HER-2 Gene Amplification, HER-2 and Epidermal Growth Factor Receptor mRNA and Protein Expression, and Lapatinib Efficacy in Women with Metastatic Breast Cancer

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Abstract

Purpose: Biomarkers from two randomized phase III trials were analyzed to optimize selection of patients for lapatinib therapy.

Experimental Design: In available breast cancer tissue from EGF30001 (paclitaxel ± lapatinib in HER-2-negative/unknown metastatic breast cancer, $n = 579$) and EGF100151 (capecitabine ± lapatinib in HER-2-positive metastatic breast cancer, $n = 399$), HER-2 gene amplification by fluorescence *in situ* hybridization (FISH), HER-2 mRNA by reverse transcription-PCR (RT-PCR), HER-2 protein expression by HercepTest immunohistochemistry (IHC), epidermal growth factor receptor (EGFR) mRNA level by RT-PCR, and EGFR protein by IHC were analyzed and compared with clinical outcome. HER-2 was determined by FISH in an academic reference/research laboratory and in a large, high-volume commercial reference laboratory.

Results: The HER-2 gene was amplified in 47% (344 of 733) and IHC was 3+ in 35% (279 of 798), with significant correlation ($P < 0.01$) between FISH and IHC. Positive EGFR immunostaining (IHC 1+, 2+, or 3+) in 28% (213 of 761) correlated with EGFR mRNA levels by RT-PCR ($r = 0.59$; $P < 0.01$). HER-2 gene amplification/overexpression was associated with improved clinical outcomes (progression-free survival; $P < 0.001$) in both trials. A significant improvement in outcome was seen in FISH-positive and IHC 0, 1+, or 2+ patients. HER-2 mRNA expression correlated with HER-2 FISH ($r = 0.83$) and IHC status ($r = 0.72$; $n = 138$). No correlation was found between EGFR expression (IHC or mRNA) and responsiveness to lapatinib regardless of HER-2 status. Although a significant correlation with lapatinib responsiveness was observed among "HER-2-negative" breast cancer patients in the large, high-volume commercial reference laboratory, this was not confirmed in the academic reference/research laboratory.

Conclusions: Women with HER-2-positive metastatic breast cancer benefit from lapatinib, whereas women with HER-2-negative metastatic breast cancer derive no incremental benefit from lapatinib.

Lapatinib (Tykerb/Tyverb) is an orally available, small-molecule inhibitor of tyrosine kinase activity of both epidermal growth factor receptor (EGFR) type 1 (ErbB1 or HER-1) and type 2 (HER-2 or ErbB2). Lapatinib has been approved in combination with capecitabine for the treatment of women with HER-2-positive metastatic breast cancer that has progressed after treatment with an anthracycline, a taxane, and trastuzumab (1).

Because lapatinib inhibits both HER-2 and EGFR (2), there are several unanswered questions about which patients with breast cancer are most likely to benefit from this form of targeted therapy and which type of HER-2 determination method is most appropriate. In addition, recent reports suggest that women with HER-2-negative breast cancer who do not meet established criteria for HER-2-positive disease on central review might benefit from adjuvant trastuzumab treatment, raising questions about the criteria used for patient selection with this targeted

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Translational Relevance

This study characterizes the association of lapatinib responsiveness with the status of two different biomarkers, HER-2 and EGFR, in two clinical trials of chemotherapy with or without added lapatinib. We show that HER-2 gene amplification/overexpression status, not EGFR status, is associated with lapatinib responsiveness in breast cancer patients. However, lapatinib responsiveness is not related to the level of HER-2 amplification among women with HER-2-amplified breast cancers. Women whose breast cancers have low-level HER-2 amplification respond similarly to women whose breast cancers have high-level HER-2 amplification. Women with HER-2-negative metastatic breast cancers do not respond to lapatinib, although initial preliminary results from a HVLab suggested the opposite conclusion. A blinded reanalysis of HER-2 amplification status determined by FISH in a ALab shows that results obtained in the HVLab misclassified ~10% of the HER-2-amplified breast cancers as not amplified. Because the only methodologic difference between FISH assays in these laboratories were the qualifications of the persons interpreting the assays, this comparison confirms the importance of having a pathologist, not a laboratory technician, perform these HER-2 assessments.

therapy (3 – 5). To address these issues, we assessed HER-2 status at the DNA, mRNA, and protein levels in breast cancer tissues from women with metastatic breast cancer who were enrolled in two large randomized phase III trials of lapatinib and chemotherapy and analyzed these data in regard to clinical outcome to determine potential associations between HER-2/EGFR status and patient responsiveness to lapatinib.

Materials and Methods

We performed a retrospective analysis of HER-2 and EGFR status in subsets of tumor specimens from 978 patients with metastatic breast cancer from two clinical trials of lapatinib plus chemotherapy versus chemotherapy alone. All laboratory analyses were done blinded to both clinical outcome information and other laboratory analyses.

Assessment of HER-2 status

The HER-2 gene, mRNA, and protein status were assessed by fluorescence *in situ* hybridization (FISH), reverse transcription-PCR (RT-PCR), and immunohistochemistry (IHC), respectively.

Fluorescence* in situ *hybridization. FISH assays were done independently by two different laboratories: one is a high-volume commercial reference laboratory (HVLab) and the other is an academic reference/research laboratory (ALab). Both laboratories processed the tissue sections using the HER-2 PathVysion FISH assay (Abbott Laboratories) as described elsewhere (6, 7); however, the method for assessment of HER-2-FISH status varied between laboratories. The ALab evaluated FISH signals by enumeration of the number of red HER-2 signals and the number of green chromosome 17 centromeres in each of at least 20 interphase carcinoma cell nuclei as approved by the U.S. Food and Drug Administration (6 – 9). The enumerations were done by a licensed clinical laboratory scientist and confirmed by a board-certified pathologist. The HVLab assessed HER-2 status by microscopic inspection of the FISH slides, with assignment of an estimated FISH ratio by a laboratory technician and reviewed, when requested, by an available pathologist. Interlaboratory variability was observed and further explored. The HER-2-FISH gene amplification results reported below are those from the ALab, except in the section where results from the two laboratories are compared.

