Disruption of Laminin-Integrin-CD151-Focal Adhesion Kinase Axis Sensitizes Breast Cancer Cells to ErbB2 Antagonists

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Abstract

Resistance to anti-ErbB2 agents is a significant problem in the treatment of human ErbB2+ breast cancers. We show here that adhesion of human ErbB2+ breast cancer cells to basement membrane laminin-5 provides substantial resistance to trastuzumab and lapatinib, agents that respectively target the extracellular and kinase domains of ErbB2. Knockdown of laminin-binding integrins (α6β4/α3β1) or associated tetraspanin protein CD151 reversed laminin-5 resistance and sensitized ErbB2+ cells to trastuzumab and lapatinib. CD151 knockdown, together with trastuzumab treatment, inhibited ErbB2 activation and downstream signaling through Akt, Erk1/2, and focal adhesion kinase (FAK). Hence, ErbB2 function in mammary tumor cells is promoted by integrin-mediated adhesion to laminin-5, with strong support by CD151, leading to signaling through FAK. Consequently, removal or inhibition of any of these components (laminin-5, integrin, CD151, FAK) markedly sensitizes cells to anti-ErbB2 agents. These new insights should be useful when devising strategies for overcoming drug resistance in ErbB2+ cancers. Cancer Res 70(6); 2256–63. ©2010 AACR.

Introduction

ErbB2/HER2, an epidermal growth factor receptor family member, is a potent oncogenic receptor kinase driving progression, malignancy, and metastasis of human breast cancer. ErbB2 activates via homodimerization or heterodimerization with other ErbB family members (1). Activated ErbB2 initiates signals through phosphoinositide 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and other pathways, thus enhancing cell proliferation and survival (2). ErbB2 gene amplification, which occurs in 15% to 25% of human breast cancers, is associated with poor patient prognosis and survival (3). ErbB2 inhibitors trastuzumab and lapatinib are clinically effective in targeting ErbB2+ breast cancers. Trastuzumab (herceptin), a HER2-specific humanized monoclonal antibody, inhibits ErbB2 signaling and triggers an antitumor antibody-dependent cellular cytotoxicity (ADCC) response (4). As a single agent, trastuzumab elicits objective tumor responses in 30% of patients with advanced ErbB2+ breast cancer and improves response rate and survival when added to chemotherapy in that patient population (5). Lapatinib, a small molecule inhibitor of ErbB2 and epidermal growth factor (EGF) receptor (EGFR) tyrosine kinase activities, induces apoptosis in ErbB2+ breast cancer cells, including those that are trastuzumab resistant (6). Consistent with this finding, lapatinib improves response rates and progression-free survival when added to chemotherapy in patients with ErbB2+ breast cancer who had previously progressed on trastuzumab (7).

Unfortunately, >60% patients with ErbB2+ cancers do not respond to trastuzumab monotherapy, and most initial responders develop resistance within 1 year (8). Resistance may arise through constitutive activation of the PI3K/Akt pathway, other ErbB family members, or alternative oncogenic pathways (4). Also, membrane-associated glycoprotein MUC4 might cause resistance by masking the ErbB2-binding site for trastuzumab (4). Potential mechanisms of lapatinib resistance include ErbB2 kinase site mutations (9), PI3K/Akt pathway hyperactivation, and increased antiapoptotic to proapoptotic protein ratio (10).

