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Context.—Human epidermal growth factor receptor 2 (HER2/neu) amplification is used as a predictive marker for trastuzumab treatment in breast cancer. Both immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) testing algorithms have been based on the 2007 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines. In late 2013, the guidelines were updated with new scoring criteria.

Objective.—To assess the impact of the revised ASCO/CAP recommendations on both IHC and FISH results by using the dual-color HER2/neu and centromeric FISH probes.

Design.—Retrospective analysis of 590 invasive carcinomas with concurrent IHC and dual-color HER2/neu and centromeric 17 (CEP17) FISH results, based on 2007 ASCO/CAP guidelines, was conducted from July 2011 to June 2013. With the revised guidelines, patients were recategorized and concordance rates between the 2 assays were recalculated.

Results.—Overall concordance rates for FISH and IHC decreased from 94.9% to 93.8% with reclassification. Negative FISH cases decreased from 79.1% to 69.3%. However, equivocal FISH cases were significantly increased from 0.7% to 9.5%, leading to more retesting. Both positive IHC and FISH cases were also noted to be increased, leading to more patients being eligible for trastuzumab treatment, especially those patients with concurrent HER2/neu and CEP17 polysomy. Approximately 1% of patients with initial FISH negative results were reclassified as having positive results when both the ratios and average copy number of HER2/neu were considered under the revised guidelines.

Conclusions.—The revised 2013 ASCO/CAP guidelines can potentially lead to more patients being eligible for trastuzumab therapy but additional retesting is to be expected owing to an increased number of equivocal FISH cases.


The human epidermal growth factor receptor 2 oncogene (HER2/neu) is amplified in approximately 20% of human breast cancers, leading to overexpression of a 185-kDa glycoprotein with tyrosine kinase activity. Both HER2/neu amplification and overexpression result in an aggressive clinical phenotype and reduced disease-free survival irrespective of nodal involvement. Trastuzumab, a humanized monoclonal antibody, was approved by the US Food and Drug Administration (FDA) for the treatment of HER2/neu-positive breast cancer in both adjuvant and metastatic settings and it is associated with improved response and progression-free survival.

The use of trastuzumab in combination with or after adjuvant chemotherapy has shown improved progression-free survival and overall survival. Several first-generation trials have confirmed overall survival benefit with 1 year of trastuzumab administration. As HER2/neu-targeted drugs such as lapatinib and pertuzumab offer no clinical benefit for patients with HER2/neu-negative metastatic breast cancer, and the toxicities of such drugs warrant accurate HER2/neu testing, it becomes imperative that the right patients be identified to receive the appropriate treatment. Thus, accurate assessment of HER2/neu amplification is important owing to its clinical utility as a predictive marker for trastuzumab response in breast cancer. Currently, immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and bright-field in situ hybridization assays are approved by the FDA for HER2/neu testing, but...
newer diagnostic techniques such as messenger RNA or DNA microarray assays are excluded owing to insufficient evidence to support their use in unselected patients. In 2007, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) jointly developed guidelines for laboratory evaluation of HER2/neu status. An algorithm that defined positive, equivocal, and negative values for HER2/neu protein expression and gene amplification was recommended. Under the 2007 guidelines, a positive HER2/neu test result was defined as IHC staining of 3+ (uniform, intense staining of >30% of invasive tumor cells) or FISH HER2/neu to centromeric 17 (CEP17) signal ratio greater than 2.2. A negative HER2/neu test result was determined as IHC staining of 0 or 1+ or FISH ratio below 1.8. An equivocal HER2/neu test result was IHC staining of 2+ (less than strong, complete membrane staining in at least 10% of cells) or 3+ in 30% of cells or less, or FISH HER2/neu to CEP17 signal ratio of 1.8 to 2.2. Unusual HER2/neu genotypic abnormalities such as chromosome 17 aneusomy and genomic heterogeneity are identified, but such aberrations are not adequately addressed and this may affect the classification.

