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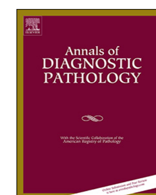
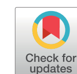


Clinicopathologic features of breast cancer reclassified as *HER2*-amplified by fluorescence in situ hybridization with alternative chromosome 17 probes



Kristen C. Blanton[a], Allison M. Deal[b], Kathleen A. Kaiser-Rogers[c], Carey K. Anders[d], Siobhan M. O'Connor[c], Johann D. Hertel[c], Benjamin C. Calhoun[b,c,*]

[a] School of Medicine, University of North Carolina, Chapel Hill, NC, USA
[b] Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA
[c] Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC, USA
[d] Department of Medicine, Division of Medical Oncology, Duke Cancer Institute, Durham, NC, USA

A R T I C L E I N F O

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A B S T R A C T

Objective: Dual probe fluorescence in situ hybridization (FISH) assays for determination of human epidermal growth factor receptor 2 (*HER2*) gene amplification in breast cancer provide a ratio of *HER2* to chromosome 17. The ratio may be skewed by copy number alterations (CNA) in the control locus for chromosome 17 (CEP17). We analyzed the impact of alternative chromosome 17 control probes on HER2 status in a series of breast cancers with an emphasis on patients reclassified as amplified.

Methods: Breast cancer patients with equivocal HER2 immunohistochemistry (2+) and equivocal FISH with CEP17 were included. Reclassification of HER2 status was assessed with alternative chromosome 17 control probes (*LIS1* and *RARA*).

Results: A total of 40 unique patients with 46 specimens reflexed to alternative chromosome 17 probe testing were identified. The majority (> 80%) of patients had pT1–2, hormone receptor-positive tumors with an intermediate or high combined histologic grade. There were 34/46 (73.9%) specimens reclassified as amplified with alternative probes, corresponding to 29/40 (72.5%) patients. Of the patients reclassified as amplified with alternative probes, 34.5% (10/29) received HER2-targeted therapy.

Conclusion: In this series, the majority of breast cancers tested with alternative chromosome 17 control probes under the 2013 ASCO/CAP Guidelines were converted to *HER2*-amplified. The treatment data and the clinicopathologic profile of the tumors suggest that most of these patients will neither receive nor benefit from HER2-targeted therapy. The findings support the recommendation in the 2018 ASCO/CAP HER2 Guidelines to discontinue the use of alternative chromosome 17 probes.

1. Introduction

Approximately 15–20% of invasive breast cancers are positive for human epidermal growth factor receptor 2 (*HER2; ERBB2*) gene amplification and protein over-expression [1-3]. The humanized anti-HER2 monoclonal antibody trastuzumab is a highly effective treatment with a favorable side effect profile [4]. Newer anti-HER2 agents, including pertuzumab, TDM1, and tucatinib, have also been shown to be effective in HER2-positive breast cancer [5-7]. Reliable assessment of patients' eligibility for HER2-targeted therapies requires accurate pathologic evaluation of HER2 status.

The Guidelines for the interpretation and reporting of HER2 testing results in breast cancer were first published in 2007 by the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) [8]. The ASCO/CAP Guidelines were updated in 2013 [9] and again in 2018 [10]. In all three versions, the Expert Panel did not make a specific recommendation for the use of immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) for first-line HER2 testing. However, most laboratories use IHC for initial HER2 testing with FISH as the reflex testing method for cases with equivocal (2+) IHC. FISH is used for first-line testing in some large academic and reference laboratories.

The most commonly used FISH assays are the US Food and Drug Administration (FDA)-approved dual probe assays. These assays employ

* Corresponding author at: Department of Pathology and Laboratory Medicine, University of North Carolina - Chapel Hill, 160 North Medical Drive, Campus Box #7525, Chapel Hill, NC 27599-7525, USA.
E-mail address: ben.calhoun@unchealth.unc.edu (B.C. Calhoun).

