Lapatinib Antitumor Activity Is Not Dependent upon Phosphatase and Tensin Homologue Deleted on Chromosome 10 in ErbB2-Overexpressing Breast Cancers

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Abstract

Trastuzumab antitumor activity in ErbB2-overexpressing breast cancers seems to be dependent upon the presence of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a phosphatase that dampens phosphatidylinositol 3-kinase-Akt signaling. Consequently, PTEN deficiency, which occurs in 50% of breast cancers, predicts for resistance to trastuzumab monotherapy. Here, we show that lapatinib, a small-molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, exerts its antitumor activity in a PTEN-independent manner. Steady-state phosphorylated ErbB2 (p-ErbB2) and p-Akt (S473) protein levels were inhibited within 30 min following lapatinib but not in response to trastuzumab in BT474 and Au565 cells (two ErbB2-overexpressing breast cancer cell lines that are sensitive to the proapoptotic effects of lapatinib). Whereas trastuzumab reportedly inhibits SRC phosphorylation (Y416), which in turn reduced SRC-ErbB2 protein interactions, lapatinib had no effect on either variable. To assess the potential functional role that PTEN might play in lapatinib antitumor activity, we selectively knocked down PTEN in BT474 and Au565 cells using small interfering RNA transfection. Loss of PTEN did not affect induction of tumor cell apoptosis by lapatinib in either cell line. In addition, lapatinib inhibited Akt phosphorylation in MDA-MB-468 cells, an ErbB1-expressing/ErbB2 non-overexpressing breast cancer line, despite their PTEN-null status. Moreover, patients with ErbB2-overexpressing inflammatory breast cancers responded to lapatinib monotherapy regardless of PTEN status. Thus, lapatinib seems to exert its antitumor activity in ErbB2-overexpressing breast cancers in a PTEN-independent manner. These data emphasize the importance of assessing PTEN status in tumors when selecting ErbB2-targeted therapies in patients with breast cancer. [Cancer Res 2007;67(3):1170–5]

Introduction

Deregulation of ErbB2 through protein overexpression or gene amplification occurs in 20% to 30% of breast cancer patients, where it predicts for a poor clinical outcome (1, 2). Consequently, ErbB2 is an attractive target for therapeutic drug development (3, 4). Trastuzumab (Herceptin), a humanized anti-ErbB2 monoclonal antibody, is approved for treating patients with ErbB2-overexpressing breast cancers (5). However, the majority of eligible patients still do not respond to trastuzumab monotherapy. In this regard, trastuzumab antitumor activity in ErbB2-overexpressing breast cancers seems to be dependent upon phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a phosphatase that dephosphorylates and inactivates phosphatidylinositol-3,4,5 trisphosphate, a lipid second messenger that recruits the serine/threonine kinase Akt to the cell membrane where Akt is then phosphorylated and activated (6–10). Phosphorylated Akt (p-Akt) in turn promotes tumor cell survival, proliferation, and angiogenesis. PTEN is therefore considered a tumor suppressor by virtue of its ability to dampen phosphatidylinositol 3-kinase (PI3K)-Akt signaling (6–9). Loss of PTEN in tumors through mutation, haploinsufficiency, and loss of heterozygosity, or epigenetic silencing (e.g., promoter hypermethylation) occurs frequently in a variety of tumors, including 50% of breast cancers (11–14). In an attempt to elucidate factors predictive of response to trastuzumab, Nagata et al. identified PTEN deficiency as a factor contributing to trastuzumab resistance in ErbB2-overexpressing breast cancer cell lines and primary tumors (10). The mechanism by which PTEN facilitates trastuzumab antitumor activity reportedly involves the inhibition of SRC phosphorylation and activation, resulting in the disruption of ErbB2-SRC protein interactions, in turn activating PTEN phosphatase activity, leading to Akt inhibition and suppression of tumor cell proliferation and survival signals.

ErbB2 has also been targeted through small-molecule tyrosine kinase inhibitors that compete with ATP for binding to the ErbB2 catalytic kinase domain. Lapatinib is a reversible inhibitor of ErbB2 and ErbB1 tyrosine kinases (15), currently in phase III clinical trials. Inhibition of ErbB2 tyrosine autophosphorylation by lapatinib blocks downstream mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK-Erk1/2) and PI3K-Akt signaling in tumor cell lines, xenografts, and patients, particularly those with ErbB2-overexpressing breast cancers (16–19).

