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PRECLINICAL STUDY

Updated 2013 College of American Pathologists/American Society of Clinical Oncology (CAP/ASCO) guideline recommendations for human epidermal growth factor receptor 2 (HER2) fluorescent in situ hybridization (FISH) testing increase HER2 positive and HER2 equivocal breast cancer cases; retrospective study of HER2 FISH results of 836 invasive breast cancers

Kamaljeet Singh[1] · Umadevi Tantravahi[2] · Michele M. Lomme[1] · Terese Pasquariello[3] · Margaret Steinhoff[1] · C. James Sung[1]

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Abstract For dual probe HER2 FISH assay, the 2013 CAP/ASCO guideline recommendations lowered the HER2/CEP17 ratio cut off for HER2 amplification to ≥2.0 and introduced an average HER2 copy number criterion for HER2 amplification (≥6.0/cell) and HER2 equivocal categories (≥4 and <6/cell). The HER2/CEP17 equivocal category is eliminated. The aim of this study is to assess the impact of 2013 HER2 FISH testing guideline recommendations update on the assignment of HER2 status with dual probe HER2 FISH assay. Dual probe HER2 FISH assay results on breast cancers from 09/2009 to 07/2015 that underwent reflex HER2 FISH testing after equivocal HER2 (2+) immunohistochemistry (IHC) were reviewed. HER2 copy number, CEP17 signals, and HER2/CEP ratios were noted. HER2 status was assigned as HER2 negative (HER2−), HER2 equivocal (HER2e), and HER2 amplified (HER2+) by applying both 2007 and 2013 CAP/ASCO HER2 FISH guideline recommendations and results were compared. New guidelines reclassified HER2 FISH status in a significant proportion of cases (8.3 %, 69/836; $p = .021$). There were 22 (2.6 %) more HER2+, 17 (2.1 %) more HER2e, and 39 (4.1 %) fewer HER2− tumors. Change of HER2 status correlated significantly with ≥3 CEP17 signals (38 vs. 2 %; $p < .001$). The 2013 CAP/ASCO guideline recommendations for HER2 FISH testing by dual probe assay increased the HER2 amplified and HER2 equivocal tumors. Increase in HER2 equivocal tumors would potentially increase the frequency of repeat HER2 testing. Tumors with ≥3 CEP17 signals, so-called chromosome 17 polysomy, are more likely to be impacted and classified as HER2 equivocal.

Keywords Breast cancer · HER2 · FISH · Equivocal · ASCO · CAP · Polysomy

Introduction

Assessment of HER2 status is a critical laboratory investigation in the management of invasive breast cancer. HER2 status is associated with clinical outcomes [1–4]. HER2 positivity is associated with poor prognosis in a newly diagnosed breast cancer in the absence of adjuvant systemic therapy [5]. HER2 positivity predicts relative as well as absolute response to systemic therapies and most importantly decides the eligibility for HER2-targeted therapy [6–9]. Approximately, 20 % of invasive breast cancers are expected to be HER2 positive [10]. Because of the stakes involved, including prognostic and predictive values of HER2 status and the cost-related to the HER2-targeted therapy, there has been a continuous effort to improve and standardize the performance of the laboratory methods employed to assess the HER2 status [11–14].

✉ Kamaljeet Singh
410jeeta@gmail.com

[1] Department of Pathology and Laboratory Medicine, Women & Infants Hospital, Alpert Medical School of Brown University, 101 Dudley Street, Providence, RI 02903, USA

[2] Pathology and Laboratory Medicine, Cytogenetics and Molecular Laboratory, Women & Infants Hospital, Alpert Medical School of Brown University, 101 Dudley Street, Providence, RI 02903, USA

[3] Immunohistochemistry Laboratory, Department of Pathology and Laboratory Medicine, Women & Infants Hospital, 101 Dudley Street, Providence, RI 02903, USA

Retrospective analysis of the data from early clinical trials of trastuzumab in which both 2+ and 3+ HER2 immunohistochemistry staining patterns were identified as HER2 positive suggested that only patients with IHC 3+ staining and/or HER2 gene amplification derived any benefit [4]. Early prospective randomized adjuvant trials of trastuzumab followed an arbitrary HER2 testing algorithm: HER2 IHC assay followed by reflex HER2 FISH for 2+ IHC staining or in situ hybridization (ISH) testing alone with amplification ratio >2 considered HER2 positive [15]. Unfortunately, the HER2 assay results performed at local laboratories, when repeated at a high volume central and reference laboratory, were discordant [16].

