An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance

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Abstract

The HER2-targeted therapy trastuzumab is widely used for the treatment of patients with metastatic breast tumors overexpressing HER2. However, an objective response is observed in only 12% to 24% of patients treated with trastuzumab as a single agent and initial responders regress in <6 months (1–3). The reason for the clinical failure of trastuzumab in this setting remains unclear. Here we show that local lymph node–positive disease progression in 89% of breast cancer patients with HER2-positive tumors involves the HER2 oncogenic variant HER2Δ16. We further show that ectopic expression of HER2Δ16, but not wild-type HER2, promotes receptor dimerization, cell invasion, and trastuzumab resistance of NIH3T3 and MCF-7 tumor cell lines. The potentiated metastatic and oncogenic properties of HER2Δ16 were mediated through direct coupling of HER2Δ16 to Src kinase. Cotargeting of HER2Δ16 and Src kinase with the single-agent tyrosine kinase inhibitor dasatinib resulted in Src inactivation, destabilization of HER2Δ16, and suppressed tumorigenicity. Activated Src kinase was also observed in 44% of HER2Δ16-expressing breast carcinomas underscoring the potential clinical implications of coupled HER2Δ16 and Src signaling. Our results suggest that HER2Δ16 expression is an important genetic event driving trastuzumab-refractory breast cancer. We propose that successful targeted therapeutics for intervention of aggressive HER2-positive breast cancers will require a strategy to suppress HER2Δ16 oncogenic signaling. One possibility involves a therapeutic strategy employing single-agent tyrosine kinase inhibitors to disengage the functionally coupled oncogenic HER2Δ16 and Src tyrosine kinase pathways. [Mol Cancer Ther 2009;8(8):2152–62]

Introduction

Recent human tumor gene expression profiling initiatives have resulted in the identification of five distinct breast cancer phenotypic subclasses underscoring the complexity of this disease (4–6). Clinical validation of the breast tumor gene expression classification scheme showed that each tumor subclass exhibited distinct clinical outcomes with the HER2/ErbB2/*neu* (HER2)–positive subtype associated with the shortest relapse-free and overall patient survival (6, 7). HER2, a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, is overexpressed in 20% to 40% of invasive breast cancers and is associated with poor prognosis in these patients (8). Furthermore, HER2-overexpressing tumors exhibit decreased response to adjuvant therapy (9). HER2 overexpression has been the target of several recent clinical interventions. Trastuzumab, a Food and Drug Administration–approved humanized monoclonal antibody directed against the HER2 cell surface receptor, has emerged as an important intervention for patients with HER2-positive tumors. However, the vast majority of patients exhibit *de novo* resistance to single-agent trastuzumab with an objective response observed in only 12% to 24% of patients with HER2-positive tumors and all initial responders develop resistance in <6 months (1–3). Improved patient responses of 40% to 50% are observed when trastuzumab is used in combination with chemotherapy; however, similar to single-agent trastuzumab, many of these patients eventually acquire trastuzumab resistance (10, 11). Early indications from clinical trials of trastuzumab and chemotherapy combinations in the adjuvant setting appear promising with impressive patient response rates, but acquired resistance to trastuzumab remains a serious problem (12, 13).

Molecular events that contribute to trastuzumab resistance have been proposed including inactivation of the PTEN phosphatase resulting in enhanced AKT signaling (14) and suppression of p27^{Kip1}, thus disengaging trastuzumab-induced G_1 cell cycle arrest (15). In addition, a unique insulin-like growth factor receptor-1 and HER2 heterodimer has been detected in trastuzumab-resistant cell lines and disrupting insulin-like growth factor receptor-1/HER2 crosstalk

sensitizes resistant cells to trastuzumab (16). Although promising, clinical verification of these molecular findings will be necessary. For example, one recent study failed to detect an association between expression of PTEN or the PTEN target AKT and clinical outcome of breast cancer patients receiving neoadjuvant trastuzumab (17).

Tumor-specific genetic or molecular events that enhance HER2 oncogenic activity and/or affect trastuzumab binding to HER2 may also contribute to trastuzumab resistance. Although the search for a somatic *HER2* oncogenic mutation in HER2-amplified breast tumors has failed to identify a promising activating genetic lesion (18–20), HER2 isoforms that may influence trastuzumab response have been observed in breast tumors. For example, a membrane-associated isoform of HER2 (p95HER2), lacking both the receptor extracellular domain and trastuzumab epitope, has been detected in up to 60% of HER2-positive tumors (21–23). Ectopic expression of p95HER2 results in an actively signaling protein, which in both preclinical and clinical studies appears to promote trastuzumab resistance (21, 23). Also of interest is a recently identified HER2 splice variant (HER2Δ16) with potent transforming activity, which was originally detected in several HER2-overexpressing breast cancer cell lines (24, 25) and primary tumors (25, 26). When overexpressed in multiple mammalian cell types, HER2Δ16 harbors enhanced transforming activity when compared with wild-type HER2 (24, 25). We reasoned that tumor expression of oncogenic HER2Δ16 would not only promote aggressive breast cancer but also may be inherently resistant to trastuzumab therapy. The HER2Δ16 isoform has not been adequately investigated in this or any clinical setting.

In this report, we show that expression of HER2Δ16 is a tumor-specific molecular event and the vast majority of women with tumor expression of HER2Δ16 develop locally disseminated node-positive breast cancer. Furthermore, tumor cell lines expressing HER2Δ16 are resistant to the HER2-targeted therapy trastuzumab. Our preclinical results suggest that single-agent targeting of tyrosine kinase pathways provides an effective strategy for suppressing HER2Δ16 tumorigenesis and trastuzumab resistance.

Materials and Methods

Human Tissues and RNA

Total RNA was extracted from institutional review board–approved normal and malignant breast samples using Trizol reagent (Invitrogen). A Human Total RNA Master Panel II containing RNA from 18 different human tissue sites was obtained from BD Biosciences.

Reverse Transcription-PCR

Reverse transcription-PCR (RT-PCR) was done using standard procedures. Primers to amplify wild-type HER2 were upstream 5′-GTGTGGACCTGGATGACAAGGG and downstream 5′-GCTCCACCAGCTCCGTTTCCTG, HER2Δ16 were 5′-CACCCACTCCCCTCTGAC spanning the junction between HER2 exons 15 and 17 in HER2Δ16 (Fig. 1A) and the downstream primer, or β-actin were 5′-CAAGGCCAACCGCGAGAAGAT and 5′-GTCCCGGCCA-GCCAGGTCCAG.

Plasmid Constructs

The pcDNA3 HER2Δ16 expression plasmid has been described previously (27). Expression plasmid for chicken Src kinase was provided by Sarah Parsons at University of Virginia.

