Quantitative Evaluation of HER-2/neu Status in Breast Cancer by Fluorescence In Situ Hybridization and by Immunohistochemistry With Image Analysis

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Abstract

We correlated quantitative results obtained in 40 invasive breast cancer cases for HER-2 gene amplification by fluorescence in situ hybridization with protein expression by immunohistochemical studies with computer-assisted image analysis. Fluorescence in situ hybridization (FISH) results were quantified as the mean number of fluorescent signals per nucleus, and immunohistochemical slides were read by semiquantitatively assessing membranous immunostaining intensity in tumor cells vs nonneoplastic breast tissue or quantitatively evaluated by image analysis. We found high correlation between immunohistochemical results by semiquantitative scoring and by image analysis. FISH results correlated with immunohistochemical results moderately when the staining intensity of only tumor cells was assessed and significantly better when the difference in staining intensity between tumor cells and nonneoplastic breast tissue was assessed. The correlation with FISH results was further improved when immunohistochemical study was combined with heat-induced epitope retrieval (HIER). Although FISH and immunohistochemical studies assess different aspects of the HER-2/neu gene (amplification vs overexpression), we found good correlation between the diagnostic techniques. The correlation was best when immunohistochemical studies were combined with HIER and assessed as the difference between tumor cells and nonneoplastic breast tissue.

The human epidermal growth factor receptor 2 (HER-2) gene on band q21 of chromosome 17, also known as neu and c-erbB-2, encodes a 185-kd transmembrane glycoprotein receptor (p185HER-2).1 HER-2/neu is overexpressed by approximately one fourth of human breast cancers2-4 and correlates with poor clinical outcome in women with node-positive and node-negative disease.3,5-8 Overexpression of HER-2/neu also is associated with adverse outcome in other cancer types, including gastric,9 ovarian,10 and pancreatic.11 In addition to its prognostic impact, the HER-2/neu protein has been targeted in novel immunotherapeutic regimens involving the Herceptin humanized monoclonal antibody (Genentech, South San Francisco, CA) with first positive clinical results in breast cancer patients with advanced, metastatic disease.12-14 In addition, tumors that overexpress HER-2/neu have been found to show resistance to certain forms of cytotoxic therapy15-20 and tamoxifen20-24 and increased sensitivity to others, eg, doxorubicin.25-28 Of particular interest was the observation that not only the presence but also the extent of HER-2/neu overexpression in individual tumors predicted the clinical outcome2,5,29 and the response to chemotherapy.25 In the latter study, only patients with tumors that showed a strong HER-2/neu overexpression (by immunohistochemical studies) profited significantly more from aggressive, dose-intensive chemotherapy with cyclophosphamide, doxorubicin, and 5-fluorouracil compared with patients with HER-2/neu–negative or weakly overexpressing tumors.25

A variety of methods are available for the detection of HER-2/neu status,7,8 but for clinical routine and research, the most widely applied techniques are fluorescence in situ hybridization (FISH) to detect HER-2/neu gene amplification4,30 and immunohistochemical studies of
paraffin-embedded tissue to detect HER-2/neu protein expression. Both techniques permit the study of small amounts of formalin-fixed, paraffin-embedded tissue and the interpretation of the findings on a cell-by-cell basis. Several studies have shown that these techniques show a high degree of concordance (>90%) for the HER-2/neu status in series of breast cancer cases. Yet, despite the clinical relevance for prognosis and therapy, none of these studies have systematically compared the extent of HER-2/neu gene amplification with protein overexpression as assessed by the 2 distinct techniques. In the present study of 40 selected breast cancer cases, we demonstrated a significant linear correlation between FISH results and immunohistochemical studies as quantified by computer-assisted image analysis.

**Materials and Methods**

We selected 40 cases from a pool of 90 invasive breast cancer cases, for which we had previously compared the status of HER-2/neu by FISH and immunohistochemical studies. All cases were accessioned consecutively at the Beth Israel Deaconess Medical Center, Boston, MA, from July 1997 through February 1998. The cases were selected based on FISH results to include a wide range of HER-2/neu amplifications. Nineteen cases were negative by FISH (average of <4 fluorescence signals per cell), and 21 cases were positive, with an average of 4 to 12 or 13 to 20 fluorescence signals per cell in 11 and 10 cases, respectively. All tumor specimens had been fixed previously in alcoholic formaldehyde (Anatech, Battle Creek, MI) followed by fixation in 10% neutral-buffered formalin. Four-micrometer-thick sections were cut from paraffin blocks onto positively charged slides.