In an effort to standardize the HER2 test result reporting, NCCN HER2 testing in breast cancer task force report and recommendations were published in 2006 [14]. In 2007, CAP and ASCO issued joint guideline recommendations for HER2 testing in breast cancer [12]. These guidelines aimed to standardize the pre-analytic, analytic, and post-analytic variables and improve the accuracy of HER2 testing in breast cancer. Based on HER2 CAP survey results from 2003 and 2004 and other studies correlating the HER2 IHC and HER2 FISH concordance, for dual probe HER2/CEP17 FISH assay, the threshold for HER2 positivity was set at >2.2 and an equivocal/borderline category with HER/CEP17 ratio between 1.8 and 2.2 was introduced. Absolute HER2 gene copy number was applicable only to assays without an internal control probe. Interestingly, in trastuzumab clinical trials, a borderline/equivocal category for HER2 FISH was never tried and the HER2/CEP17 $\geq$ 2.0 was HER2+.

The CAP/ASCO guideline recommendations for HER2 FISH testing were updated in 2013. For HER2 positivity, the dual probe HER2/CEP17 ratio was lowered to $\geq$2.0. Average HER2 copy number was added as a separate HER2 positivity criteria for both dual probe as well as single probe FISH assays: $\geq$6.0/cell is HER2 positive (HER2+) and a dual probe HER2/CEP17 ratio <2.0 with average HER2 copy number $\geq$4 and <6/cell is HER2 equivocal (HER2e) [13]. New guidelines also eliminated the dual probe HER2/CEP17 equivocal category. These recommendations are closer to the HER2 testing performed in the clinical trials; however, the clinical impact of these guidelines is still unknown. The aim of our study is to investigate the impact of new 2013 HER2 FISH guideline recommendations on determination of HER2 status in breast cancer by dual probe HER2 FISH assay and compare it with the 2007 guidelines recommendations. We also review the existing literature on this topic and summarize findings from other similar studies.

Methods

With institutional IRB approval, we retrospectively identified invasive breast cancers from 09/2009 to 07/2015 that underwent reflex dual probe HER2/CEP17 FISH testing after equivocal (2+) HER2 IHC results. At our institution, all invasive breast cancers are first tested by HER2 IHC. Equivocal (2+) IHC results are reflexed to dual probe HER2 FISH. The electronic HER2 FISH reports were reviewed and HER2 copy number, CEP17 copy number, and HER2/CEP17 were noted. All the cases were reclassified using 2007 and 2013 CAP/ASCO dual probe HER2 FISH algorithms and the results were compared. Chromosome 17 polysomy was defined as $\geq$3 CEP17 signals.

During this time, HER2 IHC was performed on formalin-fixed paraffin embedded tissue by FDA approved HercepTest™ (Dako platform). PathVysion HER2 DNA Probe Kit (PathVysion Kit) was used for HER2 FISH. In brief, the PathVysion HER2 DNA Probe Kit consists of two labeled DNA probes. The LSI HER2 probe that spans the entire HER2 gene is labeled in SpectrumOrange. The CEP 17 probe is labeled in SpectrumGreen and hybridizes to the alpha satellite DNA located at the centromere of chromosome 17 (17p11.1-q11.1). HER2 and CEP 17 signals were analyzed by two technologists. Each technologist counted 20 nuclei from two non-overlapping areas (total 80 nuclei). In cases with heterogeneity, additional 20 non-overlapping nuclei in at least 2 other foci were counted (total 120 nuclei).

Data were analyzed using IBM SPSS statistics software version 22. The frequency of HER2 status change with two guidelines and the frequency of chromosome 17 polysomy between cases with and without HER2 status change were tested for statistical significance (Chi-square and Fisher's Exact test; $p < .05$ considered significant).

