Clinical Cancer Research

Neratinib Efficacy and Circulating Tumor DNA Detection of *HER2* Mutations in *HER2* Nonamplified Metastatic Breast Cancer

Cynthia X. Ma[1], Ron Bose[1], Feng Gao[2], Rachel A. Freedman[3], Melinda L. Telli[4], Gretchen Kimmick[5], Eric Winer[3], Michael Naughton[1], Matthew P. Goetz[6], Christy Russell[7], Debu Tripathy[7], Melody Cobleigh[8], Andres Forero[9], Timothy J. Pluard[10], Carey Anders[11], Polly Ann Niravath[12], Shana Thomas[1], Jill Anderson[1], Caroline Bumb[1], Kimberly C. Banks[13], Richard B. Lanman[13], Richard Bryce[14], Alshad S. Lalani[14], John Pfeifer[15], Daniel F. Hayes[16], Mark Pegram[17], Kimberly Blackwell[5], Philippe L. Bedard[18], Hussam Al-Kateb[15], and Matthew J.C. Ellis[12]

Abstract

Purpose: Based on promising preclinical data, we conducted a single-arm phase II trial to assess the clinical benefit rate (CBR) of neratinib, defined as complete/partial response (CR/PR) or stable disease (SD) ≥24 weeks, in *HER2*^{mut} nonamplified metastatic breast cancer (MBC). Secondary endpoints included progression-free survival (PFS), toxicity, and circulating tumor DNA (ctDNA) *HER2*^{mut} detection.

Experimental Design: Tumor tissue positive for *HER2*^{mut} was required for eligibility. Neratinib was administered 240 mg daily with prophylactic loperamide. ctDNA sequencing was performed retrospectively for 54 patients (14 positive and 40 negative for tumor *HER2*^{mut}).

Results: Nine of 381 tumors (2.4%) sequenced centrally harbored *HER2*^{mut} (lobular 7.8% vs. ductal 1.6%; $P = 0.026$). Thirteen additional *HER2*^{mut} cases were identified locally. Twenty-one of these 22 *HER2*^{mut} cases were estrogen receptor positive. Sixteen patients [median age 58 (31–74) years and three (2–10) prior metastatic regimens] received neratinib. The CBR was 31% [90% confidence interval (CI), 13%–55%], including one CR, one PR, and three SD ≥24 weeks. Median PFS was 16 (90% CI, 8–31) weeks. Diarrhea (grade 2, 44%; grade 3, 25%) was the most common adverse event. Baseline ctDNA sequencing identified the same *HER2*^{mut} in 11 of 14 tumor-positive cases (sensitivity, 79%; 90% CI, 53%–94%) and correctly assigned 32 of 32 informative negative cases (specificity, 100%; 90% CI, 91%–100%). In addition, ctDNA *HER2*^{mut} variant allele frequency decreased in nine of 11 paired samples at week 4, followed by an increase upon progression.

Conclusions: Neratinib is active in *HER2*^{mut}, nonamplified MBC. ctDNA sequencing offers a noninvasive strategy to identify patients with *HER2*^{mut} cancers for clinical trial participation. *Clin Cancer Res; 23(19); 5687–95. ©2017 AACR.*

Introduction

HER2 (ERBB2) is a well-established therapeutic target in breast cancer (1–13), and HER2-negative (nonamplified) breast cancers overall do not benefit from HER2-directed drugs (14). However, the recent identification of recurrent *HER2* mutations (*HER2*^{mut}) in a subset of *HER2* gene nonamplified breast cancer suggested an additional HER2 targeting opportunity (3, 15–25). *HER2* mutations cluster in the tyrosine

[1]Division of Oncology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri. [2]Division of Public Health Science, Department of Surgery, Washington University School of Medicine, St. Louis, Missouri. [3]Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. [4]Department of Medicine, Stanford University School of Medicine, Stanford, California. [5]Department of Medicine, Duke Cancer Institute, Durham, North Carolina. [6]Medical Oncology, Mayo Clinic, Rochester, Minnesota. [7]Medical Oncology, University of Southern California, Los Angeles, California. [8]Medical Oncology, Rush University Medical Center, Chicago, Illinois. [9]Department of Medicine, University of Alabama Birmingham, Birmingham, Alabama. [10]Department of Oncology-Hematology, St. Luke's Cancer Institute, Kansas City, Missouri. [11]Department of Medicine, University of North Carolina, Chapel Hill, North Carolina. [12]Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, Texas. [13]Guardant Health Inc., Redwood City, California. [14]Puma Biotechnology, Los Angeles, California. [15]Genomic and Pathology Service, Washington University School of Medicine, St. Louis, Missouri. [16]Department of Hematology and Oncology, University of Michigan, Ann Arbor, Michigan. [17]Department of Medicine, Stanford Cancer Institute, Stanford University School of Medicine, Stanford, California. [18]Medical Oncology, Princess Margaret Cancer Centre, Toronto, Ontario, Canada.

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C.X. Ma and R. Bose contributed equally to this article.

