Fluorescence In Situ Hybridization and Immunohistochemical Assays for HER-2/neu Status Determination

Application to Node-Negative Breast Cancer

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• **Background.**—HER-2/neu (ERBB2) gene amplification and/or overexpression is a major event in human breast tumorigenesis. HER-2/neu gene alterations have been the most frequently assessed prognostic factors during the last 10 years in breast cancer and have recently emerged as a management decision tool and a therapeutic target. There is still controversy over the best method to determine whether a tumor is HER-2/neu positive. Because of the increasing demand for HER-2/neu gene status determination in clinical practice, we compared HER-2/neu gene alterations at the DNA level (gene amplification) and the protein level (overexpression) in a panel of patients with lymph node–negative breast cancer who had received local radiotherapy alone, with no adjuvant therapy.

**Methods.**—We tested 100 excised lymph node–negative breast tumors, using fluorescence in situ hybridization (FISH) with a biotinylated HER-2/neu DNA probe and immunohistochemical assays (IHC) with 2 different antibodies.

**Results.**—The FISH frequency of HER-2/neu gene amplification was 15%, and the IHC frequency of overexpression was 21%.

**Conclusion.**—Although HER-2/neu amplification by FISH and HER-2/neu overexpression by IHC correlated well in this panel of lymph node–negative breast carcinomas, there were a number of discordant cases, pointing to the important need for determining HER-2/neu alteration for the future management of HER-2/neu–based clinical applications.

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About 70% of patients with breast cancer with no lymph node involvement are cured by locoregional therapy alone. However, a substantial number of patients experience disease relapse, and new predictors of recurrence are needed to identify those patients who require more aggressive initial therapy.

The ERBB2/HER-2/neu gene, which is activated in several human carcinomas, has been described as a marker of poor prognosis. It shares sequence homology with other members of the epidermal growth factor receptor family and encodes a 185-kd (p185c-HER-2/neu) transmembrane glycoprotein. HER-2/neu protein, also commonly known as HER2, is composed of an external ligand-binding domain, a transmembrane component, and a cytoplasmic domain with intrinsic tyrosine kinase activity involved in signal transduction. No specific high-affinity ligand has so far been identified. HER-2/neu gene amplification is the most common mechanism by which the gene is activated in human tumors and has been found in 8% to 55% (average, 25%) of breast carcinomas; it has also been found in benign and in situ breast lesions.

HER-2/neu gene dysregulation has been linked to poorly differentiated breast cancer. Most prognostic studies have shown that HER-2/neu is a predictor of poorer outcome in patients with node-positive tumors. Some studies have suggested that this may be explained by a link between HER-2/neu protein and the response to therapy. HER-2/neu alterations have also been found in node-negative breast tumors, but their clinical relevance is controversial.

The HER-2/neu gene can be screened by using molecular and immunological probes that vary in their complexity, sensitivity, and specificity. Until the early 1990s, HER-2/neu amplification was mainly evaluated by Southern blotting, which was supplanted by a sensitive and rapid quantitative polymerase chain reaction method. Fluorescence in situ hybridization (FISH) is a more recent technique that enables HER-2/neu–amplified cells to be visualized within a tumor slice. HER-2/neu overexpression can be detected with various methods, including Northern blot and in situ hybridization for HER-2/neu RNA and Western blot and immunohistochemical assays for HER-2/neu protein, but the most widely used method is immunohistochemical assays (IHC). FISH and IHC can both be applied to formalin-fixed and paraffin-embedded material, which enables the visualization of amplified cells within a tumor slice and the counting of the level of gene representativity per cell, taking care of tumor heterogeneity. New candidate treat-
ments based on HER-2/neu status, such as Herceptin, require an efficient method to screen patients with breast cancer for HER-2/neu alterations. FISH and IHC are currently the most suitable techniques.\(^9\)

Herein, we analyzed HER-2/neu status by means of FISH and IHC in 100 tumors from patients with nodenegative breast cancer with lengthy disease-free survival.

**MATERIALS AND METHODS**

**Patients and Samples**

We studied 100 formalin-fixed, paraffin-embedded breast cancer tissue samples from women with primary node-negative breast cancer consecutively diagnosed at the Centre Rene Huguenin, St Cloud, France. Node negativity was based on examination of a mean of 13 nodes (range, 6–23) per patient; median size was 20.1 mm (range, 6–55 mm). The patients underwent surgery and radiotherapy from January 1988 to July 1991. Clinical data were obtained from the medical records of eligible patients (Table 1).

