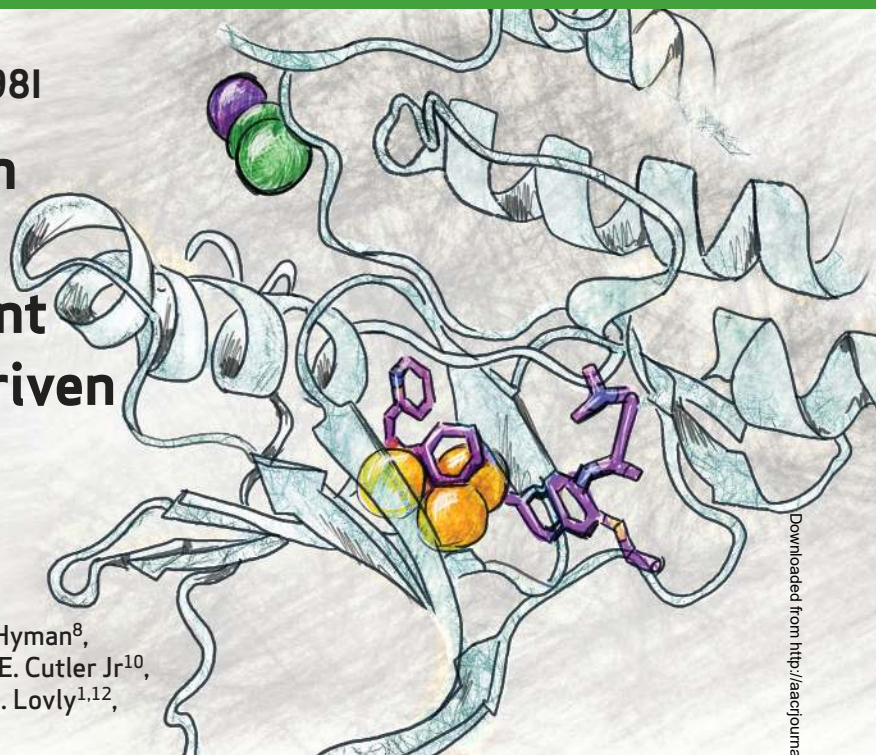


# An Acquired *HER2*<sup>T798I</sup> Gatekeeper Mutation Induces Resistance to Neratinib in a Patient with *HER2* Mutant-Driven Breast Cancer



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## ABSTRACT

We report a *HER2*<sup>T798I</sup> gatekeeper mutation in a patient with *HER2*<sup>L869R</sup>-mutant breast cancer with acquired resistance to neratinib. Laboratory studies suggested that *HER2*<sup>L869R</sup> is a neratinib-sensitive, gain-of-function mutation that upon dimerization with mutant *HER3*<sup>E928G</sup>, also present in the breast cancer, amplifies *HER2* signaling. The patient was treated with neratinib and exhibited a sustained partial response. Upon clinical progression, *HER2*<sup>T798I</sup> was detected in plasma tumor cell-free DNA. Structural modeling of this acquired mutation suggested that the increased bulk of isoleucine in *HER2*<sup>T798I</sup> reduces neratinib binding. Neratinib blocked *HER2*-mediated signaling and growth in cells expressing *HER2*<sup>L869R</sup> but not *HER2*<sup>L869R/T798I</sup>. In contrast, afatinib and the osimertinib metabolite AZ5104 strongly suppressed *HER2*<sup>L869R/T798I</sup>-induced signaling and cell growth. Acquisition of *HER2*<sup>T798I</sup> upon development of resistance to neratinib in a breast cancer with an initial activating *HER2* mutation suggests *HER2*<sup>L869R</sup> is a driver mutation. *HER2*<sup>T798I</sup>-mediated neratinib resistance may be overcome by other irreversible *HER2* inhibitors like afatinib.

**SIGNIFICANCE:** We found an acquired *HER2* gatekeeper mutation in a patient with *HER2*-mutant breast cancer upon clinical progression on neratinib. We speculate that *HER2*<sup>T798I</sup> may arise as a secondary mutation following response to effective *HER2* tyrosine kinase inhibitors (TKI) in other cancers with *HER2*-activating mutations. This resistance may be overcome by other irreversible *HER2* TKIs, such as afatinib. *Cancer Discov*; 7(6); 575-85. © 2017 AACR.

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## INTRODUCTION

DNA-sequencing efforts have revealed that *ERBB2*, the gene encoding the HER2 receptor tyrosine kinase, is mutated in a wide variety of cancer types, including 2% to 3% of primary breast cancers (1–3), with a higher incidence in lobular breast cancers (4). More than 70% of *HER2* mutations in breast cancer are found in the absence of *HER2* (*ERBB2*) gene amplification (2). Some of the common *HER2* mutations promote HER2 kinase activity and transform breast epithelial cells and other cell types (5–9). Given that irreversible EGFR/HER2 tyrosine kinase inhibitors (TKI), such as neratinib and afatinib, have shown preclinical activity against several HER2 mutants (5, 7–9), clinical trials with neratinib (SUMMIT trial; NCT01953926) and afatinib (NCI-MATCH; NCT02465060) focused on patients with *HER2*-mutant cancers are in progress. However, sustained clinical activity of ATP mimetics in patients with advanced cancer has generally been limited by the acquisition of drug resistance. Mutation of the “gatekeeper” residue within the kinase’s ATP-binding pocket, such as ABL<sup>T315I</sup>, KIT<sup>T670I</sup>, and EGFR<sup>T790M</sup>, is a common mechanism of acquired resistance. Here, we report for the first time a case of a *HER2* gatekeeper mutation in a patient with nonamplified *HER2*-mutant breast cancer with acquired resistance to neratinib.

## RESULTS

### *HER2*<sup>L869R</sup> Exhibits a Gain-of-Function Phenotype That Is Blocked by Neratinib

Targeted capture next-generation sequencing (NGS; ref. 10) of DNA from a skin metastasis in a 54-year-old female with estrogen receptor (ER)/progesterone receptor (PR)-positive, *HER2* nonamplified lobular breast carcinoma identified an *ERBB2*<sup>L869R</sup> (*HER2*<sup>L869R</sup>) somatic mutation (Supplementary Table S1). Prior therapies included chemotherapy, tamoxifen, aromatase inhibitors, everolimus, and trastuzumab. The tumor also harbored a truncation mutation in *CDH1*, *ERBB3*<sup>E928G</sup>, and amplification of *CCND1* and *FGF3/4/19*. Interrogation of the cBioPortal ( $n > 21,000$ ), Project GENIE ( $n > 18,000$ ), the Catalogue of Somatic Mutations in Cancer (COSMIC;  $n > 50,000$ ), Foundation Medicine ( $n > 40,000$ ), and Guardant Health ( $n > 17,000$ ) databases found 16 additional cancers harboring *ERBB2*<sup>L869R</sup> and one L869Q mutation, including 12 breast cancers (Supplementary Table S2). In addition, a recent study reported four instances of *ERBB2*<sup>L869R</sup> among 413 invasive lobular breast cancers (4).

