Clinical Cancer Research

Human Breast Cancer Cells Harboring a Gatekeeper T798M Mutation in HER2 Overexpress EGFR Ligands and Are Sensitive to Dual Inhibition of EGFR and HER2

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Abstract

Purpose: Mutations in receptor tyrosine kinase (RTK) genes can confer resistance to receptor-targeted therapies. A T798M mutation in the HER2 oncogene has been shown to confer resistance to the tyrosine kinase inhibitor (TKI) lapatinib. We studied the mechanisms of HER2-T798M–induced resistance to identify potential strategies to overcome that resistance.

Experimental Design: HER2-T798M was stably expressed in BT474 and MCF10A cells. Mutant cells and xenografts were evaluated for effects of the mutation on proliferation, signaling, and tumor growth after treatment with combinations of inhibitors targeting the EGFR/HER2/HER3/PI3K axis.

Results: A low 3% allelic frequency of the T798M mutant shifted 10-fold the IC_{50} of lapatinib. In mutant-expressing cells, lapatinib did not block basal phosphorylation of HER2, HER3, AKT, and ERK1/2. *In vitro* kinase assays showed increased autocatalytic activity of HER2-T798M. HER3 association with PI3K p85 was increased in mutant-expressing cells. BT474-T798M cells were also resistant to the HER2 antibody trastuzumab. These cells were sensitive to the pan-PI3K inhibitors BKM120 and XL147 and the irreversible HER2/EGFR TKI afatinib but not the MEK1/2 inhibitor CI-1040, suggesting continued dependence of the mutant cells on ErbB receptors and downstream PI3K signaling. BT474-T798M cells showed increased expression of the EGFR ligands EGF, TGFα, amphiregulin, and HB-EGF. Addition of the EGFR neutralizing antibody cetuximab or lapatinib restored trastuzumab sensitivity of BT474-T798M cells and xenografts, suggesting that increased EGFR ligand production was causally associated with drug resistance.

Conclusions: Simultaneous blockade of HER2 and EGFR should be an effective treatment strategy against HER2 gene–amplified breast cancer cells harboring T798M mutant alleles. *Clin Cancer Res; 19(19); 5390–401. ©2013 AACR.*

Introduction

The ErbB family of transmembrane receptor tyrosine kinase (RTK) comprises 4 members: EGFR (ErbB1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4; ref. 1). Several human cancers have been associated with dysregulation of ErbB receptors. Approximately 25% of invasive breast cancers exhibit *HER2* gene amplification and mRNA/protein over-expression (2). Anti-HER2 therapies such as the antibody trastuzumab are active in patients with HER2-overexpressing breast cancer (3, 4). HER2 does not have an activating ligand but can be transactivated by ligand-induced ErbB coreceptors. For example, HER2 and EGFR cooperate in the transformation of mouse fibroblasts (5). Ligand-induced EGFR forms heterodimers with HER2 (6); in turn, HER2 reduces degradation of EGFR (7) by promoting ligand binding to EGFR and inhibiting binding of EGFR to its ubiquitin ligase Cbl (8). Consistent with this mutual dependence and synergy, inhibition of EGFR can reduce the growth of HER2+ breast cancer cells both *in vitro* and *in vivo* (9–11).

The small-molecule, ATP-mimetic lapatinib blocks HER2 and EGFR kinases and downstream signaling such as PI3K/AKT and MAPK (12). Lapatinib is also approved for the treatment of HER2-overexpressing breast cancer and in combination with trastuzumab is more effective than each drug given alone (13). Activation of alternate prosurvival pathways reduces the dependence of tumors on the targeted oncogenic kinase, leading to acquired drug resistance that can be overcome by combination treatments (13). In addition, the clinical benefit of small-molecule tyrosine kinase

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Translational Relevance

Despite the effectiveness of HER2-targeted therapies such as trastuzumab and lapatinib, most patients with metastatic HER2 gene–amplified breast cancer treated with these drugs eventually progress. Mutations in receptor tyrosine kinase (RTK) genes have been shown as possible mechanisms of resistance to small-molecule tyrosine kinase inhibitors. The advent of next-generation sequencing approaches has allowed for the detection of low-frequency mutant alleles in HER2 gene–amplified breast cancers. This work investigated the effects of the "gatekeeper" (T798M) mutation in HER2. Results show that cells expressing this lapatinib- and trastuzumab-resistant mutant overexpress EGFR ligands. Simultaneous blockade of HER2 and EGFR by the combined use of trastuzumab and lapatinib or by the addition of the EGFR antibody cetuximab to trastuzumab reversed drug resistance, thus identifying combinations that may prevent the acquisition of T798M mutations in patients with HER2+ breast cancer.

inhibitors (TKI) is generally limited by acquired mutations in the targeted kinase. A common causal mechanism of acquired resistance to TKIs is the development of kinase domain mutations, such as those reported in BCR-ABL (14), cKIT (15), PDGFRα (16), and EGFR (17, 18).

Mutations in the tyrosine kinase domain of HER2 have been identified in head and neck, lung, gastric, and breast carcinomas (19–23). An *in vitro* screen using a randomly mutagenized HER2 expression library identified several kinase domain mutations associated with resistance to lapatinib (24). In this study, a T798M substitution in HER2, analogous to the gatekeeper EGFR[T790M] (17), ABL[T315I] (14), and cKIT[T670I] (15) mutations, conferred the strongest resistance to lapatinib (24). A similar random mutagenesis approach had discovered BCR-ABL mutations that were subsequently found in patients with chronic myelogenous leukemia (CML) with acquired resistance to the ABL inhibitors imatinib and dasatinib (25). Kancha and colleagues cloned eight clinically observed HER2 mutations. Some were lapatinib-sensitive whereas others, including T798M, were resistant when expressed in cells without HER2 gene amplification. Interestingly, chronic exposure to lapatinib selected cancer cells with acquired L755S and T862A drug resistant mutations (26). In The Cancer Genome Atlas (TCGA) breast cancer dataset, 8 tumors harbored mutations in HER2, one of which, D769H, occurred in a tumor that was also HER2-amplified (27, 28).

