

PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients

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Summary

The ErbB2-targeting antibody, trastuzumab (Herceptin), has remarkable therapeutic efficacy in certain patients with ErbB2-overexpressing tumors. The overall trastuzumab response rate, however, is limited and what determines trastuzumab response is poorly understood. Here we report that PTEN activation contributes to trastuzumab's antitumor activity. Trastuzumab treatment quickly increased PTEN membrane localization and phosphatase activity by reducing PTEN tyrosine phosphorylation via Src inhibition. Reducing PTEN in breast cancer cells by antisense oligonucleotides conferred trastuzumab resistance in vitro and in vivo. Patients with PTEN-deficient breast cancers had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN. Thus, PTEN deficiency is a powerful predictor for trastuzumab resistance. Additionally, PI3K inhibitors rescued PTEN loss-induced trastuzumab resistance, suggesting that PI3K-targeting therapies could overcome this resistance.

Introduction

Overexpression of ErbB2, a 185 kDa membrane receptor tyrosine kinase, is found in 20%–30% of human breast cancers and many other cancer types (Slamon et al., 1987; Yu and Hung, 2000). ErbB2 overexpression leads to a very aggressive cancer phenotype and poor patient survival (Slamon et al., 1987; Yu and Hung, 2000). Numerous efforts have been directed at developing ErbB2-targeting cancer therapies (Bange et al., 2001). One successful example is the recombinant humanized anti-ErbB2 monoclonal antibody trastuzumab (Herceptin) that specifically binds to the extracellular domain of ErbB2 (Cho et al., 2003; Shepard et al., 1991). The currently known mechanisms underlying trastuzumab's antitumor activity include the downregulation of p185^{ErbB2} and the subsequent inhibition of its downstream PI3K-Akt signaling pathway (Hudziak et al., 1989; Yakes

et al., 2002), the induction of G₁ arrest and the cyclin-dependent kinase inhibitor p27^{Kip1} (Sliwkowski et al., 1999), and the inhibition of ErbB2 ectodomain cleavage (Molina et al., 2001). Despite these and other reported functions that result from ErbB2 downregulation (Petit et al., 1997), the mechanism of trastuzumab's antitumor activity remains a fundamental question to be clearly addressed (Baselga and Albanell, 2001).

As the only FDA-approved therapeutic antibody for metastatic breast cancer, trastuzumab has demonstrated durable responses as a single agent and striking therapeutic efficacy in combination with other chemotherapeutics (Cobleigh et al., 1999; Esteva et al., 2002; Seidman et al., 2001; Slamon et al., 2001). However, less than 35% of patients with ErbB2-overexpressing metastatic breast cancer respond to trastuzumab as a single agent, whereas ~5% of patients suffer from severe side effects (e.g., cardiac dysfunction) and 40% of pa-

SIGNIFICANCE

Trastuzumab is an excellent model of rationally designed targeted cancer treatment. However, the overall trastuzumab response rate is low and the causes of trastuzumab resistance are poorly understood. Here, our data introduce the new concept that a rapid PTEN activation, before downregulation of ErbB2, is a novel mechanism underlying the antitumor activity of trastuzumab, whereas PTEN deficiency is significantly associated with trastuzumab resistance in breast cancer patients. PTEN deficiencies were reported in ~50% of breast cancers. Our findings have direct implications in the treatment of these patients with the worst clinical outcomes. PTEN deficiency may serve as an indicator for trastuzumab resistance that requires PI3K-targeting combination treatment to overcome this resistance.

tients experience other adverse effects from trastuzumab treatment (Cobleigh et al., 1999; Vogel et al., 2002). Thus, there is an urgent need to identify patients who are unlikely to respond to trastuzumab treatment to spare them the potential side effects and unnecessary cost. More importantly, trastuzumab resistance-conferring factors may serve as molecular targets for overcoming trastuzumab resistance. Unfortunately, information on the mechanisms of trastuzumab resistance in breast cancer cells is limited. Currently, there is no clinically verified factor that can be used to predict trastuzumab resistance (Albanell and Baselga, 2001).

PTEN (MMAC1/TEP) is a dual phosphatase that mainly dephosphorylates position D3 of membrane phosphatidylinositol-3,4,5 trisphosphate (PI3,4,5P3), which is the site for recruiting the pleckstrin-homology domain of Akt to the cell membrane (Parsons and Simpson, 2003). Since phosphatidylinositol 3-kinase (PI3K) catalyzes the production of PI3,4,5P3 (Cantley, 2002), PTEN antagonizes this PI3K function and negatively regulates Akt activities. Loss of PTEN function due to *PTEN* mutations, PTEN haploinsufficiency from LOH at the PTEN locus, and epigenetic downmodulation of PTEN have been reported in nearly 50% of breast cancers and in many other cancer types (Li et al., 1997; Mutter et al., 2000; Parsons and Simpson, 2003; Teng et al., 1997; Whang et al., 1998). Restoration of PTEN expression in PTEN null cells inhibits Akt activities and tumor formation (Li and Sun, 1998; Lu et al., 1999). Therefore, PTEN is an important tumor suppressor gene. However, possible functional interactions of PTEN activity with ErbB2 receptor tyrosine kinase signaling and potential roles for PTEN in the antitumor activity of trastuzumab have never been explored. In this report, we demonstrate that PTEN activation is a novel mechanism underlying trastuzumab's antitumor activity and that PTEN loss renders ErbB2-overexpressing breast cancer resistant to trastuzumab-based therapy.

Results

Trastuzumab activates PTEN in breast cancer cells

In our investigation of the mechanisms of trastuzumab's antitumor function, we observed that >20 hr trastuzumab treatment of BT474 and SKBr3 human breast cancer cell lines overexpressing endogenous ErbB2 led to ErbB2 downregulation, dephosphorylation, and receptor internalization as expected (Lee et al., 2002). However, 1 hr trastuzumab treatment (2–10 μ g/ml) did not induce ErbB2 downregulation, dephosphorylation, and receptor internalization (Figure 1A and Supplemental Figure S1 at <http://www.cancer.org/cgi/content/full/6/2/117/DC1>). Surprisingly, 1 hr trastuzumab treatment quickly reduced the level of phosphorylated and activated Akt in BT474 and SKBr3 cells while the Akt protein level remained unchanged (Figure 1A). It is known that Akt is constitutively activated in ErbB2-overexpressing cancer cells and Akt is inhibited after ErbB2 downregulation by trastuzumab, which is critical for trastuzumab's antitumor effect (Ignatoski et al., 2000; Yakes et al., 2002; Zhou et al., 2000). However, the rapid Akt dephosphorylation by trastuzumab before ErbB2 downregulation has not been reported nor has its mechanisms. Since PI3K activation is the major upstream signaling event leading to Akt phosphorylation (Cantley, 2002), we examined whether trastuzumab induced rapid Akt dephosphorylation by inhibiting PI3K. However, PI3K activity was not inhibited within 1 hr of trastuzumab treatment

(Figure 1B). Contrarily, the p85 subunit of PI3K had a transiently increased association with the ErbB2 complex (Figure 1C), which paralleled a moderate increase of ErbB2 tyrosine phosphorylation by short-time trastuzumab treatment (Figure 1A). The apparently paradoxical results indicate that, different from the previously reported mechanism of Akt dephosphorylation that is dependent on ErbB2 downregulation or PI3K inhibition (Hudziak et al., 1989; Yakes et al., 2002), the rapid Akt dephosphorylation by 1 hr trastuzumab treatment was not due to PI3K inhibition, nor by ErbB2 degradation and dephosphorylation, but by some other novel mechanism.