An Update Committee convened by ASCO and CAP conducted a systematic literature review and revised the guideline recommendations for optimal HER2/neu testing. The testing algorithms for both IHC and in situ hybridization HER2/neu assays were updated in the 2013 recommendations. Guidelines were provided for preanalytic and analytic test performance parameters as well as postanalytic interpretation of results. In addition, polysomy 17 and heterogeneity have now been addressed in the 2013 guidelines. Although the updated guidelines aimed to improve the accuracy of HER2/neu testing and its clinical utility as a predictive marker, the implications of the redefined scoring criteria on HER2/neu gene testing in routine diagnostic practice and the impact of the change in interpretation criteria need to be further assessed.

The aim of this retrospective study was to determine the potential impact of the revised 2013 HER2/neu testing recommendations on previous routine HER2/neu clinical assays at a tertiary care center.

### MATERIALS AND METHODS

#### Patient Population

Between July 2011 and June 2013 (inclusive), a total of 853 consecutive HER2/neu FISH assays were performed on invasive breast carcinomas in Singapore General Hospital (SGH). The study population included cases that were referred directly to the cytogenetics laboratory from other institutions for FISH assays (with either known HER2/neu IHC or no known record of HER2/neu IHC done) as well as in-house SGH cases. In our institution, a total of 1783 HER2/neu IHC cases were performed with 426 cases (23.9%) showing equivocal HER2/neu IHC results. At our institution, HER2/neu status is routinely tested on all invasive breast carcinomas by IHC, with borderline/equivocal results requiring reflex FISH testing for confirmation of amplification status. For this study, cases without both IHC and FISH results were excluded for analysis and a final total of 590 cases with known IHC and FISH results were included. These cases represented all the cases that were tested by both IHC and FISH HER2/neu methods.

FISH testing was carried out for 512 equivocal IHC results (86.8%), 49 negative IHC results (8.3%), and 29 positive IHC results (4.9%), based on the 2007 ASCO/CAP IHC guidelines. FISH testing that was performed on both negative IHC and positive IHC results was ordered by physicians from other institutions for various reasons. Both FISH and IHC assays were performed in the routine clinical setting.

#### Immunohistochemistry

For all IHC samples performed at SGH, the cold ischemic time for tissue fixation was within an hour. The tissue specimens were fixed in 10% neutral buffered formalin solution between 6 and 72 hours and embedded in paraffin. Sections of 4 μm were cut and immunohistochemical staining was performed with the Ventana autoimmunostainer (Roche Diagnostics, Tucson, Arizona) by using HER2/neu antibody to 1:200 (SP3 rabbit monoclonal antibody, RM-9103-R7, Thermo Fisher Scientific, Fremont, California). The intensity (0, 1+, 2+, and 3+ indicating nil, mild, moderate, and marked intensity, respectively) and the proportion of tumor cells stained were assessed on cytoplasmic membrane staining. All HER2/neu IHC results were interpreted and reviewed by pathologists.

Based on the 2007 guidelines, a positive IHC test result is defined as more than 30% of tumor cells exhibiting 3+ uniform intense circumferential membrane staining, while an equivocal IHC result includes samples with at least 10% of tumor cells with 2+ cytoplasmic membrane staining or with 30% of cells or less

### Table 1. Correlation Between HER2/neu Immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH) Results Using 2007 Guidelines

<table>
<thead>
<tr>
<th>IHC</th>
<th>FISH, No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>47 (8.0)</td>
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<tr>
<td>Equivocal</td>
<td>418 (70.8)</td>
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<tr>
<td>Positive</td>
<td>2 (0.3)</td>
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<td>Total</td>
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### Table 2. Correlation Between HER2/neu Immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH) Results Using 2013 Guidelines

