis, we propose that ErbB2/ErbB3 heterodimers, through their potent activation of PI3K signaling, modulate survivin protein expression in ErbB2-overexpressing breast cancer cells. The exact role of Akt in this process remains to be determined. However, it seems that down-regulation of survivin and tumor cell apoptosis may occur without affecting p-Akt expression (35, 44). Possible PI3K-dependent, but Akt-independent, mechanisms by which lapatinib might regulate survivin include effects on serum- and glucocorticoid-induced kinases (SGK), which are serine/threonine kinases that are highly homologous to Akt and also regulated by PI3K (45–47). Although the effects of SGK on survivin have not been studied, SGK regulates cell survival (47) and may therefore be a candidate for regulating survivin protein expression. In addition, PI3K affects cell survival through a protein kinase C–dependent pathway that is mediated by phospholipase Cγ activity (48). Thus, it is possible that lapatinib regulates survivin in part through PI3K-dependent effects on SGK or phospholipase Cγ. This might provide an explanation about why survivin was down-regulated and tumor cell apoptosis increased in some patients who responded clinically to lapatinib without a change in p-Akt expression (35).

Using His-tagged survivin under the control of a heterologous CMV promoter, we were able to show that the regulation of survivin by lapatinib was in large part posttranscriptional. Down-regulation of survivin on entry to G_1 is dependent on the ubiquitin-proteosome pathway (17). Moreover, ErbB2 regulates a number of key molecules involved in tumor metastasis, growth, and survival through ubiquitination and proteosome-dependent degradation (13, 25). Reversal of lapatinib-induced down-regulation of survivin by lactacystin, a selective inhibitor of the proteosome, is consistent with ErbB2 regulation of survivin through the ubiquitin-proteosome pathway. Elucidating the role of the proteosome in mediating the effects of ErbB2 on survivin and identifying the E3-ligase responsible for survivin ubiquitination could provide novel therapeutic strategies to more effectively treat patients with ErbB2-overexpressing tumors.

Deregulated expression of survivin occurs in tumors other than ErbB2 overexpressing breast cancers (5, 6). Lapatinib is

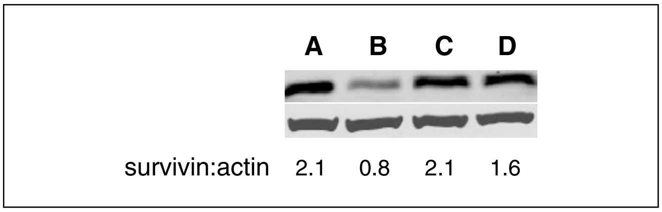

Figure 5. Lapatinib down-regulates survivin through proteosome-dependent degradation. BT474 cells were treated with lapatinib (500 nmol/L; *B*), lactacystin (5 μmol/L; *C*), and lapatinib + lactacystin (*D*). After 72 hours, cells were harvested, total cell lysate was prepared, and equal amounts of protein were separated by SDS-PAGE. Western blot analysis of survivin and actin steady-state protein levels. Untreated cells (*A*) served as controls. Blots were subjected to densitometry and absorbance values for survivin and actin were determined. The ratio of survivin/actin is indicated for each treatment condition.

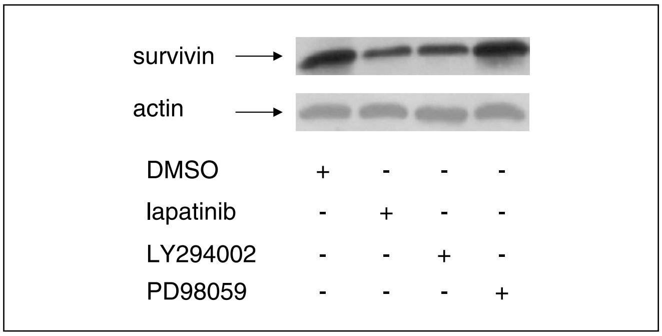

Figure 6. The effects of lapatinib on survivin seem to be mediated through inhibition of PI3K. BT474 cells were treated with DMSO (vehicle), lapatinib (500 nmol/L), LY294002 (10 μmol/L), and PD98059 (10 μmol/L). After 72 hours, cells were harvested and total cell lysates were prepared. Equal amounts of protein were separated by SDS-PAGE and Western blot analysis of survivin steady-state protein levels was done. Steady-state actin protein levels served as a control for equal loading of protein.