To study the mechanisms by which the T798M mutation confers resistance to lapatinib and strategies to reverse such resistance in *HER2* gene-amplified breast cancer, we generated BT474 cells stably expressing the mutant allele. BT474 cells stably expressing HER2[T798M] were resistant to either lapatinib or trastuzumab alone. HER2[T798M] exhibited increased autocatalytic activity compared with wild-type HER2. BT474-

HER2[T798M] cells expressed higher levels of the EGFR ligands EGF, TGFα, amphiregulin, and HB-EGF. Consistent with a causal role of these ligands, the addition of the neutralizing EGFR antibody cetuximab restored sensitivity to trastuzumab in cells and xenografts expression HER2[T798M]. Furthermore, inhibition of EGFR with lapatinib also synergized with trastuzumab against xenografts expressing HER2[T798M], suggesting simultaneous inhibition of EGFR and HER2 abrogates the resistance induced by the gatekeeper mutation.

Materials and Methods

Generation of cells stably expressing HER2[T798M]

An HER2[T798M] expression vector was generated by subcloning the mutant sequence in the *Sal*I/*Hin*dIII site of DNR Dual (BD Biosciences) and then recombined using Cre into the JP1520 retroviral vector. Retroviruses expressing HER2[T798M] were produced by transfecting Phoenix-Ampho cells using published methods (20) and then used to transduce BT474 and MCF10A cells. Stably transfected cells were selected in 1 mg/mL G418.

Cell culture and proliferation assays

BT474 cells were maintained in IMEM medium/10% FBS (Gibco). MCF10A cells were maintained in DMEM/F-12 supplemented with EGF (20 ng/mL, Invitrogen/Gibco), cholera toxin (100 ng/mL, Sigma), hydrocortisone (500 ng/mL, Sigma), insulin (10 μg/mL, Invitrogen), and 5% horse serum (Hyclone). Cell proliferation was measured either by fixing and staining cells with crystal violet (29) or by using MTT (Sigma) or premixed WST-1 reagent (Roche) according to the manufacturer's protocol. The following inhibitors were used for various proliferation assays: 1 μmol/L lapatinib; 1 μmol/L CI-1040; 1 μmol/L BIBW2992 (all from LC Laboratories); 20 μg/mL trastuzumab; 20 μg/mL cetuximab (both from the Vanderbilt University Pharmacy); 1 μmol/L BKM120 (Active Biochem); and 6 μmol/L XL-147 (provided by Exelixis). For MTT/WST-1 assays, 1×10^4 cells/well were seeded in 96-well plates. Twenty-four hours after plating, cells were treated with dimethyl sulfoxide (DMSO) or inhibitors. After 5 days of treatment, MTT/WST-1 assays were conducted according to the manufacturer's protocol. For growth assays following HER3 knockdown, pooled HER3 and control siRNA (Dharmacon) were reverse-transfected into cells using Lipofectamine RNAi Max (Invitrogen) according to manufacturer's protocol. Briefly, cells were plated in triplicate in 24-well plates and transfected with HER3 or control siRNA (50 nmol/L). Seven days posttransfection, cells were trypsinized and counted using a Coulter counter. Alternatively, cells were treated with the LNA oligonucleotides directed against HER3 (EZN3920) or control (EZN4455) at a final concentration of 5 μmol/L (30) provided by Enzon Pharmaceuticals for 7 days.

Three-dimensional Matrigel culture

Cells (5×10^3/well) were seeded in 8-well chamber slides. Before seeding, BT474 and MCF10A cells were suspended in their respective medium on growth factor–reduced Matrigel

(BD Biosciences) as described (31). Inhibitors were added at the time of cell seeding and replenished with fresh medium every 3 days. After 12 to 14 days, images were captured from at least 3 different fields. To quantify cell number, Matrigel was dissolved by treatment with dispase for 2 hours at 37°C and acini were dissociated by pipetting. Dissociated cells were treated with trypsin and pelleted by centrifugation before being resuspended in growth medium and counted using a hemocytometer.

Immunoprecipitation and immunoblotting

Immunoprecipitations were conducted with a p85 antibody (Millipore) or a HER2 antibody (Neomarkers) followed by Protein A beads (Sigma) as described (32). Immune complexes and whole-cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. For immunoblot analysis, cells were lysed in 1% NP-40 buffer containing protease and phosphatase inhibitors. Samples were sonicated for 10 seconds and centrifuged at 14,000 rpm for 5 minutes at 4°C; protein concentrations were quantitated using the BCA assay (Pierce). Primary antibodies included Y1248 P-HER2, Y1068 P-EGFR, total EGFR, Y1197 P-HER3, total AKT, S473 P-Akt, P-Erk1/2, Erk1/2, (Cell Signaling); HER3, EGFR (Santa Cruz Biotechnology); HER2 (Neomarkers); Actin (Sigma); and the 4G10 phosphotyrosine antibody (Millipore).

Cell surface biotinylation

The Cell Surface Protein Isolation Kit (Pierce) was used for biotinylation studies according to the manufacturer's protocol. After treatment with 20 μg/mL trastuzumab for 16 hours at 37°C, cells were incubated with cold acid wash buffer (0.5 mol/L NaCl, 0.2 mol/L Na acetate, pH 3.0) for 6 minutes to remove bound trastuzumab. The cell monolayers were washed 3× with ice-cold PBS (pH 8.0) before the addition of freshly prepared Sulfo-NHS-Biotin reagent (2 mmol/L; Pierce) for 30 minutes at 4°C. The reaction was quenched with 100 mmol/L glycine in PBS and cells were harvested in lysis buffer (plus protease and phosphatase inhibitors) included in the kit. After sonication for 10 seconds and centrifugation at 14,000 rpm, protein concentration in the supernatants was measured using the BCA assay (Pierce). Equal amounts of protein extracts (500 μg) were subjected to precipitation using immobilized Neutravidin gel (Pierce); eluates were next subjected to SDS-PAGE and HER2 immunoblot analysis.

