Assessment of HER-2/neu Status in Breast Cancer

Automated Cellular Imaging System (ACIS)-Assisted Quantitation of Immunohistochemical Assay Achieves High Accuracy in Comparison With Fluorescence In Situ Hybridization Assay as the Standard

Sijian Wang, MD, PhD,[1] M. Hossein Saboorian, MD,[1] Eugene P. Frenkel, MD,[2] Barbara B. Haley, MD,[2] Momin T. Siddiqui, MD,[1] Sefik Gokaslan, MD,[1] Frank H. Wians, Jr, PhD,[1] Linda Hynan, PhD,[3] and Raheela Ashfaq, MD[1,4]

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Abstract

*This retrospective study of formalin-fixed infiltrating breast cancer specimens compared manual immunohistochemical assay with a new image analyzer–assisted immunohistochemical quantitation method, using fluorescence in situ hybridization assay (FISH) as the standard. Following the manual immunohistochemical assay, 189 cases, including most manual immunohistochemically positive and some random negative cases, were analyzed by FISH assay for Her-2/*neu *gene amplification and by the Automated Cellular Imaging System (ACIS) for immunohistochemical staining. Using the FISH standard, the ACIS immunohistochemical assay attained a higher concordance rate and sensitivity than the manual immunohistochemical assay (91.0% and 88% vs 85.7% and 71%, respectively), with only a slight decrease in specificity (93% vs 96%, respectively). In particular, the ACIS immunohistochemical assay resulted in a higher correlation with the FISH assay in the manual immunohistochemical assay 2+ cases. The ACIS immunohistochemical assay achieved higher accuracy than the manual method according to receiver operating characteristic curve analysis. The ACIS method represents a substantial improvement over the manual method for objective evaluation of the HER-2/neu status.*

Breast cancer is the most common malignant tumor in women, affecting approximately 1 in 9 women in the United States during their lifetime. Although substantial progress has been made, one third of patients with breast cancer are expected to die of the disease.

HER-2/neu, also known as erbB-2, oncoprotein is overexpressed in 25% to 30% of breast cancers.[1-3] The HER-2/*neu* gene is on the long arm of chromosome 17, ie, 17q12-21.32.[4] A member of the epithelial growth factor receptors, HER-2/neu protein is located on the cytoplasmic membrane and involved in signal transduction for proliferation of epithelial cells, including mammary epithelial cells.[5,6] In more than 90% of breast cancer cases with HER-2/neu overexpression, the overexpression is attributed to amplification of the HER-2/*neu* oncogene.[1,7,8] HER-2/neu overexpression in the remaining cases may involve other mechanisms, such as transcription activation. HER-2/neu overexpression in patients with breast cancer and positive lymph nodes is linked to poor prognosis with a reduced disease-free interval and shortened survival time, and similar linkage may exist in node-negative cases.[1,2,9-12] Moreover, HER-2/neu overexpression seems to be a significant predictor for response to some therapeutic agents.[3,13,14]

Trastuzumab (Herceptin), a humanized monoclonal anti–HER-2/neu antibody, was approved by the US Food and Drug Administration as an adjuvant therapeutic agent for patients with metastatic breast cancer that overexpresses HER-2/neu protein.[15-17] As a result, evaluation of HER-2/neu status has become pivotal to determining patients' eligibility for trastuzumab treatment. In the clinical laboratory, HER-2/neu status usually is assessed in formalin-fixed and paraffin-embedded specimens using either immunohistochemical

assay (IHC) or fluorescence in situ hybridization assay (FISH) to measure HER-2/neu protein on the cell membrane and the HER-2/*neu* gene copies, respectively.

However, how to best assess HER-2/neu status still remains a hotly debated issue. This stems mainly from the facts that immunohistochemical and FISH assays measure 2 different targets, and the conventional manual immunohistochemical method may not be optimal for such a quantitative assay. In a recent comparative study, Wang et al[18] found that the 2 FISH assays, INFORM (Ventana Medical Systems, Tucson, AZ) and PathVysion (Vysis, Downers Grove, IL), achieved a high concordance rate of 98%. But the immunohistochemical assay had more complex results. All IHC negative cases and nearly all IHC low positive (1+) cases showed no gene amplification, while most IHC high positive (3+) cases had gene amplification. However, the IHC medium positive (2+) cases demonstrated significant discordance with the FISH assay, ie, some with HER-2/*neu* gene amplification and others with no amplification. Some other studies confirmed these findings,[19,20] whereas other studies found good correlation between the immunohistochemical and FISH assays.[21-23] It is apparent that an objective and reproducible immunohistochemical quantitation method is urgently needed.[24] We report the study of the usefulness of a new image analyzer system, the Automated Cellular Imaging System (ACIS, ChromaVision Medical Systems, San Juan Capistrano, CA), in quantitation of the immunohistochemical assay.

