Comparison of Central HER2 Testing With Quantitative Total HER2 Expression and HER2 Homodimer Measurements Using a Novel Proximity-Based Assay

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Abstract

The accuracy and reliability of immunohistochemical analysis and in situ hybridization for the assessment of HER2 status remains a subject of debate. We developed a novel assay (HERmark Breast Cancer Assay, Monogram Biosciences, South San Francisco, CA) that provides precise quantification of total HER2 protein expression (H2T) and HER2 homodimers (H2D) in formalin-fixed, paraffin-embedded tissue specimens. H2T and H2D results of 237 breast cancers were compared with those of immunohistochemical studies and fluorescence in situ hybridization (FISH) centrally performed at the Mayo Clinic, Rochester, MN. H2T described a continuum across a wide dynamic range (~2.5 log). Excluding the equivocal cases, HERmark showed 98% concordance with immunohistochemical studies for positive and negative assay values. For the 94 immunohistochemically equivocal cases, 67% and 39% concordance values were observed between HERmark and FISH for positive and negative assay values, respectively. Polysomy 17 in the absence of HER2 gene amplification did not result in HER2 overexpression as evaluated quantitatively using the HERmark assay.

Gene amplification or protein overexpression of the human epidermal growth factor receptor type 2 (HER2) has been reported in 25% to 30% of invasive breast cancers1,2 and is associated with shorter disease-free and overall survival, lack of responsiveness to tamoxifen antiestrogen therapy,3 and altered responsiveness to a variety of cytotoxic chemotherapy regimens.4-6 Trastuzumab (Herceptin), a recombinant humanized monoclonal anti-HER2 antibody, has been shown to offer significant disease-free and overall survival advantages in metastatic and adjuvant settings in patients with HER2-overexpressing breast cancer.7-12 Given the prognostic and predictive significance of HER2, accurate assessment of HER2 alteration in clinical materials is critical in selecting patients with breast cancer to receive the appropriate treatments, including targeted HER2 therapy.

Current methods for the determination of HER2 status include immunohistochemical analysis to detect HER2 protein overexpression and fluorescence in situ hybridization (FISH) and chromogenic hybridization to detect amplification of the HER2/neu gene. However, considerable controversy still exists regarding the accuracy, reliability, and interobserver variability of these assay methods.13 It is estimated that approximately 20% of HER2 testing performed in the field may be inaccurate when validated against central or “expert” laboratories.14,15 In an effort to improve the accuracy and consistency of HER2 testing, a joint task force of the American Society of Clinical Oncologists (ASCO) and the College of American Pathologists (CAP) recently proposed guideline recommendations for HER2 testing with immunohistochemical analysis and FISH.13
The assessment of HER2 expression by immunohistochemical analysis is inherently subjective and semiquantitative (scored as 0, 1+, 2+, and 3+), whereas the FISH test, in which the HER2 gene copy number is counted, is considered to be more quantitative analytically. However, neither test is a perfect predictor of response to trastuzumab, and both tests are affected by interlaboratory variability. Although FISH is more quantitative, multiple clinical studies have failed to demonstrate a relationship between HER2 gene copy number and response to trastuzumab. There is a need for new approaches to the assessment of HER2 status that will bring about the improved selection of patients likely to benefit from targeted HER2 therapy and the early identification of patients who are unlikely to benefit due to underlying resistance.

We have developed a novel assay (HERmark Breast Cancer Assay, Monogram Biosciences, South San Francisco, CA) that precisely quantifies total HER2 expression (H2T) and HER2 homodimers (H2D) in formalin-fixed, paraffin-embedded (FFPE) tissue samples. The HERmark assay was developed based on a proprietary proximity-based technology platform that enables accurate quantification of proteins and protein-protein complexes through the release of a fluorescent tag (VeraTag reporter, Monogram Biosciences) conjugated to a pair of monoclonal antibodies directed against their respective protein targets in molecular proximity. The aim of this study was to analytically confirm the accuracy of the new assay by comparing H2T and H2D results generated by the HERmark assay with HER2 expression by immunohistochemical analysis and HER2 gene amplification by FISH performed independently at a central laboratory (Mayo Clinic, Rochester, MN) in 237 invasive breast cancers. In addition, we investigated the impact of polysomy 17 on total HER2 protein expression and HER2 homodimers as measured quantitatively by the HERmark assay in 94 immunohistochemical 2+ (equivocal) cases that had reflex FISH results available.

