Dual HER2 Gene Protein Assay
Focused Study of Breast Cancers With 2+ Immunohistochemical Expression
Zaibo Li, MD, PhD,1 David J. Dabbs, MD,1 Kristine L. Cooper, MS,2 and Rohit Bhargava, MD1

From the 1Magee-Womens Hospital of University of Pittsburgh Medical Center, Pittsburgh, PA, and 2University of Pittsburgh Cancer Institute Biostatistics Facility, Pittsburgh, PA.

Key Words: HER2 gene; GPA; FISH; HER2 immunohistochemistry; IHC score 2+

ABSTRACT

Objectives: The combined gene protein assay (GPA) can simultaneously assess HER2 gene copy number and protein on a single slide using bright-field microscopy.

Methods: GPA was compared with a fluorescence in situ hybridization (FISH) assay on 50 invasive breast carcinomas with a 2+ score on immunohistochemistry (IHC).

Results: The cases were categorized into positive, equivocal, or negative for HER2 gene amplification using the 2013 American Society of Clinical Oncology/College of American Pathologists criteria. This resulted in 82% agreement (41 of 50) between FISH and GPA. In addition, 25 known IHC 3+ breast carcinomas analyzed by GPA showed protein overexpression and clusters of HER2 gene consistent with unequivocal amplification, and 22 known IHC-negative cases were negative for HER2 gene amplification by GPA.

Conclusions: Although GPA is an alternative to both IHC and FISH, it may be an unnecessary test for IHC 0/1+/3+ cases. The clinical utility of GPA appears to be similar to other in situ hybridization assays (ie, adjudicator of HER2 status for IHC 2+ cases).

Approximately 15% to 20% of breast cancers demonstrate unequivocal HER2 (ERBB2) gene amplification and/or protein overexpression, which enhances downstream signaling of other receptors and promotes proliferation and survival.1-3 Determination of HER2 status by HER2 assays is used as both a prognostic factor in breast cancer and a predictive factor for the effects of HER2-targeted drugs. Anti-HER2 therapy such as trastuzumab, a humanized monoclonal antibody against HER2 protein, is quite effective in HER2-positive tumors and has certainly changed the natural history of HER2-positive tumors in the past 15 years.4 However, the relative ineffectiveness of trastuzumab in HER2-normal tumors along with associated toxicity and cost of therapy requires that HER2 status be determined accurately in every clinical case.

The two common methods approved by the US Food and Drug Administration (FDA) to assess HER2 status in the clinical setting are fluorescence in situ hybridization (FISH) to evaluate HER2 gene amplification and immunohistochemistry (IHC) to detect protein overexpression. Most laboratories use IHC as a screening assay for all breast cancer cases and perform FISH testing on IHC 2+ cases. FISH has become universally accepted as a reference standard in the assessment of HER2 status; however, there are some limitations to FISH assay, mainly related to dark-field fluorescence microscopy and lack of morphologic details. To overcome some of these limitations, the bright-field in situ hybridization (ISH) methods, such as chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH), were developed. A further improvement of the ISH assay, called dual in situ hybridization (DISH), has recently become the favorite alternative to FISH since it evaluates...
both HER2 and chromosome 17 (Chr17) signals on a single slide, and silver ions provide a more discrete signal for HER2 gene compared with CISH, which uses dianobenzidine (DAB) as chromogen.

One of the main advantages of bright-field in situ hybridization (BISH) assays, like IHC, is the ease of assessment of heterogeneity. The issue of assessment of heterogeneity has recently gained more significance, and the 2013 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) HER2 testing Update Committee recently recommended reporting any discrete population of amplified cells more than 10% of the total tumor cell population. The 2013 Update Committee also suggested scanning the entire slide prior to counting and/or using an IHC HER2 test to define areas of potential amplification. Therefore, a simultaneous assessment combining IHC and ISH on one slide would facilitate evaluation and enhance test efficiency, especially for cases with HER2 heterogeneity.