Materials and Methods

Specimen Procurement and Processing

Specimens were obtained from consecutive mastectomies and breast core biopsies performed at the University of Texas Southwestern Medical Center, Dallas, between August 1998 and March 2000. All specimens were fixed in 10% neutral buffered formalin and then paraffin embedded as previously described.[18] After histologic diagnosis was made, 1 representative tumor-containing tissue block from each of the infiltrating breast carcinoma specimens was submitted for evaluation of the HER-2/neu status. The invasive component was identified on the H&E-stained slide to facilitate subsequent quantitation of the same area by the immunohistochemical and FISH assays.

Immunohistochemical Assay and the Quantitation Methods

The immunohistochemical assay for HER-2/neu protein was described previously.[18] Briefly, paraffin-embedded tissue blocks were cut to 3-μm sections, deparaffinized, and heat treated for antigen retrieval. The primary polyclonal anti–HER-2/neu antibody, A0485 (DAKO, Carpinteria, CA), was used in the optimal dilution of 1:3,500 determined in our laboratory. Biotinylated secondary antibody with streptavidin-biotin labeling and related reagents were provided in the level 2 USA UltraStreptavidin Multi-Species Detection System (Signet Laboratories, Dedham, MA). All immunohistochemical assays were performed on an automated TechMate 1000 immunostainer (Ventana Medical Systems). Positive immunohistochemical reactions were defined as a dark brown reaction product on the cell membrane.

On completion of immunohistochemical staining, invasive components in the slides were quantitated by 2 pathologists (R.A., S.W.) as negative, low (1+), medium (2+), and high positive (3+), based on both percentage of the positively stained cells and staining intensity as previously described.[18] To be more specific, negative staining (0) had either no immunostaining of the cell membrane or faint staining involving a portion of the circumference of the cell membrane in less than 10% of the invasive cell population. Low positive staining (1+) had weak but definitive staining of the entire circumference (100%) of the cell membrane in 11% to 30% of the neoplastic cell population. Medium positive staining (2+) had strong positive staining of the entire circumference (100%) of the cell membrane in 31% to 50% of the neoplastic cell population. High positive staining (3+) had strong positive staining of the entire circumference (100%) of the cell membrane in 51% to 100% of the neoplastic cell population. The scoring criteria for the HercepTest (DAKO) uses a similar principle of percentage of positively stained cells and staining intensity, but differs from ours mainly in the percentage of stained cells for each staining score. Nevertheless, the end results from our laboratory and many others seemed similar, regardless of the specific scoring system used, ie, the major discrepancy between immunohistochemical and FISH assays existed in the 2+ (medium positive) subset.

HER-2/neu protein overexpression was defined if immunohistochemical staining was 3+, while no overexpression (normal expression) was defined if immunohistochemical staining was negative, 1+, or 2+. A total of 189 cases, including most immunohistochemically positive cases (1+ to 3+) and a subset of randomly selected immunohistochemically negative cases, were further evaluated by FISH and ACIS assays.

The ACIS system consisted of an automated robotic bright-field microscope module, a computer, and a Windows NT–based software interface. The robotic microscope module scanned the immunohistochemically stained slides, and a computer monitor displayed the digitized tissue images. The ACIS system was bought and implemented for the routine operation in the OncoDiagnostic Laboratory, Parkland Hospital, University of Texas Southwestern

Medical School. Although the membrane staining was not highlighted on the image, the ACIS system, as the manufacturer described, was able to distinguish cell membrane staining from cytoplasmic staining, using so-called color-space transformation (CST) proprietary technology. ACIS then specifically quantitated HER-2/neu protein staining on cell membrane. In this study, the immunohistochemical staining was quantitated without knowledge of the manual immunohistochemical or FISH scores, using the manufacturer-preset circular marks to select the areas of invasive tumor cells. The manufacturer recommended quantitation of 5 areas with highest staining intensity. In this study, 10 areas of invasive component with highest staining intensity were quantitated to reduce potential sampling variations. The ACIS recognized 256 levels of immunohistochemical staining intensity and converted these to fractional scores for the selected individual areas. An average score for all selected areas also was calculated. As the manufacturer recommended, cases with an average score of 2.0 or higher were considered to have HER-2/neu protein overexpression, while cases with average scores lower than 2.0 were considered to have no overexpression. The cases with ACIS immunohistochemical scores between 1.8 and 2.2 were subjected to a repeated quantitation, and the average score of the 2 measurements was used as the final score.