<table>
<thead>
<tr>
<th>IHC</th>
<th>FISH, No. (%)</th>
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<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>41 (6.9)</td>
</tr>
<tr>
<td>Equivocal</td>
<td>365 (61.9)</td>
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<tr>
<td>Positive</td>
<td>3 (0.5)</td>
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<tr>
<td>Total</td>
<td>409 (69.3)</td>
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Figure 1. A, Immunohistochemistry for HER2/neu on a cell block of malignant cells from a right pleural tap showing 20% of tumor cells to demonstrate 3+ circumferential membrane staining, which would be considered as positive with the updated guidelines. B, On fluorescence in situ hybridization, the ratio was 1.1, which was not amplified (original magnifications ×200 [A] and ×1000 [B]).

Figure 2. A, Recurrent invasive breast carcinoma on the right chest wall, originally interpreted as HER2/neu positive (3+) on immunohistochemistry (IHC). On review, the intensity of cytoplasmic membrane staining of the tumor cells was 2+, thereby indicating an equivocal (2+) IHC result, with consequent reflex fluorescence in situ hybridization testing with a ratio of 1.1 (nonamplified) (B) (original magnifications ×100 [A] and ×1000 [B]).
showing 3+ membrane staining. A test result is considered negative when the results fail to fulfill the above criteria. The IHC results were compared with the FISH results on the basis of the 2007 guidelines.

The updated 2013 recommendations revised the positive IHC criteria to greater than 10% of lesional cells exhibiting 3+ uniform intense membrane staining. An equivocal result is given when more than 10% of tumor cells show 2+ cytoplasmic membrane staining. Results that fail to fulfill the above criteria are considered negative. For this study, the IHC results were recategorized according to the revised 2013 criteria and compared with the FISH results on the basis of the 2013 guidelines.

**Fluorescence In Situ Hybridization**

Freshly cut formalin-fixed, paraffin-embedded sections were baked at 56°C overnight and the slides were deparaffinized. After treatment with sodium thiocyanate and protease digestion, the slides were dehydrated in an alcohol series. The PathVysion LSI HER2/neu SpectrumOrange and CEP 17 (centromeric 17) SpectrumGreen DNA probes (Abbott Molecular, Abbott Park, Illinois) were applied to the invasive carcinoma areas marked out by the pathologists and codenatured, followed by overnight hybridization. Washes were performed and the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) anti-fade solution (Vectorshield, Vector Laboratories, Burlingame, California) and analyzed under an epifluorescence microscope. Two technologists scored every HER2/neu FISH case independently, with each technologist scoring 30 nuclei on areas of invasive tumor that were delineated by pathologists.

Signals from 60 nonoverlapping nuclei in representative fields were enumerated for the copy numbers of HER2/neu and CEP17 and the results were scored as the ratio of HER2/neu to CEP17 signal numbers. On the basis of the 2007 guidelines, amplification of the HER2/neu gene was defined as a ratio of HER2/neu to CEP17 greater than 2.2, ratios between 1.8 and 2.2 were considered as equivocal, and ratios less than 1.8 were interpreted as negative. According to the updated 2013 ASCO/CAP guideline recommendations, positive HER2/neu results were defined as ratios of at least 2.0, or ratios less than 2.0 with an average HER2/neu copy number of 6.0 signals or more per cell; equivocal results were considered as ratios less than 2.0 with an average HER2/neu copy number of at least 4.0 but less than 6.0 signals per cell; and negative results were defined as ratios less than 2.0 with an average HER2/neu copy number of less than 4.0 signals per cell. FISH results were then reclassified according to the new 2013 guidelines and compared with IHC results, also based on 2013 criteria.

**Statistical Analysis**

Overall concordance, as well as negative and positive assay concordance, was calculated from previously described formulas.6,16 Cohen's κ agreement coefficients (κ) and χ² were calculated by using the Excel 2010 (Microsoft Corp, Redmond, Washington) program. Means for each group were compared by using t test, and P < .05 was considered significant.

