Clinical Cancer Research

Dual Characteristics of Novel HER2 Kinase Domain Mutations in Response to HER2-Targeted Therapies in Human Breast Cancer

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Abstract

Purpose: Somatic mutations in the tyrosine kinase domain of human epidermal growth factor receptor 2 (HER2) may be an alternative mechanism to HER2 activation and can affect the sensitivity toward HER2-targeted therapies. We aimed to investigate the prevalence, clinicopathologic characteristics, and functional relevance of novel HER2 mutations in breast cancer.

Experimental Design: We performed Sanger sequencing of all exons of the *HER2* gene in 1,248 primary tumors and 18 paired metastatic samples. Novel HER2 mutations were functionally characterized.

Results: The total HER2 somatic mutation rate was 2.24% (28/1,248). Of the seven novel HER2 mutations, L768S and V773L were only detected in HER2-negative tumors, whereas K753E was found in HER2-positive disease. L768S and V773L mutations exhibited a significant increase in tyrosine kinase–specific activity and strongly increased the phosphorylation of signaling proteins in various cell lines. Xenograft experiments showed that NIH3T3 cells bearing the L768S and V773L mutations displayed more rapid growth. MCF10A, BT474, and MDA-MB-231 cells bearing the K753E mutation were resistant to lapatinib, but could be inhibited by neratinib. Finally, comparison of HER2 mutations in 18 pairs of primary and metastatic lesions revealed that the drug-resistant HER2 mutations (K753E and L755S) were enriched in metastatic lesions.

Conclusions: HER2-negative breast cancer with activating mutations can benefit from HER2-targeted therapies. Meanwhile, mutations in the HER2 kinase domain might be a key mechanism of resistance to HER2-targeted therapy, and irreversible tyrosine kinase inhibitors such as neratinib may offer alternative treatment options. *Clin Cancer Res; 22(19); 4859–69. ©2016 AACR.*

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Introduction

Breast cancer is the most common cancer diagnosed in women, accounting for approximately 23% of all cancer diagnoses and approximately 14% of cancer-related deaths in women worldwide (1). It is a heterogeneous disease that can be classified by molecular profiles into subtypes with unique clinicopathologic characteristics and responsiveness to targeted therapies (2, 3). As next-generation sequencing continues to reveal new information regarding the genetic make-up of different cancers, we are continuously identifying novel cancer-associated genetic alterations. However, we have yet to fully understand the functional consequences of these genetic alterations (4).

Human epidermal growth factor receptor 2 (HER2)-positive, or *HER2*-amplified, breast cancer accounts for approximately 20%–25% of invasive breast cancer and is associated with more aggressive tumor behavior, increased risk for progression, and decreased overall survival (5, 6). While HER2-targeted therapies such as trastuzumab and lapatinib have shown considerable efficacy in treating HER2-positive breast cancer patients (7–11), clinicians and researchers alike have increasingly observed primary and acquired drug resistance in HER2-positive breast cancer patients, greatly undermining the clinical value of these HER2-targeted drugs (12, 13). Extensive research has been conducted regarding the molecular mechanisms leading to trastuzumab and lapatinib resistance (11), including the overexpression or hyperexpression of other HER family receptors and their ligands, loss of PTEN resulting in the amplification of the PI3K/Akt/mTOR pathway, *PI3KCA* mutations, and Akt mutations or amplifications.

HER2 somatic mutations have been reported in various solid cancers, including breast cancer (14–20). At present, the incidence, clinicopathologic characteristics, and prognostic implications of HER2 somatic mutations are not well established, and their effects on the response of cancer to HER2-targeted treatment remains unclear. However, the study by Bose and colleagues has

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Translational Relevance

Somatic mutations in the tyrosine kinase domain of human epidermal growth factor receptor 2 (HER2) may be an alternative mechanism to HER2 activation and can affect the sensitivity toward HER2-targeted therapies. Our data imply that HER2-negative breast cancer with novel activating mutations can benefit from HER2-targeted therapies, while HER2-positive tumors harboring novel resistance mutations may not benefit from trastuzumab or lapatinib treatment, but irreversible tyrosine kinase inhibitors such as neratinib will offer alternative treatment options. Interestingly, the emergence of HER2 mutations in metastatic breast cancer indicates that mutations in the HER2 kinase domain might be a key mechanism of acquired resistance to HER2-targeted therapy. These findings will help to illustrate the potential clinical implications of HER2 somatic mutations, and assist in identifying patients who would benefit from HER2-targeted therapies and choosing the most beneficial treatment.

indicated that mutations in HER2 identify a distinctive functional phenotype of breast cancer (21), and it has been shown that gene mutations within the kinase domain of an oncoprotein can be a factor in drug sensitivity or resistance toward kinase inhibitors (22–25). Such evidence gives us cause to consider the clinical implications of HER2 mutations and their effects on HER2-targeted treatment response.

The purpose of our study was to assess the rate of HER2 somatic mutation in a Chinese breast cancer population and to functionally characterize novel HER2 mutations. In this study, we have uncovered seven novel HER2 mutations, and our characterization of these seven mutations using multiple experiment methods revealed one mutation possibly related to HER2-targeted drug resistance and two likely activating mutations. These findings were yet another step forward in determining the relationship between HER2 mutations and breast cancer characteristics.

Materials and Methods

Study cohorts

This study was approved by the Ethical Committee of Fudan University Shanghai Cancer Center (FUSCC, Shanghai, PR China), and each participant signed an informed consent document. Tumor samples were obtained from breast cancer patients treated at FUSCC between January 1, 2006 and December 31, 2010 according to the following criteria: (i) female patients diagnosed with unilateral disease; (ii) histologically confirmed invasive ductal carcinoma (IDC); (iii) patients without any evidence of metastasis at the time of diagnosis; and (iv) sufficient frozen tissues available for further research. Patients with breast carcinoma *in situ* (with or without microinvasion) or inflammatory breast cancer were excluded from our study, as were patients with any of the following circumstances: incomplete registration of patient clinical information, inability to extract required amounts of DNA from available patient blood and tissue samples, and the loss of contact with the patient to obtain follow-up data. After further exclusion of patients with an insufficient follow-up period, 102 consecutive HER2-positive breast cancer patients undergoing trastuzumab-based neoadjuvant therapy and a total of 1,146 patients (808 consecutive HER2-positive tumors and 338 HER2-negative tumors randomly selected from the 2346 consecutive patients with HER2-negative disease) receiving adjuvant therapy were included in our analysis (Supplementary Fig. S1). Pathologic examination of tumor specimens was carried out in the Department of Pathology at FUSCC. The status of ER, PR, and HER2 was reconfirmed by two experienced pathologists (Ruo-Hong Shui and Wen-Tao Yang) based on IHC and FISH. The cutoff of immunohistochemical staining for ER-negative and PR-negative status was less than 1% staining in the nuclei. HER2 status was considered negative when an IHC score was 0 or 1 or when HER2 amplification was absent (ratio<2.2), by FISH analysis.