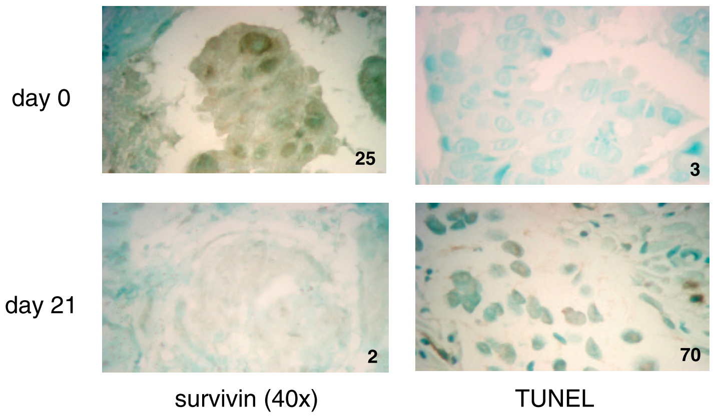

Figure 7. Survivin protein is inhibited in patients with ErbB2-overexpressing breast cancer who respond to lapatinib. Survivin protein expression in tumor tissue was assessed by quantitative immunohistochemistry before (day 0) and after 21 days of lapatinib therapy. The numbers shown indicate the absorbance value attributed to the intensity of staining (see Materials and Methods). TUNEL staining was done on the same tumor tissue. The numbers indicate the percentage of cells staining positive for TUNEL before (day 0) and after 21 days of lapatinib therapy. Representative of ErbB2-overexpressing breast cancer patients who responded clinically to lapatinib (35).

equally effective at inhibiting ErbB2 and ErbB1 tyrosine kinases. However, we were unable to establish the role of ErbB1 in regulating survivin or the role of survivin in regulating cell survival in ErbB1-dependent tumor lines. With the exception of tumors that express gain-of-function ErbB1 mutations (e.g., glioblastoma multiforme and non–small cell lung cancer with bronchioloalveolar carcinoma features), the role of ErbB1 in regulating tumor cell survival, including breast cancer, has been questioned. The relevance of ErbB2, and in particular ErbB2/ErbB3 heterodimers, rather than ErbB1 in promoting tumor cell survival seems to be a common theme in a variety of epithelial carcinomas (41). The absence of an apparent link between ErbB1, survivin, and cell survival provides a possible explanation about why the majority of breast cancers do not seem to be dependent on ErbB1 signaling for survival and why inhibitors with predominant activity against ErbB1 have thus far lacked significant clinical activity in breast cancer (49).

In a phase Ib clinical trial, the four responding patients to lapatinib monotherapy all had ErbB2-overexpressing breast cancers (35). We had sufficient tumor tissue to assess the effects of lapatinib on survivin in the four responders where marked inhibition of survivin protein expression correlated with the induction of tumor cell apoptosis and clinical response. Although intriguing, these findings are based on a small sample and will require confirmation in subsequent clinical trials with larger numbers of tissue samples.

In summary, aberrant regulation of survivin in ErbB2-overexpressing breast cancer cell lines and primary tumors is reversible using a small-molecule kinase inhibitor like lapatinib. In certain situations, down-regulation of survivin alone will be sufficient to induce spontaneous tumor cell apoptosis. In others, it might be sufficient to sensitize tumors to the killing effects of additional anticancer cytotoxic agents. The ability of ErbB-targeted therapies to modulate survivin should be taken into consideration when selecting targeted therapies to combine with anticancer cytotoxic agents because not all ErbB-targeted therapies down-regulate survivin. This is particularly relevant to combination therapies wherein many anticancer cytotoxic agents increase survivin in tumors, potentially contributing to the development of resistance (50, 51). Elucidating the regulation and role of survivin in ErbB2 and ErbB3 signaling pathways will hopefully lead to improved treatment options for patients whose tumors are dependent on these pathways for their survival.

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