Results

Totally 854 breast cancers underwent HER2 FISH testing during the study period. Our laboratory endorsed 2013 CAP/ASCO HER2 guidelines in January 2014. Out of total 854 cases, 589 cases were HER2 2+ by 2007 IHC guidelines (cases before Dec, 2013) and 265 cases were HER2 IHC 2+ by 2013 guidelines (2014 and 2015 cases). Average HER2 signals, CEP17 signals, and HER2/CEP17 ratio results were available for review in 836 cases. Eighteen cases were excluded: Four cases showed highly amplified HER2 with no recording of HER2 signals, invasive tumor was not identified in 3 cases, assay failed in 2 cases, and 9 cases did not have HER2 and CEP17 signal details.

The HER2 classification of 69 (8.3 %) cases was different with 2007 and 2013 guidelines (Table 1). Out of 741 HER2− cases by 2007 guidelines, 2 cases were reclassified as HER2+ and 42 cases were categorized as HER2e by 2013 guidelines. The two new HER2+ cases had ≥6 HER2 signals and HER2/CEP17 < 1.8. The HER2 copy numbers in 42 new HER2e cases were ≥4 and <6 and HER2/CEP17 ratio was <1.8.

Out of 38 HER2e cases by 2007 guidelines, 20 cases were reclassified as HER2+ and 5 cases were categorized as HER2− by 2013 guidelines. The new 20 HER2+ cases included 5 cases with ≥6 HER2 signals and 15 cases with HER2/CEP17 ≥2 and ≤2.2. The new 5 HER2− cases had HER2/CEP17 ratio ≥1.8 and <2.0 and <4 HER2 signals. The classification of 697 HER2−, 13 HER2e, and 57 HER2+ cases was unchanged with 2007 and 2013 guidelines. There was a shift in classification of HER2 status towards HER2e and HER2-amplified categories. Overall, there was a decrease in HER2− cases (702 vs. 741, 4.7 %), increase in both HER2e (55 vs. 38, 2.2 %) and HER2+ cases (79 vs. 57, 2.5 %) with 2013 guidelines ($p = .021$).

There were 133 (15.9 %) cases with ≥4 HER2 copy number: 40 (4.8 %) with HER2 copy number ≥6 and 93 (11.1 %) with HER2 copy ≥4 and <6 (Table 2). The HER2 copy number criteria changed the categorization of 2 HER2− cases to HER2+, 42 HER2− cases to HER2e, and 5 HER2e cases to HER2+ (Table 2). There were overall 7 cases with HER2 copy number ≥6 and HER2/CEP ratio <2.0.

There were 38 (4.5 %) cases with HER2/CEP17 ≥ 1.8 and ≤2.2: 23 (2.8 %) with HER2/CEP17 between ≥1.8 and <2 and 15 (1.8 %) with HER2/CEP17 ≥2.0 and ≤2.2. The 2013 guidelines for HER2/CEP17 ratio impacted HER2 status of 20 cases: 5 HER2e changed to HER2− and 15 HER2e changed to HER2+. There were 14 cases that were HER2+ only with the HER2/CEP criteria (HER2/CEP ≥ 2.0) and HER2− or HER2e with new HER2 copy number criteria (HER2 copy <6.0). Only one case was qualified as HER2+ with both new HER2 copy number (≥6) and HER2/CEP17 ratio criteria (≥2.0).

Overall, the number of cases in the HER2 categories with 2013 FISH guidelines was significantly different from 2007 guidelines ($p = .021$; Chi Square test). The frequency of chromosome 17 polysomy was higher in the group that underwent reclassification with new recommendations ($p < .001$; Fisher's exact test). Chromosome 17 polysomy was present in 26/69 (36 %) cases that showed HER2 status change with 2013 guidelines, as compared to 14/767 (2 %) cases without any HER2 status change (Table 3). Overall, 40/836 (4.8 %) tumors showed ≥3.0 CEP17 signals.