Because previous studies have shown FISH to be significantly more accurate at assessment of the (known) HER-2 status of molecularly characterized samples (7, 9) and because IHC status does not contribute significant new information when HER-2 FISH status is known (7, 8, 10), we defined "HER-2-positive" as HER-2 gene amplification by FISH (ratio $\geq$ 2.0) and "HER-2-negative" as a lack of HER-2 gene amplification by FISH (ratio < 2.0). If the HER-2 FISH status were unknown, we used IHC to assess HER-2 status (IHC 3+, "HER-2-positive" and IHC <3+, "HER-2-negative"). This is justified by a high rate of HER-2 gene amplification among IHC 3+ cases in this study (97%; see Results and Table 2) and in similar studies (6, 11).

Microdissection. Formalin-fixed, paraffin-embedded tumor specimens and adjacent normal tissues were cut into serial 10 μm sections. For the pathologic diagnosis, one slide was stained with H&E and evaluated by a pathologist. Other sections were stained with nuclear fast red (American MasterTech Scientific) to enable visualization of histology. Laser capture microdissection (PALM Microlaser Technologies) was done to isolate tumor cells. In some cases when larger tumor cell areas were present, malignant cells were selected under a dissecting microscope (from ×5 to ×10 magnification) and dissected from the slide with a scalpel.

RNA isolation and cDNA synthesis. RNA isolation was done according to a proprietary procedure of Response Genetics (U.S. patent no. 6,248,535) as described previously (12, 13).

Reverse transcription-PCR. Relative cDNA quantitation for HER-2 was determined using an internal reference gene (β-actin) with a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System; TaqMan; Applied Biosystems) as described previously (12, 13).

Immunohistochemistry. HER-2 protein expression status was assessed using a commercially available, Food and Drug Administration-approved IHC assay method, the HercepTest (DAKO), according to the manufacturer's instructions as summarized elsewhere (6, 7). HER-2 immunostaining was scored as 0, 1+, 2+, and 3+ by a board-certified pathologist, with 0 and 1+ considered low HER-2 expression and 3+ considered overexpression per American Society of Clinical Oncology/College of American Pathologists guidelines (14, 15).

Assessment of EGFR status

EGFR expression at the mRNA and protein levels was assessed by RT-PCR and IHC, respectively.

RT-PCR assessment of EGFR mRNA. The relative cDNA quantitation for EGFR was determined as described above for HER-2, except that a different set of primers were used for the gene of interest, EGFR.

Immunohistochemical assessment of EGFR protein expression. EGFR IHC was done using a commercially available immunohistochemical assay kit (PharmDX; DAKOCytomation). Subjective assessment of the amount of membrane staining (0, 1+, 2+, or 3+) was done according to the manufacturer's package insert.

Patients

Tissue samples used for HER-2 and EGFR analyses were from women who participated in one of two clinical trials of chemotherapy with or without lapatinib (EGF100151 or EGF30001). These two clinical trials, summarized below, are described in detail elsewhere (1, 16).

EGF100151 clinical trial. The clinical trial EGF100151 (clinicaltrials.gov registration no. NCT00078572) was a randomized, multicenter, two-arm, phase III clinical trial that compared clinical outcomes among 399 women with locally determined HER-2-positive (IHC 3+ or IHC 2+/FISH-positive) breast cancer previously treated with an anthracycline, a taxane, and trastuzumab. Patients received either standard capecitabine chemotherapy alone (2,500 mg/m^2/d, days 1-14, for 3 weeks) or capecitabine chemotherapy (2,000 mg/m^2/d, days 1-14, for 3 weeks) with lapatinib (1,250 mg/d) therapy for their metastatic disease (1).

EGF30001 clinical trial. The clinical trial EGF30001 (clinicaltrials.gov registration no. NCT00075270) was a randomized, multicenter, double-blind, placebo-controlled, two-arm, phase III clinical trial that compared clinical outcomes among 580 women with locally HER-2-negative (IHC 0 or 1+ or IHC 2+/FISH-negative) or untested metastatic breast cancer who had not received prior therapy for metastatic disease. Patients received either lapatinib (1,500 mg/d) with paclitaxel (175 mg/m^2 intravenous over 3 h every 3 weeks) or paclitaxel (175 mg/m^2 intravenous over 3 h every 3 weeks) plus placebo.

In both trials, treatment continued until disease progression or unacceptable toxicity, and patients were followed for disease progression and survival. The institutional review board for each participating institution approved the study protocol. All patients gave written informed consent. The University of Southern California institutional review board approved this retrospective sample analysis.

Statistical methods

Progression-free survival (PFS) was summarized graphically using the Kaplan-Meier method. Cox proportional hazards models were used to generate *P* values and hazard ratios (HR) for PFS. Cox models were stratified by study for pooled analyses. All *P* values are two-sided. Analyses were conducted using SAS version 9.2. HER-2 FISH and IHC assay results from the ALab were used for statistical analyses of clinical outcomes. HER-2-positive status was defined as patients with a positive (≥2.0) FISH score or IHC 3+ if FISH unknown. HER-2-negative status was defined as FISH <2 or IHC 0, 1+, or 2+ if FISH unknown.

Results

Patient and breast cancer characteristics

Trial EGF100151 enrolled 399 patients (lapatinib + capecitabine: 198; capecitabine: 201) with HER-2 status determined centrally by one or more methods in 326 (82.0%) patients. All patients had prior chemotherapy for metastatic disease as described elsewhere (1, 17). Trial EGF30001 enrolled 580 patients, but because 1 randomized subject withdrew before starting treatment, there was an intent-to-treat population of 579 patients (paclitaxel + lapatinib: 291; paclitaxel + placebo: 288). HER-2 status was centrally determined by at least one assay method in 494 (85%) of these subjects. Patient and disease characteristics were generally well-balanced within and across studies (Table 1; refs. 1, 17).

HER-2 amplification/expression of breast cancers from women in both clinical trials

HER-2 gene amplification (n = 733), HER-2 mRNA expression (n = 138; EGF100151 only), and HER-2 protein expression (n = 798) were determined from tumor tissue blocks or unstained tissue sections (Table 1; Supplementary Fig. S1).

HER-2 gene amplification status. HER-2 gene amplification was identified in 344 of 733 (47%) available breast cancer samples (EGF100151, n = 264; EGF30001, n = 80). HER-2 was not amplified in 389 (53%) breast cancers (EGF100151, n = 47; EGF30001, n = 342).

