Truncated ErbB2 Expressed in Tumor Cell Nuclei Contributes to Acquired Therapeutic Resistance to ErbB2 Kinase Inhibitors

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Abstract

ErbB2 tyrosine kinase inhibitors (TKI) block tyrosine autophosphorylation and activation of the full-length transmembrane ErbB2 receptor (p185ErbB2). In addition to p185ErbB2, truncated forms of ErbB2 exist in breast cancer cell lines and clinical tumors. The contribution of these truncated forms, specifically those expressed in tumor cell nuclei, to the development of therapeutic resistance to ErbB2 TKIs has not been previously shown. Here, we show that expression of a 95-kDa tyrosine phosphorylated form of ErbB2, herein referred to as p95L (lapatinib-induced p95L) was increased in ErbB2+)

breast cancer cells treated with potent ErbB2 TKIs (lapatinib, GW2974). Expressed in tumor cell nuclei, tyrosine phosphorylation of p95L was resistant to inhibition by ErbB2 TKIs. Furthermore, the expression of p95L was increased in ErbB2+ breast cancer models of acquired therapeutic resistance to lapatinib that mimic the clinical setting. Pretreatment with proteasome inhibitors blocked p95L induction in response to ErbB2 TKIs, implicating the role of the proteasome in the regulation of p95L expression. In addition, tyrosine phosphorylated C-terminal fragments of ErbB2, generated by alternate initiation of translation and similar in molecular weight to p95L, were expressed in tumor cell nuclei, where they too were resistant to inhibition by ErbB2 TKIs. When expressed in the nuclei of lapatinib-sensitive ErbB2+ breast cancer cells, truncated ErbB2 rendered cells resistant to lapatinib-induced apoptosis. Elucidating the function of nuclear, truncated forms of ErbB2, and developing therapeutic strategies to block their expression and/or activation may enhance the clinical efficacy of ErbB2 TKIs. Mol Cancer Ther; 10(8); 1367–74. ©2011 AACR.

Introduction

ErbB2, a 185-kDa transmembrane receptor tyrosine kinase (p185ErbB2), is deregulated in 25% of all breast cancers, where it predicts for a poor clinical outcome (1). ErbB2 activation requires autophosphorylation of tyrosine (Y) residues within the cytoplasmic domain of the receptor (e.g., Y1248; ref. 2). These phosphotyrosine residues serve as docking sites for adaptor proteins that link ErbB2 to downstream mitogen-activated protein kinase (p85) and phosphoinositide 3-kinase (PI3K) signaling networks that promote the growth and survival of breast cancer cells (2–6). In addition to p185ErbB2, truncated forms of ErbB2 lacking all or most of the N-terminus extracellular domain (ECD) exist in ErbB2+ breast cancer cell lines and clinical tumors (7–10). The most extensively studied truncated forms retain the transmembrane region and are expressed at the cell surface. Historically referred to as p95, truncated forms of ErbB2 expressed at the cell surface form heterodimers with other ErbB receptors (11) and interact with the p85 subunit of PI3K (12), thereby activating downstream signal transduction cascades in a manner similar to p185ErbB2. The generation of p95 has been shown to be dependent upon metalloproteinase activity (7). p95-positive breast cancers exhibit an aggressive clinical phenotype characterized by an increased incidence of lymph node involvement at the time of initial diagnosis (13, 14) and are more resistant to trastuzumab, as they lack the ECD (15, 16).