**RESULTS**

Of a total of 590 invasive carcinomas, which were evaluated for HER2/neu FISH tests after initial IHC testing, 4 cases were equivocal by both IHC and FISH. While retesting on another tumor block is possible, owing to cost issues, this is not routinely performed in our laboratory. A further 4 cases showed discordant IHC/FISH results, including 2 cases with negative IHC but positive FISH results and 2 cases with positive IHC but negative FISH results (Table 1). By excluding cases with equivocal immunohistochemical or FISH results, the overall concordance was 94.9% (74 of 78) and the κ test between IHC and FISH was good, namely, 0.89 (95% confidence interval [CI], 0.78–0.99). Negative assay concordance (concordance for immunohistochemically negative cases) was higher than the positive assay concordance (95.9% [47 of 49] versus 93.1% [27 of 29], respectively) (Table 1). No equivocal FISH case was observed among the negative IHC and positive IHC cases.

By recategorizing the IHC and FISH findings according to the revised 2013 ASCO/CAP recommendations (Table 2), the number of discordant IHC/FISH cases was slightly increased from 4 to 5 cases. The additional discordant case showed strong membrane staining in 20% of cells and was therefore revised from equivocal to positive IHC finding, but the FISH result was negative. By excluding cases with equivocal immunohistochemical or FISH results, the overall concordance decreased to 93.8% (75 of 80) and the κ test was still relatively good at 0.87 (95% CI, 0.77–0.98). Negative IHC assay concordance was observed to be higher than positive assay concordance (95.3% [41 of 43] versus 91.9% [34 of 37], respectively).

The IHC results of the discordant cases were retrospectively reviewed to address the discordance. Two of these negative IHC/positive FISH cases were referred from private institutions for which the original IHC material was not available. As such, we were not able to review the IHC results. For these 2 cases, the average copy number of HER2/neu was 5.9 and 13.5, while the average copy number of CEP17 was 2.2 with respective ratios of 2.7 and 6.1. Slides from the 3 positive IHC/negative FISH SGH cases were retrieved and reviewed. One of the discordant cases, which was interpreted as IHC 3+ 20%, was done on a cell block from a pleural tap. On further evaluation, no change was made to the IHC interpretation (Figure 1, A). The FISH ratio was 1.1 with an average HER2/neu copy number of 2.8 per cell (Figure 1, B). The second false-positive IHC case was originally interpreted as 3+ membrane staining intensity in 60% to 70% of tumor cells. On review, the IHC staining intensity was regarded as 2+, implying an equivocal IHC result (Figure 2, A). The FISH result showed a ratio of 1.1 with an average HER2/neu copy number of 2.4 (Figure 2, B). The third positive IHC/negative FISH discordant case was initially interpreted as 3+ membrane staining intensity in 60% to 70% of tumor cells. On review, the overall concordance rate after further review was increased to 96.2% (75 of 78).

Among the equivocal IHC cases according to 2007 guidelines, 17.6% (90 of 512) of cases were positive for FISH (Table 1). With the reclassification of the testing criteria, the percentage of positive FISH cases was 17.7% (89 of 503) (Table 2). As for negative FISH cases among the equivocal IHC cases, there were 418 of 512 cases (81.6%) when using the 2007 guidelines as compared to 365 of 503 cases (72.6%) with the new classification. The number of

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**Figure 3.** A, Immunohistochemistry (IHC) for HER2/neu on a left supraclavicular lymph node that disclosed metastatic breast carcinoma. HER2/neu IHC was originally interpreted as 3+ (30% to 40% of tumor cells staining with 3+ intensity). On review however, the intensity of staining was regarded as 2+, indicating an equivocal result. B, Fluorescence in situ hybridization for this case was nonsampled with a ratio of 1.1 (original magnifications ×40 [A] and ×1000 [B]).
cases with dual equivocal IHC and FISH results increased from 4 of 590 cases (0.7%) to 49 of 590 cases (8.3%) with implementation of the 2013 recommendations. As for negative FISH cases, the number of such cases decreased from 467 of 590 (79.1%) to 409 of 590 (69.3%) with the new guidelines.

