VOLUME 28 · NUMBER 28 · OCTOBER 1 2010

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Human Epidermal Growth Factor Receptor 2 Assessment in a Case-Control Study: Comparison of Fluorescence In Situ Hybridization and Quantitative Reverse Transcription Polymerase Chain Reaction Performed by Central Laboratories

Frederick L. Baehner, Ninah Achacoso, Tara Maddala, Steve Shak, Charles P. Quesenberry Jr, Lynn C. Goldstein, Allen M. Gown, and Laurel A. Habel

See accompanying editorials on pages 4289 and 4293 and article on page 4307

From the University of California, San Francisco, San Francisco; Genomic Health, Redwood City; Kaiser Permanente, Oakland, CA; and PhenoPath Laboratories, Seattle, WA.

Submitted July 15, 2009; accepted June 10, 2010; published online ahead of print at www.jco.org on August 9, 2010.

Supported by Genomic Health.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Corresponding author: Frederick L. Baehner, MD, University of California, San Francisco, 1600 Divisadero St, Rm R200, San Francisco, CA 94063; e-mail: rbaehner@genomichealth.com.

0732-183X/10/2828-4300/$20.00

DOI: 10.1200/JCO.2009.24.8211

ABSTRACT

Purpose
The optimal method to assess human epidermal growth factor receptor 2 (HER2) status remains highly controversial. Before reporting patient HER2 results, American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines mandate that laboratories demonstrate ≥ 95% concordance to another approved laboratory or methodology. Here, we compare central laboratory HER2 assessed by fluorescence in situ hybridization (FISH) and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using Onco*type* DX in lymph node–negative, chemotherapy-untreated patients from a large Kaiser Permanente case-control study.

Patients and Methods
Breast cancer specimens from the Kaiser–Genomic Health study were examined. Central FISH assessment of HER2 amplification and polysomy 17 was conducted by PhenoPath Laboratories (ratios > 2.2, 1.8 to 2.2, and < 1.8 define HER2 positive, HER2 equivocal, and HER2 negative, respectively). HER2 expression by RT-PCR was conducted using Onco*type* DX by Genomic Health (normalized expression units ≥ 11.5, 10.7 to < 11.5, and < 10.7 define HER2 positive, HER2 equivocal, and HER2 negative, respectively). Concordance analyses followed ASCO/CAP guidelines.

Results
HER2 concordance by central FISH and central RT-PCR was 97% (95% CI, 96% to 99%). Twelve percent (67 of 568 patients) and 11% (60 of 568 patients) of patients were HER2 positive by RT-PCR and FISH, respectively. HER2-positive patients had increased odds of dying from breast cancer compared with HER2-negative patients. Polysomy 17 was demonstrated in 12.5% of all patients and 33% of FISH-positive patients. Nineteen of 20 FISH-positive patients with polysomy 17 were also RT-PCR HER2 positive. Although not statistically significantly different, HER2-positive/polysomy 17 patients tended to have the worst prognosis, followed by HER2-positive/eusomic, HER2-negative/polysomy 17, and HER2-negative/eusomic patients.

Conclusion
There is a high degree of concordance between central FISH and quantitative RT-PCR using Onco*type* DX for HER2 status, and the assay warrants additional study in a trastuzumab-treated population.

J Clin Oncol 28:4300-4306. © 2010 by American Society of Clinical Oncology

INTRODUCTION

The human epidermal growth factor receptor 2 gene (*HER2*) is reportedly amplified in 20% to 25% of human breast cancers; however, recent data suggest that it may be amplified in 10% to 11% of lymph node–negative disease.[1,2] HER2 gene amplification and protein overexpression is a prognostic marker for aggressive disease and an important predictive marker for specific therapies, including trastuzumab (Herceptin; Genentech, South San Francisco, CA) and lapatinib (Tykerb; GlaxoSmithKline, London, United Kingdom).[3,4] The US Food and Drug Administration (FDA) has approved two immunohistochemical (IHC) assays and three fluorescent in situ hybridization (FISH) assays for HER2 assessment; however, considerable debate persists about which assay is best for establishing HER2 status.[5]

The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) developed guidelines for laboratory evaluation of HER2 status.[6] These enumerate requirements for HER2 analyses and recommend either using IHC assays for initial evaluation of HER2 status followed by reflex testing by FISH of some IHC categories or the primary use of FISH in initial testing. Even with these guidelines, concern remains about the accuracy of HER2 testing.[6-10] Two independent cooperative groups reported that up to 20% of locally performed HER2 assays reported as positive could not be confirmed by central laboratories.[9,10]

HER2 gene amplification is highly associated with mRNA overexpression and increased protein levels, and small studies have compared mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR) with FISH.[1,11,12] Quantitative RT-PCR by the Onco*type* DX assay (Genomic Health, Redwood City, CA) quantifies gene expression using RNA extracted from fixed paraffin-embedded tumor tissue and has been shown to be 95% concordant with IHC.[13,14] Herein, we compare central laboratory HER2 assessment by FISH and quantitative RT-PCR using Onco*type* DX in patients from a large Kaiser Permanente case-control study according to the ASCO/CAP guidelines and explore the association between HER2 assessed by both assays and chromosome 17 polysomy status with risk of breast cancer death.[15]

PATIENTS AND METHODS

Tumor Samples and Patient Clinical Data

This is a case-control study nested within a cohort of women diagnosed with lymph node–negative breast cancer who were not treated with chemotherapy. Eligible women, identified using the Kaiser Permanente Northern California Cancer Registry, were diagnosed from 1985 to 1994 and were younger than age 75 years at diagnosis. The study was approved by the Kaiser Permanente Institutional Review Board. Patients were excluded for inflammatory carcinoma, bilateral breast carcinoma, evidence of metastasis (including lymph nodes) at initial diagnosis, prior invasive cancer at diagnosis, or unknown/unconfirmed tamoxifen treatment. Cohort eligibility was confirmed by medical record review, and members were observed until breast cancer death, contralateral breast cancer, departure from health plan (including any death cause), or December 2002, whichever came first.[15]