**Materials and Methods**

**Samples**

FFPE tissue blocks from 251 patients with invasive breast cancer were obtained from the archives of the Anatomic Pathology Division, Mayo Clinic, from 1998 to 2006. This study was performed in accordance with institutional and national guidelines and was approved by the Mayo Clinic Institutional Review Board. Of the 251 samples, 11 tissue blocks were determined to have inadequate invasive tumors for the HERmark assay, and 3 samples did not pass the quality control requirements of the HERmark assay. Therefore, 237 samples were adequate for data analysis in this study. Immunohistochemical studies had already been performed at the time of initial diagnosis in all of the cases. Patient tissue specimens were broadly distributed by immunohistochemical staining category (0, 1+, 2+, or 3+). Reflex FISH for HER2 gene amplification had also been performed at the time of initial diagnosis on all 94 of the immunohistochemical 2+ cases. FISH was performed on sections from the same paraffin blocks on which immunohistochemical analysis had been done. Serial 5-μm tissue sections on positively charged glass slides were freshly prepared and sent to Monogram Biosciences for performance of the HERmark assay.

**Immunohistochemical Analysis**

Immunohistochemical tests were repeated for all cases using freshly cut sections from the archival tissue blocks in the Tissue and Cell Molecular Analysis Shared Resource, Mayo Clinic, using the HercepTest (DAKO, Carpinteria, CA) according to the manufacturer’s recommendations. To validate the staining procedure, positive and negative control cell lines were tested in parallel. Benign epithelial cells were also examined. The immunohistochemical staining was scored (B.C.) and categorized as negative, equivocal, or positive according to the ASCO/CAP guideline recommendations for HER2 testing.

**Fluorescence In Situ Hybridization**

HER2 FISH tests were performed on deparaffinized 5-μm tissue sections in the Clinical Cytogenetics Laboratory at the Mayo Clinic using the PathVysion HER2 DNA probe kit and the HER2/cen tromere 17 (HER2/CEP17) probe mixture (Vysis, Downers Grove, IL) as previously described. For each case, a parallel H&E-stained slide was examined for regions of invasive carcinoma by a board-certified pathologist (R.B.J.). The complete tissue section was scanned by 2 certified cytogenetic technologists to detect any subpopulation of amplified cells. Each technologist scored 30 representative nuclei from the invasive tumor (60 nuclei total), with an overall evaluation performed by a board-certified pathologist (R.B.J.). HER2 amplification was defined as an HER2/CEP17 ratio of more than 2.2 according to the ASCO/CAP guideline recommendations for HER2 testing. HER2 amplification in a polysomy 17 background was defined by an HER2/CEP17 ratio of more than 2.2 and 3 or more CEP17 signals in more than 30% of nuclei.

Quality control of the HER2 FISH test is assessed routinely according to standard CAP and the American College of Medical Genetics guidelines. The performance of the assay as assessed on a monthly basis has been stable according to the Westgard rules. Each lot of HER2 probe is tested against normal metaphases and against amplified and nonamplified paraffin-embedded primary breast cancer specimens to ensure that the probe is hybridizing correctly. In
addition, each FISH assay includes a known positive control paraffin-embedded primary breast cancer specimen with HER2 amplification.