Lapatinib elicits antitumor activity in preclinical models of trastuzumab resistance and in patients whose breast cancers have progressed on trastuzumab therapy (19, 20). Because lapatinib and trastuzumab have non–cross-resistant mechanisms of action (19), we sought to determine whether PTEN deficiency affects lapatinib antitumor activity in ErbB2-overexpressing breast cancers. Here, we show that lapatinib differs in its effects on ErbB2 phosphorylation and ErbB2-SRC protein interactions compared with trastuzumab. Importantly, loss of PTEN protein expression did not affect lapatinib-induced apoptosis in ErbB2-overexpressing breast cancer cell lines, nor was PTEN deficiency associated with...
resistance to lapatinib monotherapy in patients with ErbB2-overexpressing breast cancers. These findings provide a rationale for selecting ErbB2-targeted therapies based on a molecular characterization of tumors that includes PTEN status to guide therapeutic options in breast cancer.

Materials and Methods

Cell culture and reagents. BT474, Au565, and MDA-MB-468 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and l-glutamine from Life Technologies (Carlsbad, CA) in a humidified atmosphere of 5% CO2 at 37°C. IRDye 800–conjugated affinity-purified anti-rib IgG and anti-mouse IgG were from Rockland (Gilbertsville, PA). Alexa Fluor 680 goat anti-rib IgG was purchased from Molecular Probes (Eugene, OR). Antibodies to SRC and p-SRC (Y416) were from Biosource (Camarillo, CA) and Cell Signaling Technologies (Beverly, MA), respectively. Anti-human survivin antibody was from R&D Systems (Minneapolis, MN). p-tyrosine antibody was purchased from Sigma Chemical (St. Louis, MO). ErbB2 (Ab-11) antibody was from Neomarkers (Union City, CA). p-Akt (Ser437), Akt1,2, p-Erk1,2, and Erk1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PTEN antibody (sc-7974) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). p-Akt (Ser437), Akt1/2, p-Erk1/2, and Erk1/2 antibodies were purchased from Cell Signaling Technologies (Beverly, MA), respectively. Anti-human survivin antibody was from R&D Systems (Minneapolis, MN).

Methods

SDS-PAGE and Western blot analysis. Whole-cell extracts were prepared by scraping cells off Petri dishes, washing cell pellets 2× in PBS, and then resuspending pellets in two-packed-cell volumes of radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.25% (w/v) deoxycholate, 1% NP40, 5 mmol/L sodium orthovanadate, 2 mmol/L sodium fluoride, and a protease inhibitor cocktail]. Protein concentrations were determined using a modification of the Bradford method (Bio-Rad Labs, Hercules, CA). Protein concentration standards were purchased from Pierce (Rockford, IL). Protein concentration standards were purchased from Pierce (Rockford, IL). Protein concentration standards were purchased from Pierce (Rockford, IL).

Immunohistochemistry. Fresh tumor biopsies were collected before initiating lapatinib therapy as part of a clinical trial in which patients with recurrent inflammatory breast cancer early were treated with lapatinib therapy alone (1.500 mg/d) conducted in accordance with the 1996 version of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards at the participating institutions, and all patients provided a signed informed consent. Biopsies were stained with H&E to verify the presence of tumor. PTEN immunostaining was done using the PTEN antibody obtained from Cascade BioScience, Inc. (Winchester, MA). PTEN was processed with antigen retrieval using citrate buffer (pH 6. DakoCytomation, Glostrup, Denmark) in the "decolorizer" (Biocare Corp., Walnut Creek, CA). PTEN marker was immunostained using the Autostainer (DakoCytomation). Envision + dual link polymer-HRP (DakoCytomation) was used as the detection chemistry, and DAB + (DakoCytomation) was used as the chromagen. After immunostaining, all slides were counterstained manually with methyl green (DakoCytomation). The association between PTEN-deficient (0,1+) and non-deficient (2,+) and clinical response to lapatinib was analyzed using χ2 test generating χ2, degree of freedom, and P.