Follow-up for the patients was completed on December 31, 2014. The median length of follow-up was 32.0 months [interquartile range (IQR), 16.8–42.4 months]. All of the patients were monitored every month during adjuvant therapy and then with physical examination and radiologic assessments every 6 months for the first two years and yearly thereafter. Relapse-free survival (RFS) events included the following: the first recurrence of invasive disease at a local, regional, or distant site; contralateral breast cancer; and death from any cause. Patients without RFS events were censored at the last follow-up.

Sample processing and mutational analysis

Pretreatment tumor samples were obtained via core-needle biopsy from HER2-positive patients undergoing neoadjuvant therapy, while surgical tumor samples were obtained from both HER2-positive and HER2-negative patients undergoing surgery at FUSCC. Tumor samples were obtained in accordance with the appropriate institutional review boards and well preserved in liquid nitrogen. Generally, tumor tissues were macrodissected to avoid the influence of stromal tissues (<20%). We used QIAamp DNA Mini Kits (Qiagen) to extract DNA from the tissues and paired blood samples. The quality and concentration of extracted DNA were determined using NanoDrop 2000 (Thermo Fisher Scientific). The extracted DNA was then used for mutation analysis.

We performed Sanger sequencing of all 27 exons of *HER2* in frozen tumor tissues and paired blood DNA for mutation detection. The primers for Sanger sequencing are listed in Supplementary Table S1. PCR amplification, purification of products, and sequencing were performed as described previously (26). The detected somatic mutations were confirmed by resequencing of the tumor and matched normal blood DNA from new PCR products.

Cell lines

MCF7, MCF10A, MDA-MB-231, NIH3T3, and BT474 cells were purchased from the ATCC and authenticated by short tandem repeat profiling conducted by ATCC on October 15, 2013. Liquid nitrogen stocks were created upon receipt, and the cells were stored in liquid nitrogen and used for no more than 6 months after being thawed. The cells were maintained in complete growth medium as described previously (21, 27). Inhibition of cell growth by lapatinib and neratinib was measured by the metabolic reduction of WST-8 [Cell Counting Kit-8 (CCK-8) cell proliferation assay; Dojindo Laboratories) as described previously (26), and a six-parameter nonlinear regression was used to calculate the

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half maximal inhibitory concentrations (IC_{50}) values using Sigma Plot 2001 software (Systat Software).

Antibodies and inhibitors

The following antibodies were purchased from Cell Signaling Technology: HER2, phospho-HER2 (Thr1221/Tyr1222), HER3, phospho-HER3 (Y1289), EGFR, phospho-EGFR (Tyr1173, Tyr1068), PLCγ, phospho-PLCγ (Tyr783), and GAPDH. Trastuzumab was obtained from Roche. Lapatinib and Neratinib were obtained from Selleck Chemicals.

In vitro kinase assays and protein structure visualization

HER2 somatic mutations were introduced into the HER2 kinase domain using the Quik Change II kit (Agilent Technologies). The desired constructs were verified by Sanger sequencing. Using baculovirus vectors, His 6–tagged HER2 wild-type (WT) and mutant (mut) kinase domain constructs were expressed as per the Bac-to-Bac system (Life Technologies). Using Ni-NTA-agarose beads, recombinant HER2 kinase domain proteins were purified to 70% to 80% purity (28). Radiometric kinase assays were conducted either with kinases in solution (monomers) or attached to the surface of liposomes to induce *in vitro* dimerization as detailed in published works (28). Statistical analysis of the *in vitro* kinase assays was conducted using Student *t* test. PyMol (Schrödinger) was used for protein structural alignments and visualizations.

Cloning and transfection of mutant and WT HER2 into cell lines

HER2 somatic mutations were introduced into the GV219 vector bearing HER2 cDNA using primers synthesized by GenePharma Co. Ltd. and *Pfx*50 DNA Polymerase (Life Technologies). These constructs were then shuttled into the pCDH1-CMV-EF1-Puro lentivector (System Biosciences) containing a puromycin-resistant marker using the In-Fusion HD Cloning System Kit (Clontech). The desired constructs were verified using Sanger sequencing. Transfection of pCDH constructs into target cells was carried out according to the reagent protocol for Lipofectamine 2000 (Thermo Fisher Scientific). Cell lines were subjected to one week of puromycin selection and were analyzed for transfection efficacy.

Three-dimensional culture in Matrigel

Basically, 4.0×10^3 MCF10A cells expressing mutant or HER2 WT were first seeded in growth factor–reduced Matrigel (BD Biosciences) in 8-well chamber slides (29). Lapatinib, neratinib, trastuzumab, or dimethyl sulfoxide (DMSO) vehicle was added at the time of seeding and was replaced with fresh media containing the corresponding drugs every four days. Photographs were obtained on day eight after initial seeding.

Soft agar colony forming assay

Six-well plates were first layered with 0.6% agar in MCF10A growth medium. A top layer of 1.0×10^4 HER2 WT or mutant MCF10A cells suspended in MCF10A growth media containing 0.4% agar was added per well after the solidification of the bottom layer. Cells were allowed to form colonies for seven days and then were photographed. Subsequently, lapatinib (0.5 μmol/L), neratinib (0.5 μmol/L), or DMSO vehicle (0.5%) was added to the cultures on day eight, and the cells were incubated for an additional seven days. The plates were stained with crystal violet (Sigma-Aldrich) for one hour and photographed with a Bio-Rad ChemiDoc XRS system.