In vitro kinase assays

Five hundred micrograms of total protein from cells was precipitated with an HER2 antibody overnight at 4°C. Precipitates were subjected to an *in vitro* kinase assay as described (33). Briefly, precipitates were washed twice with NP-40 lysis buffer followed by 1 washes with kinase buffer (20 mmol/L HEPES, pH 7.5, 10 mmol/L MgCl$_2$, 10 mmol/L MnCl$_2$, 1 mmol/L dithiothreitol, 0.1 mmol/L Na$_3$VO$_4$). The immune complexes were next divided on ice into 2 equal aliquots and ATP (final concentration, 0.1 mmol/L) was added to one of the kinase reactions, which were carried out

for 5 minutes at 30°C and terminated by adding 5× loading buffer followed by boiling for 3 minutes. Kinase reaction products were then separated on a 7.5% SDS-PAGE gel and subjected to immunoblot analysis.

Real-time quantitative PCR

RNA isolation and real-time quantitative PCR (RT-PCR) were carried out as described (34, 35). Primer sequences for ErbB ligands are as follows: Heregulin (HRG): forward 5'-TGGCTGACAGCAGGACTAAC-3', reverse 5'-CTGGCCTGG-ATTTCTTC-3'; EGF: forward 5'-AGCTAACCCATTATGGCAA-CA-3', reverse 5'-AGTTTTCACTGAGTCAGCTCCAT-3'; TGFα: forward 5'-GGACAGCACTGCCAGAGA-3', reverse 5'-CAGG-TGATTACAGGCCAAGTAG-3'; amphiregulin (AREG): forward 5'-ATATCACATTGGAGTCACTGCCCA-3', reverse 5'-GGGTCCATTGTCTTATGATCCAC-3'; HB-EGF: forward 5'-GAAAGACTTCCATCTAGTCACAAAGA-3', reverse 5'-GGGA-GGCCCAATCCTAGA-3'; epiregulin (EREG): forward 5'-TGCATGCAATTTAAAGTAACTTATTTGACTA-3', reverse 5'-ATCTTAAGGTACACAATTATCAAAGCTGA-3'; and betacellulin (BTC): forward 5'-TGCCCCAAGCAATACAAGC-3', reverse 5'-CGTCTGCTCGGCCACC-3'.

HER2^{T798M} sequencing

Exons 19-20 of HER2 were amplified from reverse-transcribed RNA isolated from BT474^{T798M} using the primer pair forward 5'-GTAGGATCCAGCCCACGCTC-3' and reverse 5'-CTAGACACCACTCCACCCAG-3'. The RT-PCR product was cloned into the pCR Blunt vector (Invitrogen). A total of 96 colonies were picked to inoculate individual cultures in 96-well blocks, which were then pooled into 12 groups of 8 individual clones. These 12 pools were screened for the presence of HER2^{T798M} by direct (Sanger) sequencing. Individual plasmids from positive pools were resequenced to determine the allelic frequency of HER2^{T798M}.
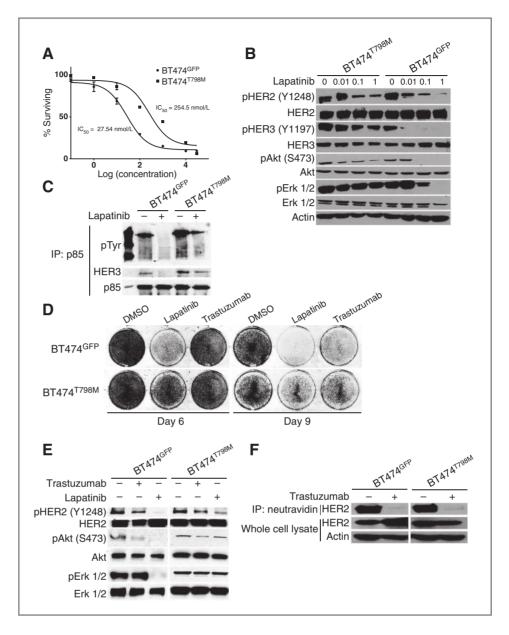
Xenograft studies

Mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee. Mice were housed in the Vanderbilt Animal Care Facility. A 17β-estradiol pellet (Innovative Research of America) was injected subcutaneously in the dorsum of 5- to 6-week-old athymic female mice (Harlan Sprague-Dawley) the day before tumor cell injection. BT474 cells (5×10^6) mixed 1:1 with Matrigel (BD Biosciences) were injected s.c. in the right flank of each mouse. Tumor diameters were measured with calipers twice per week and volume in mm^3 calculated by the formula volume = width^2 × length/2. When tumors reached a volume ≥200 mm^3, mice were treated for 4 to 5 weeks with the following, either alone or in combination: trastuzumab 30 mg/kg twice per week intraperitoneally (i. p.), lapatinib 100 mg/kg daily via orogastric gavage, and cetuximab 1 mg twice per week i.p.