Tumor-microenvironment interactions markedly affect antitumor drug responses. For example, ECM proteins, including laminin-5, protect malignant mammary cells (11) and other cancer cells (12) from chemically induced apoptosis. In nearly all epithelial tissues, laminin-5 regulates cell organization, gene expression, and survival (13). Although laminin-5 levels diminish upon malignant transformation of breast epithelium (14), it still can support mammary tumor survival (15) and tumor metastasis to lung (16), lymph node (17), and likely other tissues.
Integrins, at the tumor-ECM microenvironment interface, can promote tumor cell survival and protection from chemically induced apoptosis (18). The laminin-binding integrin α6β4 promotes breast tumor survival (11, 15). Furthermore, deletion of the β4 signaling domain sensitized ErbB2+ mouse mammary tumors to gefitinib/irressa (19), a tyrosine kinase domain inhibitor. Survival promotion by α6β4 sometimes may (20) or may not (21) involve activation of Akt, a key determinant of drug resistance (4). Laminin-binding integrins (α3β1, α6β1, α6β4) associate closely with CD151, a tetraspanin family member (22). CD151 minimally affects integrin-dependent cell adhesion to laminin but rather influences adhesion strengthening, cell invasion, and migration and three-dimensional cell morphology (22). CD151 expression correlates with poor prognosis in colon (23) and non–small cell lung cancers (24) and with invasiveness in mammary carcinoma cells (25). Ablation of CD151 protein affects tumor cell growth, invasion, migration, and EGF sensitivity in human basal-like breast cancer (26). Because α6β4 affects ErbB2+ breast tumor progression (19) and CD151 is elevated in 32% of ErbB2+ human tumors (26), we hypothesized that laminin-5, a laminin-5, α6β4 integrins in Breast Cancer

Materials and Methods

Cells and antibodies. All ErbB2+ human mammary tumor cell lines BT474, ZR-75-1, SKBR3, and MD-MB-453 cells, from American Type Culture Collection, were cultured in RPMI supplemented with 10% fetal bovine serum (FBS). Monoclonal antibodies to CD151 (5C11), CD9 (MM2/57), CD81 (M38), integrin α2 (IE10), α5 (B1I26), α3 (A3-X8), α6 (A6-EL1), and β1 (TS2/16) were referenced elsewhere (26). Monoclonal antibodies against α6 (GoH3) and β4 integrins and against FAK were obtained from BD Bioscience. Antibodies against E-cadherin and phosphorylated FAK (Y397) were from Santa Cruz Biotechnology, Inc. Claudin 3–specific antibody was from Invitrogen. Rabbit polyclonal antibodies against total and phosphorylated PTEN, MAPK, Akt, ErbB2, and EGFR were purchased from Cell Signaling Technology. Anti–β1-actin was from Sigma.

siRNA and shRNA targeting. For siRNA targeting, cells (~2–4 × 10^5 per well in six-well plates) were preincubated with siRNAs using RNAiMAX (Invitrogen), at days ~3 and ~1 before assay. Before drug addition, siRNA-treated cells were essentially indistinguishable with respect to physical appearance and viability. All siRNAs were purchased from Dharmacon. Sequences of siRNAs targeting CD151, α6, α3, and CD9 were described (26). Independent siRNAs for FAK are UAGUACACUCUUUGCAUUAU and GGCAUAUAUGGC-CACUGU. PTEN was targeted using four oligo pools (GAUGACACUAACACAAUAU, GACUUGACUUGACUUAU, GAUCUUGACAUUGCUUAAU, CGAUAAGCUUUGAUAU). For stable knockdown of human CD151, ErbB2+ breast cancer cells were infected with lentivirus as described (26). CD151-null cells were negatively selected by a two-color flow cytometry. Knockdown efficiency was evaluated by flow cytometry and immunoblotting.

Drug sensitivity assays. Matrix proteins collagen, fibronectin, and Matrigel were from BD Bioscience. Laminin-5 was from human A431 cells. Plates (24-well) were precoated with ~5 μg/mL laminin-5 or Matrigel (overnight at 4°C) or with 20 μg/mL collagen I or 10 μg/mL fibronectin (1 h at 7°C) or left uncoated. Cells (~5 × 10^4 per well) were plated for 24 h and then treated with trastuzumab (0–512 μg/mL) or lapatinib (0–500 nmol/L). After another 48 to 72 h, cells were quantitated using PicoGreen (Invitrogen). Each data point represents the mean of three separate experiments, each with triplicate measurements. Variability between experiments was typically <15%. Trastuzumab (herceptin) was from Genentech, lapatinib was from commercial sources and anti-FAK inhibitor TAE226 was from Novartis Co. Seven different ErbB2-negative cell lines showed no drug sensitivity, except at the highest doses of trastuzumab (256–512 μg/mL yielded mean decrease in cell number of 2–3%) and lapatinib (2.5–10 μmol/L yielded a mean decrease in cell number of 3–10%).

