An Acquired HER2\textsuperscript{T798I} Gatekeeper Mutation Induces Resistance to Neratinib in a Patient with HER2 Mutant–Driven Breast Cancer

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ABSTRACT

We report a HER2\textsuperscript{T798I} gatekeeper mutation in a patient with HER2\textsuperscript{L869R}-mutant breast cancer with acquired resistance to neratinib. Laboratory studies suggested that HER2\textsuperscript{L869R} is a neratinib-sensitive, gain-of-function mutation that upon dimerization with mutant HER3\textsuperscript{E928G}, 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\textsuperscript{T798I} was detected in plasma tumor cell-free DNA. Structural modeling of this acquired mutation suggested that the increased bulk of isoleucine in HER2\textsuperscript{T798I} reduces neratinib binding. Neratinib blocked HER2-mediated signaling and growth in cells expressing HER2\textsuperscript{L869R} but not HER2\textsuperscript{L869R/T798I}. In contrast, afatinib and the osimertinib metabolite AZ5104 strongly suppressed HER2\textsuperscript{L869R/T798I}-induced signaling and cell growth. Acquisition of HER2\textsuperscript{T798I} upon development of resistance to neratinib in a breast cancer with an initial activating HER2 mutation suggests HER2\textsuperscript{L869R} is a driver mutation. HER2\textsuperscript{T798I}-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\textsuperscript{T798I} 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.

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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** (HER2) 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**-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 ABL1T315I, KIT1926E, and EGFR2779M, 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**

**HER2L869R 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**L869R (**HER2**L869R) 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**E928G, and amplification of **CCND1** and **FGF3**/4/19. Interrogation of the eBioPortal (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**L869R and one **L869Q** mutation, including 12 breast cancers (Supplementary Table S2). In addition, a recent study reported four instances of **ERBB2**L869R 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**L869R is homologous to BRAFV600E, a gain-of-function mutation found in >50% of melanomas (11), and EGFRL858R/R, 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 EGFRL858R (14).

On the basis of these structural data, we hypothesized that **HER2**L869R 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**L869R. Cells expressing **HER2**L869R exhibited increased phosphorylation of AKT, ERK, and S6, which were blocked by neratinib (Fig. 1D). Phosphorylation of **HER2**WT, but not **HER2**L869R, was blocked by the reversible HER2/EGFR TKI lapatinib, whereas neratinib inhibited phosphorylation of both WT and mutant receptors. Expression of **HER2**L869R 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/HER2WT cells. Growth of MCF10A/HER2L869R cells was inhibited by neratinib but not by lapatinib, whereas the **HER2**WT 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**E928G mutation, a known activating mutation in **HER3** (15), we next asked whether **HER2**L869R and **HER3**E928G 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 (16) database (>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 × 10−12; www.cbioportal.org/genie). **ERBB2**L869R and **ERBB3**E928G were found to co-occur in another breast cancer case in the METABRIC dataset (16). Structural modeling of the HER2L869R/HER3E928G 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**L869R and **HER3**E928G 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 HER2T779E 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
HER2T798I Mediates Acquired Resistance to Neratinib

**Figure 1.** HER2L869R 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. BRAFV600, ERBB2L869, and EGFR-L861 residues are highlighted in yellow. **B,** The structure of HER2L869R 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 HER2L869R model. **D,** MCF10A cells stably expressing HER2WT or HER2L869R 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 HER2L869R at baseline and 20 days after starting treatment with neratinib.
tissue-based NGS analysis of DNA from a new metastasis and plasma tumor, cell-free DNA (cfDNA; Guardant360) revealed that ERBB2 T798I remained (44% allele frequency and 8.7% cfDNA, respectively; Supplementary Table S3). ERBB3 T928I remained in the post-treatment biopsy as well. ERBB2 T798I 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 T798I 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 L860R 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 T798I was acquired during neratinib therapy.

HER2 T798I is homologous to EGFR T790M and imatinib-resistant KIT T670I in gastrointestinal stromal tumors (Fig. 2B). EGFR T790M 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 T798I functions in a similar manner, we constructed computational models of HER2 WT and HER2 T798I 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 WT, whereas this distance is reduced to 3.6 Å in HER2 T798I, 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 WT, HER2 L860R, HER2 T798I, or HER2 L860R/T798I (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 WT or HER2 L860R, but not in cells expressing HER2 T798I or HER2 L860R/T798I 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 T798I and HER2 T798I/L860R 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 L860R, HER2 L860R/T798I but not HER2 T798I alone, displayed enhanced pAKT, pERK, and pS6 (Fig. 2E). Furthermore, although HER2 L860R and HER2 L860R/T798I induced EGFR-independent MCF10A cell proliferation, HER2 T798I did not (Fig. 2F). Although untreated MCF10A/HER2 L860R/T798I cells did not proliferate as fast as cells expressing HER2 L860R, 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 T790M (18). MCF10A cells expressing both mutations displayed reduced sensitivity to neratinib (IC50 = 154 nmol/L) compared with cells expressing HER2 L860R (IC50 = 23.9 nmol/L; Fig. 2G). These results suggest that, like EGFR T790M for gefitinib and erlotinib, HER2 T798I confers a growth advantage in the presence of neratinib.