HER-2 mRNA expression. HER-2 mRNA levels were determined for 138 samples from EGF100151. There was a direct and statistically significant correlation between increasing HER-2 gene amplification ratio and HER-2 mRNA expression level (r = 0.83; P < 0.001; Supplementary Fig. S2). A correlation was also observed between HER-2 mRNA and HER-2 protein determined by IHC (r = 0.72; P < 0.001; Supplementary Fig. S2B).

HER-2 protein expression status. Of 798 samples, strong (IHC 3+) HER-2 protein immunostaining was observed in 279 (35%) samples, moderate (IHC 2+) in 83 (10%) samples,

Table 1. Distribution of patient and tumor characteristics

	EGF100151 clinical trial					
	All		HER-2 positive*		HER-2 negative †	
	Capecitabine and lapatinib (n = 198)	Capecitabine alone (n = 201)	Capecitabine and lapatinib (n = 139)	Capecitabine alone (n = 132)	Capecitabine and lapatinib (n = 28)	Capecitabine alone (n = 27)
Mean (range) age, y	54 (26-80)	52 (28-83)	53 (32-80)	51 (30-83)	55 (29-79)	56 (40 - 80)
Stage						
IIIb-c (%)	7 (4)	8 (4)	7 (5)	5 (4)	0	0
IV (%)	191 (96)	193 (96)	132 (95)	127 (96)	28 (100)	27 (100)
Visceral metastasis (%) ‡	153 (77)	158 (79)	104 (75)	100 (76)	21 (75)	24 (89)
	EGF30001 clinical trial					
	All		HER-2 positive*		HER-2 negative †	
	Paclitaxel and lapatinib (n = 291)	Paclitaxel alone (n = 287)	Paclitaxel and lapatinib (n = 49)	Paclitaxel alone (n = 37)	Paclitaxel and lapatinib (n = 202)	Paclitaxel alone (n = 206)
Mean (range) age, y	51.3 (23-87)	52.4 (25-78)	51 (34-75)	51 (28-78)	52 (23-74)	52 (26-78)
Stage						
IIIb-c (%)	37 (13)	40 (14)	6 (12)	8 (22)	26 (13)	21 (10)
IV (%)	254 (87)	247 (86)	43 (88)	29 (78)	168 (87)	182 (90)
Visceral metastasis (%) ‡	183 (63)	184 (64)	34 (69)	19 (51)	125 (62)	138 (67)

*HER-2-positive status: FISH-positive (n = 344) or if FISH unknown, IHC 3+ (n = 13).
† HER-2-negative status: FISH-negative (n = 389) or if FISH unknown, IHC 2+, 1+, or 0 (n = 74).
‡ With or without other metastases.

Table 2. Comparison of HER-2 gene amplification by FISH with HER-2 protein expression by IHC

FISH	IHC				
	0 (%)	1+ (%)	2+ (%)	3+ (%)	Total
Negative	237 (94.8)	99 (81.8)	36 (48)	8 (3)	380
Positive	13 (5.2)	21 (18.2)	39 (52)	258 (97)	331
Total	250 (100)	120 (100)	75 (100)	266 (100)	711

weak (IHC 1+) in 137 (17%) samples, and no immunostaining (IHC 0) in 299 (37%) samples. Among breast cancers assessed for both HER-2 gene status by FISH and HER-2 protein by IHC (n = 711), there was a significant correlation between HER-2 gene amplification and HER-2 protein overexpression whether IHC 2+ was considered overexpression (P < 0.0001) or whether IHC 2+ breast cancers were eliminated as indeterminate (P < 0.0001; Table 2).

EGFR status of breast cancers from women in both clinical trials

EGFR expression (IHC 1+, 2+, or 3+) was identified in 213 of 761 (28%) breast cancers from both clinical trials (Supplementary Table S1). Among 134 samples from EGF100151 analyzed for EGFR mRNA expression by RT-PCR and for EGFR protein expression by IHC, there was a significant correlation between expression levels identified by these two methods (r = 0.59; P < 0.001; Supplementary Fig. 2C). This correlation was similar to the correlation observed between RT-PCR and IHC for HER-2 (r = 0.72; P < 0.001; Supplementary Fig. 2B). No correlation was observed between EGFR expression and either HER-2 gene amplification or HER-2 gene expression (data not shown). Of the 761 samples tested for both EGFR expression and HER-2 status, only 100 both expressed EGFR (IHC 1+, 2+, or 3+) and had HER-2 gene amplification/overexpression compared with 112 that only had EGFR expression, prevalences that are consistent with two independent events.

Association of HER-2 status with clinical outcomes

Improvements in clinical outcome were observed for HER-2-positive (FISH-positive or FISH-unknown and IHC 3+) patients who received lapatinib with chemotherapy compared with patients who received chemotherapy alone (either capecitabine or paclitaxel). Improvements in clinical outcome were observed for PFS (HR, 0.47; P < 0.0001; Fig. 1A), response rate (EGF100151 = 28% versus 15%; EGF30001 = 63% versus 38%; P < 0.05), and clinical benefit rate (EGF100151 = 34% versus 17%; EGF30001 = 69% versus 41%; P < 0.05). Although overall survival was numerically improved for HER-2-positive patients receiving lapatinib, the difference in survival was not statistically significant. Clinical outcomes, as illustrated by PFS (Fig. 1A-F), were not improved for FISH-negative patients (Fig. 1E and F) who received lapatinib-containing treatment compared with chemotherapy alone treatment.

Association of clinical outcome with lapatinib treatment and HER-2 status determined by FISH or IHC. We assessed the potential for increasing levels of HER-2 gene amplification to be associated with increased responsiveness to lapatinib. We found that HER-2 gene amplification was associated with improved clinical outcome among those patients treated with lapatinib, as described above, and that this improvement was similar regardless of the degree of HER-2 gene amplification (Table 3).