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Corresponding Authors: C.X. Ma, Washington University School of Medicine, 3rd Floor, 4515 McKinley Research Bldg, Campus Box 8076, St Louis, MO 63110. Phone: 314-362-9383; Fax: 314-747-9320; E-mail: cynthiaxma@wustl.edu; R. Bose, Washington University School of Medicine, 3rd Floor, 4515 McKinley Research Bldg, Campus Box 8076, St Louis, MO 63110. Phone: 314-747-3096; Fax: 314-747-9320; E-mail: rbose@wustl.edu; and M.J.C. Ellis, Lester and Sue Smith Breast Center, Baylor College of Medicine, One Baylor Plaza, 320A Cullen, MS 600 Houston, TX 77030. Phone: 713-798-1640; Fax: 713-798-8884; E-mail: mjellis@bcm.edu

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Translational Relevance

Prospective trials in genomically defined populations are needed for next-generation sequencing to guide individualized cancer care. We report a phase II trial of the pan-HER inhibitor neratinib for patients with *HER2*-mutated (*HER2*^{mut}), nonamplified metastatic breast cancer (MBC). The study demonstrated a clinical benefit rate of 31% (90% confidence interval, 13%–55%) with manageable toxicities. The screening process was challenged by the low frequency of *HER2*^{mut} (2.4%) and a high rate of sequencing failure (26%) using archival tumor material. Retrospective plasma ctDNA sequencing showed high specificity and good sensitivity in detecting *HER2*^{mut}, supporting its use as a screening tool to identify future trial patients. The on-target effect of neratinib was supported by early decreases of ctDNA *HER2*^{mut} variant allele frequency, which then increased upon clinical progression. The predominance of estrogen receptor positivity of *HER2*^{mut} MBC provided the rationale for testing fulvestrant plus neratinib in in the next phase of this trial.

kinase and extracellular dimerization domains of HER2, leading to enhanced kinase activity and tumorigenesis in preclinical models (16, 26, 27). Importantly *HER2*^{mut} render tumor cells sensitive to HER2-targeted agents, especially neratinib (16, 26, 27), a potent irreversible pan-HER inhibitor (28–34). We therefore conducted a phase II trial of neratinib in patients with *HER2*^{mut}, nonamplified metastatic breast cancer (MBC; Mutant HER2 trial: MutHER). The primary endpoint was clinical benefit rate (CBR). Secondary endpoints included progression-free survival (PFS), toxicity profile, *HER2*^{mut} frequency in MBC, and analysis of ctDNA for *HER2*^{mut} detection and response monitoring. The Clinicaltrials.gov# is NCT01670877.

Materials and Methods

Patients

Patients with HER2-negative (0 or 1+ by immunohistochemistry or nonamplified by FISH) MBC, at least 18 years old, Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) $\leq$2, measurable or evaluable disease by RECIST 1.1, and adequate organ function were preregistered for *HER2*^{mut} screening by DNA sequencing of archival primary or metastatic tumor performed centrally at the Clinical Laboratory Improvement Amendment (CLIA)-certified Washington University Genomic and Pathology Service (GPS). Patients were eligible for screening while receiving other treatment. The initial requirement of at least one prior systemic therapy was subsequently removed to improve accrual. Local *HER2* mutation testing in a local CLIA laboratory was also allowed.

Eligibility criteria for registration included tumor positive for somatic *HER2*^{mut} identified by CLIA-certified laboratories, recent disease progression, adequate organ function, QTc interval $\leq$450 msec (men) or $\leq$470 msec (women), and left ventricular ejection fraction $\geq$ institutional lower limit of normal, $\geq$1 week wash-out from radiotherapy or systemic therapy. Patients receiving other cancer therapy, strong CYP3A4 inducers or inhibitors, uncontrolled concurrent illness, $\geq$grade 2 diarrhea, being pregnant, or breastfeeding were not eligible. Treated brain metastases stable for $\geq$3 months without steroids were allowed. This study was conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice, and was approved by each center's Regulatory and Ethics Committees. All participants provided written informed consent.

Study design and treatment

The primary objective was rate of CB defined as complete/partial response (CR/PR) or stable disease (SD) $\geq$24 weeks. Secondary objectives included *HER2*^{mut} frequency and clinicopathologic characteristics of these patients, PFS on neratinib, toxicity profile, and analysis of ctDNA for *HER2*^{mut} detection and response monitoring.

Sample size was calculated based on Simon's Optimal two-stage design to enroll 10 patients in the first stage and 19 patients in the second stage to allow 80% power and a one-sided 0.05 significance level to detect an anticipated 20% CBR against the null hypothesis of 5%. At least one CB was required to proceed to the second stage. The primary endpoint is met if at least four of 29 achieved CB. After observing that almost all *HER2*^{mut} tumors were ER^{+}, the protocol was amended to add fulvestrant to the regimen (if ER^{+}). This pragmatic decision led to an early stop of enrollment to neratinib monotherapy. Here, we report the results from the 16 patients who received neratinib monotherapy prior to the activation of this amendment.

Patients were started with neratinib orally at 240 mg daily in a 28-day cycle. Diarrhea prophylaxis with loperamide was mandatory during the first cycle of therapy. Loperamide was administered at an initial dose of 4 mg with the first dose of neratinib on cycle 1 day 1, followed by 2 mg every 4 hours for 3 days, then 2 mg every 6 to 8 hours during the first cycle of therapy, and as needed. Subjects were allowed to escalate neratinib dose to 320 mg daily if no intolerable grade 2 or higher treatment-related adverse events (AE) were experienced during a complete cycle of treatment. A maximum of three dose reductions in 40-mg decrements were permitted for toxicities. AEs were accessed by NCI CTCAE 4.0 weekly during the first cycle and day 1 of subsequent cycles. Patients underwent tumor evaluation by RECIST 1.1 every two cycles and echocardiograms every four cycles.