Lapatinib is a highly selective small molecule inhibitor of the ErbB2 and EGFR tyrosine kinases. Inhibition of ErbB2 tyrosine autophosphorylation by lapatinib leads to the inactivation of downstream cell growth and survival signals (17–19). Although a significant advancement in the treatment of breast cancer, the clinical efficacy of lapatinib has been limited by the development of acquired therapeutic resistance (20, 21). To address this problem, we generated clinically relevant models of acquired resistance to lapatinib using human ErbB2+ breast cancer cell lines (22, 23). We now show that treatment with ErbB2 tyrosine kinase inhibitors (TKI) increased the expression of a tyrosine phosphorylated, truncated form of ErbB2 that...
was expressed in the nuclei of ErbB2+ breast cancer cells, which will herein be referred to as p95L (lapatinib-induced p95). In contrast to truncated forms of ErbB2 expressed at the cell surface, the phosphorylation of p95L, and similar truncated forms that were also expressed in tumor cell nuclei, was resistant to ErbB2 TKI. The data supporting the activation and nuclear localization of p95L in response to ErbB2 TKI, and the role of nuclear, truncated forms of ErbB2 in the development of therapeutic resistance to ErbB2 TKIs, will be discussed.

Materials and Methods

Cell culture and reagents

BT474, SKBR3, Au565, MCF7, and T47D breast cancer cell lines were obtained from the American Type Culture Collection. Lapatinib-resistant breast cancer cells were generated as previously described (22). All cells were cultured as previously described (11, 17, 22). No independent authentication of these cells was done by the authors. Anti-phosphotyrosine (p-tyr) antibody, GW2974, and calpain inhibitor 1 were purchased from Sigma-Aldrich. Anti-c-ErbB2 (Ab-11) monoclonal antibody was from Neo Markers. Anti-ErbB2 (AA1243-02) IgG was obtained from Molecular Probes. Lapatinib IgG were from Rockland. Alexa Fluor 680 goat anti-rabbit IgG was from Life Technologies. Lapatinib (GW572016) and GW2974, and calpain inhibitor 1 were from Calbiochem. BB94 (batastat) was from Kimia Corp. Protein G agarose was from Proliance. Rapa and lactacystin were from Calbiochem. Lapatinib for cell culture work was from Upstate Biotechnology. MG132, 1,10-phenanthroline, and rBT474 cells were suspended in HBSS and mixed with Matrigel (BD Biosciences) at 1:1 ratio to make final concentrations of 1 × 10^6 cells per 50 mL. A 50-μL tumor cell suspension was inoculated into bilateral mammary fat pads of female NOD/SCID mice (5 ~ 6 weeks old, 4 mice per group). Animals were treated with lapatinib (75 mg/kg/d) by oral gavage until they were sacrificed. Tumor volumes were measured serially, and tumor volumes calculated using the following formula: long axis × (short axis)^2 × 0.52. The mice were euthanized with CO₂ inhalation and tumor xenografts excised 59 days after implantation of tumor cells. All animal studies were conducted in compliance with Duke animal care regulations. Human biopsies were collected from breast cancer skin metastasis after informed written consent was obtained as part of an Institutional Review Board–approved tissue collection protocol. Tumor specimens were flash frozen in liquid nitrogen and stored at −80°C. Tissue extracts were prepared for Western blot analysis by homogenization in radioimmunoprecipitation assay buffer at 4°C.

Expression of truncated forms of ErbB2 in human breast cancer cell lines

C-terminal fragments (CTF; c-611, c-676, and c-678) were generated on the basis of ErbB2 open reading frames from LTR-2/ErbB2 (8) and subcloned into the pcDNA3.1 vector (Invitrogen) with forward primers: 5'-ACAAGCTT ACCATGCCCATCTGGAG-3', 5'-ACAAGCTTACCATGAAGCGACGGCAGCA-3' and 5'-ACAAGCTT ACCATGCCGAACCTTGCTG-3', and reverse primer: 5'-AACTCGAG TCACATCGG-3'. CTFs were transfected with empty vector alone (controls) or the same vector containing p185^ErbB2 or the various CTFs using the Lipofectamine 2000 Reagent from Invitrogen according to the manufacturer’s protocol. Stably transfected cells were selected using G418 (400 μg/mL), and the expression levels of CTFs were confirmed by Western blot analysis.

Immunofluorescence microscopy

Cells were cultured in 6-well plates with or without the indicated treatments. After washing with PBS, cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 for 20 minutes, and blocked with 2% bovine serum albumin in PBS for 60 minutes protected from light. After washing in PBS + 0.1% Tween-20, the membranes were scanned using an Odyssey imaging system.