The tumor suppressor PTEN is a dual-phosphatase that negatively regulates Akt activity (Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000; Leslie and Downes, 2002). We thus asked whether PTEN activation may possibly be involved in the trastuzumab-mediated rapid Akt dephosphorylation before PI3K inhibition and ErbB2 downregulation. We examined the PTEN phosphatase activity in untreated and trastuzumab-treated (20 or 60 min) SKBr3 cells after immunoprecipitation (IP) of a similar amount of PTEN using the Ab-2 PTEN antibody (Figure 1D, insert). Compared with untreated cells, 20 min trastuzumab treatment dramatically increased PTEN activity, which continued to increase 60 min after treatment (Figure 1D). Similar results were obtained using another PTEN antibody (A2B1, not shown). Therefore, trastuzumab treatment indeed led to a rapid increase of PTEN phosphatase activity that could account for the rapid Akt dephosphorylation before ErbB2 is downregulated and before PI3K is inhibited.

Membrane-associated PTEN is known to be biologically active in cells (Comer and Parent, 2002; Iijima and Devreotes, 2002; Wu et al., 2000). To investigate whether PTEN activation by trastuzumab is due to recruitment of PTEN to its biologically active subcellular location, we examined whether membrane localization of PTEN is increased by trastuzumab. After treating BT474 cells with or without trastuzumab for 20 min, we separated membrane bound proteins from cytosolic proteins in cell lysates and examined PTEN proteins by Western blotting (Figure 1E). In the untreated cells, the majority of PTEN protein was in the cytosolic pool of the lysates, while a very low level of PTEN was detected in the membrane bound pool. This is consistent with the notion that PTEN is functionally inactivated in ErbB2-overexpressing cancer cells (Cantley and Neel, 1999; Lu et al., 1999). However, upon trastuzumab treatment, PTEN protein in the membrane bound pool was noticeably enriched. Interestingly, the MCF7 breast cancer cells with normal ErbB2 expression had more membrane bound PTEN than the ErbB2-overexpressing BT474 cells before trastuzumab treatment, yet a further increase of the membrane bound PTEN was not induced by trastuzumab in MCF7 cells (Supplemental Figure S2). These data suggest that in ErbB2-overexpressing breast cancer cells, trastuzumab treatment specifically facilitates PTEN localization to the cell membrane, where this phosphatase is active and functions to dephosphorylate PIP₃ to PIP₂, leading to Akt dephosphorylation.

PTEN binds to the cellular membrane through its C2 domain and there are two tyrosine phosphorylation sites in the C2 domain of PTEN (Y240 and Y315) important for PTEN phosphatase activity and tumor inhibition function (Koul et al., 2002). Tyrosine phosphorylation of PTEN reduces the capacity of the PTEN C2 domain to bind to the cellular membrane (Lu et al., 2003). Since we observed increased membrane localization of PTEN by tras-

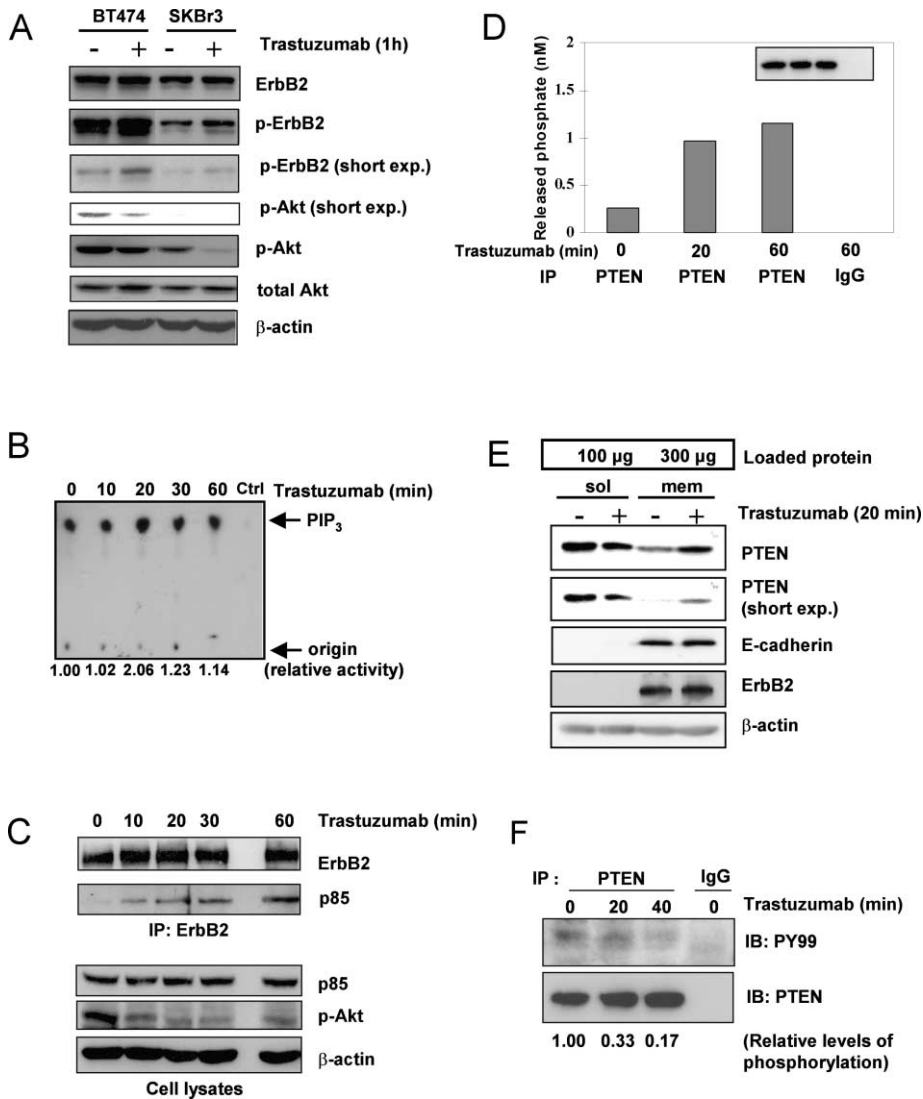


Figure 1. Rapid Akt dephosphorylation and PTEN activation by trastuzumab

A: Immunoblotting (IB) of ErbB2, Tyr1248-phosphorylated ErbB2 (p-ErbB2), phosphorylated Akt (p-Akt), and total Akt in trastuzumab (+, 2 μ g/ml) or control IgG-treated (-, 2 μ g/ml) BT474 cells and SKBr3 cells. β -actin blot served as loading controls in all IB experiments. All in vitro experiments were repeated at least three times. Reproducible representative results are shown.

B: PI3K assay after 2 μ g/ml of trastuzumab treatment of SKBr3 cells for the indicated time. Lysates were immunoprecipitated with the PY20 antibody followed by incubation in kinase buffer. Numbers below the panel indicate relative PI3K activity to untreated cells. IP with IgG served as a control.

C: Trastuzumab transiently increased association of the p85 subunit of PI3K with the ErbB2 complex. ErbB2 immunoprecipitates and total cell lysates from untreated or trastuzumab-treated (2 μ g/ml) cells were immunoblotted with indicated antibodies.

D: Increased PTEN phosphatase activities in trastuzumab-treated (2 μ g/ml) SKBr3 cells. Upper right insert shows, from left to right, immunoprecipitated PTEN proteins from untreated and trastuzumab-treated (20 or 60 min) cells. Immunoprecipitates by rabbit IgG from trastuzumab-treated cells were the negative control.