There have been prior attempts to combine the IHC and ISH assay, and the gene protein assay (GPA) described in this study reflects methodologic improvements in such technique with automation of the staining process. The GPA combines the anti-HER2 rabbit monoclonal primary antibody (PATHWAY HER2, clone 4B5; Ventana Medical Systems, Tucson, AZ) for IHC and the HER2 DNA probe cocktail assay (INFORM HER2 Dual ISH; Ventana Medical Systems) for ISH on a single slide. The ratio of the HER2 gene to Chr17 can be obtained in formalin-fixed, paraffin-embedded tissue specimens by light microscopy following staining on the BenchMark XT (Ventana Medical Systems). Few pilot studies comparing GPA with IHC alone and FISH alone have shown concordance exceeding 90% by using tissue microarray (TMA) slides on both breast cancer and gastric cancer cases. However, experience with GPA is limited, and previous studies have included all types of cases and therefore contained a large number of cases with clearly negative or unequivocally positive cases. Therefore, the comparative data on cases in which the ISH assay is clinically needed are quite limited. The main goal of the current study was to compare this new GPA method with standard FISH on the most challenging group of breast cancers (ie, HER2 IHC 2+ cases) to assess the clinical utility of GPA.

**Materials and Methods**

**Cases**

This study included whole tissue sections of 50 invasive breast cancer cases with equivocal HER2 IHC results (IHC score 2+) on clinical testing. FISH and GPA were performed on the same tumor blocks. In addition, 25 known HER2 IHC 3+ invasive carcinoma cases (represented on TMA) and 22 known IHC-negative cases (scores 0 and 1+ also represented on TMA) were also evaluated by GPA.

**GPA Staining**

GPA was performed on the BenchMark XT platform (Ventana Medical Systems) according to the manufacturer’s recommendation. The gene and protein detection combines PATHWAY anti-HER2/neu (4B5) rabbit monoclonal primary antibody (Ventana Medical Systems) for IHC and the INFORM HER2 Dual ISH DNA probe cocktail assay (Ventana Medical Systems) for ISH on a single slide. As recommended, HER2 IHC was performed first using the ultraView DAB IHC detection kit (Ventana Medical Systems). Thereafter, hybridization was performed using a cocktail of the 2,4 dinitrophenyl (DNP)–labeled HER2 probe and digoxigenin (DIG)–labeled Chr17. The HER2 gene and Chr17 signals were detected using the ultraView SISH DNP detection kit and ultraView Red ISH DIG detection kit (Ventana Medical Systems), respectively. The HER2 gene signals were detected prior to Chr17 detection. The slides were counterstained with hematoxylin II (Ventana Medical Systems). Signal visualization was performed via light microscopy in which HER2 appears as discrete black signals and Chr17 as red signals in the nuclei, and the HER2 protein shows brown staining in the cell membranes.

**FISH**

At our institution, HER2 FISH is guided by a pathologist at all stages of testing, interpretation, and sign-out, with careful attention to morphologic details. Specifically, the area of invasive carcinoma is circled on an H&E-stained slide by a pathologist. This slide is superimposed on the 4-μm parallel section to be used for FISH, and the FISH slide is etched at the back with the diamond point pencil in the same area where the counts are performed. The signals for HER2 gene and Chr17 are visualized under a fluorescence microscope using appropriate filters. For the current study, the average numbers of HER2 and Chr17 signals per cell were recorded, and the HER2:Chr17 ratio was calculated for each case. FISH analysis was performed using the dual-color Vyysis FDA-approved PathVysion HER2 DNA Probe Kit (Abbott Molecular, Des Plaines, IL). The details of the FISH assay performed in our laboratory have been described previously.

**Interpretation**

GPA slides were interpreted for both HER2 IHC and HER2 ISH. The IHC portion of the GPA was interpreted according to the 2013 HER2 ASCO/CAP guidelines. For the ISH portion of the GPA, the staining results were enumerated by counting at least 20 nuclei by one pathologist (R.B.) blinded to FISH results. Similar to FISH, areas with
stronger IHC staining were counted in heterogeneous cases. Both FISH and GPA-ISH were interpreted using the 2013 HER2 ASCO/CAP updated guidelines.5

Statistical Analysis

Agreement of FISH and GPA quantitative measures was estimated by Pearson correlation coefficient, and comparisons were made by the paired t test for HER2 copy number, Chr17 copy number, and HER2:Chr17 ratio. Agreement of HER2 gene amplification status was estimated by Cohen’s κ, weighted by the severity of the disagreement. Confidence intervals are reported, and all tests are two sided. Statistical analysis was performed using R version 2.15.2 (2012; R Foundation for Statistical Computing, Vienna, Austria).