**FISH for HER-2/neu Gene Amplification**

FISH for HER-2/neu gene amplification was performed using the Ventana INFORM Her-2/neu Gene Detection System (Ventana Medical Systems, Tucson, AZ) as described in the guide accompanying the kit. Tissue sections (on slides) were baked overnight at 65°C, deparaffinized in three 5-minute changes of xylene, transferred through two 2-minute changes of 100% ethanol, and allowed to air dry. Slides were immersed for 15 minutes in 30% Oncor Pretreatment Solution (Oncor, Gaithersburg, MD) at 43°C, briefly washed in 2× sodium chloride/sodium citrate (SSC) at room temperature, dehydrated through 100% ethanol, and air dried. Slides were then incubated for 60 minutes in Oncor Protein Digesting Enzyme Working Solution at 37°C. Slides were briefly washed in 2× SSC at room temperature, dehydrated through 100% ethanol, and allowed to air dry. Tissue sections were denatured by immersing slides in Oncor Denaturation Solution for 8 minutes at 75°C, followed by rinsing through 100% ethanol at ~20°C and air drying. Oncor biotinylated HER-2/neu DNA probe was p Freshly cut paraffin sections of cases known to be amplified for the HER-2/neu gene by FISH.

**Immunohistochemical Studies for HER-2/neu Protein Expression**

Immunohistochemical studies for HER-2/neu protein expression were performed at PhenoPath Laboratories, Seattle WA, using a polyclonal antibody to the HER-2/neu protein. Four-micrometer sections from each case were deparaffinized and rehydrated in graded alcohols. The slides were subjected to heat-induced epitope retrieval (HIER) by immersing them in a 0.01-mol/L concentration of citrate buffer (pH 6.0) preheated to greater than 90°C and then heated in a vegetable steamer (Black & Decker, Towson, MD) for 20 minutes, followed by a 20-minute cooldown period at room temperature. Slides then were incubated with an anti–HER-2/neu polyclonal antibody (1:1,000 dilution; DAKO, Carpinteria, CA) on an autostainer (DAKO) for 30 minutes at room temperature. The primary antibody used in
this study is the same anti–HER-2/neu antibody currently available in the HercepTest kit (DAKO). However, in the HercepTest kit, the antibody is provided in a prediluted form. For each case, 1 slide was incubated with phosphate-buffered saline instead of the primary antibody as a negative control. For positive controls, a composite slide composed of formalin-fixed cell pellets of the following 4 cell lines (obtained from Nora Disis, PhD, Fred Hutchinson Cancer Research Center, Seattle, WA) was used: MCF-7 (a cell line negative for HER-2/neu overexpression) and 3 human carcinoma cell lines showing increasing levels of overexpression of HER-2/neu (BT-20 [low overexpressor], SKBR-3 [intermediate overexpressor], and SKOV-3 [high overexpressor]). Antibody was localized using the LSAB+ Detection System (labeled streptavidin biotin immunoperoxidase, DAKO) according to the manufacturer’s instructions using the autostainer and counterstained with hematoxylin. All immunostains were run with and without HIER (immersion of the slides in a 0.01-mol/L concentration of citrate buffer, pH 6.0; preheated to greater than 90°C; and then heated in the vegetable steamer for 20 minutes, followed by a 20-minute cooldown period at room temperature).

**Interpretation of FISH and Immunohistochemical Results**

Interpretation of FISH (T.W.J., S.J.S.) and immunohistochemical (H.A.L., H.Y., A.M.G.) studies was performed by investigators blinded to the results of the other assay. The technical procedures for the detection systems have been described in detail. For evaluation of gene amplification (FISH procedure), 20 randomly selected invasive tumor nuclei in 2 separate, distinct tumor areas (ie, a total of 40 tumor nuclei) were scored for the number of fluorescent signals. Cases were considered amplified if the mean number of fluorescent signals was greater than 4. Cases with more than 20 signals were scored as 20. For the semiquantitative evaluation of protein expression (immunohistochemical procedure), all slides were scored by 1 pathologist (A.M.G.). The extent of membranous immunostaining intensity (in multiple, representative 20× fields per case) was scored on a 5-tier scale (0, completely negative; 1+, faint membranous positivity; 2+, moderate membranous positivity; 3+, strong, circumferential membranous positivity; and 4+, extremely strong positivity). For each case, infiltrating carcinoma and adjacent nonneoplastic breast tissue were scored separately, and a final subtracted score of tumor minus nonneoplastic tissue was used to correct for variability in background staining of nonneoplastic tissue (which should not overexpress the HER-2/neu protein). Cases in which there was a marked locoregional difference in terms of HER-2/neu expression in various areas of the slide were not entered into the evaluation.