Immunohistochemistry

Tumors were harvested at the end of treatment (after 4 to 5 weeks), fixed in formalin, and paraffin embedded. Tumor sections (5 μm) were stained with a S473 P-Akt antibody

Figure 1. Cells expressing HER2^{T798M} are resistant to lapatinib and trastuzumab. A, BT474^{GFP} and BT474^{T798M} cells were treated with increasing concentrations of lapatinib for 5 days. MTT assay was conducted at the end of treatment and dose inhibition curves produced by Graphpad Prism software. IC$_{50}$ values were calculated according to the formula $Y = 100/[1 + 10^{(X - \log IC_{50})}]$. B, BT474^{GFP} and BT474^{T798M} cells were treated with increasing concentrations of lapatinib for 3 hours. Protein extracts were prepared and subjected to immunoblot analyses with the antibodies indicated on the left. C, cells were treated with lapatinib for 3 hours, and protein extracts were subjected to immunoprecipitation with a p85 antibody. Immune complexes associated with p85 were separated by SDS-PAGE followed by immunoblot with the indicated antibodies. D, cells were treated with either 1 μmol/L lapatinib or 20 μg/mL trastuzumab for 9 days. Crystal violet assays were conducted on days 6 and 9, and images captured using the Li-Cor Odyssey System. E, cells were treated with 1 μmol/L lapatinib or 20 μg/mL trastuzumab for 3 hours. Protein extracts were prepared and subjected to immunoblot analyses with antibodies indicated on the left. F, cells were treated with 20 μg/mL trastuzumab overnight and then cell surface proteins were biotinylated as described in Methods. Biotinylated proteins were captured with Neutravidin gel and analyzed by immunoblot with a HER2 antibody. Bottom, HER2 and actin immunoblots of whole-cell lysates to control for gel loading.

(Cell Signaling) and staining intensity was scored by an expert pathologist (M.V. Estrada) blinded to treatment groups. Intensity of cytoplasmic staining was scored from 0 to 3+, in 5 different fields at ×400 magnification for each section. The average score from those 5 fields was then used to calculate an H score by the following formula: $3 \times$ (% of 3+ cells) + $2 \times$ (% of 2+ cells) + $1 \times$ (% of 1+ cells). The mean H scores ± SEM for each treatment group were compared by ANOVA.

Results

T798M mutation in HER2 confers resistance to HER2 antagonists in HER2-amplified breast cancer cells

BT474 cells were stably transduced with a retroviral vector encoding either HER2^{T798M} or GFP. Expression of the T798M mutant isoform increased the IC$_{50}$ to lapati-nib by 10-fold compared with GFP-expressing cells (Fig. 1A). Treatment with 1 μmol/L lapatinib blocked phosphorylation of HER2, HER3, AKT, and Erk1/2 in BT474^{GFP} but not in BT474^{T798M} cells (Fig. 1B). Presence of the mutation also resulted in continued association of HER3 with the p85 regulatory subunit of PI3K in the presence of lapatinib (Fig. 1C). Lapatinib or trastuzumab inhibited growth of BT474^{GFP} cells, but BT474^{T798M} cells were resistant to both inhibitors (Fig. 1D). Treatment with trastuzumab inhibited AKT phosphorylation in BT474^{GFP} but not in BT474^{T798M} cells (Fig. 1E). Trastu-zumab binds to domain IV of HER2 (36) and induces receptor internalization and downregulation from the cell surface, thus attenuating downstream signal trans-duction (37). As trastuzumab did not inhibit down-stream signaling in cells expressing T798M (Fig. 1E), we

investigated whether this mutation would impair trastuzumab-induced receptor internalization. Cell surface biotinylation followed by precipitation of labeled proteins with neutravidin showed that trastuzumab markedly downregulated cell surface HER2 in both BT474^{GFP} and BT474^{T798M} cells (Fig. 1F).

We next examined whether the T798M mutant allele was required for growth and survival of mutant-expressing cells by using the covalent irreversible HER2/EGFR small molecule inhibitor BIBW2992 (afatinib). Afatinib has been shown to bind to and inhibit the EGFR T790M gatekeeper mutant (38). Treatment with BIBW2992 but not with lapatinib eliminated phosphorylation of HER2 and EGFR as measured with site-specific receptor antibodies (Fig. 2A) and potently inhibited growth of both BT474^{GFP} and BT474^{T798M} cells (Fig. 2B). BIBW2992 also disrupted the association of p85 with HER3 and inhibited P-AKT levels in both cell lines (Fig. 2C). Similar results were observed with treatment with the pan-ErbB irreversible inhibitor CI-1033 (39), which inhibited HER2 phosphorylation and growth of both BT474^{GFP} and BT474^{T798M} cells (Supplementary Fig. S1). These results suggest that HER2^{T798M}-expressing cells rely on the mutant allele for activation of the PI3K/AKT pathway and their survival.

HER2^{T798M} has increased autocatalytic activity compared to wild-type HER2

Expression of T798M resulted in increased HER3 phosphorylation and its association with the p85 subunit of PI3K (Supplementary Fig. S2), suggesting that the mutant HER2 might be catalytically more active than wild-type HER2. To examine this possible gain of function, we stably expressed HER2^{WT} or HER2^{T798M} in MCF10A human mammary epithelial cells that normally express low levels of HER2. Expression of both HER2^{WT} and HER2^{T798M} resulted in increased phosphorylation of HER2, HER3, AKT and ERK1/2 compared to controls (Fig. 3A). Treatment with lapatinib inhibited pHER2 and pHER3 as well as growth in 3-dimensional (3D) Matrigel of MCF10A^{WT} but not MCF10A^{T798M} cells (Fig. 3B and C). In addition, MCF10A^{T798M} acini were larger and more invasive than MCF10A^{WT} acini and proliferated faster than MCF10A^{WT} cells (Fig. 3D) in both full-serum and serum-free conditions, further supporting a gain of HER2 function conferred by the mutant allele. Finally, to test HER2 kinase activity, HER2^{WT} and HER2^{T798M} were immunoprecipitated from both cell types and the pull-downs were tested in an in vitro kinase reaction (Fig. 3E). Immunoprecipitates from parental MCF10A were used as controls. HER2^{T798M} showed markedly higher tyrosine phosphorylation than HER2^{WT}, suggesting that the mutant allele has higher catalytic activity (Fig. 3E).