The patients (mean age, 58 years; range, 33–86 years) met the following criteria: primary, unilateral, nonmetastatic, lymph node–negative breast carcinoma for which complete clinical, histological, and biological data were available and no neoadjuvant therapy (hormone therapy or chemotherapy). The median follow-up was 7.9 years (range, 1–10 years). Twenty-one patients experienced disease relapse (8 locoregional relapses: 3 skin, 4 breast, 1 supraclavicular recurrences; 12 metastases; and 1 contralateral breast cancer, which was an invasive tubular carcinoma grade 1 according to the Scarff, Bloom, and Richardson grading system [SBR] and different from the initial tumor, which was a ductal carcinoma, SBR 2).

**Clinical and Pathological Data**

The histological type and steroid hormone receptor status of each tumor were established at the time of surgery, together with node status. The malignancy of infiltrating carcinomas was scored according to Bloom and Richardson’s histoprognostic system.\(^10\) Estrogen and progesterone receptors were determined biochemically, using the Dextran-coated charcoal method as described by the European Organisation for Research and Treatment of Cancer.\(^11\) Receptor concentrations below 14 fmol/mg of cytosolic protein were considered negative. The main classic prognostic factors are shown in Table 1.

**Fluorescence In Situ Hybridization**

HER-2/neu amplification was examined in 4-μm paraffin-embedded tissue sections on silanized slides with the FISH technique on interphase tumor cells using the Oncor/Ventana INFORM Gene Amplification Detection System (Ventana Medical Systems, Tucson, Ariz; formerly sold by Oncor Inc, Gaithersburg, Md) and processed according to the manufacturer’s recommendations; positive (SKBR-3 cell line) and negative (MDA-MB-468 cell line) control cases were provided by the manufacturer (Oncor). A digoxigenin-labeled centromeric probe for chromosome 17 (D17Z1) was also processed on consecutive slides from amplified cases to detect abnormal copy number for chromosome 17 (Oncor). Briefly, after deparaffinization in xylene and drying, the slides were treated in a 30% Na2SO3 and protein was digested overnight in a humidiﬁed chamber at 37°C. Posthybridization washes with agitation were performed in 50% formamide at 78°C for 8 minutes. Hybridization with a biotinylated probe was performed overnight in a humidified chamber at 37°C. Posthybridization washes were performed in 50% formamide at 43°C for 15 minutes. The HER-2/neu probe was visualized with fluorescein-labeled avidin solution, and the signal was amplified by

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**Table 1. Relationship Between Clinical and Pathological Characteristics and Incidence of HER-2/neu Amplification and Overexpression**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. (%) of Cases (n = 100)</th>
<th>No. (%) of Amplified Cases: FISH Positive (n = 15)</th>
<th>No. (%) of Overexpressed Cases: IHC Positive (n = 21)</th>
<th>P Value†</th>
<th>P Value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>26 (26)</td>
<td>5 (33.3)</td>
<td>7 (33.3)</td>
<td>.70</td>
<td>.39</td>
</tr>
<tr>
<td>&gt;50</td>
<td>74 (74)</td>
<td>10 (66.7)</td>
<td>14 (66.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Node size, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤30</td>
<td>84 (87.5)</td>
<td>13 (86.6)</td>
<td>17 (80.9)</td>
<td>.75</td>
<td>.51</td>
</tr>
<tr>
<td>&gt;30</td>
<td>12 (12.5)</td>
<td>2 (13.4)</td>
<td>4 (19.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>92 (92)</td>
<td>14 (93.4)</td>
<td>20 (95.2)</td>
<td>.73</td>
<td>.67</td>
</tr>
<tr>
<td>Lobular</td>
<td>3 (3)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular</td>
<td>5 (5)</td>
<td>1 (6.6)</td>
<td>1 (4.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>24 (24.5)</td>
<td>1 (6.6)</td>
<td>3 (14.3)</td>
<td>.11</td>
<td>.33</td>
</tr>
<tr>
<td>2</td>
<td>51 (52)</td>
<td>8 (53.4)</td>
<td>11 (52.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23 (23.5)</td>
<td>6 (40)</td>
<td>7 (33.3)</td>
<td>.020</td>
<td>.055</td>
</tr>
<tr>
<td>Estrogen receptor‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>73 (81.1)</td>
<td>7 (53.8)</td>
<td>12 (63.1)</td>
<td>.21</td>
<td>.015</td>
</tr>
<tr>
<td>Negative</td>
<td>17 (18.9)</td>
<td>6 (46.2)</td>
<td>7 (36.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Progesterone receptor‡</td>
<td></td>
<td></td>
<td></td>
<td>.020</td>
<td>.055</td>
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<tr>
<td>Positive</td>
<td>59 (65.5)</td>
<td>6 (46.2)</td>
<td>8 (42.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>31 (34.5)</td>
<td>7 (53.8)</td>
<td>11 (57.9)</td>
<td>.20</td>
<td></td>
</tr>
</tbody>
</table>