Discussion

Recently, many workers have reported the impact of 2013 CAP/ASCO guideline recommendations on HER2 testing in breast cancer (Table 4). Stoss et al. applied the 2013 and 2007 CAP/ASCO guidelines on the HER2 FISH data of tumors from screening population of the HERceptin Adjuvant (HERA) trial. Authors reported an increase in HER2 positive cases with 2013 CAP/ASCO guidelines (3380, 56.2 % vs. 3339, 55.5 %) [17]. In a similarly designed study, Long et al. reclassified 717 HER2 FISH results with both 2007 and 2013 guidelines. Total 55 (7.7 %) cases were reclassified when reassessed using 2013 guidelines. Nineteen of 25 cases in the 2007 HER2 equivocal category were reclassified as HER2 positive ($n = 13$) or negative ($n = 6$). Thirty-five previously negative cases became equivocal with 2013 guidelines. The HER2 positive case numbers increased from 71 to 85. Authors predicted an increase in HER2 positive and HER2 equivocal cases with 2013 updated guidelines [18]. In a similar study of re-evaluation and reclassification of dual probe HER2/CEP17 FISH results of 904 cases with 2013 guidelines, Bethune et al. noted similar trend in the reshuffling of the HER2 categories [19]. There was an increase in HER2 positive cases by 1.6 %, increase in HER2 equivocal by 5.9 %, and decrease in HER2 negative cases by 7.6 %. The authors expressed uncertainty regarding the clinical impact of this reclassification in the majority of the cases and predicted an increase in resource utilization due to retesting.

Similarly designed studies published as abstracts have also reported similar changes in HER2 status with 2013

Table 1 Cross-tabulation of HER2 classification with 2007 and 2013 CAP/ASCO guideline recommendations

	2007 CAP/ASCO (%)			Total
	Amplified	Equivocal	Non-amplified	
2013 CAP/ASCO*				
Amplified	57 (6.8)	20 (2.4)	2 (0.2)	79 (9.4)
Equivocal	0	13 (1.6)	42 (5.0)	55 (6.6)
Non-amplified	0	5 (0.6)	697 (83.4)	702 (84.0)
Total	57 (6.8)	38 (4.5)	741 (88.6)	836

* Significant change in the number of cases in the HER2 categories with 2013 and 2007 guideline recommendations ($p = .021$; Chi-Square test)

Table 2 Cross-tabulation of HER2 copy number and the HER2/CEP ratio groups

	2013 HER2 copy number criteria			Total
	<4	≥6	4 < 6	
HER2/CEP categories				
<1.8	697 (83.4 %)	2 (0.2 %)*	42 (5.0 %)#	741 (88.6 %)
≥2.2	0	32 (3.8 %)	25 (3.0 %	57 (6.8 %)
1.8–<2.0	5 (0.6 %)	5 (0.6 %)*	13 (1.6 %)	23 (2.8 %)
2.0–≤2.2	1 (0.1 %)*	1 (0.1 %)*	13 (1.6 %)*	15 (1.8 %)
Total	703 (84.1 %)	40 (4.8 %)	93 (11.1 %)	836 (100 %)

* HER2 positive with new 2013 HER2 copy number and/or HER2/CEP17 criteria

HER2 equivocal with new 2013 HER2 copy number criteria

Table 3 Relationship of HER2 status change with 2007 and 2013 guideline recommendations and CEP17 ≥ 3 (chromosome 17 polysomy)

	CEP17 signals ≥ 3		Total
	Absent	Present*	
HER2 status			
Change	43 (62 %)	26 (38 %)	69 (8.3 %)
No change	753 (98 %)	14 (2 %)	767 (91.7 %)
Total	796 (95 %)	40 (5 %)	836 (100 %)

* HER2 status change and CEP17 signals ≥ 3, $p < .001$

HER2 FISH guideline recommendations [20, 21]. Espinet et al. reported an identical trend with 26 new positive, 58 new equivocal, and 4 new negative cases when updated guidelines were applied retrospectively to 622 HER2 FISH results. Most of the new HER2 positives were in chromosome 17 polysomy setting with CEP17 copy numbers ranging from 4 to 5 and these cases were negative by HER2 IHC. In a study of 1893 HER FISH assay results, Andrade et al. reported reclassification of HER2 status in 74 (3.9 %) cases which included 17 new HER2 positive cases.