Central tumor DNA sequencing for HER2 mutation

Central tumor DNA sequencing for *HER2* mutation was performed at the GPS laboratory. Sanger technology was initially used to sequence exons 8 and 18 to 24 of *HER2*, which applied to approximately 20% of samples by October 2014. Subsequently, a PCR based next-generation sequencing (NGS) assay was used to sequence all *HER2* coding exons. PCR was performed using the 48.48 high-throughput access array system (Fluidigm Corp.). Cluster generation and sequencing were performed using Illumina's HiSeq2500 Reagent Kit (200 cycles), and 2 $\times$ 101 paired-end sequence reads were generated. Each patient's DNA was processed in three independent technical replicates to generate and sequence three independent amplicon libraries. Variant call was performed on all BAM files together by a combination of commercially available and custom-developed scripts to generate a multi-sample variant call file. Variants were called if at least three of the four BAM files had evidence for the variant, and the average variant frequency was $\geq$10%.

Cell-free tumor DNA sequencing

Digital sequencing of cell-free DNA was performed by Guardant Health, Inc. (Guardant360, www.guardanthealth.com/guardant360/), a CLIA-certified and College of American Pathologists (CAP)-accredited clinical laboratory. Note that 5 to 30 ng of ctDNA was isolated from plasma (two 10-mL Streck tubes drawn for each patient), and sequencing libraries were prepared with custom in-line barcode molecular tagging and complete sequencing at 15,000× read depth. The panel utilizes hybrid capture followed by NGS of all exons in 30 genes, including *HER2*, and critical exons (those reported as having a somatic mutation in COSMIC) of 40 additional genes to detect and report single-nucleotide variants and small indels in 70 genes, copy-number amplifications in 18 genes, and select fusions (Supplementary Fig. S1). Postsequencing bioinformatics matches the complementary strands of each barcoded DNA fragment to remove false-positive results (35). The variant allele fraction (VAF) was computed as the number of mutated DNA molecules divided by the total number (mutated plus wild type) of DNA fragments at that allele; VAF was reported as a percentage.

Statistical analysis

The CBR was calculated as the proportion of CR, PR, or SD≥24 weeks with 90% exact binomial confidence intervals (CIs) by RECIST 1.1. Response duration was defined as the duration between the first scan demonstrating disease response and that at progression. PFS was defined as weeks from treatment initiation to progression or death. The associations of *HER2* mutations with histology subtype (lobular vs. ductal), hormone receptor status (ER^+/PR^+ vs. ER^-/PR^-), and the source of sample (primary vs. metastatic) were assessed using Fisher exact tests. The diagnostic ability of ctDNA sequencing for *HER2* mutations was summarized by sensitivity and specificity, with corresponding 90% CI. The association between ctDNA-mutant VAF and clinical outcomes (PFS and tumor size change) was also assessed using Spearman correlation coefficients. The data were analyzed using the standard package of SAS (Version 9.3, SAS Institute).

Table 1. Incidence of *HER2*-activating mutations by histology, hormone receptor status, and tumor tissue source for patients whose tumor samples successfully sequenced at GPS laboratory

	Positive for *HER2* mutation, *N* (%)	*P*
Histology		
Invasive ductal carcinoma ($n = 309$)	5 (1.6%)	0.026[a]
Invasive lobular carcinoma ($n = 51$)	4 (7.8%)	
Invasive ductal/lobular carcinoma ($n = 12$)	0 (0%)	
Other[b] ($n = 9$)	0 (0%)	
Hormone receptor status		
ER^+ and/or PR^+ ($n = 277$)	9 (3.2%)	0.219
ER^- PR^- $HER2^-$ ($n = 82$)	0 (0%)	
Unknown ($n = 17$)	0 (0%)	
Samples tested		
Primary breast cancer ($n = 256$)	6 (2.3%)	>0.99
Metastatic site ($n = 125$)	3 (2.4%)	
Total ($n = 381$)	9	

[a]Comparison between ductal carcinoma ($n = 309$) vs. lobular carcinoma ($n = 51$).
[b]Others include metaplastic ($n = 2$); mucinous ($n = 3$); and unknown ($n = 4$).

Results

Screening for HER2 mutation

Between December 1, 2013, and August 15, 2015, 636 women with *HER2* nonamplified MBC, median age 56 (range, 23–87) years, were consented for central *HER2* sequencing at Washington University CLIA-certified GPS laboratory (Supplementary Fig. S2). Among the 579 patients eligible for preregistration, 517 had tissue available for testing (Table 1). Adequate quantity and quality of tumor DNA were extracted and successfully sequenced for 381 of 517 (74%) samples (primary