Human tumor xenografts, animal treatment, and human tumor biopsies

NOD.CB17-Prkdc<sup>scid</sup>/J (NOD/SCID) mice were purchased from Jackson Laboratory and bred in the Duke Comprehensive Cancer Center Isolation Facility. BT474 and rBT474 cells were suspended in HBSS and mixed with Matrigel (BD Biosciences) at 1:1 ratio to make final concentrations of 1 × 10^6 cells per 50 mL. A 50-μL tumor cell suspension was inoculated into bilateral mammary fat pads of female NOD/SCID mice (5 ~ 6 weeks old, 4 mice per group). Animals were treated with lapatinib (75 mg/kg/d) by oral gavage until they were sacrificed. Tumor volumes were measured serially, and tumor volumes calculated using the following formula: long axis × (short axis)^2 × 0.52. The mice were euthanized with CO₂ inhalation and tumor xenografts excised 59 days after implantation of tumor cells. All animal studies were conducted in compliance with Duke animal care regulations. Human biopsies were collected from breast cancer skin metastasis after informed written consent was obtained as part of an Institutional Review Board–approved tissue collection protocol. Tumor specimens were flash frozen in liquid nitrogen and stored at −80°C. Tissue extracts were prepared for Western blot analysis by homogenization in radioimmunoprecipitation assay buffer at 4°C.

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Proliferation and apoptosis assay

The proliferation assay was carried out in a 96-well plate format in a final volume of 100 μL per well cell culture medium with the cell proliferation reagent WST-1 from Roche Diagnostics. Details of the WST-1 proliferation and Annexin V/nexin 7-AAD apoptosis assays were previously published (17, 22).

Statistical analysis

Data were expressed as means with standard error bars included. Student’s t test was used to determine statistical significance between 2 groups. P < 0.05 was considered a statistically significant difference.

Results

ErbB2 TKIs increase the expression of phospho-p95L in tumor cell nuclei

The effects of ErbB2 TKI on ErbB2 tyrosine phosphorylation were determined in BT474 cells, a human ErbB2 breast cancer cell line, using immunofluorescence microscopy. Total ErbB2 and phosphotyrosine (p-tyr) signals were visualized by immunofluorescence microscopy using an ErbB2 phosphotyrosine–specific primary antibody and an FITC-conjugated secondary antibody. D, steady-state protein levels of p185ErbB2 and p95L were determined in nuclear extracts from BT474 and Au565 cells treated for 24 hours with lapatinib (500 nmol/L) or vehicle alone (control). Steady-state protein levels of Oct 1, IκB, and E-cadherin, which represent nuclear, cytoplasmic, and cell membrane proteins, respectively, were used to confirm the purity of nuclear extracts. E, Au565 cells were treated with GW2974 (1 μmol/L) or vehicle alone (−) for 24 hours. Steady-state levels of total and phosphorylated p185ErbB2 and p95L were analyzed by ErbB2 IP/Western blot (WB) analysis from nuclear extracts. Total (green) and phosphorylated (red) forms of the indicated proteins are shown. Cells treated with vehicle alone (0.01% DMSO) served as controls for all of the experiments in Fig. 1.