E: Trastuzumab increased membrane bound PTEN in BT474 cells. PTEN protein in the cytosolic pool (sol) and the membrane bound pool (mem) of untreated (-) or trastuzumab-treated (+, 2 μ g/ml) cell lysates were blotted with indicated antibodies. E-cadherin and ErbB2 were membrane protein markers.

F: Reduction of PTEN tyrosine phosphorylation after trastuzumab treatment in BT474 cells. BT474 cells were treated without or with 2 μ g/ml of trastuzumab for the indicated time. PTEN IP using the A2B1 PTEN antibody was followed by IB with PY99 phosphotyrosine antibody (top). The membrane was then stripped and reprobed with A2B1 PTEN antibody to show the IP efficiency. IgG was used as a negative control. Numbers below the panel indicate relative levels of PTEN tyrosine phosphorylation with 0 min trastuzumab treatment defined as 1.

tuzumab treatment in ErbB2-overexpressing cells, we investigated whether this may result from reduced PTEN tyrosine phosphorylation. We immunoprecipitated PTEN from untreated or trastuzumab-treated BT474 cells with the A2B1 PTEN antibody and immunoblotted with the PY99 phosphotyrosine antibody (Figure 1F). Consistent with the notion that PTEN is functionally inactive in ErbB2-overexpressing cells (Lu et al., 1999), we found that PTEN is highly phosphorylated on tyrosine in untreated BT474 cells. However, trastuzumab treatment rapidly reduced PTEN tyrosine phosphorylation in BT474 cells (Figure 1F), which paralleled the increased PTEN membrane localization and PTEN activation by trastuzumab in these cells (Figures 1D and 1E). Thus, trastuzumab induces PTEN activation by increasing the translocation of PTEN from the cytoplasm to the membrane through reducing the inhibitory tyrosine phosphorylation of PTEN. Notably, these events occur before ErbB2 is downregulated and PI3K is inhibited. Therefore, activation of

PTEN is an early molecular event after trastuzumab treatment independent of ErbB2 downregulation and PI3K inhibition.

Trastuzumab activates PTEN by inhibiting Src association with ErbB2

To investigate how trastuzumab inhibits PTEN tyrosine phosphorylation, we examined whether trastuzumab may inhibit Src tyrosine kinase, since Src activation has recently been reported to increase PTEN tyrosine phosphorylation (Lu et al., 2003). Compared to untreated cells, trastuzumab treatment rapidly inhibited Src kinase activity (Figure 2A) and rapidly reduced Src phosphorylation on Y416 (Figure 2B), an indicator of Src activity (Frame, 2002). Reduced Src-Y416 phosphorylation corresponded to reduced PTEN tyrosine phosphorylation (Figure 2B).

Src is known to bind to ErbB2 and is thus activated in ErbB2-overexpressing cancer cells (Belsches-Jablonski et al., 2001; Muthuswamy and Muller, 1995). To explore the mechanisms of

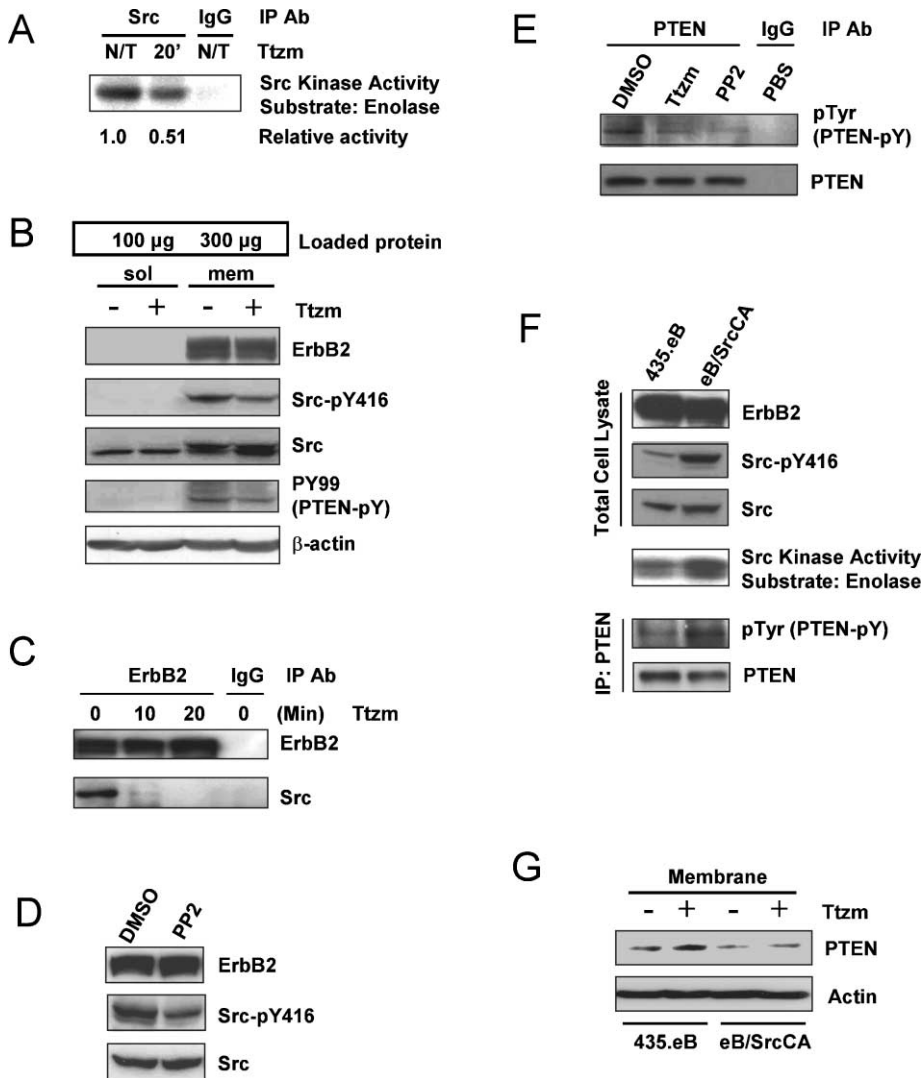


Figure 2. Trastuzumab inhibited Src by dissociating Src from ErbB2, leading to reduced PTEN tyrosine phosphorylation

A: Trastuzumab treatment inhibits Src kinase activity. BT474 cells were either untreated (N/T) or treated with 2 µg/ml trastuzumab (Ttzm) for the indicated time. Src kinase activity was assayed as in Experimental Procedures.

B: Trastuzumab treatment inhibits Src-Y416 phosphorylation. BT474 cells were treated similarly as in Figure 1E. Cytosolic pool (sol) and membrane bound pool (mem) lysates were blotted with the indicated antibodies.

C: Trastuzumab induces Src dissociation from ErbB2. BT474 cells were treated with trastuzumab for the indicated time. Anti-ErbB2 and control IgG immunoprecipitates were blotted with Src antibody to detect ErbB2 bound Src.

D: The Src inhibitor PP2 decreases Src-Y416 phosphorylation. ErbB2, Src-Y416 phosphorylation, and Src proteins were blotted with the indicated antibodies in cell lysates.

E: Src inhibition results in PTEN dephosphorylation/activation. ErbB2 transfectants of MDA-MB-435 cells (435.eB) were treated with 10% DMSO, 2 µg/ml trastuzumab (Ttzm), 10 µM PP2, or PBS buffer. IP was done with PTEN antibody or control IgG followed by IB of total PTEN and tyrosine-phosphorylated PTEN.