Cell Lines and Transfections

The NIH3T3 and MCF-7 cell lines were purchased from the American Type Culture Collection and cultured according to their instructions. Stable cell lines expressing pcDNA3, pcDNA3-HER2, or pcDNA3-HER2Δ16 have been described elsewhere (27). Transfection of cells with nonspecific control or Src kinase RNA interference (RNAi; Dharmacon) was done using methods described elsewhere (28).

Cell Invasion/Migration Assay

Cell invasion/migration was determined using the 24-well BD BioCoat Invasion Chamber with an insert containing an 8 μm pore size PET membrane with or without Matrigel Basement Membrane Matrix (BD Biosciences). Cells were seeded at 2.5×10^4 per well in medium with 0.2% fetal bovine serum and medium containing 10% fetal bovine serum as a chemotactic. Cells were incubated at 37°C for 22 h and stained with crystal violet.

Cell Proliferation Assay

Cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and/or colony formation assays exactly as described elsewhere (29).

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation and Western blot analysis were done as described previously (30). All antibodies were obtained from Cell Signaling, except HER2 P-Y1248 (Upstate), P-Tyr PY99 (Santa Cruz Biotechnology), P-Tyr 4G10 (Upstate), HER2 (Neomarkers), α-tubulin (Upstate), β-actin (Abcam), goat anti-mouse IRDye 800CW, and goat anti-rabbit IRDye 800CW (Li-Cor).

Nondenaturing PAGE

Total cell lysates were prepared and analyzed by gradient nondenaturing PAGE as described elsewhere (31).

Immunofluorescence and Immunohistochemistry

Deconvolution microscopy and immunofluorescent detection of HER2/HER2Δ16 and Src kinase were done exactly as described elsewhere (32) using rabbit anti-HER2 (Lab Vision) with Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes) and mouse anti-Src kinase (Invitrogen) with Alexa Fluor 568–conjugated goat anti-mouse IgG (Molecular Probes). Immunohistochemistry of primary human breast tumors for phospho-Src kinase was done essentially as described elsewhere (30) with the addition of an antigen retrieval step by incubating the slides in 100 mmol/L citric buffer (pH 6.2) in a steam chamber for 30 min. Primary antibody was P-Src Y416 (Cell Signaling) used at a 1:100 dilution.

Results

HER2Δ16 Is a Tumor-Specific HER2 Oncogenic Isoform

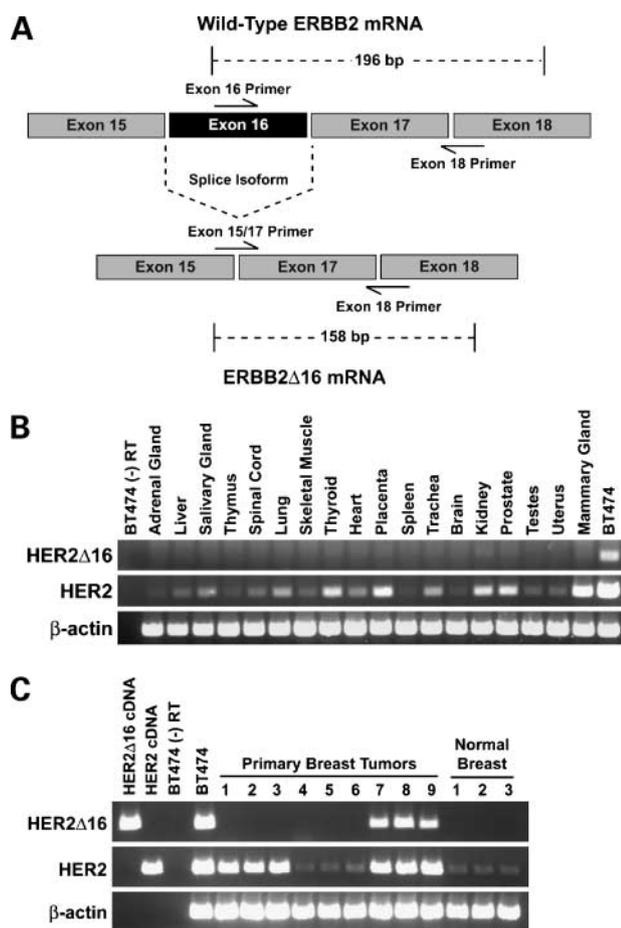Expression of an oncogenic HER2 splice isoform with an in-frame deletion of exon 16 (HER2Δ16) has been noted in

Figure 1. Expression of HER2 and HER2Δ16 in human tissues and breast tumors. **A,** schematic of mRNA from wild-type HER2 and the exon 16 deletion isoform HER2Δ16 with relative positions of RT-PCR primers and predicted size of each PCR-amplified product indicated. **B,** RT-PCR detection of HER2 and HER2Δ16 expression in normal human tissue RNA. **C,** RT-PCR detection of HER2 and HER2Δ16 expression in RNA isolated from normal and malignant breast tissue. Representative examples of tumors with HER2 overexpression (1–3), tumors lacking HER2 overexpression (4–6), and tumors with HER2 and HER2Δ16 overexpression (7–9).

several breast cancer cell lines (24) and most recently in a limited survey of primary breast carcinomas (26). Loss of HER2 exon 16 results in a constitutively active HER2 protein with an in-frame deletion of residues 691 to 707 within one of the ectodomain cysteine-rich regions (24–26). To facilitate detection of HER2Δ16 expression in human tissue samples, we designed a highly sensitive HER2Δ16-specific RT-PCR assay using an oligonucleotide primer that traverses the unique exons 15 and 17 junction of HER2Δ16 (Fig. 1A). For specific RT-PCR of wild-type HER2, we replaced the exon 15/17 primer with an oligonucleotide primer within exon 16 (Fig. 1A). As predicted, the exon 15/17 primer only amplified the 158-bp PCR product from the HER2Δ16 cDNA, whereas the exon 16 primer amplified the predicted 196-bp product from a HER2 cDNA, thereby showing the specificity of our assay (Fig. 1B and C).