Results

The HER2 IHC signal on the GPA slide was similar to the original separately performed HER2 IHC. All 22 previously tested HER2 IHC-negative cases represented on TMA were again scored as 0 or 1+ on GPA, and all 25 previously tested HER2 IHC 3+ cases (also represented on TMA) showed intense complete membrane staining (“chicken wire”) on GPA. All 50 previously tested IHC 2+ cases on whole-slide sections (the main focus of this study) were again scored as 2+ on GPA Image 1.

The overall agreement between FISH and GPA with respect to HER2 gene status on IHC 2+ cases was 82% (41 of 50) using the updated 2013 ASCO/CAP HER2 criteria Table 1. Four FISH-equivocal cases were classified as amplified by GPA. Four FISH-negative cases were classified as equivocal by GPA. One FISH-“amplified” case was classified as negative by GPA. This latter case showed a ratio of 2.02 and average HER2 copies of 2.92 by FISH. Most physicians consider such cases negative for amplification in routine clinical practice, but these cases are now categorized as equivocal by the 2013 ASCO/CAP guidelines. Details for all nine discordant cases are provided in Table 2.

For the 50 IHC 2+ cases, the mean HER2 copies per cell, Chr17 signals per cell, and HER2:Chr17 ratios were also compared between GPA and FISH. Correlation estimates and 95% confidence intervals (CIs) between GPA and FISH measurements were 0.91 (0.85-0.95), 0.78 (0.65-0.87), and 0.89 (0.81-0.94) for HER2 copy number Figure 1, Chr17 copy number Figure 2, and HER2:Chr17 ratio Figure 3, respectively. The mean HER2 copy number was slightly higher by GPA compared with FISH (3.51 vs 3.12), with a mean difference of 0.39 (95% CI, 0.17-0.61; P < .001). The mean Chr17 copy number was not significantly different for GPA compared with FISH (2.30 vs 2.21), with a mean difference of 0.09 (95% CI, –0.03 to 0.21; P = .124).

The HER2:Chr17 ratio was slightly higher by GPA than by FISH (1.49 vs 1.39), with a mean difference of 0.10 (95% CI, 0.01-0.19; P = .028).

None of 22 known IHC-negative cases showed HER2 gene amplification on GPA Image 2. All 25 (100%) of IHC 3+ cases showed clusters of the HER2 gene consistent with HER2 amplification by GPA Image 3.

Discussion

Due to its prognostic and predictive value in breast cancer, determination of HER2 status is now standard of care for all newly diagnosed invasive breast cancers. IHC for HER2 protein expression and FISH for determination of HER2 gene status are the preferred assays recommended by the ASCO/CAP.5,9 IHC is relatively inexpensive, with a short turnaround time. Assessment of HER2 protein expression heterogeneity is quite quick and easy with IHC. However, IHC can be affected to a significant degree due to preanalytic, analytic, and even postanalytic (interpretational issue) variables. IHC also results in a relatively higher number of equivocal (ie, IHC score 2+) results.10-17 In contrast, FISH appears to be more quantitative and to have less interobserver variability and a low percentage of equivocal results (~ 5%).10,11,14 However, FISH has a much longer turnaround time (≥3 days) and uses dark-field microscopy, under which the tissue morphologic details and HER2 expression heterogeneity are not well appreciated. Furthermore, the fluorescent signals fade over time; therefore, the slides cannot be stored for a long time. To overcome some of these limitations of the FISH assay, bright-field methods such as CISH, SISH, and DISH have been developed and are now used clinically to assess HER2 gene status.15-20 However, these BISH methods (including the most popular, DISH) still have some limitations such as difficulty in differentiating tumor cells (especially low-grade nuclei) from surrounding stromal cells or lymphocytes, difficulty in recognizing cell boundaries, and background staining such as “silver dust” appearing as the true signal.21,22 Assessment of HER2 heterogeneity is somewhat easier with these BISH assays compared with FISH but still not optimal since slides need to be scanned using a ×40 objective. The 2013 ASCO/CAP HER2 testing Update Committee suggested that cases containing more than 10% of the total tumor cell population with amplified cells should be reported as positive for HER2.5 Since HER2 heterogeneity is much more easily appreciated on IHC, a simultaneous assessment combining IHC and BISH on one slide would facilitate evaluation and enhance test efficiency. Previously, Downs-Kelly et al23 described a bright-field assay called Enz-Met GenePro that simultaneously detected HER2 gene and protein. In the study, Downs-Kelly et al
analyzed 94 cases of invasive breast carcinoma and found the test to be 92.6% accurate. The assay used silver ions for HER2 gene detection and an alkaline phosphate method with fast red as chromogen for protein detection using the CB11 antibody. Chr17 signals were not detected. Despite these excellent results, the assay never became popular. The exact reason for this is not clear but could be related to lack of complete automation in performing the assay, deviation from the HER2:Chr17 ratio method for classification, and using fast red as chromogen for protein detection, which may alter semiquantification of IHC results. Similarly, Ni et al described a HER2 protein and gene double-staining...
Table 2
Details for Nine Discordant Cases Between FISH and GPA Using 2013 ASCO/CAP Criteria for Categorization