Figure 1. Phosphorylation of nuclear, truncated ErbB2 is resistant to ErbB2 TKI. A, the chemical structure of lapatinib. B, BT474 cells were treated for 48 hours with GW2974 (1 μmol/L) or vehicle alone (−). Total ErbB2 and phosphotyrosine (p-tyr) signals (green) were visualized by immunofluorescence microscopy as described in Materials and Methods. Cell nuclei were counterstained blue with DAPI. The bottom row merges FITC and DAPI signals. C, Au565 cells were treated with lapatinib (1 μmol/L) or vehicle alone (control) for 24 hours, and p-ErbB2 was assessed by immunofluorescence microscopy using an ErbB2 phosphotyrosine–specific primary antibody and an FITC-conjugated secondary antibody. D, steady-state protein levels of p185ErbB2 and p95L were determined in nuclear extracts from BT474 and Au565 cells treated for 24 hours with lapatinib (500 nmol/L) or vehicle alone (control).
In Fig. 1E, total ErbB2 protein was immunoprecipitated (IP) from nuclear extracts isolated from Au565 cells treated with vehicle alone (control) or GW2974. Steady-state levels of total (green) and phosphorylated (red) p185ErbB2 and p95L were determined by Western blot analysis. Although both p185ErbB2 and p95L were expressed in a phosphorylated state, GW2974 inhibited phosphorylation of p185ErbB2 but not p95L. Similar results were seen in other ErbB2− breast cancer cell lines (data not shown).

Increased expression of p95L in lapatinib-resistant breast cancer cell lines and tumor xenografts

P95L protein levels were increased in models of acquired resistance to lapatinib (e.g., rBT474; rAu565; ref. 22) compared with their lapatinib-sensitive cell counterparts (Fig. 2A). In Fig. 2B, the growth of tumor xenografts established from resistant cells (rBT474) was significantly increased compared with tumors derived from parental cells (BT474), in animals treated with lapatinib (P < 0.05). Steady-state p95L protein levels were increased in rBT474 compared with BT474 tumor xenografts (data not shown).

To determine whether a truncated form(s) of ErbB2 similar to p95L could be detected in clinical tumors, we analyzed steady-state ErbB2 protein levels in biopsies from metastatic breast cancer sites that had developed while patients were on lapatinib therapy. A truncated form of ErbB2, similar in molecular weight to p95L in rBT474 cells, was seen in both clinical samples (Fig. 2C, MCB1 and MCB2).

Comparison of p95L with CTFs of ErbB2 generated by alternate initiation of translation

CTFs of ErbB2 generated by alternate initiation of translation (e.g., c-611, c-676, c-687) have been reported (8). c-611 lacks most of the ECD, whereas c-676 and c-687 lack the ECD and transmembrane regions. We synthesized CTFs by alternate initiation of translation from methionines 611 (c-611) and 676 (c-676) and used the pcDNA3.1 vector to express them in non–ErbB2-overexpressing MCF7 and T47D breast cancer cells. In Fig. 3A, the subcellular localization of p185ErbB2, c-611, c-676, and c-687 expressed in MCF7 transfected cells was determined by immunofluorescence microscopy using an ErbB2-specific primary and FITC-conjugated secondary antibody (green). While c-611 localized to the cell membrane and cytoplasm, c-676 was seen primarily in tumor cell nuclei (blue). In Fig. 3B, the effects of GW2974 on the phosphorylation of c-676 expressed in MCF7 transfected cells were examined by immunofluorescence microscopy using a phosphotyrosine (p-tyr) antibody and FITC-conjugated secondary antibody (green). Phosphorylation of nuclear c-676 was not inhibited by GW2974. The effect of GW2974 on steady-state phosphoprotein levels of the indicated CTFs was next determined by Western blot analysis using an ErbB2 phosphotyrosine-specific antibody in whole cell extracts from T47D cells transfected with c-611, c-676, and c-687.

Figure 2. Increased expression of p95L in ErbB2− breast cancer models of acquired lapatinib resistance and clinical tumor samples. A, steady-state protein levels of p185ErbB2 and p95L in rBT474 and rAu565 cells and their lapatinib-sensitive parental cell counterparts (BT474 and Au565) were determined by Western blot analysis. Actin steady-state protein levels served as a control for equal loading of protein. The results are representative of 3 independent experiments. B, tumor xenografts from resistant cell lines (rBT474 and rAu565) were significantly increased compared with tumors derived from parental cells (BT474), in animals treated with lapatinib (P < 0.05). Steady-state p95L protein levels were increased in rBT474 compared with BT474 tumor xenografts (data not shown).