We next analyzed HER2 and HER2Δ16 expression in a panel of normal human tissues and primary human breast tumors. Interestingly, we failed to detect HER2Δ16 transcripts in RNA samples from a panel of 18 different normal human tissues (Fig. 1B) including several normal breast samples (Fig. 1B and C). Consistent with other reports, HER2 was expressed at higher levels in normal thyroid, placenta, trachea, kidney, prostate, and mammary gland (Fig. 1B and C; refs. 33, 34). We next analyzed HER2 and HER2Δ16 expression in a patient cohort of 85 primary invasive breast tumors (Supplementary Table S1). The 33 HER2-negative tumors analyzed in this study also lacked HER2Δ16 expression (Fig. 1C). We therefore selected the remainder of our cohort to include only patients with HER2-positive breast tumors. We observed a direct concordance between HER2-positive invasive tumors determined by fluorescent *in situ* hybridization or immunohistochemistry and our RT-PCR expression analysis and all HER2 fluorescent *in situ* hybridization–negative or immunohistochemistry-negative tumors were also negative by RT-PCR (data not shown). We also detected HER2Δ16 expression in 52% (27 of 52) of HER2-positive breast tumors (Fig. 1C; Supplementary Table S2). Amplification by RT-PCR of the HER2Δ16 isoform was confirmed by direct sequencing of the PCR products (data not shown). In summary, HER2Δ16 appears to be a tumor-specific HER2 oncogenic isoform coexpressed with HER2 in nearly half of the HER2-positive invasive breast tumors analyzed in this study.

HER2Δ16 Expression Is Significantly Associated with Lymph Node–Positive Breast Cancer

Expression of HER2 with and without HER2Δ16 expression was compared with common clinicopathologic parameters to identify possible associations. Consistent with numerous published studies, the HER2-negative patients in our cohort tended to have lower-grade tumors and lacked significant associations with lymph node involvement and estrogen receptor status (9). Also consistent with published clinical studies, HER2-positive patients tended to have higher-grade tumors, but once again significant associations between lymph node involvement and estrogen receptor status were not detected (Supplementary Table S2).

To determine the effect of HER2Δ16 expression on clinicopathologic parameters, we divided the HER2-positive patients into two groups based on the presence or absence of HER2Δ16 coexpression. Although patients with HER2-positive and HER2Δ16-negative tumors tended to have higher-grade tumors, we observed a surprising inverse correlation with lymph node involvement with only 12% (3 of 25) of these patients presenting with affected nodes (Supplementary Table S2). In striking contrast, 89% (24 of 27) of patients with HER2-positive tumors coexpressing HER2Δ16 presented with positive lymph nodes. These patients also tended to have higher-grade estrogen receptor–negative tumors (Supplementary Table S2). Positive lymph nodes at the time of diagnosis remains the most reliable clinical predictor of negative prognosis in the breast cancer clinic. The highly significant association of HER2Δ16 expression with positive lymph nodes ($P < 0.0001$) observed in this limited analysis

strongly implicates HER2Δ16 as a clinically important and tumor-specific HER2 molecular alteration promoting aggressive locally disseminated metastatic breast cancer.

HER2Δ16 Forms Stable Dimers and Couples to Multiple Oncogenic Signaling Pathways

We next compared the signaling activities of wild-type HER2 and HER2Δ16 ectopically expressed in the MCF-7 breast cancer cell line. Despite equivalent levels of receptor expression, basal levels of HER2 site-specific and total phosphorylation were greater than HER2Δ16 (Fig. 2A). However, nearly all of MCF-7 expressed HER2Δ16 was stably dimerized, whereas HER2 was predominantly expressed as a monomer (Fig. 2B), suggesting that HER2Δ16 forms a stable constitutively active homodimer. We next determined if the HER2Δ16 homodimer coupled with other members of the EGFR family. We examined expression and basal phosphorylation of each EGFR family member in the MCF-7 cell lines. Ectopic expression of HER2 or HER2Δ16 resulted in a slight but equivalent increase in EGFR expression and phosphorylation when compared with vector control cells (Fig. 2C). Dramatic activation of HER3 was observed in the HER2 but not the HER2Δ16-expressing MCF-7 cells (Fig. 2C). In addition, enhanced expression of HER4 was observed in the HER2 cells, whereas HER2Δ16-expressing MCF-7 cells lacked HER4 expression altogether (Fig. 2C; ref. 27). These results indicate that wild-type HER2 and HER2Δ16 differentially couple to other EGFR family members, nevertheless it seems unlikely that additional EGFR family members will significantly contribute to HER2Δ16 oncogenic activity.

We next determined if HER2Δ16 expression resulted in activation of oncogenic signal transduction pathways known to function downstream of constitutively activated HER2, that is, FAK, Src kinase, phosphatidylinositol 3-kinase/AKT, and mitogen-activated protein kinase. Despite high levels of autophosphorylation and an association with activated HER3, ectopic HER2 expression in MCF-7 cells only marginally activated each signal transduction pathway examined (Fig. 2D). In contrast, ectopic HER2Δ16 expression resulted in increased activation of multiple oncogenic pathways examined (Fig. 2D).

HER2Δ16 Expression Enhances Cell Tumorigenicity

We next compared the biological activities of ectopically expressed HER2 and HER2Δ16 in NIH3T3 and MCF-7 cells. Expression of HER2Δ16 significantly enhanced the proliferation of NIH3T3 and MCF-7 cells in growth kinetic and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, respectively, whereas HER2 expression failed to affect cell proliferation in these assays (Fig. 3A and B). Both HER2 and HER2Δ16 enhanced colony formation of MCF-7 cells (Fig. 3C). Although HER2Δ16 expression resulted in an insignificant increase in colony number when compared with HER2-expressing cells, the colonies formed by HER2Δ16-expressing MCF-7 cells were significantly larger (Fig. 3C).

We next examined the ability of HER2 and HER2Δ16 to promote MCF-7 cell migration and invasion. MCF-7 cells lack the ability to migrate and invade, and as predicted, MCF-7 cells exhibited minimal activity in the *in vitro* cell migration/invasion assay (Fig. 3D). Although HER2 enhanced the colony formation activity of MCF-7 cells, overexpression of HER2 failed to enhance MCF-7 cell migration or invasion, whereas HER2Δ16 dramatically potentiated MCF-7 cell migration and invasion (Fig. 3D). These results suggest that HER2Δ16-expressing cells have enhanced metastatic potential and corroborate our clinical observations where HER2Δ16 expression was significantly associated with locally disseminated breast cancer (Supplementary Table S2).

HER2Δ16-Expressing Cells Are Trastuzumab Resistant

We next determined if HER2Δ16 expression influences tumor cell response to the HER2-targeted therapy, trastuzumab. Although the trastuzumab binding site is amino-terminal to the exon 16 deletion of HER2Δ16 (Fig. 4A; ref. 35), one report suggests that the HER2Δ16 deletion affects trastuzumab binding (26). Using a cell surface immunofluorescence approach, we observe equivalent levels of trastuzumab binding to both HER2 and HER2Δ16 at the cell surface of expressing MCF-7 cells. In addition, trastuzumab binding was similar to the binding of a control HER2 polyclonal antibody directed against a HER2 extracellular domain peptide that does not overlap with the trastuzumab binding region (Fig. 4B). These results indicate that trastuzumab binds similarly to HER2Δ16 and wild-type HER2 at the cell surface of breast tumor cells.