The L869R mutation is located within the activation loop of the HER2 kinase domain. Sequence alignment of the HER2, EGFR, and BRAF kinase domains showed that HER2<sup>L869R</sup> is homologous to BRAF<sup>V600E</sup>, a gain-of-function mutation found in >50% of melanomas (11), and EGFR<sup>L861R/Q</sup>, an activating mutation in non-small cell lung cancer (NSCLC; Fig. 1A; ref. 12). We performed structural modeling of the L869R mutation using Rosetta (13) and examined the residue pair energies involving L869. The mutation resulted in the addition of a strong attractive interaction between R869 and D769 (Fig. 1B and C). This interaction potentially stabilizes the active conformation of the C helix. We also predict that mutating L869 to a polar residue (Arg) disrupts the autoinhibitory contacts between the C helix and the activation loop

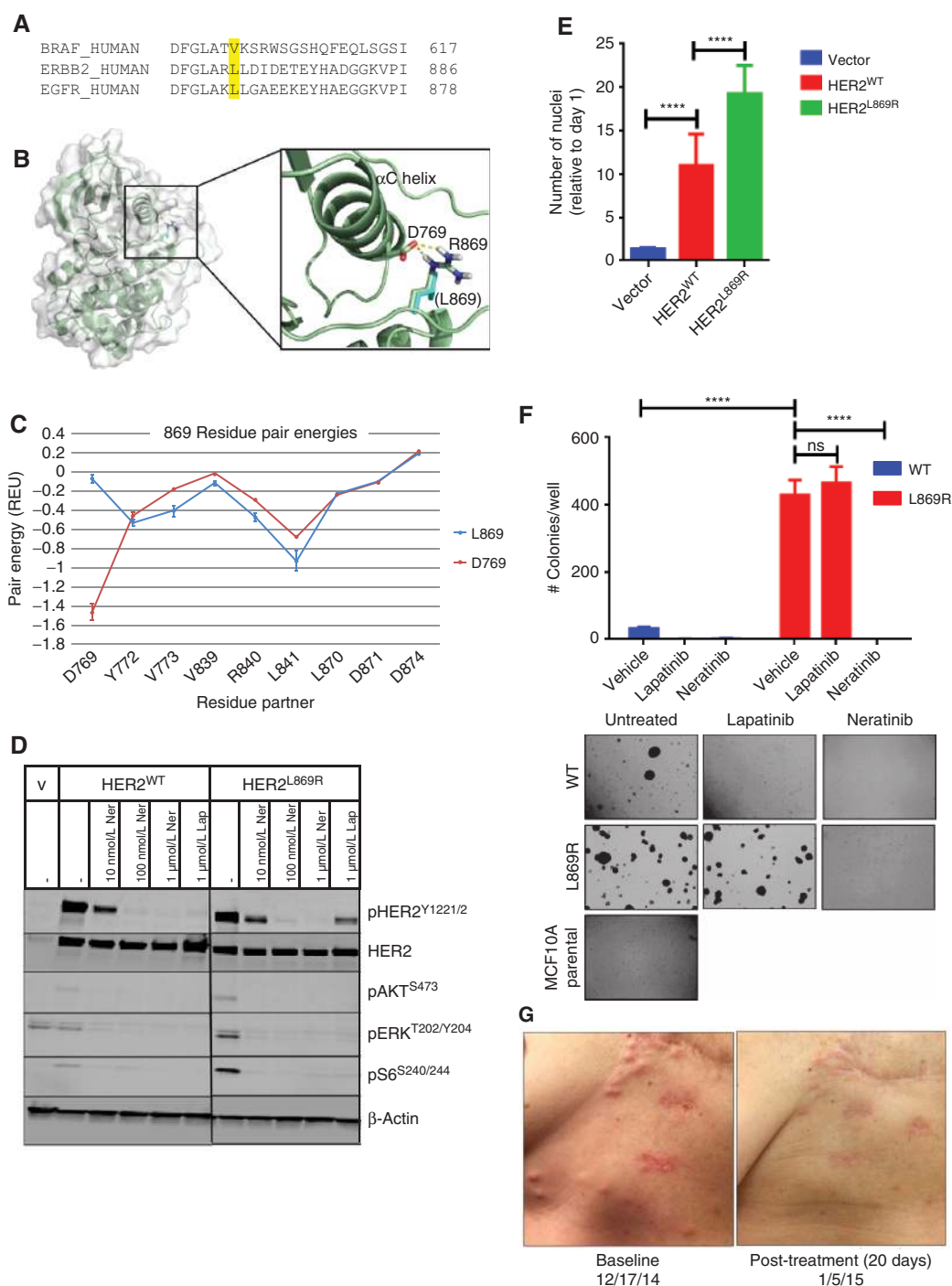
helix, resulting in destabilization of the inactive conformation of the kinase, similar to EGFR<sup>L858R</sup> (14).

On the basis of these structural data, we hypothesized that HER2<sup>L869R</sup> would display increased signaling and transforming capacity. To test this, we stably transduced MCF10A breast epithelial cells with lentiviral vectors encoding HER2 wild-type (WT) or HER2<sup>L869R</sup>. Cells expressing HER2<sup>L869R</sup> exhibited increased phosphorylation of AKT, ERK, and S6, which were blocked by neratinib (Fig. 1D). Phosphorylation of HER2<sup>WT</sup>, but not HER2<sup>L869R</sup>, was blocked by the reversible HER2/EGFR TKI lapatinib, whereas neratinib inhibited phosphorylation of both WT and mutant receptors. Expression of HER2<sup>L869R</sup> enhanced MCF10A cell proliferation in growth factor-depleted media (Fig. 1E) and colony formation in three-dimensional (3-D) Matrigel in the absence of EGF and insulin (Fig. 1F) compared with MCF10A/HER2<sup>WT</sup> cells. Growth of MCF10A/HER2<sup>L869R</sup> cells was inhibited by neratinib but not by lapatinib, whereas the HER2<sup>WT</sup> cells were sensitive to both TKIs. With these supporting data, the patient was enrolled in a clinical trial with single-agent neratinib (NCT01953926). Upon treatment, the patient exhibited an excellent clinical response, showing near disappearance of multiple skin metastases after 20 days (Fig. 1G), and a 77% reduction in marker lesions by RECIST criteria after 8 weeks.

Because the patient harbored a co-occurring *ERBB3*<sup>E928G</sup> mutation, a known activating mutation in *HER3* (15), we next asked whether HER2<sup>L869R</sup> and HER3<sup>E928G</sup> might co-operate to drive HER2 signaling. Mutations in *ERBB2* and *ERBB3* often co-occur in cancer. In the American Association for Cancer Research (AACR) Project GENIE dataset (>18,000 sequenced tumors), 8.3% of *ERBB2*-mutated cancers also harbor mutations in *ERBB3*, whereas only 2.3% of *ERBB2* WT cancers have *ERBB3* mutations ( $q$  value =  $1.3 \times 10^{-10}$ ; www.cbioportal.org/genie). *ERBB2*<sup>L869R</sup> and *ERBB3*<sup>E928G</sup> were found to co-occur in another breast cancer case in the METABRIC dataset (16). Structural modeling of the HER2<sup>L869R</sup>/HER3<sup>E928G</sup> double mutant predicted that the HER3 mutation, located at the dimer interface, may enhance heterodimerization of the kinase domains through decreased bulk and electrostatic repulsion (Supplementary Fig. S1A). Calculating the change in free energy of WT heterodimers compared with mutant heterodimers demonstrated a significant difference in the capacity of the latter to bind to one another (Supplementary Fig. S1B). Furthermore, coexpression of the HER2<sup>L869R</sup> and HER3<sup>E928G</sup> intracellular domains resulted in enhanced transphosphorylation of HER3 and ERK as substrates compared with that induced by expression of either mutant alone (Supplementary Fig. S1C). Phosphorylation of mutant HER2 and HER3, as well as the elevated downstream signaling induced by the expression of both mutants, was blocked by treatment with neratinib (Supplementary Fig. S1D). These data suggest that these co-occurring mutations in *ERBB2* and *ERBB3* enhance ERBB signaling output, which, in turn, can be blocked by neratinib.