**Image Analysis**

For the quantitative evaluation of HER-2/neu expression, 3 representative 20× fields of each tumor were digitized and imported into a G3 Macintosh computer (with a built-in 24-bit capture board; Apple, Cupertino, CA) and a standard one chip color CCD camera (TK-C138, JVC, Tokyo, Japan). The auto mode on the camera was turned off, and manual controls were used to adjust the image intensity, which was kept at an identical level during the entire study. Images were imported using the Acquire command in Photoshop (Adobe Systems, San Jose, CA; version 5.0). The digitized images were stored in Photoshop or PICT file format on the hard drive or on an external storage device (ZIP drive, Iomega, Roy, UT). The procedure for determining the immunostaining intensity was performed as previously described and is shown in schematic form in Figure II. By using the lasso tool in the toolbox, a cohesive group of invasive tumor cells was selected and the background removed from the image and thus from simultaneous analysis. The magic wand tool and the Select Similar command were used to select the immunohistochemical chromogen, and an optical density plot of the selected pixels was generated using the Histogram command in the image menu (Figure 1). Subsequently, a field of nonneoplastic breast tissue (lobular unit or duct epithelium) was selected in the same way and its optical density plot generated in analogy (Figure 1, lower panels). The mean immunostaining intensity was indicated by the Histogram command as arbitrary units (0–255, where 0 = dark and 255 = white) and the gray level calculated as the difference between the indicated value and the maximum “white” level of 255.

**Statistical Analysis**

Quantitative immunostaining of HER-2/neu protein expression was correlated with the results obtained for HER-2/neu gene amplification as assessed by FISH. Correlations were determined by linear regression, and the strength of the correlation was judged by the linear regression coefficient r. P values were assigned on the basis of the value of the linear correlation coefficient and the number of data points used in the linear regression.

**Results**

**Immunohistochemical Staining Pattern**

All positive breast tumors exhibited membranous HER-2/neu immunostaining, in most cases evenly distributed throughout the slide. Since cases with strong locoregional differences in HER-2/neu expression were not entered into the study (see the “Materials and Methods” section), we
found no noteworthy differences in HER-2/neu immunostaining in the 3 representative tumor areas in any of the examined cases (data not shown). Both the tumor cells and the nonneoplastic breast tissue showed various extents of cytoplasmic HER-2/neu immunostaining, ranging from entirely negative (most cases) to a faint blush or even homogeneously distributed distinct cytoplasmic positivity. Cytoplasmic HER-2/neu immunostaining was not influenced by the extent of HER-2/neu gene amplification, but seemed to depend on other variables (eg, differences in tissue fixation and/or processing, pretreatment, specimen thickness). Also, the extent of cytoplasmic HER-2/neu immunostaining was not affected by HIER (Figure 2A).

**Correlation of Image Analysis and Semiquantitative Score**

We found a highly significant correlation between HER-2/neu protein overexpression as assessed by Photoshop-based image analysis of immunostained slides and by the semiquantitative HER-2/neu immunostaining score. The correlation was further improved by the use of HIER; $r$ values ranged between 0.76 (without HIER, Figure 3) and 0.80 (with HIER, Figure 3). One case called HER-2/neu–positive based on the semiquantitative score had low immunostaining intensity by image analysis (Figure 3). This case was negative for gene amplification (<4 fluorescent signals), suggesting that the image analysis of the immunohistochemical data was more accurate in this case.