Results

Early inhibition of ErbB2 autophosphorylation and downstream PI3K-Akt signaling by lapatinib. Trastuzumab reportedly affects SRC phosphorylation, SRC-ErbB2 protein interactions, PTEN activation, and Akt phosphorylation within 30 min of treatment, before ErbB2 receptor internalization and downregulation (10). To determine whether lapatinib elicits similar biological effects, we assessed ErbB2 phosphorylation, SRC activation, and SRC-ErbB2 protein interactions in lapatinib-treated BT474 and Au565 cells, two ErbB2-overexpressing breast cancer lines that are highly sensitive to lapatinib-induced apoptosis (17, 18). Cells were treated with 1 µmol/L lapatinib, a clinically relevant drug concentration previously shown to induce apoptosis in BT474 and Au565 cells. After 40 min, cells were harvested, and steady-state p-ErbB2 protein levels were assessed by Western blot analysis. p-ErbB2 was assessed by Western blot after immunoprecipitating ErbB2 (Fig. 1A, lanes 1–4) or directly from total cell extracts (lanes 6–9). In both cases, steady-state p-ErbB2 protein levels were inhibited in lapatinib-treated cells compared with vehicle-treated controls (Fig. 1A). In contrast, expression of p-ErbB2 increased in cells treated with trastuzumab (10 µg/mL) for 40 min (Fig. 1A). Total ErbB2 protein remained unchanged in both lapatinib and trastuzumab-treated cells (Fig. 1A). Consistent with their respective effects on p-ErbB2, steady-state p-Akt protein levels were inhibited after 40 min in lapatinib-treated but not trastuzumab-treated cells (Fig. 1B).

We next examined the effect of lapatinib on the phosphorylation of SRC at tyrosine residue 416 (Y416), a phosphorylation site previously shown to be relevant to trastuzumab antitumor activity (10). Steady-state p-SRC (Y416) protein levels were examined by Western blot from cells treated with either lapatinib or trastuzumab, as described above. p-SRC steady-state protein levels remained unchanged in both lapatinib- and trastuzumab-treated cells (Fig. 1C).
SRC-ErbB2 protein interactions are regulated by ErbB2 phosphorylation state (21). We next examined the effect of lapatinib on ErbB2-SRC protein interactions. ErbB2 was immunoprecipitated from equal amounts of protein prepared from total cell extracts, and then steady-state protein levels of total ErbB2, total SRC, p-ErbB2, and p-SRC (Y416) were analyzed by Western blot analysis. As shown in Fig. 2, lapatinib did not affect ErbB2-SRC protein interactions. In contrast, the amount of SRC protein associated with ErbB2 increased in trastuzumab-treated BT474 and Au565 cells (Fig. 2), consistent with the increased expression of p-ErbB2 following trastuzumab treatment. Vehicle-treated cells served as controls.

Genetic PTEN knockdown does not abrogate lapatinib-induced apoptosis. We next sought to determine whether the loss of PTEN protein would affect the sensitivity of ErbB2-overexpressing breast cancer cells to lapatinib. BT474 and Au565 cells were transfected with an siRNA construct specifically targeting PTEN. As shown, PTEN mRNA and protein levels were effectively inhibited following transfection with PTEN siRNA compared with a pooled set of control siRNA constructs (siPool; Fig. 3A and B). The effect of PTEN knockdown on lapatinib-induced apoptosis was then assessed by cell cycle analysis using propidium iodide staining and flow cytometry; cells containing <2N DNA content represented an apoptotic cell population. Lapatinib was added to cell culture 48 h after siRNA transfection, when PTEN protein expression had reached its nadir. Lapatinib did not affect PTEN protein expression in siRNA-transfected cells (Fig. 3C). Loss of PTEN protein did not affect the ability of lapatinib to induce apoptosis in BT474 or Au565 cells (Fig. 3D and E). Consistent with work that we previously published (22, 23), trastuzumab did not induce apoptosis in BT474 or Au565 cells, making it difficult to assess the effects of PTEN knockdown (data not shown). In addition, siRNA transfection did not activate IFN-regulated gene expression, a potential nonspecific effect of siRNA (data not shown).

PTEN status did not affect clinical response to lapatinib in patients with ErbB2-overexpressing inflammatory breast cancers. The clinical relevance of the previous cell-based observations was examined in a phase II clinical trial of lapatinib monotherapy in patients with recurrent inflammatory breast cancer. Patients were administered 1,500 mg lapatinib orally once a day on a continuous basis. Clinical response was assessed after...
Figure 3. PTEN knockdown does not affect the sensitivity of ErbB2-overexpressing breast cancer cells to lapatinib-induced apoptosis. Efficacy of PTEN knockdown in BT474 (A) and Au565 (B) cells. Inhibition of PTEN steady-state protein levels (top) and mRNA (bottom) in BT474 (A) and Au565 (B) cells at 48, 72, 96, and 120 h (5 d) after transfection with PTEN siRNA (100 nmol/L). Transfection with a control siRNA construct (siPool) served as a control.

