Impact of 2013 American Society of Clinical Oncology/College of American Pathologists Guidelines on HER2 Fluorescent In Situ Hybridization Testing in Breast Cancers

Experience From a National Reference Laboratory

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ABSTRACT

Objectives: We compared the impact of 2013 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines on human epidermal growth factor receptor 2 (HER2) fluorescence in situ hybridization (FISH) testing results on breast cancers.

Methods: HER2 FISH testing performed between May 2015 and April 2016 following 2013 ASCO/CAP guidelines was included. HER2 to control probe ratios, mean HER2, and control probe copy numbers were used to reassign HER2 status using 2007 ASCO/CAP and US Food and Drug Administration (FDA) guidelines.

Results: HER2 FISH results were available in 2,017 cases. A total of 342 (17.0%) cases were amplified, 301 (14.9%) were equivocal, and 1,374 (68.1%) were nonamplified. After additional testing with the alternate probe, amplified cases increased to 21.6%. HER2 positivity rates following the 2013 ASCO/CAP guidelines were significantly higher compared with the 2007 ASCO/CAP and FDA guidelines.

Conclusions: The 2013 ASCO/CAP guidelines lead to a higher number of HER2 FISH positive and equivocal cases. In a reference laboratory setting where an alternative control probe was used to resolve equivocal FISH cases, 31.2% of patients with initial equivocal results became HER2 positive.

Since human epidermal growth factor receptor 2 (HER2)-directed therapies became available for treatment of breast cancer, determining HER2 status of breast cancers has been an area of challenge and controversy. Despite improvements in the standardization of preanalytical and analytical phases of HER2 testing, there is a significant lack of clinical outcome information validating the current classification guidelines. Initially, HER2-targeted therapy (trastuzumab) was used in metastatic breast cancer, which used immunohistochemistry (IHC) to determine HER2 positivity.1,2 Adjuvant trials showed the benefits of targeted therapies in nonmetastatic breast cancer using IHC and/or fluorescent in situ hybridization (FISH) following the US Food and Drug Administration (FDA) criteria to evaluate protein overexpression and/or gene amplification, respectively, for determining HER2 status.3-5 The initial FDA-approved HER2 testing for amplification of ERB-B2/neu by FISH was dichotomous (ie, results were categorized as negative or positive). HER2 to control probe ratio of 2.0 or higher was considered positive, and these patients were eligible for targeted therapies. Like many biologic processes, HER2 protein expression and gene amplification are a continuum. The 2007 American Society of Clinical Oncologists (ASCO) and the College of American Pathologists (CAP) guidelines added an equivocal category when the ratio was between 1.8 and 2.2.6 However, a revision of the 2007 ASCO/CAP guidelines emphasized that patients with a ratio of 2.0 to 2.2 should not be excluded from targeted therapies.7 In 2013, ASCO/CAP revised the guidelines in an attempt...
to decrease false-negative results and reverted to the FDA-approved ratio of 2.0 or more for HER2 positivity.\(^8,9\) We reviewed the HER2 FISH testing results after the implementation of the 2013 ASCO/CAP guidelines to determine the effects on HER2 reporting from a large national reference laboratory.

**Materials and Methods**

HER2 FISH testing performed between May 2015 and April 2016, after implementation of the 2013 ASCO/CAP guidelines and when the alternate probe (RAI1) was available in our laboratory for retesting of equivocal HER2 FISH cases, was identified. Information on HER2 to control probe ratio, HER2 and control probe copy numbers per cell, and prior HER2 IHC testing, if performed in our laboratory, was recorded. Our laboratory used the FDA-approved dual-probe HER2 IQFISH (DAKO, Carpentaria, CA) for initial FISH testing. Cases with an equivocal HER2 FISH result were retested with HER2 and RAI1 probes (both Agilent Technologies, Santa Clara, CA). At least 40 cells were counted in equivocal FISH cases and reviewed by both a technician and a board-certified pathologist trained in anatomic pathology. The 2013 ASCO/CAP guidelines were used for interpretation of the FDA-approved dual-probe set (HER2, CEN17). The HER2 FISH assay with RAI1 control probe was interpreted in the following manner: it was reported as nonamplified if the HER2 to RAI1 ratio was less than 2.0 and the average number of RAI1 per cell was 1.5 or more, and there were 16 or fewer cells out of 40 with only one RAI1 signal; it was reported as amplified if the HER2 to RAI1 ratio was 2.0 or more. If the RAI1 was deleted (defined as RAI1 copy number per cell <1.5 and 17 or more out of 40 cells with only one RAI1 signal per cell), the HER2 status was considered unresolved/indeterminate, and additional testing was recommended. A cutoff for RAI1 gains was not used due to the assumption that cases with gains of HER2, the centromere, and RAI1 were unlikely to represent true HER2 amplification. The same set of cases was reclassified following the 2007 ASCO/CAP guidelines and the FDA criteria using information on HER2 to control probe ratios and HER2 and control probe copy numbers per cell for each case.

Each HER2 category following different guidelines was compared by using the \(\chi^2\) test with a significance of 5% \((P = .05)\). This work was approved by the University of Utah Institutional Review Board (IRB 77507).