Signaling assays. Control and CD151-deficient BT474 or ZR75 cells were detached using EDTA, washed, incubated at 37°C for 30 min, and then plated on laminin-5 ± trastuzumab. Cells were lysed in buffer containing 1 mmol/L Na2VO4, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS and protease cocktail (Roche). After reducing electrophoresis, proteins were transferred to nitrocellulose and blotted for phosphorylated ErbB2 (Y1221/1222), Akt (S473), MAPK (T202/Y204), PTEN (T382/383) and FAK (Y397) and for total ErbB2, MAPK, Akt, PTEN, and FAK proteins.

Results

Laminin-5, α3β1 and α6β4 integrins, and CD151 affect ErbB2 drug sensitivity. We hypothesized that laminin-5, a key epithelial cell microenvironment component (31), might...
affect sensitivity to ErbB2-targeted drugs. Indeed, four different ErbB2-positive mammary cell lines (SKBR3, BT474, MDA-MB-231, ZR75) were markedly more resistant to trastuzumab when grown on laminin-5, compared with fibronectin, collagen, noncoated plastic (Fig. 1A), or Matrigel (Supplementary Fig. S1). These results suggest that drug resistance is supported by receptors for laminin-5 (integrins α6β4 and/or α3β1) but not by receptors for collagen (α2β1), fibronectin (α5β1), or Matrigel (α6β1). These integrins are variably present in the ErbB2+ cells (Supplementary Figs. S2 and S3B). Consistent with the laminin-5 results, simultaneous ablation of integrin α3 and α6 subunits (by 78–95%; Supplementary Figs. S3A, B) markedly sensitized ZR75 cells to trastuzumab (Fig. 1B), as the IC50 decreased by a factor of ∼16 (−12.1–0.76 μg/mL). By contrast, for ZR75 cells on noncoated plastic, the IC50 decreased by a factor of only ∼4.5 (4.9–1.1 μg/mL; Fig. 1B).

The α3 and α6 integrins associate closely with tetraspanin CD151 whenever it is coexpressed. As an alternative to ablating α3 and α6 integrins, which disrupts adhesion to laminin-5, we stably knocked down CD151 (by >90%; Supplementary Fig. S3C), which minimally affects cell adhesion. Nonetheless, stable CD151 knockdown markedly enhanced trastuzumab sensitivity of SKBR3, BT474, MDA-MB-453, and ZR75 cells on laminin-5 (Fig. 2A). By contrast, CD151 knockdown minimally affected sensitivity for cells on noncoated tissue culture plastic (Fig. 2A), Matrigel (Supplementary Fig. S1) or collagen (Supplementary Fig. S1). For representative ZR75 cells on laminin-5, trastuzumab sensitivity was enhanced similarly by siRNA knockdown of either CD151 or α6 integrin (IC50, 6–8 to 0.9–1.0 μg/mL; Fig. 2B). Knockdown of α3 integrin increased trastuzumab sensitivity to a lesser extent (IC50, 2.5 μg/mL; Fig. 2B). Knockdown of CD151 did not affect surface levels of α3, α6, β1, or β4 integrins (Supplementary Fig. S3B).