F: Src activation leads to increased PTEN tyrosine phosphorylation. Lysates from 435.eB and 435.eB stably expressing constitutively activated Src (eB/SrcCA) were immunoblotted with the indicated antibodies. The Src kinase activities in both cell lines were evaluated as in **A**.

G: Constitutive Src activation abolishes trastuzumab-mediated PTEN membrane translocation. 435.eB and eB/SrcCA cells were untreated (-) or treated (+) with trastuzumab. The membrane bound pool of cell lysates was blotted with indicated antibodies.

trastuzumab-mediated Src inhibition, we examined whether Src binding to ErbB2 is inhibited by trastuzumab treatment in BT474 cells. Trastuzumab induced a very rapid and dramatic reduction of ErbB2 bound Src (Figure 2C), indicating that trastuzumab inhibited Src activity in ErbB2-overexpressing cells most likely by inhibiting Src binding to ErbB2.

To further confirm that Src inhibition can reduce PTEN tyrosine phosphorylation in ErbB2-overexpressing breast cancer cells, we examined whether the Src kinase inhibitor PP2 may reduce PTEN tyrosine phosphorylation in the ErbB2-overexpressing 435.eB breast cancer cells (Yu et al., 1998a). Compared to the solvent 10% dimethyl sulfoxide (DMSO)-treated 435.eB cells, PP2 treatment effectively inhibited Src phosphorylation on Y416 (Figure 2D) and also dramatically reduced tyrosine phosphorylation of PTEN, similar to trastuzumab's effect (Figure 2E). Contrarily, the eB/SrcCA cells, which are 435.eB cells stably expressing a constitutively activated Src (Chan et al., 2003) and having higher levels of Src-Y416 phosphorylation and Src activity, showed a dramatic increase of PTEN tyrosine phosphorylation compared to the 435.eB cells (Figure 2F). Furthermore, although trastuzumab treatment effectively increased

the PTEN membrane translocation in the 435.eB cells, PTEN membrane translocation by trastuzumab in the eB/SrcCA cells expressing constitutively activated Src was less effective (Figure 2G). Together, our data indicate that trastuzumab treatment inhibits Src binding to ErbB2 in ErbB2-overexpressing breast cancer cells, thus inhibiting Src kinase activity, which leads to reduced PTEN tyrosine phosphorylation and increased PTEN membrane localization and activity.

PTEN activation contributes to trastuzumab's antiproliferation function

Trastuzumab is known to inhibit the growth of ErbB2-overexpressing breast cancer cells through the downregulation of ErbB2 and the subsequent inhibition of its downstream PI3K-Akt signaling pathway (Hudziak et al., 1989; Yakes et al., 2002). Since we found that trastuzumab activates PTEN before ErbB2 is downregulated and before PI3K is inhibited, it is imperative to determine whether PTEN activation indeed caused the rapid Akt dephosphorylation and contributes to the antiproliferation function of trastuzumab. To achieve this, we transfected SKBr3 breast cancer cells with PTEN antisense (AS) oligonucleotides

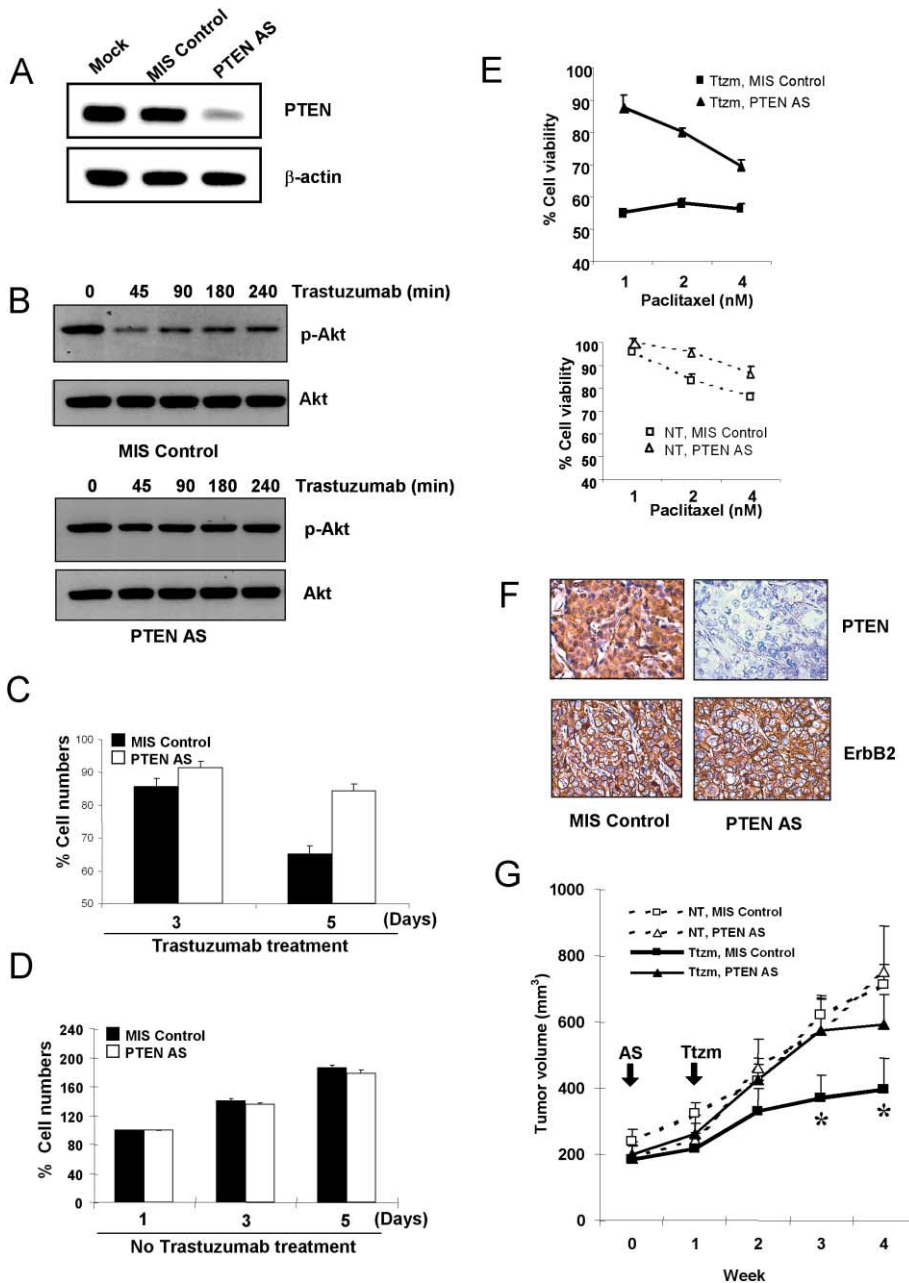


Figure 3. PTEN reduction by PTEN AS oligonucleotides confers resistance to trastuzumab's antiproliferative effects in vitro and in vivo

A: PTEN protein is reduced in SKBr3 cells transfected with PTEN AS (25 nM) compared to mock or mismatched (MIS) oligonucleotide-transfected cells.

B: Trastuzumab-mediated Akt dephosphorylation is attenuated in PTEN AS-transfected SKBr3 cells. MIS or PTEN AS-transfected SKBr3 cells (as in **A**) were treated with trastuzumab (2 μ g/ml) for the indicated times, and cell lysates were immunoblotted.

C: Trastuzumab inhibited cell proliferation less in PTEN AS-treated SKBr3 cells compared to MIS-transfected cells. MIS or PTEN AS-transfected SKBr3 cells (as in **A**) were treated with trastuzumab (2 μ g/ml) for 3 and 5 days. Cell mass was determined by MTS assay. Percent cell number represents % cell counts under each treatment relative to untreated cells.