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Table 1
Clinicopathologic Features of Breast Cancer with HER2 FISH and Reflex testing with Alternative Chromosome 17 probes (n = 40 patients)

Pt	Specimen Type	IHC	CEP17 Probe			HER2 Status	Alternative Probes				HER2 Status	Hormone Receptor Status[f]		HER2 Therapy
			Avg. copy number and ratio				*Avg. copy number and ratio*							
			HER2	CEP17	Ratio		LIS1	Ratio	RARA	Ratio		ER	PR	
1	**Met**	2+	4.0	2.5	1.6	Equivocal	2.6	1.5	3.9	1.0	Equivocal	Positive	Positive	
2	**Met**	2+	4.9	2.6	1.9	Equivocal	1.7	2.9	4.6	1.1	Amplified	Positive	Negative	Yes
3	**Core**	2+	5.7	4.0	1.4	Equivocal	2.3	2.5	7.2	0.8	Amplified	Positive	Positive	
4	**Met**	2+	5.5	3.9	1.4	Equivocal	1.7	3.2	3.4	1.6	Amplified	Negative	Negative	
5	**Core**	2+	4.8	2.9	1.6	Equivocal	2.1	2.2	4.4	1.1	Amplified	Positive	Positive	Yes
6	**Exc**	1+[a]	5.3	4.8	1.1	Equivocal	3.4	1.6	3.5	1.5	Equivocal	Negative	Negative	Yes
7	**Core**	2+	6.4	3.5	1.8	Equivocal[b]	3.0	2.1	NR	NR	Amplified	Positive	Positive	
8	**Core**	2+	4.3	2.3	1.8	Equivocal	3.6	1.2	3.8	1.1	Equivocal	Positive	Positive	Yes
9	**Met**	2+	4.2	NR[c]	NR[c]	Equivocal	2.9	1.4	3.8	1.1	Equivocal	Negative	Negative	Yes
10	**Core**	2+	4.8	3.5	1.3	Equivocal	1.8	2.6	NR	NR	Amplified	Positive	Negative	
10	**Exc**	2+	5.8	2.9	2.0	Equivocal[b]	1.8	3.2	NR	NR	Amplified	NR	NR	
11	**Core**	2+	5.6	3.9	1.4	Equivocal	3.6	1.6	5.9	1.0	Equivocal	Positive	Positive	Yes
12	**Core**	2+	4.9	2.9	1.7	Equivocal	1.6	3.1	3.7	1.3	Amplified	Positive	Positive	
13	**Core**	2+	4.7	2.6	1.8	Equivocal	1.8	2.6	3.9	1.2	Amplified	Positive	Positive	
14	**Core**	2+	4.3	3.1	1.4	Equivocal	1.8	2.4	3.8	1.1	Amplified	Negative	Positive	
15	**Core**	2+	5.2	3.6	1.5	Equivocal	1.8	2.9	4.0	1.3	Amplified	Positive	Positive	
16	**Met**	2+	4.3	3.7	1.2	Equivocal	1.8	2.4	4.3	1.0	Amplified	Negative	Negative	Yes
17	**Met**	2+	4.2	2.7	1.6	Equivocal	1.9	2.2	4.8	0.9	Amplified	Positive	Negative	
18	**Core**	2+	4.5	2.6	1.7	Equivocal	2.1	2.1	2.2	2.0	Amplified	Negative	Negative	Yes
19	**Core**	2+	4.8	3.5	1.4	Equivocal	1.9	2.6	4.2	1.1	Amplified	Positive	Positive	Yes