We next isolated nuclear extracts from BT474 and Au565 cells treated with vehicle alone (controls) or lapatinib. The purity of nuclear extracts was confirmed using Oct 1, 1kB, and E-cadherin, which represent nuclear, cytoplasmic, and cell membrane proteins, respectively. Steady-state levels of total p95L protein increased in lapatinib-treated cells without an appreciable change in p185ErbB2 (Fig. 1D). Additional molecular weight bands above 98 kDa, which have been seen previously in ErbB2 blots, were observed.

In Fig. 2A, total ErbB2 protein was immunoprecipitated (IP) from nuclear extracts isolated from Au565 cells treated with vehicle alone (control) or GW2974. Steady-state levels of total (green) and phosphorylated (red) p185ErbB2 and p95L were determined by Western blot analysis. Although both p185ErbB2 and p95L were expressed in a phosphorylated state, GW2974 inhibited phosphorylation of p185ErbB2 but not p95L. Similar results were seen in other ErbB2− breast cancer cell lines (data not shown).

Comparison of p95L with CTFs of ErbB2 generated by alternate initiation of translation

CTFs of ErbB2 generated by alternate initiation of translation (e.g., c-611, c-676, c-687) have been reported (8). c-611 lacks most of the ECD, whereas c-676 and c-687 lack the ECD and transmembrane regions. We synthesized CTFs by alternate initiation of translation from methionines 611 (c-611) and 676 (c-676) and used the pcDNA3.1 vector to express them in non–ErbB2-overexpressing MCF7 and T47D breast cancer cells. In Fig. 3A, the subcellular localization of p185ErbB2, c-611, c-676, and c-687 expressed in MCF7 transfected cells was determined by immunofluorescence microscopy using an ErbB2-specific primary and FITC-conjugated secondary antibody (green). While c-611 localized to the cell membrane and cytoplasm, c-676 was seen primarily in tumor cell nuclei (blue). In Fig. 3B, the effects of GW2974 on the phosphorylation of c-676 expressed in MCF7 transfected cells were examined by immunofluorescence microscopy using a phosphotyrosine (p-tyr) antibody and FITC-conjugated secondary antibody (green). Phosphorylation of nuclear c-676 was not inhibited by GW2974. The effect of GW2974 on steady-state phosphoprotein levels of the indicated CTFs was next determined by Western blot analysis using an ErbB2 phosphotyrosine-specific antibody in whole cell extracts from T47D cells transfected with c-611, c-676, and c-687.

Figure 2. Increased expression of p95L in ErbB2− breast cancer models of acquired lapatinib resistance and clinical tumor samples. A, steady-state protein levels of p185ErbB2 and p95L in rBT474 and rAu565 cells and their lapatinib-sensitive parental cell counterparts (BT474 and Au565) were determined by Western blot analysis. Actin steady-state protein levels served as a control for equal loading of protein. The results are representative of 3 independent experiments. B, tumor xenografts from resistant cell lines (rBT474 and rAu565) were significantly increased compared with tumors derived from parental cells (BT474), in animals treated with lapatinib (P < 0.05). Steady-state p95L protein levels were increased in rBT474 compared with BT474 tumor xenografts (data not shown).
with c-611, c-676, c-687, or vector alone (Fig. 3C). GW2974 inhibited tyrosine phosphorylation of c-611 but not c-676 or c-687. Expression of p95L in BT474 cells treated with GW2974 was included as a reference. Similar results were observed in MCF7 transfected cells (data not shown).

Proteasome inhibitors block p95L induction by ErbB2 TKI

We examined the effects of protease inhibitors on p95L expression in lapatinib-treated Au565 cells. Cells were treated as indicated in Fig. 4A. Briefly, cells were treated with lapatinib alone, the indicated protease inhibitors alone, or (24) a combination of lapatinib plus protease inhibitor. Included among the protease inhibitors were BB-94, a metalloproteinase inhibitor that blocked phorbol ester–induced p95 expression (7), and a γ-secretase inhibitor that reduced ErbB4 truncation (25). The BB-94 and the γ-secretase inhibitor had little effect on the induction of p95L by lapatinib (Fig. 4A). However, inhibitors of the 20S proteasomal subunit (lactacystin, MG132, calpain I inhibitor) blocked the induction of p95L in lapatinib-treated Au565 cells (Fig. 4A). Cells treated with vehicle alone served as controls.