With the revised 2013 ASCO/CAP recommendations, there was an increment of 9 cases for positive IHC cases from 29 of 590 (4.9%) to 38 of 590 cases (6.4%), although this finding was not significant ($P = .26$) (Table 3). The number of equivocal IHC cases decreased from 512 of 590 (86.8%) to 503 of 590 cases (85.3%) ($P = .45$).

With the revised 2013 recommendations, there was a decrease in the number of negative FISH cases from 467 of 590 (79.1%) to 409 of 590 (69.3%) ($P < .001$) (Table 4). There was an increase in the number of positive FISH cases from 119 of 590 (20.2%) to 125 of 590 (21.2%), although this finding was not significant ($P = .67$). However, the number of equivocal FISH cases increased significantly from just 4 of 590 cases (0.7%) to 56 of 590 (9.5%) ($P < .001$).

Among the FISH cases, when using the 2007 scoring criteria, the average $HER2/neu$ to $CEP17$ ratio was approximating $1.36 \pm 0.75$ in the negative IHC group, while equivocal IHC cases showed a higher ratio of $1.63 \pm 1.04$ ($P = .02$) (Table 5). As expected, the ratio was the highest in the positive IHC group ($5.29 \pm 2.71$, $P < .001$). A similar trend was observed for average copy number of $HER2/neu$ signals with statistical significance seen among the IHC groups. No statistical significance was observed for average copy number of $CEP17$ signals among the IHC groups although there was a slight increase in the average copy number of $CEP17$ for negative IHC compared to positive IHC groups.

Based on the 2013 guidelines, the average $HER2/neu$ to $CEP17$ ratio was $1.36 \pm 0.75$ in the negative IHC group (Table 6). In comparison, the ratio in the equivocal IHC group was higher ($1.59 \pm 1.00$, $P = .02$) and the highest ratio was demonstrated in the positive IHC cases ($4.86 \pm 2.59$, $P < .001$). Increasing $HER2/neu$ copy numbers were observed from negative IHC to positive IHC groups, and statistical significances of $P < .001$ were seen among the groups except for negative IHC compared to equivocal IHC groups where $P = .005$. As for $CEP17$ copy numbers, no statistical significances were observed among the IHC groups although a slight increase in the copy numbers was seen from negative IHC to positive IHC cases. When the $HER2/neu$ and $CEP17$ copy numbers as well as the ratio of $HER2/neu$ to $CEP17$ signals were compared between the IHC 2007 and IHC 2013 groups (Tables 5 and 6), no statistical differences among the IHC groups were detected.

Table 7 shows the FISH results, using both the 2007 and 2013 guidelines, independently of IHC results. When comparing the $HER2/neu$ ratios and the $HER2/neu$ and $CEP17$ copy numbers between the 2 guidelines, the values for all the variables were found to be lower with the revised 2013 recommendations except for $CEP17$ copy numbers in the equivocal and positive FISH categories, which demonstrated a slight increase ($P = .05$ and $P = .23$, respectively).

A total of 54 FISH cases, which were classified as negative on the basis of the 2007 guidelines, were reclassified as equivocal with the revised 2013 recommendations (Table 8). Increased copy numbers of $HER2/neu$ and $CEP17$ signals as well as ratios were noted with the reclassification ($P < .001$). In this cohort, 87% (47 of 54 cases) showed IHC equivocal results, 11.1% (6 of 54 cases) showed negative IHC results, and 1 case had positive IHC result when based on 2013 testing guidelines. Therefore, only 1 IHC 3+ 25% case was reclassified from equivocal IHC to positive IHC case with the revised 2013 recommendations. A total of 5 negative FISH cases were reclassified as positive on the basis of the 2013 guidelines, and the IHC scores were equivocal for all the cases with both 2007 and 2013 testing guidelines.