20	**Core**	1+[d]	4.0	2.7	1.5	Equivocal	2.1	1.9	4.2	0.9	Equivocal	Positive	Positive	
21	**Met**	2+	4.3	3.4	1.2	Equivocal	3.6	1.2	3.9	1.1	Equivocal	Positive	Positive	
22	**Core**	2+	4.4	3.9	1.1	Equivocal	2.0	2.2	4.1	1.1	Amplified	Positive	Positive	Yes
23	**Core**	2+	4.1	4.2	1.0	Equivocal	4.0	1.0	3.8	1.1	Equivocal	Negative	Negative	
23	**Exc**	0	4.3	3.3	1.3	Equivocal	3.8	1.1	3.5	1.2	Equivocal	NR	NR	
24	**Exc**	NR[e]	4.8	3.8	1.3	Equivocal	1.5	3.2	4.9	1.0	Amplified	Positive	Positive	
25	**Core**	2+	5.0	2.7	1.8	Equivocal	1.6	3.2	5.3	0.9	Amplified	Positive	Positive	Yes
26	**Exc**	2+	5.5	4.1	1.4	Equivocal	2.5	2.2	5.3	1.0	Amplified	Positive	Positive	
27	**Core**	2+	4.9	3.0	1.7	Equivocal	1.8	2.7	4.7	1.1	Amplified	Positive	Positive	
27	**Exc**	NR	4.6	2.6	1.8	Equivocal	1.7	2.7	5.4	0.8	Amplified	NR	NR	
28	**Exc**	2+	4.4	3.0	1.5	Equivocal	2.2	2.0	4.7	1.0	Amplified	Positive	Positive	
28	**Met**	2+	5.3	3.8	1.4	Equivocal	1.7	3.1	5.3	1.0	Amplified	NR	NR	
29	**Met**	2+	4.3	3.9	1.1	Equivocal	2.6	1.7	3.3	1.3	Equivocal	Positive	Positive	
30	**Core**	2+	4.8	3.3	1.5	Equivocal	1.8	2.7	4.1	1.2	Amplified	Positive	Positive	Yes
30	**Exc**	2+	5.1	3.5	1.4	Equivocal	1.6	3.1	4.7	1.1	Amplified	NR	NR	
31	**Core**	2+	5.0	3.2	1.6	Equivocal	1.8	2.9	4.8	1.0	Amplified	Positive	Negative	
32	**Met**	2+	5.0	2.8	1.8	Equivocal	2.7	1.9	4.7	1.1	Equivocal	Positive	Positive	Yes
33	**Core**	2+	5.1	3.1	1.6	Equivocal	1.5	3.3	4.2	1.2	Amplified	Positive	Positive	
33	**Exc**	NR	5.0	3.7	1.4	Equivocal	1.7	2.9	4.9	1.0	Amplified	NR	NR	
34	**Core**	2+	4.1	2.8	1.5	Equivocal	1.6	2.5	3.9	1.1	Amplified	Positive	Positive	
35	**Core**	2+	3.1	2.7	1.1	Equivocal	1.5	2.1	3.0	1.1	Amplified	Positive	Positive	
36	**Core**	2+	4.0	3.1	1.3	Equivocal	1.9	2.1	1.9	2.2	Amplified	Positive	Positive	Yes
37	**Met**	2+	4.5	3.9	1.2	Equivocal	3.2	1.4	4.0	1.1	Equivocal	Negative	Negative	
38	**Core**	2+	4.9	3.9	1.3	Equivocal	1.9	2.6	4.4	1.1	Amplified	Positive	Positive	
39	**Met**	2+	4.4	2.6	1.7	Equivocal	1.8	2.5	3.4	1.3	Amplified	Positive	Negative	Yes
40	**Core**	2+	6.4	3.5	1.8	Equivocal[b]	1.9	3.5	NR	NR	Amplified	Positive	Positive	