Treatment with lactacystin alone, at the same concentration that blocked induction of p95L, had relatively little antitumor activity in Au565 cells (Fig. 4B). However, there was enhanced antitumor activity when lactacystin was combined with a sublethal concentration of lapatinib (0.1 μmol/L) that was otherwise sufficient to induce p95L.

Expression of truncated ErbB2 reduces the antitumor activity of lapatinib

To determine the impact of nuclear, truncated forms of ErbB2 on the antitumor activity of lapatinib, we expressed c-676 in BT474 cells (Fig. 5A). We chose c-676 because of its
Discussion

The development of acquired therapeutic resistance represents a significant barrier limiting the clinical efficacy of lapatinib. Acquired resistance to lapatinib does not appear to be related to loss of target sensitivity as tyrosine phosphorylation of p185ErbB2 remains inhibited in ErbB2+ breast cancer cells that have developed resistance to lapatinib (22, 26). We now show that p95L is expressed in the nuclei of ErbB2+ breast cancer cells, in a tyrosine phosphorylated, presumably activated state. In contrast to truncated forms of ErbB2 expressed at the cell surface, the phosphorylation of truncated ErbB2 in the nucleus was resistant to ErbB2 TKIs. Importantly, expression of a truncated form of ErbB2 (c-676) in the nuclei of ErbB2+ breast cancer cells rendered cells resistant to the antitumor effects of lapatinib (Fig. 5).

Distinct forms of ErbB2 differing in their (i) subcellular localization, (ii) regulation of expression, and (iii) sensitivity to ErbB2 targeted therapies exist in breast cancer cells. Expressed at the cell surface, p95 and c-611 mediate resistance to trastuzumab but not ErbB2 TKIs (11, 15). In contrast, truncated forms of ErbB2 expressed in tumor cell nuclei in a tyrosine phosphorylated state are resistant to inhibition by ErbB2 TKIs. Whereas some nuclear, truncated forms of ErbB2 are generated through alternate initiation of translation (8), p95L appears to be mediated by the activation of the proteasome, as proteasome inhibitors block its induction (Fig. 4A). As summarized in Fig. 6, cleavage at a putative proteasome recognition site located within the intracellular domain (see *) would generate a truncated form of ErbB2 with a predicted molecular weight similar to p95L. This truncated form contains tyrosine...
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autophosphorylation sites (e.g., Y1248) and the nuclear localization signal (NLS). In the nucleus, tyrosine phosphorylation (Y1248) of p95L is resistant to lapatinib (Y1248+). In contrast, the tyrosine phosphorylation of nuclear p185ErbB2 is inhibited by lapatinib (Y1248−). C, truncated forms of ErbB2 expressed at the cell surface (e.g., p95 and c-676) are inhibited by lapatinib (Y1248).

It is not clear why the phosphorylation of truncated ErbB2 in the nucleus is resistant to ErbB2 TKI. One potential explanation is that the structural conformation of p95L and c-676 differs from p185ErbB2, preventing ErbB2 TKIs to gain access to their active sites. Studies to elucidate the structural conformation of nuclear, truncated forms of ErbB2 should help answer this question.

Although the function of nuclear, truncated forms of ErbB2 is unknown, insight from studies of p185ErbB2 include therapies that prevent induction of p95L and/or inactivate other truncated forms of ErbB2 that are resistant to lapatinib and GW2974 (Fig. 5B). Strategies to enhance the clinical efficacy of ErbB2 TKIs may now include therapies that prevent induction of p95L and/or inactivate other truncated forms of ErbB2 that are expressed in tumor cell nuclei.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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