HER2^{T798M} expressed at low frequency is sufficient to confer resistance

It has been shown that a very low allele frequency of the EGFR^{T790M} gatekeeper mutation is enough to confer resistance to the EGFR-TKI gefitinib (40). Using dilutional cloning, we found that approximately 3.1% of HER2 alleles contain T798M in lapatinib-resistant BT474^{T798M} cells (Supplementary Fig. S3). This is consistent with earlier reports which showed that T790M mutation in EGFR can render cells resistant to gefitinib with an allele frequency of
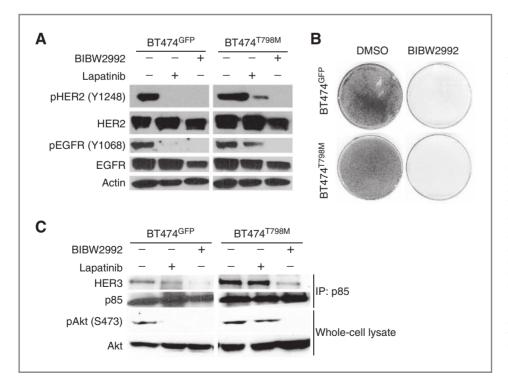

Figure 2. Cells expressing HER2^{T798M} remain dependent on the ErbB pathway to activate PI3K/AKT. A, cells were treated with 1 μmol/L lapatinib or 1 μmol/L BIBW2992 for 3 hours. Protein extracts were then prepared and subjected to immunoblot analyses with the indicated antibodies. B, cells were treated with 1 μmol/L BIBW2992. Monolayers were fixed and stained with crystal violet when DMSO-treated cells reached confluence (day 6). C, cells were treated with 1 μmol/L lapatinib or 1 μmol/L BIBW2992 for 3 hours; protein extracts were precipitated with a p85 antibody. Antibody pull-downs were next washed, separated by SDS-PAGE, and subjected to immunoblot analysis as described in Methods using the indicated antibodies (top 2 panels). Immunoblot analyses on whole-cell extracts were conducted using the antibodies indicated on the left (bottom 2 panels).
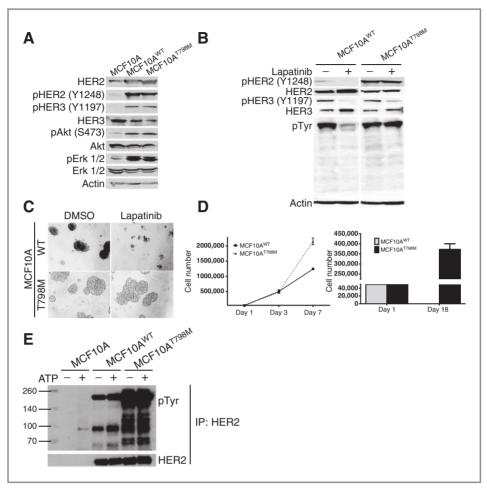
Figure 3. HER2[T798M] exhibits a gain of function compared to HER2[WT]. A, retroviral infection was used to produce MCF10A cells stably expressing either HER2[WT] or HER2[T798M]. Whole-cell lysates were subjected to immunoblot analyses with antibodies indicated on the left. B, cells were treated with 1 μmol/L lapatinib for 3 hours, and whole lysates were subjected to immunoblot analyses with the indicated antibodies. C, cells were plated in Matrigel and treated with DMSO or 1 μmol/L lapatinib. Cells were plated in triplicates and images captured on day 14. Fresh medium and inhibitor were replenished every 3 days. D, MCF10A[WT] or MCF10A[T798M] (5 × 10⁴/well) were plated in triplicates in 6-well plates in absence or presence of 10% serum. For growth assay in serum-containing media, cells were counted on days 1, 3, and 7. For growth assays in serum-free condition, cells were counted on days 1 and 18. E, HER2 was immunoprecipitated from 500 μg of protein extract from MCF10A cells with a C-terminus HER2 antibody where indicated. Immobilized HER2 was used for *in vitro* kinase assays as described in Methods (26). Kinase reactions were eluted from beads and subjected to SDS-PAGE and immunoblot analysis with HER2 and anti-phosphotyrosine antibodies.

3.3% (40). To support further that this low frequency of expression was sufficient to confer resistance, we carried out co-culture experiments wherein varying proportions of BT474[GFP] (green) and BT474[T798M] cells (unlabeled) were mixed and then subjected to selection with lapatinib. We found that lapatinib-resistant acini emerge when as low as 5% of the total cell population is BT474[T798M] (Supplementary Fig. S4). In this experiment, expansion of the mutant population of cells was indicated by loss of the GFP fluorescence from the resistant acini.

HER2[T798M]-expressing cells rely on HER3-PI3K for survival

In HER2 gene–amplified breast cancer cells, HER2 potently activates the PI3K prosurvival pathway mainly by dimerizing with and phosphorylating the HER3 coreceptor (41,