**Correlation of Image Analysis and FISH Results**

A significant correlation was noted when comparing the quantitative HER-2/neu immunohistochemical score (as assessed by image analysis) with HER-2/neu gene expression as assessed by FISH (mean number of fluorescent signals per tumor cell) and this correlation was improved only marginally by HIER ($r = 0.65$ with HIER vs $r = 0.62$ without HIER). However, the correlation between immunohistochemical and FISH results was much better when immunohistochemical results were calculated as the subtracted value of tumor tissue minus nonneoplastic breast tissue, suggesting that this resulted in effective correction of cytoplasmic immunostaining. By far the best correlation ($r = 0.76$) was seen when slides were exposed to HIER before immunohistochemical studies. With only one or two exceptions, most cases judged negative by FISH (<4 fluorescent signals per cell) had corrected immunostaining scores of less than 20 (mean ± SD), while almost all FISH-positive cases had immunostaining scores of more than 20 (mean ± SD, $P < .01$, Wilcoxon). When cases were grouped by gene amplification into no amplification (<4 fluorescent signals per nucleus), low amplification (4-12 signals), or high amplification (12-20 signals), the average immunostaining intensity was 6.1 ± 14.0, 39.2 ± 26.5, and 55.9 ± 26.2, respectively (Figure 2C, with HIER). However, owing to large interindividual variations in protein expression, these differences did not reach statistical significance. Similar differences were observed.
Figure 2
Correlation of HER-2/neu status of 40 breast cancer cases as assessed by immunohistochemical studies and fluorescence in situ hybridization (FISH). Immunohistochemical intensity was quantified as chromogen gray levels in nonneoplastic breast tissue (A) or tumor tissue (B) using Photoshop-based image analysis (values are given as arbitrary units [AU]). FISH data are given as the number of fluorescent signals per nucleus (mean of 40 nuclei per cancer case). C, Correlation of FISH data with immunohistochemical data after correction for background immunostaining intensity (given as difference for tumor minus nonneoplastic breast tissue). The study was performed with and without heat-induced epitope retrieval (HIER). For statistical analysis, we used the Spearman test for nonzero correlation and corresponding P values. Shown also is the regression line demonstrating slope and intercept (thick gray line). A, Without HIER: \( r = 0.09; P = .50 \). With HIER: \( r = 0.18; P = .43 \). B, Without HIER: \( r = 0.62; P < .001 \). With HIER: \( r = 0.65; P < .001 \). C, Without HIER: \( r = 0.72; P < .001 \). With HIER: \( r = 0.76; P < .001 \). See the legend for Figure 1 for proprietary information.
when the cases were run without HIER (12.3 ± 24.2, 41.6 ± 35.2, and 74.9 ± 27.1 for negative, low, and high gene amplification, respectively; Figure 2C).

**Discussion**

Based on several landmark articles,\textsuperscript{14,16,38-40} it is generally accepted that only distinct membranous immunostaining of HER-2/neu bears prognostic value in breast cancer, while the occasional cytoplasmic immunostaining is without such relevance. Only membranous immunostaining correlates with gene amplification\textsuperscript{14,38} and with raised protein levels.\textsuperscript{39,40} Jacobs et al\textsuperscript{34} reported that the diagnostic accuracy of HER-2/neu immunohistochemical studies (compared with gene amplification by FISH) is improved significantly when cytoplasmic HER-2/neu immunostaining of nonneoplastic breast tissue is taken into consideration and immunohistochemical findings are expressed as a subtracted score of neoplastic minus nonneoplastic immunostaining. These findings are supported by the data presented in this article. When compared with gene amplification by FISH, the \( r \) value as a measure of correlation reliability was improved when the immunostaining intensity of nonneoplastic breast tissue was subtracted from the immunostaining intensity of the invasive carcinoma (Figure 2C). However, it should be emphasized that the immunostaining pattern in the nonneoplastic breast tissue was absent in most cases and, when present, was found in a faint, usually cytoplasmic but occasionally membranous distribution. Thus, subtraction of cytoplasmic immunostaining in nonneoplastic breast tissue from the immunostaining intensity of invasive carcinoma emphasizes the specific membranous immunostaining signal in the tumor cells. In this respect, it should be mentioned that we routinely use only tissue blocks for HER-2/neu immunohistochemical studies that contain both tumor and nonneoplastic breast tissue.

In our image analysis, the hematoxylin counterstain did not interfere with the assessment of immunostaining since only the brown chromogen was selected and quantified (\textit{magic wand} tool; Figure 1). We found an improved concordance between FISH and immunohistochemical studies when cases were run with HIER (Figures 2B, 2C, and 3). This is in agreement with a previous study that demonstrated optimal HER-2/neu immunostaining of formalin-fixed, paraffin-embedded tissue with the use of epitope retrieval.\textsuperscript{41} In this respect, it is noteworthy that in our present analysis, HIER did not result in increased cytoplasmic immunostaining in nonneoplastic breast tissue (Figure 2A). While the findings...
of our present study provide no further insight into the exact nature of “nonspecific” HER-2/neu expression in nonneoplastic tissue, the data support the observation\textsuperscript{33} that the extent of nonspecific HER-2/neu expression in nonneoplastic breast tissue does not correlate with the extent of HER-2/neu amplification in tumor cells as assessed by FISH (Figure 2A).