D: Similar cell growth in MIS- or PTEN AS-transfected SKBr3 cells without trastuzumab. Percent cell numbers represents % cell counts compared to Day 1 (defined as 100%).

E: PTEN-reduced BT474 cells are more resistant to trastuzumab plus paclitaxel than PTEN normal cells but have a similar paclitaxel response to PTEN normal cells. BT474 cells were transfected with 25 nM MIS or PTEN AS, treated by paclitaxel (1, 2, and 4 nM) without or with 2 μ g/ml of trastuzumab (Ttzm) for 3 days. Percent cell viability are cell mass as a % of untreated MIS-transfected cells.

F: PTEN expression is reduced in BT474 xenografts injected with PTEN AS. BT474 cells were inoculated into the mfp of female nude mice. After tumors reached 150 mm³, MIS or PTEN AS were injected intratumor (15 μ g/injection, twice a week) for 1 week. Removed tumors were stained with antibodies to PTEN and ErbB2.

G: PTEN-deficient BT474 xenografts are more resistant to trastuzumab than PTEN normal BT474 xenografts. After 1 week of PTEN AS or MIS treatment (as in **F**), mice were treated with trastuzumab (10 mg/kg) or vehicle twice a week. The arrows indicate the starting days of AS/MIS treatment (AS) and trastuzumab/vehicle treatment (Ttzm). The results shown are the mean tumor volume \pm SE; * p < 0.05.

(Butler et al., 2002), which effectively reduced endogenous PTEN expression compared to mock-transfected cells or cells transfected with control mismatched (MIS) oligonucleotides (Figure 3A). Compared with MIS control-treated cells showing rapid Akt dephosphorylation by trastuzumab (Figure 3B, top), Akt dephosphorylation was attenuated in PTEN AS-treated cells (Figure 3B, bottom). This indicates that PTEN is required for the rapid Akt dephosphorylation by trastuzumab. To investigate whether PTEN activation contributes to trastuzumab's antiproliferation function, we compared cell growth between MIS control and PTEN AS-transfected SKBr3 cells after trastuzumab treatment. PTEN AS-treated cells with reduced PTEN showed significantly less growth inhibition by trastuzumab than MIS control-transfected SKBr3 cells having normal PTEN expression

(Figure 3C). We observed similar results in BT474 cells (Supplemental Figure S3). These data demonstrate that normal PTEN levels are required for the antiproliferation function of trastuzumab. Without trastuzumab treatment, PTEN reduction by PTEN AS alone had no significant effect on cell proliferation (Figure 3D), which was consistent with the similar Akt phosphorylation levels in cells treated with PTEN AS alone to that treated with MIS control (Figure 3B, time 0). That blocking PTEN only reduced trastuzumab's antiproliferative function but had no effect on cells without trastuzumab treatment reiterated that PTEN activity is important for trastuzumab function, which reconciles well with our finding that PTEN is activated by trastuzumab (Figures 1D–1F). Thus, PTEN reduction led to resistance to the antiproliferation effect of trastuzumab in these cells.

Trastuzumab is mostly used in combination with other chemotherapeutics in the clinic (Slamon et al., 2001). Therefore, we further examined whether PTEN reduction may confer breast cancer cell resistance to trastuzumab plus paclitaxel chemotherapy. We transfected BT474 cells with PTEN AS or MIS control oligonucleotides, then treated them with or without trastuzumab (2 $\mu\text{g/ml}$) plus different concentrations of paclitaxel (1, 2, and 4 nM) and measured cell viability 3 days later. MIS control-treated cells with normal PTEN expression showed significant growth inhibition by trastuzumab plus paclitaxel ($p < 0.05$), whereas PTEN AS-transfected cells were not inhibited (Figure 3E, top). However, paclitaxel alone without trastuzumab showed no statistically significant difference in cell viability between PTEN AS- and MIS control-transfected cells (Figure 3E, bottom), indicating that PTEN status does not significantly impact breast cancer cell sensitivity to paclitaxel but specifically impacts trastuzumab sensitivity. These results indicate that breast cancer cells with reduced PTEN are resistant to growth inhibition by trastuzumab as a single agent (Figure 3C) as well as in combination with paclitaxel (Figure 3E).

PTEN deficiency contributes to trastuzumab resistance in vivo

To investigate if PTEN deficiency may confer breast cancer cell resistance to trastuzumab in vivo, we injected a tumorigenic subline of BT474 cells into the mammary fat pad (mfp) of athymic nude mice. When tumor xenografts reached $>150 \text{ mm}^3$, we injected PTEN AS or MIS oligonucleotides into the tumor xenografts. One week later, we treated mice with trastuzumab (10 mg/kg) or vehicle twice per week. PTEN expression in tumor xenografts was effectively inhibited by PTEN AS when ErbB2 expression remained the same (Figure 3F). Trastuzumab only inhibited MIS control-treated tumors expressing normal PTEN, but not PTEN AS-treated tumors with reduced PTEN (Figure 3G). Without trastuzumab treatment, PTEN AS- and MIS-treated tumors had similar tumor growth rates (Figure 3G). These results demonstrated that PTEN reduction confers breast tumor xenografts resistance to trastuzumab's antitumor function.

PI3K inhibitors rescue PTEN loss-induced trastuzumab resistance

The above data clearly demonstrate that PTEN deficiency confers trastuzumab resistance in breast cancer. In search of strategies for overcoming trastuzumab resistance from the loss of PTEN, we reasoned that since PI3K antagonizes PTEN function, inactivation of PI3K may rescue trastuzumab resistance from PTEN deficiency. We investigated whether PI3K inhibitors that are known to quickly induce Akt dephosphorylation in ErbB2-overexpressing cells may reverse trastuzumab resistance in PTEN-deficient breast cancer cells (Xiong et al., 2001). We transfected BT474 cells with MIS and PTEN AS oligonucleotides, then treated them with trastuzumab, the PI3K inhibitor LY294002, or trastuzumab plus LY294002. We then determined cell viability 3 days later (Figure 4A). The MIS-treated cells are more sensitive to trastuzumab-mediated growth inhibition than the PTEN AS-treated cells, and the combination treatment inhibited the viability of MIS-treated cells slightly better than trastuzumab alone or LY294002 alone ($p > 0.05$). However, the LY294002 plus trastuzumab combination inhibited cell viability significantly better than trastuzumab alone or LY294002 alone in PTEN AS-treated cells ($p < 0.01$). Similarly, another PI3K inhibitor, Wort-

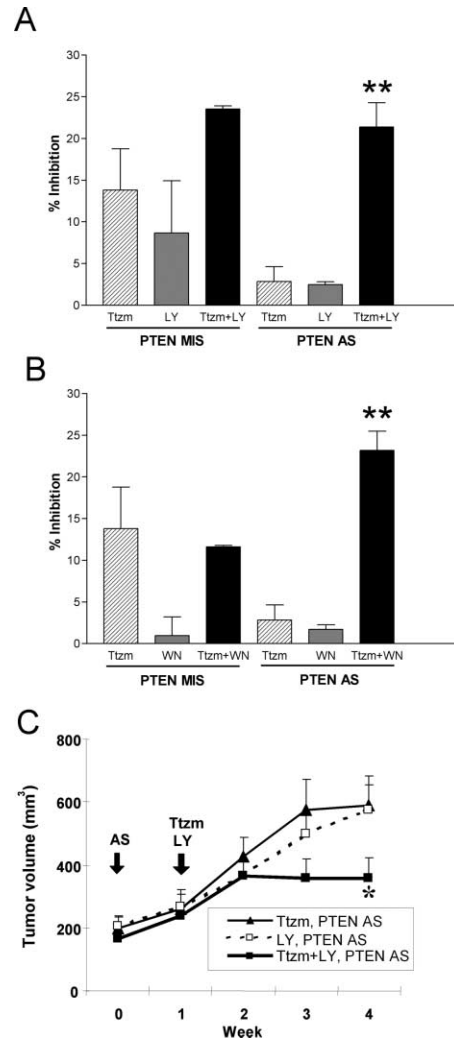


Figure 4. PI3K inhibitors enhance trastuzumab's antiproliferation function in PTEN-reduced BT474 cells in vitro and in vivo