COMMENT

Accurate $HER2/neu$ assessment for clinical decision making for patients with invasive breast carcinoma is integral to patient care. The ASCO/CAP 2007 guidelines recommended that laboratories performing $HER2/neu$ IHC studies ensure that there is high concordance, typically 95%, with another validated assay. In our review of 590 cases, the overall concordance achieved for combined positive and negative cases was 94.9% (74 of 78), which closely conformed to the ASCO/CAP requirements. Recategorizing the IHC and FISH results, using the revised 2013 guidelines, resulted in a decrease in the overall concordance rate to 93.8% (75 of 80). Negative assay concordance was found to be higher than positive concordance with both 2007 and 2013 guidelines. As this is a retrospective study of clinical cases, the samples consisted mainly of equivocal IHC cases that underwent reflex FISH assays. Thus, the concordance rate derived from this study may not be reflective of the true rate between IHC and FISH. This is a main limitation of our study and subjecting all IHC cases for FISH would give the true concordance rate.

Possible causes for the discordance between IHC and FISH include analytic and postanalytic technical issues such as sample handling (eg, delayed or prolonged fixation, method of tissue processing, and type of fixative$^{14}$), $HER2/neu$ antibody reagent limitations and different antibodies used,$^{15}$ limited assay sensitivity or specificity, interpretative errors, and intrinsic biological properties such as true protein overexpression without gene amplification.$^{16}$

Slamon et al$^{15}$ reported that breast cancer cases with gene amplification and overexpression may lose membrane staining after paraffin embedding. Poor standardization of fixative methods can lead to loss of antigenicity, resulting in false-negative IHC results. Reduction of $HER2/neu$ immunoreactivity in paraffin sections may also be due to prolonged fixation, although some studies have shown that long fixation period beyond 96 hours, which is beyond the ASCO/CAP recommendation of 6 to 72 hours, does not
The impact of 2013 ASCO/CAP guidelines resulted in underscoring the sensitivity to cold ischemic time than CEP17 and this may affect biomarker results. Times greater than 1 hour negatively affected breast cancer fixation may affect the IHC and FISH results, and ischemic reflex FISH testing. Equivocal IHC cases would result in fewer cases undergoing the revised 2013 guidelines, the reduction in the number of negative IHC and FISH tests. While 2 of the false-positive IHC cases were regarded as interpretive in nature, the third case of false-positive IHC was discovered in a cell block that had been prepared from cytologic material (pleural fluid). It is possible that initial handling of the fluid and the process of conversion to cell block may have contributed to the discordance. A study of immunohistochemically determined receptor status on cell blocks and histologic sections of breast cancer found a concordance rate of 90% for HER2/neu results. Interestingly, a more recent report indicated reliability of HER2/neu assessment by dual in situ hybridization on thrombin and formalin cell block material of breast cancers, compared with IHC and FISH on histologic specimens.

FISH assays, which are less dependent on tissue fixation methods than IHC tests, are thought to be more reproducible, and their strong correlation with response to trastuzumab treatment has led some authors to suggest that FISH should be used as the primary HER2/neu test. Similarly to some European countries, IHC testing remains the primary HER2/neu screening test at our center with equivocal IHC cases undergoing reflex FISH testing. With the revised 2013 guidelines, the reduction in the number of equivocal IHC cases would result in fewer cases undergoing reflex FISH testing.

A study by Khoury et al showed that a delay to formalin fixation may affect the IHC and FISH results, and ischemic times greater than 1 hour negatively affected breast cancer biomarker results. HER2/neu signals were found to be more sensitive to cold ischemic time than CEP17 and this may result in underscoring the HER2/neu to CEP17 ratio. Cold ischemic time exceeding 3 hours can cause significant FISH signal intensity degradation, leading to false-negative FISH results. Thus, proper handling of specimens is crucial to optimal IHC and FISH testing. In our cohort, only 0.3% (2 of 590) showed IHC positivity but had FISH negativity (Table 1). The lower incidence of false-negative FISH results may be attributed to the robustness of HER2/neu DNA to tissue alterations caused by preanalytic processes as compared to HER2/neu protein.