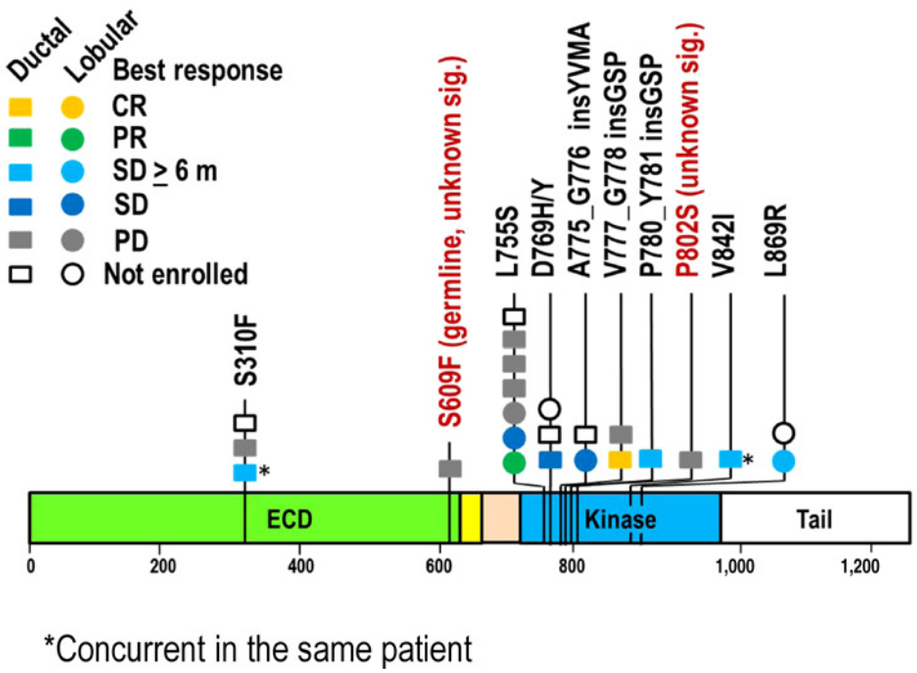

Figure 1.
HER2 mutations identified by tumor DNA sequencing at Central and other laboratories. Each circle (lobular) or square (ductal) represents a single patient except in a case of concurrent S310F and V842I mutations (indicated by *).

Table 2. Characteristics of the 16 patients who received neratinib (*N*, %)[a]

Age, years	
Median (range)	58 (31–74)
Race	
White/African American/Asian	13 (81%)/1 (6%)/2 (13%)
ECOG PS	
0/1/2	9 (56%)/7 (44%)/0 (0%)
Menopausal status	
Pre-/postmenopausal	2 (12%)/14(88%)
Histology	
Ductal/lobular	11 (67%)/5 (31.3%)
Hormone receptor status	
$ER^+PR^+/ER^+PR^-/ER^-PR^-HER2^-$	7 (44%)/8 (50%)/1 (6%)
HER2 status	
Nonamplified/amplified	16 (100%)/0 (0%)
Evaluable disease by RECIST	
Measurable/nonmeasurable	13 (81%)/3 (19%)
Disease magnitude	
Visceral/nonvisceral	11 (69%)/5 (31%)
Prior number of metastatic regimens	
Any/endocrine/chemo (median, range)	3 (2–10)/2 (0–3)/2 (0–6)
Reason off study	
Adverse event/progressive disease	1 (6%)/15 (94%)

[a]Unless otherwise specified.

$n = 256$, metastasis $n = 125$) with nine of 381 (2.4%) samples positive for at least one *HER2* mutation. Notably, all nine cases with *HER2* mutations were also positive for ER and/or PR. The incidence of *HER2* mutation was nine of 277 (3.2%) in hormone receptor–positive cases in contrast with the 0 of 82 (0%) in triple-negative breast cancers. However, the difference in *HER2* mutation incidence by hormone receptor status did not reach statistical significance ($P = 0.219$), likely because of the small sample size. Lobular cancers had a significantly higher incidence of *HER2* mutations, 7.8% (4/51), compared with ductal histology, 1.6% (5/309), ($P = 0.026$; Table 1). There was no difference in the detection rate of *HER2* mutations in tumors from primary (2.3%) or metastatic sites (2.4%).

An additional 13 patients with $HER2^{mut}$ were identified by an outside CLIA-lab (Supplementary Fig. S2). Figure 1 shows the distribution of the *HER2* mutations identified in all 22 cases. L755S was the most common mutation ($n = 7$, 32%). The median patient age was 58 (range, 31–72) years, 15 of 22 (68%) were ductal, seven of 22 (32%) were lobular cancers, and 21 of 22 (95%) were ER^+. Retrospectively, 21 of 22 patients presented initially with early-stage disease. The median recurrence-free survival from surgery to recurrence was 35.9 (90% CI, 15.4–93.8) months.

Clinical characteristics for patients receiving neratinib

Sixteen patients received neratinib monotherapy, including 14 with known-activating mutations and two of unknown significance (no preclinical data; Fig. 1; Supplementary Fig. S2). Table 2 details the patient characteristics. As of data cutoff, all patients had stopped study therapy due to progression ($n = 15$, 94%) or AE ($n = 1$, 6%).

Adverse events

All 16 patients were evaluable for AE (Table 3). Treatment was well tolerated, and most AEs were grades 1 and 2, with one patient who discontinued therapy due to AE (grade 3 fatigue and dehydration). The most common grade 2 and above AEs included diarrhea (69%), anorexia (44%), and fatigue (31%), and there were no grade 4 AEs. Diarrhea ($n = 4$, 25%) was the only treatment-related grade 3 AE that occurred in more than one patient, but the duration was short, lasting a median of 1.5 days (range, 1–3 days). Neratinib was reduced to 200 mg daily in four (25%) patients due to grade 3 diarrhea ($n = 2$), grade 2 nausea/anorexia ($n = 1$), and grade 3 fatigue/dehydration leading to neratinib discontinuation ($n = 1$). Neratinib was escalated to 320 mg daily in five patients (three PD, two SD $\geq$24 weeks) in cycles two ($n = 4$) and four ($n = 1$) and continued until disease progression, except in one patient who subsequently reduced to 240 mg 10 days after due to grade 2 nausea/anorexia and diarrhea.