A: BT474 cells were treated with MIS or PTEN AS oligonucleotides as in Figure 3A. Cells were then treated with trastuzumab (Ttzm, 2 $\mu\text{g/ml}$), LY294002 (LY, 0.8 μM), or trastuzumab plus LY294002 (Ttzm + LY) for 3 days. Percent inhibition is % growth reduction under each treatment relative to untreated cells. $**p < 0.01$.

B: BT474 cells were treated with MIS or PTEN AS as in Figure 3A. Cells were then treated with trastuzumab (2 $\mu\text{g/ml}$), Wortmannin (WN, 1 μM), or trastuzumab plus Wortmannin (Ttzm + WN) for 3 days. Percent inhibition is % growth reduction under each treatment relative to untreated cells. $**p < 0.01$.

C: BT474 xenografts were treated with PTEN AS as in Figure 3G. Mice were treated with trastuzumab (10 mg/kg) twice a week or/and LY294002 (100 mg/kg) 3 times a week for 3 weeks. The arrows indicate the starting days of AS treatment (AS) and trastuzumab and/or LY294002 treatment (Ttzm, LY). The results are the mean tumor volume \pm SE. $*p < 0.05$.

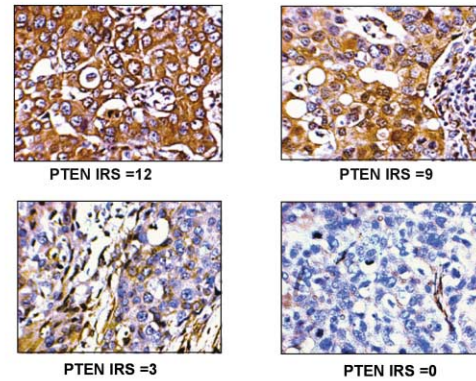
mannin, in combination with trastuzumab, also inhibited PTEN AS-treated BT474 cells significantly better than either treatment alone ($p < 0.01$), whereas the combination had a similar inhibitory effect as trastuzumab alone in MIS-treated cells (Figure 4B). In vivo, trastuzumab combined with the PI3K inhibitor LY294002 was also significantly ($p < 0.05$) more effective than trastuzumab alone or LY294002 alone in inhibiting PTEN AS-treated BT474 tumor xenografts in mice (Figure 4C). These results indicate

that PI3K inhibitors could overcome trastuzumab resistance in PTEN-deficient breast cancer cells.

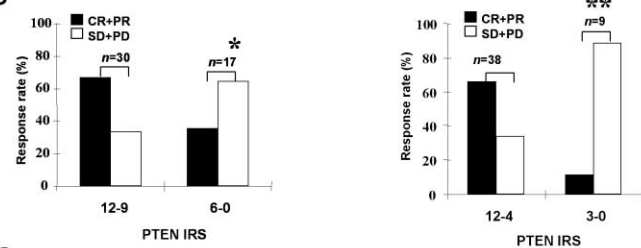
Patients with PTEN-deficient breast cancers have a poor clinical response to trastuzumab

To explore the clinical significance of PTEN status in predicting response to trastuzumab-based therapy in patients, we evaluated PTEN expression in 47 ErbB2-overexpressing primary breast carcinomas from patients who subsequently developed metastatic breast cancer and received trastuzumab plus taxane (paclitaxel or docetaxel) therapy (Esteva et al., 2002; Seidman et al., 2001). As controls, we evaluated PTEN expression in primary breast carcinomas not selected for ErbB2 overexpression from 37 patients who subsequently developed metastatic breast cancer and received taxane without trastuzumab (Holmes et al., 1991; Valero et al., 1995). Immunohistochemical staining using PTEN antibodies revealed that PTEN expression in these tumors was heterogeneous with different intensities. Thus, PTEN expression levels were semiquantified using immunoreactive scores (IRS) calculated by multiplying the percentage of PTEN-positive cells (scored 0 to 4) with the PTEN staining intensity (1 to 3) (Chui et al., 1996). IRS of 0 to 12 represents PTEN staining from a nondetectable level in PTEN-lost tumors to full expressions in normal individuals (Figure 5A). We observed PTEN loss (IRS < 9) in 36.2%–43.2% of the tumors examined (17/47 and 16/37), which is consistent with previous reports on loss of PTEN expression in ~40% breast cancers using immunohistochemistry (IHC) staining (Depowski et al., 2001; Perren et al., 1999). Remarkably, among the 47 breast cancer patients who received trastuzumab plus taxane, patients with PTEN-deficient tumors (IRS 0–6) had significantly lower complete and partial response (CR+PR) rates to trastuzumab plus taxane therapy than patients with PTEN-positive tumors (35.7% versus 66.7%, $p < 0.05$) (Figure 5B, left). Using more stringent criteria for PTEN loss (IRS < 4), patients with PTEN-negative tumors demonstrated a striking, highly significant ($p < 0.01$) worse response to trastuzumab plus taxane therapy than patients with PTEN-positive tumors (11.1% versus 65.8% CR+PR) (Figure 5B, right). Furthermore, we identified a statistically significant trend suggesting that the probability of response to trastuzumab decreases as PTEN IRS decreases ($p < 0.01$) (Supplemental Figure S4). However, among the 37 patients treated by taxane without trastuzumab, patients with PTEN-deficient tumors had similar response (CR+PR) rates to those with PTEN-positive tumors ($p = 0.40$ when PTEN loss is defined as IRS 0–6; $p = 0.74$ when PTEN loss is defined as IRS < 4; Supplemental Figure S5). These data clearly demonstrate that PTEN loss does not confer breast cancer resistance to taxane but specifically to trastuzumab. To confirm that PTEN is a specific marker for trastuzumab response, we examined whether Ki67, a known prognostic marker for breast cancer (Faneyte et al., 2003), may predict trastuzumab response in patients who received trastuzumab plus taxane. Ki67 did not correlate with clinical responses to trastuzumab plus taxane treatment (Supplemental Figure S6). It has been reported that patients with ErbB2 FISH-positive and/or IHC 3+ breast cancers respond to trastuzumab better than patients with ErbB2 FISH-negative and/or IHC 2+ cancers (Vogel et al., 2002). Although we see a similar tendency (59% for FISH-positive and/or IHC 3+ versus 37% for FISH-negative and/or IHC 2+) among the 47 patients treated with trastuzumab plus taxane, the difference was not statistically

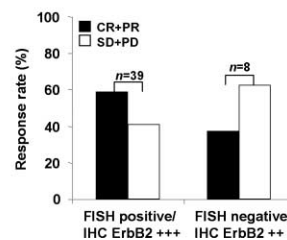
A



B



C



D

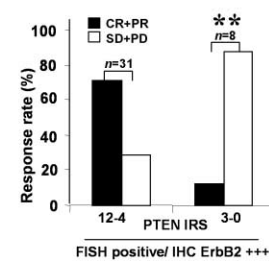


Figure 5. Patients with PTEN-deficient breast tumors have a poor response to trastuzumab-based therapy

A: PTEN expression patterns in human breast tumors. ErbB2-overexpressing (IHC 3+, 2+, and/or FISH-positive) primary breast carcinomas were collected from 47 patients who subsequently received trastuzumab plus taxane (paclitaxel or docetaxel) on IRB-approved clinical trials conducted at the UT M.D. Anderson Cancer Center (Esteva et al., 2002; Seidman et al., 2001). PTEN expression in these tumors was examined by IHC and semiquantified using immunoreactive scores (IRS, see Experimental Procedures). Representative tumor PTEN stainings (IRS 12, 9, 3, and 0) are shown.

