Comparison of Fluorescence In Situ Hybridization (FISH) and Dual-ISH (DISH) in the Determination of HER2 Status in Breast Cancer

Aaron S. Mansfield, MD,1 William R. Sukov, MD,2 Jeanette E. Eckel-Passow, PhD,3 Yuta Sakai,2 Frank J. Walsh,2 Melissa Lonzo,2 Anne E. Wiktor,2 Ahmet Dogan, MD, PhD,2 and Robert B. Jenkins, MD, PhD2

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Abstract

The determination of HER2 amplification is critical to selecting appropriate patients for HER2 targeted therapy in breast cancer. Dual in situ hybridization (DISH), an alternative to fluorescence in situ hybridization (FISH) and immunohistochemistry, is now available. To compare the FISH and DISH methods, we tested 251 samples enriched for common or difficult-to-assess HER2 anomalies. Seven samples failed DISH testing. There was a 64% (156/244) concordance between FISH and DISH by anomaly (κ = 0.58, 95% confidence interval, 0.51-0.65; P < .0001) and an 83% (203/244) concordance by amplification status (κ = 0.58, 95% confidence interval, 0.47-0.69; P < .0001). DISH resulted in lower estimates of HER2/chromosome 17 ratios than FISH, and many cases that were equivocal with FISH were normal with DISH. DISH did not detect any case with coamplification of HER2 and centromere 17. Using a cohort of difficult specimens, we observed less than 95% concordance between FISH and DISH. DISH may underestimate the HER2/chromosome 17 ratio, or FISH may overestimate this ratio.

Breast cancer is the most common female malignancy,1 with 10% to 20% of these invasive carcinomas demonstrating HER2 protein overexpression or gene amplification.2-4 The appropriate assessment of HER2 expression or amplification status is critical in determining which patients should receive HER2-targeted therapy. The clinical trials that used HER2-targeted therapy in the adjuvant5-8 or metastatic9-13 settings used both immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) to determine HER2 status and to select patients for enrollment and treatment. Since then significantly variable results have been demonstrated among laboratories.14,15 The American Society of Clinical Oncologists and College of American Pathologists (ASCO/CAP) have recommended guidelines for the use of IHC and FISH.16 Although FISH remains the “gold standard” to determine HER2 gene amplification, an alternative to FISH is dual in situ hybridization (DISH), which uses chromogenic HER2 and chromosome 17 probes. Both FISH and DISH use formalin-fixed, paraffin-embedded human breast cancer tissue specimens. DISH has 2 principal advantages over FISH. First, visualization via DISH uses light microscopy; second, the specimens can be archived and retrieved indefinitely. DISH was recently approved by the US Food and Drug Administration (FDA) for use in determining HER2 gene status. Although DISH appears to perform comparably with FISH in breast cancers in which HER2 is normal or highly amplified, these test methods have not been formally compared in specimens exhibiting other common and difficult-to-assess centromere 17 (cen17) or HER2 anomalies. Such specimens represent a substantial proportion of cases encountered in a typical...
cytogenetics or pathology laboratory performing HER2 testing, especially those that perform reflex FISH after IHC detects a 2+ result. Therefore, in the current study, we tested a selected cohort of specimens enriched for cases with common and/or difficult-to-assess centromere 17 and HER2 anomalies. Our goal was to determine the concordance of these methods in the context of such common but complicated clinical scenarios.

**Materials and Methods**

**Patients**

The validated HER2 FISH assay was used to test 7,872 mammary carcinoma samples at Mayo Clinic (Rochester, MN) between January 1, 2010, and December 31, 2011. The distribution of cases was as follows: (1) 2,953 (37.5%) cases with no HER2 or chromosome 17 abnormality; (2) 2,066 (26.2%) cases with a gain of 17 (0.8 < HER2/cen17 ratio <1.3, and gain of both HER2 and cen17); (3) 1,027 (11.8%) cases with duplication of HER2 (HER2/cen17 ratio 1.3-1.8); (4) 1,050 (13.3%) cases with HER2 amplification (HER2/cen17 ratio >2.2); (5) 271 (3.4%) cases with equivocal HER2/cen17 ratio (1.8 ≤ HER2/cen17 ratio <2.2); (6) 219 (2.8%) cases with monosomy 17 (0.8 < HER2/cen17 ratio <1.3, and loss of both HER2 and cen17); (7) 195 (2.5%) cases with heterogeneous HER2 amplification (5%-50% of tumor nuclei with HER2 amplification); (8) 285 (3.6%) cases with HER2 deletion (HER2/cen17 ratio <0.8); and (9) 98 (1.2%) cases with HER2 ratio 1.3 to 2.2 and monosomy 17 centromere.