Patient cases were patients whose first event was breast cancer death. At each death, up to three controls were randomly selected from patients who were alive and under follow-up for at least as long as the patient case's time to breast cancer death (incidence density sampling). A patient could be both a control (up until time of death from breast cancer) and patient case. Controls were matched to their patient case on age, race, calendar year of diagnosis, and treatment of index breast cancer with tamoxifen.[15] Eligibility and case-control selection were performed before laboratory analyses. Tamoxifen treatment was defined as treatment for $\geq$ 3 months within 2 years of surgery and before breast cancer recurrence. Matching on tamoxifen treatment facilitated analyses stratified by tamoxifen treatment.

Pathology was conducted blinded to clinical outcome using the Nottingham system.[16-18] Microscopic tumor size was from pathology reports. Tumors with less than 5% carcinoma were excluded.

Central Laboratory IHC and FISH

PhenoPath Laboratories (Seattle, WA) performed and interpreted IHC for estrogen receptor (ER) and progesterone receptor (PR) and FISH for HER2. ER (SP1; 1:250; LabVision, Fremont, CA) and PR (636; 1:200; Dako, Carpinteria, CA) were performed and scored by two pathologists as published.[19,20] For FISH, deparaffinized tissue sections were pretreated using a modification of the vendor's standard protocol and then incubated with the FDA-approved Vysis PathVysion probe set (Abbott Diagnostics, Chicago, IL). Morphometric analysis used MetaSystems image analysis system, with Metafer software with extended focus/tile sampling (MetaSystems, Altlussheim, Germany). Manual counting was performed when autofluorescence and/or artifact prevented sufficient cell counting. All samples with ratios of HER2/CEP17 between 1.5 and 2.5 by morphometric analysis were scored manually by counting more than 60 nonoverlapping cells. A ratio of more than 2.2, 1.8 to 2.2, or less than 1.8 is positive, equivocal, or negative for amplification, respectively. For primary concordance analysis, equivocal and negative groups were combined.[6] Polysomy 17 was defined as a CEP17 signal $\geq$ three copies.[21-23]

Sample Preparation and Gene Expression Analysis Using Quantitative RT-PCR by Oncotype DX

Gene expression used whole tumor sections and the Onco*type* DX assay.[24] RNA was extracted from $3 \times 10\ \mu m$ or $6 \times 10\ \mu m$ whole sections when manual microdissection was required. Manual microdissection was performed when tumor constituted less than 50% of the total epithelium or when ductal carcinoma in situ grade and invasive carcinoma nuclear grade differed. After DNase I treatment, total RNA content was measured, and absence of DNA contamination was verified. Gene-specific reverse transcription was performed followed by quantitative PCR (TaqMan) using Prism 7900HT instruments (Applied Biosystems, Foster City, CA). The expression of 16 cancer-related genes, including ER (*ESR1*) and PR (*PGR*), were normalized relative to five reference genes (*ACTB*, *GAPDH*, *GUSB*, *RPLP0*, and *TFRC*); reference normalized expression ranged from 2 to 16 units (each 1-unit increase reflects approximately a two-fold increase in RNA). HER2 status was classified as negative ($<$ 10.7 normalized expression units), equivocal (10.7 to $<$ 11.5 units), and positive ($\geq$ 11.5 units); ER status was classified as negative ($<$ 6.5 units) and positive ($\geq$ 6.5 units), and PR status was classified as negative ($<$ 5.5 units) and positive ($\geq$ 5.5 units; expression relative to reference genes; $\log_2$). All cut points were prespecified.[25-27]

Statistical Analyses

Analyses were conducted by Kaiser Permanente. For concordance analysis, only unique patients were included. Equivocal samples from both methods were excluded (ASCO/CAP guidelines).[6] Measures of agreement include overall concordance (number of samples that agree divided by the total number of samples) and κ statistics (agreement adjusted for chance).[28] Exact 95% CIs for the concordance statistic were calculated using the *F*-distribution method using the FREQ procedure in SAS (SAS Institute, Cary, NC).[29] Percent positive agreement was calculated as the number of samples positive by both assays divided by the number of samples positive by the FISH assay. Percent negative agreement was calculated similarly.

Outcome analyses were performed on all patients and separately in patients who were and were not treated with tamoxifen. Odds ratios (ORs) for breast cancer death associated with HER2 status and breast cancer subtypes were calculated by conditional logistic regression using maximum likelihood estimation. HER2 status by FISH and hormone receptor (HR) status by IHC were compared with HER2 and HR status by RT-PCR. *P* values using the likelihood ratio test and 95% Wald CIs for the OR were calculated. All statistical tests were performed at the two-sided $\alpha = .05$ significance level.