Because there was a strong correlation between HER-2 status determined by FISH and IHC, we observed, as expected, a significant association of improved clinical outcome with HER-2-positive disease determined by either method (Fig. 1B and C). We also compared the subsets of patients whose samples were classified differently by the two methods [FISH-positive but IHC-negative (IHC <3+) and FISH-negative but IHC-positive] for outcome by treatment arm. Patients (n = 73) with FISH-positive but IHC-negative (0, 1+, or 2+) breast cancers who were treated in a lapatinib-containing treatment arm (n = 43) had a significant improvement in PFS (HR, 0.45; P = 0.0143) compared with patients with FISH-positive, IHC-negative samples who were treated in a chemotherapy alone arm (n = 30; Fig. 1D). Similar observations were made for those patients (n = 34) with FISH-positive, IHC 0 or 1+ breast cancers (HR, 0.34; P = 0.033). In contrast, patients with breast cancers that are FISH-negative and had any degree of IHC-positive expression above 0 (IHC 3+, 2+, or 1+; n = 143) did not show a similar improvement in PFS (HR, 0.97; P = 0.882; Fig. 1E).

There were too few (n = 8) women with FISH-negative, IHC 3+ positive breast cancers to perform statistical analysis of this patient subset. However, all 4 such women treated with lapatinib and chemotherapy experienced shorter PFS (12.4, 15.1, 9.0, and 0.1 weeks) than the median PFS for FISH-positive women treated with lapatinib (34 weeks) and also shorter PFS than the median without lapatinib (20 weeks), suggesting no benefit of lapatinib in these patients. The 4 women with FISH-negative, IHC 3+ cancers treated with chemotherapy alone had PFS of 33.5, 24.0, 19.0, and 1.0 weeks, similar to results obtained in patients with FISH-negative, IHC <3+ tumors treated with chemotherapy alone. Likewise, those women whose breast cancers were negative by both FISH and IHC (IHC 0; n = 237) also did not benefit from lapatinib treatment (PFS HR, 1.13; P = 0.43; Fig. 1F).

Lack of association of EGFR expression status with responsiveness to lapatinib

Analysis of EGFR expression regarding clinical outcome showed no correlation between EGFR status and responsiveness to lapatinib treatment (Supplementary Fig. S3). HER-2-amplified patients benefited from lapatinib treatment regardless of EGFR status (Supplementary Fig. S3A-C), whereas HER-2-nonamplified patients showed no association with benefit from lapatinib treatment regardless of EGFR status (Supplementary Fig. S3D-F).

Potential for patients with "HER-2-negative" breast cancer to respond to lapatinib

Because a preliminary analysis of HER-2 FISH status done in a HVLab from EGF100151 suggested that patients with "HER-2-negative" (FISH-negative and IHC 0, 1+, or 2+) breast cancer might also respond to lapatinib, a blinded reanalysis of samples from both trials was done by the ALab to evaluate the level of concordance in FISH testing and assess whether HER-2-negative patients respond to lapatinib. The HER-2 and EGFR status, described above, was the retrospectively determined analyses done in the ALab. The HVLab also had retrospectively and independently evaluated the HER-2 FISH status in many of the same cases. Results of central HER-2 testing by both HVLab and

ALab were available in 263 cases from the EGF100151 trial. Comparison of the HER-2 FISH status (amplified versus nonamplified) from these two laboratories for EGF100151 showed a relatively favorable level (89%) of concordance between these two laboratories ($P < 0.001$; Table 4). Using the HER-2 status from either laboratory showed that, among HER-2-amplified patients, there were significant improvements in PFS (HVLab: $P < 0.001$; ALab: $P < 0.001$; Table 3), response rate (HVLab: $P < 0.01$; ALab: $P < 0.05$), and clinical benefit rate (HVLab: $P < 0.01$; ALab: $P < 0.01$) associated with lapatinib and chemotherapy versus chemotherapy alone.

In contrast, different conclusions were suggested by analysis of the HER-2-negative populations from these two laboratories for EGF100151. Using the HVLab HER-2 status for assessment of lapatinib efficacy, it was observed that patients with HER-2 FISH-negative breast cancers treated with lapatinib and chemotherapy had a significantly prolonged PFS ($P = 0.046$) compared with chemotherapy alone. In contrast, those breast cancer cases characterized as HER-2 FISH-negative in the ALab showed no such association between improved outcome and lapatinib treatment ($P = 0.888$; Table 3). Furthermore, among the HER-2-negative populations from these two laboratories, similar associations/lack of associations were also observed for response rate (HVLab: $P = 0.13$; ALab: $P = 0.67$).

Direct comparison of the HER-2 FISH results from the two laboratories showed that more than a third of the cases considered to be HER-2 FISH-negative in the HVLab were assessed as HER-2 FISH-positive in the ALab (Table 4). These FISH-negative (HVLab)/FISH-positive (ALab) cases were largely IHC 3+ (14 of 20 with available IHC 3+, 70%), consistent with a HER-2-FISH-positive status. In contrast, the FISH-positive (HVLab)/FISH-negative (ALab) cases had relatively few HER-2 IHC 3+ cases (1 of 6 with IHC 3+, 17%) as did the

