Analysis of Intratumoral Heterogeneity and Amplification Status in Breast Carcinomas With Equivocal (2+) HER-2 Immunostaining

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Abstract

Fluorescence in situ hybridization (FISH) and immunohistochemical analysis for assessment of HER-2 status in breast carcinomas are discordant in a significant proportion of cases with equivocal (2+) immunostaining.

To evaluate the role of intratumoral heterogeneity and degree of amplification, we performed additional HER-2 immunostains and FISH on tumor-bearing blocks from 20 invasive breast carcinomas with immunohistochemical scores of 2+ with gene amplification and in 18 cases without amplification.

Of the amplified cases, 11 (55%) had a 3+ immunohistochemical score on at least 1 additional slide, 8 (40%) remained 2+, and 1 (5%) had a slide scored 1+. All cases rescored 3+ showed high-level amplification in original and repeated FISH; cases remaining 2+ had a heterogeneous FISH profile (low-level amplification or a mosaic mixture of high-level amplified and nonamplified cells) in original and repeated FISH. Of nonamplified cases, 13 (72%) had a 1+ score on at least 1 additional slide, 4 (22%) remained 2+, and 1 (6%) had 1 slide scored 3+. In the nonamplified cases, 17 (94%) showed no amplification in repeated FISH.

Significant intratumoral heterogeneity and minimal (low-level) HER-2 amplification account for many breast cancers with 2+ HER-2 protein expression.

Many laboratories evaluate HER-2 status in breast carcinomas by performing immunohistochemical stains to detect protein overexpression. A number of previous studies have shown that strong (3+) and weak or absent (0-1+) staining predict the presence or absence of HER-2 amplification, respectively, in tumor cells, with 90% to 95% sensitivity and specificity.1-7 Hence, a 3+ and a 0 to 1+ stain result are considered diagnostic from a clinical standpoint. Approximately 15% of breast carcinomas are characterized by moderate protein overexpression; by convention or by HercepTest (DAKO, Carpinteria, CA) kit instructions, these cases are graded as equivocal, or 2+.8-10 Because some but not all of these cases are characterized by amplification of the HER-2 gene, a 2+ result is considered equivocal and, thus, clinically nondiagnostic.6 Most experts would recommend referring 2+ cases for HER-2 gene status evaluation with interphase cytogenetics (fluorescence in situ hybridization [FISH]).11-15

The nature of breast carcinomas with 2+ immunostaining is poorly understood. A large proportion likely constitutes tumors with insufficient gene amplification, such that there is an increase in gene copy number resulting in low-level protein overexpression.16 Alternatively, equivocal immunohistochemical results also might result from processing artifact17,18 or a misinterpretation on the part of the individual examining the slides.19,20 Thus, errors in interpretation potentially could represent an overestimate of “minimal” (ie, 0-1+) staining or an underestimate of “strong” (ie, 3+) staining.

It has been shown that genetic heterogeneity arises in breast carcinomas as they undergo clonal evolution.21 We hypothesize that at least some 2+ immunoreactive breast tumors are heterogeneous in HER-2 gene status. Hence, equivocal expression levels in such cases might represent an
artifact of partial sampling such that a portion of the tumor with HER-2 overexpression or amplification is underrepresented or missed. To test this hypothesis, we performed 1 or more additional HER-2 immunostains and FISH on a series of breast carcinomas with 2+ immunostaining and previously defined amplification status.

Materials and Methods

Case Selection

We searched our surgical pathology files to identify cases of invasive breast carcinoma that initially were scored 2+ by immunohistochemical stains and in which amplification subsequently was detected by FISH. In addition, a negative control group (ie, HER-2 score of 2+ but no amplification by FISH) also was selected. Between June 1999 and September 2003 we identified 22 cases scored 2+ by immunohistochemical analysis that had amplification by FISH. Two of the cases were ovarian carcinomas and, thus, were excluded, resulting in 20 cases for inclusion in the study group. Eighteen cases were chosen as the amplification-negative control series. The clinical and histologic characteristics of both groups were similar.

Immunohistochemical Analysis

Initial and additional immunohistochemical stains were performed using HercepTest on an automated immunostaining system (Autostainer, DAKO). Paraffin sections were cut at 4 µm and mounted on silanized slides (SuperFrost Plus, Fisher Scientific, Pittsburgh, PA). Heat-induced epitope retrieval, using diluted Epitope Retrieval Solution supplied with the HercepTest Kit, was performed in a water bath (40 minutes, 95°C-99°C).

Of the 38 original HER-2 slides, 37 were available for review. Small tumor volumes were present in 5 cases. Of these, 4 were needle core biopsy samples and 1 was from a patient who had received neoadjuvant therapy. Although initial slides had been interpreted by a variety of pathologists, review of the original material and evaluation of additional stains was performed by one of us (D.W.V.) according to the manufacturer’s guidelines for HercepTest, with scoring of 0, 1+, 2+, or 3+. Tumors were considered to have 2+ staining if there was weak to moderate complete membrane staining in greater than 10% of the invasive carcinoma cells. Only membranous staining was considered, and cytoplasmic staining was ignored.

A total of 32 additional stains were performed on additional material from the 20 cases with HER-2 amplification. Seven were done on the same block, 13 on different blocks from the same site, and 12 on tissue samples from a secondary site or different procedure (axillary lymph nodes in 10 cases, previous breast biopsy in 2 cases). There was insufficient invasive tumor in 2 blocks to perform an evaluation, resulting in 30 stains for review. Thirty-four stains were performed on the 18 cases in the negative control group. Sixteen were performed on the same site (ie, breast), and 18 were performed on axillary lymph node metastases. As in the study group, 2 had insufficient residual tumor after trimming, yielding a total of 32 stains for evaluation.

Fluorescence In Situ Hybridization

FISH for HER-2 gene amplification was performed using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL); the technique is described in detail elsewhere. To ensure that the correct area of tissue is evaluated, a pathologist reviews the H&E-stained slide and circles the invasive tumor for scoring. After appropriate processing and hybridization, the number of CEP17 (chromosome 17 centromere–specific probe) and HER-2 signals in each of 60 nuclei from an invasive area of the tumor are counted and averaged together. The mean HER-2/CEP17 signal ratio then is calculated for each tumor. According to the manufacturer’s guidelines, a ratio greater than 2.0 is used as the criterion for amplification. For comparison and validation, FISH studies also were performed on the additional material studied by immunohistochemical analysis.

HER-2 gene amplification is a cyogenetically complex phenomenon. Therefore, for purposes of this study, we further qualified the chromosomal abnormalities to include the degree of HER-2 amplification (high- vs low-level amplification). First, as a general rule whenever there are cells with more than 10...
copies of the *HER-2* gene, high-level amplification is considered to be present regardless of the number of CEP17 signals.  

This nearly always results in a signal ratio of more than 2.0. Second, if significant mosaicism (i.e., a mixture of amplified and nonamplified tumor cells) is present, the tumor will be interpreted as amplified even if the overall ratio is less than 2.0. Third, an interpretation of low-level amplification may be made if a subpopulation of cells exhibits low-level amplification even though the overall signal ratio is greater than 2.0. This situation often occurs when the HER-2 copy number is between 4 and 10 per cell and the CEP17 copy number is 2 or fewer per cell.

### Results

**Immunohistochemical Analysis**

There was a high level of agreement in retrospective review of the original HER-2 immunostains (35/37 [95%]). The 2 discrepant cases were interpreted as having strong enough membranous staining to be classified as 3+. Although most of the cases had homogeneous, moderate immunoreactivity throughout the tumor, a substantial proportion (14/37 [38%]) had heterogeneous expression. These cases were characterized by occasional cells with strong staining directly adjacent to others with minimal to moderate staining in Image 1.

The results from the additional immunohistochemical stains are summarized in Table 2. In the amplified study group, 11 (55%) of 20 cases had a score of 3+ on at least 1 additional sample in Image 2. Three cases (15%) were scored 3+ on all stained sections. Of the 20, 8 (40%) remained 2+ on all additional samples. One was scored 1+ in 1 section but remained 2+ on the other block that was tested.

In the nonamplified control group, 13 (72%) of 18 cases were scored 1+ when at least 1 additional block was stained in Image 3. And 8 of them (44%) were scored 1+ on slides from all additionally stained sections. Four remained at 2+ with staining of additional sections. Only 1 of the 18 changed to 3+, but it remained 2+ on the second block that was retested.

Overall, 55% (34/62) of the immunohistochemical stains performed on the additional block(s) had a score that was different from the original 2+ interpretation. These changes would have altered the interpretations in 68% (26/38) of the cases. A change in score was independent of the tissue site (i.e., breast primary vs axillary metastasis), and none of the cases

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Results of HER-2 Immunohistochemical Staining on Additional Tumor Samples*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>By block, score</strong></td>
<td><strong>Amplified</strong></td>
</tr>
<tr>
<td>0</td>
<td>n = 30</td>
</tr>
<tr>
<td>1+</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2+</td>
<td>1 (3)</td>
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<tr>
<td>3+</td>
<td>14 (47)</td>
</tr>
<tr>
<td><strong>By case</strong></td>
<td>n = 20</td>
</tr>
<tr>
<td>Any score 1+</td>
<td>1 (5)</td>
</tr>
<tr>
<td>All remaining 2+</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Any score 3+</td>
<td>11 (35)</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage) unless otherwise indicated. Any score 1+ cases are those with at least 1 block graded 1+; all remaining 2+, cases with every additional block scored 2+; any score 3+, cases with at least 1 additional block scored 3+. For a description of the scoring, see the text.

**Image 1** Immunohistochemical staining patterns observed in HER-2 2+ cases. A, Diffuse, weak to moderate, complete membrane staining in the majority of tumor cells (HER-2, ×400). B, Heterogeneous staining among the tumor cells, varying from barely perceptible to moderate or strong (HER-2, ×400).
was scored 0 in retesting. In the amplified group, 20 immunohistochemical stains were performed on the same tissue source as the original. Of these, none (0%) were scored 1+, 9 (45%) were scored 2+, and 11 (55%) were scored 3+. Of the 10 stains performed on different sites, 1 (10%) was scored 1+, 5 (50%) were scored 2+, and 4 (40%) were scored 3+. Changes in score also were independent of tissue site in the nonamplified group. Of the 15 immunohistochemical stains performed on blocks from the same site as the original, 8 (53%) were scored 1+, 6 (40%) were scored 2+, and 1 (7%) was scored 3+. Seventeen stains were performed on blocks from axillary lymph nodes, and 9 (53%) were scored 1+, 8 (47%) were scored 2+, and none (0%) were scored 3+.

**Fluorescence In Situ Hybridization**

Table 3 lists the original HER-2/CEP17 signal ratios and cytogenetic interpretations of the amplified cases. The mean ratio in the amplified group was 5.94 (range, 2.04-13.41), compared with a mean ratio in the control group of 1.23 (range, 0.62-1.93). In the amplified study group, the mean ratio of the cases scored 3+ on at least 1 additional immunohistochemical stain was 8.13. Nearly all (10/11 [91%]) of these cases showed high-level amplification by FISH. One was interpreted as a mosaic, being composed of a mixture of amplified and normal cells. The mosaic tumor was the only case with a signal ratio less than 5.0. The mean ratio of cases that remained 2+ with additional immunohistochemical
stains was 3.27, and all but 1 of these cases had a signal ratio less than 5.0. Unlike the cases that had immunohistochemical scores of 3+ on a second block, the majority of the 2+ cases (6/9 [67%]) did not exhibit homogeneous high-level amplification. Five were interpreted as having low-level amplification, and 1 was a mosaic, comprising a mixture of cells with high- and low-level HER-2 amplification.

Image 4 illustrates representative FISH amplification patterns for high- and low-level cases.

FISH also was performed on the additional blocks evaluated by immunohistochemical analysis. Results are given in Table 4. Within the amplified group, all cases (10/10 [100%]) in which additional immunohistochemical stains revealed a change to 3+ were interpreted as having high-level amplification by FISH on the additional block(s). Cases that remained 2+ with additional immunohistochemical stains had a heterogeneous FISH profile. One case showed high-level amplification, and 3 were mosaic, composed of a mixture of cells with high- and low-level amplification. Another case (No. 13) revealed mosaicism in 1 block. The other block (scored 1+ by immunohistochemical testing) had a HER-2/CEP17 ratio of 1.53 with an interpretation of low-level amplification (see the “Discussion” section).

In the nonamplified control group, additional FISH testing revealed no amplification in 17 (94%) of 18 cases. Case 22 showed high-level amplification in 1 block and no amplification in the other. Case 23 was the only one in the negative control group with a block scored 3+ in additional immunohistochemical stains. Although that block showed no amplification based on the 2.0 ratio cutoff used in this study, it was interpreted as having low-level amplification by our cytogenetics laboratory (see the “Discussion” section). As mentioned, the other block from that case remained 2+ and showed no amplification in FISH testing.

Discussion

The purpose of the present study was to characterize the immunophenotypic and cytogenetic features of breast carcinomas with weak (ie, 2+) HER-2 overexpression. Our results show that a high proportion of these cases are characterized by a significant degree of intratumoral heterogeneity with respect to HER-2 protein expression. Overall, 68% of the 2+ cases we studied would have had a different immunohistochemical score if another slide(s) had been examined. This variability was independent of whether the additional stains were performed on samples from the same tissue site as the...
There are conflicting reports in the literature concerning the degree of intratumoral heterogeneity of HER-2 expression among breast carcinomas.8,24-26 To our knowledge, our study is the first to specifically address this issue in 2+, or equivocal, cases. We have observed that staining heterogeneity, within a slide and in different blocks of a tumor, is a common feature of 2+-staining tumors and one that adds to the difficulty with their interpretation. These results support the findings of Wang et al,27 who compared the Automated Cellular Imaging System with manual interpretation of HER-2 immunohistochemical stains. In their study, even the more sensitive automated technique, which quantitates membranous staining, resulted in widespread immunohistochemical scores in the manual 2+ cases.28

Staining heterogeneity in 2+ cases likely has a biologic basis. Even in highly amplified cell lines, there is considerable cell-to-cell variation in HER-2 gene copy number and protein content.28 Furthermore, it has been demonstrated that amplification within a breast carcinoma may be limited to regional subpopulations.29 These impressions were corroborated by our study. Among the amplified cases, 10% exhibited mosaic profiles by FISH in the originally analyzed blocks, with some of the tumor cells showing high-level amplification and others showing low-level or no amplification. This same phenomenon also was seen in the repeated FISH studies. Among the 9 cases that remained 2+ with additional immunohistochemical stains, 7 contained enough residual tumor for repeated FISH testing. Of the 8 FISH studies (1 case had 2 FISH tests) performed, 4 (50%) were interpreted as mosaic. Hence, it is not surprising that such focally amplified clonal populations might be missed or underrepresented in a single, randomly chosen tissue section.

Another interesting feature of HER-2 2+-staining breast carcinomas is that many seem to have “low-level” HER-2 gene amplification by FISH. Previous studies using Southern blot analysis and FISH have demonstrated that HER-2 amplification correlates strongly with the degree of protein overexpression observed by immunohistochemical analysis.23,30 By using FISH, Pauletti et al23 identified 42 breast carcinomas with HER-2 overexpression (defined as a score of 2+ or 3+ by frozen section immunohistochemical analysis) that had an HER-2/CEP17 ratio greater than 2.0 (ie, amplified). Of these, 39 (93%) had greater than 10 HER-2 copies per cell. In contrast, our collection of tumors specifically selected for 2+ cases contained a significant number (5/20 [25% of total]) with low-level amplification (3-10 copies). As would be expected, these cases had low HER-2/CEP17 signal ratios (<5.0). It is important that this was seen only in amplified tumors that lacked any 3+ staining in additional block(s). Because gene copy number and degree of amplification are correlated,23,31 we interpret our data as an indication that low-level amplification is responsible for the 2+ staining observed in some breast carcinomas.
When there are fewer than 10 HER-2 copies per cell, the presence of CEP17 gains (ie, 3 or more copies of CEP17) typically results in HER-2/CEP17 signal ratios between 1.3 and 2.0. These tumors have abnormal ratios, but the actual HER-2 copy number is not high enough to be classified as amplification by the Vysis PathVysion criteria. At some institutions, these tumors are considered to have low-level amplification, or duplication. Many of our 2+ cases exhibited abnormal HER-2/CEP17 ratios between 1.0 and 2.0. This feature may represent another subgroup of weakly overexpressed breast carcinomas.

The clinical relevance (eg, response to treatment and prognosis) of various HER-2 gene amplification subsets (ie, high- vs low-level amplified cases) remains unclear. One recent study demonstrated that patients with 2+ immunohistochemical staining do not respond to single-agent trastuzumab therapy. However, another study has shown that survival is stratified by level of HER-2 amplification as determined by FISH. Consequently, cases with focally amplified clones or low-level amplification might represent a subset of 2+ cases that will, in fact, respond to therapy.

In our study, the initial HER-2 stain results were reported by multiple pathologists and performed during a 2-year interval. The repeated HER-2 stains, on the other hand, were done simultaneously and interpreted by 1 pathologist (D.W.V.). Hence, at least some discrepant results could be related to technical artifacts (ie, daily technical variation in staining) and interpretation variability. We do not intend to refute the well-established fact that HER-2 immunostains, particularly 2+.
cases, are subject to significant interobserver variation in their interpretation.\(^3\)\(^,\)\(^2\)\(^,\)\(^3\)\(^,\)\(^4\) In our study, there was a disagreement with the HER-2 interpretation in 2 of the original 37 slides available for review, although this retrospective evaluation was biased by knowledge of the original score. Although we agree with Wang et al\(^2\)\(^7\) that heterogeneous expression likely contributes to the problem of evaluating weakly positive HER-2 stains, our data indicate that low-level amplification and intratumoral heterogeneity of HER-2 amplification also might account for a significant fraction of the breast cancer cases that show 2+ expression by immunohistochemical analysis.

We continue to advocate an algorithm for assessment of HER-2 status that starts with immunohistochemical analysis and reflexes 2+ cases to FISH.\(^2\)\(^)\(^2\) However, our results emphasize the need for judicious choice of blocks for immunohistochemical analysis; those having optimal representation of invasive tumor should be selected. Furthermore, we think it would be superior to perform staining on a nodal metastasis rather than microscopic foci of primary tumor after a biopsy has been performed. Some may question whether restaining a second block in 2+ cases potentially might save the additional expense of FISH testing. Although we would not rule out this practice, particularly in selected “borderline” cases, we identified no specific histologic or staining clues to predict when restaining might be efficacious. Further studies are required to determine whether intratumoral heterogeneity or degree of amplification (high- vs low-level amplification) has clinical significance.

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