We also tested breast cancer cell sensitivity to lapatinib, a membrane permeable ErbB2 kinase inhibitor (34). Again, control IC50 (120–190 nmol/L) were markedly diminished upon knockdown of α6 or CD151 (9–11 nmol/L), α3 (∼16 nmol/L), or both integrin subunits together (∼8 nmol/L) in ZR75 cells plated on laminin-5 (Fig. 3A). Knockdown of tetraspanin protein CD9 had negligible effect (IC50, ∼150 nmol/L; Fig. 3A). In BT474 cells, CD151 knockdown (by >90%; Supplementary Fig. S4) again had more effect for cells on laminin-5 (IC50, decreased from ∼14 to 2 nmol/L) compared with tissue culture plastic (6.5–3.5 nmol/L; Fig. 3B).

ErbB2 and CD151 signaling. CD151 ablation did not affect ErbB2 oligomerization in BT474 cells, as seen by chemical cross-linking (Supplementary Fig. S5). For BT474 cells on laminin-5 in the presence of 1% serum, ErbB2 phosphorylation was reduced upon adding trastuzumab (by ∼32%) or ablation of CD151 (by ∼46%), whereas both together had a roughly additive effect (∼85% reduction; Fig. 4A). Trastuzumab treatment and CD151 ablation similarly diminished AKT phosphorylation in an additive manner (trastuzumab by 59%; CD151 by 42%; both by 91%; Fig. 4B). For Erk1/2, another signaling molecule downstream of ErbB2, inhibition was synergistic. Erk1/2 phosphorylation was diminished by trastuzumab (45%) and CD151 ablation (10%), with both together yielding 88% reduction (Fig. 4C). Interference with PTEN, an upstream phosphatase that suppresses PI3K and AKT, can enhance trastuzumab resistance (35). However, for BT474 cells already resistant due to plating on laminin-5, knockdown of PTEN yielded little or no further increase in resistance to trastuzumab or lapatinib (Supplementary Fig. S6). Furthermore, trastuzumab treatment and/or CD151 ablation minimally affected phosphorylation of PTEN at S380 (Fig. 4D).

A central role for FAK. For BT474 cells on laminin-5, FAK activation (Y937 phosphorylation) was slightly diminished by trastuzumab treatment (12%), and CD151 knockdown had a modest effect (43% reduction; Fig. 4E). However, both together reduced FAK activation by 83% (Fig. 4E). Consistent with ErbB2 signaling through FAK, knockdown of FAK (>90%; Fig. 5C) markedly increased trastuzumab sensitivity of BT474 cells on laminin-5 (IC50, ∼210 to 13–16 μg/mL; Fig. 5A). Knockdown of CD151 or FAK, alone or together,
Similarly enhanced trastuzumab sensitivity (Fig. 5A). By contrast, BT474 cells on tissue culture plastic did not show resistance to trastuzumab and were relatively unaffected by FAK knockdown (Fig. 5B). Hence, laminin-5 and CD151 may promote trastuzumab resistance entirely by acting through FAK.

A small molecule inhibitor of FAK, TAE226, inhibits multiple tumor cell types (36, 37). Titration of TAE226 up to 1.0 μmol/L caused only a slight (27–29%) inhibition of BT474, SKBR3, ZR75, and MDA-MB-453 cell growth during a 48-h assay (not shown). However when combined with trastuzumab, the effects of TAE226 were more dramatic. At 0.5 to 1.0 μmol/L, TAE226 markedly increased trastuzumab sensitivity of BT474 (Fig. 6A) and SKBR3 (Fig. 6B), as IC50 shifted downwards by a factor of ~4 to 7. FAK ablation (by siRNA) and inhibition (by TAE226) yielded similar sensitization to trastuzumab. Hence, the secondary ability of TAE226 to inhibit insulin-like growth factor-1R (38) seems not to be a factor in these experiments.