* FISH indicates fluorescence in situ hybridization; IHC, immunohistochemical assays; NS, not significant; and SBR, Scarff, Bloom, and Richardson grade. For SBR category, 2 cases were not done; estrogen receptor, 3 cases were not done; and progesterone receptor, 10 cases were not done.

† χ² Test.

‡ Estrogen receptor positive, >14 fmol/mg of cytosolic protein; progesterone receptor positive, >10 fmol/mg of cytosolic protein.
**Table 2. Correlation Between HER-2/neu Gene Amplification Evaluated by FISH and HER-2/neu Overexpression Assessed by IHC in 100 Node-Negative Breast Malignant Tumors**

<table>
<thead>
<tr>
<th>HER-2/neu Expression (IHC), No (%)</th>
<th>HER-2/neu Gene Amplification (FISH), No. (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overexpression</td>
<td>21 (21)</td>
<td>8 (9.4)</td>
</tr>
<tr>
<td>Normal expression</td>
<td>79 (79)</td>
<td>77 (90.6)</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>85</td>
</tr>
</tbody>
</table>

* FISH indicates fluorescence in situ; IHC, immunohistochemical assays.

A. Immunohistochemical assay showing membranous staining for HER-2/neu protein on invasive ductal carcinoma scored as 3+ (original magnification ×40). B. Fluorescence in situ hybridization showing amplification for HER-2/neu, more than 18 signals per nucleus (oil, original magnification ×100).

successive incubations with an antiavidin antibody. For the chromosome 17 α-satellite probe, a rhodamine-labeled antidigoxigenin was used (Boehringer Mannheim, Germany). The slides were counterstained with DAPI in an antifade medium. Slides were kept at 4°C in the dark before examination with a Zeiss fluorescence microscope. The slides were scored double-blind by 2 operators within 48 hours of the end of the technical procedure.

Two different areas containing invasive carcinoma cells were selected in each case. Amplification was considered to be present when more than 4 green fluorescence signals were identified in each nucleus and amplified tumors had a minimum of 20 nuclei with more than 4 copies of the HER-2 gene.

**Immunohistochemistry**

Overexpression of HER-2/neu protein was detected automatically by IHC using 2 different antibodies in the standard avidin-biotin-peroxidase complex detection system (Chem mate, Dako, Glostrup, Denmark).

The primary antibodies used in this assay were a rabbit anti-human HER-2/neu polyclonal antibody (c-erbB-2 Oncoprotein, A 0485) from Dako at 1:1000 dilution, and the monoclonal antibody Ab-3 (Calbiochem-Novabiochem, Cambridge, England) at 1:1000 dilution, incubated in a Dako Autostainer for 30 minutes at room temperature, after antigen-retrieval microwave treatment (4 times 5 minutes, 750 W) in 0.01 mmol/L citrate buffer, pH 6. Positive controls have been done on cases showing Paget disease on nipple and in situ carcinoma on breast tissue. Normal breast tissue near these lesions negative in IHC has been considered a negative control.

Overexpression of HER-2/neu was defined by the presence of brown staining of the surface membrane of the cancer cell. We used the Dako standardized system for IHC interpretation, available at their Web site (www.dakousa.com/herinfo).

**Statistical Analysis**

The χ² test was used to test the relationship between HER-2/neu gene dysregulation and clinical and pathological characteristics, such as age, tumor size, stage, histological status, and estrogen and progesterone receptors, and to identify a correlation between the results of FISH and IHC.