<table>
<thead>
<tr>
<th>Case No.</th>
<th>GPA HER2/Cell</th>
<th>FISH HER2/Cell</th>
<th>GPA Chr17/Cell</th>
<th>FISH Chr17/Cell</th>
<th>GPA Ratio</th>
<th>FISH Ratio</th>
<th>GPA Result</th>
<th>FISH Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>7.8</td>
<td>5.0</td>
<td>3.6</td>
<td>3.58</td>
<td>2.16</td>
<td>1.4</td>
<td>Amplified</td>
<td>Equivocal</td>
</tr>
<tr>
<td>29</td>
<td>5.5</td>
<td>5.66</td>
<td>2.35</td>
<td>2.91</td>
<td>2.34</td>
<td>1.95</td>
<td>Amplified</td>
<td>Equivocal</td>
</tr>
<tr>
<td>28</td>
<td>6.1</td>
<td>4.25</td>
<td>3.35</td>
<td>2.72</td>
<td>1.82</td>
<td>1.56</td>
<td>Amplified</td>
<td>Equivocal</td>
</tr>
<tr>
<td>10</td>
<td>5.7</td>
<td>4.5</td>
<td>2.85</td>
<td>3.04</td>
<td>2.0</td>
<td>1.48</td>
<td>Amplified</td>
<td>Equivocal</td>
</tr>
<tr>
<td>34</td>
<td>4.5</td>
<td>3.5</td>
<td>3.3</td>
<td>2.82</td>
<td>1.36</td>
<td>1.24</td>
<td>Equivocal</td>
<td>Not amplified</td>
</tr>
<tr>
<td>33</td>
<td>4.7</td>
<td>3.35</td>
<td>3.5</td>
<td>2.42</td>
<td>1.34</td>
<td>1.38</td>
<td>Equivocal</td>
<td>Not amplified</td>
</tr>
<tr>
<td>22</td>
<td>4.0</td>
<td>2.58</td>
<td>3.07</td>
<td>2.5</td>
<td>1.3</td>
<td>1.03</td>
<td>Equivocal</td>
<td>Not amplified</td>
</tr>
<tr>
<td>9</td>
<td>4.65</td>
<td>3.15</td>
<td>2.65</td>
<td>1.85</td>
<td>1.75</td>
<td>1.7</td>
<td>Equivocal</td>
<td>Not amplified</td>
</tr>
<tr>
<td>44</td>
<td>2.1</td>
<td>2.92</td>
<td>1.4</td>
<td>1.45</td>
<td>1.5</td>
<td>2.02</td>
<td>Not amplified</td>
<td>Amplified</td>
</tr>
</tbody>
</table>

ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists; Chr17, chromosome 17; FISH, fluorescence in situ hybridization; GPA, gene protein assay.

Figure 1
Graphical representation of average HER2 gene copy number by fluorescence in situ hybridization (FISH) and gene protein assay (GPA) in all 50 cases.

(PGDS) assay using the anti-ErbB2 polyclonal antibody A0485 from DAKO (Glostrup, Denmark) and the CISH ErbB2 probe from Zymed Laboratories (South San Francisco, CA). The ISH component of the PGDS was compared with FISH, which revealed an overall concordance of 90% for full face sections (81 cases) and 92% for TMA cases (53 cases). However, the number of IHC 2+ cases included was 15% for full face sections and only 6% for TMA cases. Similar to Downs-Kelley et al,23 Ni et al24 also did not determine the Chr17 copy number in the PGDS assay.