Trastuzumab treatment of MCF-7/HER2 cells down-regulated wild-type HER2 protein levels and suppressed HER2-mediated signaling in MCF-7 cells to basal levels. Significantly, the PTEN tumor suppressor was dephosphorylated and therefore activated following trastuzumab treatment of HER2-expressing MCF-7 cells (Fig. 4C, *asterisks*). The antitumor activity of dephosphorylated PTEN is emerging as an important mechanism of trastuzumab action (14). Trastuzumab treatment also significantly reduced cell proliferation and colony formation of each HER2-expressing MCF-7 and NIH3T3 cell line (Fig. 4D; Supplementary Fig. S1A). Despite similar levels of trastuzumab-induced HER2Δ16 down-regulation, oncogenic signaling was sustained in trastuzumab-treated HER2Δ16-expressing cells. Most notably, PTEN remained in the inactive phosphorylated state (Fig. 4C, *asterisks*). Consistent with this sustained oncogenic signaling, HER2Δ16-expressing MCF-7 and NIH3T3 cell lines were refractory to trastuzumab treatment in both cell proliferation and invasion assays (Fig. 4D; Supplementary Fig. S1A). Surprisingly, under several experimental conditions, trastuzumab appears to behave as a HER2Δ16 agonist and promotes HER2Δ16 cell growth and invasion (Fig. 4D; Supplementary Fig. S1A).

HER2Δ16 Cooperates with Src Kinase to Promote Cell Invasion

Despite the high levels of autophosphorylation, HER2 only marginally activated the multiple signal transduction pathways examined and each of these pathways was suppressed by trastuzumab treatment. HER2Δ16-expressing cells, on the other hand, were trastuzumab-resistant and retained activation of multiple oncogenic and cell invasion


Figure 2. HER2Δ16 forms stable dimers and activates multiple signaling pathways in breast tumor cells. **A,** Western blot analysis of total and site-specific HER2 and HER2Δ16 phosphorylated activation in stable MCF-7 cell lines. **B,** assay of constitutive HER2 or HER2Δ16 receptor dimerization from indicated stable MCF-7 cell line lysates analyzed by native nonreducing PAGE and Western blot. **C,** expression and phosphorylated activation of HER/ErbB family members in response to ectopic HER2 and HER2Δ16 expression in stable MCF-7 cell lines. Each HER receptor was immunoprecipitated (*IP*) from the indicated MCF-7 cell line and analyzed for phosphorylated activation by anti-P-Tyr Western immunoblot (*IB*) analysis. **D,** protein lysates from the indicated MCF-7 stable cell lines were analyzed by Western blot for activation of multiple oncogenic signaling pathways.

pathways following trastuzumab treatment (Fig. 4). With the ultimate goal of disengaging downstream HER2Δ16 oncogenic signaling, we used pharmacologic inhibitors to characterize the hierarchy of HER2Δ16 signaling. Using this strategy, we identified Src kinase as a critical upstream effector of AKT, mitogen-activated protein kinase, and FAK in HER2Δ16 cells (Supplementary Fig. S1B). We therefore examined potential cooperation between HER2Δ16 and Src kinase signaling in the HER2Δ16-expressing MCF-7 cell line.

Translocation of cytosolic Src kinase to the cell membrane where Src activates multiple signaling pathways including FAK and mitogen-activated protein kinase is considered an important mechanism of Src action (36, 37). We therefore examined localization of Src kinase by deconvolution microscopy in the HER2- and HER2Δ16-expressing MCF-7 cells. Prominent membrane staining of both HER2 and HER2Δ16 was observed in the cell lines (Fig. 5A, α-*HER2*). However, Src was predominantly localized to perinuclear regions in HER2-expressing cells where inactive Src resides (Fig. 5A, α-*Src*; ref. 37). In contrast, Src was colocalized to its active site at the cell membrane with HER2Δ16 (Fig. 5A). Using a coimmunoprecipitation assay, we also show a physical interaction between Src kinase and HER2Δ16; however, an interaction between Src and wild-type HER2 was not observed (Fig. 5B). Taken together, these results suggest that HER2Δ16 facilitates recruitment and activation of Src kinase through direct interaction at the cell membrane. Src kinase

is known to facilitate cell motility and invasion, in part, through activation of FAK (36). To determine if Src mediates HER2Δ16 cell invasion, we suppressed Src activity in HER2Δ16-expressing MCF-7 cells using pharmacologic inhibitors or Src targeting RNAi. Interestingly, RNAi-mediated suppression of Src kinase also resulted in a loss of HER2Δ16 protein levels, whereas wild-type HER2 levels were unaffected by Src suppression (Fig. 5C). Furthermore, RNAi or pharmacologic suppression of Src resulted in a dramatic reduction in HER2Δ16-induced cell invasion (Fig. 5C; Supplementary Fig. S1C).

We next examined a small cohort of HER2Δ16-expressing primary breast tumors for coactivation of Src kinase by immunohistochemistry using an activation-specific Src kinase antibody (Src P-Y416). Importantly, activated Src appeared to be a common feature of HER2Δ16-expressing breast tumors, with 44% of HER2Δ16-expressing tumors also expressing activated Src kinase (Fig. 5D). These results suggest that HER2Δ16 cooperates with Src kinase in preclinical models as well as in invasive breast tumors to drive the metastatic potential of breast tumor cells.

HER2Δ16-Expressing Cell Lines Are Sensitive to a Single-Agent Tyrosine Kinase Inhibitor

Src kinase appears to be uniquely coupled to HER2Δ16 oncogenic signaling and has been associated with trastuzumab resistance, in part, through its ability to phosphorylate and inactivate PTEN (14, 38). Furthermore, suppression of Src kinase activity in HER2Δ16-expressing cells results in a

loss of HER2Δ16 protein and disengages HER2Δ16-mediated cell invasion. We therefore reasoned that a tyrosine kinase inhibitor may directly or indirectly affect both Src kinase and HER2Δ16, thereby suppressing HER2Δ16 oncogenic activity and reversing therapeutic resistance. The tyrosine kinase inhibitor dasatinib has been recently approved for the treatment of patients with imatinib-refractory leukemias (39, 40). Dasatinib is currently in clinical trials for the treatment of solid tumors and has recently been shown to inhibit the growth and proliferation of multiple breast tumor cell lines (41, 42). Although originally designed as a Src kinase family inhibitor, dasatinib displays broader than anticipated tyrosine kinase inhibitor activity including direct inhibition of receptor tyrosine kinases (42–45).