Antitumor activity

One of the 10 patients enrolled in the first stage achieved CB; therefore, the study continued to the second stage until the activation of the protocol amendment for subsequent patients to receive the combination of fulvestrant and neratinib if ER^+. In total, 16 patients received neratinib monotherapy. The median number of metastatic regimens received prior to study entry was three (range, 2–10). Five patients experienced CB including one CR (6%), one PR (6%), and three SD $\geq$24 weeks (19%). The CBR was 31% (90% CI, 13%–55%), which met the primary endpoint. The median response duration in patients who achieved CB was 24 (range, 24–66) weeks. PFS and changes in tumor size were shown in Figure 2A and B, respectively. An example of response is illustrated in Figure 2C. Among the 15 patients with ER^+ breast cancer, only one (pt 16) received a CDK4/6 inhibitor (ribociclib) for metastatic disease prior to study enrollment. This patient experienced prolonged disease stabilization on neratinib (PFS 37 weeks). Treatment benefit was observed across different *HER2* mutations including L755S, V777_G778 insGSP, P780_Y781 insGSP, V842I, S310F, and L869R. Multi-gene panel next-generation tumor sequencing results were available for 13 patients: common co-occurring mutations included *CDH1* (5/13), *PIK3CA* (4/13), and *TP53* (6/13).

Plasma cell-free tumor DNA sequencing

ctDNA sequencing for $HER2^{mut}$ detection and response monitoring was performed using a 70-gene digital sequencing assay (Guardant Health; Supplementary Fig. S1). Plasma collected from patients with *HER2*-activating mutations was analyzed at baseline as positive controls ($n = 14$) and additionally tested following 4 weeks on neratinib ($n = 13$) and upon progression ($n = 9$). Because a sample size of at least 30 negative controls was required to ensure 90% confidence of >90% testing specificity, plasma collected at preregistration for 40 patients negative for $HER2^{mut}$ by tumor sequencing at GPS was analyzed.

Table 3. Grade 2 and above AEs at least possibly related to neratinib ($N = 16$)

AE	Grade 2, *N* (%)	Grade 3, *N* (%)	Total, *N* (%)
Diarrhea	7 (44%)	4 (25%)*	11 (69%)
Anorexia	7 (44%)	0	7 (44%)
Fatigue	4 (25%)	1 (6%)	5 (31%)
Anemia	3 (19%)	0	3 (19%)
Nausea	3 (19%)	0	3 (19%)
Dehydration	1 (6%)	1 (6%)	2 (13%)
Dyspepsia	2 (13%)	0	2 (13%)
Hypophosphatemia	2 (13%)	0	2 (13%)
Vomiting	2 (13%)	0	2 (13%)
Syncope	0	1 (6%)	1 (6%)

NOTE: AEs that are grade 2 or above experienced by more than one patient or any occurrence of grade 3 AEs are included.

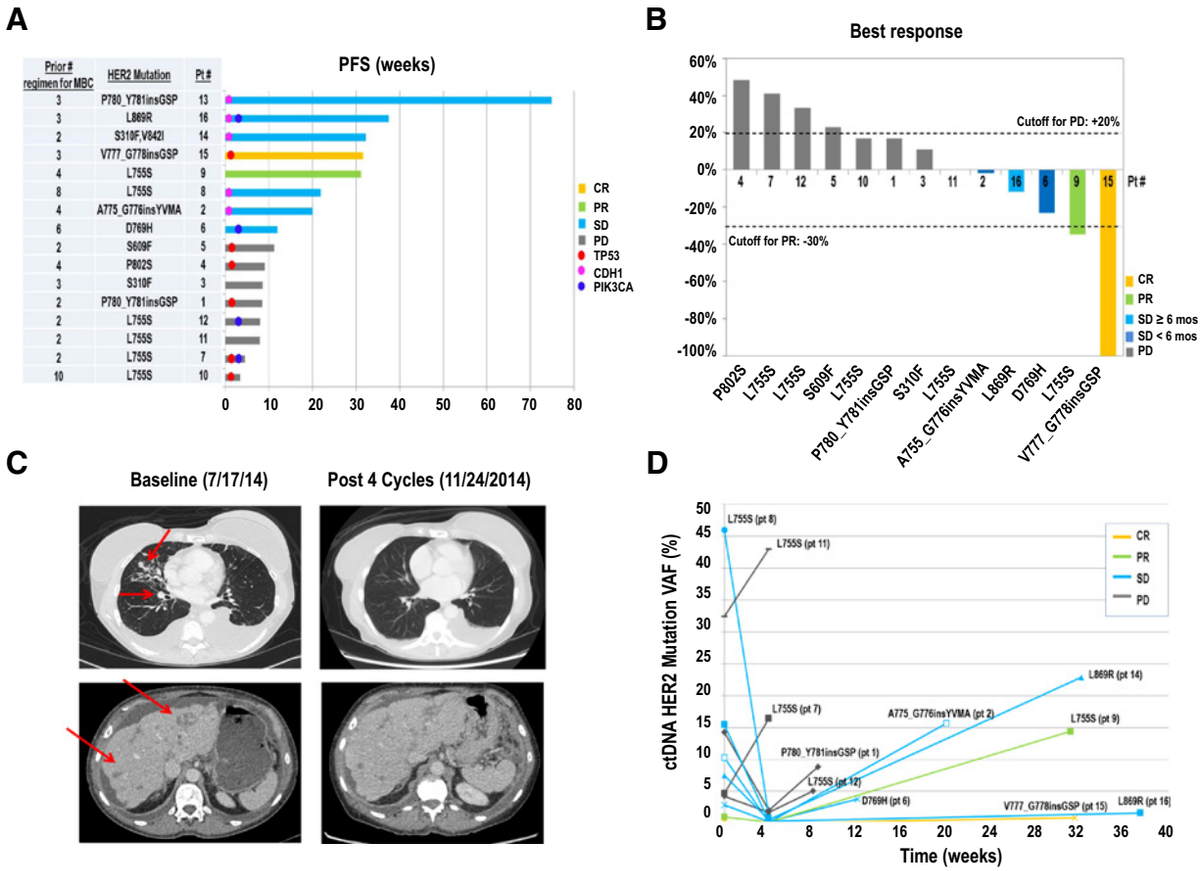

Figure 2.