### Acquired HER2<sup>T798I</sup> Mediates Neratinib Resistance

After 5 months on therapy, the patient developed a painful metastasis in the sternum. The addition of the ER antagonist fulvestrant to neratinib induced a prompt symptomatic and clinical response. After 10 additional months on the combination, the patient progressed with new skin metastases. Targeted



**Figure 1.** HER2<sup>L869R</sup> exhibits a gain-of-function phenotype that is blocked by neratinib. **A**, The amino acid sequences of human BRAF, ERBB2, and EGFR were aligned using Clustal Omega. BRAF<sup>V600</sup>, ERBB2<sup>L869</sup>, and EGFR<sup>L861</sup> residues are highlighted in yellow. **B**, The structure of HER2<sup>L869R</sup> was modeled. The mutation from leucine (cyan) to arginine (highlighted in blue) permits favorable charge interaction (dashed yellow lines) with Asp769. **C**, Residue pair energies involving residue 869 reveal the addition of a strong attractive (negative) interaction at Asp769 in the HER2<sup>L869R</sup> model. **D**, MCF10A cells stably expressing HER2<sup>WT</sup> or HER2<sup>L869R</sup> were treated with vehicle (V; DMSO), 0.01 to 1.0 μmol/L neratinib (Ner), or 1 μmol/L lapatinib (Lap) for 4 hours in serum-free media. Cell lysates were probed with the indicated antibodies. Scans are all from the same gel/film; the vertical black line indicates an irrelevant lane that was removed from the figure for clarity. **E**, Stably transduced MCF10A cells were seeded in 96-well plates in MCF10A starvation media (1% charcoal-stripped serum, no EGF). After 7 days, nuclei were stained with Hoechst and scored using the ImageXpress system. Data points represent the average ± SD of four replicate wells (\*\*\*\*,  $P < 0.0001$ , ANOVA followed by Tukey multiple comparisons test). **F**, Stably transduced MCF10A cells were plated in 3-D Matrigel in the presence of the indicated drugs (100 nmol/L). Colonies were grown in media containing 5% charcoal-stripped serum without EGF and insulin. After approximately 2 weeks, colonies were stained with MTT and counted using the GelCount system. ns, not significant. Data represent the average ± SD of three replicates. Representative fields (10× objective) of wells are shown below (\*\*\*\*,  $P < 0.0001$ , ANOVA followed by Tukey multiple comparisons test). **G**, Chest wall skin metastases of patient with invasive lobular breast cancer harboring HER2<sup>L869R</sup> at baseline and 20 days after starting treatment with neratinib.



tissue-based NGS analysis of DNA from a new skin metastasis and plasma tumor, cell-free DNA (cfDNA; Guardant360) revealed that *ERBB2*<sup>L869R</sup> remained (44% allele frequency and 8.7% cfDNA, respectively; Supplementary Table S3). *ERBB3*<sup>E928G</sup> remained in the post-treatment biopsy as well. *ERBB2*<sup>T798I</sup> was found in plasma (1.3% cfDNA), but not in DNA from the synchronous skin metastasis. Additional single-gene deep sequencing of plasma *ERBB2* using two rounds of targeted capture (average >4,000 reads per sample) in an independent plasma sample from that used for the Guardant360 test failed to identify *ERBB2*<sup>T798I</sup> in any of the plasma samples obtained at study enrollment or during the first 9 cycles of neratinib, but increased to 1.0% of reads at the time of clinical progression (Fig. 2A; Supplementary Table S4). In contrast, *ERBB2*<sup>L869R</sup> was detected in 6.8% of reads in the pretreatment sample, decreasing considerably during therapy, and rebounding up to 15.2% at progression. These data suggest that *ERBB2*<sup>T798I</sup> was acquired during neratinib therapy.

*HER2*<sup>T798I</sup> is homologous to *EGFR*<sup>T790M</sup> and imatinib-resistant *KIT*<sup>T670I</sup> in gastrointestinal stromal tumors (Fig. 2B). *EGFR*<sup>T790M</sup> drives resistance to first- and second-generation *EGFR* TKIs in NSCLC by two mechanisms: first, by mediating steric hindrance of ATP-competitive drugs, and second, by increasing the affinity of ATP, resulting in enhanced phosphotransfer and kinase activity (17). To determine whether *HER2*<sup>T798I</sup> functions in a similar manner, we constructed computational models of *HER2*<sup>WT</sup> and *HER2*<sup>T798I</sup> bound to neratinib. We found that the increased bulk of the isoleucine at position 798 would result in steric hindrance when neratinib binds (Fig. 2C). The closest approach between nonhydrogen atoms from residue T798 to neratinib is 4.1 Å in *HER2*<sup>WT</sup>, whereas this distance is reduced to 3.6 Å in *HER2*<sup>T798I</sup>, resulting in a reduced size of the binding pocket. Therefore, the isoleucine substitution at position 798 is expected to reduce neratinib binding.

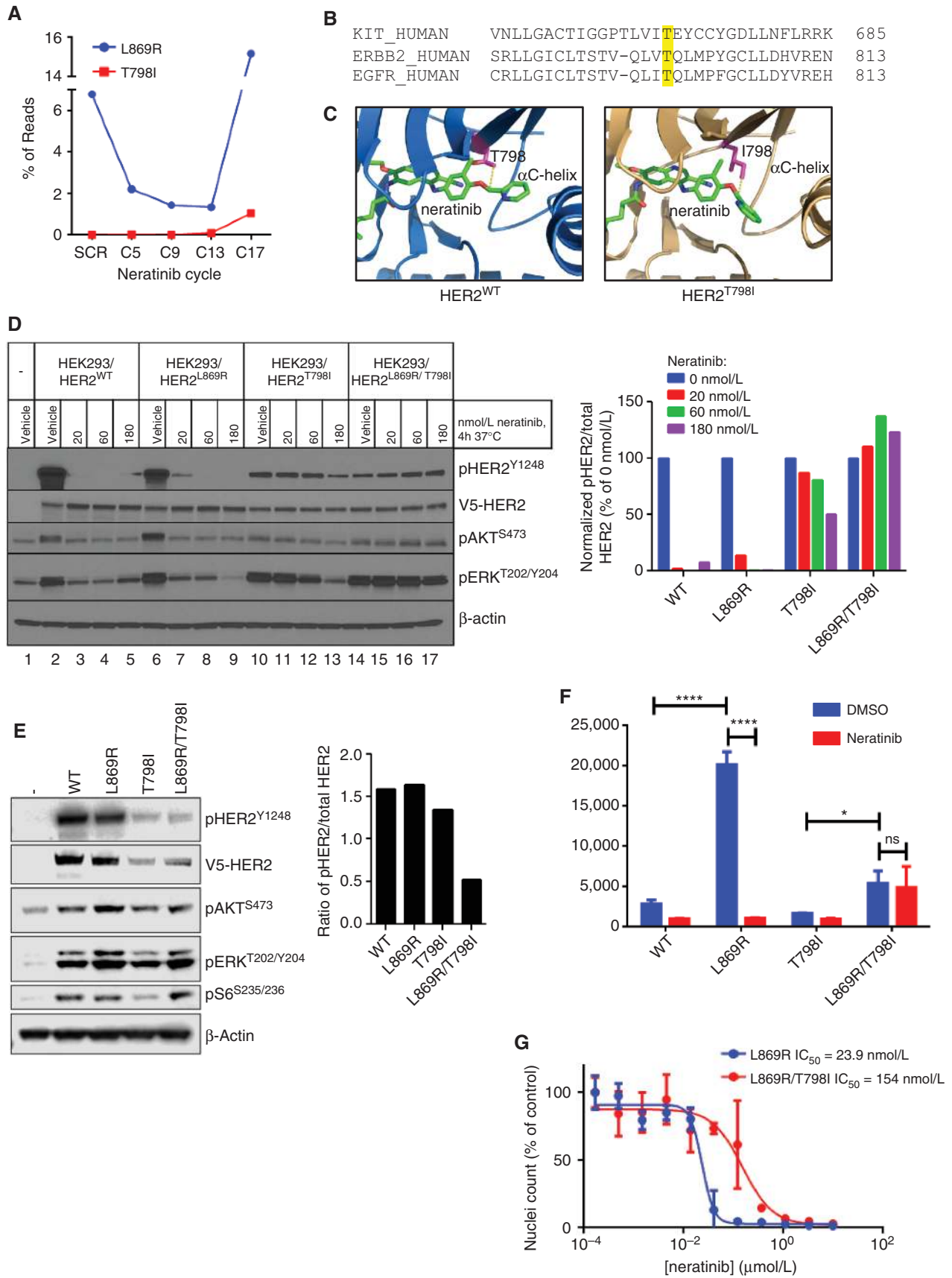
Next, we asked whether the T798I mutation would block neratinib action. HEK293 cells transfected with *HER2*<sup>WT</sup>, *HER2*<sup>L869R</sup>, *HER2*<sup>T798I</sup>, or *HER2*<sup>L869R/T798I</sup> (both mutations *in cis*) were treated with increasing doses of neratinib for 4 hours. Low doses of neratinib (20 nmol/L) blocked pHER2, pAKT, and pERK in cells expressing *HER2*<sup>WT</sup> or *HER2*<sup>L869R</sup>, but not in cells expressing *HER2*<sup>T798I</sup> or *HER2*<sup>L869R/T798I</sup> treated with up to 180 nmol/L neratinib (Fig. 2D). To confirm these findings, we stably transduced MCF10A cells with WT and mutant *HER2*. We noted that *HER2*<sup>T798I</sup> and *HER2*<sup>L869R</sup>