Considering the potent tyrosine kinase inhibitor activity of dasatinib, we next determined the effect of dasatinib treatment on HER2Δ16-expressing cell lines. Dasatinib effectively suppressed the activation of multiple signaling molecules, including Src kinase, at a concentration of 100 nmol/L in each MCF-7 cell line tested (Fig. 6A, *asterisks*). Interestingly, the loss of activated Src was accompanied by a specific and dramatic loss of HER2Δ16 protein, whereas HER2 activation and protein levels were only marginally affected by dasatinib treatment (Fig. 6A, *asterisks*). We observed a similar loss of HER2Δ16 protein when Src expression was suppressed by RNAi (Fig. 5C). Taken together, these results suggest that Src loss-of-function results in specific destabilization of HER2Δ16. We are currently investigating the molecular basis of HER2Δ16 destabilization in response to dasatinib.

Consistent with dasatinib-induced suppression of multiple signaling pathways, each cell line tested was sensitive to dasatinib treatment with an IC_{50} of 10 nmol/L observed for MCF-7 cells and HER2- and HER2Δ16-expressing cells were equally sensitive to dasatinib with an IC_{50} of ~50 nmol/L (Fig. 6B). Furthermore, as a single-agent, dasatinib effectively suppressed cell growth and proliferation in each cell line tested including trastuzumab-resistant

Figure 3. HER2Δ16 promotes proliferation and invasion of tumor cells. **A,** cell growth assay of NIH3T3 cells expressing HER2 or HER2Δ16. Each cell line was plated in duplicate at 0.5×10^5 cells per plate and cell numbers were counted. *Inset,* Western blot analysis of each stable cell line. **B,** indicated MCF-7 stable cell lines were plated in triplicate in a 96-well plate at 2×10^3 cells per well and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay was done after 7 d using standard procedures. **C,** colony formation assay with each cell line seeded in duplicate into a 6-well plate at 1×10^3 cells per well and incubated for 7 to 10 d. Colony number and size were calculated using a ColCount colony counter (Oxford Optronix). Data are normalized to the MCF-7/pcDNA vector control. Representative colony formation results. **D,** invasion and migration of each stable MCF-7 cell line was determined using a BD BioCoat Invasion Chamber with (invasion) or without (migration) Matrigel insert. Invading/migrating cells were counted after 22 h. **B** to **D,** mean ± SE. *Asterisk,* HER2Δ16 cells significantly different from both vector control and HER2-expressing cells.
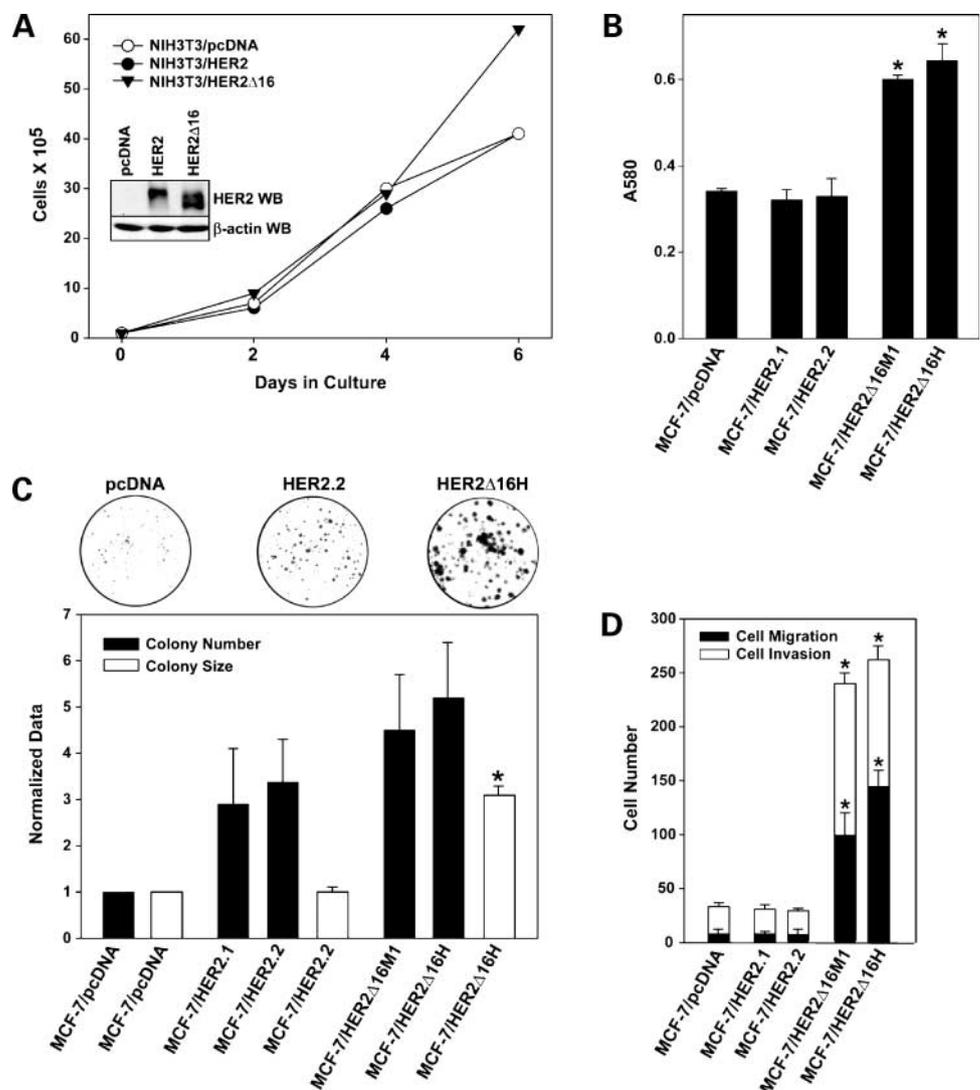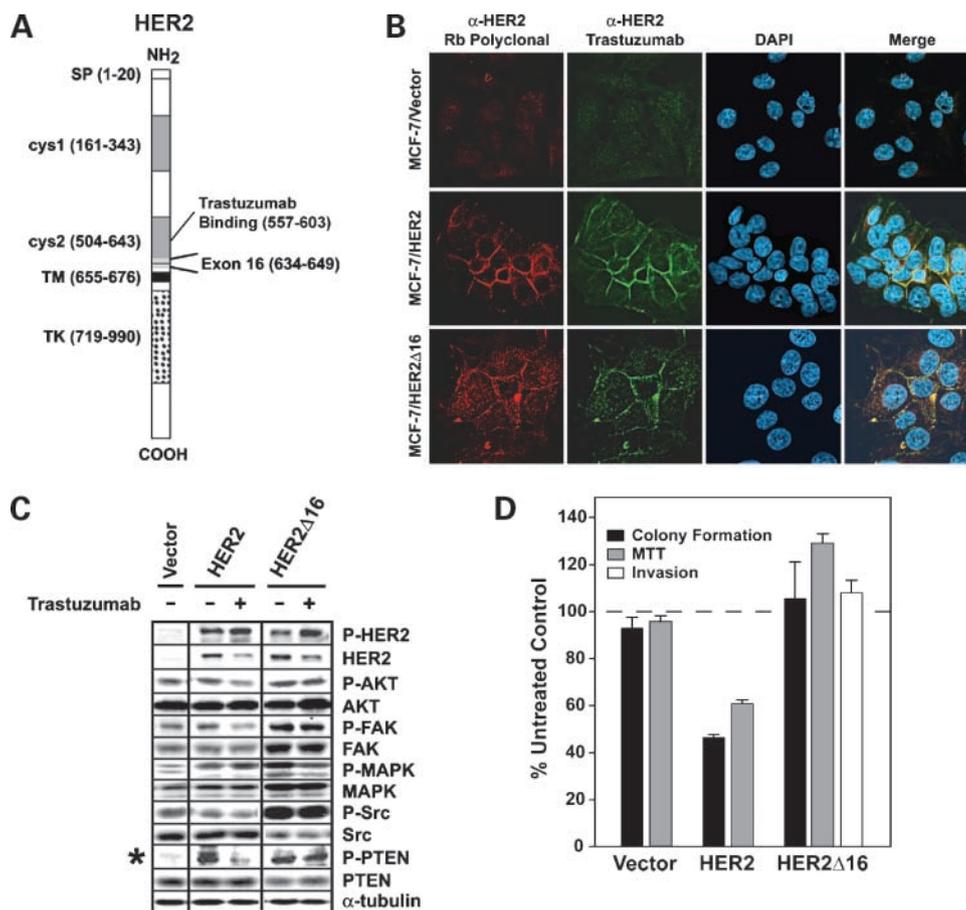
Figure 4. HER2Δ16 promotes trastuzumab resistance. **A,** schematic of HER2 protein with positions of putative signal peptide (*SP*), two cysteine-rich regions (*cys1* and *cys2*), transmembrane region (*TM*), and intracellular tyrosine kinase domain (*TK*). The exon 16 deletion in HER2Δ16 (*light gray*) is positioned in a region carboxyl to the binding site of trastuzumab. **B,** trastuzumab binds to the cell surface of HER2Δ16-expressing MCF-7 cells. Immunofluorescence assay of the indicated stable MCF-7 cell lines showing overlap between cell surface binding of a HER2 polyclonal antibody (*red*) and the humanized HER2 antibody trastuzumab (*green*) in both HER2- and HER2Δ16-expressing cells. Binding of HER2 antibody or trastuzumab was not observed in the MCF-7/vector control cells. **C,** indicated stable MCF-7 cell lines were treated with 10 µg/mL trastuzumab for 48 h and protein lysates were analyzed by Western blot. *Asterisk,* trastuzumab dephosphorylated activation of PTEN in HER2-expressing cells. **D,** HER2Δ16-expressing MCF-7 cells are trastuzumab resistant. Each stable MCF-7 cell line was treated with 10 µg/mL trastuzumab in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay or colony formation assay. Only the invasive HER2Δ16 cell line was treated with 10 µg/mL trastuzumab in an invasion assay using a BD BioCoat Invasion Chamber. Each entire experiment was repeated at least three times. Mean ± SE of percent inhibition of treated versus untreated cells.