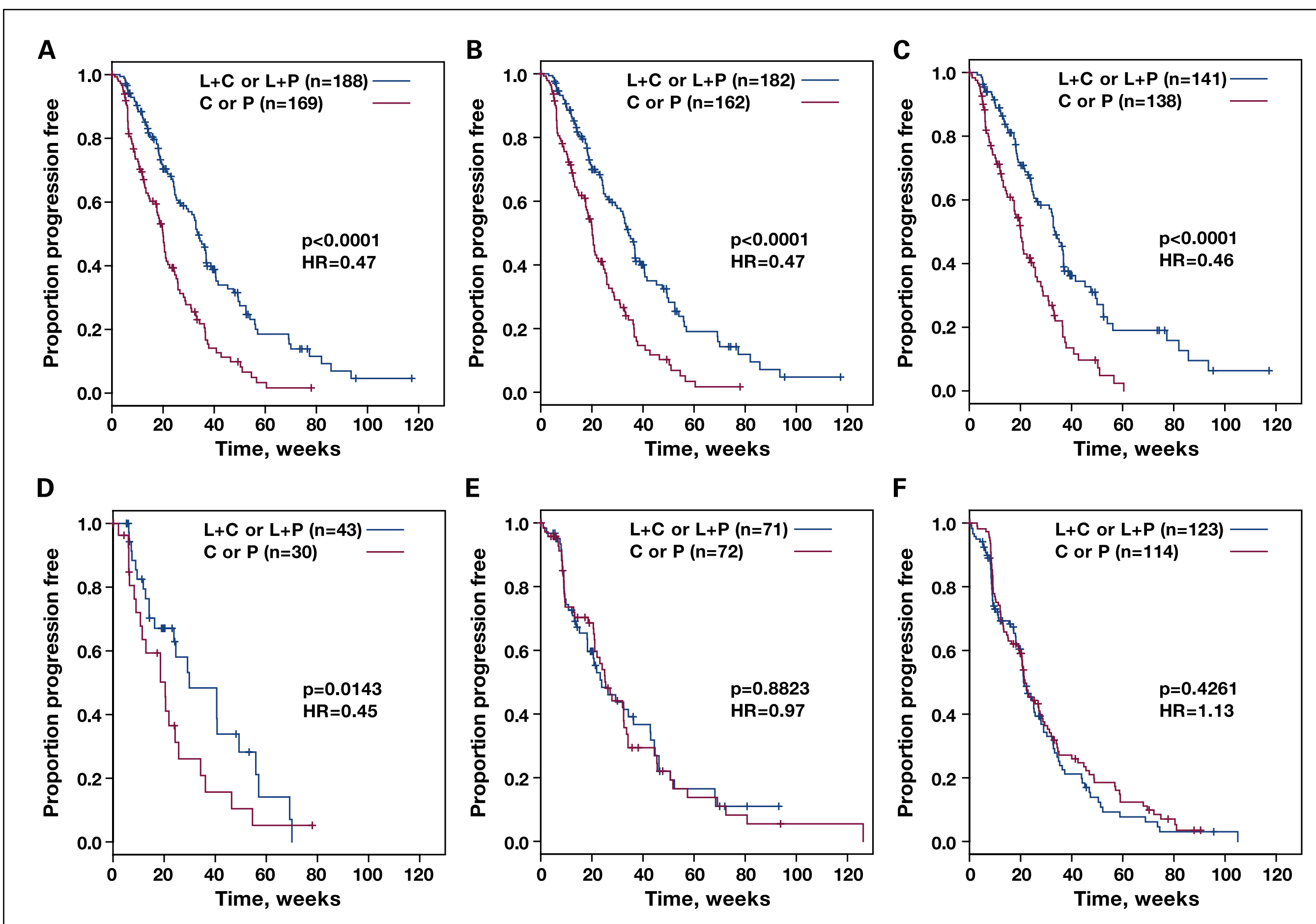

Fig. 1. Association of clinical outcomes with lapatinib treatment and HER-2 status in a combined analysis of PFS in EGF100151 and EGF30001 trials. *A,* analysis of PFS for women with HER-2-positive (FISH-positive or IHC 3+ if FISH unknown). Among women whose breast cancers were HER-2-positive, there was a significant improvement in PFS for women receiving lapatinib ($n = 188$) compared with those receiving capecitabine or paclitaxel chemotherapy alone ($n = 169$; HR, 0.47; $P < 0.0001$). *B,* analysis of PFS for women with HER-2 gene amplification by FISH. Among women whose breast cancers had HER-2 gene amplification (FISH $\geq$ 2.00), there was a significant improvement in PFS for women receiving lapatinib ($n = 182$; HR, 0.47; $P < 0.0001$) compared with those receiving capecitabine or paclitaxel chemotherapy alone ($n = 162$). *C,* analysis of PFS for women with HER-2 protein overexpression by IHC (3+). Among women whose breast cancers had HER-2 IHC 3+, there was a significant improvement in PFS for women receiving lapatinib ($n = 141$; HR, 0.46; $P < 0.0001$) compared with those receiving capecitabine or paclitaxel chemotherapy alone ($n = 138$). *D,* analysis of PFS for women whose breast cancers where FISH-positive, IHC-negative (IHC 2+, 1+, or 0) by lapatinib-containing treatment arm. There was a significant improvement in PFS for women receiving lapatinib ($n = 43$; HR, 0.45; $P = 0.0143$) compared with those receiving capecitabine or paclitaxel chemotherapy alone ($n = 30$). *E,* analysis of PFS for women whose breast cancers were FISH-negative, IHC-positive (IHC >0). Among women with HER-2 FISH-negative, IHC-positive breast cancers, there was no significant difference between PFS of women receiving chemotherapy with lapatinib ($n = 71$; HR, 0.97; $P = 0.88$) and women receiving chemotherapy alone ($n = 72$). *F,* analysis of PFS for women whose breast cancers were FISH-negative, IHC-negative (IHC 0). Women with HER-2 FISH-negative, IHC 0 breast cancers who were treated with chemotherapy and lapatinib ($n = 123$) did not show a significant difference in PFS compared with women treated with chemotherapy alone ($n = 114$; HR, 1.13; $P = 0.4261$).

FISH-negative (HVLab)/FISH-negative (ALab) cases (2 of 35, 5.7%). Six cases reported as HER-2 amplified in the HVLab were reported as not amplified in the ALab. Five of these 6 had HER-2 ratios between 2.0 and 2.4 in the HVLab; the sixth was reported as having a HER-2 FISH ratio of 4 by the HVLab. None of the 5 cases with HVLab FISH ratios between 2.0 and 2.4 had IHC 3+ immunostaining, consistent with the ALab FISH-negative status, whereas the case with HVLab FISH ratio of 4.0 did show IHC 3+ immunostaining, consistent with an amplified HER-2 status. However, this case had been excluded from clinical outcome evaluation by the ALab because two different areas of the tumor showed different results (heterogeneity), with carcinoma cells in one area showing HER-2 gene amplification (FISH ratio, 9.06) and overexpression (IHC 3+) and carcinoma cells in the other area of the same tumor showing a lack of amplification (FISH ratio, 1.07) and low expression (IHC 0).

Detailed analysis of the FISH ratios for all cases for which there were ratios determined in both laboratories (EGF100151, n = 264; EGF30001, n = 88) showed marked differences in the actual FISH ratios reported by these two laboratories (Fig. 2). Whereas the ALab reported FISH ratios that showed continuous variation across the entire range of FISH ratios, the HVLab reported amplified FISH ratios that were limited to a few whole number integer ratios (Fig. 2). Using FISH ratios from the ALab to assess lapatinib efficacy showed a lack of association between improved outcomes and lapatinib treatment in the FISH-negative population (P = 0.888).