Another contributing factor to false-negative FISH results includes intratumoral genetic heterogeneity. Genetic heterogeneity is thought to be either rare or underestimated in breast carcinomas and its presence can lead to genetic instability of subclonal cell population and genetic diversity. Since HER2/neu-amplified cells can occur as scattered cells or focal amplified regions, any intraobserver variability can lead to interpretive inconsistency. Thus, the most intense IHC-stained tumor areas should be marked out by the pathologists for FISH testing if heterogeneous IHC staining is encountered, a practice followed at our institution. Areas such as ductal carcinoma in situ and necrotic, sclerotic, and hemorrhagic areas should be avoided, and FISH testing should only be carried out on the delineated invasive tumor areas.

On the other hand, IHC/FISH discrepancies, especially false-positive IHC results, were thought likely to be due to an interpretive error rather than a technical error. Concurring with this observation, the positive concordance in our study was noted to be slightly lower than the negative concordance. Further review of our positive IHC but negative FISH cases indeed revealed that 2 of the 3 cases were due to interpretation of 2+ intensity of IHC circumferential staining as 3+ intense staining (Figures 2A, A, and 3A). Thus, strict adherence to the ASCO/CAP interpretative criteria for positive IHC staining can help to reduce such discrepancies.

Under the new 2013 ASCO/CAP guidelines, the percentage of tumor cells showing IHC 3+ staining was revised from more than 30% to more than 10%. In our study, 9 cases showing IHC 3+ between 10% to 30% would be reclassified to positive IHC status from an initial equivocal status. Among this cohort, at least 7 cases had positive HER2/neu FISH results, which rendered patients eligible for trastuzumab therapy. Although the stricter criteria of 30%
numbers with higher HER2/neu membranous immunoreactivity. Thus, the rationale for reverting to just more than 10% is to allow this small cohort of patients to qualify for trastuzumab adjuvant trials. Consequently, the positive IHC cases in our study increased from 4.9% (29 of 590) to 6.4% (38 of 590) (Table 3), implying more patients could benefit from the targeted therapy. Indeed, only 1 case had negative results for HER2/neu FISH despite IHC 3+ with 20% immunoreactivity. This discordant case could require further FISH or IHC retesting on a new specimen.

The close association between HER2/neu gene amplification and protein overexpression in breast carcinomas was evident by the higher FISH ratios as well as HER2/neu copy numbers with higher HER2/neu membranous immunoreactivity as confirmed by other studies (Tables 5 and 6). In addition, most IHC2+ cases were negative for HER2/neu FISH amplification (81.6% (418 of 512; Table 1), which was a finding similar to that of others. However, the number of equivocal IHC/negative FISH cases decreased with the revised 2013 ASCO/CAP guidelines because of an increase in the number of negative FISH cases with an average HER2/neu copy number of at least 4.0 but less than 6.0 that were reclassified to equivocal. In fact, the number of equivocal FISH cases increased from 0.7% (4 of 590) to 8.3% (49 of 590) (Tables 1 and 2), implying more patients could benefit from the targeted therapy. Indeed, only 1 case had negative results for HER2/neu FISH despite IHC 3+ with 20% immunoreactivity. This discordant case could require further FISH or IHC retesting on a new specimen.

Although coamplification of the pericentromeric region of CEP17 is uncommon, such cases were encountered in our study, leading to a ratio below 2.0. At least 54 FISH cases were reclassified from negative to equivocal, as the average HER2/neu copy number was at least 4.0 but not more than 6.0 signals per cell. In addition, 5 cases were reclassified from negative to positive, as the average copy HER2/neu numbers were at least 6.0 (Table 8). A mean of at least 3 CEP17 signals per nucleus is generally used as a common threshold to define elevated CEP17 or polysomy status.