HER2Δ16-expressing cells (Fig. 6C; Supplementary Fig. S1A). Significantly, dasatinib also suppressed HER2Δ16-mediated cell invasion (Fig. 6C; Supplementary Fig. S1A). The addition of trastuzumab failed to enhance the inhibitory effects of single-agent dasatinib treatments, suggesting that dasatinib alone is sufficient to fully suppress HER2 and HER2Δ16 signaling. Taken together, our results indicate that the proliferative and aggressive metastatic properties of trastuzumab-resistant HER2Δ16-expressing breast tumor cells can be suppressed by single-agent treatment using the tyrosine kinase inhibitor dasatinib.

Discussion

Patients with HER2-overexpressing breast tumors have a greater tendency to develop incurable and often lethal metastatic disease. For reasons that are poorly understood, the HER2-targeted therapy trastuzumab fails as a single agent to provide durable response for patients with metastatic breast cancer involving HER2. In fact, up to 88% of these patients exhibited *de novo* resistance to trastuzumab monotherapy (1–3). In this communication, we show that ~90% of women with HER2-positive tumors and locally disseminated disease, and thus candidates for trastuzumab therapy, presented with tumor expression of HER2Δ16, an oncogenic isoform of HER2. We further show that HER2Δ16 expression is a tumor-specific event that promotes trastuzumab resistance in multiple tumor cell lines. Current clinical procedures for evaluating HER2 in breast cancer patients rely on immunohistochemistry and/or fluorescent *in situ* hybridization. These assays lack the necessary specificity to distinguish between wild-type HER2 and expression of

the trastuzumab-resistant HER2Δ16 oncogenic isoform. The high prevalence of covert HER2Δ16 expression in patients with aggressive HER2-positive breast cancer may be one reason why the overwhelming majority of these patients fail single-agent trastuzumab therapy.

Several mechanisms of trastuzumab resistance have been proposed including inactivation of PTEN, down-regulation of p27[Kip1], HER2 crosstalk with insulin-like growth factor receptor-1, and tumor expression of p95HER2 (14, 15, 21–23, 46). Of these potential markers of trastuzumab response, only PTEN expression has been adequately tested clinically and PTEN expression failed to predict patient response to trastuzumab. Clinical verification of our findings would potentially have a significant effect on the management of patients with HER2-positive breast cancer. Our results suggest that HER2-positive patients can be further stratified into a trastuzumab-resistant population based on HER2Δ16 coexpression. We predict that patients expressing HER2 alone may benefit from single-agent trastuzumab, whereas patients coexpressing HER2 with oncogenic HER2Δ16 would require a more aggressive therapeutic regimen. Obtaining clinical support for our hypothesis has been proven difficult. Early single-agent trastuzumab trials were discontinued in favor of trastuzumab and chemotherapy combinations. The insufficient number of available patient tumor RNA samples from single-agent trastuzumab trials has prevented us from evaluating the effect of HER2Δ16 expression in these trials.