The lack of transforming capacity of HER2 T798I alone suggests that it is not a driver oncogene, but an acquired alteration as a result of therapeutic pressure. Consistent with this speculation, HER2 T798I 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 T798I 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 L860R/T798I. 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 L860R/T798I 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
**HER2T798I Mediates Acquired Resistance to Neratinib**

**A**

% of Reads vs. Neratinib cycle

**B**

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**G**

**HER2T798I** IC50 = 23.9 nmol/L

**L869R/T798I** IC50 = 154 nmol/L
Figure 3. Afatinib and AZ5104 block HER2L869R/T798I 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 HER2WT, HER2L869R, HER2T798I, or HER2L869R/T798I (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*798I 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 more deeply on the lip of the pocket (Fig. 3C and D). On the basis of these studies, HER2*798I 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 HER2WT or HER2*L869R compared with cells expressing HER2*L869R/T798I (Fig. 3E and F). Treatment with afatinib and AZ5104 blocked phosphorylation of HER2WT as well as both HER2 mutants. In contrast, osimertinib failed to inhibit HER2WT, HER2*L869R, or HER2*798I. Inhibition of pAKT, pERK, and pS6 with all small molecules mirrored that of pHER2 in MCF10A cells (Fig. 3F). MCF10A/HER2*L869R and MCF10A/HER2*L869R/T798I 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 IC50 values in HER2*469M-expressing cells, whereas neratinib was less effective against 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 IC50 values in HER2*469M-expressing cells, whereas neratinib was less effective against afatinib and AZ5104, suggesting that the latter two inhibitors are able to overcome HER2*798I-mediated drug resistance.

Recent reports have proposed the acquisition of HER2 mutations in patients with HER2 WT amplification treated with anti-HER2 therapies (22). In addition, neratinib has shown clinical activity and is being used in patients with HER2 WT amplification (23). Thus, we tested whether a HER2 gatekeeper mutation would confer resistance to neratinib when present in a background of HER2 WT amplification. We used HER2-amplified BT474 cells stably expressing HER2*798M, which we previously reported to be lapatinib resistant (24). BT474*GFP control cells and BT474/HER2*798M cells were treated with vehicle (DMSO), lapatinib, neratinib, afatinib, osimertinib, or AZ5104. Lapatinib failed to suppress pHER2, pAKT, pERK, and pS6 in HER2*798M-expressing cells (Fig. 4C). Treatment with neratinib inhibited pHER2, pAKT, and pS6 in BT474*GFP cells but not in BT474/HER2*798M cells. Consistent with the findings in MCF10A cells, afatinib and AZ5104, but not osimertinib, blocked pAKT, pERK, and pS6 in both BT474*GFP and BT474/HER2*798M cells. As only approximately 3% of the ERBB2 alleles in the BT474/HER2*798M cells harbor the mutation (24), these data suggest that just a few HER2*798M alleles can confer resistance to neratinib, but not afatinib, in cells with HER2 WT gene amplification.

**DISCUSSION**

We report herein the identification of a HER2*798I 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*798I directly promoted resistance to neratinib in lentivirally transduced cell lines. In contrast to neratinib, afatinib and the metabolite of osimertinib, AZ5104, blocked HER2*798I-induced signaling and cell growth.

Although the initial neratinib-sensitizing HER2*469M 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 WT (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 HER2 E928K 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 23 patients who did not benefit harbored ERBB3 alterations in their cancer (27).

Our findings parallel the identification of the EGFR*790M 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*790M is reported to promote resistance by simultaneously increasing ATP affinity and decreasing drug binding (28). Although our data suggest that the HER2*798I 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*798I and EGFR*790M also differ in that the former is exceedingly rare in untreated tumors (Supplementary

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We report herein the identification of a HER2*798I 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*798I directly promoted resistance to neratinib in lentivirally transduced cell lines. In contrast to neratinib, afatinib and the metabolite of osimertinib, AZ5104, blocked HER2*798I-induced signaling and cell growth.

Although the initial neratinib-sensitizing HER2*469M 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 WT (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 HER2 E928K 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 23 patients who did not benefit harbored ERBB3 alterations in their cancer (27).

Our findings parallel the identification of the EGFR*790M 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*790M is reported to promote resistance by simultaneously increasing ATP affinity and decreasing drug binding (28). Although our data suggest that the HER2*798I 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*798I and EGFR*790M also differ in that the former is exceedingly rare in untreated tumors (Supplementary
Table S2), whereas EGFR<sub>T790M</sub> also occurs in germline DNA and can promote lung cancer formation (29), suggesting that EGFR<sub>T790M</sub> itself is oncogenic. This is also consistent with the notion that HER2<sub>T798M</sub> 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<sub>T798M</sub> gatekeeper mutation increased HER2 autophosphorylation and association of HER3 with the p85-regulatory subunit of PI3K (24). In the current study, HER2<sub>T798M</sub> alone did not appear to enhance HER2 signaling or HER2-induced proliferation more than HER2<sub>WT</sub> (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 HER2<sub>T798M</sub>.