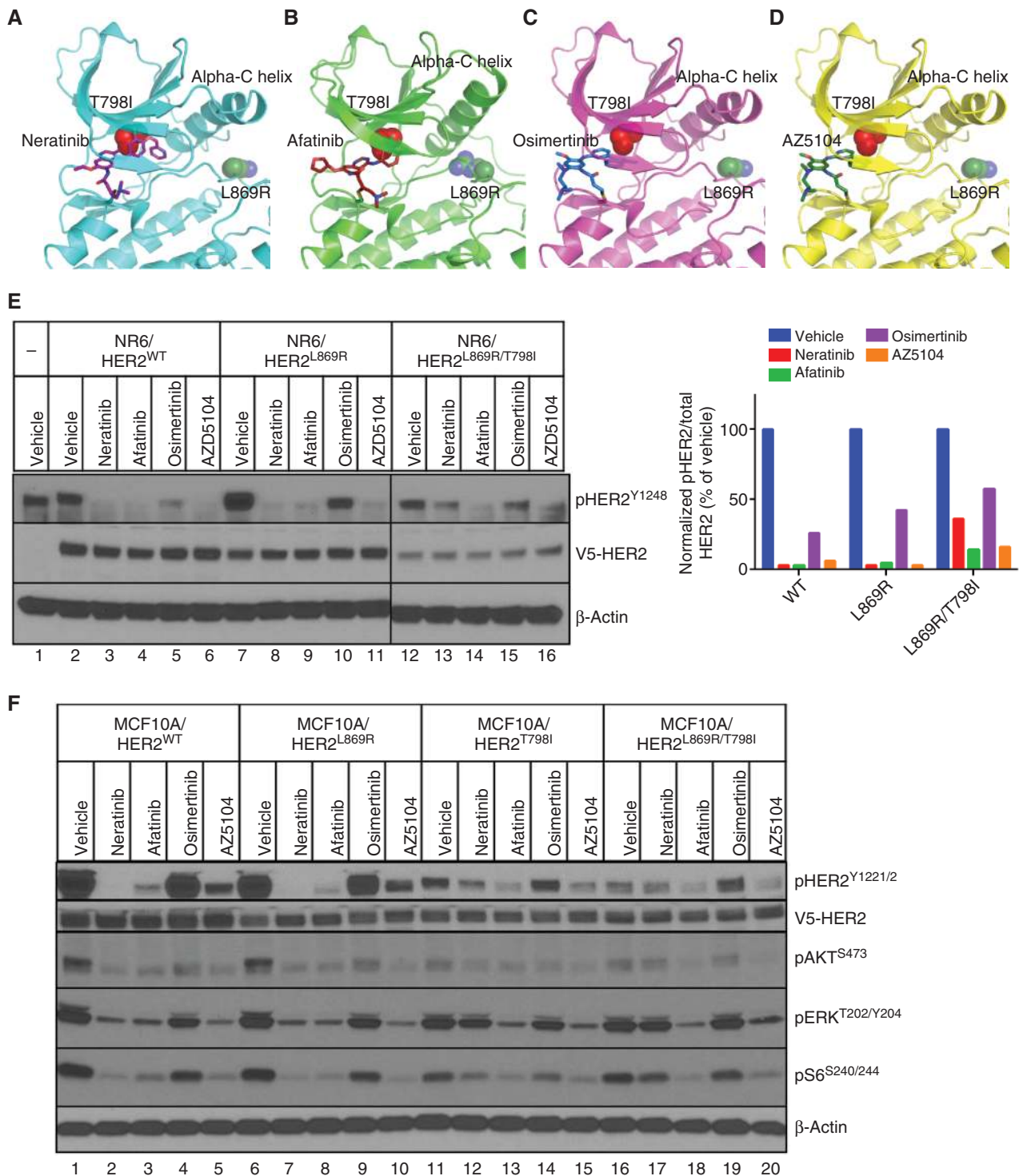
were poorly expressed in HEK293 and MCF10A cells (Fig. 2D and E). Treatment with the proteasome inhibitor MG132 for 24 hours restored expression of the T798I mutants (Supplementary Fig. S2), suggesting that this mutation decreases protein stability. Cells expressing *HER2*<sup>L869R</sup> or *HER2*<sup>L869R/T798I</sup>, but not *HER2*<sup>T798I</sup> alone, displayed enhanced pAKT, pERK, and pS6 (Fig. 2E). Furthermore, although *HER2*<sup>L869R</sup> and *HER2*<sup>L869R/T798I</sup> induced EGF-independent MCF10A cell proliferation, *HER2*<sup>T798I</sup> did not (Fig. 2F). Although untreated MCF10A/*HER2*<sup>L869R/T798I</sup> cells did not proliferate as fast as cells expressing *HER2*<sup>L869R</sup>, they were the only cells that grew in the presence of neratinib. A similar slow growth rate has been reported in *EGFR* TKI-resistant cell lines and patients' tumors harboring *EGFR*<sup>T790M</sup> (18). MCF10A cells expressing both mutations displayed reduced sensitivity to neratinib (IC<sub>50</sub> = 154 nmol/L) compared with cells expressing *HER2*<sup>L869R</sup> (IC<sub>50</sub> = 23.9 nmol/L; Fig. 2G). These results suggest that, like *EGFR*<sup>T790M</sup> for gefitinib and erlotinib, *HER2*<sup>T798I</sup> confers a growth advantage in the presence of neratinib.