RESULTS

Patient Characteristics

Of the 402 patient cases and 989 controls identified as eligible, 234 patient cases and 631 controls who were eligible after chart review met matching case-control criteria and had tumor blocks. Two hundred twenty patient cases and 570 controls (647 unique patients) were RT-PCR evaluable; a subset of 203 patient cases and 490 controls (568 unique patients) had sufficient tumor for FISH. Data are presented for the evaluable 568 unique patients. Patient demographics and clinical characteristics are listed in Table 1. Patient cases and controls, as

Table 1. Patient Demographics and Baseline Clinical Characteristics

Demographic or Clinical Characteristic	Patient Cases (n = 203)		Controls (n = 365)		Total Patients for Concordance Analysis (N = 568)	
	No.	%	No.	%	No.	%
ER status and Tam treatment strata*						
ER positive, Tam = yes	50	25	114	31	164	29
ER positive, Tam = no	105	52	216	59	321	57
ER negative, Tam = yes	9	4	2	< 1	11	2
ER negative, Tam = no	39	19	33	9	72	13
Age, years						
< 40	16	8	14	4	30	5
40-49	40	20	77	21	117	21
50-59	56	28	100	27	156	27
≥ 60	91	45	174	48	265	47
Race						
White (non-Hispanic)	159	78	299	82	458	81
White (Hispanic)	5	3	10	3	15	3
Black	19	9	25	7	44	8
Asian	20	10	31	8	51	9
Menopausal status						
Premenopausal	59	29	88	24	147	26
Postmenopausal	135	67	261	72	396	70
Missing	9	4	16	4	25	4
Adjuvant Tam						
No	144	71	249	68	393	69
Yes	59	29	116	32	175	31
Tumor size, cm						
≤ 1.0	46	23	122	33	168	30
> 1.0-2.0	87	43	166	45	253	45
> 2.0-4.0	67	33	74	20	141	25
> 4.0	3	1	3	1	6	1
Tumor grade† (all patients)						
Well	23	11	111	30	134	24
Moderate	84	41	175	48	259	46
Poor	96	47	79	22	175	31
Tumor grade† (HER2 positive by FISH)						
Well	1	3	2	8	3	5
Moderate	12	34	10	40	22	37
Poor	22	63	13	52	35	58
Tumor grade† (HER2 positive by RT-PCR)						
Well	2	6	4	13	6	9
Moderate	15	42	14	45	29	43
Poor	19	53	13	42	32	48

NOTE. Percentages may not sum to 100% exactly as a result of rounding.
Abbreviations: ER, estrogen receptor; Tam, tamoxifen; HER2, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization; RT-PCR, reverse transcriptase polymerase chain reaction.
*ER status by RT-PCR. The concordance between ER by RT-PCR and ER by immunohistochemistry is 96%.
†Tumor grade as assessed by pathologist 1.

Table 2. HER2 Concordance Excluding Equivocal Results (2007 ASCO/CAP Guidelines)

Oncotype DX Status	Central FISH Positive		Central FISH Negative		Total No. of Patients
	No. of Patients	%	No. of Patients	%	
Oncotype DX positive	55	98	11	3	66
Oncotype DX negative	1	2	408	97	409
Total	56		419		475

NOTE. Percentages are column percentages. FISH HER2 status is used as the denominator. Concordance is 97% (95% CI, 96% to 99%); κ is 89% (95% CI, 83% to 95%).
Abbreviations: HER2, human epidermal growth factor receptor 2; ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; FISH, fluorescence in situ hybridization.

expected, were similar by matching factors. One third of patients were treated with adjuvant tamoxifen (not all ER-positive patients received tamoxifen because this cohort includes patients from the 1980s). Compared with controls, patient cases more commonly had larger/more poorly differentiated tumors. HER2-positive tumors were more commonly moderately or poorly differentiated than HER2-equivocal and HER2-negative tumors by both methods (Table 1).

Distribution of HER2

Among the 568 evaluable patients, 12% and 11% were HER2 positive by quantitative RT-PCR and FISH, respectively (Appendix Fig A1, online only). There were more equivocal results by RT-PCR than FISH (15% *v* 2%, respectively). The majority (87%) of the 88 RT-PCR–equivocal samples were FISH negative (Appendix Fig A2, online only). This relatively high number of RT-PCR–equivocal samples has been noted among ER-positive patients.[30] Appendix Table A1 (online only) presents cross-tabulated results for HER2 status by FISH and RT-PCR.

Concordance Between FISH and RT-PCR

Central FISH and RT-PCR concordance was 97% (95% CI, 96% to 99%), percent positive agreement was 98% (95% CI, 90% to 99%), and percent negative agreement was 97% (95% CI, 95% to 99%; Table 2). Lacking a gold standard, the terms percent positive agreement and percent negative agreement are preferred by the FDA over sensitivity and specificity.[31] We conducted additional analyses of concordance excluding FISH-equivocal results, treating equivocal results by RT-PCR as negative, and excluding equivocal results by both assays. In all cases, concordance for these comparisons was 97% (95% CI, 95% to 98%; Appendix Tables A2 and A3, online only).

The distribution of FISH HER2/CEP17 ratio compared with central RT-PCR HER2 is shown in Figure 1. Spearman rank correlation of the relationship between the two assays is 0.45. One observation was FISH HER2 positive but RT-PCR HER2 negative.

HER2 Expression and Breast Cancer Death

Among all patients, by FISH (OR = 1.95; 95% CI, 1.19 to 3.19) and RT-PCR (OR = 1.72; 95% CI, 1.04 to 2.84), HER2-positive patients had increased odds of breast cancer death compared with HER2-negative patients. OR estimates and overlapping 95% CIs for the comparisons by FISH and RT-PCR are shown in Figure 2. By both assays, equivocal patients did not have significantly different odds of dying from breast cancer compared with HER2-negative patients (FISH: OR = 0.84; 95% CI, 0.22 to 3.13; RT-PCR: OR = 0.66; 95% CI, 0.40 to 1.09). Similar results were obtained in the groups that did and did not receive tamoxifen (Fig 2).