FISH Assay

The FISH assay, PathVysion, was used to evaluate the status of HER-2/*neu* gene amplification as previously described.[18] The PathVysion assay was performed using the manufacturer's recommended protocol after the laboratory had received proficiency certification for performing the PathVysion assay. Four-micrometer sections were prepared from paraffin-embedded tissue blocks. Two directly labeled probes, LSI HER-2/neu SpectrumOrange and CEP 17 SpectrumGreen (Vysis), were used for the HER-2/*neu* gene and the internal control alpha satellite sequence of the chromosome 17 centromere, respectively. The fluorescent signals were quantitated independently by 2 cytotechnologists under the supervision of 2 pathologists (R.A., S.W.). At least 60 cells of the invasive component were counted in each specimen for distinct fluorescent signals for these 2 sequences. The result was finalized by a pathologist and reported as the ratio of average copy number of HER-2/*neu* gene to average copy number of chromosome 17 centromere. Specimens with a ratio of 2.0 or higher were designated as having HER-2/*neu* gene amplification and those with a ratio lower than 2.0 as having no gene amplification.

Data Analyses

Statistical analyses were conducted to compare the ACIS and manual immunohistochemical methods using the FISH assay standard. The frequencies were compared using chi-square tests for 2 related samples with correction for continuity.[25] The phi coefficients, a correlation coefficient used for pairs of dichotomous variables, were calculated, and *t* tests were performed to compare each of these dependent samples.[26]

Receiver operating characteristic (ROC) curve analysis was used to compare accuracy of the manual and ACIS methods, using the FISH assay as the standard. The HER-2/neu status of each case was identified as either concordant (with both protein overexpression and gene amplification or with neither overexpression nor amplification) or discordant (with overexpression but not amplification or with amplification but not overexpression). We used a software program (MedCalc, Mariakerke, Belgium) to perform the ROC curve analysis. We compared the discriminatory power of the manual and ACIS methods for identifying cases of HER-2/neu overexpression with the use of values for the area-under-the-curve (AUC) for each ROC curve. When making comparisons between AUC values, we used $P < .05$ as the indicator of statistical significance.

Results

Case Selection

All infiltrating breast cancer specimens consecutively accessioned between August 1998 and March 2000 were submitted to the immunohistochemical assay, and HER-2/neu expression was evaluated by the manual method. Of these specimens, 199 cases were analyzed subsequently by the PathVysion FISH assay. These 199 cases were composed of 2 groups. The first group of 48 specimens was selected randomly from immunohistochemically stratified cases accessioned between August 1998 and February 1999 and, thus, was a representative sample of all cases analyzed by immunohistochemical assay during that period. The second group consisted of 151 specimens from the cases accessioned between March 1999 and March 2000. Following the manual immunohistochemical assay, all 136 cases with positive staining (1+ to 3+) and a random subset of 15 negative cases were analyzed by the FISH assay. Most of the immunohistochemically negative cases during this period were excluded from the FISH assay because the study by Wang et al[18] showed that none of the immunohistochemically negative cases had HER-2/*neu* gene amplification. The ACIS quantitation of immunohistochemical staining was conducted in 189 of the 199 FISH-analyzed cases, using the same immunohistochemical slides that originally were quantitated by the manual immunohistochemical method. The immunohistochemical slides for the remaining 10 cases,

including 2 cases of the first group and 8 cases of the second group, were not available for the ACIS quantitation.

ACIS Method Had Higher Correlation With the FISH Assay

We compared the manual and ACIS immunohistochemical methods, using the PathVysion FISH assay as the standard. **Table 1** and **Table 2** summarize the immunohistochemical and FISH scores (the scores of individual cases are not listed). Of the 189 cases, 75 cases (39.7%) had HER-2/*neu* gene amplification, while 114 cases (60.3%) had no gene amplification.

The ACIS immunohistochemical scores ranged from 0 to 4.6. HER-2/neu overexpression was defined as a score of 2.0 or more. To better evaluate the ACIS method, the scores were divided into 4 subsets, ie, 0 to 0.9 (46 cases), 1.0 to 1.9 (69 cases), 2.0 to 2.9 (36 cases), and 3.0 or more (38 cases) (Table 1). Each subset tended to show mainly gene amplification or no amplification, with little overlap. It is also informative to note that HER-2/*neu* gene amplification was found in all 38 cases with ACIS scores of 3 or higher, but in none of the 46 cases with ACIS scores lower than 1.