C, lapatinib treatment does not induce PTEN protein in BT474 cells transfected with PTEN siRNA. BT474 cells were transfected with PTEN or siPool siRNA constructs. Lapatinib (500 nmol/L) was added 48 h after transfection, and cells were harvested 72 and 120 h later. Equal amounts of protein from total cell lysate collected at each time point were separated by SDS-PAGE, and PTEN steady-state protein levels were assessed by Western blot.

D, effects of PTEN knockdown on lapatinib-induced apoptosis in BT474 (D) and Au565 (E) cells. Cells were transfected with PTEN siRNA (100 nmol/L). Lapatinib (500 nmol/L/BT474 and 1 µmol/L/Au565) was added 48 h after transfection, and cell cycle analysis using propidium iodide staining and flow cytometry was done 48 h later. The 2N population represents cells undergoing apoptosis. Untransfected cells treated with DMSO (vehicle) and those transfected with control siRNA (siPool) served as controls.

F, inhibition of p-Akt by lapatinib in cells transfected with PTEN siRNA. BT474 cells were transfected with PTEN siRNA. After 48 h, lapatinib (500 nmol/L) was added to cell culture, and p-Akt and p-Erk1/2 steady-state protein levels were assessed by Western blot 48 h after lapatinib (lane 2). Cells treated with DMSO (vehicle) served as controls (lane 1). Untransfected and cells transfected with control siRNA (siPool) served as controls. Representative of three independent experiments.
8 weeks of therapy according to the Response Evaluation Criteria in Solid Tumors and/or using Canfield digital photography to document response in non-target inflammatory skin changes. Response rate was the primary objective of the study rather than time to disease progression. PTEN protein expression was analyzed by immunohistochemistry in fresh tumor tissue obtained before therapy. Scoring of PTEN staining by immunohistochemistry (0, 1, 2, and 3+) was conducted in a blinded fashion by a centralized reference pathology laboratory (Targeted Molecular Diagnostics, Westmont, IL). Shown are representative examples of tumors expressing high (3+), low (1+), positive endothelial cell staining, and a PTEN-negative tumor with positive staining in adjacent perineural tissue (Fig. 4). Preliminary analysis of 38 evaluable patients, of which 15 responded, indicates that 73% (11 of 15) exhibited low (1+) or absent (0) PTEN protein expression (Table 1), consistent with PTEN deficiency not having an effect on response status.

**Discussion**

Decisions regarding optimal cancer treatment will increasingly be dependent upon molecular characterization of tumors rather than histology alone. Establishing a tumor molecular profile for predicting response to targeted therapies, such as small-molecule inhibitors of ErbB receptor tyrosine kinases, will likely require multiple variables due to the highly complex nature of the signaling networks that regulate cell proliferation and survival in epithelial carcinomas. For example, the majority of ErbB2-overexpressing breast cancers do not respond to trastuzumab therapy alone. PTEN deficiency seems to predict for resistance to trastuzumab monotherapy in patients with ErbB2-overexpressing metastatic breast cancers (10). The mechanism by which PTEN contributes to the trastuzumab antitumor activity reportedly involves the inhibition of SRC phosphorylation (Y416), disruption of ErbB2-SRC protein interactions leading to PTEN activation, and abrogation of PI3K-Akt signaling (10). This cascade of events reportedly occurs within 30 min of trastuzumab therapy, before ErbB2 receptor internalization and down-regulation. We now show that PTEN deficiency does not abrogate the antitumor activity of lapatinib in ErbB2-overexpressing breast cancer cell lines, or in ErbB2-overexpressing inflammatory breast cancers.

Despite targeting ErbB2, lapatinib and trastuzumab elicit distinct biological effects on ErbB2 signaling. Lapatinib blocks ErbB2 tyrosine phosphorylation in breast cancer cell lines, tumor xenografts, and in patients with ErbB2-overexpressing breast cancers (16–19). Inhibition of ErbB2 signaling and downstream pathways by lapatinib occurs within 30 min following treatment with clinically relevant drug concentrations. Rather than inhibiting ErbB2 phosphorylation, trastuzumab increased p-ErbB2 within 30 min without affecting Akt phosphorylation, consistent with an early agonist effect on ErbB2 signaling. Interestingly, although lapatinib markedly inhibited p-ErbB2, it did not affect SRC phosphorylation (Y416) or ErbB2-SRC protein interactions. The explanation for this apparent discrepancy may be related to the fact that ErbB2-SRC protein interactions are regulated by ErbB2 phosphorylation on amino acid residues distinct from the tyrosine autophosphorylation sites located in the COOH-terminal region of the receptor (21). It is not clear that these phosphorylation sites are sensitive to the inhibitory effects of lapatinib. Although the phosphorylation sites that mediate SRC-ErbB2 protein interactions might be insensitive to lapatinib, they may be sensitive to increased ErbB2 phosphorylation in response to trastuzumab, providing a possible explanation for the increased association of SRC with ErbB2 observed in trastuzumab-treated cells.