Abbreviations: Pt, patient; IHC, Immunohistochemistry; CEP17, chromosome 17 centromere enumerator probe; HER2, human epidermal growth factor receptor 2; ER, estrogen recepror; PR, progesterone receptor; Avg, Average; RARA, retinoic acid receptor-α; LIS1, lissencephaly gene; Exc, Excision; Met, Metastasis; NR, Not performed/not reported.

[a] Patient 6: Positive (3+) HER2 IHC on a breast core biopsy at an outside laboratory. Negative (1+) HER2 IHC on a skin punch biopsy at UNC. HER2 FISH on a post-treatment breast specimen was equivocal and reflexed to alternative probe testing.

[b] Patients 7, 10, 40: Original results were reported prior to implementation of 2013 Guidelines. Equivocal based on 2007 Guidelines and classified as amplified by 2013 Guidelines.

[c] Patient 9: Absent CEP17 signals in tumor cells; disomic hybridization pattern for CEP17 in adjacent normal tissue. CEP17 and HER2:CEP17 could not be reported (NR) for this specimen.

[d] Patient 20: Prior negative (1+) IHC and equivocal HER2 FISH at an outside laboratory. Repeat HER2 FISH at UNC was equivocal, prompting alternative probe testing.

[e] Patient 24: Amplified HER2 FISH at an outside institution based on HER2 copy number (mean HER2 6.4, mean CEP17 4.7) and an equivocal HERmark result. Alternative probe testing was performed at our institution to exclude co-amplification

[f] For patients with more than one specimen tested with alternative chromosome 17 probes, hormone receptor studies were not repeated on the subsequent specimens (i.e., NR).

fluorochrome-labeled probes for *HER2*, located on the long arm of chromosome 17, and a separate probe for chromosome 17 that hybridizes to alpha satellite sequences at the centromere of chromosome 17 (CEP17; *D17Z1*). *HER2* and CEP17 signals are counted in a subset of tumor cells to generate a *HER2:*CEP17 ratio. *HER2:*CEP17 ratios ≥2.0 are classified as amplified [9,10]. The chromosome 17 centromere probe/control probes are used to control for aneusomy (especially polysomy) for chromosome 17. However, several studies have shown that whole chromosome polysomy for chromosome 17 is uncommon in breast tumors [11,12]. Copy number alterations (CNA) in the DNA that

encompass the hybridization site for the control probe may explain the increased number of chromosome 17 signals in many cases [13]. The increased CEP17 copy number seen in some cases may skew the *HER2:*CEP17 ratio toward normal, while loss of one or more copies of this locus can produce a falsely positive *HER2*:CEP17 ratio. In both situations, the *HER2* FISH results may not appear to correlate with *HER2* mRNA or protein expression levels [13,14].

The 2013 ASCO/CAP Guidelines [9] recommended testing with an alternative chromosome 17 probe in cases with equivocal FISH results (i.e., ISH Group 4 with *HER2:*CEP17 ratio < 2.0 and mean *HER2* copy number ≥ 4.0 and < 6.0) [10,15]. However, the 2018 ASCO/CAP HER2 Guidelines essentially eliminated the equivocal FISH category and the use of alternative chromosome 17 probes [10]. The recommendation for the use of alternative chromosome probes in the 2013 Guidelines was based on the idea that these probes may "correct" the estimation of chromosome 17 copy number, but some studies have shown CNA in the hybridization sites for alternative probes as well as CEP17 [16,17]. The goal of this study was to evaluate the impact of alternative chromosome 17 probes [*LIS1 (PAFAH1B1)* and *RARA*] on the assessment of *HER2* gene amplification by FISH. The classification of these cases based on the updated 2018 ASCO/CAP Guidelines [10] is emphasized and data on treatment with HER2-targeted therapies are also reported.

2. Materials and methods

This study was approved by the University of North Carolina Institutional Review Board. The study population was selected from primary and metastatic breast cancer cases undergoing dual probe FISH for HER2 gene status at the UNC Cytogenetics Laboratory from 2013 to 2018. Cases with equivocal IHC and FISH results were reflexed to a second FISH assay with an alternative chromosome 17 probe, including *LIS1* and/or *RARA*. The distribution of cases assigned to each HER2 category by *LIS1* and/or *RARA* with the 2013 ASCP/CAP Guidelines was recorded.

During the study period, the HER2 status of breast carcinomas was determined by FISH using the PathVysion *HER2* DNA Probe Kit, (Abbott Molecular, Inc., Des Plaines, IL). Tumors were tested with FISH using a dual probe assay with a *HER2* gene probe labeled in Spectrum Orange and a chromosome 17 centromeric enumeration probe (CEP17; *D17Z1*) labeled in Spectrum Green. To confirm invasive carcinoma, 4-μm-thick sections stained with hematoxylin and eosin (H&E) were evaluated, and separate cut sections that remained unstained were analyzed by *HER2:*CEP17 FISH. For all FISH cases, two areas of 30 cells each in a region designated by a breast pathologist as invasive carcinoma, are routinely analyzed by two independent observers. If the results of the initial analysis were equivocal, FISH with probes for *LIS1* and/or *RARA* was performed.

Surgical pathology and *HER2* FISH data were retrieved from the Anatomic Pathology Laboratory Information System (Epic Beaker, Epic Systems Corporation, Verona, WI) and reviewed by a dedicated breast pathologist (BCC). Patient demographics and treatment data were obtained from the electronic medical record in Epic (Epic Systems Corporation, Verona, WI).

3. Results

3.1. HER2 FISH with alternative chromosome 17 probes

A total of 46 specimens from 40 unique patients were tested with *HER2:*CEP17 FISH and reflexed to *LIS1* and/or *RARA* FISH (Table 1). A schematic representation of the positions of the CEP17, *LIS1*, *RARA* and *HER2* probes on chromosome 17 is shown in Fig. 1. The specimens tested included 25 (54.3%) core biopsies of the primary tumor in the breast, 9 (19.5%) surgical excision breast specimens, and 12 (26.1%) specimens collected from local or distant metastatic sites. There were 6 patients with alternative probe results on 2 specimens and hormone receptor studies were not repeated on the second specimen.