The manual immunohistochemical scores were in 4 separate levels, ie, negative (31 cases), 1+ (45 cases), 2+ (55 cases), and 3+ (58 cases) (Table 1). In contrast with the ACIS subsets, the manual subsets displayed more overlap of HER-2/*neu* gene amplification and no amplification, particularly in the subset of 55 cases with 2+ immunohistochemical staining that included 34 cases with no gene amplification and 21 cases with gene amplification.

The difference between the immunohistochemical methods is highlighted in Table 2. Compared with the 39.7% of cases with HER-2/*neu* gene amplification, the ACIS quantitation detected HER-2/neu overexpression in 39.2% of cases, whereas the manual method detected overexpression in only 30.7% of cases. With HER-2/neu overexpression defined as an ACIS score of 2 or higher or a manual score of 3+, the ACIS quantitation detected 106 cases with neither gene

Table 1
Summary of Scores for Immunohistochemical and Fluorescence In Situ Hybridization (FISH) Assays

	ACIS Immunohistochemical Scores				
	0-0.9	**1.0-1.9**	**2.0-2.9**	**3.0 or Higher**	**Subtotal (% of Total)**
FISH*					
No amplification	46	60	8	0	114 (60.3)
Amplification	0	9	28	38	75 (39.7)
Subtotal (% of total)	46 (24.3)	69 (36.5)	36 (19.0)	38 (20.1)	189 (100.0)
	Manual Immunohistochemical Scores				
	0	**1+**	**2+**	**3+**	**Subtotal (% of Total)**
FISH*					
No amplification	31	44	34	5	114 (60.3)
Amplification	0	1	21	53	75 (39.7)
Subtotal (% of total)	31 (16.4)	45 (23.8)	55 (29.1)	58 (30.7)	189 (100.0)

ACIS, Automated Cellular Imaging System (see text for proprietary information).
* For HER-2/*neu* gene amplification.

Table 2
Comparison of Immunohistochemical Methods Using the Fluorescence In Situ Hybridization (FISH) Assay Standard*

	Immunohistochemical Method				
	ACIS		Manual		
	Normal Expression (0-1.9)	**Overexpression (2.0 or Higher)**	**Normal Expression (0-2+)**	**Overexpression (3+)**	**Subtotal (% of Total)**
FISH†					
No amplification	106	8	109	5	114 (60.3)
Amplification	9	66	22	53	75 (39.7)
Subtotal (% of total)	115 (60.8)	74 (39.2)	131 (69.3)	58 (30.7)	189 (100.0)

ACIS, Automated Cellular Imaging System (see text for proprietary information).
* The sensitivity and specificity for the ACIS method were 88% and 93%, respectively; for the manual method, they were 71% and 96%, respectively. For the ACIS method, chi square = 121.16_1 ($P < .0001$); for the manual method, chi square = 90.3_1 ($P < .0001$) (chi square values were calculated with continuity adjustments); the phi coefficients for the ACIS and manual methods were 0.81 and 0.70, respectively; $t = 3.714_{186}$ ($P = .0003$).
† For HER-2/*neu* gene amplification.

Table 3
Summary of ACIS Immunohistochemical Scores in the Manual Immunohistochemical Subsets

		ACIS			
		Normal Expression (0-1.9)		Overexpression (2.0 or Higher)	
	Manual	0-0.9	1.0-1.9	2.0-2.9	3.0 or Higher
FISH*					
Negative subset (n = 31)					
No amplification	31	23	8	0	0
Amplification	0	0	0	0	0
Subtotal (discordant)	31	31 (0)		0 (0)	
1+ subset (n = 45)					
No amplification	44	15	28	1	0
Amplification	1	0	1	0	0
Subtotal (discordant)	45	44 (1)		1 (1)	
2+ subset (n = 55)					
No amplification	34	8	21	5	0
Amplification	21	0	8	9	4
Subtotal (discordant)	55	37 (8)		18 (5)	
3+ subset (n = 58)					
No amplification	5	0	3	2	0
Amplification	53	0	0	19	34
Subtotal (discordant)	58	3 (0)		55 (2)	

ACIS, Automated Cellular Imaging System (see text for proprietary information); FISH, fluorescence in situ hybridization.
* For HER-2/*neu* gene amplification.

amplification nor overexpression, 66 cases with both gene amplification and overexpression, and 17 cases with discordant gene and protein status. In parallel, the manual immunohistochemical method showed 109 cases with neither gene amplification nor overexpression, 53 cases with both gene amplification and overexpression, and 27 cases with discordant gene and protein status. Using the FISH assay as the standard, the ACIS and manual immunohistochemical methods achieved respective concordance rates of 91.0% (106 + 66 of 189 cases) and 85.7% (109 + 53 of 189 cases).