Antitumor activity of neratinib. **A,** Swimmer Plot of time to progression (TTP) for patients with *HER2*-activating mutations. Co-occurring mutations in *TP53, CDH1,* and *PIK3CA* by tumor DNA sequencing are indicated. **B,** Percentage change in target lesion at best response compared with baseline. Patients with measurable diseases are included. **C,** Representative CT images of a patient on study. Sixty-four-year old woman (Pt 9) with $ER^+/HER2^-$ metastatic breast cancer, *HER2* L755S, prior disease progression on fulvestrant, and exemestane plus everolimus in the metastatic setting, achieved a partial response on neratinib. **D,** ctDNA *HER2* mutation variant allele frequency in response to neratinib. *HER2* mutation VAFs at baseline, 4 weeks, and progression are plotted for individual patients ($n = 11$).

Among the 14 positive control cases, two were negative for any $HER2^{mut}$ by ctDNA sequencing despite the detection of other genetic alterations in the same sample, and 11 were positive for the same $HER2^{mut}$ as by tumor testing (sensitivity: 11/14; 79%, 90% CI, 53%–94%; Supplementary Table S1). One other patient (Pt 14), SD $\geq$ 24 weeks on neratinib, had discrepant $HER2^{mut}$ alleles by tumor and ctDNA sequencing. ctDNA sequencing identified *HER2* L869R and D769Y at baseline, in contrast to the S310F and V842I detected in the breast cancer specimen collected from several years prior to trial enrollment. Interestingly, the ctDNA VAFs for both *HER2* L869R and D769Y were reduced at week 4, and increased at progression, accompanied by the emergence of several other *HER2* mutations, including the T798I mutation in the kinase domain of HER2 analogous to the *EGFR* T790M "gate-keeper" drug resistance mutation (Fig. 3A; refs. 36–38).

Among the 40 negative control cases, 32 were informative for ctDNA sequencing interpretation (eight had no detectable ctDNA mutations), and all 32 were negative for $HER2^{mut}$ despite the detection of other somatic mutations in each case. Therefore, the specificity of ctDNA for $HER2^{mut}$ detection was 32 of 32 (100%; 90% CI, 91%–100%; Supplementary Table S1). The overall concordance rate between plasma and tumor *HER2* sequencing results was 43 of 46 (93.5%; 90% CI, 87%–100%; Supplementary Tables S1 and S2).

We further queried the ctDNA sequencing data from 1,834 advanced breast cancer patients clinically tested at Guardant Health between October 2015 and August 2016 using the same assay. *HER2*-activating mutations in the absence of *HER2* amplification were identified in 48 of 1,584 (3.0%) evaluable patients. The incidence and distribution (Supplementary Fig. S3) were similar to prior tumor-based analyses (16).

Among the 11 patients (four PD and seven non-PD as best response by RECIST) with baseline *HER2* mutation detected by ctDNA who also had subsequent blood collections, $HER2^{mut}$ VAF decreased at week 4 in nine patients, including all seven patients with non-PD as their best response. The $HER2^{mut}$ VAF at week 4 was reduced to nondetectable in the patient with CR (Pt 15). Although two patients with PD as the best response (Pts 1 and 12) demonstrated a decrease in $HER2^{mut}$ VAFs at week 4, their $HER2^{mut}$ VAFs subsequently increased at week 8, when progression was radiographically detected. In contrast, the other two of four patients with PD as the best response (Pts 7 and 11) had a rise