Xenograft experiments

Exponentially growing, lentivirally transduced HER2 WT or mutant NIH3T3 cells were trypsinized and resuspended in serum-free medium, and then 1.0×10^6 cells were mixed at a 1:2 ratio (v/v) with growth factor–reduced Matrigel. Next, 0.1 mL of cell suspension was injected subcutaneously into 6- to 8-week-old female nude mice (FUSCC Animal Facility, Shanghai, China). The tumor volume was calculated as $\pi/6 \times (\text{width})^2 \times \text{length}$ using digital calipers. These experiments were conducted under institutional guidelines and with Institutional Animal Care and Use Committee approval in accordance with the FUSCC Animal Studies Committee.

Statistical analysis

Frequency tabulation and summary statistics were performed to describe the data distribution. Pearson χ^2 test was performed to compare categorical variables. One-way ANOVA and Student *t* test were used to compare continuous variables. Survival curves were constructed using the Kaplan–Meier method, and the univariate survival difference was determined using the log-rank test. All of the statistical analyses were performed using Stata statistical software, version 19.0 (Stata Corp). A two-sided *P* value less than 0.05 was considered to be statistically significant.

Results

Identification of HER2 somatic mutations

Using Sanger sequencing, we analyzed the entire exons of the *HER2* gene of 1,248 DNA samples from primary breast cancer patients treated at FUSCC. The neoadjuvant chemotherapy cohort included 102 HER2-positive tumors, while the adjuvant chemotherapy cohort consisted of 1,146 tumors that were either HER2-positive or HER2-negative. The clinicopathologic characteristics of the patients are shown in Supplementary Table S2.

Our analysis identified 28 breast cancer patients with HER2 somatic mutations; clinical information regarding the HER2-mutant tumors is shown in Supplementary Table S3, and the details of the 28 tumors are described in Supplementary Table S4. The overall HER2 mutation rate was 2.24% (28/1248). In the 910 HER2-positive breast cancers, the HER2 mutation rate was 2.31% (21/910); in the 338 HER2-negative breast cancers, the HER2 mutation rate was 2.07% (7/338). Each of the 28 patients had one HER2 somatic mutation; among those, 13 patients had previously reported HER2 mutations, and the remaining 15 patients had novel HER2 mutations. Compared with the HER2 WT group, tumors with HER2 mutations were associated with higher grade ($P = 0.043$, Supplementary Table S3). However, no statistical significance was observed in other clinicopathologic factors, including age, menopausal status, tumor size, lymph node status, and receptor status.

Further examination of the location of these novel mutations in the HER2 domain structure showed that these mutations were clustered in two areas, with 40.0% of the patients (6/15) having transmembrane mutations between residues 647 and 680, and 60.0% of patients (9/15) having kinase domain mutations between residues 753 and 773 (Fig. 1A). Notably, the L768S and V773L mutations, located in the kinase domain

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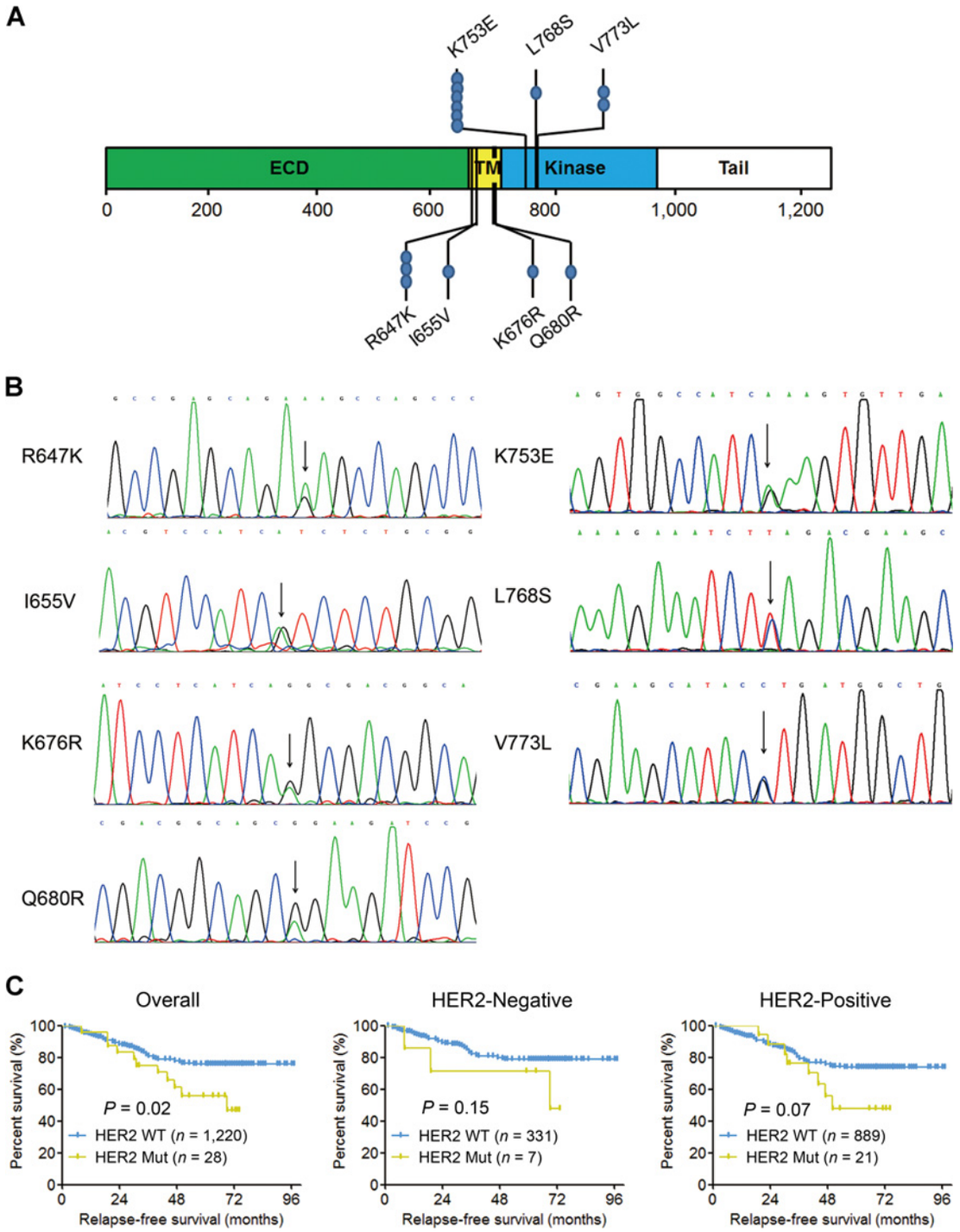

Figure 1.