With the goal of identifying an alternative targeting strategy in HER2Δ16-expressing tumor cells, we found that Src kinase is a critical effector of HER2Δ16 tumorigenic properties. In fact, Src kinase appears to function as a "master regulator" in our system stabilizing HER2Δ16 protein expression and coupling HER2Δ16 to multiple mitogenic and cell motility pathways. For example, suppression of Src activity resulted in disengaged mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT oncogenic pathways and loss of FAK activation, an essential
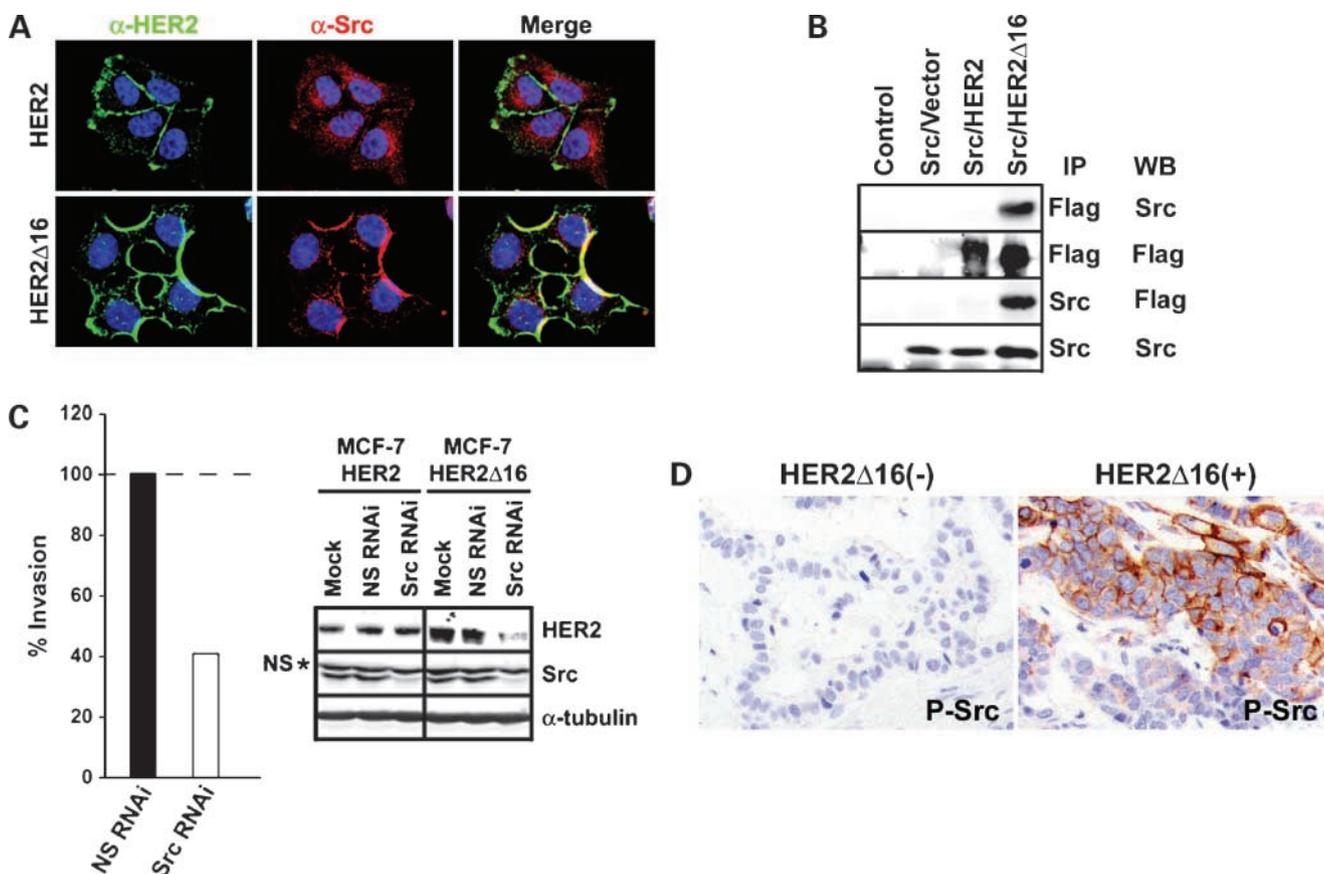