Discussion

HER-2 and EGFR have been shown to act synergistically to transform NIH3T3 cells (18), with HER-2 potentiating the signaling of EGFR through increased affinity of ligand binding to EGFR, suppression of EGFR degradation, and enhancement of EGFR recycling (19–21). These observations suggest that both HER-2 and EGFR may play a role in promoting tumor cell growth in selected breast cancers; therefore, both may represent targets for anti-receptor-targeted therapy. From this perspective, inhibition of both receptors could be more effective cancer therapy than inhibition of either receptor alone. To assess the potential clinical utility of HER-2 and EGFR as molecular markers of responsiveness to lapatinib, we retrospectively evaluated the association between these receptors and clinical outcome in two trials of lapatinib treatment for women with metastatic breast cancer. These trials were designed to treat patients with, respectively, HER-2-positive breast cancer (EGF100151) and HER-2-negative/untested breast cancer (EGF30001) with chemotherapy, either capecitabine or paclitaxel, and to assess the potential for added lapatinib treatment to improve clinical outcome (1, 16).

Table 3. Correlation of PFS with responsiveness to lapatinib

Blinded analysis by HVLab*

HER-2 status	n	HR (95% CI)	P
Positive	255	0.47 (0.32-0.67)	<0.001
Negative	86	0.54 (0.30-0.99)	0.046

Blinded reanalysis by ALab*

HER-2 status	n	HR (95% CI)	P
Positive	271	0.46 (0.33-0.65)	<0.001
Negative	47	0.94 (0.39-2.28)	0.888

HR by FISH ratios in blinded reanalysis †

FISH ratios	n	HR (95% CI)	P
<2.0	390	1.09 (0.86-1.37)	0.486
2.0-5.5	82	0.48 (0.28-0.83)	0.009
5.5-7.6	89	0.35 (0.18-0.69)	0.002
7.6-10.1	87	0.58 (0.33-1.05)	0.072
≥10.1	88	0.42 (0.24-0.74)	0.003

*Analysis of cases with results from the EGF100151 clinical trial.
† Analysis of all cases in the ALab for patients in either clinical trial.

The focus of this study is on central laboratory analyses of HER-2 and EGFR as molecular markers and the potential association of these markers with responsiveness to lapatinib treatment. We found that ~85% (264 of 311) of "HER-2-positive" breast cancers in the EGF100151 trial did have HER-2 gene amplification and ~19% (80 of 422) of "HER-2-negative/unknown" breast cancers in the EGF30001 trial also had HER-2 gene amplification. Analysis of either of these two clinical trials separately showed that lapatinib was associated with a significantly more favorable clinical outcome (improved time to progression, PFS, response rate, and clinical benefit rate) only among women whose breast cancers had HER-2-positive disease (analyses not shown; refs. 16, 17). Women with HER-2-positive breast cancers, as measured by local laboratories, had an improved PFS [median, 6.2 versus 4.3 months; HR, 0.57; 95% confidence interval (95% CI), 0.43-0.77; P = 0.00013] when treated with lapatinib plus capecitabine compared with capecitabine alone (EGF100151; refs. 1, 17). Similarly, a retrospective analysis of centrally confirmed HER-2 status in the EGF30001 clinical trial showed that only women with HER-2-positive breast cancers appeared to benefit from lapatinib plus paclitaxel as first-line treatment compared with placebo plus paclitaxel (median PFS, 8.1 versus 5.0 months; HR, 0.52; 95% CI, 0.31-0.86; P = 0.004). Analysis of either of these clinical trials separately had shown that lapatinib had no effect on women whose breast cancers were HER-2-negative by FISH (analyses not shown; refs. 16, 17). Likewise, analysis of the pooled data from both clinical trials showed similar associations with regard to HER-2 status and clinical benefit from lapatinib treatment regardless of which chemotherapy was used. Similar to data presented recently for the HERA adjuvant trial (22), this benefit was independent of the level of HER-2 gene amplification (Table 3). Women whose breast cancers had low-level HER-2 amplification showed a lapatinib-related improved outcome that was similar to the improved outcome observed in high-level HER-2 amplification breast cancer cases. The analyses of both HER-2 status and EGFR status using FISH, IHC, and RT-PCR in both clinical trials permitted us to explore additional questions related to lapatinib treatment, including the potential role of EGFR status on selection of patients for lapatinib treatment and the potential for "HER-2-negative" breast cancer patients to respond to lapatinib treatment. Finally, pooling of the findings permitted us to make some estimates of the prevalence of heterogeneity in HER-2 status among breast cancer patients.

Table 4. Comparison of FISH determinations at two different reference laboratories

ALab	HVLab		
	FISH amplified	FISH nonamplified	Total
FISH-positive	199	23	221
FISH-negative	6	35	43
Total	205	58	263

NOTE: Concordance rate: 234/263 = 89% (P < 0.0001; 95% CI, 85-93%).

Our findings support an association between HER-2-positive (HER-2-amplified/overexpressed), but not EGFR-positive, breast cancers and responsiveness to lapatinib. Although our findings show that only women with HER-2-amplified/overexpressed breast cancers respond to lapatinib, not all women with this genetic alteration do respond. Some genetic alterations in these cancers are likely responsible for this primary resistance to lapatinib; indeed, work to identify additional genes that can improve the predictive value of existing molecular markers is ongoing (23). Further, our findings indicate that EGFR status is generally not useful for selecting patients with breast cancer who will respond to lapatinib treatment despite the significant EGFR inhibitory potency of lapatinib (2). Our results support similar observations that have been reported previously with gefitinib (24) and erlotinib (25), both small-molecule inhibitors of EGFR. However, our investigations did not include functional measurements of EGFR inhibition by lapatinib; hence, no information is available regarding inhibition of EGFR phosphorylation status or downstream signaling. In addition, we have not done any assessment of EGFR gene amplification or mutation. Our findings in human breast cancers are not surprising, because the EGFR gene is seldom altered in this disease. Less than 1% of breast cancers have EGFR gene mutations, and EGFR gene amplifications are, likewise, infrequent (26).[7] The level of EGFR expression observed by IHC in the EGF100151 and EGF30001 clinical trials cases is consistently less than the level of EGFR immunostaining observed in normal ductal and lobular epithelium present in the same biopsy specimen. In fact, nearly every breast cancer specimen that contained normal ductal or lobular epithelium showed EGFR immunostaining in the normal epithelium, even when the tumor cells showed no EGFR immunostaining (data not shown). Similar findings related to EGFR expression in normal and breast carcinoma cells have been reported by others (27, 28). Because the EGFR expression levels in breast cancer are actually lower than the EGFR expression levels in normal breast epithelium, it is not surprising that this decreased level of expression was not a factor in selecting women who might respond to lapatinib treatment. However, these findings in breast cancer clearly do not exclude the possibility that EGFR expression levels or EGFR gene mutations might prove to be an important target for lapatinib in other cancers dependent on EGFR signaling or where the EGFR gene is known to be mutated or amplified (29–31).