**RESULTS**

**HER-2/neu Gene Amplification**

Fifteen (15%) of the 100 cases showed HER-2/neu gene amplification. Each case had a minimum of 20 malignant cells with a count of 7 to 23 signals per cell. The mean signal count of the positive samples was low (<10 signals per cell) in 3 cases and high in 12 cases (>9 copies per cell).

**HER-2/neu Protein Expression**

Overexpression of erbB-2/neu oncoprotein was screened for with both the polyclonal and the monoclonal antibody in each case. Twenty-one samples (21%) were positive with both antibodies (Table 2), and none of the samples was positive with one antibody and not the other.

**Comparison of HER-2/neu Amplification and Protein Overexpression**

We found very strong agreement between HER-2/neu gene amplification and HER-2/neu protein overexpression (P < 10⁻²⁷) (Table 2 and Figure).

Two of the 15 HER-2/neu gene-amplified cases were negative with both antibodies in IHC. The HER-2/neu DNA and protein status of these 2 tumors was confirmed...
by conducting a second FISH and IHC analysis of adjacent tumor sections. Interestingly, using a satellite probe for the 17 centromere, we found that these 2 cases were monosomic for chromosome 17q, whereas most of the other samples were diploid or polyploid (data not shown). In addition, 8 cases were IHC positive but FISH negative.

**Prognostic Significance**

We sought links between HER-2/neu amplification and/or overexpression (negative/positive) and standard clinical, pathological, and biological factors in breast cancer (Table 1). HER-2/neu amplified or overexpressed status was not significantly associated (χ² test) with age or standard prognostic factors, such as microscopic tumor size and histopathological grade, but was associated with steroid receptor status (positive or negative), particularly with estrogen receptor status and progesterone receptor only for overexpressed status.

Among the 21 patients who experienced disease relapse, 3 were both amplified and overexpressed for HER-2/neu in their tumors, 1 was amplified with no overexpression, and 2 overexpressed HER-2/neu with no amplification (Table 3). The other 15 patients with recurrent disease were negative both for amplification and overexpression. The presence of metastasis is clearly independent on HER-2/neu activation, as shown in Table 3. Nevertheless, patients with an HER-2/neu-activated tumor showed a trend toward a higher rate of local recurrence (17%, 4/23) than those with an HER-2/neu-negative tumor (5%, 4/77). This was not observed between HER-2/neu activation status and rate of distal metastatic relapse (9% vs 13%; 2/23 vs 10/77).

**COMMENT**

There is considerable interest in the potential value of HER-2/neu amplification and overexpression as a marker of poor prognosis and a predictor of response or resistance to chemotherapy and hormone therapy. HER-2/neu status is now used to select patients for treatment with a monoclonal antibody to HER-2/neu protein (Herceptin, Genentech Inc, South San Francisco, Calif). The choice of the method used to identify patients who may benefit from this therapy is crucial.

HER-2/neu gene amplification was detected on homogeneous DNA solution extracted from tumor piece, including the Southern blot technique, the dot-blot, and more recently the quantitative polymerase chain reaction. The previous molecular methods were hampered by their inability to differentiate tumor tissue from surrounding stromal, thus distorting the results. FISH is a more recently developed method that can visualize the number of gene copies present in tumor cells and provide a sensitive, accurate, and reproducible measure of HER-2/neu gene amplification, even with archival material. The IHC method can also easily be carried out on formalin-fixed, paraffin-embedded tissue, and the technique is more familiar, less expensive, and simpler compared with FISH, which measures HER-2/neu gene amplification. Moreover, IHC identifies cases in which the gene product is overexpressed in the absence of gene amplification. Nevertheless, it is not easy to navigate among the many published trials, in which more than 20 different monoclonal and polyclonal antibodies were used for IHC. Although these 2 techniques can measure alterations on an individual cell basis, they are complex and subject to considerable variations in the hands of different teams, posing problems for reproducibility and widespread use.