HER2 somatic mutations in breast cancer. **A,** a novel HER2 mutation spectrum in 15 patients as indicated by lollipop mutation plots. Blue circles represent each case of the indicated mutation. Each patient had one HER2 somatic mutation, resulting in a total of 15 novel HER2 mutations in 15 patients. ECD, extracellular domain; TM, transmembrane region. **B,** detection of the mutant allele (indicated by arrows) in tumors harboring HER2 mutations by Sanger sequencing of tumor DNA samples. **C,** Kaplan–Meier estimates of relapse-free survival (RFS) according to HER2 mutation status in the overall (n = 1,248), HER2-negative (n = 338), and HER2-positive (n = 910) groups. Mut, mutant; WT, wild-type.

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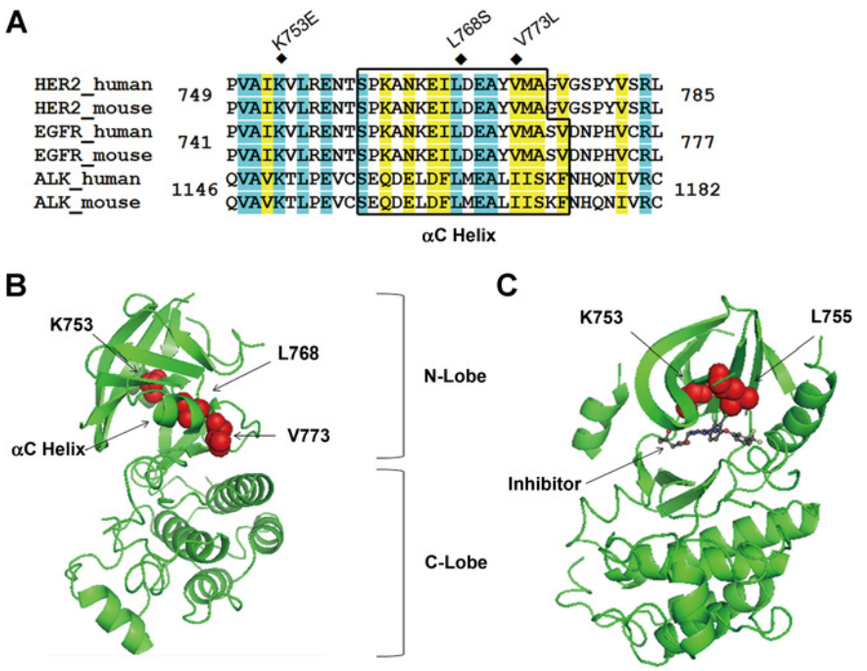

Figure 2.

Protein structure visualization of the HER2 somatic mutations. **A,** multisequence alignment of HER2, EGFR, and ALK tyrosine kinases. The locations of the somatic mutations are marked with diamonds. Identical residues are shaded blue, and similar residues are shaded yellow. **B,** HER2 kinase domain structure (PDB: 3PP0) showing the locations of point mutations (red spheres). **C,** the proximity of the HER2-K753 to L755 and the tyrosine kinase inhibitor is shown. The HER2 L755S mutation is a known lapatinib-resistant mutation.

of HER2, were only detected in HER2-negative breast cancer. Meanwhile, kinase domain mutation K753E was only observed in HER2-positive tumors. Fig. 1B shows the results of Sanger sequencing, in which the novel mutant alleles were clearly detectable.

Finally, we performed survival analysis of the overall population, as well as the HER2-negative and HER2-positive subgroups (Fig. 1C). Kaplan-Meier plots clearly showed that, in the overall population, breast cancer with HER2 mutations exhibited decreased RFS ($P = 0.02$) compared with breast cancer bearing WT HER2. In HER2-negative breast cancers, there was no significant difference in RFS between HER2-mutant and wild-type breast cancer ($P = 0.15$). However, in the HER2-positive subgroup, the HER2-mutant cohort tended to have worse RFS than the HER2 wild-type cohort ($P = 0.07$). Further analysis found that when broken out from the overall dataset, TM mutations alone did not have significant impact on RFS (Supplementary Fig. S2A, $P = 0.74$), while kinase domain mutations showed significant decrease of RFS compared with HER2 WT (Supplementary Fig. S2B, $P = 0.01$), and seemed to account for the impact on RFS seen in Fig. 1C. Because of the limited sample size of patients bearing HER2 mutations, we did not perform survival analysis of each individual mutation. Regardless, these data hinted that HER2 mutations, especially kinase domain mutations, might play a pivotal role in the progression and treatment of breast cancer, especially in the HER2-positive subgroup.

Protein structure visualization of novel HER2 somatic mutations

We further investigated the influence of these novel mutations on the structure of the HER2 protein. Multisequence alignment and protein structure visualization indicated that novel activating mutations (L768S and V773L) and previously reported activating mutations (V777L and D769H) either flanked or were within the αC helix of the tyrosine kinase domain of HER2 (Fig. 2A and B and Supplementary Fig. S3). Past studies have identified the HER2 L755S mutation to be a lapatinib resistance mutation (23, 30), and the proximity of the HER2 L755 side chain to the binding site for small-molecule kinase inhibitors such as lapatinib may explain how a mutation at this amino acid residue may cause drug resistance (Fig. 2C). The novel HER2 mutation K753E is in close proximity to L755 and the binding point of lapatinib and was thought to produce resistance to the tyrosine kinase inhibitor.

Differential activation of HER2 signaling by HER2 somatic mutations

We next conducted *in vitro* kinase assays on the HER2 kinase domain mutations using recombinant expression of the isolated kinase domain, and the kinase activity of monomeric and dimeric HER2 was compared. WT HER2 showed a 2-fold increase in kinase activity upon *in vitro* dimerization. Of the seven novel HER2 mutations tested here, L768S and V773L showed a significant increase in tyrosine kinase-specific activity compared with WT, with further increases observed after *in vitro* dimer formation (Fig. 3A). The HER2 K753E mutation also moderately increased tyrosine kinase-specific activity but was of no statistical significance.