B: Worse responses to trastuzumab-based therapy in patients with PTEN-deficient tumors than those with PTEN-positive tumors. PTEN deficiency were defined by PTEN IRS < 9 (left panel) or < 4 (right panel). * $p < 0.05$; ** $p < 0.01$. Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). n, number of patients studied.

C: Trastuzumab plus taxane response rates in patients with ErbB2 FISH-positive and/or IHC 3+ breast cancers versus patients with ErbB2 FISH-negative and/or IHC 2+ cancers ($p > 0.05$).

D: Among patients with ErbB2 FISH-positive and/or IHC 3+ breast cancers, patients with PTEN-deficient tumors responded less to trastuzumab-based therapy than those with PTEN normal tumors. ** $p < 0.01$. Data (A–D) were obtained in a double blind manner.

significant (Figure 5C). However, among patients with ErbB2 FISH-positive and/or IHC 3+ breast cancers, patients with PTEN-deficient tumors (IRS 0–3) had significantly lower response (CR+PR) rates than patients with PTEN-positive tumors

(12.5% versus 71%, $p < 0.01$) (Figure 5D). The data indicate that PTEN status is a unique and powerful marker for predicting clinical response to trastuzumab-based therapy.

Discussion

Trastuzumab has been a model of a rationally designed, highly specific, targeted cancer therapy and has brought valuable therapeutic benefits to patients with ErbB2-overexpressing cancers (Albanell and Baselga, 2001; Dickman, 1998). However, the limited response rates of trastuzumab, even in patients with tumors expressing very high levels of ErbB2, raises questions on the mechanisms of trastuzumab function (other than those related to the inhibition of ErbB2 signaling) and on the mechanisms of trastuzumab resistance in patients. Here, we report that the phosphatase function of the tumor suppressor PTEN is rapidly activated by trastuzumab before it downregulates the ErbB2 receptor. This finding led to the new concept that PTEN activation is a novel mechanism that contributes to trastuzumab's antitumor activity independent of its well-known function of ErbB2 downregulation. We also found that trastuzumab activated PTEN by inhibiting PTEN tyrosine phosphorylation through reducing the ErbB2 receptor bound Src—thus inhibiting Src, a tyrosine kinase for PTEN (Lu et al., 2003). This indicates a functional crosstalk between the ErbB2 receptor and the PTEN tumor suppressor in breast cancer cells via Src: Src plays important oncogenic functions (e.g., inactivates PTEN) when bound to and activated by overexpressed ErbB2, whereas trastuzumab dissociates Src from ErbB2, inhibits Src, and thus activates PTEN, which contributes to the trastuzumab antitumor function.

This newly identified mechanism of trastuzumab function clearly indicates that trastuzumab responsiveness depends not only on the downregulation of ErbB2 and the inhibition of ErbB2-related downstream events, but also on the status of PTEN. Indeed, our data from cultured breast cancer cell lines, mouse tumor xenografts, and most importantly, from clinical samples of breast cancer patients consistently demonstrated that PTEN deficiency is a molecular mechanism that confers ErbB2-overexpressing breast cancers resistance to trastuzumab-based therapy and that PTEN loss was associated with trastuzumab resistance in patients. The response rate of trastuzumab as a single agent was reported higher in patients with ErbB2 FISH-positive and/or IHC 3+ breast cancers than in patients with ErbB2 FISH-negative and/or 2+ tumors (Vogel et al., 2002). We observed a similar, but not significant, trend in our small cohort of 47 patients treated with trastuzumab plus taxane. Notably, among these 47 patients, there was an even smaller cohort of patients ($n = 39$) with ErbB2 FISH-positive and/or IHC 3+ breast cancers, who, based on the previous reports (Vogel et al., 2002), should have better response rates to trastuzumab. However, among the 39 patients, those with PTEN-negative tumors had significantly worse response to trastuzumab-based therapy than those with PTEN-positive tumors ($p = 0.004$). Thus, the PTEN status seems to be a more sensitive and specific predictor for trastuzumab-based therapy.

Insulin-like growth factor-I receptor (IGF-IR) signaling was implicated in trastuzumab resistance, but it has not been validated with clinical data (Lu et al., 2001). Remarkably, our finding that PTEN deficiency confers trastuzumab resistance is strongly supported by clinical data that patients with PTEN-deficient tumors did not respond to trastuzumab as well as those with

normal PTEN, and the difference was statistically highly significant ($p < 0.01$) even in this small cohort. Thus, the PTEN status of ErbB2-overexpressing breast tumors may be used as a predictive factor for patients' trastuzumab response in the clinic. PTEN loss in an ErbB2-overexpressing breast tumor can also serve as an indication that the patient needs combination therapy with PI3K inhibitors to overcome the PTEN loss-mediated trastuzumab resistance to receive the maximum therapeutic benefit of trastuzumab. Large-scale clinical investigations will be performed to further validate these ideas.

Loss of PTEN function from *PTEN* mutations was reported in 5%–10% of human breast cancers, and PTEN haploinsufficiency due to loss of heterozygosity at the PTEN locus can be found in nearly 50% of breast tumors (Li et al., 1997; Teng et al., 1997). In addition, epigenetic downmodulation of PTEN has also been reported (Mutter et al., 2000; Whang et al., 1998). Consistent with these reports, we identified PTEN deficiencies in ~40% of breast cancers in our two small cohorts, indicating that the PTEN loss rate is similar among patients with ErbB2-overexpressing tumors and breast cancer patients in general. Our finding that PTEN deficiency confers trastuzumab resistance in ErbB2-overexpressing breast cancers could directly impact the clinical management of these patients with PTEN-deficient tumors. In light of this, we have shown that PI3K inhibitors could overcome PTEN loss-induced trastuzumab resistance in ErbB2-overexpressing breast cancer cells in vitro and in vivo. This suggests that members of the PI3K pathway, as well as PTEN, are molecular targets for overcoming trastuzumab resistance. These new insights will guide the future development of agents for overcoming trastuzumab resistance and novel targeted cancer therapies to benefit cancer patients.

Experimental procedures

Antibodies and reagents

PTEN antibodies were from Santa Cruz Biotechnology (A2B1, Santa Cruz, CA) and Lab Vision Corp. (Ab2, Fremont, CA); antibodies to Akt, phospho-Akt, and Y416-phosphorylated Src were from Cell Signaling Technology (Beverly, MA); antibodies to ErbB2 and v-Src (Ab-1) were from Calbiochem (La Jolla, CA); antibodies to the 85 kDa subunit of PI3K, phospho-ErbB2, and phospho-tyrosine (4G10) were from Upstate Biotechnology, Inc. (Walsham, MA); antibody to phosphotyrosine (PY20) was from BD Biosciences (San Diego, CA); and trastuzumab was from Genentech, Inc. (San Francisco, CA). LY294002 was from Cell Signaling Technology; Wortmannin, PP2, and AG1879 were from Calbiochem; Enolase was from Sigma (St. Louis, MO); and taxol was from Bristol Myers-Squibb (Princeton, NJ). Src/CA mutant expression vector was provided by Dr. David Shalloway (Cornell University).