The GPA described in this study combines the anti-HER2 rabbit monoclonal primary antibody (PATHWAY HER2 clone 4B5; Ventana Medical Systems) for IHC and the HER2 Dual ISH DNA probe cocktail assay (INFORM HER2 Dual ISH; Ventana Medical Systems) for ISH on a single slide. Two pilot studies had evaluated this combined GPA in breast and gastric carcinomas using TMA slides.

Nitta et al27 evaluated 183 breast tissue cores on TMA and compared single IHC and single DISH with GPA-IHC and GPA-ISH. Three readers evaluated the slides, and the results showed more than 95% agreement between single assays vs GPA. Hirschmann et al26 evaluated 82 breast carcinomas and 25 gastric carcinomas using TMA. The agreement between FISH and GPA-ISH for both breast and gastric cancers was above 95%. However, both Nitta et al27 and Hirschmann et al26 included all types of cases and therefore had a large number of cases at the extremes (ie, clearly negative or unequivocally positive cases). Nitta et al27 had only 5% (ie, 10 cases) of the total cases that were scored as 2+ on IHC. Therefore, the comparative data on cases in which ISH assay is clinically needed (ie, equivocal IHC 2+ cases) are quite limited.

In the current study, we investigated GPA’s performance with a focus on equivocal IHC 2+ cases. Of the 50 cases tested, categorization by FISH and GPA was similar
and Chr17 signals and to differentiate tumor cells from current FISH vs GPA study. It is much easier to count nevertheless, we used a similar type of cases (IHC 2+) for the cases, and the current study used prospective cases. Nevertheless, many tumor remained within tissue blocks in many cases, and the current study used prospective cases. However, we used a similar type of cases (IHC 2+) for the current FISH vs GPA study. It is much easier to count HER2 and Chr17 signals and to differentiate tumor cells from surrounding stromal cells and lymphocytes based on clear cell boundaries marked by IHC staining on GPA slides than on DISH slides. In addition, “silver dust” artifact, which can sometimes make the DISH slide difficult to interpret, was not seen on any GPA slide. The exact reason for this technical improvement is not known, but this is likely due to the addition of naphthol phosphate in the hybridization buffer (by the manufacturer) during hybridization, which is also supposed to block DNP-DAB interaction. Compared with the FISH method, GPA shows similar accuracy and faster turnaround time (less than 24 hours). Also, there is no need to set up a fluorescent microscope, and no special training is required to interpret GPA slides.

With IHC staining and ISH on one single slide, tumor cell boundaries are clearly noted (especially with 2+ cases), and the intensity of IHC staining can be used to choose an area for counting HER2 gene signals. This is not possible with FISH and DISH. Since the GPA slide combines two tests (IHC and ISH) on one slide, it is reasonable to question how it should be used in clinical practice. Currently, most laboratories use IHC as a screening assay. If the IHC score is 0 or 1+, no further testing is performed, and the HER2 status is considered negative. If the IHC score is 3+, then again no further testing is performed, and
the HER2 status is considered positive. Reflex FISH testing is performed if the IHC score is 2+ and HER2 status is determined based on FISH results. This is a reasonable approach if the laboratory’s IHC-FISH concordance for negatives and positives is high. In our opinion, GPA should be used in a manner similar to the FISH assay in clinical practice (ie, adjudicator of HER2 status when separately performed IHC is interpreted as a score of 2+). This practice can keep costs down, and unnecessary signal counting (in clearly IHC-positive and IHC-negative cases) can be avoided. It should also be noted that the benefit of GPA is minimal for laboratories that target their FISH based on HER2 IHC expression and where the FISH process is tightly controlled by pathologists. However, many laboratories outsource FISH or just perform FISH only without IHC. GPA is particularly beneficial in such a setting where it is helpful in selecting an area for performing the counts. Our study does not argue against the current practice of IHC screening followed by FISH on IHC 2+ cases but rather for making HER2 interpretation easier and faster using a bright-field ISH assay, which is as accurate as the current method.

In summary, this new assay performs accurately not only for unequivocally positive and negative cases but also for the most challenging (IHC 2+) cases. It combines the advantages of both IHC and FISH—that is, accuracy similar to FISH for IHC-equivocal cases, faster turnaround time (less than 24 hours) like IHC, and evaluation under bright-field microscopy where the tissue morphology is well preserved and heterogeneity is better appreciated.

References