HERmark Assay

The HERmark Breast Cancer Assay is an application of the VeraTag technology platform designed specifically for breast cancer and currently includes 2 quantitative measurements: H2T and H2D. VeraTag is a proximity-based method designed to accurately and reproducibly quantitate protein expression and protein-protein complexes, including cell surface dimers, in FFPE specimens. The detailed method of VeraTag platform technology was previously published. Briefly, H2T and H2D are detected through the release of a fluorescent tag (VeraTag reporter) conjugated to a monoclonal antibody directed against the cytoplasmic domain of HER2 (Ab8, LabVision, Fremont, CA). For the H2T assay, this antibody is paired with a biotinylated second antibody directed against the C-terminus of HER2 (Ab15, LabVision) or with biotinylated Ab8 for the H2D assay. The “photosensitizer molecule” (streptavidin-conjugated methylene blue) that is subsequently added and bound to the biotinylated antibody liberates singlet oxygen on illumination with 670-nm light. The release of VeraTag reporter molecules requires molecular proximity of the VeraTag antibody to the HER2 photosensitizer molecule antibody (proximity-based assay). Signal quantified by capillary electrophoresis is normalized to invasive tumor area on the FFPE tissue section. The continuous H2T results are also grouped as HERmark negative, HERmark equivocal, and HERmark positive. The assay has been validated according to the specifications prescribed by the Clinical Laboratory Improvement Amendments and is performed in a CAP-certified clinical reference laboratory at Monogram Biosciences.

Statistical Analyses

Differences in continuous H2T and H2D measurements among various immunohistochemical and FISH subgroups were analyzed by using the Mann-Whitney and Kruskal-Wallis tests. Concordance in immunohistochemical, FISH,
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Results

Continuous H2T and H2D Measurements

The dot plots (Figure 2) describe variable H2T and H2D levels by the HERmark assay and their distributions among the 3 immunohistochemical subgroups (negative, equivocal, and positive) as defined according to the ASCO/CAP guidelines for HER2 testing. For the entire data set, H2T values ranged from 1.5 to 295.7, and H2D values ranged from 0 to 130.7. H2T and H2D were significantly correlated ($r^2 = 0.85; P < .0001$). The H2T and H2D levels of the immunohistochemically negative subgroup ranged from 1.5 to 64.5 (mean ± SD, 5.9 ± 7.2) and 0.0 to 28.5 (mean ± SD, 2.0 ± 3.2), respectively. The H2T and H2D levels of the immunohistochemically equivocal subgroup ranged from 1.5 to 133.6 (mean ± SD, 20.6 ± 21.4) and 0.7 to 66.7 (mean ± SD, 8.7 ± 11.0), respectively. The H2T and H2D levels of the immunohistochemically positive subgroup ranged from 9.7 to 295.7 (mean ± SD, 81.6 ± 65.8) and 2.3 to 130.7 (mean ± SD, 36.0 ± 32.7), respectively. The H2T and H2D levels in the 3 immunohistochemical subgroups were significantly different ($P < .0001$; Kruskal-Wallis test).

Concordance Between H2T and Central Immunohistochemical Studies

The continuous H2T measurements were also categorized as HERmark negative (H2T < 10.5), HERmark equivocal (10.5 ≤ H2T ≤ 17.8), and HERmark positive (H2T > 17.8) as shown in Figure 2A. H2D correlated significantly with H2T and showed a distribution pattern similar to H2T but was not used to define HERmark categories (Figure 2B). The concordance between the HERmark categories and the central immunohistochemical testing categories is shown in Table I. Of the 84 cases in the immunohistochemically negative subgroup, 80 (95%), 2 (2%), and 2 (2%) were classified as negative, equivocal, and positive by HERmark, respectively. Of the 101 cases in the immunohistochemically equivocal subgroup, 53 (52.5%), 2 (2%), and 46 (45.5%) were classified as negative, equivocal, and positive by HERmark, respectively. Of the 52 cases in the immunohistochemically positive subgroup, 3 (6%), 48 (92%) were classified as negative, equivocal, and positive by HERmark, respectively. The overall concordance was 67%, with a weighted $\kappa$ of 63% (95% confidence interval, 55%-70%) calculated using the $\kappa$ statistic.
When the equivocal cases were excluded from the HERmark and immunohistochemical results, the positive and negative concordance between HERmark and central immunohistochemical testing was 98% **Table 2**. The overall concordance was 98%, with a $\kappa$ of 95% (95% confidence interval, 89%-100%).