We explored the relationship between breast cancer death and breast cancer subtypes defined as combinations of HER2 and HR

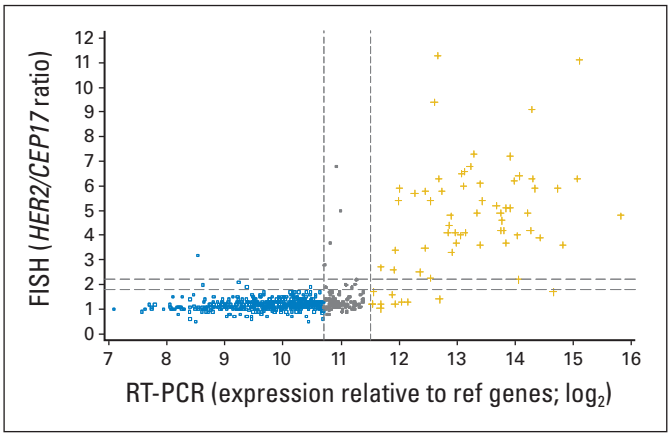

Fig 1. Distribution of human epidermal growth factor receptor 2 (HER2): reverse transcriptase polymerase chain reaction (RT-PCR; cycle threshold) by central laboratory fluorescence in situ hybridization (FISH; ratio). Blue squares indicate HER2 negative by RT-PCR, gray squares indicate HER2 equivocal by RT-PCR, and gold plus signs indicate HER2 positive by RT-PCR. Note that only one HER2 FISH-positive patient is RT-PCR negative. FISH diagnostic categories were as follows: negative, ratio of less than 1.8; equivocal, ratio of 1.8 to 2.2; and positive, ratio of more than 2.2. RT-PCR diagnostic categories were as follows: negative, less than 10.7 normalized expression units; equivocal, 10.7 to less than 11.5 units; and positive, ≥ 11.5 units.

status (assessed by RT-PCR or FISH/IHC). HR-positive, HER2-negative status is the reference category for all comparisons. Although the 95% CIs are wide and overlap, the point estimates trend toward HR-negative/HER2-positive patients having the worst prognosis (OR = 4.21 by IHC/FISH and 3.91 by RT-PCR), followed by HR-negative/HER2-negative patients (OR = 2.13 by IHC/FISH and 2.42 by RT-PCR), HR-positive/HER2-positive patients (OR = 1.58 by IHC/FISH and 1.79 by RT-PCR), and HR-positive/HER2-negative patients.

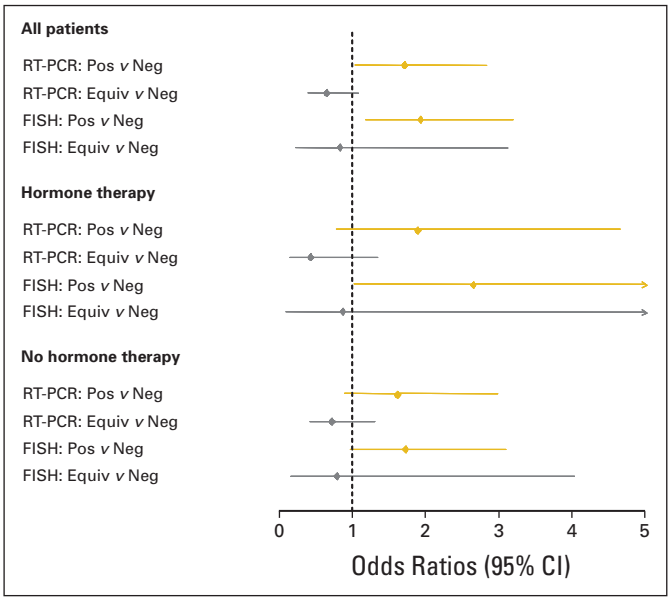

Fig 2. Forest plot of human epidermal growth factor receptor 2 (HER2) univariate analyses. Odds ratios of breast cancer death for HER2 status as assessed by reverse transcriptase polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). A higher odds ratio is associated with a worse outcome. Pos, positive; Neg, negative; Equiv, equivocal.

Chromosome 17 Polysomy Status and HER2 Amplification

Among all patients, seventy-one patients (12.5%) showed chromosome 17 polysomy (polysomy 17; Table 3). Among FISH HER2-positive patients, 33% showed polysomy 17. In FISH-negative patients, 10% showed polysomy 17. Among FISH-positive patients with polysomy 17, 19 of 20 were RT-PCR HER2 positive. The majority of FISH-negative polysomy 17 patients were RT-PCR HER2 negative (32 of 49 patients; 65%), but rare patients (four of 49 patients; 8%) were RT-PCR HER2 positive.

Breast Cancer Death: Chromosome 17 Polysomy Status and HER2 Amplification

By FISH, we evaluated polysomy status and breast cancer death (Fig 3). Compared with HER2-negative, chromosome 17 eusomic patients, HER2-positive patients with polysomy 17 had 2.77 times (95% CI, 1.21 to 6.33 times) the odds of dying from breast cancer; HER2-positive, chromosome 17 eusomic patients had 1.78 times (95% CI, 0.97 to 3.25 times) the odds of dying from breast cancer; and HER2-negative, polysomy 17 patients had 1.50 times (95% CI, 0.83 to 2.72 times) the odds of dying from breast cancer. Similar results were obtained when HER2 status was measured by RT-PCR, although the results were not statistically significant (OR = 2.14, 1.41, and 1.22 for HER2-positive, polysomy 17; HER2-positive, chromosome 17 eusomic; and HER2-negative, polysomy 17 patients, respectively, compared with HER2-negative, chromosome 17 eusomic patients).