PTEN dampens PI3K-Akt signaling in tumor cells by affecting phosphorylation of Akt primarily on Ser473, although Thr308 may also be modulated in some tumor cell lines (24, 25). Although lapatinib inhibits Akt phosphorylation (S473) in ErbB2-overexpressing breast cancer cells, the dependence of lapatinib antitumor activity on the inhibition of Akt phosphorylation is unclear. First, lapatinib induces tumor cell apoptosis even in the absence of reduced p-Akt expression (22). Second, tumor cells continue to proliferate despite complete inhibition of p-Akt by lapatinib (23). And third, patients respond to lapatinib monotherapy in the absence of reduced p-Akt expression (16, 20). If the inhibition of Akt phosphorylation is not essential for lapatinib antitumor activity, then loss of PTEN protein should not render cells resistant to lapatinib. In contrast, inhibition of survivin, a member of the inhibitor of apoptosis family of proteins, seems to be a better correlate of lapatinib antitumor activity (22).

PTEN is not the only regulator of Akt phosphorylation. The protein phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP) inactivates Akt by directly dephosphorylating Akt (Ser473), inhibiting tumor cell proliferation and survival in vitro and in vivo (26). Although expression of PHLPP in primary tumor tissue has not been reported, the prediction is that PHLPP deficiency may contribute to tumor progression, independent of

<table>
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<th>PTEN expression</th>
<th>Clinical response (n = 15)</th>
<th>Progressive disease (n = 23)</th>
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<tr>
<td>0/1+</td>
<td>11 (73%)</td>
<td>18 (78%)</td>
</tr>
<tr>
<td>2/3+</td>
<td>4 (27%)</td>
<td>5 (22%)</td>
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NOTE: $\chi^2 (1, N = 38) = 0.122, P = 0.73.$ *Immunohistochemistry.
regulation of Akt phosphorylation by lapatinib. MDA-MB-468 cells are insensitive to lapatinib-induced apoptosis in part because they do not overexpress ErbB2 (19). Survivin is not inhibited by lapatinib in MDA-MB-468 cells (Fig. 5f), consistent with the regulation of survivin being predominantly ErbB2 mediated in breast cancer cells (22). However, inhibition of ErbB1 signaling in lapatinib-treated MDA-MD-468 cells abrogates downstream Ras-Raf-MAPK-Erk1/2 signaling. Ras directly activates PISK-Akt (27), which might help explain how lapatinib inhibits PISK-Akt even in PTEN-null MDA-MB-468 cells.

In summary, lapatinib and trastuzumab not only differ in their biological effects on signaling pathways in ErbB2-overexpressing breast cancer cells but also in their dependence upon PTEN for antitumor activity. Whereas PTEN deficiency reportedly predicts for resistance to trastuzumab (10), it does not seem to play a role in mediating lapatinib antitumor activity in ErbB2-overexpressing breast cancers. Our clinical data are based on a small series of patients with response rate as the primary objective and will require confirmation in larger clinical trials with survival outcomes. Nonetheless, because PTEN deficiency occurs in ~50% of breast cancers, its status should be included in the molecular characterization of ErbB2-overexpressing breast cancers to improve clinical outcome in patients whose breast cancers are PTEN deficient by selecting ErbB2-targeted therapies whose antitumor activity is PTEN independent.

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Sensitivity of PTEN-Deficient Breast Cancers to Lapatinib

Figure 5. Lapatinib inhibits p-Akt in a dose- and time-dependent manner in PTEN-null MDA-MB-468 breast cancer cells. A, MDA-MB-468 cells were treated with lapatinib at the indicated concentrations (μM, mL/L). Cells were harvested after 72 h, and steady-state protein p-Akt and p-Erk1/2 protein levels were assessed by Western blot analysis. Cells treated with DMSO (vehicle) served as a control (C). Total Erk1/2 protein served as a control for equal loading of protein. B, MDA-MB-468 cells were treated with lapatinib (2.5 μM/L) for the indicated times (hrs). Equal amounts of protein were separated by SDS-PAGE, and steady-state protein levels of p-Akt, p-Erk1/2, and survivin were assessed by Western blot. Total Erk1/2 protein served as a control for equal loading of protein. Representative of three independent experiments.

References

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