With this definition, 74.6% (44 of 59) of our FISH cases that were reclassified to positive or equivocal from initial negative status demonstrated more than 3 mean copies of CEP17 per nucleus. A concurrent increase in the average HER2 copy numbers was noted, indicating CEP17 coamplification. In fact, CEP17 coamplification may be a prominent cause of IHC/FISH discrepancy. Increased copy numbers of both HER2/neu and CEP17 may be associated with polysomy 17, but the clinical significance of polysomy in negative IHC cases is unknown, and the evidence so far has shown an absence of a relationship between polysomy and HER2/neu protein status or benefit from HER2/neu-targeted therapy. Array-comparative genomic hybridization has shown that true polysomy 17 is rare and elevated CEP17 copy numbers are usually due to focal centromeric amplification or gain of long arm of chromosome 17. Studies have suggested that CEP17 coamplification may be associated with strong protein expression without HER2/neu gene amplification. In our cohort, only 1 case showing IHC 3+ 25% demonstrated coamplification of CEP17 and the HER2/neu FISH (average HER2/neu copy number was 4.4) result was eventually classified as an equivocal FISH case with the revised recommendations. Otherwise, most cases showed equivocal IHC results even though there was a concurrent increase in the CEP17 copy numbers. Indeed, no statistical significance was observed for CEP17 among the IHC groups despite the slight increase in the average copy number of CEP17 signals for negative IHC compared to positive IHC groups (Tables 5 and 6). Therefore, elevated CEP17 signals may not contribute significantly to HER2/neu overexpression as confirmed by other studies. The use of an alternative probe for CEP17 can help to address the issue of polysomy 17 or pericentromeric gain of chromosome 17.

**CONCLUSIONS**

The overall IHC and FISH concordance was slightly reduced with the 2013 revised criteria, with an increase in the number of cases with both equivocal IHC and FISH results. The number of equivocal FISH cases was increased, leading to the need for more retesting. Overall, however, both positive IHC and FISH cases were increased, leading to more patients being eligible for trastuzumab treatment, especially those patients with concurrent HER2/neu and CEP17 polysomy. However, the number of equivocal IHC cases was slightly reduced and this led to fewer cases.

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**Table 7.** HER2/neu/Centromeric (CEP17) Ratios and Average Copy Numbers of HER2/neu and CEP17 Signals of Fluorescence In Situ Hybridization (FISH) Assays Using 2007 and 2013 Guidelines

<table>
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<tr>
<th>FISH Results</th>
<th>2007 Guidelines</th>
<th>2013 Guidelines</th>
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<td></td>
<td>HER2/neu</td>
<td>CEP17</td>
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<tr>
<td>Negative</td>
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<td>2.43 ± 0.46</td>
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<td>Equivocal</td>
<td>4.70 ± 0.73</td>
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<td>Positive</td>
<td>9.79 ± 4.71</td>
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**Table 8.** Fluorescence In Situ Hybridization (FISH) Results of HER2/neu/Centromeric 17 (CEP17) Ratios and Average Copy Numbers of HER2/neu and CEP17 Signals in Cases With a Change in the FISH Status Based on the 2013 Guidelines

<table>
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<th>Changes in FISH Status</th>
<th>HER2/neu</th>
<th>CEP17</th>
<th>Ratio</th>
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<td>Negative to equivocal (n = 54)</td>
<td>4.67 ± 0.51</td>
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<td>Negative to positive (n = 5)</td>
<td>6.22 ± 0.26</td>
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<td>1.60 ± 0.12</td>
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</tbody>
</table>
undergoing reflex HER2/neu FISH testing if IHC was used as the primary HER2/neu screening modality.

References