**Evaluation of Immunohistochemically Equivocal Cases by FISH and HERmark**

Reflex FISH was performed on 94 breast cancers that had been determined as 2+ immunohistochemically at the time of initial diagnosis. Variable H2T and H2D levels were correlated to corresponding results for the HER2/CEP17 ratio **Figure 3**. The HER2/CEP17 ratio showed weak to moderate positive correlation with H2T (Spearman $\rho$, 0.43) and H2D (Spearman $\rho$, 0.30), and the correlations were statistically significant (H2T, $P < .0001$; H2D, $P = .003$; Spearman rank correlation coefficient test). Of the 94 cases that were 2+ immunohistochemically, 62 (66%), 5 (5%), and 27 (29%) were determined at the same central laboratory as negative, equivocal, and positive by FISH, respectively **Figure 4**. Of the 62 FISH-negative cases, 24 (39%), 21 (34%), and 17 (27%) were determined as negative, equivocal, and positive by HERmark, respectively. Of the 5 FISH-equivocal cases, 1 (20%), 2 (40%), and 2 (40%) were determined as negative, equivocal, and positive by HERmark, respectively. Of the 27 FISH-positive cases, 3 (11%), 6 (22%), and 18 (67%) were determined as negative, equivocal, and positive by HERmark, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Central IHC</th>
<th>HER2 Negative</th>
<th>Equivocal</th>
<th>Positive</th>
<th>Total IHC</th>
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<tr>
<td>HERmark</td>
<td>80 (95)</td>
<td>33 (32.7)</td>
<td>1 (2)</td>
<td>114</td>
</tr>
<tr>
<td>Equivocal</td>
<td>2 (2)</td>
<td>31 (30.7)</td>
<td>3 (6)</td>
<td>36</td>
</tr>
<tr>
<td>Positive</td>
<td>2 (2)</td>
<td>37 (36.6)</td>
<td>48 (92)</td>
<td>87</td>
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<tr>
<td>Total central IHC</td>
<td>84</td>
<td>101</td>
<td>52</td>
<td>237</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage) except for the totals. Concordance, 67%; 95% confidence interval (CI), 61%-73%. Weighted $\kappa$, 63%; 95% CI, 55%-70%. Concordance was defined as (80 + 31 + 48)/237. IHC was performed at the Mayo Clinic, Rochester, MN; the HERmark assay was performed by Monogram Biosciences, South San Francisco, CA.

**Table 2**

<table>
<thead>
<tr>
<th>Central IHC</th>
<th>HER2 Negative</th>
<th>Equivocal</th>
<th>Positive</th>
<th>Total IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HERmark</td>
<td>80 (98)</td>
<td>1 (2)</td>
<td>81</td>
<td></td>
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<tr>
<td>Positive</td>
<td>2 (2)</td>
<td>48 (98)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Total central IHC</td>
<td>82</td>
<td>49</td>
<td>131</td>
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</tr>
</tbody>
</table>

* Data are given as number (percentage) except for the totals. Concordance, 98%; 95% confidence interval (CI), 95%-100%. Weighted $\kappa$, 95%; 95% CI, 89%-100%. Concordance was defined as (80 + 48)/131. IHC was performed at the Mayo Clinic, Rochester, MN; the HERmark assay was performed by Monogram Biosciences, South San Francisco, CA.

**Figure 3** Correlation between the HER2 gene/chromosome 17 copy number (HER2/CEP17) ratio by fluorescence in situ hybridization and total HER2 expression (H2T) **A** and HER2 homodimers (H2D) **B** by the HERmark assay (Monogram Biosciences, South San Francisco, CA). Spearman rank correlation coefficient was used to describe the relationship between the variables. **A**, Spearman $\rho$, 0.43; $P < .0001$. **B**, Spearman $\rho$, 0.30; $P < .003$. 