42). To determine whether cells bearing the T798M mutant remain dependent on PI3K/AKT, we treated BT474[GFP] and BT474[T798M] cells with the pan-PI3K inhibitor BKM120 (43). Treatment with BKM120 blocked phosphorylation of AKT in S473 (Fig. 4A) and inhibited growth of both BT474[GFP] and BT474[T798M] cells (Fig. 4B and C). Amplified HER2 signaling also hyperactivates the RAS/MEK/ERK pathway (44). Treatment with the MEK1/2 inhibitor CI-1040 (45) blocked phosphorylation of ERK1/2 in both cell types (Fig. 4A) but did not inhibit their growth (Fig. 4B and C). These data suggest that expression of HER2[T798M] does not dispense with the dependence of HER2-overexpressing cells on PI3K. We next examined whether treatment with a second pan-PI3K inhibitor, XL-147 (46), would overcome resistance to trastuzumab of cells expressing HER2[T798M]. The combination of trastuzumab and XL-147 was more potent than either drug
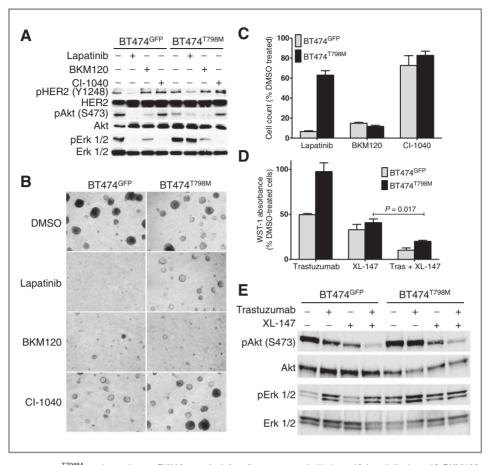
Figure 4. Cells expressing HER2^{T798M} are dependent on PI3K for survival. A, cells were treated with 1 μmol/L lapatinib, 1 μmol/L BKM120, or 1 μmol/L CI-1040 for 3 hours. Protein extracts were prepared and subjected to immunoblot analyses with the indicated antibodies. B, cells in 3D Matrigel growth assays were treated with DMSO, 1 μmol/L lapatinib, 1 μmol/L BKM120, and 1 μmol/L CI-1040. Cells were plated in triplicates and images captured on day 14. Media and inhibitors were replenished every 3 days. C, quantification of 3D growth assays was conducted by dissolution of the Matrigel and mechanical disruption of acini into single-cell suspensions for cell counting as described in Methods. The bars represent the mean of 3 wells ± SEM. D, cells (10^4/well) were seeded in 96-well plates and 24 hours after plating treatment with 20 μg/mL trastuzumab, 6 μmol/L XL-147, or the combination was begun. WST-1 absorbance was measured on day 5. Fresh medium and inhibitors were replenished after 3 days. The bars represent the mean of 4 wells ± SEM. E, cells were treated with 20 μg/mL trastuzumab, 6 μmol/L XL-147, or the combination for 3 hours. Protein extracts prepared were prepared and subjected to immunoblot analyses using antibodies indicated on the left.

alone at inhibiting BT474^{T798M} cell growth (Fig. 4D). Immunoblot analyses confirmed that XL-147 inhibited S473 P-AKT in both BT474^{GFP} and BT474^{T798M} cells (Fig. 4E). Because HER3 is the major activator of PI3K in HER2-dependent cells, we next tested the effects of HER3 inhibition. RNA interference-induced knockdown of HER3 resulted in growth inhibition of both BT474^{GFP} and BT474^{T798M} cells (Supplementary Fig. S5A). Furthermore, knockdown of HER3 using an HER3-specific locked nucleic acid (LNA) antisense oligonucleotide (30) markedly reduced S473 P-AKT in BT474^{T798M} cells (Supplementary Fig. S5B). These results suggest BT474^{T798M} cells remain dependent on the HER3-PI3K axis for survival.

BT474^{T798M} cells overexpress EGFR ligands and combined blockade of EGFR and HER2 inhibits their growth

We speculated that another explanation for both the increased intracellular signaling generated by HER2^{T798M}

(Figs. 1C and 3B; Supplementary Fig. S2) and the acquired resistance to trastuzumab would be enhanced activation of HER2 by ligand-induced ErbB coreceptors. Therefore, we carried out quantitative PCR with mRNA isolated from BT474^{GFP} and BT474^{T798M} cells using primers specific for the ErbB receptor ligands heregulin, EGF, TGFα, amphiregulin, HB-EGF, epiregulin, and betacellulin. BT474^{T798M} cells expressed >2-fold higher levels of the EGFR ligands EGF, TGFα, amphiregulin, and HB-EGF compared with BT474^{GFP} cells (Fig. 5A). Consistent with enhanced ErbB ligand production, HER2 antibody pull-downs contained more HER3 and EGFR in BT474^{T798M} than in BT474^{GFP} cells in the absence of exogenous TGFα (Fig. 5B and C). If overexpression of EGFR ligands is causal to the resistance to HER2 inhibitors, we proposed that blockade of ligand binding with the EGFR antibody cetuximab should overcome resistance to lapatinib and trastuzumab. However, treatment of BT474^{T798M} cells with cetuximab did not add to the modest growth inhibition by lapatinib, although
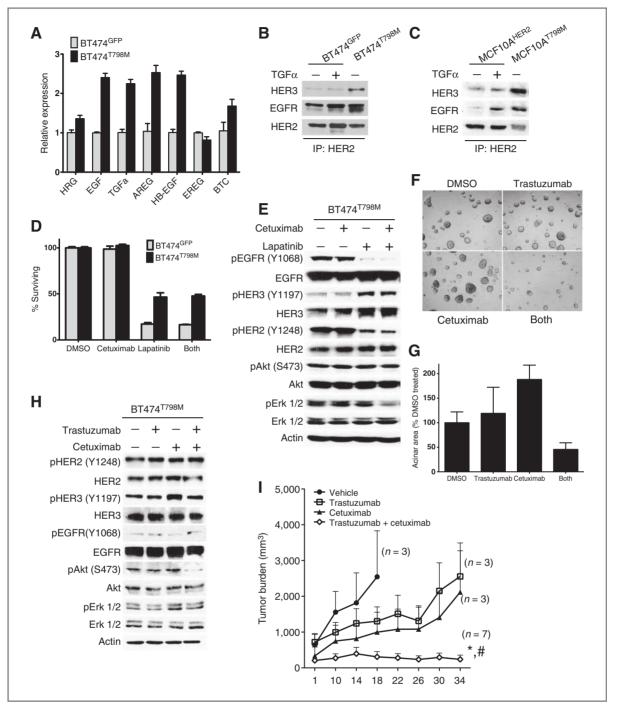
Figure 5. HER2[T798M] cells express higher levels of EGFR ligands and HER2-containing heterodimers. A, RT-qPCR analysis for ErbB receptor ligand expression was conducted using RNA template from BT474[GFP] and BT474[T798M] cells, normalized to expression in GFP cells for each ligand. B, lysates prepared from BT474[GFP] ($\pm$40 ng/mL TGFα) and BT474[T798M] were immunoprecipitated with a HER2 C-terminal antibody followed by immunoblot analyses with the indicated antibodies. C, lysates prepared from MCF10A[WT] ($\pm$40 ng/mL TGFα) and MCF10A[T798M] were analyzed as in B. D, BT474[T798M] cells were treated with 20 μg/mL cetuximab and 1 μmol/L lapatinib, either alone or in combination for 5 days. MTT assays were conducted at the end of treatment. E, cells were treated with 20 μg/mL cetuximab and/or 1 μmol/L lapatinib for 3 hours. Lysates were prepared and subjected to immunoblot analyses with the indicated antibodies. F, for 3D Matrigel growth assays, cells were treated with 20 μg/mL trastuzumab, 20 μg/mL cetuximab, or the combination. Cells were plated in duplicates and images captured on day 14. Fresh medium and inhibitors were replenished every 3 days. G, average acinar area was quantified with the ImageJ software. Each bar represents the mean acinar area $\pm$ SEM ($n = 2$). H, cells were treated with 20 μg/mL cetuximab and/or and 20 μg/mL trastuzumab and lysates analyzed as in E. I, BT474[T798M] xenografts were established in female athymic mice as indicated in Methods. Once tumors reached at least $\geq$200 mm[3] in volume, mice were treated with trastuzumab (30 mg/kg i.p. twice per week), cetuximab (1 mg i.p. twice per week), or both antibodies. Each data point represents the mean tumor volume $\pm$ SEM. *, $P < 0.005$ versus trastuzumab; #, $P < 0.05$ versus cetuximab. The number of mice in each treatment arm is indicated next to each curve.