It should be noted that the proportion of amplified cases is 11%. This proportion is less than the known prevalence of HER2 amplification (~20%) because more than 80% of our practice involves reflex FISH testing of cases with 2+ findings on IHC. The number of cases listed herein is more than 7,872 because of the overlap among some categories (eg, cases with heterogeneous HER2 amplification often have overall ratios less than 2.2).

We selected 251 samples from the aforementioned cohort. This included a “control” group of 50 samples composed of 25 cases without HER2 or centromere 17 abnormalities, and 25 cases with high-level HER2 amplification. The 201 test cases included 52 cases with chromosome 17 aneusomy (gain or monosomy), 50 cases with HER2 deletion, 50 cases with HER2 duplication, and 49 cases with an equivocal result. Cases with HER2 duplication or deletion were sometimes also aneusomic. Two of the high-level amplified cases contained heterogeneous HER2 amplification.

**HER2 Testing Methods**

All testing (FISH and DISH) was performed on serially cut 5-μm paraffin-embedded tissue sections. One slide was stained with H&E and reviewed by a board-certified pathologist (W.R.S.) to identify areas of invasive tumor. For both assays, the H&E-stained slide was used as a template to mark the remaining unstained slides to ensure that only invasive tumor cells were analyzed.

FISH testing was performed using the PathVysion HER2 DNA Probe Assay (Abbott Molecular, Abbott Park, IL) according to previously defined protocols. If the sample could not be analyzed because of weak hybridization signals, overdigestion of the tissue, or hybridization failure, another slide was processed using a clinically validated modified protocol. Samples exhibiting centromere 17 amplification (≥6 centromere 17 signals) were retested with a laboratory-developed probe set using probes directed toward HER2 and D17S122. D17S122 is rarely coamplified when HER2 and centromere 17 are coamplified (unpublished data, April 2012). The HER2/D17S122 probe set allows a more accurate determination of the ratio of HER2 number to overall chromosome 17 copy number when HER2/centromere 17 coamplification is present.

The INFORM HER2 Dual ISH DNA Probe Assay was used on a 5-μm tissue section using the BenchMark XT Staining Platform (Ventana Medical Systems, Tucson, AZ).

All samples were processed following the FDA-approved protocol. Two technologists blindly assessed each sample for both FISH and DISH and scored 30 nuclei for the number of HER2 and centromere 17 signals in each cell. The HER2/cen17 probe signal ratio was determined based on analysis of 60 cells. If the HER2/cen17 ratio was within the equivocal range (1.8-2.2), 2 additional technologists scored 30 cells with the final ratio based on 120 nuclei.

**Statistical Methods**

We used SAS (Cary, NC) statistical software to test for agreement with the κ statistic and create a Bland-Altman (limits of agreement) plot. The 7 samples that failed analysis were excluded from the analysis for agreement. This study was conducted in accord with the ethical standards established by Mayo Clinic.

**Results**

We evaluated the HER2 status for 251 samples using both FISH and DISH. The initial FISH failure rate was 8.4%. Repeat testing of all failed FISH samples was successful. The initial DISH failure rate was 11%; 7 samples (2.8%) failed repeat testing. Therefore, these 7 specimens were excluded from the following comparisons (a total of 244 specimens were included in the study).

The overall concordance between FISH and DISH based on abnormality was 64% (156/244) **Table 1**. Compared with FISH, DISH detected 17 (59%) of 29 cases with HER2...
duplication, 8 (38%) of 21 cases with HER2 duplication and aneusomy, 41 (79%) of 52 cases with HER2 deletion and aneusomy, and 9 (60%) of 15 cases with HER2 deletion. The \( \kappa \) coefficient was 0.58 (95% confidence interval [CI], 0.51-0.65; \( P < .0001 \)) for agreement between FISH and DISH based on abnormality.

When tumors were categorized according to ASCO/CAP criteria\(^{16} \) as amplified, equivocal, or nonamplified, a concordance rate of 83% was found between test modalities Table 2. Fifteen (60%) of 25 tumors characterized as amplified with FISH were also amplified with DISH. However, of the 25 tumors considered HER2 amplified with FISH, DISH classified 7 (28%) as not amplified and 3 (12%) as equivocal. Conversely, of 17 tumors characterized with DISH as HER2 amplified, 15 (88%) were also shown to be amplified with FISH and 2 (12%) were equivocal with FISH. All cases of HER2 amplification with DISH were amplified with FISH. The 2 cases with heterogeneous HER2 amplification with FISH were also found to be heterogeneous with DISH. The \( \kappa \) coefficient was 0.58 (95% CI, 0.47-0.69; \( P < .0001 \)) for agreement between FISH and DISH based on amplification status.