**Results**

Of the 2,038 patient samples tested for HER2 FISH, 21 (1.0%) were excluded due to hybridization failure or absence of invasive carcinoma in the deeper sections obtained for FISH testing. Of those cases, 552 were received for HER2 IHC testing with reflex FISH if the IHC result was equivocal \((\geq 2+).\) In total, 1,465 cases were received for HER2 FISH testing. We did not have knowledge of prior HER2 testing (IHC or FISH), if done, at the referring institution.

Following the 2013 ASCO/CAP criteria and using the FDA-approved HER2/CEN17 probes, 1,374 (68.1%) cases were interpreted as negative, 301 (14.9%) as equivocal, and 342 (17.0%) as positive.\(^{11}\) Three of the 301 cases with an equivocal FISH result did not hybridize when retested with the alternate RAI1 probe. Ninety-three (31.2%) of 298 equivocal cases were reclassified as amplified with a HER2 to RAI1 ratio of 2.0 or more, and 184 (61.7%) were nonamplified with a HER2 to RAI1 ratio of less than 2.0. Twenty-one (7.0%) of the original equivocal cases remained unresolved due to an RAI1 deletion.\(^{12}\) After the recommended additional testing of equivocal cases with the alternate RAI1 probe, 1,558 (77.2%) were negative, 21 (1.0%) remained equivocal, and 435 (21.6%) were positive. Additional testing with the alternate probe resulted in a 27% increase in HER2 positivity from 17.0% to 21.6%.

The HER2 probe used in the reflex test (Agilent Technologies) is dimmer and smaller (191 kb vs 218 kb) compared with the FDA-approved (DAKO IQFISH) probe. However, correlation between the two HER2 probes was good, with close average copy numbers per cell (4.6 and 4.3, respectively), whereas correlation between the two control probes (centromeric CEN17 and noncentromeric RAI1) was low to medium, with average copy numbers of 3.3 and 2.9, respectively.\(^{13}\)

For patients with equivocal HER2 FISH testing and an average HER2 copy number between 4.0 and 4.9, retesting with the alternate probe RAI1 reclassified these cases as FISH positive, FISH negative, and FISH equivocal in 26.6%, 69.2%, and 4.2% of the cases, respectively. Among patients with an equivocal HER2 FISH result and an average HER2 copy number per cell between 5.0 and 5.9, additional testing with the alternate probe RAI1 reclassified cases as FISH positive, FISH negative, and FISH equivocal in 42.9%, 48.8%, and 8.3% of the cases, respectively. The number of cases classified as amplified following the alternate probe testing was significantly higher in cases with an average HER2 copy number between 5.0 and 5.9 compared with cases with an average HER2 copy number between 4.0 and 4.9 \((P < .01).\)
When the cases were classified following the 2007 ASCO/CAP guidelines, 1,634 (81.0%) were negative, 149 (7.4%) were equivocal, and 234 (11.6%) were positive. When the cases were reassigned a HER2 status following the FDA guidelines, 1,711 (84.8%) were negative and 306 (15.2%) were positive (Figure 1).

Twenty-eight (1.4%) of the patients had a ratio of 2 or more, with an average HER2 copy number less than 4 consistent with a deletion of the CEN17 area.

Discussion

We report a 21% HER2 FISH positivity rate following the 2013 ASCO/CAP guidelines, after additional testing with the alternate chromosome 17 reference probe RAI1, similar to what is reported from another large reference laboratory. Both HER2 positive and equivocal rates were significantly higher following the 2013 guidelines compared with the 2007 guidelines before additional testing. After additional testing with the alternate probe, 31% of formerly FISH-equivocal patients were reinterpreted as positive, all of whom showed a low HER2 to RAI1 ratio (range, 2.0-3.6; mean, 2.3) (data not shown). Our equivocal rates were significantly decreased after additional testing with the alternate control probe because a HER2 to RAI1 ratio of less than 2.0 is defined as “negative,” whereas some other laboratories define a HER2 to alternate probe ratio of less than 2.0 as “equivocal” due to lack of specific guidelines. Regardless of how HER2 amplification is defined using a secondary in situ hybridization assay, the rate of HER2 overexpression...
in HER2 FISH-equivocal cases has been reported to be low.\textsuperscript{10, 11}

Of the 435 (21.6\% of 2,017) patients who were HER2 positive by the 2013 ASCO/CAP guidelines and following additional testing with an alternate probe, 357 (17.7\%) (278 after initial and 79 after alternate probe testing) were positive with a ratio of 2.0 or more and an average HER2 copy number of 4.0 or more per cell, 42 (2.1\%) (28 after initial and 14 after alternate probe testing) were positive with a ratio of 2.0 or more and an average HER2 copy number of less than 4.0 per cell, 36 (8.3\%) were positive with a FISH ratio of less than 2.0 and a HER2 signal of 6 or more, and 93 (21.4\%) were positive following alternate probe RAI1 testing. A total of 129 (29.7\%) patients were HER2 positive by FISH with a ratio of less than 2.0. This is an additional 6.4\% of women who would be eligible for targeted therapy, although currently it is not known if they would benefit from it as they would not have been included in N9831 or Herceptin adjuvant trials.\textsuperscript{4, 5}