Our findings of a lack of lapatinib responsiveness among patients with HER-2-negative metastatic breast cancer are consistent with findings showing lack of benefit of trastuzumab in HER-2-negative patients in the metastatic setting and at odds with recent suggestions that assessment of HER-2 status may not be important in predicting responsiveness to trastuzumab in the adjuvant treatment of breast cancer (3, 5). These results provide a note of caution when reviewing recent findings from adjuvant trials evaluating trastuzumab that suggest benefits of trastuzumab may not be limited to FISH-positive or IHC 3+ patients in that setting (3–5). Several possibilities could explain apparent responsiveness to HER-2-targeted therapy among women with tumors classified as "HER-2-negative." The data we report comparing the analyses by HER-2 status as assessed by the two different laboratories (HVLab and ALab) confirm the potential importance of disagreements in the classification of HER-2 FISH status despite significant overall agreement between results from two laboratories. One explanation is that false-negative HER-2 FISH results could be reported in situations where the FISH results are not directly assessed by a pathologist. Although the fluorescence signals are easily recognized and counted in cellular nuclei, some breast cancer specimens may have changes making identification of tumor cell nuclei difficult to recognize, especially when reactive changes due to prior needle core biopsy are present. In these situations, cells with large, reactive nuclei may be mistaken for tumor cells and (correctly) scored as not amplified, missing the opportunity to score the status of the tumor cell nuclei. Other possibilities, besides testing errors, should be considered.

There may be biological differences in patient response to treatment in an adjuvant setting compared with a metastatic disease setting. However, this appears to be unlikely. Preclinical *in vitro* and *in vivo* models have consistently shown trastuzumab to be effective treatment against only HER-2-overexpressing cells, not HER-2-low-expressing cells (32, 33). Likewise, clinical trials of metastatic disease have consistently shown that the benefit of trastuzumab is limited to those women with HER-2-amplified/overexpressed disease (8, 10). In clinical trials of trastuzumab in women with metastatic breast cancer, no significant improvement is observed among women whose breast cancers lack the HER-2 alteration relative to control treatment (8), an outcome similar to that described here for lapatinib.

Some have suggested that an anti-HER-2 antibody such as trastuzumab could trigger a host immune response in the adjuvant setting that may not be triggered late in the disease process (3). However, there are no data supporting such an immune response limited to "early" breast cancers.

Breast cancer "heterogeneity" of HER-2 status within a single tumor cell population has also been suggested as a possible reason for apparent responsiveness among "HER-2-negative" breast cancers (3). However, our findings reported here do not support that possibility. HER-2 "heterogeneity" was observed in 3 of the 714 (0.4%) cases in these clinical trials for which both FISH and IHC data were available. Because the breast cancer tumor cells in these 3 cases were composed of two different populations of cells, one population HER-2-amplified/overexpressed and the other population HER-2-nonamplified/low expressed, we excluded these 3 cases from the statistical analyses assessing an association between HER-2, EGFR, and

[7] Sanger Institute. Catalogue of Somatic Mutations in Cancer. 2007. Available at: http://www.sanger.ac.uk/genetics/CGP/cosmic/. Accessed April 16, 2008.