DISCUSSION

HER2 positivity in breast cancer has a significantly worse prognosis with an increased risk of recurrence, and large randomized trials have established trastuzumab as the standard of care in patients with early HER2-positive breast cancer, who are also suitable for treatment with chemotherapy.[1,2] Accurate and precise measurement of HER2 status is of marked therapeutic importance, and new methodologies for HER2 assessment must be rigorously compared with the current measurement standards.[6] The primary objective of this study was to determine the HER2 concordance using central quantitative RT-PCR by Onco*type* DX and central FISH in a large cohort of patients with early breast cancer with long follow-up (> 8 years).

Overall, there was a high level of concordance between quantitative RT-PCR and FISH (97%; 95% CI, 96% to 99%), and the number of discordant samples was small. There was an imbalance in the distribution of the HER2-discordant tumors between central FISH and central RT-PCR. FISH-negative but RT-PCR–positive tumors were more common than FISH-positive but RT-PCR–negative tumors. Importantly, there was only a single tumor that was FISH positive (low-level amplification) but RT-PCR negative. The causes of these discordances are likely multifactorial. True biologic differences between RNA levels and DNA gene amplification offer one possibility.[5] Sources of analytic variability are another possibility; not all FISH or RT-PCR assays are the same.[32] Differences in RNA extraction, reverse transcription, PCR protocols, instruments, primer/probe selection, and reagent manufacturing can contribute to assay variation. The Onco*type* DX assay uses controls, calibrators, reference ranges (for quantitative single-gene ER, PR, and HER2 results), and normalization to address differences in RNA quality.[14,33,34,35] The variability

Table 3. Incidence of Polysomy 17 by HER2 Status (FISH and RT-PCR)

HER2 Status	Central FISH Positive		Central FISH Equivocal		Central FISH Negative		Total RT-PCR	
	No. of Polysomy-Positive Patients/ No. of HER2-Positive Patients*	% of Polysomy-Positive Patients	No. of Polysomy-Positive Patients/ No. of HER2-Equivocal Patients	% of Polysomy-Positive Patients	No. of Polysomy-Positive Patients/ No. of HER2-Negative Patients†	% of Polysomy-Positive Patients	No. of Polysomy-Positive Patients/ Total No.	% of Polysomy-Positive Patients
RT-PCR positive	19/55*	35	1/1	100	4/11	36	24/67	36
RT-PCR equivocal	0/4	0	1/5	20	13/79	17	14/88	16
RT-PCR negative	1/1	100	0/4	0	32/408†	8	33/413	8
Total FISH	20/60	33	2/10	20	49/498	10	71/568	13

Abbreviations: HER2, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization; RT-PCR, reverse transcriptase polymerase chain reaction.
*Patients show both polysomy and chromosome 17 amplification by FISH.
†Patients show polysomy but no chromosome 17 amplification.

(standard deviation) contributed by instruments, operators, reagents, and day-to-day variation for RT-PCR using Onco*type* DX is less than 0.5 expression units.[14,36] Sources of preanalytic variability (eg, delay to fixation, choice of fixative, or duration of fixation) may also play a role, and their impact in HER2 assessment by FISH is well described.[37,38]

The use of the equivocal range is mandated by the ASCO/CAP guidelines for the reporting of HER2 results.[5,24] Consistent with the reported literature, in this study, there were 2% FISH-equivocal tumors in contrast to 15% RT-PCR–equivocal tumors.[39] The majority of RT-PCR–equivocal tumors were negative by central FISH. This is dissimilar to the recently reported results from Eastern Cooperative Oncology Group trial E2197 comparing HER2 by RT-PCR and IHC (N = 755), where overall concordance was 95% (95% CI, 92% to 96%) and only 3% (26 of 755 tumors) of RT-PCR tumors compared with 23% (175 of 755 tumors) of IHC tumors were equivocal.[6,40] One explanation for this difference may be the inverse relationship between HER2 and ER protein levels in breast cancer.[41,42] This Kaiser Permanente cohort is primarily ER positive (77%), whereas in E2197, there were fewer ER-positive tumors (53%); this may suggest that tumors with equivocal levels of HER2 mRNA seem to be more frequent among ER-positive than ER-negative patients (Appendix Fig A2).[30,41,43] This study suggests that RT-PCR HER2-equivocal patients seem to be similar to HER2-negative patients with respect to grade and clinical outcomes; hence, it would be reasonable to consider them as HER2 negative. Comparing FISH to RT-PCR, when RT-PCR–equivocal patients are considered negative, the concordance remains high at 97% (Appendix Tables A2 and A3). Of note, ASCO/CAP guidelines recommend use of FISH for determination of HER2 status in equivocal patients.

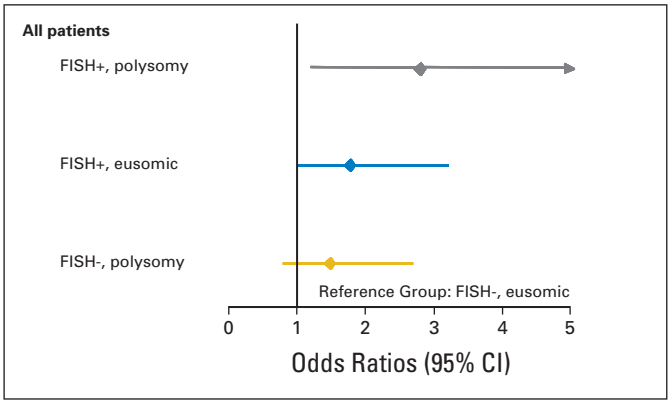

Fig 3. Forest plot of polysomy 17 status and human epidermal growth factor receptor 2 status. Odds ratios of breast cancer death for fluorescence in situ hybridization (FISH). A higher odds ratio is associated with a worse outcome.