Lentiviral vectors were used to transduce mutant or WT HER2 into MCF10A (Fig. 3B) and MCF7 (Fig. 3C) cells to assess the effect of HER2 mutations on signal transduction pathways. Using these two cell lines, we measured the HER2 autophosphorylation and phosphorylation of HER2's dimerization partners (HER3 and EGFR) and downstream signaling protein PLCγ via Western blotting. The L768S and V773L mutations strongly increased the phosphorylation of all signaling proteins in both MCF10A and MCF7 cell lines, indicating that they were activating mutations. It has been reported that HER2 mutations that strongly activate signaling showed lower amounts of total HER2 than HER2 WT (21, 31), and we

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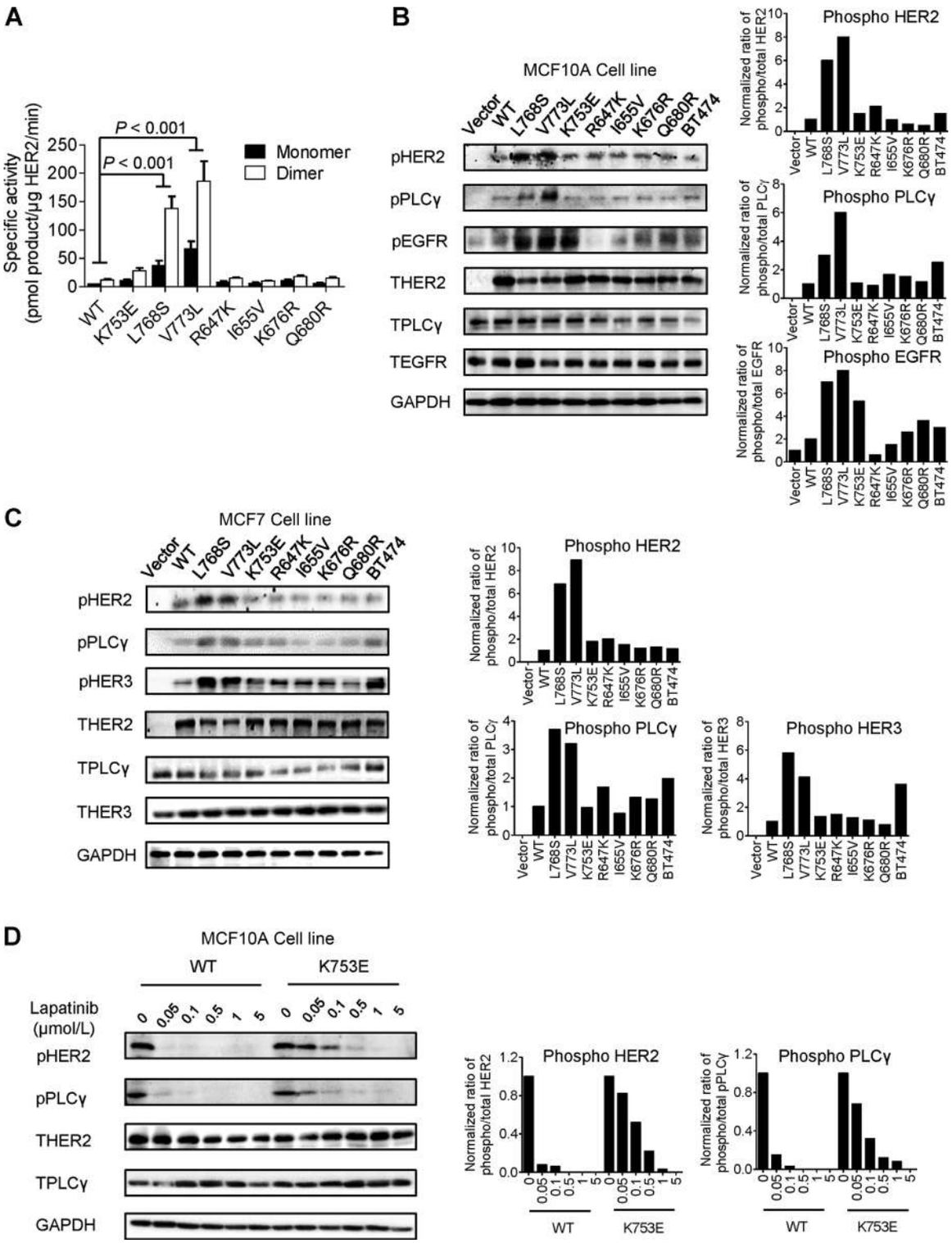

Figure 3.

HER2 mutations differentially activate HER2 signaling. **A,** HER2 WT or mutant kinase domain constructs were recombinantly expressed and assayed *in vitro*. **B,** MCF10A cells were lentivirally transduced with HER2 WT or the respective mutants, and the lysates were probed with the indicated antibodies. **C,** MCF7 cells were lentivirally transduced with HER2 WT or the respective mutants, and the lysates were probed with the indicated antibodies. **D,** MCF10A cells expressing HER2 WT or K753E were treated with lapatinib at the indicated concentrations for 4 hours and were then analyzed by Western blotting using the indicated antibodies. The results are the mean ± SD from three independent experiments. WT, wild-type.

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observed in both MCF10A and MCF7 cell lines that cells bearing L768S or V773L mutation had a slightly lower HER2 expression compared with cells bearing WT HER2 or other HER2 mutations. Immunofluorescence experiments using MCF10A cells bearing L768S or V773L mutation confirmed that the two mutants showed significant internalization of HER2 compared with HER2 WT (Supplementary Fig. S4), our results being consistent with the past studies.

Meanwhile, the K753E mutation strongly increased the phosphorylation of EGFR in MCF10A cells but did not increase the autophosphorylation of HER2 in MCF10A cells or increase the phosphorylation of PLCγ in MCF7 cells. Previous study reported HER2 kinase domain mutation to result in constitutive phosphorylation and activation of HER2 and EGFR, which was thought to be the cause of resistance to tyrosine kinase inhibitors (TKI; 32), and we sought to explore the relation between our novel HER2 mutations and TKI resistance in latter experiments.