Statistical analyses revealed that compared with the FISH standard, the ACIS and manual methods had respective chi-square results of 121.16 and 90.3, both statistically significant ($P < .0001$). However, the t test of the 2 phi coefficients was 3.714 ($P = .0003$), suggesting that the correlation between FISH and the ACIS method was statistically higher than that between FISH and the manual method.

Higher ACIS/FISH Correlation Was From the Manual Immunohistochemical 2+ Subset

To further study the higher correlation between the ACIS method and FISH assay, the 189 cases were first divided into 4 subsets based on manual immunohistochemical scores, ie, negative, 1+, 2+, and 3+. The cases then were subdivided according to gene amplification status and ACIS immunohistochemical scores **Table 3**.

In the manual negative subset, none of the 31 cases had HER-2/*neu* gene amplification or overexpression by the ACIS method (all scores lower than 2.0), therefore achieving 100% concordance between the FISH assay and the ACIS method. Similarly, only 2 of 45 cases in the 1+ subset showed discordance between the FISH assay and the ACIS method, ie, 1 case with overexpression by the ACIS method but no gene amplification and 1 case with gene amplification but no overexpression. The concordance rate between the ACIS method and the FISH assay was 96%. The 3+ subset revealed discordance in only 2 of 58 cases, both of which had overexpression by ACIS but no gene amplification. The concordance rate between the ACIS method and the FISH assay was 97%. Overall, the concordance rate between the ACIS method and the FISH assay in all 3 subsets was 100% or close to 100%.

On the other hand, previous studies demonstrated that the manual immunohistochemical method and FISH could have significant discordance in the manual 2+ cases.[18-20] Indeed, the present study revealed widespread ACIS immunohistochemical scores in the 55 cases with manual 2+ scores. Nevertheless, the 34 cases with no gene amplification tended to have lower ACIS scores (0-2.9), whereas the 21 cases with gene amplification tended to have higher ACIS scores (1.0 to 3.0 or higher). The concordance rate between the ACIS method and FISH assay was still 76% (42 of 55 cases), with 13 discordant cases. In contrast, the concordance rate between the manual method and the FISH assay was 62% (34 of 55 cases).

The ACIS Method Achieved Higher Accuracy Than the Manual Method

Accuracy of an assay is reflected by its sensitivity and specificity in comparison with a standard assay. Using the FISH assay as the standard, the ACIS immunohistochemical

method achieved a sensitivity of 88% and a specificity of 93% (HER-2/neu overexpression defined as a score of 2.0 or higher), whereas the manual method attained a sensitivity of 71% and a specificity of 96% (HER-2/neu overexpression defined as 3+ staining; Table 2).

ROC curve analysis then was used to compare the accuracy of the immunohistochemical methods against the FISH standard. As the ACIS method is capable of detecting essentially continuous levels of immunohistochemical staining intensity, all scores (0-4.6) were used as the varying cutoff levels. In contrast, the manual method had very limited scores for such cutoff levels, ie, negative, 1+, 2+, and 3+. Based on these varying cutoff levels, the corresponding sensitivity and specificity values were calculated and the ROC graphs obtained **Figure 1**. The ROC curve for the manual immunohistochemical method showed that sensitivity and specificity would be 99% and 66%, respectively, if HER-2/neu overexpression were defined as 2+ or higher (2+ and 3+). However, sensitivity would decrease to 71% and specificity would increase to 96% when HER-2/neu overexpression was defined as 3+ or higher (3+ only). In contrast, the ROC curve for the ACIS method demonstrated a segment of the ROC curve with increased sensitivity and specificity, ie, 95% and 90% (cutoff 1.8 or higher) and 85% and 97% (cutoff 2.2 or higher). The cutoff score of 2.0 or higher used in this study to define HER-2/neu overexpression fell within this segment with a sensitivity of 88% and a specificity of 93%. The difference between these 2 immunohistochemical quantitation methods was further highlighted in the AUC values. The AUC values representing the ACIS and manual immunohistochemical ROC curves were 0.971 (95% confidence interval, 0.936-0.990) and 0.923 (95% confidence interval, 0.876-0.957), respectively. The difference between these AUC values was statistically significant ($P = .013$).