EGFR phosphorylation was inhibited. In addition, P-HER2, P-HER3, P-AKT, and P-Erk1/2 were maintained following treatment with this combination (Fig. 5D and E). We speculate that this activation reflects EGFR-independent HER2 signaling, which is maintained because of the inability of lapatinib to bind HER2^{T798M} with high affinity and inhibit its kinase activity.

In 3D Matrigel, the HER2 T798M mutant–expressing cells were resistant to single-agent cetuximab or trastuzumab. However, the combination of trastuzumab and cetuximab blocked BT474^{T798M} cell growth (Fig. 5F and G). Consistent with the growth-inhibitory effect of the combination, immunoblot analysis showed that treatment with both antibodies markedly inhibited S473 P-AKT (Fig. 5H). To test the effect of the combination *in vivo*, we injected BT474^{T798M} cells into athymic nude mice and treated established tumors with trastuzumab, cetuximab, or the combination for 5 weeks. Trastuzumab or cetuximab alone had no effect on tumor growth. However, treatment with both antibodies resulted in complete response in 3 of 7 (43%) mice (Fig. 5I). Immunohistochemical analysis of tumors

after about 5 weeks of treatment showed that cetuximab alone or in combination with trastuzumab resulted in a slight decrease in cytoplasmic P-Akt intensity (Supplementary Fig. S6). These observations suggest that dual inhibition of HER2 and EGFR limits the growth of trastuzumab-resistant cells in tumors bearing HER2^{T798M}.

Finally, we hypothesized that as lapatinib also inhibits the EGFR tyrosine kinase, BT474^{T798M} cells should be sensitive to dual HER2 blockade with trastuzumab and lapatinib. In this combination, trastuzumab should partially downregulate mutant HER2 and lapatinib should inhibit transactivation of HER2 (and HER3) by ligand-induced EGFR. Indeed, the combination of trastuzumab and lapatinib inhibited BT474^{T798M} cell growth in 3D Matrigel even though the cells were resistant to each drug alone (Fig. 6A and B). Treatment with the combination also inhibited Y1068-pEGFR and Y1197-pHER3. Treatment with trastuzumab had no effect, whereas lapatinib modestly inhibited HER2/HER3 heterodimers. However, treatment with both inhibitors markedly reduced HER2/HER3 heterodimers potentially explaining the inhibition of S473 P-AKT (Fig. 6C). Furthermore,
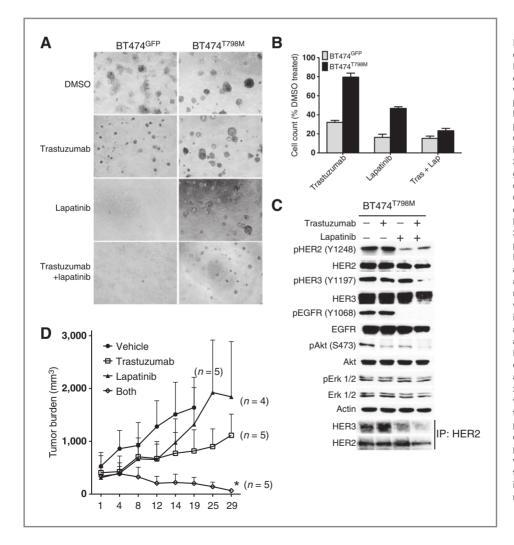

Figure 6. Simultaneous blockade of EGFR and HER2 inhibits HER2^{T798M}-expressing tumors. A, cells in 3D Matrigel growth assays were treated with DMSO, 20 μg/mL trastuzumab, 1 μmol/L lapatinib, or both drugs. Cells were plated in duplicates and images captured on day 14. Media and inhibitors were replenished every 3 days. B, quantification of 3D growth assays was conducted as described earlier and in Methods. C, cells were treated with similar concentrations of trastuzumab, lapatinib, or the combination for 3 hours; whole-cell lysates were prepared and subjected to SDS-PAGE followed by immunoblot analyses with the indicated antibodies. Bottom 2 panels represent immunoprecipitation with an HER2 antibody followed by immunoblot analyses of the antibody pull-downs with HER2 and HER3 antibodies. D, BT474^{T798M} xenografts were established in female athymic mice as indicated in Methods. Once xenografts reached ≥200 mm^3 in volume, mice were treated with trastuzumab (30 mg/kg i.p. twice per week), lapatinib (100 mg/kg/d), or the combination. Each data point represents the mean tumor volume ± SEM. *, $P < 0.05$ versus trastuzumab. The number of mice in each treatment arm is indicated next to the curves.

treatment with both inhibitors but not each drug alone induced regression of BT474[T798M] xenografts established in athymic mice (Fig. 6D). There was a modest reduction in S473 P-Akt levels as measured by immunohistochemistry (IHC) in tumors in all treatment arms at the end of 4 weeks of therapy (Supplementary Fig. S7). These results suggest that even though HER2[T798M] confers resistance to lapatinib or trastuzumab when used as single agents, simultaneous blockade of HER2 and EGFR with both of these inhibitors is effective against HER2-overexpressing breast cancer cells bearing HER2[T798M] alleles.