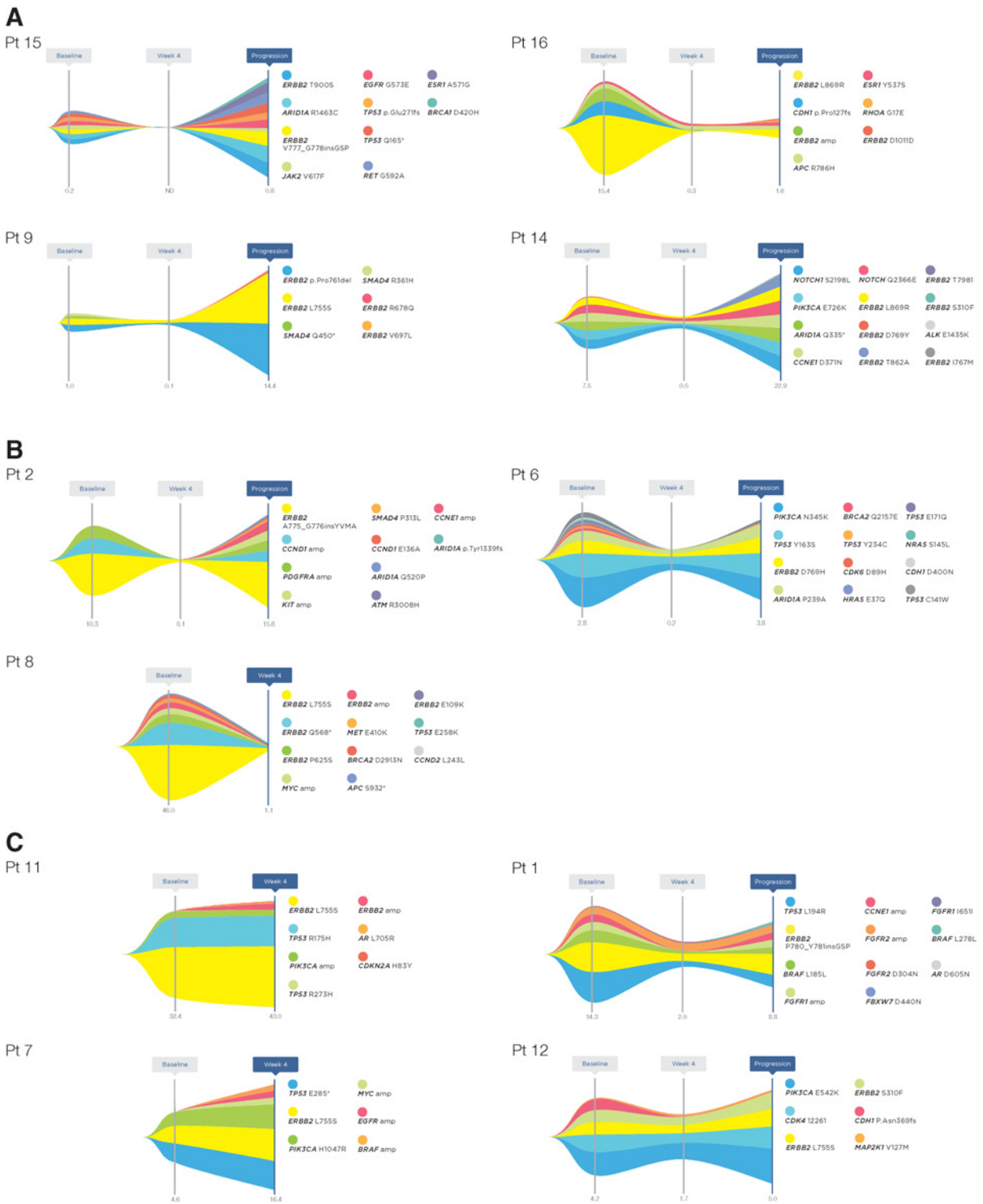

Figure 3.

Variant allele frequency maps of ctDNA-detected mutations in individual patients in response to neratinib treatment. Variant allele frequencies (VAFs) of ctDNA-detected mutations over time are shown for the 11 patients with detectable *HER2* mutation at baseline and with available blood samples at subsequent time points ($n = 11$ at 4 weeks, and $n = 8$ at progression). **A** includes four patients who achieved clinical benefit (CR in Pt 15, PR in Pt 9, SD $\geq$24 weeks in Pts 16 and 14). **B,** three patients who had best response of SD but lasted less than 24 weeks (Pt 2, Pt 6, and Pt 8). **C,** four patients who had PD as the best response (Pt 11, Pt 7, Pt 1, and Pt 12). Numbers at the bottom of each graph represent the VAFs of the *HER2* mutation colored in yellow, which were also used to plot Fig. 2D. All ctDNA alterations are shown except for patients 14 and 6 due to space limits in the figure. The complete list of mutations and their VAFs is detailed in Supplementary Table S3.

in *HER2*^{mut} VAFs at week 4, consistent with early progression (Fig. 2D and 3; Supplementary Table S2). The absolute levels of *HER2*^{mut} VAFs at week 4 were significantly associated with PFS (Spearman correlation coefficient rho = –0.69, $P = 0.017$, $n = 11$) and tumor size change (rho = 0.67, $P = 0.05$, $n = 9$). All patients had a rise in *HER2*^{mut} VAFs upon disease progression.

A number of genetic alterations were also identified in ctDNA sequencing, which co-occur with *HER2* mutations (Supplementary Table S3), with the VAFs of *HER2* mutations among the highest in each case. The VAFs of these co-occurring genetic alterations trended similarly to that of *HER2* mutations (Fig. 3). These data further support *HER2* mutations being a driver event in *HER2*^{mut} breast cancers which can be treated with neratinib.

Discussion

HER2-targeted agents have markedly improved outcomes for patients with HER2-positive breast cancer (2, 5–9). In this phase II study, single-agent neratinib, an irreversible pan-HER inhibitor, demonstrated a CBR of 31% (90% CI, 13%–55%), in a heavily pretreated patient population with *HER2*-mutated nonamplified MBC. Neratinib was well tolerated with limited duration of grade 3 diarrhea (median = 1.5 days) and only one patient discontinuing therapy due to AE. The incidence of *HER2*-activating mutations in primary breast cancer approximates 2% to 3% overall, with an approximately threefold higher incidence in lobular cancers (3, 15–17). Therefore, approximately 5,000 to 7,500 (2%–3% of 250,000; ref. 39) new breast cancers each year in the United States likely have a *HER2* mutation at diagnosis. The overall prevalence of *HER2* mutation in MBC can only be estimated, but in the United States, at least 200,000 patients are likely to be living with advanced disease today based on a relapse risk of 30% and median survival of 3 years (40). Based on the incidence of 2% to 3%, 4,000 to 6,000 patients have a *HER2*-mutant metastatic tumor. This prevalence is sufficient to conduct randomized trials to establish a standard of care if *HER2* mutation screening were widely available.