Functional analysis of the activating HER2 mutations L768S and V773L in HER2-negative breast cancer

As previous experiments showed that HER2 mutations L768S and V773L play a role in the activation of the HER2 signaling pathway, we further explored the functional relevance of these two mutations. Growth effects in three-dimensional cultures were assessed by seeding MCF10A cells transduced with WT and mutant HER2 into Matrigel culture in the presence of DMSO vehicle (0.5%), trastuzumab (100 μg/mL), lapatinib (0.5 μmol/L), or neratinib (0.5 μmol/L). HER2 WT cells seeded into Matrigel culture containing DMSO vehicle formed spherical structures (Fig. 4A) in accordance with a previous report (33). On the eighth day after seeding, MCF10A cells bearing HER2 V768S and V773L mutations formed acini or invasive structures. The spherical morphology was maintained in these two mutations when the same cells were seeded in Matrigel culture containing trastuzumab. Whereas, MCF10A cells bearing HER2 K753E, R647K, I655V, K676R, and Q680R mutations formed spherical structures similar to WT HER2 in both cultures (Fig. 4A, top 2 rows).

The ability of HER2 mutations to increase tumor formation in xenografts was tested using the NIH3T3 cell line. NIH3T3 cells bearing HER2 WT was used as the control cell line. NIH3T3 cells bearing HER2 L768S and V773L mutations displayed more rapid growth than the HER2 WT controls ($P < 0.01$, respectively), while the HER2 K753E mutation showed tumor growth indistinguishable from that of the HER2 WT control (Fig. 4C). These findings were in concordance with our clinical data (Supplementary Table S4), where we can see that tumors (CT2571 and CT2283) with HER2 L768S- and V773L-activating mutations were both diagnosed at T3 stage with large tumor sizes and lymph node metastasis.

Functional analysis of lapatinib-resistant HER2 mutation K753E in HER2-positive breast cancer

Previous studies have shown that HER2 L755S mutations led to lapatinib resistance in mammalian cells (23, 30). Thus, we first compared the sensitivity of MCF10A cells carrying the HER2 WT or HER2 K753E mutation toward lapatinib (Fig. 3D). MCF10A cells with HER2 WT showed significant reduction in HER2 autophosphorylation at 50 nmol/L lapatinib. MCF10A cells with HER2 K753E mutations were less sensitive to lapatinib, showing significant reduction in HER2 autophosphorylation at 500 nmol/L and complete inhibition at 5 μmol/L lapatinib.

MCF10A cells with HER2 WT or mutants were also used to conduct soft agar colony assays. The cells were seeded in soft agar, and, after 7 days of growth, they were treated with DMSO vehicle (0.5%), trastuzumab (100 μg/mL), lapatinib (0.5 μmol/L), or neratinib (0.5 μmol/L) for an additional week before the number of colonies formed was quantified manually. We used L755S as a lapatinib-resistant control, and observed a small increase in its colony number with trastuzumab or lapatinib treatment compared with vehicle. Activating mutations L768S and V773L significantly increased the number of colonies formed in soft agar compared with WT (Fig. 4B). Trastuzumab and lapatinib treatment significantly decreased the number of colonies formed by MCF10A cells bearing HER2 L768S or V773L mutation but did not significantly reduce the number of colonies formed by MCF10A cells with HER2 K753E mutation. While L768S and V773L have similar colony number with K753E after being treated with trastuzumab and lapatinib, the drugs significantly decreased the number of colonies formed, and we conclude that L768S and V773L mutations were sensitive toward trastuzumab and lapatinib treatment. Meanwhile, neither drug was able to significantly reduce the number of colonies formed by MCF10A cells with HER2 K753E mutation, indicating that K753E was resistant to these drugs. In contrast, neratinib produced a strong inhibition of colony formation for all eight mutations, including K753E and L755S.

To measure the inhibition of cell growth by lapatinib and neratinib, we measured the IC_{50} values of these two tyrosine inhibitors in MCF10A, BT474, and MDA-MB-231 cells bearing HER2 WT or mutants (Table 1). The parental *HER2* gene-amplified cell line BT474 was used as a positive control, and IC_{50} values of 72 nmol/L with lapatinib and less than 2 nmol/L with neratinib, similar to previously published values, were observed (34, 35). The MCF7 cell line, which is insensitive to either tyrosine inhibitor, was used as a negative control. MCF10A cells carrying HER2 WT were inhibited by 480 nmol/L lapatinib, while doses of 500–1,000 nmol/L were required to inhibit most of these HER2 mutations. In contrast, the growth of MCF10A cells was potently inhibited by neratinib, regardless of the presence of HER2 WT or mutants. Notably, MCF10A K753E cells were resistant to lapatinib (IC_{50}>10,000 nmol/L) but could be inhibited by neratinib, although they required a relatively higher dose (IC_{50} of 32 nmol/L) than HER2 WT and other HER2 mutations. The inhibition of cell growth by lapatinib and neratinib in BT474 cells and MDA-MB-231 was in concordance with that in MCF10A cells, with K753E mutation showing relative resistance to lapatinib (IC_{50} of 1240 nmol/L and >10,000 nmol/L, respectively) compared with WT and other mutations, but with overall sensitivity to neratinib.

Enriched HER2 mutations in HER2-positive metastatic lesions indicate trastuzumab resistance

Finally, we collected 18 samples of metastatic HER2-positive breast cancers, 16 of which had received one year of adjuvant treatment with trastuzumab. We examined all of the exons of HER2 in the 18 pairs of samples using Sanger sequencing. Interestingly, we discovered that although none of the paired primary tumors harbored HER2 mutations, the rate of HER2 mutation in metastatic lesions was 27.8% (5/18, Table 2).