The lack of transforming capacity of *HER2*<sup>T798I</sup> alone suggests that it is not a driver oncogene, but an acquired alteration as a result of therapeutic pressure. Consistent with this speculation, *HER2*<sup>T798I</sup> is exceedingly rare in tumors from patients not treated with *HER2* TKIs (Supplementary Table S2). Of all of the tumors sequenced in the cBioPortal, COSMIC, Foundation Medicine, and Guardant Health databases (more than 100,000 samples sequenced in all), *HER2*<sup>T798I</sup> was found in only one colorectal cancer cell line (Foundation Medicine) and one endometrial cancer cell line (Cancer Cell Line Encyclopedia), strongly suggesting that in the patient reported herein, T798I was acquired due to selective pressure of neratinib treatment.

We next examined a panel of other irreversible *EGFR*/*HER2* TKIs for their ability to block *HER2*<sup>L869R/T798I</sup>. These included afatinib, a covalent *EGFR*/*HER2* inhibitor, the *EGFR* inhibitor osimertinib (AZD9291), which exhibits selectivity against mutant *EGFR* (including T790M) but does not block WT *HER2* (19), and AZ5104, an osimertinib metabolite that inhibits WT *HER2* and *EGFR* (20). We performed computational modeling of *HER2*<sup>L869R/T798I</sup> bound to neratinib, afatinib, osimertinib, and AZ5104 (Fig. 3A–D). These small molecules are expected to bind *HER2* using the same mechanism and position by which they bind *EGFR*. By analogy with *EGFR*, the *HER2* kinase is predicted to adopt distinct conformations when bound by each inhibitor. Afatinib and neratinib have covalent binding modes that project deeply into the

**Figure 2.** *HER2*<sup>T798I</sup> induces acquired resistance to neratinib. **A**, The patient's plasma was drawn at the time of clinical trial screening (SCR) and the indicated cycles of neratinib therapy (1 cycle = 28 days). Plasma cfDNA was subjected to *ERBB2* targeted capture and sequenced. **B**, The amino acid sequences of human *KIT*, *ERBB2*, and *EGFR* were aligned using Clustal Omega. *KIT*<sup>T670I</sup>, *ERBB2*<sup>T798I</sup>, and *EGFR*<sup>T790M</sup> gatekeeper residues are highlighted in yellow. **C**, The structure of *HER2*<sup>WT</sup> and a model of *HER2*<sup>T798I</sup> are shown with neratinib bound to the kinase pocket. The threonine (WT) or isoleucine (mutant) residue at position 798 is shown in pink. **D**, HEK293 cells were transiently transfected with V5-tagged *HER2*<sup>WT</sup>, *HER2*<sup>L869R</sup>, *HER2*<sup>T798I</sup>, or *HER2*<sup>L869R/T798I</sup> and treated with the indicated concentrations of neratinib for 4 hours in serum-free media. Cell lysates were subjected to immunoblot analyses with the indicated antibodies. The bar graph represents quantification of immunoblot bands using ImageJ software. **E**, MCF10A cells stably expressing V5-tagged *HER2*<sup>WT</sup>, *HER2*<sup>L869R</sup>, *HER2*<sup>T798I</sup>, or *HER2*<sup>L869R/T798I</sup> were cultured in MCF10A growth factor–depleted media. Cell lysates were subjected to immunoblot analyses with the indicated antibodies. The bar graph represents quantification of immunoblot bands using ImageJ software. **F**, MCF10A cells from **E** were treated with ±123 nmol/L neratinib in growth factor–depleted media for 6 days. Nuclei were stained with Hoechst and scored using the ImageXpress system. ns, not significant. Data represent the average ± SD of four replicate wells (\*, *P* < 0.05; \*\*\*\*, *P* < 0.0001, ANOVA followed by Tukey multiple comparisons test). **G**, MCF10A cells stably expressing *HER2*<sup>L869R</sup> (blue) or *HER2*<sup>L869R/T798I</sup> (red) were treated with increasing concentrations of neratinib for 6 days. Nuclei were stained with Hoechst and scored using the ImageXpress system. Data represent the average ± SD of four replicate wells. IC<sub>50</sub> values were calculated using GraphPad Prism.





**Figure 3.** Afatinib and AZ5104 block HER2<sup>L869R/T798I</sup> signaling. **A–D**, Computational modeling of the HER2 kinase domain in complex with neratinib (**A**), afatinib (**B**), osimertinib (**C**), and AZ5104 (**D**) was performed. The N-terminal lobe and part of the C-terminal lobe of the tyrosine kinase domain (TKD) is shown in ribbon style. Each inhibitor is represented as sticks bound in the substrate-binding pocket. The T798I mutation is shown as red spheres deep in the pocket. The L869R mutation is shown as blue and green spheres on the far side of the alpha-C helix. **E**, NR6 cells stably expressing V5-tagged HER2<sup>WT</sup>, HER2<sup>L869R</sup>, HER2<sup>T798I</sup>, or HER2<sup>L869R/T798I</sup> (LR/TI) were treated with the indicated drugs at 100 nmol/L for 4 hours in serum-free media. Cell lysates were subjected to immunoblot analyses with the indicated antibodies. Scans are all from the same gel/film; the vertical black line indicates an irrelevant lane that was removed from the figure for clarity. The bar graph represents quantification of immunoblot bands using ImageJ software. **F**, Stably transduced MCF10A cells were treated with the indicated drugs at 100 nmol/L for 4 hours in EGF- and serum-free media. Cell lysates were subjected to immunoblot analyses with the indicated antibodies as described in Methods.

substrate binding pocket of the HER2 kinase (Fig. 3A and B). The sterically larger side chain of HER2<sup>T798I</sup> decreases the available space and decreases the polar character of the binding pocket. This is predicted to affect neratinib binding, which, by being the largest of these small molecules, extends the deepest into the pocket. Although afatinib is predicted to make slight contact with T798I, it does not insert as far into the tunnel as neratinib does. Osimertinib and AZ5104 are predicted to bind much less deeply on the lip of the pocket (Fig. 3C and D). On the basis of these studies, HER2<sup>T798I</sup> is predicted to disrupt neratinib binding, but is not expected to significantly affect the binding of afatinib, osimertinib, or AZ5104.

We next tested the ability of the panel of inhibitors to block mutant HER2 in stably transduced NR6 mouse fibroblasts, which lack endogenous EGFR (21), and MCF10A cells. In both cell types, neratinib more efficiently blocked HER2 phosphorylation in cells expressing HER2<sup>WT</sup> or HER2<sup>L869R</sup> compared with cells expressing HER2<sup>L869R/T798I</sup> (Fig. 3E and F). Treatment with afatinib and AZ5104 blocked phosphorylation of HER2<sup>WT</sup> as well as both HER2 mutants. In contrast, osimertinib failed to inhibit HER2<sup>WT</sup>, HER2<sup>L869R</sup>, or HER2<sup>T798I</sup>. Inhibition of pAKT, pERK, and pS6 with all small molecules mirrored that of pHER2 in MCF10A cells (Fig. 3F).

MCF10A/HER2<sup>L869R</sup> and MCF10A/HER2<sup>L869R/T798I</sup> were highly sensitive to afatinib and AZ5104 in growth factor–depleted media, whereas higher doses of osimertinib were required to block the growth of both cell types (Fig. 4A). Neratinib and AZ5104 showed similar IC<sub>50</sub> values in HER2<sup>L869R</sup>-expressing cells, whereas neratinib was less effective against HER2<sup>L869R/T798I</sup>-expressing cells. In 3-D Matrigel, 100 nmol/L of neratinib, afatinib, or AZ5104 completely blocked acini formation by MCF10A/HER2<sup>L869R</sup> cells, whereas 100 nmol/L of osimertinib only slightly suppressed acini growth (Fig. 4B). Both neratinib and osimertinib failed to suppress growth of MCF10A/HER2<sup>L869R/T798I</sup> cells in 3-D Matrigel, whereas this was completely blocked by afatinib and AZ5104, suggesting that the latter two inhibitors are able to overcome HER2<sup>T798I</sup>-mediated drug resistance.