Eligibility for targeted therapies that showed benefit was based on a HER2 ratio of 2.0 or more.\textsuperscript{4, 5, 12} The 2007 ASCO/CAP guidelines added an equivocal category with a ratio between 1.8 and 2.2. Although shortly after publication of the guidelines, it was recommended that patients

![Figure 2](https://academic.oup.com/ajcp/article-abstract/148/4/308/4108065)

**Figure 2** Distribution of 2,017 patients following 2013 American Society of Clinical Oncology/College of American Pathologists guidelines according to human epidermal growth factor receptor 2 (HER2) to control probe ratio and average HER2 copy number per cell. a Three cases with equivocal FISH testing with FDA-approved probes showed hybridization failure with alternate probe RAI1. b RAI1 deletion is defined as an average copy number per cell of less than 1.5 and 17 or more out of 40 cells with only one RAI1 copy.

![Figure 3](https://academic.oup.com/ajcp/article-abstract/148/4/308/4108065)

**Figure 3** The correlation between average human epidermal growth factor receptor 2 copy numbers (A) and control probes (CEN17 and RAI1) (B) at initial and reflex testing. A, $R^2 = 0.55$. B, $R^2 = 0.2$. 

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with a ratio between 2.0 and 2.2 should not be excluded from targeted therapies, patients with a ratio between 1.8 and less than 2.0 were not eligible. Thirty-six of 1,713 patients with a ratio of less than 2.0 and nine of 77 patients with a ratio between 1.8 and less than 2.0 who had a HER2 copy number of 6 or more were positive following the 2013 ASCO/CAP guidelines but would have been negative with the FDA and the 2007 ASCO/CAP guidelines, respectively. Fifteen cases with a HER2 copy number of 4 or more and less than 6 converted to positive following additional testing, resulting in a total of 24 (31%) patients with a ratio of 1.8 to less than 2.0 (equivocal by the 2007 ASCO/CAP guidelines) classified as positive by the 2013 ASCO/CAP guidelines, making them eligible for targeted therapies.

This study is from a large national reference laboratory and has limitations. Although some of the cases were received for IHC testing to be followed by FISH if equivocal (2+), most cases were sent for HER2 FISH as the first line of testing at our laboratory. The results of IHC testing at the referring institution, if performed, were not available to us. Seventy (12.7%) of 553 cases received for IHC followed by reflex FISH testing if equivocal (2+) showed HER2 amplification by FISH, and 259 (18.5%) of 1,455 cases received for only HER2 FISH testing showed amplification (data not shown), suggestive of referral of some IHC 3+ cases for FISH confirmation, resulting in increased positivity rates seen in tests received for FISH only.

We show in a reference laboratory setting that HER2 positivity is significantly increased following 2013 ASCO/CAP guidelines, confirming findings of Shah et al.10 Positivity rates were similar (23.6% vs 21.6%) despite different probe sets being used in these two reference laboratories. Both studies showed an increase in positivity rate compared with both the FDA criteria and the 2017 ASCO/CAP guidelines. Additional testing was performed by Shah et al using the D17S122 alternate probe, which converted 52% of equivocal cases to positive compared with 31% with RAI1 in our laboratory. Different client basis, known inconsistent results with different secondary reflex assays, and nonuniform interpretation of results with alternate probes (as seen with unresolved equivocal case rates following the reflex test from our laboratory and what is reported by Shah et al)10 may have contributed to a difference in final equivocal rates in these two studies.10,11

The increase in HER2 FISH positivity experienced in a reference laboratory—with the use of an alternate probe in equivocal cases—may not be reflective of positivity rates at other institutions where equivocal cases are more likely to be retested on different tumor block(s) or retested with IHC. Single-probe rather than dual-probe HER2 FISH testing might also yield different positivity rates as it will not identify cases with ratios of 2.0 or more and a HER2 copy number of less than 6.0. However, results from smaller studies have also shown increases in FISH positivity rates.13,14 The increase in HER2 FISH positivity was noted irrespective of IHC results by Shah et al,10 which was broadly distributed across all 139 referring institutions.19 Implementation of the 2013 ASCO/CAP guidelines resulted in an increase in equivocal cases, which are likely handled differently at different centers. In primary testing laboratories, retesting of a different block with IHC and/or FISH may be the testing of choice. At reference laboratories, the best additional testing has been alternate probes in the absence of information on availability of another tumor block or prior IHC testing at the referring institutions. However, because of the lack of guidelines for reporting HER2 status with the alternate probes, these laboratories face challenges in reporting the results. There might also be differences in positivity rates due to reporting bias from larger centers and reference laboratories.

The 2013 ASCO/CAP guidelines resulted in an increase in the number of positive as well as equivocal cases, which require additional testing with an associated cost and increased turnaround times. The National Surgical Adjuvant Breast and Bowel Project B-47 trial may provide information about the benefits of targeted therapies to low HER2 expressers, but currently it is not known if patients with newly identified, all low-level amplified HER2-positive tumors will equally benefit from targeted therapies.

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References