Discussion

In this report, we studied cellular and biochemical effects of the gatekeeper T798M mutation in the HER2 kinase domain in breast cancer cells. Expression of T798M conferred resistance to both lapatinib and trastuzumab. As the T790M gatekeeper mutation in EGFR has increased affinity for ATP (47), we speculate the corresponding T798M mutation in HER2 may similarly stabilize HER2 in an active conformation (26). Consistent with this, we observed that HER2[T798M] has increased catalytic activity compared to HER2[WT]. In addition, structural modeling of T798M suggests that the methionine substitution at T798 sterically inhibits drug binding and/or further destabilizes the inactive conformation of HER2. As lapatinib binds preferentially the inactive confirmation of HER2, this further contributes to drug resistance (24). This may also explain the marked shift in the IC_{50} of lapatinib against HER2[T798M] cells.

Trastuzumab is unable to block transactivation of HER2 by ligand-induced ErbB coreceptors (48). Exogenous and endogenous ligands of EGFR and HER3 have been shown to rescue from the growth-inhibitory effect of the antibody (9, 35, 49). Furthermore, HER2+ xenografts selected *in vivo* for acquired resistance to trastuzumab exhibit higher levels of P-EGFR and EGFR/HER2 heterodimers as well as overexpression of EGFR, TGFα, HB-EGF, and heregulin RNAs compared with parental trastuzumab-sensitive tumors (50). Finally, expression of drug-resistant mutants of EGFR (38) and HER2 (33, 51) also results in overproduction of ErbB receptor ligands. Thus, we examined expression of ErbB receptor ligands in cells expressing the HER2 mutant. BT474[T798M] cells overexpressed EGF, TGFα, amphiregulin, and HB-EGF mRNAs and exhibited higher levels of HER2-containing heterodimers compared to BT474[GFP] cells. Addition of cetuximab, an antibody that blocks ligand binding to EGFR, overcame the resistance to trastuzumab (Fig. 5), suggesting that ligand overexpression was causal to this resistance. These data suggest that although HER2[T798M] is intrinsically sensitive to trastuzumab-induced downregulation similar to HER2[WT] (Fig. 1F), its expression increases ligand-induced HER2-containing heterodimers (Fig. 5B and C), potentially explaining how T798M contributes to trastuzumab resistance. Further studies are required to determine the mechanisms by which amplification of HER2 signaling results in enhanced production of ErbB receptor ligands.

The HER2[T798M] kinase was exquisitely sensitive to submicromolar concentrations of BIBW2992 (afatinib). This is an irreversible small-molecule inhibitor that covalently binds Cys^{805} and Cys^{773} in the ATP pocket of HER2 and EGFR, respectively, and potently inhibits the receptors' kinase activity (52). Afatinib is also effective against the analogous drug-resistant T790M mutation in EGFR and has shown clinical activity in lung cancers harboring this mutation (53). Cells expressing HER2[T798M] were also sensitive to HER3 knockdown and to 2 pan-PI3K inhibitors currently in clinical development. These results suggest that cells expressing the mutant continue to rely on the HER2/HER3/PI3K axis for growth and survival. Therefore, afatinib and other irreversible HER2 TKIs, HER3 antibodies, PI3K inhibitors, and drugs that disrupt ligand-induced HER2-HER3 dimers (i.e., pertuzumab) represent clinical approaches and combinations currently used in patients with HER2-overexpressing cancers that may prevent the acquisition or the effects of HER2[T798M].

Mutations in HER2 are present in breast tumors, but, to our knowledge, with very few exceptions, they have all been reported in breast cancers without HER2 gene amplification (23, 27, 52). One possibility is that allelic dilution may render it difficult to identify this drug resistant mutant by conventional direct sequencing in cancers with HER2 gene amplification. It is also possible that this mutant may require enrichment after treatment of a clinically sensitive tumor with lapatinib, analogous to the enrichment of imatinib-resistant gatekeeper mutants in BCR-ABL in patients with CML (14). To detect low-frequency mutations, direct sequencing will need to be combined with methodologies which are more sensitive to detect rare mutations, especially in highly amplified genes such as *HER2*. This has been observed in lung cancer, where use of SURVEYOR but not Sanger sequencing (40) detected the EGFR T790M mutation in gefitinib-resistant primary lung tumors. This suggests next-generation massively parallel sequencing that can provide deep gene coverage may be required to detect HER2 mutations in patients with HER2 gene–amplified breast cancer. This is also consistent with our findings that a low frequency of expression of the mutant allele (∼3%) was sufficient to confer drug resistance. Finally, sequencing efforts to date have focused on pretreatment samples. We speculate that many resistance-associated mutations are acquired (or enriched from a small and undetectable pre-existing population). As such, they may only be detected following treatment and progression of clinically resistant disease.

Authors' Contributions

Conception and design: B.N. Rexer, R. Ghosh, A. Narasanna, J.A. Engelman, C.L. Arteaga
Development of methodology: R. Ghosh, A. Narasanna, A. Chakrabarty, C.L. Arteaga
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.N. Rexer, R. Ghosh, A. Narasanna, M.V. Estrada, A. Chakrabarty, Y. Song, J.A. Engelman, C.L. Arteaga

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Human Breast Cancer Cells Harboring a Gatekeeper T798M Mutation in HER2 Overexpress EGFR Ligands and Are Sensitive to Dual Inhibition of EGFR and HER2

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