Recent reports have proposed the acquisition of HER2 mutations in patients with HER2<sup>WT</sup> amplification treated with anti-HER2 therapies (22). In addition, neratinib has shown clinical activity and is being used in patients with HER2<sup>WT</sup> amplification (23). Thus, we tested whether a HER2 gatekeeper mutation would confer resistance to neratinib when present in a background of HER2<sup>WT</sup> amplification. We used HER2-amplified BT474 cells stably expressing HER2<sup>T798M</sup>, which we previously reported to be lapatinib resistant (24). BT474<sup>GFP</sup> control cells and BT474/HER2<sup>T798M</sup> cells were treated with vehicle (DMSO), lapatinib, neratinib, afatinib, osimertinib, or AZ5104. Lapatinib failed to suppress pHER2, pAKT, pERK, and pS6 in HER2<sup>T798M</sup>-expressing cells (Fig. 4C). Treatment with neratinib inhibited pHER2, pAKT, and pS6 in BT474<sup>GFP</sup> cells but not in BT474/HER2<sup>T798M</sup> cells. Consistent with the findings in MCF10A cells, afatinib and AZ5104, but not osimertinib, blocked pAKT, pERK, and pS6 in both BT474<sup>GFP</sup> and BT474/HER2<sup>T798M</sup> cells. As only approximately 3% of the ERBB2 alleles in the BT474/HER2<sup>T798M</sup> cells harbor the mutation (24), these data suggest that just a few HER2<sup>T798M</sup> alleles can confer resistance to neratinib, but not afatinib, in cells with HER2<sup>WT</sup> gene amplification.

## DISCUSSION

We report herein the identification of a HER2<sup>T798I</sup> gatekeeper mutation in a patient with HER2-mutant, nonamplified breast cancer with acquired resistance to neratinib. Structural modeling showed that the T798I mutation results in a steric clash with neratinib, which would reduce drug binding. HER2<sup>T798I</sup> directly promoted resistance to neratinib in lentivirally transduced cell lines. In contrast to neratinib, afatinib and the metabolite of osimertinib, AZ5104, blocked HER2<sup>T798I</sup>-induced signaling and cell growth.

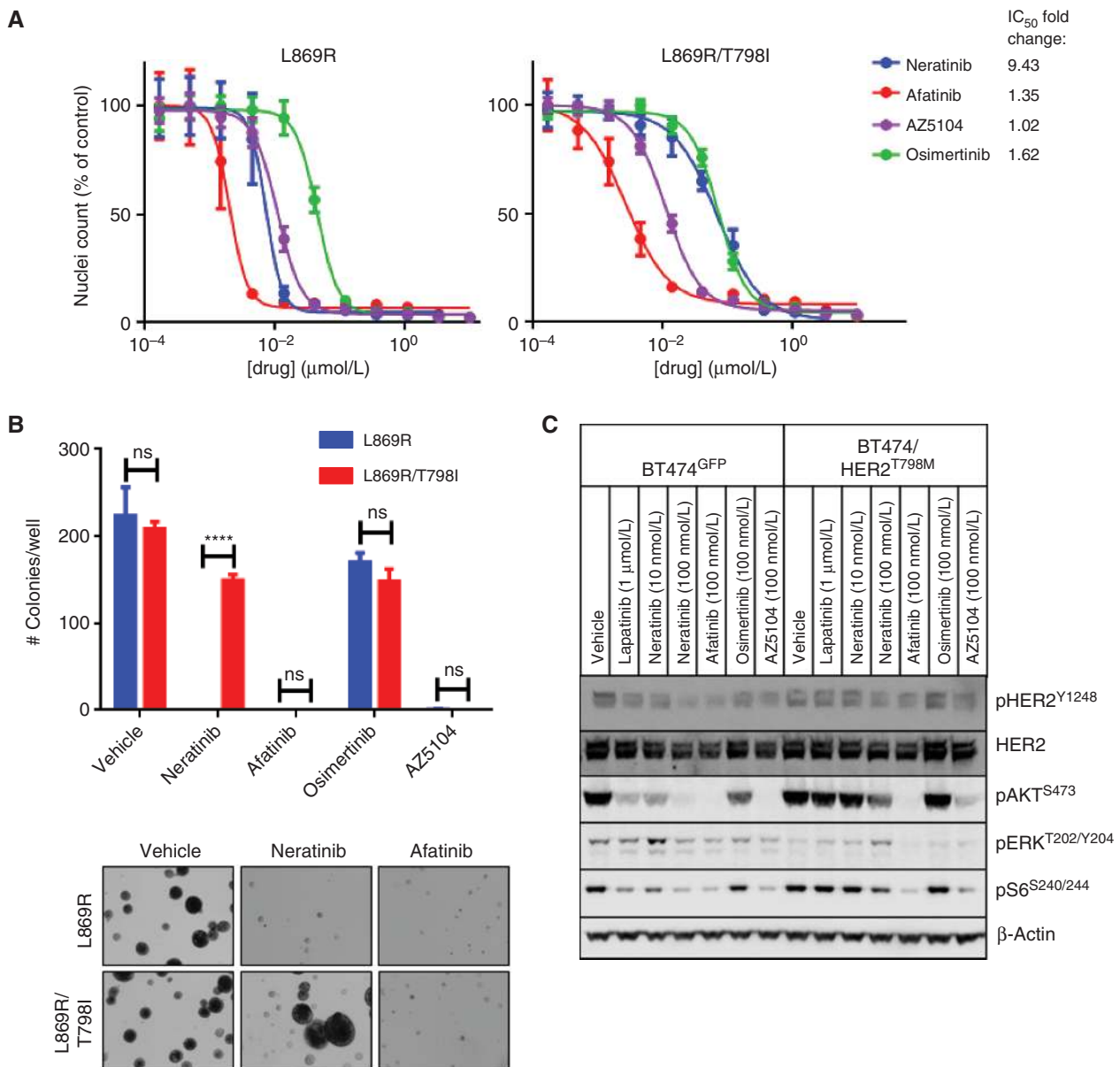
Although the initial neratinib-sensitizing HER2<sup>L869R</sup> mutation induced constitutive phosphorylation of AKT, ERK, and S6 and displayed gain-of-function activity when expressed in breast epithelial cells (Fig. 1), we failed to observe increased phosphorylation of this mutant compared with HER2<sup>WT</sup> (Figs. 1D and 2D and E). We speculate that the L869R mutation likely removes autoinhibitory interactions, thus placing the kinase in a better position to interact with other ERBB receptors and adaptor proteins/downstream substrates (25, 26). Notably, the HER2-mutant cancer also harbored a known activating HER3<sup>E928G</sup> mutation (15). We speculate these comutations resulted in increased dependence on the ERBB pathway and contributed to the tumor's initial sensitivity to neratinib. Consistent with this speculation, preliminary results from the SUMMIT trial show that among 17 patients who exhibited clinical benefit from neratinib, 2 patients harbored ERBB3 missense mutations, whereas none of the 25 patients who did not benefit harbored ERBB3 alterations in their cancer (27).