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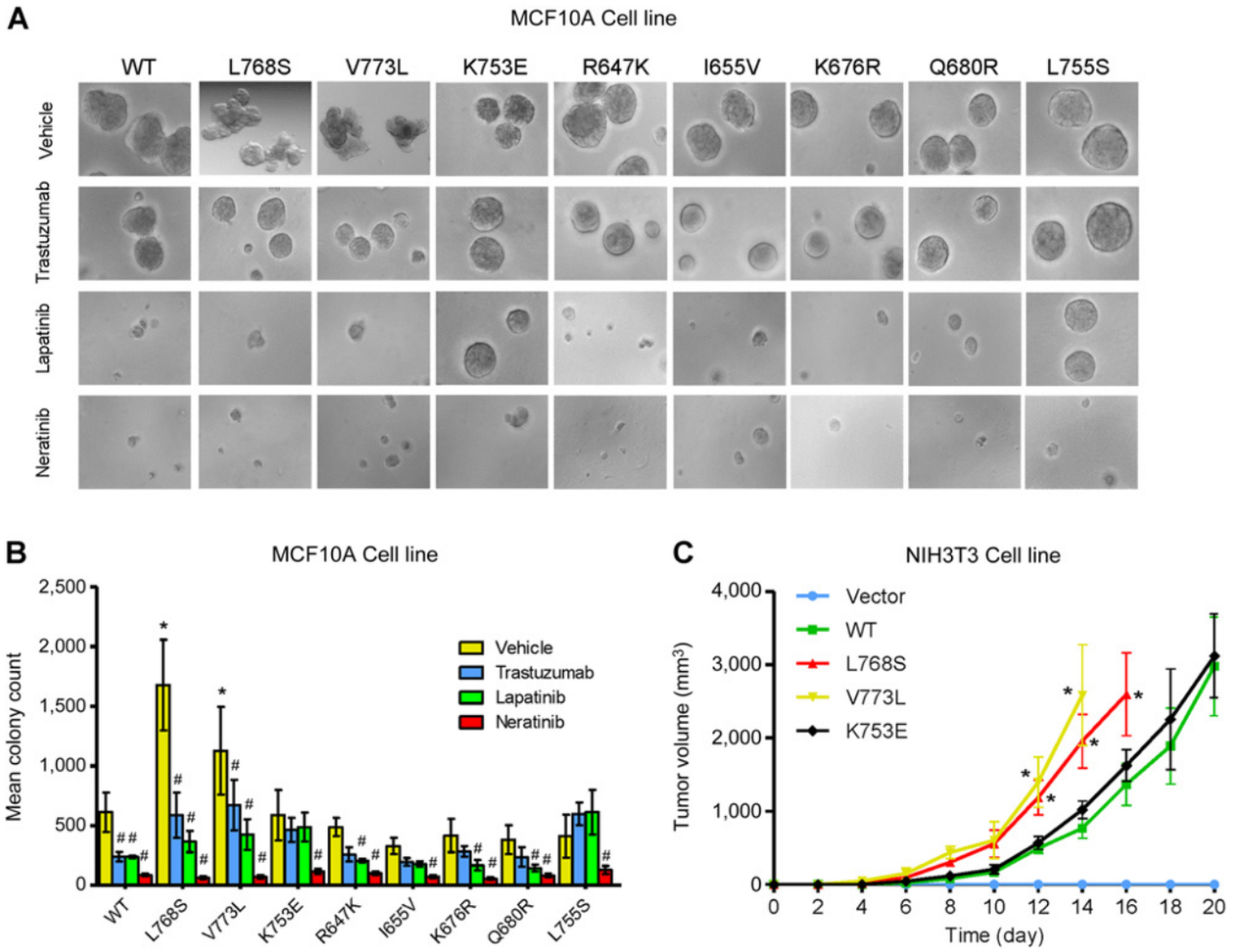

Figure 4.

HER2 mutations promote oncogenesis and lead to lapatinib resistance. **A,** MCF10A-HER2 WT or mutants were seeded on 3D Matrigel culture in the presence or absence of DMSO vehicle (0.5%), trastuzumab (100 μg/mL), lapatinib (0.5 μmol/L), or neratinib (0.5 μmol/L). Phase contrast images of acini or invasive structures were acquired at ×200 magnification on day eight. **B,** MCF10A-HER2 WT or mutants were seeded in soft agar. After seven days of growth, they were treated with DMSO vehicle (0.5%), trastuzumab (100 μg/mL), lapatinib (0.5 μmol/L) or neratinib (0.5 μmol/L) for an additional week. Asterisk (*) indicates a significant difference between the HER2 mutant and HER2 WT ($P < 0.05$). # indicates that the effect of inhibitor treatment was significant ($P < 0.05$). **C,** NIH3T3 cells expressing HER2 mutants L768S and V773L formed tumors more rapidly than HER2 WT. $n = 4$ mice for each group. Tumor size was measured every two days. *, $P < 0.05$. The results are the mean ± SD from three independent experiments. WT, wild-type.

Among the five cases with emerged HER2 mutations in metastatic lesions after one year of trastuzumab adjuvant treatment, two cases were identified with the K753E mutation, and the other three had the L755S mutation. K753E and L755S were confirmed to be drug-resistant mutations (resistant to trastuzumab and lapatinib). The mutation rate of HER2 was much higher than that in the primary tumor (2.24%, 28/1248).

Discussion

In this study, we uncovered seven novel HER2 mutations and have functionally characterized them according to their response toward HER2-targeted treatments. We discovered one novel mutation from HER2-positive patients, K753E, to produce a lapatinib-resistant phenotype, while two other novel mutations found in HER2-negative patients, L768S and V773L, were activating mutations. The remaining four novel HER2 mutations showed no functional effect in our assay. Furthermore, we noticed an enrichment of drug-resistant mutations (K753E and L755S) in HER2-positive metastatic lesions.

The HER2 mutation K753E occurring in the flank of the αC helix was found in one patient (HER2-positive) receiving neoadjuvant therapy and five patients (HER2-positive) receiving adjuvant therapy. We originally hypothesized that the HER2 K753E mutation may induce lapatinib resistance due to its proximity to known the lapatinib-resistant mutation L755S (23, 30). Our experiments confirmed that cell lines with the HER2 K753E mutation overexpression were indeed resistant to lapatinib but were sensitive to the irreversible TKI neratinib. Previous reports concerning HER2 kinase domain mutations have described

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Table 1. Inhibition of cell growth by lapatinib and neratinib