In a different study design evaluating the results of combined HER2 IHC and FISH assays, Varga et al. compared the HER2 results 1 year after implementation of 2013 guidelines. Authors reported an increase in overall HER2 positivity (2 %) after implementing new guidelines. This higher frequency of HER2 positive cases was observed more with HER2 FISH assay than with the IHC (3.5 vs.1 %). HER2 equivocal results also increased and resulted in delay in assignment of a final HER2 status [22]. In a similarly designed study, published as an abstract, Fulton et al. reported a shift in cases from IHC 0 to IHC 1+, IHC1+ to IHC 2+, and a slight increase in positive IHC-FISH concordance (95.7–97.3 %). In contrast to other studies, authors reported an increase in HER2 FISH negative (79–87 %) and decrease in HER2 positive rate (12–9 %). Authors also predicted the new guidelines might result in the increased overall testing costs [23]. In a study looking at both IHC and FISH results after implementing new guidelines, contrary to Fulton et al. findings, Onguru et al. observed a higher discordance rate between IHC and FISH results with new guidelines [24]. The impact of the new guidelines also depends on the HER2 testing protocol. In a setting like our institution, IHC followed by FISH, updated IHC guidelines also contribute to the overall impact. We did not investigate the contribution of updated HER2 IHC guidelines in our study. Briefly, it appears that a relatively higher number of cases, in a shorter interval of time, were classified as 2+ by 2013 (265 cases in 18 months) guidelines as compared to 2007 guidelines (589 in 52 months).

Investigating new guidelines in a primary HER2 FISH testing followed by IHC for equivocal FISH results, Muller et al. compared the frequency of HER2 FISH results before and after implementing the updated guidelines. Authors noted an increase in HER2 equivocal cases, which they attributed to the new HER2 copy number criteria [25]. Authors also noted that performing reflex HER2 IHC after equivocal FISH results is helpful in some cases (5 of 17 cases).

With 2013 guidelines, the most significant change in the HER2 status assignment is noticed in tumors with CEP17 signals ≥3, the so-called chromosome 17 polysomy group. Out of the 6018 cases enrolled in HERA trial, 61.1 % (69/113) new HER2 equivocal cases showed ≥3 CEP17 signals. All 21 cases that switched from HER2 negative to HER2 positive had mean CEP17 count of 5. In our cohort too, frequency of tumors with CEP17 signal ≥3 was significantly higher in the group that underwent HER2 status change with 2013 guidelines (36 vs. 2 %). Bethune et al. reported that 77 % of their cohort that underwent classification change with new guidelines had ≥3 CEP17 signals and all the 66 new HER2 equivocal tumors showed ≥3 CEP17 signals. Authors also noted that the pathological features of this group were intermediate between HER2 negative and HER2 positive tumors, which led them to propose that these might represent "luminal B" group at the molecular level.

The updated CAP/ASCO guidelines inadvertently cluster these HER2 non-amplified and chromosome 17 polysomy tumors into a new HER2 equivocal group. This

Table 4 Studies reporting impact of 2013 CAP/ASCO guideline recommendations on HER2 FISH results in breast cancer

Study	HER2 testing protocol	HER2 assay(s) compared	Total cases	HER2 status change, *n* (%)	HER2+	HER2 e	HER2−
Long et al. [18]	IHC followed by FISH	FISH	719	55 (7.6 %)	↑ (1.9 %)	↑ (2.2 %)	↓ (4.2 %)
Kos et al. [37]	ISH (mostly FISH)	ISH (mostly FISH)	306	42 (14 %)	↑ (6.8 %)	↑ (0.3 %)	↓ (7.1 %)
Espinet et al. [20]	ISH (FISH or SISH) followed by IHC	ISH	622	88 (14 %)	↑ (5 %)	↑*	↓*
Bethune et al. [19]	IHC followed by FISH	FISH	904	85 (9.5 %)	↑ (1.6 %)	↑ (5.9 %)	↓ (7.6 %)
Andrade et al. [21]	Not known	FISH	1893	74 (3.9 %)	↑ (0.9 %)	↑ (1.5 %)	↓ (1.9 %)
Singh et al. (this study)	IHC followed by FISH	FISH	836	69 (8.3 %)	↑ (2.6 %)	↑ (2.1 %)	↓ (4.6 %)