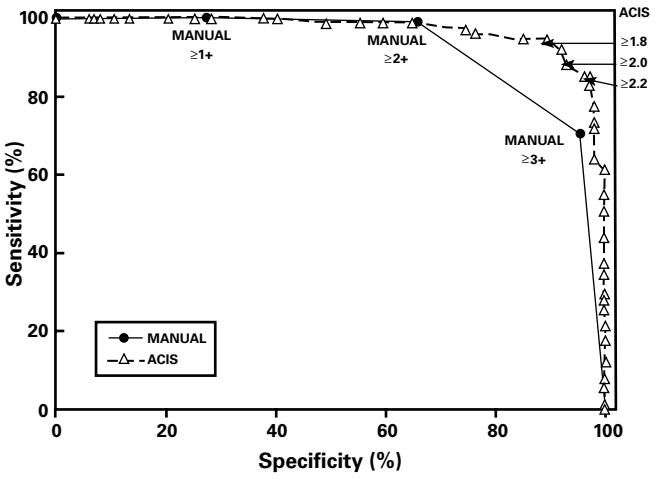

Figure 1 Receiver operating characteristic curves for the manual and ACIS (Automated Cellular Imaging System; see text for proprietary information) immunohistochemical methods.

Discussion

In a continuing effort to develop an objective, accurate, and reproducible assay for evaluation of the HER-2/neu status in breast cancer specimens, we studied the usefulness of the ACIS system in immunohistochemical quantitation. In this retrospective study of 189 invasive breast cancer cases, 39.2% of the cases showed HER-2/neu overexpression by the ACIS method, very close to 39.7% of cases with gene amplification by the FISH assay (Table 1). This relatively high rate of HER-2/neu overexpression or gene amplification rate was apparently due to the exclusion of most manual immunohistochemically negative cases from the second group (see "Case Selection"). Nevertheless, this exclusion did not seem to affect the subsequent analyses.

The manual immunohistochemical method detected 30.7% of cases with overexpression (3+ staining) and 29.1% of cases with 2+ staining, consistent with a previous finding of a high rate for the immunohistochemical 2+ cases.[18] Moreover, the FISH assay had an overall concordance rate of 91.0% with the ACIS method, higher than the 85.7% with the manual method. The difference in concordance rates was statistically significant. Indeed, FISH and the ACIS method achieved a 100% or nearly a 100% concordance rate in the subsets of manual negative, 1+, and 3+ cases. The exception was in the manual 2+ subset in which the ACIS quantitation revealed widespread scores (Table 3), suggesting the presence of highly heterogeneous HER-2/neu protein expression in this subset. This finding also underscores the intrinsic weakness of the manual immunohistochemical method for such a challenging quantitation task. Although ACIS quantitation still resulted in 13 discordant cases of the 55 cases in the 2+ subset, these discordant cases had a narrower range of ACIS scores, between 1.0 and 2.9, ie, near the cutoff score of 2.0, in contrast with widespread ACIS scores found for the entire manual 2+ subset (Table 3). Therefore, it is conceivable that at least some of these discordant cases actually may express borderline levels of HER-2/neu protein.

ROC curve analysis (Figure 1) revealed that the manual method produced a jagged ROC curve owing to very limited cutoff scores. Either sensitivity or specificity would have to be compromised when either 2+ or higher or 3+ or higher was chosen as the cutoff value. In fact, this observation supports the previously advocated 2-step approach, ie, to use the manual immunohistochemical method as a screening assay, followed by the FISH assay to analyze the immunohistochemically positive cases, particularly the

manual 2+ cases.[18] In sharp contrast, the ACIS immunohistochemical method produced continuous variable scores, resulting in a much smoother ROC curve. The difference between the 2 AUC values was 0.048 and was statistically significant ($P = .013$). Such a difference did not seem dramatic at first glance. However, considering the 2 ROC curves had large overlap except for the segment corresponding to the manual immunohistochemical cutoff points between 2+ or higher and 3+ or higher, the difference in AUC values strongly suggests that, when compared with the FISH standard, the ACIS method is more accurate than the manual method, particularly for the manual 2+ cases. The immunohistochemical assay used the polyclonal A0485 antibody, the primary antibody in the HercepTest kit, but not the kit itself. But both the manual and ACIS immunohistochemical methods in the study produced consistent results.

How to best assess HER-2/neu status in breast cancer has been a hotly debated issue, particularly after the US Food and Drug Administration approved the use of trastuzumab that targets HER-2/neu protein. The hallmark of HER-2/neu abnormality in breast cancer is protein overexpression, which in most cases apparently occurs as the result of the corresponding gene amplification. Consequently, either immunohistochemical assay or FISH has been explored as a single assay to evaluate respective HER-2/neu protein or gene status.