Cell		IC_{50} (nmol/L)[a]	
		Lapatinib	Neratinib
MCF10A	HER2 WT	480 ± 50	<2
	K753E	>10,000	32 ± 8
	L768S	1,050 ± 480	<2
	V773L	960 ± 380	<2
	R647K	650 ± 370	<2
	I655V	520 ± 420	<2
	K676R	385 ± 270	<2
	Q680R	550 ± 190	<2
BT474		72 ± 24	<2
	HER2 WT	109 ± 36	<2
	K753E	1,240 ± 460	40 ± 8
	L768S	286 ± 110	<2
	V773L	218 ± 85	<2
	R647K	86 ± 40	<2
	I655V	120 ± 52	<2
	K676R	64 ± 36	<2
	Q680R	78 ± 5	<2
MDA-MB-231	HER2 WT	3,620 ± 860	410 ± 140
	K753E	>10,000	889 ± 215
	L768S	4,890 ±1110	590 ± 165
	V773L	4,980 ± 785	780 ± 184
	R647K	3,578 ± 759	472 ± 106
	I655V	3,496 ± 808	508 ± 108
	K676R	3,730 ± 960	537 ± 124
	Q680R	3,778 ± 845	496 ± 98
MCF7		>10,000	>3,000

NOTE: Cells were incubated with certain drugs for six days, and cell viability was measured using CCK8 assay.

[a]Mean IC_{50} (nmol/L) ± SD from three independent determinations.

tumor resistance toward TKIs. For example, *in vivo* studies showed that, in lung adenocarcinoma, tumor cells harboring a common mutation subtype of HER2, $HER2^{YVMA}$ (12 bp duplication/insertion of the amino acid sequence YVMA in exon 20 at codon 776), were demonstrated to be resistant to reversible EGFR-TKIs such as gefitinib and erlotinib but were sensitive to HER2 and dual EGFR/HER2 inhibitors (32).

Furthermore, the emergence of HER2 K753E and L755S mutations in paired metastatic breast cancer lesions after trastuzumab treatment compared with paired primary lesions also indicates that these mutations may play a vital role in the development of resistance, suggesting that mutations in the HER2 kinase domain may be a key mechanism of resistance to HER2-targeted therapy. The selection of functional HER2 mutations might act as potential drivers of trastuzumab resistance during the progression of HER2-positive disease. Our results hint that more potent HER2 inhibitors, such as neratinib, may be of substantial therapeutic benefit for patients who develop trastuzumab-resistant breast cancer.

The activating HER2 mutation L768S was found in one patient (HER2-negative) receiving adjuvant therapy, while the V773L mutation was found in two patients (HER2-negative) receiving adjuvant therapy. A previous study by Bose and colleagues (21) identified HER2 D769Y and D769H mutations to be activating mutations, and they have postulated that the D769H/Y mutations may increase hydrophobic contacts and possibly increase HER2-HER3 dimerization. Similar mechanisms may exist for the novel L768S mutation occurring in the αC helix. Meanwhile, the HER2 mutation V773L was found in close proximity to the known activating mutation V777L (21).

A previous report regarding HER2 mutations estimated an overall HER2 mutation rate of approximately 1.6% in breast cancer patients, translating into approximately 4,000 HER2 mutation–positive patients annually in the United States alone (21). However, relative studies were conducted in Western populations, consisting mainly of Caucasian patients, with only a very low percentage of patients with an Asian background. In our study of Chinese breast cancer patients, we found the overall HER2 mutation rate to be 2.24% (28/1248), with 1.12% (14/1248) of patients presenting with novel HER2 mutations.

The significance of our study is that we present a comprehensive functional analysis of HER2 mutations in breast cancer; we screened both HER2-positive and HER2-negative breast cancer patients for somatic mutations in all exons of the *HER2* gene, and we discovered novel mutations in both groups that may have clinical implications regarding the treatment of these patients. In addition, ours is one of the few studies conducted on *HER2* gene mutations in the Asian breast cancer population. At present, racial differences in the pathophysiology of breast cancer is yet unclear, but previous studies have reported the prevalence and spectrum of *BRCA1* and *BRCA2* mutations to vary among different races (36–39), and it has been shown that the incidence of HER2 mutations in lung cancer varies depending on the ethnicity of the patients and affects the response to HER2-targeted treatment (15, 20, 32, 40, 41). However, our study observed similar patterns in hotspot regions and types of HER2 mutation to previously reported data, together providing future directions for mutation screening and the development of novel targeted therapy.

Nevertheless, our study has several limitations. First, our samples were nearly all newly diagnosed breast cancer patients, with only 18 available cases of metastatic tumors. Although the frequency of HER2 mutations in recurrent or metastatic patients (27.8%) was much higher than the 2.24% mutation rate observed in the primary breast cancer population, a larger patient sample of metastatic breast cancer would better support our conclusions. Second, our study focused on the effect of HER2 mutations upon HER2-targeted therapies, and did not conduct experiments regarding the effect of these mutations upon treatment with non-HER2 targeted therapies. However, further analysis of HER2-negative patients who did not undergo HER2-targeted therapies found that there was no significant

Table 2. Enrichment of drug-resistant HER2 mutations (K753E and L755S) in metastatic lesions

Cohort	Patient code	HER2 mutation in metastatic tumor	Primary tumor	ER	PR	HER2	HER2-targeted therapy
Metastatic	CT1980	K753E	WT	-	-	+	Trastuzumab for one year
	CT2685	L755S	WT	+	+	+	Trastuzumab for one year
	CT3478	L755S	WT	-	-	+	Trastuzumab for one year
	CT3799	K753E	WT	+	+	+	Trastuzumab for one year
	CT4167	L755S	WT	+	-	+	Trastuzumab for one year

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difference in RFS between HER2 WT and HER2-mutant cohorts, suggesting that HER2 mutations may have little or no effects on treatment with non-HER2–targeted therapies. Furthermore, because the mechanism of action for trastuzumab includes the activation of immune cells, cell culture assays conducted in our study could not completely mimic the tumor microenvironment of breast cancer undergoing trastuzumab treatment. Future clinical applications would require a prospective analysis of a larger patient population conducted at multiple institutions to correctly appraise the benefits of genetic screening of HER2 mutations for patient treatment.

In conclusion, we revealed seven novel HER2 mutations and functionally characterized them according to their biologic functions and response toward HER2-targeted treatment. We also identified enriched drug-resistant mutations in HER2-positive metastatic tumors. Further studies based on these findings will help to illustrate the potential clinical implications of HER2 somatic mutations and assist in identifying patients who would benefit from HER2-targeted therapies and in choosing the most beneficial therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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