Cell culture

SKBr3 cells were from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A containing 15% fetal bovine serum (FBS), and the BT474 subline was from Dajun Yang (Georgetown University) and maintained in DMEM/F12 with 10% FBS. Both lines have the wild-type *PTEN* gene (Li et al., 1997).

PI3K activity assay

PI3K activities were determined in the PY20 antibody immunoprecipitates of untreated or trastuzumab-treated lysates as we did previously (Tan et al., 1999).

Src kinase assay

Equal amounts of cell lysates were immunoprecipitated with anti-Src antibody (OP07, Oncogene Research Products, Boston, MA). The precipitates were washed and incubated with 250 $\mu\text{g/ml}$ enolase, 2 μCi of [γ - ^{32}P]ATP,

and 3 μ l of 100 mM ATP in 40 μ l of kinase buffer. After 30 min at 37°C, the samples were boiled in 15 μ l of 6 \times SDS sample buffer for 5 min and then subjected to SDS-PAGE. The ³²P-labeled enolase was visualized by autoradiography.

Antisense delivery in vitro

Antisense (AS) oligonucleotides against PTEN (ISIS 116847: 5'-CTGCTAGC CTCTGGATTTGA-3') and control oligonucleotides (ISIS 116848: 5'-CTTCTG GCATCCGGTTTAGA-3') have been described (Butler et al., 2002). Cells were plated in 100 mm dishes at 30% confluence and transfected with oligonucleotides (12.5 nM for BT474 and 25 nM for SKBr3 cells) twice using Oligofectamine (Life Technologies, Rockville, MD) 24 and 72 hr postplating. Cells were replated for individual assays described in this report 96 hr postplating. PTEN expression was determined 120 hr postplating (Figure 3A).

Cell viability assay

1–5 \times 10³ cells/well were seeded in 96-well plates after transfection. Viable cells were determined by CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI).

Cell lysates preparation, IP, and IB

Cells at 70%–80% confluence were treated with each reagent indicated in the individual experiments. For PTEN phosphotyrosine immunoblots, ErbB2 IP, and Src IP, cells were treated with 30 μ M pervanadate for 10 min as described (Lu et al., 2003). Cells were washed with PBS and lysed in IP buffer with protease inhibitor cocktail (Sigma). IP and IB were performed as described (Yu et al., 1998b).

PTEN phosphatase assay

PTEN phosphatase activity was measured as described with minor modifications (Georgescu et al., 1999). Lysates were precleared with protein G-agarose beads to eliminate trastuzumab used as treatments prior to IP with PTEN antibodies (Ab-2 or A2B1). After IP, the beads were washed once in lysis buffer; five times in low-stringency buffer containing 20 mM HEPES (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, and 2.5 mM MgCl₂; and once in phosphatase assay buffer lacking PIP₃. Assays were performed for 40 min at 37°C in 50 μ l buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM DTT, and 100 μ M water-soluble diC8-PIP₃ substrate (Echelon, Salt Lake City, UT) in a 96-well plate. Release of phosphate from substrate was measured using Biomol Green Reagent (Biomol Research Laboratories, Inc., Plymouth Meeting, PA).

Separation of membrane bound and cytosolic proteins

Cells were lysed in saponin buffer (0.01% saponin, 10 mM Tris-HCl at pH 7.4, 140 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.2 mM sodium vanadate, 50 mM NaF, 25 mM β -glycerophosphate) containing protease inhibitor cocktail (Sigma). Proteins were extracted for 20 min on ice, and samples were centrifuged at 14,000 \times g for 30 min at 4°C. The saponin soluble (cytosolic) pool was collected (Palka and Green, 1997). The remaining pellet was rinsed with saponin buffer once, extracted in IP buffer, and centrifuged to remove insoluble materials. The supernatant was collected (membrane bound pool).

Analysis of internalization using flow cytometry

Immunofluorescent flow cytometry was performed as described (Drebin et al., 1985) using ErbB2 antibody (Ab5, Calbiochem).

BrdU incorporation assay

S phase cells were detected using 5-bromo-2'-deoxyuridine (BrdUrd) Labeling and Detection Kit I (Roche Molecular Biochemicals, Indianapolis, IN).

Immunofluorescence staining

It was done as described (Tan et al., 2002).

Animal experiment

Female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN) were implanted with 0.72 mg 60 day release 17 β -estradiol pellets (Innovative Research, Sarasota, FL) and irradiated at 3.5 Gy prior to inoculation. BT474 cells (8 \times 10⁶) in 200 μ l of PBS and Matrigel (BD Biosciences) mixture (1:1 ratio) were injected subcutaneously into a mouse mfp. When the mfp tumor reached >150 mm³, PTEN AS or control MIS oligonucleotides (30 μ g/week)

in saline was delivered by multiple intratumor injection as described (Urano et al., 2002). A week later, mice received trastuzumab (10 mg/kg) or vehicle (PBS) twice/week, combined with LY294002 (100 mg/kg) or vehicle (DMSO) three times/week. Tumor diameters were serially measured with calipers and tumor volumes calculated: volume = width² \times length/2. Each treatment group had 8–12 mice. Unpaired Student's *t* test was used to assess statistical significance.

Patient samples

ErbB2-overexpressing primary breast carcinomas were collected from 47 patients who subsequently developed metastatic breast cancer and received trastuzumab plus taxane (paclitaxel or docetaxel) (Esteva et al., 2002; Seidman et al., 2001). Primary breast carcinomas not selected for ErbB2-overexpressing tumors from 37 patients who subsequently developed metastatic breast cancer and received taxane without trastuzumab were collected as controls (Holmes et al., 1991; Valero et al., 1995). The 47 patients were treated between 1998 and 2001 and the 37 patients were treated between 1990 and 1991 under IRB-approved clinical trials at The University of Texas M.D. Anderson Cancer Center. Association between clinical response to therapy and PTEN status was tested using chi-square and Fisher's tests.

PTEN IHC and evaluation

Slides (4 μ m) of formalin-fixed, paraffin-embedded tissue sections were incubated with PTEN antibody (Ab-2, 1:500) as described (Podsypanina et al., 2001). IHC was performed with LSAB2 kit (DAKO, Carpinteria, CA), color development with 3-3'-diaminobenzidine, and counterstaining with hematoxylin. PTEN expression level was scored semiquantitatively based on staining intensity and distribution using the immunoreactive score (IRS) as described (Chui et al., 1996; Friedrichs et al., 1993) and as following: IRS = SI (staining intensity) \times PP (percentage of positive cells). SI was determined as 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. PP was defined as 0, <1%; 1, 1%–10%; 2, 11%–50%; 3, 51%–80%; and 4, >80% positive cells. Ten visual fields from different areas of each tumor were used for the IRS evaluation. Negative control slides without primary antibody were included for each staining. Normal breast epithelium or vascular endothelium known to express normal PTEN was used as positive controls.

ErbB2 status

ErbB2 gene amplification in patients' samples was assayed by fluorescence in situ hybridization (FISH) using PathVysion FISH assay (Vysis, Inc., IL). ErbB2 protein was determined by IHC as described (Jacobs et al., 1999).

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