The significance of HER2 status should be considered in terms of prognosis and prediction of treatment benefit. As previously reported for patients not treated with trastuzumab, HER2 positivity by both methods was a weakly prognostic factor in the present chemotherapy- and trastuzumab-naive cohort; HER2-positive patients had increased odds of dying from breast cancer compared with HER2-negative patients.[44-46] By both assays, the HER2-equivocal patients did not have significantly different odds of dying from breast cancer compared with HER2-negative patients. These results were independent of tamoxifen treatment. Although HER2 positivity has been associated with tamoxifen resistance in HR-positive patients, we could not directly examine this question because patient cases and controls were matched on tamoxifen therapy.[41] We observed a slightly stronger association between HER2 status and risk of breast cancer death among patients treated with tamoxifen compared with those not treated with tamoxifen (Fig 2). Consistent with reported observations regarding the intrinsic subtypes of breast cancer, in this cohort, HR-negative/HER2-positive patients tended to have the worst prognosis, followed by HR-negative/HER2-negative (triple-negative) patients, HR-positive/HER2-positive patients, and finally, HR-positive/HER2-negative patients.[47] This relationship was similar between patients who were and were not treated with tamoxifen.

Depending on study population and polysomy 17 definition, the frequency of polysomy 17 in breast cancer ranges from 13% to 46%.[48-52] Using the common definition of ≥ three CEP17 copies, 12.5% of tumors showed polysomy 17 by FISH.[22] Consistent with reports that patients with polysomy 17 and HER2 amplification have clinical outcomes similar to HER2-positive patients, one third of HER2-positive patients showed polysomy 17 and had the worst prognosis. Patients with polysomy 17 without HER2 amplification (HER2 negative), often equivocal by IHC, are reported to have outcomes similar to HER2-negative, chromosome 17 eusomic patients, and this was observed in our cohort.[23,53,54] In contrast to reports that polysomy 17 tumors without HER2 amplification invariably show no increase in HER2 RNA levels, in this study, 9% (four of 49 tumors) did show expression levels consistent with HER2 amplification, as previously

described by IHC.[23,55,56] This may support recent reports that standard FISH probes may misclassify rare HER2-amplified tumors as polysomy 17 when the amplicon extends into the centrometric region of chromosome 17.[57]

The strengths of this study include the large study cohort of lymph node–negative, chemotherapy- and trastuzumab-naive patients and the use of central laboratories with standardized methods for FISH and RT-PCR assays.[14,32,36,58] Not all FISH or all RT-PCR assays are the same.[59] There are also some limitations to consider when interpreting these study results. Because of the cohort age, only approximately 30% of patients were treated with tamoxifen, although this is consistent with other reported populations during this period. HER2 IHC was not available for review. Finally, the cohort is chemotherapy and trastuzumab naive and thus shows prognosis with respect to HER2 status but not prediction of trastuzumab benefit. Assessment of HER2 status by IHC and/or FISH should continue as the approach for making trastuzumab or lapatinib treatment decisions.[14,33,34] Although with regard to trastuzumab benefit, recent quantitation of *HER2* gene amplification by FISH did not correlate with trastuzumab benefit, *HER2* gene expression in a trastuzumab-treated population has not been studied with quantitative RT-PCR and warrants further study.[22]

Recent reports that patients with discordant local and central laboratory HER2 results responded to trastuzumab highlight the need for standardized, robust methods that are less affected by sources of variability.[6] These data show that RT-PCR is highly concordant with high-quality, central laboratory–performed FISH and may be of use to clinicians and pathologists who are uncertain about select HER2 assay results obtained by FISH and IHC. Furthermore, these standardized HER2 results may prove useful for laboratories performing mandatory ASCO/CAP concordance studies.[58,60,61] In conclusion, this study demonstrated a high level of concordance for HER2 between central FISH and central RT-PCR using the Onco*type* DX assay, and the assay warrants further study in a trastuzumab-treated population.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Frederick L. Baehner, Genomic Health (C); Steve Shak, Genomic Health (C); Tara Maddala, Genomic Health (C) **Consultant or Advisory Role:** None **Stock Ownership:** Steve Shak, Genomic Health; Tara Maddala, Genomic Health **Honoraria:** None **Research Funding:** Laurel A. Habel, Genomic Health, bioTheranostics **Expert Testimony:** None **Other Remuneration:** None

AUTHOR CONTRIBUTIONS

Conception and design: Frederick L. Baehner, Tara Maddala, Steve Shak, Charles P. Quesenberry Jr, Laurel A. Habel
Provision of study materials or patients: Ninah Achacoso, Laurel A. Habel
Collection and assembly of data: Frederick L. Baehner, Ninah Achacoso, Tara Maddala, Charles P. Quesenberry Jr, Lynn C. Goldstein, Allen M. Gown, Laurel A. Habel
Data analysis and interpretation: Frederick L. Baehner, Ninah Achacoso, Tara Maddala, Charles P. Quesenberry Jr, Lynn C. Goldstein, Allen M. Gown, Laurel A. Habel
Manuscript writing: Frederick L. Baehner, Ninah Achacoso, Tara Maddala, Steve Shak, Charles P. Quesenberry Jr, Lynn C. Goldstein, Allen M. Gown, Laurel A. Habel
Final approval of manuscript: Frederick L. Baehner, Ninah Achacoso, Tara Maddala, Steve Shak, Charles P. Quesenberry Jr, Lynn C. Goldstein, Allen M. Gown, Laurel A. Habel