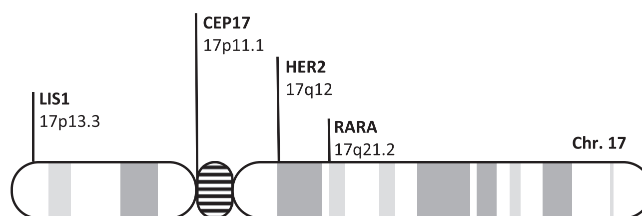

Fig. 1. Schematic of Chromosome 17 and Associated Probe Locations. Pictured are the probe locations for the human epidermal growth factor receptor-2 (*HER2*), chromosome 17 centromere enumerator (CEP17; D17Z1), lissencephaly (*LIS1*), and retinoic acid receptor-α (*RARA*) loci.

HER2 immunohistochemistry (IHC) results were available for 43 specimens: 40 were equivocal (2+) and the scores for the remaining 3 cases were 1+ (Patients 6 and 20), and 0 (Patient 23). The indication for *HER2* FISH in 40/46 (87.0%) specimens was an equivocal (2+) IHC result. In 6/46 (13.0%) the indication for *HER2* FISH testing was an equivocal FISH result from an outside laboratory, conflicting IHC and FISH results from an outside laboratory, or a prior equivocal FISH result at our institution. *HER2* FISH was performed on 1 excision specimen (Patient 6 in Table 1) due to conflicting IHC results: 3+ at an outside laboratory on a pretreatment breast core biopsy and 1+ at UNC on an ipsilateral skin punch biopsy. Testing with alternative chromosome 17 probes was performed on 3 specimens (Patients 7, 10 and 40 in Table 1) based on an equivocal FISH result using the 2007 ASCO/CAP Guidelines (i.e., just prior to implementation of the 2013 Guidelines).

A total of 34/46 (73.9%) specimens tested with alternative chromosome 17 probes were reclassified as amplified using the 2013 ASCO/CAP guidelines (Fig. 2). Of these reclassified specimens, all 34 were amplified based on reflex testing with the *LIS1* probe and 2 of these 34 cases also were amplified with the *RARA* probe. In 1 of 46 specimens, reflex testing with alternative probes was performed to investigate an unusual hybridization pattern for CEP17. In this single case, there were few or no CEP17 signals in the tumor cells, but the expected disomic pattern of hybridization in adjacent normal tissue was preserved (patient 9 in Table 1). In 41 (91.1%) of the remaining 45 specimens, the chromosome 17 copy number obtained with *LIS1* was lower (closer to the disomic number 2.0) than the number obtained with CEP17. However as indicated above, in only 34 of these cases was the number of chromosome 17 control probe signals low enough to yield a *HER2*:control locus ratio of greater than or equal to 2.

3.2. Clinicopathological features

There was no significant difference in the clinicopathologic features of those patients whose tumors were reclassified from equivocal to amplified with alternative chromosome 17 probes (n = 29) when compared to the rest of the cohort (n = 11) (all $p > .09$). The median age was 57.4 years (range: 28–90). Of the 31/40 patients with available pT or pN data, 27/31 (87.1%) were pT1 or pT2, 15 (48.4%) were lymph node-negative (pN0), 7 (22.6%) had 1–3 positive lymph nodes (pN1) and 2 (6.5%) had 4–9 positive lymph nodes (pN2). Of the patients with alternative probe testing, 33/40 (82.5%) had hormone receptor-positive tumors and 35/40 (87.5%) had an intermediate or high combined histologic grade.

3.3. Treatment and follow-up

The results for each pathologic specimen described above correspond to re-classification of 29/40 (72.5%) patients as amplified. Of the 36/40 (90.0%) patients with available treatment data, 15/36 (41.7%) received HER2-targeted therapy, 10 of whom were reclassified as amplified with alternative chromosome 17 probes. Follow-up data was