Figure 4. Afatinib and AZ5104 block HER2<sub>L869R/T798I</sub>-induced growth. A, MCF10A cells stably expressing HER2<sub>L869R</sub> and HER2<sub>L869R/T798I</sub> 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 ± SD of four replicate wells. The fold change in IC<sub>50</sub> values of MCF10A<sub>L869R/T798I</sub> 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 ± SD of three replicates. Representative fields (10× 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<sub>GFP</sub> (control) and BT474/HER2<sub>T798M</sub> were treated with the indicated drugs for 4 hours in serum-free media. Cell lysates were tested in immunoblot analyses using the indicated antibodies.
HER2 T798I Mediates Acquired Resistance to Neratinib

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 T798I/L869R displayed increased phosphorylation of HER2 signaling targets and EGF-independent proliferation compared with MCF10A/HER2WT, as well as robust growth in the presence of neratinib (Figs. 2E and 4B), altogether suggesting that even low levels of HER2 T798I can promote neratinib resistance.

We are unable to determine whether ERBB2 L869R and ERBB2 T798I 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, EGF R T790M is usually found on the same allele as the initial TKI-sensitizing EGF R mutation (30), suggesting that the two ERBB2 mutations may also occur in cis. In addition, the allele frequency of ERBB2 T798I in plasma tumor cfDNA in the patient progressing on neratinib was lower than the frequency of ERBB2 L869R (Fig. 2A; Supplementary Table S3) consistent with HER2 T798I being the initial driver mutation, and HER2 T798I representing an acquired subclonal drug-resistant mutation. A similar relationship is typically seen with somatic EGF R T790M 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 T798I 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 EGF R T790M. For example, a subset of patients with EGFR TKI-resistant NSCLC with EGF R T790M detected in plasma but not in a tumor biopsy still responded to osimertinib (32).

PIK3CA M1043I, an activating mutation in the p110α catalytic subunit of PI3K (33), was found at 0.1% frequency in the same plasma sample where HER2 T798I was first detected (Supplementary Table S3). PIK3CA mutations are associated with resistance to anti-HER2 therapy in HER2-overexpressing breast cancers (34). Whether PIK3CA M1043I 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 L869R/T798I 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 T798I (Fig. 3A and B). Treatment with low doses of afatinib (10 nmol/L), easily achievable in patients (35), completely blocked growth of MCF10A/HER2 L869R/T798I 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 L869R cfDNA dropped to 0.4% and ERBB2 T798I 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 T798I may be observed more frequently. We propose that afatinib is active against HER2 T798I and is an alternative worthy of clinical investigation in cancers harboring the HER2 gatekeeper mutation. Finally, this report supports the development of HER2 T798I 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 QIAseq platform, QiAseq SP, and QiAseq SP 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 35°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 WT, HER2 L869R/HER3 T792G, and HER2 T798I/L869R 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 BT474GFP and BT474/HER2 T798M (24). MCF10A/HER2 T798I 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 1x antibiotic-antimycotic (Gibco). BT474 cells were maintained in Improved Minimum Essential Media supplemented with 10% FBS, 1x antibiotic-antimycotic, and 100 μg/mL G418. MCF10A cells were maintained in MCF10A complete media (DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, and 1x antibiotic-antimycotic). For experiments under growth factor-depleted conditions, MCF10A cells were grown in

Detailed procedures are available in Supplementary Methods.
DMEM/F12 supplemented with 1% charcoal/dextran-stripped serum, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.1 μg/mL chola
toxin, and 1x antibiotic-antimycotic. Cell lines were routinely evalu-
ated 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 AZS104 (Astra-
Zeneca 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 chemi-
luminescence 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 fac-
tor–depleted media. The next day, media were replaced with 100 μL
media containing increasing amounts of inhibitor (0.17 μmol/L–10
μmol/L in 3-fold dilutions). After 5 to 6 days, nuclei were stained with
10 μg/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 pro-
tocols (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 ≥100 μm
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 accord-
ance with the Declaration of Helsinki and approved by an Insti-
tutional Review Board.

**Statistical Analysis**

All experiments were performed using at least three technical repli-
cates and at least two independent times. P values were generated by
ANOVA followed by Tukey multiple comparisons test unless other-
wise indicated. Data are presented as mean ± SD. IC50 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. Lowy is a consultant/advisory board
member for Atraiad, Clovis, Genoptix, Novartis, Pfizer, and Sequenom.
C.L. Arteaga is a consultant for Puma Biotechnology. Inc. No poten-
tial conflicts of interest were disclosed by the other authors.

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et al. Oncogenic alterations in ERBB2/HER2 represent potential
therapeutic targets across tumors from diverse anatomic sites of
HER2T798I Mediates Acquired Resistance to Neratinib

Correction: An Acquired $\text{HER2}^{T798I}$ Gatekeeper Mutation Induces Resistance to Neratinib in a Patient with HER2 Mutant–Driven Breast Cancer


In the original version of this article (1), the stated disclosure of the author Carlos L. Arteaga is incorrect. The error has been corrected in the latest online HTML and PDF versions of the article. The authors regret this error.

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