REFERENCES

1. Slamon DJ, Clark GM, Wong SG, et al: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177-182, 1987
2. Chia S, Norris B, Speers C, et al: Human epidermal growth factor receptor 2 overexpression as a prognostic factor in a large tissue microarray series of node-negative breast cancers. J Clin Oncol 26:5697-5704, 2008
3. Slamon DJ, Leyland-Jones B, Shak S, et al: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783-792, 2001
4. Geyer CE, Forster J, Lindquist D, et al: Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 355:2733-2743, 2006
5. Sauter G, Lee J, Bartlett JM, et al: Guidelines for human epidermal growth factor receptor 2 testing: Biologic and methodologic considerations. J Clin Oncol 27:1323-1333, 2009
6. Wolff AC, Hammond ME, Schwartz JN, et al: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 25:118-145, 2007
7. Press MF, Sauter G, Bernstein L, et al: Diagnostic evaluation of HER-2 as a molecular target: An assessment of accuracy and reproducibility of laboratory testing in large, prospective, randomized clinical trials. Clin Cancer Res 11:6598-6607, 2005
8. Perez EA, Suman VJ, Davidson NE, et al: HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial. J Clin Oncol 24:3032-3038, 2006
9. Paik S, Bryant J, Tan-Chiu E, et al: Real-world performance of HER2 testing: National Surgical Adjuvant Breast and Bowel Project experience. J Natl Cancer Inst 94:852-854, 2002
10. Roche PC, Suman VJ, Jenkins RB, et al: Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. J Natl Cancer Inst 94:855-857, 2002
11. Benöhr P, Henkel V, Speer R, et al: Her-2/neu expression in breast cancer: A comparison of different diagnostic methods. Anticancer Res 25:1895-1900, 2005
12. Gjerdrum LM, Sorensen BS, Kjeldsen E, et al: Real-time quantitative PCR of microdissected paraffin-embedded breast carcinoma: An alternative method for HER-2/neu analysis. J Mol Diagn 6:42-51, 2004
13. Baehner FL, Gray R, Maddala T, et al: HER2 concordance between central laboratory immunohistochemistry and quantitative reverse transcription polymerase chain reaction in Intergroup Trial E2197. Presented at the 2008 American Society of Clinical Oncology Breast Cancer Symposium, Washington, DC, September 5-7, 2008
14. Cronin M, Pho M, Dutta D, et al: Measurement of gene expression in archival paraffin-embedded tissues: Development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol 164:35-42, 2004
15. Habel LA, Shak S, Jacobs MK, et al: A population-based study of tumor gene expression and risk of breast cancer death among lymph node-negative patients. Breast Cancer Res 8:R25, 2006
16. Elston CW, Ellis IO: Pathological prognostic factors in breast cancer: I. The value of histological grade in breast cancer: Experience from a large study with long-term follow-up. Histopathology 19: 403-410, 1991
17. Elston CW, Ellis IO: The Breast (ed 3). London, United Kingdom, Churchill Livingstone, 1999
18. Tavassoli FA, Devilee P (eds): World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Breast and Female Genital Organs. Lyon, France, IARC Press, 2003
19. Farinola MA, Gown AM, Judson K, et al: Estrogen receptor alpha and progesterone receptor expression in ovarian adult granulosa cell tumors

and Sertoli-Leydig cell tumors. Int J Gynecol Pathol 26:375-382, 2007

20. Cheang MC, Treaba DO, Speers CH, et al: Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. J Clin Oncol 24:5637-5644, 2006

21. Reinholz MM, Bruzek AK, Visscher DW, et al: Breast cancer and aneusomy 17: Implications for carcinogenesis and therapeutic response. Lancet Oncol 10:267-277, 2009

22. Dowsett M, Procter M, McCaskill-Stevens W, et al: Disease-free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 year of trastuzumab: The HERA Trial. J Clin Oncol 27:2962-2969, 2009

23. Vanden Bempt I, Van Loo P, Drijkoningen M, et al: Polysomy 17 in breast cancer: Clinicopathologic significance and impact on HER-2 testing. J Clin Oncol 26:4869-4874, 2008

24. Paik S, Shak S, Tang G, et al: A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 351: 2817-2826, 2004

25. Esteva FJ, Sahin AA, Cristofanilli M, et al: Prognostic role of a multigene reverse transcriptase-PCR assay in patients with node-negative breast cancer not receiving adjuvant systemic therapy. Clin Cancer Res 11:3315-3319, 2005

26. Mina L, Soule SE, Badve S, et al: Predicting response to primary chemotherapy: Gene expression profiling of paraffin-embedded core biopsy tissue. Breast Cancer Res Treat 103:197-208, 2007

27. Cobleigh MA, Tabesh B, Bitterman P, et al: Tumor gene expression and prognosis in breast cancer patients with 10 or more positive lymph nodes. Clin Cancer Res 11:8623-8631, 2005

28. Cohen J: A coefficient of agreement for nominal scales. Educ Psychol Measure 20:27-46, 1960

29. Collett D: Modeling Binary Data. London, United Kingdom, Chapman and Hall, 1991

30. Shak S, Baehner FL, Palmer G, et al: Subtypes of breast cancer defined by standardized quantitative RT-PCR analysis of 10,618 tumors: Updated analysis with 20,050 tumors. Presented at the 29th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, December 14-17, 2006

31. US Department of Health and Human Services: Guidance for industry and FDA staff: Statistical guidance on reporting results from studies evaluating diagnostic tests. http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm071287.pdf

32. Gown AM, Goldstein LC, Barry TS, et al: High concordance between immunohistochemistry and fluorescence in situ hybridization testing for HER2 status in breast cancer requires a normalized IHC scoring system. Mod Pathol 21:1271-1277, 2008

33. Ming Z, Bronner M, Magi-Galluzzi C, et al: Optimized RNA extraction and RT-PCR assays provide successful molecular analysis on a wide variety of archival fixed tissues. Presented at the Annual Meeting of the American Association for Cancer Research, Los Angeles, CA, April 14-17, 2007