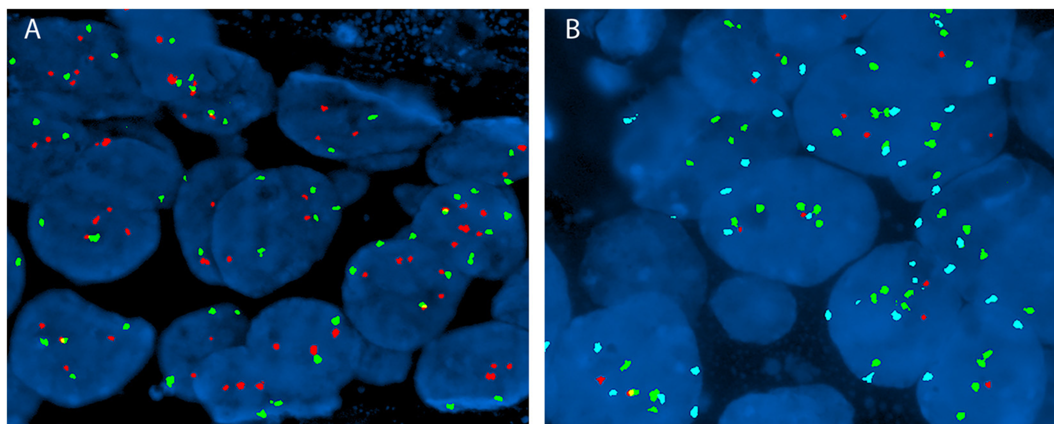

Fig. 2. Representative images of an equivocal *HER2* FISH result reclassified as amplified with alternative chromosome 17 probes and the 2013 ASCO/CAP Guidelines (patient 34 in Table 1). Dual-probe FISH yielded a mean *HER2* (red) copy number of 4.1, a mean CEP17 (green) copy number of 2.8 and a *HER2*:CEP17 ratio of 1.5 (A). Alternative chromosome 17 probes demonstrated a mean number of 3.9 *RARA* (green) and 1.6 *PAFAH1B1/LIS1* (red) signals per cell; CEP17 (aqua) (B). The *HER2:PAFAH1B1/LIS1* ratio was 2.5 (amplified) and the *HER2:RARA* ratio was 1.1 (not amplified). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

available for 37/40 patients at a median of 16 months (range: 0.3–62 months). At the time of last follow-up, 17 patients were alive with disease, 16 had no evidence of disease (NED) and 4 were deceased. All 4 of the patients who were deceased at last follow up had tumors that were reclassified as amplified with alternative chromosome 17 probes. Three (3) of the deceased patients were referred to our institution for evaluation and treatment of metastatic disease and 2 had received HER2-targeted therapy.

4. Discussion

The 2013 ASCO/CAP HER2 Guidelines classified cases based on a combination of the *HER2:*CEP17 ratio and the mean *HER2* copy number, and allowed the use of alternative chromosome 17 probes [9]. The emphasis on *HER2* signal number and alternative chromosome 17 probes represented attempts to address issues related to the potential for a skewed *HER2*:CEP17 ratio in cases with CEP17 CNA. One of the most significant changes in the 2013 guidelines was the classification of cases with a mean *HER2* copy number ≥ 4.0 and < 6.0 and a *HER2:*CEP17 ratio < 2.0 as equivocal [9]. Several subsequent studies showed that the new equivocal category and the use of alternative chromosome 17 probes resulted in an increase in the proportion of equivocal and amplified *HER2* FISH results, particularly among cases with equivocal (2+) IHC results and CEP17 CNA [17-32]. However, copy number variations also have been reported for the most commonly used alternative chromosome 17 control probes [i.e., *RAI1, LIS1 (PAFAH1B1), RARA, and TP53*] [16,17]. Therefore, it is not clear which probe provides the most accurate reflection of the chromosome 17 copy number and ultimately the most accurate *HER2*:control ratio.

The emphasis on *HER2* copy number in the 2013 Guidelines effectively created 5 categories of FISH results [15]. The group of FISH cases defined as equivocal in the 2013 Guidelines [9] corresponds to ISH Group 4 in the scheme developed by Press et al. and adopted in the most recent 2018 ACCO/CAP HER2 FISH Guidelines [10,15,33]. Based on the data from patients screened for enrollment in three Breast Cancer International Research Group (BCIRG) clinical trials [15] and the NSABP-B47 trial of "Trastuzumab for Women with HER2-low Breast Cancer" [34], patients in ISH Group 4 are unlikely to benefit from HER2-targeted therapy. However, it should be noted that patients with any *HER2* FISH result with a mean *HER2* copy number ≥ 4.0 may have been excluded from NSABP-B47 [20].