* Absolute number not known/not available

ISH in situ hybridization; *SISH* silver in situ hybridization

phenomenon has previously been reported in series investigating breast cancers that show equivocal HER2 amplification [26]. Interestingly, polysomy 17 is not associated with HER2 overexpression on IHC or increased HER2 mRNA level by RT-PCR [26]. Tumors carrying chromosome 17 polysomy in the absence of HER2 gene amplification resemble HER2 negative than HER2 positive tumors [26]. Recent reports question the existence of true chromosome 17 polysomy in breast cancer. Many molecular techniques suggest that true chromosome 17 polysomy is a rare event in breast cancer and most of the elevated CEP17 signals detected by dual probe ISH HER2 testing are local gain/amplification in the peri-centromeric region of chromosome 17 [27–30].

It is not entirely clear how HER2 non-amplified and chromosome 17 polysomy predict the response to HER2-targeted therapy. Response to HER2-targeted therapy is seen only in chromosome 17 polysomy tumors with 3+ HER2 IHC score [31]. Findings from at least two studies, WO16229 trastuzumab trial and CLAGB 9840, suggest that chromosome 17 polysomy was associated with HER2 overexpression in the absence of Her2 amplification [31]. In the chemotherapy-only group of N9831, an adjuvant trastuzumab trial, a longer 5-year disease-free survival rate (78 vs. 68 %) was observed in HER2 non-amplified and chromosome 17 polysomy tumors versus HER2 amplified and chromosome 17 disomy tumors [32]. The predictive biomarker analysis of National epirubicin adjuvant trial/BR9601 cases revealed CEP17 probe duplication as the most powerful predictor of benefit from anthracyclines [33]. This new HER2 equivocal group with elevated CEP17 signals needs further investigations to assess if there is a subgroup within this cohort that would be worth treating with HER2-targeted therapy.

The borderline/equivocal category for HER2 amplification was reported in earlier studies comparing the HER2 immunohistochemistry and FISH tests [34]. Most of these tumors showing equivocal HER2 amplification comprise low HER2 copy numbers and show chromosome 17 polysomy. An equivocal category was introduced in 2003 CAP HER2 FISH proficiency survey program after the participants' responses to a case (CYH-01) from 2002 CAP HER2 FISH proficiency survey program were reported as HER2 amplified by 56 % (50/89) participants and HER2 not amplified by 44 % (39/50). This was a case of low-level HER2 amplification. In 2003, the same challenge was reported as amplified by (76 %) 94/124, non -amplified by 12 % (15/124), and equivocal by 12 % (15/124) participants [35]. Interestingly, none of the first generation randomized trials of adjuvant trastuzumab ever used the equivocal category based on IHC, HER2 copy number, or HER2/CEP17. Instead a HER/CEP17 $\geq$ 2 was used as a cut off for HER2 amplification in these trials. Updated HER2 FISH guidelines for dual probe assay, by removing the HER2/CEP17 equivocal category, level the HER2 testing in the clinical practice and trial settings. New guidelines also lower the HER2/CEP17 ratio cut off for HER2 amplification to $\geq$2.0, which was practiced in the early clinical trials of trastuzumab. Addition of absolute HER2 copy number criteria for dual probe ISH assays for HER2 negative, equivocal, and amplified categories also brings the updated ASCO/CAP guidelines closer to the clinical practice and published evidence [36].

Conclusion

The 2013 CAP/ASCO guideline recommendations for HER2 testing by dual probe HER2 FISH assay classify more breast cancer cases as HER2 positive and HER2 equivocal and fewer cases as HER2 negative. An increase in HER2 positive cases would potentially increase the number of patients eligible for HER2-targeted therapy. Increase in HER2 FISH equivocal cases will increase HER2 repeat testing. Breast cancers with so-called chromosome 17 polysomy, defined by CEP17 copy number $\geq$3.0, are more likely to be reclassified using new

guidelines. The chromosome 17 polysomy tumors comprise a significant proportion of new HER2 equivocal category.

Compliance with ethical standards

Conflict of Interest None.

Financial disclosure None.

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