Our findings parallel the identification of the EGFR<sup>T790M</sup> gatekeeper mutation in NSCLC resistant to EGFR inhibitors. We note that for EGFR, two nucleotides would need to be mutated to change the threonine codon at position 790 to an isoleucine [ACG (Thr) > ATA, ATC, or ATT (Ile)], whereas only one nucleotide change is needed for the T790M mutation (ACG > ATG). The opposite is true for ERBB2 [ACA (Thr) > ATA (Ile) vs. ACA > ATG (Met)]. Thus, it is easier for the tumor to mutate ERBB2 codon 798 to an isoleucine rather than a methionine.

EGFR<sup>T790M</sup> is reported to promote resistance by simultaneously increasing ATP affinity and decreasing drug binding (28). Although our data suggest that the HER2<sup>T798I</sup> mutation could affect neratinib binding through steric interactions, it could similarly affect ATP binding and kinase activity. Although the change in distance (0.5 Å) from residue 798 to neratinib could theoretically be accommodated by conformational changes, the structural evidence suggests that replacing a polar amino acid (Thr) with a hydrophobic residue (Ile) would decrease ATP affinity. The WT Thr side chain contains an -OH group that faces the ATP-binding site. In the AMP-PNP-bound crystal structure of EGFR (2GS7.pdb), that -OH group is within 3.4 Å of the N6 of AMP-PNP. Replacing the Thr with Ile would remove that favorable interaction and is expected to decrease ATP affinity. These structural assessments are consistent with our cell-based findings that T798I-expressing cells do not show increased HER2 phosphorylation, even when corrected for expression levels (Fig. 2D and E).

HER2<sup>T798I</sup> and EGFR<sup>T790M</sup> also differ in that the former is exceedingly rare in untreated tumors (Supplementary





**Figure 4.** Afatinib and AZ5104 block HER2<sup>L869R/T798I</sup>-induced growth. **A**, MCF10A cells stably expressing HER2<sup>L869R</sup> and HER2<sup>L869R/T798I</sup> were treated with increasing concentrations of neratinib, afatinib, osimertinib, or AZ5104. After 5 days, nuclei were stained with Hoechst and scored using the ImageXpress system. Data represent the average  $\pm$  SD of four replicate wells. The fold change in IC<sub>50</sub> values of MCF10A<sup>L869R/T798I</sup> cells relative to L869R cells is shown. **B**, Stably transduced MCF10A cells were plated in 3-D Matrigel in the presence of the indicated drugs (100 nmol/L). ns, not significant. After 9 days, colonies were stained with MTT and counted using the GelCount system. Data represent the average  $\pm$  SD of three replicates. Representative fields (10 $\times$  objective) of wells treated with vehicle (DMSO), 100 nmol/L neratinib, and 100 nmol/L afatinib are shown (\*\*\*\*,  $P < 0.0001$ , ANOVA followed by Tukey multiple comparisons test). **C**, BT474<sup>GFP</sup> (control) and BT474/HER2<sup>T798M</sup> were treated with the indicated drugs for 4 hours in serum-free media. Cell lysates were tested in immunoblot analyses using the indicated antibodies.

Table S2), whereas *EGFR*<sup>T790M</sup> also occurs in germline DNA and can promote lung cancer formation (29), suggesting that *EGFR*<sup>T790M</sup> itself is oncogenic. This is also consistent with the notion that *HER2*<sup>T798I</sup> alone is not oncogenic, but requires another activating mutation *in cis* (e.g., L869R) to promote HER2 signaling and oncogenic growth (Fig. 2).

We previously reported that a *HER2*<sup>T798M</sup> gatekeeper mutation increased HER2 autophosphorylation and association

of HER3 with the p85-regulatory subunit of PI3K (24). In the current study, HER2<sup>T798I</sup> alone did not appear to enhance HER2 signaling or HER2-induced proliferation more than HER2<sup>WT</sup> (Fig. 2E and F). This discrepancy may be due to differences in experimental conditions (i.e., serum starvation), differences between the Met and Ile residues, or lower expression of the mutant receptor compared with WT, which we observed in multiple cell lines expressing



T798I alone or *in cis* with L869R (Figs. 2E and 3E). We speculate that the decreased expression of the mutant may be due to decreased protein stability (Supplementary Fig. S2). Despite this decreased expression, MCF10A cells expressing HER2<sup>T798I/L869R</sup> displayed increased phosphorylation of HER2 signaling targets and EGF-independent proliferation compared with MCF10A/HER2<sup>WT</sup> cells, as well as robust growth in the presence of neratinib (Figs. 2E–G and 4B), altogether suggesting that even low levels of HER2<sup>T798I</sup> can promote neratinib resistance.

We are unable to determine whether *ERBB2*<sup>L869R</sup> and *ERBB2*<sup>T798I</sup> occur *in cis* in the patient's plasma, as these two mutations are 213 bp apart, longer than the length of cfDNA fragments shed from tumor cells. In NSCLC, *EGFR*<sup>T790M</sup> is usually found on the same allele as the initial TKI-sensitizing *EGFR* mutation (30), suggesting that the two *ERBB2* mutations may also occur *in cis*. In addition, the allele frequency of *ERBB2*<sup>T798I</sup> in plasma tumor cfDNA in the patient progressing on neratinib was lower than the frequency of *ERBB2*<sup>L869R</sup> (Fig. 2A; Supplementary Table S3) consistent with *HER2*<sup>L869R</sup> being the initial driver mutation, and *HER2*<sup>T798I</sup> representing an acquired subclonal drug-resistant mutation. A similar relationship is typically seen with somatic *EGFR*<sup>T790M</sup> in the plasma of patients progressing on EGFR inhibitors compared with the level of the original drug-sensitive EGFR mutation (31). We also note that *HER2*<sup>T798I</sup> was not found in a new skin metastasis synchronous with the progression on neratinib, suggesting spatially heterogeneous mechanisms of drug resistance. This finding is consistent with other reports where plasma may serve as a repository of different acquired drug-resistant mutations found in some but not all metastatic sites, whereas a tissue biopsy of a single lesion may produce a less complete picture, as suggested by studies with drug-resistant NSCLC expressing *EGFR*<sup>T790M</sup>. For example, a subset of patients with EGFR TKI-resistant NSCLC with *EGFR*<sup>T790M</sup> detected in plasma but not in a tumor biopsy still responded to osimertinib (32).

*PIK3CA*<sup>M1043I</sup>, an activating mutation in the p110 $\alpha$  catalytic subunit of PI3K (33), was found at 0.1% frequency in the same plasma sample where *HER2*<sup>T798I</sup> was first detected (Supplementary Table S3). *PIK3CA* mutations are associated with resistance to anti-HER2 therapy in HER2-overexpressing breast cancers (34). Whether *PIK3CA*<sup>M1043I</sup> contributes to a multifactorial resistance to neratinib is also possible but beyond the scope of this report. Although afatinib and neratinib are both irreversible covalent EGFR/HER2 TKIs, we found that afatinib, but not neratinib, was able to block *HER2*<sup>L869R/T798I</sup> activity. We speculate that because neratinib is larger than afatinib, the former is more likely to be affected by a steric clash with the bulkier isoleucine residue in *HER2*<sup>T798I</sup> (Fig. 3A and B). Treatment with low doses of afatinib (10 nmol/L), easily achievable in patients (35), completely blocked growth of MCF10A/*HER2*<sup>L869R/T798I</sup> cells, whereas treatment with neratinib at clinically achievable concentrations (36) failed to do so (Fig. 4A and B). We also observed moderate activity of the osimertinib metabolite AZ5104 (Fig. 4). However, this drug is not being developed independently of osimertinib, and only approximately 10% of osimertinib is metabolized into AZ5104 in humans (20).