**Discussion**

Targeting of ErbB2 by trastuzumab (extracellularly) and by lapatinib (intracellularly) can effectively inhibit ErbB2+ breast cancer. Removal, replacement, or inhibition of laminin-5, integrin α6β4 (or α3β1), CD151, or FAK sensitized cells to trastuzumab and lapatinib and diminished ErbB2 signaling. Hence, there is a functional collaboration between ErbB2 and laminin-5-integrin-CD151 complexes, with FAK playing a key downstream role. Our cells were sensitized to respond to trastuzumab at doses (~4–128 μg/mL) comparable with steady-state levels (~80 μg/mL) obtained in human patients.
The tumor microenvironment can dramatically affect drug sensitivity (39), and integrin-dependent adhesion generally supports tumor cell drug resistance (40). Indeed, plating on laminin-5 enhanced ErbB2+ breast cancer cell resistance to trastuzumab and lapatinib. However, ErbB2+ cell resistance was not enhanced by collagen I, Matrigel, fibronectin, or non-coated plastic. There are few reports of "matrix-specific" drug resistance. Adhesion to laminin-1 promoted small cell lung cancer drug resistance (41), contrasting with our results, in which Matrigel/laminin-1 was not protective. Elsewhere, laminin-5, but not other ECM proteins, was modestly protective when added to already adherent hepatocellular carcinoma cells treated with Iressa/gefitinib (42), an EGFR tyrosine kinase inhibitor. Ours may be the first study to show a specific protective effect for laminin-5 adhesion on tumor cell drug resistance in general and drugs targeting ErbB2 in particular. Although laminin-5 (laminin 332) may be lost as breast cancers become malignant (14), it has been suggested that laminin-5 present in normal breast tissue may support the initial transition to invasive cancer (43). In addition, laminin-5 can appear at the invasive front in some breast carcinomas (44), and its appearance may correlate with decreased breast cancer patient survival (45).

Whereas this study focused on laminin-5, the results are relevant to other laminins. For example, laminin-10 (laminin 511), which contributes to breast cancer progression (46) and is elevated in \( \sim 35\% \) of ErbB2+ human breast cancer samples, also supports trastuzumab resistance (not shown). Other notable similarities between laminin 10 and laminin 5 include (a) recognition by integrins \( \alpha 3\beta 1 \) and \( \alpha 6\beta 4 \), (b) regulatory involvement of CD151, and (c) signaling through FAK (47).

Consistent with the role of laminin-5, major laminin receptors (integrins \( \alpha 6\beta 4 \) and \( \alpha 3\beta 1 \)) also contribute to trastuzumab resistance. In a prior study, the \( \beta 4 \) integrin cytoplasmic tail enhanced murine HER2/ErbB2+ cell resistance to gefitinib (19). However, our results point not only to \( \alpha 6\beta 4 \) but also \( \alpha 3\beta 1 \), acting together with laminin-5 and tetraspanin CD151 to enhance ErbB2+ cell resistance to both extracellular (trastuzumab) and intracellular (lapatinib) agents. Matrigel, containing laminin-1, did not support drug resistance, most likely because its receptor (\( \alpha 6\beta 1 \)) was minimally present on our ErbB2+ cells.

Ablation of CD151 (but not tetraspanin CD9) was nearly as effective as \( \alpha 6 \) and \( \alpha 3 \) ablation, with respect to drug sensitization. Removal of CD151 does not affect cell surface integrin levels and minimally affects cell adhesion to laminin. Although we did not obtain evidence for a direct association between ErbB2, integrins, and/or FAK, we suspect that the presence or absence of CD151 may nonetheless affect drug resistance by influencing the distributions of ErbB2, laminin-binding integrins, and/or FAK relative to one another. In this regard, CD151 does indeed affect the distribution of laminin-binding integrins (26). Furthermore, CD151 recruits...
laminin-binding integrins into tetraspanin-enriched microdomains containing a variety of tetraspanins and other proteins (22). Through one or more of these proteins, CD151-integrin complexes could indirectly affect ErbB2 functional efficiency. Indeed, CD151 ablation did partly diminish ErbB2 phosphorylation in cells on laminin-5. Consistent with CD151 support of ErbB2 functional efficiency (and therefore drug resistance), mice lacking CD151 showed a marked delay in the spontaneous appearance of ErbB2-driven mammary tumors.5 Hence, the integrin-CD151-ErbB2 collaboration supporting trastuzumab resistance in vitro is likely to be also relevant in vivo.