In the majority of cases in this series, the indication for *HER2* FISH with an alternative chromosome 17 probe was an equivocal result (as defined in the 2013 Guidelines) obtained from a dual probe assay using CEP17. When patients with known metastases are excluded, the majority in this series had pathological Stage I-II, intermediate to high combined histologic grade, hormone receptor-positive, lymph node-negative tumors. The clinicopathological characteristics of these tumors are not typical for *HER2*-amplified tumors but they are similar to the "excess" equivocal cases identified using the 2013 Guidelines [19,25,28,35]. These tumors also were unlikely to have positive (3+) IHC results, similar to the data from other studies of the equivocal category in the 2013 Guidelines [15,18,19,21,22]. The clinicopathologic features and HER2 IHC data may account for the fact that approximately 60% of patients in this series who were re-classified as amplified with alternative probes were not treated with HER2-targeted therapy. In studies using gene expression analysis [36,37] and surrogate immunohistochemistry markers [25] for intrinsic molecular subtypes, the majority of *HER2* FISH equivocal tumors (as defined in the 2013 Guidelines) are luminal-type, and not *HER2*-enriched. In the series reported by Desai et al., most of the tumors reclassified as amplified with alternative chromosome 17 probes were Luminal A or B subtype, indicating that these are not *HER2*-driven tumors [36].

Three specimens in this series were tested with alternative chromosome 17 probes based on an equivocal FISH result using the 2007 Guidelines, just prior to the implementation of the 2013 Guidelines (Patients 7, 10 and 40 in Table 1). The core biopsies for Patients 7 and 40, with mean *HER2* copy numbers of 6.4 and *HER2*:CEP17 ratios of 1.8, would be classified as amplified using the 2013 Guidelines and presumably would be classified as amplified (ISH Group 3) in the 2018 Guidelines. The excision specimen from Patient 10, with a mean *HER2* copy number of 5.8 and a *HER2*:CEP17 ratio of 2.0 would be classified as amplified in the 2013 Guidelines and amplified (ISH Group 1) in the 2018 Guidelines. It should be noted that Patient 10 also had a core biopsy that was converted to amplified with alternative chromosome 17 probes. That specimen, with a mean *HER2* copy number of 4.8 and a *HER2*:CEP17 ratio of 1.3, would have been classified as equivocal by the 2013 Guidelines. Using the 2018 Guidelines, it would correspond to ISH Group 4 and would be classified as not amplified in the absence of a 3+ HER2 IHC result. Taking all of this into consideration, if the 3 specimens classified as equivocal under the 2007 Guidelines were excluded from analysis, the rate of conversion to amplified (with alternative probes and the 2013 Guidelines) would remain high: 31/43 (72.1%) specimens and 27/38 (71.5%) patients.

The use of alternative chromosome 17 probes in this series increased the number of patients potentially eligible for HER2-targeted therapy. However, there is no well-established benefit from HER2-targeted therapy in this patient population and many of these "amplified"

cases may in fact represent false-positives [38]. In a study of over 10,000 patients screened for enrollment in three BCIRG trials, there was no significant difference in DFS or OS when the patients in ISH Group 4 were compared to those with clearly negative FISH results (i.e., ISH Group 5; *HER2:*CEP17 ratio < 2.0 and a mean *HER2* copy number of < 4.0) [15]. Based on the outcomes data and the absence of HER2 protein overexpression in ISH Group 4, Press et al. concluded that tumors with a *HER2:*CEP17 ratio < 2.0 and a mean *HER2* copy number ≥ 4.0 and < 6.0 should be classified as not amplified [15]. This recommendation was incorporated into the updated 2018 ASCO/CAP HER2 Guidelines [10].

Limitations of this study include the single-institution retrospective design and limited sample size. Strengths of the study include the focus on tumors reclassified as amplified with alternative probes, the detailed characterization of the FISH and IHC findings, and thorough clinicopathologic characterization with data on treatment with HER2-targeted therapy. Although the sample size is limited, the detailed characterization of this group of patients provides an accurate reflection of the impact of alternative chromosome 17 probes in complex breast cancer cases at a tertiary referral center. No prospective clinical trials using alternative chromosome 17 probes to select patients for HER2-targeted therapy have been reported.

In summary, HER2 FISH with alternative chromosome 17 probes reclassified the tumors in 29/40 (72.5%) patients as amplified. The majority of these patients were not treated with HER2-targeted therapy. The best available retrospective data derived from large clinical trials indicate that this group of patients is unlikely to benefit from trastuzumab [15]. Our findings support the classification of these cases as not amplified in the 2018 ASCO/CAP HER2 Guidelines [10].

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Prior presentation

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Protection of human and animal subjects

This study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

Declaration of competing interest

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