Some studies have assessed both gene and protein alterations of HER-2/neu in breast cancer.12,14,19 The use of cell-cell techniques on consecutive tumor sections can overcome problems of tissue heterogeneity. In the present study, we analyzed the incidence of ERBB-2 alterations by FISH and IHC in a homogeneous panel of 100 patients with lymph node–negative breast cancer with no systemic adjuvant therapy to evaluate its prognostic interest, with no interaction of any chemotherapeutic agents or hormoneregulators. To optimize the overexpression level by IHC, we used 2 antibodies—the monoclonal Ab3 and a polyclonal (c-erbB-2 Oncoprotein, A 0485)—which have frequently been used in previous studies. HER-2/neu protein overexpression was observed in 21 of the 100 cases studied herein, in agreement with published frequencies, and the 2 antibodies yielded totally concordant results. Fifteen cases showed HER-2/neu gene amplification. Thirteen cases were both amplified and overexpressed, 8 cases were overexpressed but not amplified, and 2 cases were amplified but not overexpressed. The remaining 77 tumors showed no overexpression or amplification. The frequency of HER-2/neu–amplified tumors among HER-2/neu–overexpressing tumors observed herein (62%) is similar to that reported in the literature, and amplification is clearly associated with overexpression. Several studies have shown that HER-2/neu protein overexpression can be present without detectable HER-2/neu amplification.12,16,22,23 HER-2/neu overexpression is controlled not only by the degree of gene amplification but also by the rate of gene transcription and protein degradation. In a previous study,
we found a good correlation between amplification status and HER-2/neu RNA levels. Taken together, our results confirm the existence of translational and/or posttranscriptional regulation of HER-2/neu suggested by Oshima et al.

Two amplified cases showed a low level of immunostaining (Table 2) in agreement with reports of tumors with gene amplification but no protein overexpression. Divergent data can be obtained when different parts of the tumor are used to detect amplification and overexpression, partly because of tumor heterogeneity. The advantage of the FISH versus IHC comparative study is the ability to analyze gene integrity and protein expression on 2 consecutive tumor sections, thus manipulating the same cells. Nevertheless, we observed 2 tumors with HER-2/neu amplification but no corresponding protein overexpression.

The 2 techniques combined identified 23 HER-2/neu–activated tumors (23%). FISH alone would have missed 8 of 23 cases and detected 2 amplified tumors without overexpression. This implies that FISH can select patients for HER-2/neu–specific treatment on the basis of normal HER-2/neu expression.

Conflicting data have been reported on the prognostic value of HER-2/neu status in node-negative breast cancer, possibly because of divergent definitions of node negativity. In our study, a mean of 13 nodes (range, 6–23) per patient were examined. The risk of metastasis was not influenced by HER-2/neu activation in our series, as shown in Table 3. Nevertheless, patients with HER-2/neu–activated tumors tended to have a higher rate of local recurrences (17%) than those with HER-2/neu–negative tumors (5%). A slightly higher frequency of local relapses was found in patients with HER-2/neu–amplified and overexpressed tumors, suggesting a role of the HER-2/neu gene in local proliferation. HER-2/neu activation has frequently been described in tumors with a high proliferative rate and a low degree of differentiation (ie, high histoprognostic grade).

The HER-2/neu gene status may be useful not only as a prognostic factor in determining clinical outcome, but also as a predictive factor in projecting the response to adjuvant therapies.

More recent data suggest that HER-2/neu status of tumors is becoming important with the availability of new therapeutic strategies based on humanized antibodies, such as Herceptin therapy. Recent studies have shown efficacy in clinical trials in patients with metastatic breast cancer, which confirms the importance and the increasing interest in this oncogene.

In view of these increasing applications, an international consensus regarding the appropriate method to evaluate HER-2/neu status for routine use (ie, HER-2/neu gene amplification and/or HER-2/neu protein overexpression) is necessary.

FISH, one of the most recent technical approaches, should be superior to other methods, such as Southern blotting, for detecting amplification for clinical application, but the most widely used technique to detect HER-2/neu activation remains IHC. Ross and Fletcher argued in favor of FISH, not only because of differences in the sensitivity of the antibodies used for IHC, but also because of disagreements among pathologists on how to score IHC positivity. Our results confirm that FISH and IHC do not provide precisely the same information and suggest that both techniques should be used in clinical trials to establish the best method for each specific treatment; nevertheless, the cost per case is substantially higher for FISH.

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References


HER-2/neu Status in Node-Negative Breast Cancer Patients—Onody et al