Although others suggested direct association between ErbB2 and laminin-binding integrins (48, 49), we failed to coimmunoprecipitate ErbB2 with α3β1 or α6β4, either in the presence or absence of CD151. Elsewhere, we showed that CD151 supports α6 integrin–dependent functions and promotes EGFR efficiency in human basal-like breast cancer cells (26). We suspect that support of ErbB2 and EGFR functions by laminin-5-integrin-CD151 complexes will be mechanistically similar.

FAK plays a key role in breast cancer initiation and progression (29) but was not known to affect resistance to ErbB2 (or EGFR) targeting agents. Here we show that FAK plays a key role downstream of both CD151-integrin complexes and ErbB2.

CD151 ablation plus ErbB2 inhibition synergized to prevent FAK activation, suggesting that both independently activate the same FAK. CD151 ablation plus ErbB2 inhibition also synergized to prevent activation of extracellular signal-regulated kinase 1/2, a key regulator of cell proliferation, downstream of FAK. Furthermore, FAK and CD151 knockdowns yielded similar trastuzumab sensitization (without additive effects), and drug sensitization was seen on laminin-5, but not on other substrates. Hence, drug resistance promoted by CD151-integrin-laminin complexes seems to be entirely FAK dependent. Although ablation of FAK can sensitize cells to various other anticancer agents (36, 50), we provide the first evidence for sensitization to ErbB2-targeting agents. Although others saw activated FAK physically associate with ErbB2 and α6β4, but not with α3β1 (48), we did not see FAK coimmunoprecipitation with either α6β4 or α3β1.

The AKT kinase cascade typically regulates drug-resistant cancer cell survival (35). For cells on laminin-5, trastuzumab treatment or CD151 ablation each partly diminished AKT activation, consistent with diminished cell survival. Activation

![Figure 5. Ablation of FAK affects trastuzumab sensitivity. A, BT474 cells, transfected with siRNAs, were plated on laminin-5 (1 x 10⁴ per well; A) or noncoated plastic (B) treated with trastuzumab for 2 d, and cell numbers were assessed. C, efficiency of FAK and CD151 knockdown was determined by immunoblotting.](image)

5 Manuscript in preparation.
of PTEN, a phosphatase that suppresses the AKT pathway, can enhance trastuzumab sensitivity, whereas PTEN loss is associated with resistance (35). However, for ErbB2+ cells on laminin-5, PTEN ablation minimally affected trastuzumab resistance. Also, neither trastuzumab nor CD151 ablation activated PTEN, despite increasing trastuzumab sensitivity. Hence, the role of PTEN is minimized for cells on laminin-5.

Inhibition of laminin-5, α3β1, α6β4, CD151, or FAK should enhance breast cancer sensitivity to drugs targeting ErbB2. Indeed, we show that an inhibitor of FAK (TAE226) can enhance trastuzumab sensitivity. Elsewhere, the same FAK inhibitor reduced cell survival in imatinib-resistant gastrointestinal stromal tumor cells (36) and enhanced ovarian cancer inhibition by the chemotherapeutic agent docetaxel (50). Our new insights into CD151 effects on drug sensitivity add to the emergence of CD151 and other tetraspanins as potential targets in cancer, infectious disease, and other pathologies (51). Targeting of CD151 offers potential advantages. First, it should provide drug sensitization while minimally disrupting vital integrin-mediated cell adhesion and signaling processes. Second, it affects functions of multiple laminin-binding integrins at once. Third, it could affect other growth factor receptors, such as EGFR, which may be supported by CD151 (26). In conclusion, we have identified novel regulators of ErbB2 drug sensitivity, which may provide a new approach to sensitizing cells to drugs targeting ErbB2 and possibly other receptor tyrosine kinases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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