Overall, DISH resulted in lower HER2/centromere 17 ratios than FISH as illustrated by regression analysis Figure 1A. Limits of agreement analysis demonstrated a bias of 0.12 (95% CI, 1.23-1.46) when the differences between FISH and DISH were compared with the average value for each paired result Figure 1B. That is, the HER2/chromosome 17 ratio determined with FISH in the majority of samples was on average 0.12 higher than that of DISH. The agreement between FISH and DISH appeared worse at higher ratios.

Twenty samples required testing using the D17S122 FISH probe because amplification of centromere 17 was detected on initial HER2/D17Z1 FISH analysis Image 1. Using this FISH reflex assay, the HER2 status of all 20 cases was resolved, with 4 cases ultimately demonstrating HER2 amplification. The DISH assay did not detect HER2 amplification in any of these 4 cases. Of the remaining 16 samples that underwent reflex D17S122 testing with FISH, 2 failed DISH testing and results from only 2 of the remaining 14 samples (14%) were concordant with those obtained with FISH.

Our study included 12 specimens with IHC findings of 3+. Eight, two, and one of these specimens were amplified, duplicated, or equivocal, respectively, by FISH. DISH detected 6 of the 8 IHC 3+ FISH-amplified cases.

### Discussion

Despite decades of use, HER2 testing remains controversial.\(^ {17} \) The ASCO/CAP guidelines for HER2 testing recommend a 95% concordance with another validated test for positive and negative assay values.\(^ {16} \) One large randomized

### Table 1
Concordance of FISH and DISH as Classified by Anomaly Type\(^ a \)

<table>
<thead>
<tr>
<th>DISH Anomaly</th>
<th>FISH Amplification</th>
<th>HER2 Equivocal</th>
<th>HER2 Duplication</th>
<th>HER2 Duplication with Cen17 Aneusomy</th>
<th>HER2 Deletion with Cen17 Aneusomy</th>
<th>HER2 Deletion</th>
<th>Normal HER2 and Cen17 Fail</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 amplification</td>
<td>15</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HER2 equivocal</td>
<td>2</td>
<td>21</td>
<td>5</td>
<td>10</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HER2 duplication</td>
<td>0</td>
<td>1</td>
<td>17</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>HER2 duplication with Cen17 aneusomy</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Cen17 aneusomy</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>41</td>
<td>3</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>24</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>HER2 deletion</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>5</td>
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<tr>
<td>Normal HER2 and Cen17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<td>3</td>
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<tr>
<td>Total</td>
<td>17</td>
<td>28</td>
<td>25</td>
<td>28</td>
<td>67</td>
<td>31</td>
<td>11</td>
<td>37</td>
</tr>
</tbody>
</table>

DISH, dual in situ hybridization; FISH, fluorescence in situ hybridization. \(^ a \) See Materials and Methods section for definitions of anomaly types.

### Table 2
Concordance of FISH and DISH HER2 Amplification Status Based on ASCO/CAP Criteria\(^ {16, a} \)

<table>
<thead>
<tr>
<th>DISH</th>
<th>HER2 Amplification</th>
<th>HER2 Equivocal</th>
<th>HER2 Not Amplified</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>15</td>
<td>3</td>
<td>7</td>
<td>25</td>
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<tr>
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<td>21</td>
<td>25</td>
<td>48</td>
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<tr>
<td>HER2 not amplified</td>
<td>0</td>
<td>4</td>
<td>167</td>
<td>171</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>28</td>
<td>199</td>
<td>244</td>
</tr>
</tbody>
</table>

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; DISH, dual in situ hybridization; FISH, fluorescence in situ hybridization. \(^ a \) The cases that failed DISH testing are not included.
A clinical trial demonstrated a high degree of discordance between local and central laboratories performing HER2 IHC and FISH testing. Because IHC is more susceptible to variation in preanalytic procedures, it is thought that FISH is a more reliable method of HER2 analysis and currently represents the gold standard for HER2 gene status assessment. Nonetheless, issues still remain that influence FISH analysis and interpretation. One difficulty that can arise in HER2 FISH analysis is the distinction of invasive tumor nuclei from noninvasive tumor nuclei or non-neoplastic nuclei, because FISH tends to obscure tissue architecture. To reduce misinterpretation of HER2 results arising from this difficulty, the DISH assay was developed to allow probe signal interpretation without destroying histologic features, thus allowing for a more accurate evaluation of the relevant nuclei. DISH also has the advantage of using a standard bright-field microscope rather than needing a fluorescence-capable microscope.