Immediately following progression on neratinib, the patient was treated with capecitabine chemotherapy. The patient responded well and remains in a partial response approximately 1 year later. We repeated NGS of her plasma tumor DNA after approximately 6 months on capecitabine; *ERBB2*<sup>L869R</sup> cfDNA dropped to 0.4% and *ERBB2*<sup>T798I</sup> and *CCND1* amplification were no longer detectable, consistent with the decrease in tumor burden and the patient's clinical response. If the patient progresses on capecitabine and the *ERBB2* mutations are once again detectable, there will be strong consideration for treatment with afatinib at that time. As more patients with *HER2*-mutant cancers are treated with *HER2* TKIs such as neratinib, we expect that acquired *HER2*<sup>T798I</sup> may be observed more frequently. We propose that afatinib is active against *HER2*<sup>T798I</sup> and is an alternative worthy of clinical investigation in cancers harboring the *HER2* gatekeeper mutation. Finally, this report supports the development of *HER2*<sup>T798I</sup>-selective inhibitors that would spare the toxicity associated with therapeutic inhibition of WT ERBB receptors.

## METHODS

### *ERBB2* Single-Gene Targeted Capture

Extraction of cfDNA from plasma was performed using a fully automated QIAGEN platform, QIAAsymphony SP, and QIAAsymphony DSP Virus/Pathogen Midi Kit following centrifugation. Sequence libraries were prepared according to the KAPA Hyper protocol (Kapa Biosystems) with the ligation of Illumina sequence adaptors, followed by PCR amplification and clean-up. Barcoded libraries were hybridized with DNA probes targeting all coding exons of *ERBB2* (Integrated DNA Technologies) in two successive captures, using a protocol modified from the NimbleGen SeqCap Target Enrichment system. The first capture was incubated at 55°C for 16 hours, followed by postcapture washes and 16 cycles of PCR amplification. The second capture was incubated at 65°C for 4 hours, followed by postcapture washes and 3 to 5 cycles of PCR amplification. Captured libraries were sequenced on an Illumina HiSeq as paired-end 100-bp reads.

### Computational Modeling

Structural modeling of inhibitor-bound *HER2*<sup>WT</sup>, *HER2*<sup>L869R</sup>/*HER3*<sup>E928G</sup>, and *HER2*<sup>L869R/T798I</sup> was performed using Rosetta. Detailed procedures are available in Supplementary Methods.

### Cell Lines and Inhibitors

The MCF10A breast epithelial cells (ATCC CRL-10317; purchased in 2012) and HEK293 human embryonic kidney cells (ATCC CRL-1573; purchased in 2006) were from ATCC. Cell lines were authenticated by ATCC prior to purchase by the short tandem repeat method. The 293FT cells were purchased from Invitrogen (cat. no. R70007). The NR6 cells have been described previously (21), as have BT474<sup>GFP</sup> and BT474/*HER2*<sup>T798M</sup> (24). *ERBB2*<sup>T798M</sup> was verified by sequencing cDNA using primers for *ERBB2*. Other than routinely checking cell morphology for consistency with published images, no other authentication was performed.

The 293FT, HEK293, and NR6 cells were maintained in DMEM supplemented with 10% FBS and 1 $\times$  antibiotic-antimycotic (Gibco). BT474 cells were maintained in Improved Minimum Essential Media supplemented with 10% FBS, 1 $\times$  antibiotic-antimycotic, and 100  $\mu$ g/mL G418. MCF10A cells were maintained in MCF10A complete media (DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 10  $\mu$ g/mL insulin, 0.5  $\mu$ g/mL hydrocortisone, 0.1  $\mu$ g/mL cholera toxin, and 1 $\times$  antibiotic-antimycotic). For experiments under growth factor-depleted conditions, MCF10A cells were grown in

DMEM/F12 supplemented with 1% charcoal/dextran-stripped serum, 10  $\mu\text{g}/\text{mL}$  insulin, 0.5  $\mu\text{g}/\text{mL}$  hydrocortisone, 0.1  $\mu\text{g}/\text{mL}$  cholera toxin, and 1 $\times$  antibiotic-antimycotic. Cell lines were routinely evaluated for *Mycoplasma* contamination. All experiments were completed less than 2 months after thawing early-passage cells.

The following inhibitors were used: MG132 (Selleck Chemicals), lapatinib (LC Laboratories), neratinib (PUMA Biotechnology), afatinib (Selleck Chemicals), and osimertinib and AZ5104 (AstraZeneca Pharmaceuticals).

### Immunoblot Analysis

Cells were washed with PBS and lysed on ice in RIPA lysis buffer plus protease and phosphatase inhibitors. Protein concentration was measured using the BCA protein assay reagent (Pierce). Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Immunoreactive bands were detected by enhanced chemiluminescence following incubation with horseradish peroxidase-conjugated secondary antibodies (Promega). Detailed information on antibodies is available in Supplementary Methods. Immunoblot bands were quantified from inverted images using ImageJ software.

### Cell Growth Assays

MCF10A cells were seeded in black clear-bottom 96-well plates (Greiner Bio-One) at a density of 1,000 cells per well in growth factor-depleted media. The next day, media were replaced with 100  $\mu\text{L}$  media containing increasing amounts of inhibitor (0.17 nmol/L–10  $\mu\text{mol}/\text{L}$  in 3-fold dilutions). After 5 to 6 days, nuclei were stained with 10  $\mu\text{g}/\text{mL}$  Hoechst 33342 (Thermo Fisher Scientific) at 37°C for 20 minutes. Fluorescent nuclei were counted using the ImageXpress Micro XL automated microscope imager (Molecular Devices).

For 3-D growth assays, cells were seeded on growth factor-reduced Matrigel (BD Biosciences) in 48-well plates following published protocols (37). Inhibitors were added to the medium at the time of cell seeding. Fresh media and inhibitors were replenished every 3 days. Following 9 to 14 days, colonies were stained with 5 mg/mL MTT for 20 minutes. Plates were scanned and colonies measuring  $\geq 100 \mu\text{m}$  were counted using GelCount software (Oxford Optronix). Colonies were photographed using an Olympus DP10 camera mounted in an inverted microscope.

### Patient Studies

Informed consent was obtained from the patient described in this study. The clinical trial (NCT01953926) was conducted in accordance with the Declaration of Helsinki and approved by an Institutional Review Board.

### Statistical Analysis

All experiments were performed using at least three technical replicates and at least two independent times. *P* values were generated by ANOVA followed by Tukey multiple comparisons test unless otherwise indicated. Data are presented as mean  $\pm$  SD. IC<sub>50</sub> values were generated through GraphPad Prism (version 6.0).

Detailed descriptions of NGS, multiple sequence alignment, determination of mutation frequencies, transient transfections, and generation of stable cell lines are available in Supplementary Methods.

### Disclosure of Potential Conflicts of Interest

R. Lanman has ownership interest (including patents) in Guardant Health, Inc. D.M. Hyman reports receiving commercial research grants from AstraZeneca and Puma Biotechnology and is a consultant/advisory board member for Atara Biotherapeutics, Chugai, and CytomX. A.S. Lalani has ownership interest (including patents) in

Puma Biotechnology. C.M. Lovly is a consultant/advisory board member for Ariad, Clovis, Genoptix, Novartis, Pfizer, and Sequenom. C.L. Arteaga is a consultant for Puma Biotechnology, Inc. No potential conflicts of interest were disclosed by the other authors.

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