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Polysomy 17 was observed in 23 (24%) of the 94 breast cancers scored immunohistochemically as 2+, either on its own (15 of 23 cases) or in combination with HER2 gene amplification (8 of 23 cases). Figure 5 shows variable H2T and H2D levels and their distributions among various FISH and polysomy 17 subgroups. The H2T and H2D levels of the FISH-negative group ranged from 3.4 to 54.1 (mean ± SD, 14.2 ± 9.1) and 1.5 to 27.3 (mean ± SD, 6.2 ± 4.9), respectively. The H2T and H2D levels of the FISH-positive group ranged from 4.0 to 133.6 (mean ± SD, 39.2 ± 33.3) and 0.9 to 66.7 (mean ± SD, 17.2 ± 17.9), respectively. The H2T and H2D levels between the FISH-negative and FISH-positive groups were significantly different (H2T and H2D, P < .001; Mann-Whitney test). Breast cancers with polysomy 17 without HER2 gene amplification (polysomy 17–positive, FISH-negative group, Figure 5) did not show higher H2T or H2D levels compared with those of the polysomy 17–negative, FISH-negative group (H2T, P = .96; H2D, P = .94; Mann-Whitney test). These 2 FISH-negative subgroups were not significantly different in H2T or H2D levels from those of the overall FISH-negative group regardless of polysomy 17 status (H2T, P = .98; H2D, P = .90; Kruskal-Wallis test). On the other hand, the HER2 amplified cancers, with or without polysomy 17, showed significantly higher H2T and H2D levels than cancers without HER2 amplification (H2T and H2D, P < .001; Kruskal-Wallis test).

Figure 4 HERmark (HM; Monogram Biosciences, South San Francisco, CA) results for 94 breast cancers that had been previously determined as immunohistochemically (IHC) equivocal and subsequently evaluated by reflex fluorescence in situ hybridization (FISH). –, negative; –/+ , equivocal; +, positive.

Figure 5 Impact of polysomy 17 on total HER2 expression (H2T) (A) and HER2 homodimers (H2D) (B) by the HERmark assay (HM; Monogram Biosciences, South San Francisco, CA). The median value for each group is indicated by a short red line within the data distribution. FISH (–), HER2 gene not amplified; FISH (+), HER2 gene amplified; polysomy 17 (–), polysomy 17 not displayed; polysomy 17 (+), polysomy 17 displayed. FISH, fluorescence in situ hybridization.
Discussion

The HERmark assay provides precise quantification of total HER2 protein expression and homodimerization in breast cancer samples prepared as FFPE specimens. Generation of the assay signal requires 2 distinct epitope-specific monoclonal antibodies, resulting in significantly better sensitivity and specificity for HER2 measurements than the immunohistochemical method. As a result, HERmark is able to measure a continuous distribution of HER2 expression extending over a broad dynamic range corresponding to approximately 2,500 to 2 million receptors per cell, based on the study of breast cancer cell line controls as previously published. Assays that measure gene copy number or messenger RNA (mRNA) rely on the assumption that the amount of DNA or RNA detected will accurately reflect the amount of protein that is ultimately translated. This is likely an oversimplification given the complexity of the potential regulatory influences involved in the expression of receptor tyrosine kinases like HER2. Because of its ability to measure at the protein level, the HERmark assay can capture the impact of transcriptional and/or translational regulatory events that may influence HER2 protein expression, unlike assays that measure gene copy number or quantitative mRNA.

In this study, we demonstrated a continuum of HER2 expression and homodimerization that extended over approximately a 2.5-log dynamic range in the 237 FFPE breast cancer specimens. Negative and positive concordance values between HERmark and central immunohistochemical studies were high (95% and 92%, respectively), whereas concordance within the equivocal group was low (30.7%). The concordance was excellent (98%) when equivocal cases were excluded from both assays, which meets the ASCO/CAP guidelines requiring that laboratories show at least 95% concordance within the equivocal group was low (30.7%). The concordance was excellent (98%) when equivocal cases were excluded from both assays, which meets the ASCO/CAP guidelines requiring that laboratories show at least 95% concordance with another validated test for positive and negative results. One such factor may be that all of the cases were selected as 2+ immunohistochemically (weak overexpression) to begin with. It is conceivable that HER2 results are inherently more variable in immunohistochemically equivocal cases when the HER2 status is determined by different HER2 testing methods. In addition, the biologic regulation of HER2 expression in tumor cells is complex, and HER2 gene amplification may not always correlate quantitatively with HER2 protein expression, although gene amplification is generally considered the main mechanism of HER2 overexpression in breast cancer.