Recent studies have compared the performance of DISH relative to FISH and largely identified a high degree of concordance between the methods. Brugmann et al. in a comparison of FISH and DISH methods in 201 tumors, found that only 5 (2.3%) cases were discordant based on ASCO/CAP criteria, and 2 were critically misclassified. However, it is important to note that the 201 tumors tested represented consecutively obtained samples and consisted primarily of nonamplified tumors (89%) with a smaller subset of amplified tumors (11%) and very few equivocal cases (<1%). Although such a cohort would be useful to assess the performance of an assay on a general population, it does little to assess performance on more complex cases. Indeed, based on IHC results showing 0 to 1+ staining, most of the cases would not have been tested using FISH or DISH in clinical practice. Similarly, another study using the INFORM HER2 silver in situ hybridization assay (Ventana Medical Systems) identified a high degree of correlation between test methods, but suffered because the total number of cases analyzed was small.

The current study compared the performance of FISH and DISH for assessing HER2 status in a group of breast adenocarcinomas enriched for common but difficult HER2 and chromosome 17 anomalies. This subset of tumors represents a substantial proportion of tumors encountered in a typical cytogenetics or pathology laboratory that performs clinical HER2 testing. However, studies comparing the performance of FISH and DISH in such tumors have not been undertaken. We observed a 64% concordance by anomaly type and 83% concordance when categorized by ASCO/CAP amplification.
status. Overall, DISH tended to underestimate the HER2/chromosome 17 ratio relative to FISH. Most significantly, 28% of tumors that would have been considered HER2 amplified with FISH would have been considered normal with DISH. Assuming that FISH is the “gold standard” for assessing HER2 amplification, this observation suggests that a significant number of women would not have been offered HER2-targeted therapy had their samples been tested using DISH instead of FISH. Our findings also suggest that DISH does not reliably detect HER2 amplification when the centromere of chromosome 17 is coamplified.

Our clinical practice is largely (>80%) based on reflex FISH testing of IHC 2+ specimens. Relatively unusual HER2 and chromosome 17 anomalies are likely to be enriched in such populations. Thus, any reflex assay—such as FISH or DISH—will have to be robust enough to correctly classify such specimens.

Our current results (demonstrating that DISH would have misclassified 28% of tumors as nonamplified but which were amplified with FISH) are of concern because these potentially false-negative results would generate a lost treatment opportunity for patients. It should be noted that the current study does not reflect the overall case distribution of a laboratory that tests all high-risk breast cancer cases (e.g., IHC 0, 1+, 2+, and 3+ cases) because the number of cases resulting in misclassification would likely be smaller in such a laboratory. However, our results suggest that a significant subset of tumors would be incorrectly characterized as nonamplified if DISH were the primary means of assessing HER2 gene copy status.

Several factors likely contribute to misclassification of the HER2 gene status relative to DISH testing. The first issue is an underestimation of HER2 and centromere 17 copy number, which is likely because of the simultaneous use of 2 chromogens. Because more signals are present in the nucleus,
the signals have an increased tendency to overlap and obscure one another, making accurate enumeration of each probe more difficult. FISH is not immune to this problem; however, FISH has the advantage that probes can be assessed independently through different fluorescent microscope filters. The second major issue is the inability to conduct reflex testing using alternate methods of enumerating chromosome 17 in cases in which centromere 17 is coamplified with HER2. It was recently shown that almost 45% of 171 cases classified as nonamplified based on a HER2/centromere 17 probe ratio were amplified when an alternative chromosome 17 probe was used. These findings suggest that the centromere of 17 often is coamplified with HER2 and that reflex testing with an alternate chromosome 17 probe is indicated in such cases. At Mayo Clinic, tumors showing 6 or more centromere 17 signals are reflexively tested with a probe that includes D17S122 (which is rarely amplified when the centromere of 17 is amplified). In the current study, we observed only an 11% concordance between FISH and DISH when reflex D17S122 FISH testing was required, and DISH did not detect any of the 4 coamplified HER2 and centromere 17 cases.

In summary, we report herein the performance of DISH relative to FISH in determining HER2 gene status in a group of breast adenocarcinomas enriched for tumors with complex HER2 or centromere 17 abnormalities. We observed an 83% concordance rate between FISH and DISH in determining HER2 amplification status. Importantly, we identified a large proportion of breast carcinomas that showed HER2 amplification with FISH but were classified as nonamplified with DISH. Furthermore, DISH does not reliably detect HER2 amplification when the centromere of chromosome 17 is also coamplified. We conclude that the reported advantages of DISH, such as reduced reporting turnaround time, sample processing time, and increased ease of analysis may not outweigh its apparently high false-negative rate.

From the 1Departments of Oncology and 2Laboratory Medicine and Pathology, and 3Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN.

Address reprint requests to Dr Jenkins: Mayo Clinic, 200 First Street SW, Rochester, MN 55905; rjenkins@mayo.edu.

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