The concern has been raised that the interpretation of HER-2/neu immunostained slides is subject to considerable interobserver variability, because of subjective assessment of staining intensity and of membranous vs cytoplasmic staining.\textsuperscript{28,31,42,43} However, in our study, we found a high degree of correlation when the computer-assessed immunostaining data were compared with the data as assessed by semiquantitative estimation of immunostaining intensity on a 5-tier scale (Figure 3) on slides with or without HIER pretreatment. This high degree of correlation may have been accomplished in part by the fact that both the computer-based and the semiquantitative assessment were based on a subtraction of neoplastic minus nonneoplastic breast tissue, thus effectively correcting for cytoplasmic background immunostaining. Since the 40 cases on which this study is based were selected from a larger pool of cases with the aim of covering as wide a spectrum of gene amplifications as possible, calculations of HER-2/neu incidence among this selected population, as well as concordance of the individual techniques, may not be representative for larger series of routinely diagnosed cancer cases.

All previous studies that have examined the diagnostic value of FISH and immunohistochemical results have used distinct cutoff values to distinguish positive from negative cases. One of the aims of our present study was to examine whether immunohistochemical studies, when combined with computer-based image analysis, could distinguish cancer cases with weak HER-2/neu gene amplification from those with strong amplification. Even though the reliability values for the correlation between gene amplification and protein expression were rather high (r values higher than 0.7), there still was considerable variability of immunohistochemical results within cases with no, low, or high gene amplification (Figure 2C). Rather than pointing toward problems in the accuracy of gene amplification (FISH) or protein expression (immunohistochemical studies), this finding reflects differences in the biology of the 2 aspects of HER-2/neu status. Indeed, several earlier studies have repeatedly shown discordance between the 2 techniques, with 5% to 10% of cases negative by immunohistochemical study despite gene amplification by FISH.\textsuperscript{3,34,44,45}

It is possible that in cases with low-level gene amplification, gene transcription and posttranslational and posttranscriptional events could be abnormal or down-regulated, leading to low HER-2/neu oncoprotein expression or abnormal protein or epitope production. Alternatively, the low gene copy number (<10 signals per nucleus) could represent aneuploidy for chromosome 17 rather than gene amplification per se.\textsuperscript{34} However, the data presented in the present study (Figure 2C) demonstrate that discrepancies between the 2 techniques were not limited to cases with low gene amplification, but that a similar number of discordant or ambiguous cases was seen with high gene amplification. When only the tumor cells were considered (Figure 2B), 3 cases that were scored as nonamplified by FISH had intense HER-2/neu immunoreactivity by immunohistochemical studies. This could represent single-copy overexpression of the HER-2/neu gene at the messenger RNA transcription level or beyond, with resultant overexpression of protein. Alternatively, it may be due to gene amplification that is below the detection level of the FISH assay. These scenarios were alluded to in the study by Slamon and coworkers,\textsuperscript{3} in which low HER-2/neu DNA levels were found with overexpression of messenger RNA and protein levels in 10% of cases. Similar findings of absent HER-2/neu gene amplification with protein expression have been reported in 3% to 7% of cases in other studies.\textsuperscript{4,46,47} However, in the 3 cases in our study, the cytoplasmic background reactivity also was very high, and background correction resulted in an immunohistochemical score that was no longer different from the other nonamplified cases (Figure 2C). Thus, it becomes obvious that further clinicopathologic studies are required to demonstrate whether HER-2/neu gene amplification or protein expression or a combination of the two techniques is the ideal predictor of clinical outcome and therapy response in breast cancer. Since the gene product is being targeted with therapies such as anti–HER-2/neu antibodies, immunohistochemical studies may well be the more biologically relevant assay. Finally, consideration may have to be given to the fact that the estimated cost of the FISH technique is by far higher than that of immunohistochemical HER-2/neu detection.\textsuperscript{34}

It should be noted that we used only a single anti–HER-2/neu antibody in the present study, the aim of which was to demonstrate that image analysis is feasible and yields biologically significant results, rather than to compare different antibodies, as has been done by Press and coworkers.\textsuperscript{31}

We have shown that image analysis with immunohistochemical studies of HER-2/neu is feasible and yields data that correlate well with semiquantitative estimates of immunostaining intensity and with HER-2/neu gene amplification by FISH. Consistent with a previous analysis of FISH vs immunohistochemical data,\textsuperscript{8} our data emphasize that diagnostic accuracy of HER-2/neu immunohistochemical studies can be improved markedly by subtracting the immunostaining intensity of nonneoplastic breast tissue from the values assessed in tumor cells, thus effectively correcting for nonspecific background immunoreactivity.
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