34. Vandesompele J, De Preter K, Pattyn F, et al: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034, 2002

35. Suzuki T, Higgins PJ, Crawford DR: Control selection for RNA quantitation. Biotechniques 29: 332-337, 2000

36. Cronin M, Sangli C, Liu ML, et al: Analytical validation of the Oncotype DX genomic diagnostic test for recurrence prognosis and therapeutic response prediction in node-negative, estrogen receptor-positive breast cancer. Clin Chem 53:1084-1091, 2007

37. Tapia C, Schraml P, Simon R, et al: HER2 analysis in breast cancer: Reduced immunoreactivity in FISH non-informative cancer biopsies. Int J Oncol 25:1551-1557, 2004

38. Khoury T, Sait S, Hwang H, et al: Delay to formalin fixation effect on breast biomarkers. Mod Pathol 22:1457-1467, 2009

39. Owens MA, Horten BC, Da Silva MM: HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. Clin Breast Cancer 5:63-69, 2004

40. Badve S, Gray R, Childs BH, et al: HER2 concordance between central laboratory immunohistochemistry and quantitative reverse transcription polymerase chain reaction in Intergroup Trial E2197. Presented at the 2008 American Society of Clinical Oncology Breast Cancer Symposium, Washington, DC, September 5-7, 2008

41. Konecny G, Pauletti G, Pegram M, et al: Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J Natl Cancer Inst 95:142-153, 2003

42. Desmedt C, Haibe-Kains B, Wirapati P, et al: Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. Clin Cancer Res 14:5158-5165, 2008

43. Wirapati P, Sotiriou C, Kunkel S, et al: Meta-analysis of gene expression profiles in breast cancer: Toward a unified understanding of breast cancer subtyping and prognosis signatures. Breast Cancer Res 10:R65, 2008

44. Press MF, Bernstein L, Thomas PA, et al: HER-2/neu gene amplification characterized by fluorescence in situ hybridization: Poor prognosis in node-negative breast carcinomas. J Clin Oncol 15: 2894-2904, 1997

45. Press MF, Pike MC, Chazin VR, et al: Her-2/neu expression in node-negative breast cancer: Direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res 53:4960-4970, 1993

46. Yamauchi H, Stearns V, Hayes DF: When is a tumor marker ready for prime time? A case study of c-erbB-2 as a predictive factor in breast cancer. J Clin Oncol 19:2334-2356, 2001

47. Perou CM, Srølie T, Eisen MB, et al: Molecular portraits of human breast tumours. Nature 406:747-752, 2000

48. Hyun CL, Lee HE, Kim KS, et al: The effect of chromosome 17 polysomy on HER-2/neu status in breast cancer. J Clin Pathol 61:317-321, 2008

49. Merola R, Mottolese M, Orlandi G, et al: Analysis of aneusomy level and HER-2 gene copy number and their effect on amplification rate in breast cancer specimens read as 2+ in immunohistochemical analysis. Eur J Cancer 42:1501-1506, 2006

50. Salido M, Tusquets I, Corominas JM, et al: Polysomy of chromosome 17 in breast cancer tumors showing an overexpression of ERBB2: A study of 175 cases using fluorescence in situ hybridization and immunohistochemistry. Breast Cancer Res 7:R267-R273, 2005

51. Torrisi R, Rotmensz N, Bagnardi V, et al: HER2 status in early breast cancer: Relevance of cell staining patterns, gene amplification and polysomy 17. Eur J Cancer 43:2339-2344, 2007

52. Ma Y, Lespagnard L, Durbecq V, et al: Polysomy 17 in HER-2/neu status elaboration in breast cancer: Effect on daily practice. Clin Cancer Res 11:4393-4399, 2005

53. Barberis M, Pellegrini C, Cannone M, et al: Quantitative PCR and HER2 testing in breast cancer: A technical and cost-effectiveness analysis. Am J Clin Pathol 129:563-570, 2008

54. Dal Lago L, Durbecq V, Desmedt C, et al: Correction for chromosome-17 is critical for the determination of true Her-2/neu gene amplification status in breast cancer. Mol Cancer Ther 5:2572-2579, 2006

55. Nistor A, Watson PH, Pettigrew N, et al: Real-time PCR complements immunohistochemistry in the determination of HER-2/neu status in breast cancer. BMC Clin Pathol 6:2, 2006

56. Downs-Kelly E, Yoder BJ, Stoler M, et al: The influence of polysomy 17 on HER2 gene and protein expression in adenocarcinoma of the breast: A fluorescent in situ hybridization, immunohistochemical, and isotopic mRNA in situ hybridization study. Am J Surg Pathol 29:1221-1227, 2005

57. Tse CH, Gown AM, Goldstein LC, et al: Amplification involving the CEP17 region may lead to false negative results of HER2 gene amplification by FISH. Cancer Res 69:1079, 2009 (abstr)

58. Yaziji H, Goldstein LC, Barry TS, et al: HER-2 testing in breast cancer using parallel tissue-based methods. JAMA 291:1972-1977, 2004

59. Harlan LC, Abrams J, Warren JL, et al: Adjuvant therapy for breast cancer: Practice patterns of community physicians. J Clin Oncol 20:1809-1817, 2002

60. Yaziji H, Gown AM: Testing for epidermal growth factor receptor in lung cancer: Have we learned anything from HER-2 testing? J Clin Oncol 22:3646, 2004

61. Yaziji H, Gown AM: Accuracy and precision in HER2/neu testing in breast cancer: Are we there yet? Hum Pathol 35:143-146, 2004