Immunohistochemical analysis has been used widely in the diagnostic laboratory, but mainly as a qualitative assay to produce positive or negative results. It is easy to perform and inexpensive. Most important for HER-2/neu evaluation, the immunohistochemical assay directly measures HER-2/neu protein, the target of trastuzumab. Until recently, the immunohistochemical assay often had been used as the single assay to evaluate HER-2/neu status. However, protein quantitation by immunohistochemical analysis may be affected considerably by a number of factors, such as specimen fixation and processing, antigen retrieval, and antibody specificity and sensitivity. Another important factor is that manual immunohistochemical quantitation is intrinsically subjective and crude. These problems probably have contributed substantially to conflicting reports in literature.

As an alternative, FISH recently became widely used. FISH is less prone to variations in specimen handling because DNA is very stable. Also, FISH quantitation involves counting the number of punctate fluorescent signals, which is more objective than estimating immunohistochemical staining intensity. But FISH is more time consuming and expensive. Moreover, it evaluates HER-2/neu status indirectly as far as the target of trastuzumab treatment is concerned. In fact, a small but definite number of cases overexpress HER-2/neu protein without concurrent gene amplification. Comparative studies revealed high discordance in the 2+ (medium positive) subset between the immunohistochemical and FISH assays. As a result, we, among others, have advocated a combinatory approach to evaluate HER-2/neu status, ie, use the immunohistochemical assay to screen all invasive breast cancer cases and FISH to further analyze immunohistochemically positive cases, particularly 2+ cases. This approach has made best use of the current technology because the subsets of manual negative, 1+, and 3+ cases showed very good correlation with FISH results; therefore, the subset of 2+ cases seemed to be the major one to which the FISH assay would add important information. In fact, the approach is in agreement with the updated 2000 American Society of Clinical Oncology recommendations.[27]

Lately, some studies seemed to suggest that FISH-detected HER-2/*neu* gene status may provide better prognostic information than immunohistochemically detected HER-2/neu protein status did[28] or may have a better predictive value for trastuzumab treatment compared with the HercepTest immunohistochemical assay.[29,30] These studies potentially could be interpreted as the basis to sway the pendulum from the aforementioned combinatory approach to the other end, ie, FISH as the primary or even the only assay to evaluate HER-2/neu status. However, we believe that by comparing the manual immunohistochemical assay with FISH, these studies mainly highlighted the weakness of the widely used immunohistochemical method instead of proving conclusively that the immunohistochemical assay as a whole is inferior to FISH for evaluation of HER-2/neu status. As previously discussed, the immunohistochemical assay is easy to perform and inexpensive, and, most important, it directly measures HER-2/neu protein, the biologic target of trastuzumab. Therefore, a FISH assay probably should not be construed as a permanent "gold standard" for the evaluation of HER-2/neu status, but rather a current working standard. These new studies just gave additional impetus to optimizing the immunohistochemical assay rather than replacing it.

Manual quantitation is one of the major factors that potentially could affect the outcome of an immunohistochemical assay. The inherently subjective, crude estimation of immunohistochemical staining means that the manual method can be only semiquantitative, even in the best scenario, rather than truly quantitative. To that end, the use of image analysis in immunohistochemical quantitation is promising. Image analysis has been used to quantitate biologic markers, such as nuclear hormone receptors[31] and MIB-1.[32] The present study demonstrates that an image analyzer, such as ACIS, can be applied to quantitate protein on cell membrane. Compared with the manual immunohistochemical method, there are major benefits of using such an image analyzer to quantitate immunohistochemical staining.

First, it converts a qualitative or semiquantitative assay to a truly quantitative assay. Second, it can improve assay objectivity and reproducibility. Even though the 2-step combinatory approach with the primary immunohistochemical assay–FISH discordance in the 2+ immunohistochemical group is arguably the best at present, such an approach still is affected by the inherently arbitrary immunohistochemical scoring system. The introduction of image analyzer–assisted immunohistochemical quantitation conceivably will result in more consistent assay results and improve intraobserver and interobserver reproducibility. Third, such image analyzer–assisted immunohistochemical quantitation does not need much additional training and is simple to perform. Instead of adding a totally new assay to laboratory operation, it modifies only the quantitation step while keeping the previous immunohistochemical assay steps unchanged. Fourth, the image analyzer–assisted immunohistochemical quantitation may provide more information about HER-2/neu protein for potential clinical exploitation. By detecting essentially continuous staining intensity, it can provide more refined information about HER-2/neu protein status. Such information conceivably may be used to stratify the patient population for prognostic and predictive studies. It also may help us to better understand the biologic behavior of some tumors. For example, it reveals wide-ranging staining scores in the manual 2+ cases. A logical question that can be asked is whether these cases would have the same or a different response to trastuzumab treatment. As more molecular-based diagnostic and therapeutic agents become available, such image analyzer–assisted immunohistochemical quantitation may be applied to evaluate other biologic markers.