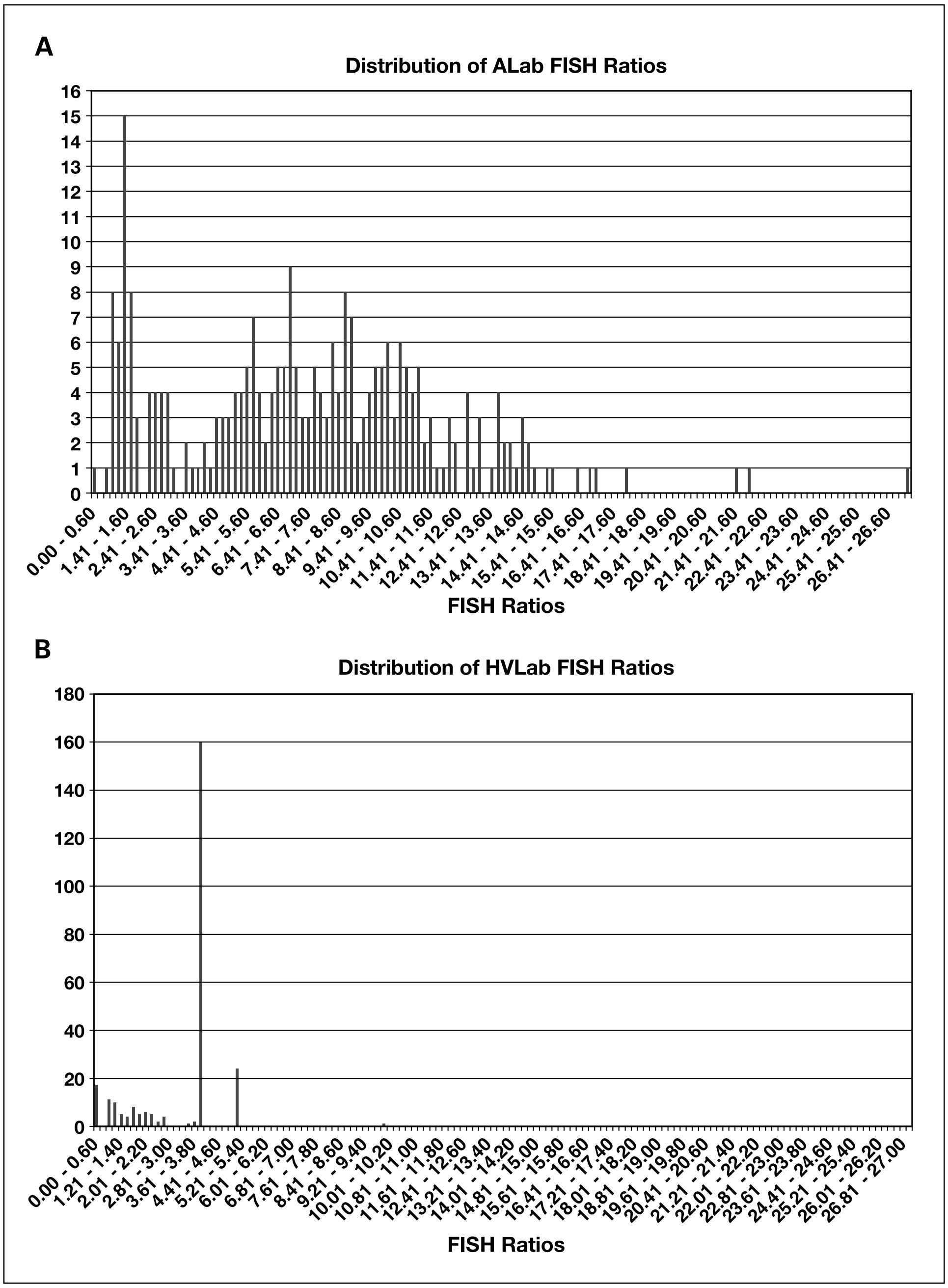

Fig. 2. Comparison of HER-2 gene amplification data determined by FISH in two different reference laboratories for women in the EGF100151 and EGF30001 clinical trials. *A,* plot of HER-2 FISH ratios from ALab, where the HER-2 and chromosome 17 centromere signals were scored in individual tumor cell nuclei and a pathologist assessed the HER-2 status based on FISH ratios. *B,* plot of HER-2 FISH ratios from a HVLab, where a technician estimated the FISH ratios without a pathologist scoring the FISH signals of each individual case.

clinical outcome related to lapatinib treatment. Although some have suggested that "heterogeneity" for HER-2 status among the tumor cell nuclei of a single carcinoma is frequent and responsible for "HER-2-negative" breast cancers that respond to trastuzumab-targeted therapy (3), our findings contradict those opinions (3, 5). Although we do confirm that a few breast cancers show "heterogeneity" for HER-2 status, we also show that the prevalence of cases with true HER-2 "heterogeneity" is quite low (<1%). These findings are similar to our observations in previous studies of HER-2 gene amplification in breast cancer (5–8, 32–37), although the remarkable "homogeneity" of HER-2 status was explicitly discussed in only two of our previous publications (9, 34).

Our study provides support for the idea that apparent responsiveness to HER-2-targeted therapy among "HER-2-negative" breast cancer patients in the adjuvant setting (3–5) is probably related to errors in testing, especially because patients were entered in trials based on the presence of "HER-2-positive" disease by local laboratories. Our findings also raise questions about current HER-2 testing practices. Although women entered in the EGF100151 trial were originally selected as having "HER-2-positive" disease based on local laboratory IHC testing, central laboratory reanalysis of these breast cancers showed that ~14% of these patients had tumors that lacked HER-2 amplification/overexpression (Table 1). The variable error rate associated with IHC analysis of paraffin-embedded tissues is well-recognized and is known to show both false-negative and false-positive results (6–8, 11, 14, 15, 35–41). A 14% false-positive IHC is reflected by the women entered in EGF100151 who did not have HER-2 gene amplification (or overexpression) on central laboratory reanalysis. This is typical of HER-2 IHC assays of paraffin-embedded tissues when antigen retrieval is used in the procedure as in both DAKO HercepTest and Ventana CB11 Pathway IHC assays (6). Because HER-2 FISH assays are the most accurate assays for assessment of HER-2 status in paraffin-embedded tissues (7, 37), as well as for several other reasons detailed elsewhere (42), we have strongly recommended performance of primary FISH testing for HER-2 status to avoid these errors.

Here, we also call attention to a potential problem with HER-2 FISH testing in high-volume central laboratories, that is, the use of laboratory technicians to assess HER-2 status by FISH in some of these high-volume commercial laboratories. Consistent with the Food and Drug Administration-approved guidelines for HER-2 FISH testing, we strongly recommend that a board-certified pathologist interpret FISH assay results, not a laboratory technician who may have difficulty identifying which cells are tumor cells and which cells are benign, reactive cells in a small percentage of the breast cancer cases.

HER-2-targeted therapies such as trastuzumab and lapatinib have markedly improved the clinical outcomes of women with breast cancer (1, 8, 17, 43–45). Several questions remain about how best to use (or not use) molecular markers to select patients who are most likely to benefit from such potent targeted therapies, especially because they are associated with significant side effects and expense. This is particularly true of lapatinib, because it is a dual-kinase inhibitor of both EGFR and HER-2. To have sufficient numbers to perform exploratory analyses of the potential association of both HER-2 and EGFR with responsiveness to lapatinib therapy, these two trials were analyzed together as treatment arms of chemotherapy with or without lapatinib. Here, we report that, in breast cancer, HER-2 amplification/overexpression but not EGFR expression is correlated with responsiveness to lapatinib treatment and show that careful assessment of HER-2 status by a pathologist may play an important role in patient selection.

Disclosure of Potential Conflicts of Interest

M.F. Press: Genentech, commercial research grant; M.F. Press: GlaxoSmithKline, Inc., other commercial support; R.S. Finn: research funding from GlaxoSmithKline; R.S. Finn: consultant and honoraria and GlaxoSmithKline research grant; D. Cameron: lectures and consultancy fees GlaxoSmithKline; C.E. Geyer: consultant, GlaxoSmithKline Administrative Board; A. Di Leo: consultant and honoraria; K. Danenberg: Response Genetics, ownership interest (including patents).

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HER-2 Gene Amplification, HER-2 and Epidermal Growth Factor Receptor mRNA and Protein Expression, and Lapatinib Efficacy in Women with Metastatic Breast Cancer

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