Figure 5. HER2Δ16 directly couples to Src kinase in breast tumor cells. **A,** immunofluorescent detection of HER2 or HER2Δ16 (*green*) and Src kinase (*red*) in HER2Δ16- and HER2-expressing MCF-7 cells and analyzed by deconvolution microscopy. **B,** Src kinase coimmunoprecipitates with HER2Δ16 but not HER2. HEK293T cells were cotransfected with chicken Src kinase and HER2-Flag or HER2Δ16-Flag. Src and Flag immunoprecipitates were probed by Western blot for Src kinase or Flag-tagged HER2 and HER2Δ16. **C,** suppression of Src kinase destabilizes HER2Δ16 and inhibits cell invasion. Invasion assay of MCF-7/HER2Δ16 cells using a BD BioCoat Invasion Chamber following treatment with nonspecific (*NS*) or Src targeting RNAi. Control Western blot showing suppression of Src expression and loss of HER2Δ16 protein following treatment with Src RNAi. *Asterisks,* nonspecific band observed in Src kinase Western blot. **D,** activated Src kinase is coexpressed with HER2Δ16 in invasive breast tumors. HER2Δ16(+) and HER2Δ16(−) primary invasive breast tumors (Supplementary Tables S1 and S2) were stained by immunohistochemistry for activated Src kinase P-Src Y416. Membrane associated and activated Src kinase was observed in 44% (4 of 9) of HER2Δ16(+) tumors and 25% (1 of 4) of HER2Δ16(−) tumors.
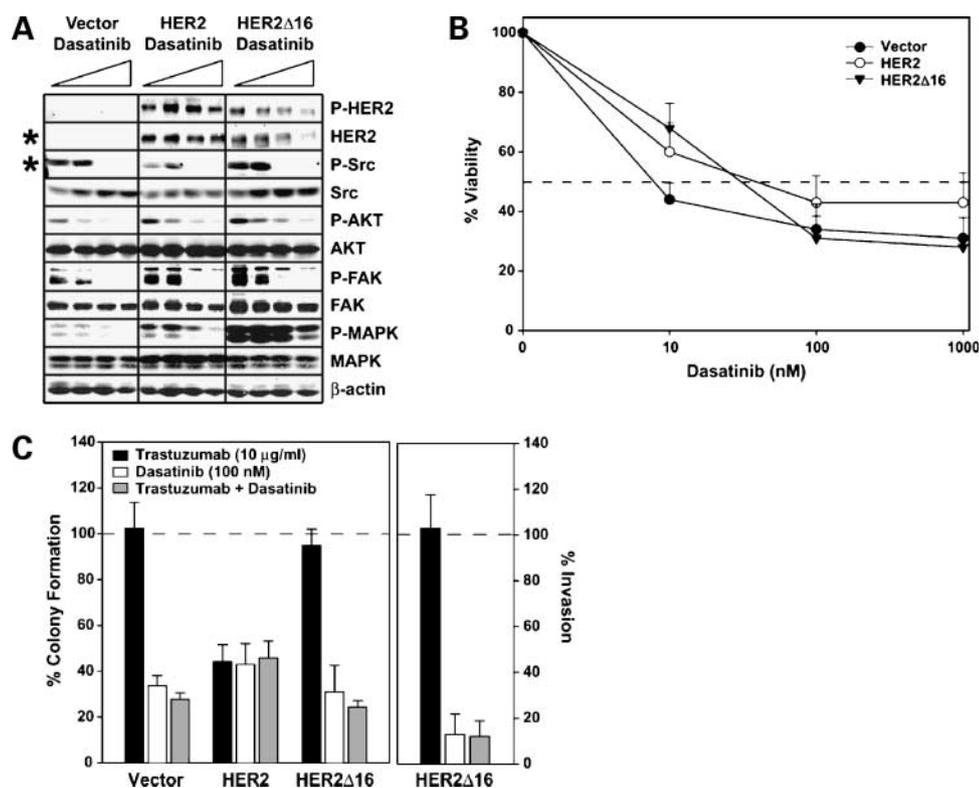
Figure 6. Trastuzumab-resistant HER2Δ16 cells are sensitive to the tyrosine kinase inhibitor dasatinib. **A,** each stable MCF-7 cell line was treated with increasing amounts of dasatinib (0, 10, 100, or 1,000 nmol/L), and after 24 h, protein lysates were analyzed by Western blot. *Asterisks,* dasatinib-induced suppression of Src kinase and loss of HER2Δ16. **B,** dasatinib dose-response. Each stable MCF-7 cell line was treated with the indicated concentration of dasatinib and viability was determined using a colony formation assay. **C,** trastuzumab-resistant and trastuzumab-sensitive tumor cell lines are sensitive to dasatinib. Each indicated stable MCF-7 cell line was treated with 10 μg/mL trastuzumab and/or 100 nmol/L of the tyrosine kinase inhibitor dasatinib in a colony formation assay and the invasive MCF-7/HER2Δ16 was similarly treated in an invasion assay using a BD BioCoat Invasion Chamber. **B** and **C,** each experiment was repeated at least three times. Mean and ± SE of percent viability of treated versus untreated cell lines.

mediator of Src promoted cell motility and invasion (36). In addition, Src kinase may contribute to trastuzumab resistance of HER2Δ16-expressing cells through phosphorylated inactivation of the PTEN tumor suppressor (38). We observed both sustained Src activation and PTEN phosphorylation following trastuzumab treatment of resistant HER2Δ16-expressing breast tumor cells. Consistent with other reports, PTEN was dephosphorylated to an active form following trastuzumab treatment of responsive HER2-expressing breast tumor cells (14). As an important clinical correlate, we observed expression of activated Src kinase in a large percentage of HER2Δ16-expressing invasive breast tumors, raising the possibility that coupled HER2Δ16 and Src kinase signaling contributes to trastuzumab-refractory disseminated breast cancer observed clinically.

Preclinical and clinical evidence of cooperation between the oncogenic tyrosine kinases HER2Δ16 and Src to promote breast tumorigenesis implies that treatment with a tyrosine kinase inhibitor may suppress the oncogenic HER2Δ16/Src signaling axis. We investigated the efficacy of the tyrosine kinase inhibitor dasatinib, currently in clinical use for the treatment of patients with refractory leukemias, as a single-agent in our preclinical assays. Although originally designed as a Src kinase family inhibitor, dasatinib appears to have broad tyrosine kinase specificity (43, 45) and effectively inhibits breast tumor cell lines with the most aggressive phenotypes (41, 42). Likewise, dasatinib suppressed each tumor cell line tested here including HER2Δ16-expressing trastuzumab-refractory tumor cell

lines. Dasatinib at a dose of 100 nmol/L was sufficient as a single agent to fully suppress HER2Δ16-expressing cells and the addition of trastuzumab had no effect or only marginally improved responses. Interestingly, dasatinib sensitively did not appear to correlate with the levels of Src activation in each cell line. Consistent with these findings, Src kinase failed to emerge as a marker predicting dasatinib sensitivity in breast cancer cell lines (41, 42), underscoring the potential broad target specificity of this tyrosine kinase inhibitor (43, 45). Although promising candidates have been identified, the direct mediators of dasatinib action in breast tumor cells remain to be confirmed.

Significantly, we also found that knockdown of Src kinase by RNAi or potent dasatinib inhibition of Src kinase activity resulted in an unexpected and specific loss of HER2Δ16 protein, whereas wild-type HER2 levels were largely unaffected. The exact mechanism triggering HER2Δ16 degradation is under investigation but most likely involves enhanced HER2Δ16 ubiquitination and proteosomal degradation in the absence of Src activation.[8] Nevertheless, the dramatic preclinical efficacy of dasatinib in HER2Δ16-expressing cells appears to be a result of direct inactivation of Src kinase and subsequent HER2Δ16 destabilization. Interestingly, Src kinase has been shown to phosphorylate Cbl ubiquitin ligase, thereby suppressing Cbl-mediated ubiquitination and proteosomal degradation of EGFR family members (47–50). By inactivating Src kinase, dasatinib

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[8] Mitra D, Parvani JG, Jones FE, unpublished observations.

may reverse Cbl suppression and promote HER2Δ16 degradation. The essential contribution of Src kinase to HER2Δ16 stability and tumorigenesis and the high prevalence of activated Src observed in HER2Δ16-expressing breast carcinomas supports a clinical role for single-agent dasatinib intervention in patients with trastuzumab-resistant HER2Δ16-expressing tumors.

In conclusion, our clinical findings indicate that HER2Δ16 represents the first highly penetrate HER2 oncogenic alteration identified in human cancer. We propose that the success of therapeutic intervention in HER2-positive breast cancer will require a paradigm shift from the molecular targeting of wild-type HER2 to a strategy that suppresses HER2Δ16 oncogenic signaling. One exciting possibility is a therapeutic regimen employing tyrosine kinase inhibitors as single agents to suppress trastuzumab-refractory disease. Because HER2Δ16 expression is a tumor-specific oncogenic event, we predict that, in contrast to wild-type HER2, HER2Δ16-targeted therapeutics would have few, if any, treatment-induced side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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