However, as a relatively new immunohistochemical quantitation method, the ACIS system still needs improvements before widespread clinical use. For example, in technical aspects, we noticed that when the computer monitor displayed a tissue image, individual cells were fuzzy with a resolution near a low-power view under a light microscope. Such resolution made it difficult to identify areas of invasive component when both invasive and in situ components were present. Sometimes, the invasive component had to be confirmed in a corresponding tissue slide under a light microscope. Cost is another major factor. The implementation of the ACIS system is likely to be an expensive process. In addition, the cost of continuous use and maintenance of such an image analyzer may be substantial. Software and hardware upgrades also may be needed. Because we have used the ACIS system for only a limited time, a comprehensive cost analysis is not feasible yet. Nevertheless, an immunohistochemical assay with such an image analyzer–assisted quantitation inevitably will be more expensive to the patient. It is very likely that the high cost will limit the use of image analyzer–assisted immunohistochemical quantitation of HER-2/neu protein to high volume laboratories, at least for the near future. Another important issue is technical and clinical validation of the ACIS system in immunohistochemical quantitation of HER-2/neu protein. More extensive studies certainly are required.

We studied the usefulness of the ACIS system in immunohistochemical quantitation of HER-2/neu protein expression. This study showed that such an image analyzer–assisted immunohistochemical assay may have great potential to supplement or replace the manual immunohistochemical method to evaluate HER-2/neu status, due to its inherently better objectivity and reproducibility.

From the [1]Department of Pathology, [2]Department of Internal Medicine, and [3]Academic Computing Services, the University of Texas Southwestern Medical Center, Dallas; and the [4]Laboratory of Cytopathology, Parkland Memorial Hospital, Dallas, TX.

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Address reprint requests to Dr Ashfaq: Director of Cytopathology, Dept of Pathology, the University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-9072.

References

1. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*. 1987;235:177-181.
2. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the Her/*neu* proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244:707-712.
3. Revillion F, Bonneterre J, Peyrat JP. *ERBB2* oncogene in human breast cancer and its clinical significance. *Eur J Cancer*. 1998;34:791-808.
4. Popescu NC, King CR, Kraus MH. Localization of the human *erbB-2* gene on normal and rearranged chromosomes 17 to bands q12-21.32. *Genomics*. 1989;4:362-366.
5. Alroy I, Yarden Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett*. 1997;410:83-86.
6. Reese D, Slamon DJ. HER-2/neu signal transduction in human breast and ovarian cancer. *Stem Cells*. 1997;15:1-8.
7. Naber SP, Tsutsumi Y, Yin S, et al. Strategies for the analysis of oncogene overexpression: studies of the *neu* oncogene in breast carcinoma. *Am J Clin Pathol*. 1990;94:125-136.
8. Kallioniemi O-P, Kallioniemi A, Kurisu W, et al. *ERBB2* amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci U S A*. 1992;89:5321-5325.
9. McGann AH, Dervan PA, O'Regan M, et al. Prognostic significance of c-erbB-2 and estrogen receptor status in human breast cancer. *Cancer Res*. 1991;51:3296-3303.

10. Gusterson, BA, Gelber RD, Goldhirsch A, et al. Prognostic importance of c-erbB-2 expression in breast cancer. *J Clin Oncol.* 1992;10:1049-1056.
11. Quenel N, Wafflart J, Bonichon F, et al. The prognostic value of c-erbB2 in primary breast carcinomas: a study on 942 cases. *Breast Cancer Res Treat.* 1995;35:283-291.
12. Press MF, Bernstein L, Thomas PA, et al. HER-2/*neu* gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J Clin Oncol.* 1997;15:2894-2904.
13. Allred DC, Harvey JM, Berardo M, et al. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol.* 1998;11:155-168.
14. Muss HB, Thor AD, Berry DA, et al. C-erbB-2 expression and response to adjuvant therapy in women with node-positive breast cancer. *N Engl J Med.* 1994;330:1260-1266.
15. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A.* 1992;89:4285-4289.
16. