Detection of HER-2/neu (c-erbB-2) Overexpression and Amplification in Breast Carcinomas with Ambiguous Immunohistochemical Results. A Further Contribution to Defining the Role of Fluorescent In Situ Hybridization

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Abstract. Background: The assessment of HER-2/neu status is a prerequisite in the clinical management of patients with breast cancer in order to obtain prognostic and predictive information, including Herceptin® sensitivity. Immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) are the techniques recommended to detect HER-2/neu alterations and considerable attention is currently being focused on the standardization of these techniques. Intrinsic limitations of IHC, such as antigen preservation and antibody specificity, may make it difficult to score the membrane staining thereby resulting in inconclusive results. Patients and Methods: In this study, 65 invasive breast carcinomas, with doubtful IHC results using the monoclonal antibody CB11, were reanalyzed with two recently licensed assays, the Hercep Test™ (IHC) and the Path Vision™ (FISH). Results: IHC with Hercep Test™ detected HER-2 protein overexpression in 72% of cases, including nine (14%) strongly positive (3+), 13 (20%) medium positive (2+) and 25 (38%) weakly positive (1+) specimens. FISH testing, with interpretable results in 48 cases, showed a moderate HER-2 gene amplification in only 22% of carcinomas with 2+ or 3+ overexpression. Conclusion: These data indicated an excessive sensitivity of the Hercep Test™ and suggested that, in the case of indeterminate results after standard IHC, the FISH technique is the best approach to establish HER-2 status.

The HER-2/neu oncogene, also known as c-erbB-2, is amplified and/or overexpressed in 15-30% of breast cancers. Since 1987 (1), several reports have demonstrated that the amplification of HER-2/neu is an indicator of high clinical tumor aggressiveness and poor prognosis in both node-positive and node-negative patients (2, 3). Moreover, HER-2/neu overexpression/amplification appears to be a significant predictor of increased benefit from anthracycline-based treatments (4) and of less benefit from non-anthracycline chemotherapy and hormonal therapy.

Recently, HER-2/neu assumed further therapeutic implications when the United States Food and Drug Administration (FDA) approved trastuzumab (Herceptin®), a humanized monoclonal antibody directed against the HER-2/neu protein, as the therapy for metastatic breast cancer. Therefore, HER-2 status is of great clinical value because it represents a prognostic parameter, a predictive factor and a therapeutic target. There is, however, considerable debate about the relative advantages and disadvantages of when, how and where to conduct HER-2 testing (5-11).

Several studies have shown an almost complete concordance between gene amplification detected in tissue sections by fluorescent in situ hybridization (FISH) and overexpression determined by immunohistochemistry (IHC). Therefore, both these techniques have been approved by the FDA for HER-2 status determination with formalin-fixed paraffin-embedded breast cancer tissue sections. FISH is a gene-based technique with good reproducibility and excellent prognostic correlations (12), but requires specialized and more expensive equipment. IHC has been the only technique used in Herceptin® clinical trials. It is relatively inexpensive and the reagents are readily available, but there is considerable variability in results due to deficiencies in standardization of tissue fixation and handling, variability in antibody sensitivity and specificity with about 20 polyclonal and monoclonal antibodies currently available, irregular use of positive and negative controls and lack of consistency in interpreting and reporting results.

The interpretation of immunohistochemical results is, in part, facilitated by adopting the Hercep Test™ scoring system (13). However, comparing IHC and FISH results, the highest degrees of concordance were observed for negative cases.

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(0-1+) and cases with high expression levels (3+), while for 2+ cases, gene amplification alone was observed in percentages varying between 25% and 81% (14, 15) depending on the antibody used. The problem of false positives for category 2+ is a significant shortcoming of IHC and accounts for the great variability of overexpression, ranging from 9% to 60%, in cases reported in the literature (15).

For these reasons, currently particularly in Europe, tumors with an IHC 2+ score are tested with FISH to confirm or exclude HER-2 gene amplification. CB11 is one of the most widely used, FDA approved, monoclonal anti-HER-2 antibodies. Staining with this antibody tends to be stronger than other antibodies, with significant cytoplasmic staining which often makes plasma membrane positivity difficult or impossible to score (16).

In this study, two "gold standard" assays were applied: Hercep Test™ (IHC) and Path Vysion™ (FISH), in order to verify the HER-2/neu status in cases of breast cancer with inconclusive results after immunohistochemical staining with the CB11 antibody.

**Patients and Methods**

The present study is based on retrospective retrieval specimens of invasive breast carcinoma cases sharing the common characteristics of uninterpretable immunohistochemical results with the CB11 monoclonal antibody at the first cancer diagnosis, due to an excess of cytoplasmic staining impeding a correct evaluation of the cell membrane (Figure 1). Of the 266 primary invasive breast carcinomas consecutively observed in our Institute from October 2000 to December 2001, 65 cases (24%) satisfied the inclusion criteria even after repetition of IHC staining with CB11.

All testing was performed on adjacent sections, 4-μm thick, obtained from the most representative formalin-fixed, paraffin-embedded tumor block of each case.

**Immunohistochemistry.** The IHC staining of specimens with inconclusive results was performed on formalin-fixed paraffin-embedded breast cancer tissues using the monoclonal antibody CB11, which targets the intracellular domain of HER-2/neu protein (BioGenex, San Ramon, CA, USA). After deparaffinization, endogenous peroxidase was blocked using 10% hydrogen peroxide. No antigen retrieval procedure was used. The primary antibody was applied at a 1:10 dilution for 30 min at room temperature, followed by the biotin-streptavidin peroxidase complex (BioGenex). Diaminobenzidine was used as the chromogen. The slides were counterstained with Harris’s hematoxylin.

The Hercep Test™ (anti-HER-2 polyclonal antibody, DAKO) was applied exactly according to the commercial instructions (17), with epitope retrieval in 10 mmol/l citrate buffer in a water bath at 95°C for 40 min. According to the Hercep Test™ criteria (13), immunoreaction was scored as 3+ if more than 10% of the tumor cells showed strong and complete membrane staining, as 2+ if membrane positivity was moderate and complete in more than 10% cells, as 1+ if membrane positivity was weak and incomplete in more than 10% cells and 0 if membrane staining was absent or present in less than 10% cells.

**Fluorescent in situ hybridization (FISH).** For FISH analysis, the Abbott-Vysis Path Vysion™ HER-2 DNA Probe Kit (Abbott Laboratories, Abbott Park, IL, USA) was used, strictly following the manufacturer’s recommended protocol (18). Briefly, after deparaffinization, the sections were pretreated with the paraffin reagent kit (Abbott-Vysis). After pretreatment, enzyme digestion and fixation, the sections were denatured at 72°C (±1°C). Subsequently, 10 μl of a mixture of two directly labelled probes, the Her-2/neu-specific sequence probe (LSI HER-2/neu SpectrumOrange) and a probe for a satellite sequence at chromosome 17 (CEP 17 SpectrumGreen), were added to the tissue sections and hybridization was carried out at 37°C overnight (14-18 h) in a moist chamber. The slides were then washed, counterstained with DAPI, and covered with a cover slip using cement rubber. Slides of formalin-fixed, paraffin-embedded, cultured human cell lines with HER-2/neu gene amplified and not amplified, obtained from Vysis, were used as controls in the same bath.

The slides were evaluated for HER-2/neu gene amplification using a Nikon ECLIPSE E1000 (Nikon Instruments) fluorescence microscope equipped with three filter combinations (DAPI/Orange dual bandpass, DAPI/Green dual bandpass and DAPI/Green/Orange triple bandpass) for signal detection. Signal enumeration was conducted under oil immersion at 1,000 x magnification. The results were reported as the ratio between the average copy number of the HER-2/neu gene and that of the chromosome 17 centromere, analyzing 60 neoplastic nuclei. Specimens with a signal ratio of less than 2.0 were considered non-amplified and 2.0 or greater as amplified.

**Statistical analysis.** Statistical differences were calculated by the Chi-square test. Regression analysis (Spearman’s test), for comparison of HER-2/neu overexpression, was carried out by the Hercep Test™ and gene amplification by FISH.

**Results**

The mean age of the 65 patients was 59 years (range, 39-87). The main pathological features of the cases included in the study are provided in Table I. No significant differences in the distribution of these characteristics were evident when compared to the total consecutive series of breast cancers in our Institute.

In the first step of this study, the 65 specimens were submitted to immunohistochemical staining with the Hercep Test™. Of these, 22 (34%) were positive with a score of 2+ or 3+, while 43 (66%) were negative with a score of 0 or 1+ (Table II).

The second step was based on HER-2/neu gene amplification by FISH. A HER-2/chromosome 17 ratio was successfully detected in 48 out of the 65 specimens (74%). In the 17 remaining specimens, evaluation was not possible, even after a second hybridization attempt, due to DNA damage or absence of signal because of inadequate linking of one or both the DNA probes.

Using the criteria previously outlined, five cases (10%) showed low-level amplification with a HER-2 neu/centromere 17 ratio ≤5 (range 2.32-4.00).

Of the 18 cases with HER-2 overexpression of 2+ or 3+ by IHC with the Hercep Test™, only four (22%) were amplified...
They included one out of the twelve 2+ cases (8%), with a slight amplification (ratio 2.32), and three of the six 3+ cases (50%), with ratios of 2.37, 3.09 and 4.00, respectively. A slight amplification (ratio 2.34) was also observed in a case with a 1+ score, while none of the 13 cases with 0 score was amplified.

The overall concordance between the Hercep Test™ IHC scores of 2+ and 3+ and a HER-2/CEP 17 ratio of >2 was poor, with a correlation coefficient of 0.209 (Figure 2).

**Discussion**

Accurate detection of the HER-2/neu gene alteration in human breast cancer has become increasingly important for determining patient prognosis, as well as response to standard chemotherapeutic agents. Moreover, it is currently the sole criteria for selection of patients for HER-2/neu-target therapy with Herceptin®.

For these reasons, testing for HER-2/neu status is of great clinical value for the management of patients with breast cancer and the pathologist plays a pivotal role in providing...
consistent and reliable information in order to identify those patients most likely to benefit from Herceptin® therapy, as well as avoiding inappropriate treatment (8, 9).

Despite the variety of laboratory methods available, controversy exists over which assay should be used in clinical practice. Thus, standardization and validation of these methods are mandatory in order to obtain maximum benefit for the patient.

Most recent debate has focused on the comparison of HER-2 protein expression, determined by the widely available IHC, and HER-2 gene amplification, evaluated with the more specialized FISH tests. Discrepancies between FISH and IHC may be explained by referring to the tissue handling methods applied and evaluation criteria adopted. However, for a subset of cases with discordant results no reasonable explanation can be given based on pre-analytical factors. In these cases, functional inefficiencies in amplification, changes at the mRNA level or post-transcriptional dysregulations with accumulation of protein may play a role.

In the present study, we were faced with an additional problem not previously treated in the literature, concerning the routine determination of the HER-2 status with CB11, one of the most widely available antibodies which, in paraffin sections, offers a good balance between sensitivity and specificity. With this antibody, excessive cytoplasmic staining may develop in a consistent proportion of cases (24% in our series).

The significance of cytoplasmic staining with CB11 is controversial, although most studies agree that it is a non-specific finding, possibly due to the intrinsic characteristics of this antibody and/or to the inappropriate pre-analytical handling and fixing of the tissues. However, a few studies demonstrated concordance between strong cytoplasmic staining and poor prognosis in a subset of node-positive breast cancer patients (19).

In the presence of this artefact, results should be interpreted with caution and membrane-staining scoring may be impossible. Interms of deciding on the best therapeutic option for these patients, an alternative assessment method for the HER-2 status must be found. To resolve this problem, we applied two FDA-approved "gold standard" tests: the Hercep Test™, an IHC test kit using a rabbit polyclonal antibody with standardized procedures and evaluation criteria included, and the FISH test, based on gene copy enumeration and less prone to subjective interpretation and possible variability.

Our results indicated that, in about one-third of the cases with inconclusive results after IHC using the monoclonal antibody CB11, there was HER-2/neu overexpression (2+ or 3+) after IHC with the Hercep Test™. At first sight this seems to indicate a possible superiority of the Hercep Test™ for antigen demonstration in tissues with inappropriate pre-analytical handling as well. However, out of the 18 cases demonstrating HER-2 overexpression 2+ or 3+ by IHC with the Hercep Test™, only four (22%) were amplified. These included one out of the twelve 2+ cases (8%), with a slight amplification (ratio 2.32), and three out of the six 3+ cases (50%) with ratios of 2.37, 3.09 and 4.00.

These data confirmed previous doubts related to the high incidence of false-positive results by the Hercep Test™ (20, 21), particularly in the 2+ category, and are consistent with the fact that the specificity and the positive predictive value of the Hercep Test™ were lower with respect to those of CB11 (80% vs. 93% and 57% vs. 76%, respectively) (22).

On the other hand, the results of this study demonstrated that, in the presence of an excessive cytoplasmic background after IHC with CB11, the probability of finding HER-2/neu amplification is low and that, when present, the entity of gene amplification is very limited. This latter aspect reflects the high sensitivity of FISH (16) and, thus, there is temptation to use only FISH for HER-2/neu evaluation, as recently suggested by some authors (9, 11).

However, in the "real world" of the new targeted therapies, the routine use of FISH is not practical since the test is expensive and only available in large specialized laboratories.

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Table III. Relationship between Her-2/neu overexpression detected by the Hercep Test™ and Her-2/neu gene amplification detected by Path Vysion™.

<table>
<thead>
<tr>
<th>IHC</th>
<th>≤2</th>
<th>2-5</th>
<th>≥ 5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13 (100%)</td>
<td>-</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>1+</td>
<td>16 (94%)</td>
<td>1 (6%)</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>2+</td>
<td>11 (92%)</td>
<td>1 (6%)</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>3+</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

Total 43 5 0 48

Figure 2. Correlation between HER-2/neu overexpression and amplification (n=48, Spearman’s r=0.209, p=0.153).
(23); thus, accurate testing for HER-2/neu can be achieved using immunohistochemistry with complementary FISH testing (24).

In conclusion, based on our results, we propose that both in the systematic evaluation of the HER-2/neu status at the time of initial breast cancer diagnosis and at the time of relapse, each laboratory should continue to perform IHC with CB11 or other antibodies, for which experience has been consolidated using adequate tissue and standardized methodologies (25). In this context, IHC with the Hercep Test™ is attractive because of the advantage of a unified methodology. However, it is relatively expensive and does not resolve doubts regarding the clinical significance of the 2+ cases due to the high rate of false-positive results.

Therefore, in a metastatic setting in which an excess of cytoplasmatic background hinders accurate membrane evaluation, the final decision concerning eligibility for Herceptin™ therapy should depend directly on FISH results, preferably assessed in a reference laboratory with a high volume of HER-2/neu testing (26).

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References


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