This study demonstrates the feasibility of targeting a rare breast cancer patient population with a multi-institutional, collaborative effort. The logistics of large-scale screening efforts were the major challenge, hampered by insufficient tumor tissue in approximately one-third of patients. ctDNA-based *HER2* sequencing provides a noninvasive, highly specific and sufficiently sensitive alternative to invasive approaches to be recommended in a clinical trial setting to assist in patient identification.

This study was proof of concept and had a small sample size. However, the investigation met the predefined primary endpoint and provided strong evidence that *HER2* mutation is a valid therapeutic target. Although there have been several single case reports of patients with *HER2* mutated, nonamplified MBC responding to HER2-targeted agents, this is the first report of a formal study addressing this question (22, 25). Although the CBR of 31% (90% CI, 13%–55%) is modest, it is significant in a heavily pretreated patient population that received a median of 3 (range, 2–10) prior treatment regimens in the metastatic setting. As an example, single-agent palbociclib showed a CBR of 19% overall, and 29% in the HR$^+$ HER2$^-$ subset, in patients with Rb+ advanced breast cancer who had progressed through at least two prior lines of hormonal therapy (41).

In summary, our data indicate efficacy of neratinib for *HER2*-mutated nonamplified breast cancer and provide a rationale for a large-scale ctDNA-based screening program to identify a sufficient number of patients with *HER2*-mutant breast cancer to establish a new standard of care. The universal, and in many cases, quite rapid development of acquired resistance despite a clear initial molecular response by *HER2* mutation VAF in ctDNA indicates that rational combination therapy is the next step. The strong association between ER positivity and *HER2*-activating mutations demands dual targeting of ER and HER2 pathways based on the success of combining ER and HER2-targeted agents in *HER2*-amplified disease (42–44). This phase II trial has therefore been amended to investigate the combination of fulvestrant and neratinib in subsequent patients with ER-positive *HER2*-mutated nonamplified breast cancer and to allow ctDNA determination of *HER2* mutation status as an eligibility criterion (NCT01670877). Other ongoing studies that target *HER2*-mutated advanced solid tumors include the SUMMIT neratinib basket study (NCT01953926), the NCI-MATCH trial with afatinib (NCT02465060), and My Pathway trial with trastuzumab plus pertuzumab (NCT02091141).

Disclosure of Potential Conflicts of Interest

C.X. Ma reports receiving commercial research grants from and is a consultant/advisory board member for Novartis, Pfizer, and Puma Biotechnology. R. Bose is a consultant/advisory board member for Genetech. G. Kimmick reports receiving commercial research grants from BioNovo, Bristol-Myers Squibb, GlaxoSmithKlein, Novartis, and Puma Biotechnology. E. Winer is a consultant/advisory board member for Genetech, LEAP, and Tesaro. M. Naughton reports receiving speakers bureau honoraria from Genetech and Novartis. D. Tripathy is a consultant/advisory board member for Puma Biotechnology. M. Cobleigh is a consultant/advisory board member for Puma Biotechnology. C. Anders is a consultant/advisory board member for Angiochem, Eli Lilly, Genentech, Geron, Kadmon, Merrimack, Nektar, Novartis, Sanofi, and to BBB. K.C. Banks holds ownership interest (including patents) in Guardant Health. R.B. Lanman holds ownership interest (including patents) in Guardant Health. R. Bryce and A. Lalani are employees of PUMA Biotechnology. D.F. Hayes reports receiving commercial research grants from Puma Biotechnology, reports receiving other commercial research support from AstraZeneca, Janssen, and Pfizer, and holds ownership interest (including patents) in OncImmune. K. Blackwell is a consultant/advisory board member for Puma Biotechnology. M.J.C. Ellis is an employee of and holds ownership interest (including patents) in Bioclassifier LLC, and is a consultant/advisory board member of AstraZeneca, Novartis, Pfizer, and Puma Technology. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: C.X. Ma, R. Bose, E. Winer, M. Naughton, R. Bryce, K. Blackwell, M.J.C. Ellis

Development of methodology: C.X. Ma, J. Pfeifer, M. Pegram, H. Al-Kateb, M.J.C. Ellis

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.X. Ma, R.A. Freedman, M.L. Telli, G. Kimmick, M. Naughton, M. Goetz, C. Russell, D. Tripathy, M. Cobleigh, A. Forero, T.J. Pluard, C. Anders, P.A. Niravath, C. Bumb, R.B. Lanman, J. Pfeifer, D.F. Hayes, M. Pegram, K. Blackwell, P.L. Bedard, M.J.C. Ellis

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.X. Ma, R. Bose, F. Gao, R.A. Freedman, G. Kimmick, M. Cobleigh, A. Forero, K.C. Banks, R.B. Lanman, M. Pegram, K. Blackwell, P.L. Bedard, H. Al-Kateb, M.J.C. Ellis

Writing, review, and/or revision of the manuscript: C.X. Ma, R. Bose, F. Gao, R.A. Freedman, M.L. Telli, G. Kimmick, E. Winer, M. Naughton, M. Goetz, C. Russell, D. Tripathy, M. Cobleigh, A. Forero, T.J. Pluard, C. Anders, P.A. Niravath, S. Thomas, J. Anderson, K.C. Banks, R.B. Lanman, R. Bryce,

A.S. Lalani, J. Pfeifer, D.F. Hayes, M. Pegram, K. Blackwell, P.L. Bedard, H. Al-Kateb, M.J.C. Ellis

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.X. Ma, S. Thomas, J. Anderson, K.C. Banks, M.J.C. Ellis

Study supervision: C.X. Ma, D. Tripathy, A. Forero, K. Blackwell, M.J.C. Ellis

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