Understanding these relationships in tumors currently defined as equivocal is very important to correctly assess these cases with HERmark in future studies because the patients are the ones with the greatest need for improvements in HER2 testing. Studies comparing HERmark and FISH results in cases that are scored immunohistochemically as negative (0, 1+), equivocal (2+), and positive (3+) are needed to further investigate this issue.

The increase in HER2 gene copy number in breast cancer can be attributed to HER2 gene amplification (replication of a segment of a chromosome) and chromosome 17 aneuploidy (a change in the number of whole chromosomes). Gene amplification is by far the most common mechanism generating excess HER2 gene copies; however, polysomy 17 is reported to occur in 13% to 46% of breast cancers. It is conceivable that protein overexpression can result not only from the increased number of genes secondary to gene amplification but also as a result of concomitant increased numbers of chromosomes. It is interesting that polysomy 17 is commonly found in breast cancers with weak HER2 protein expression (immunohistochemical score, 2+) without gene amplification. Some immunohistochemically positive breast cancers do not show HER2 gene amplification by FISH. It has been suggested that some of these “false-positive” immunohistochemical results may be due in part to polysomy 17, resulting in increased HER2 protein expression. It remains unclear whether polysomy 17 tumors without HER2 gene amplification should be regarded as HER2+ and, thus, expected to respond to trastuzumab treatment. In this study, we sought to evaluate the impact of polysomy 17 on HER2 protein expression using the more quantitative HERmark assay.

Polysomy 17 was observed in 23 (24%) of the 94 cancers scored immunohistochemically as 2+ in this study, on its own (15/94 [16%]) or in combination with HER2 gene amplification (8/94 [9%]). The distributions of continuous H2T and H2D values were very similar among the FISH-negative subgroups (Figure 5), regardless of polysomy 17 status. On the other hand, H2T and H2D values in the FISH-positive subgroups were significantly higher than those of the FISH-negative subgroups, regardless of the polysomy 17 status. The
results indicate that polysomy 17 does not result in higher HER2 expression or HER2 homodimer values in the absence of HER2 gene amplification. These results are consistent with a recent report by Vanden Bempt et al.27 that polysomy 17 on its own was not associated with increased HER2 mRNA levels by reverse transcription–polymerase chain reaction or HER2 overexpression by immunohistochemical analysis and that polysomy 17 tumors in the absence of HER2 gene amplification resemble HER2– tumors.27 Polyosmy 17 appears biologically and functionally distinct from HER2 gene amplification.30

Although the accuracy of the HERmark assay was confirmed in this comparative study with validated immunohistochemical and FISH tests independently performed at a central laboratory, clinical studies are clearly needed to understand the relationship between quantitative HER2 expression and homodimer measurements with clinical outcomes in patients with breast cancer treated with anti-HER2 therapy. Most of the tumor tissues in this study were procured before US Food and Drug Administration approval of adjuvant trastuzumab therapy for early-stage breast cancer. The limited number of trastuzumab-treated cases in this study does not provide adequate statistical power for sufficient analysis of correlating the HERmark status and patient response to trastuzumab. The relationship between quantitative HER2 expression as measured by HERmark and clinical outcome of patients with metastatic breast cancer treated with trastuzumab-containing therapy was previously published for 2 metastatic breast cancer cohorts.31,32 Recently, Lipton et al.33 reported that quantitative HER2 expression or homodimer levels by the HERmark assay correlated with clinical outcome of trastuzumab therapy better than immunohistochemical or central FISH studies in patients with metastatic breast cancer. In particular, patients with HER2 gene amplification by FISH but low HER2 protein expression or homodimer levels as measured by HERmark responded poorly to trastuzumab-containing therapy, suggesting that not all gene-amplified tumors overexpress the target of trastuzumab.33 Several additional larger clinical studies are ongoing to further confirm and validate the clinical usefulness of the HERmark assay in metastatic and adjuvant breast cancer treatment settings.

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References


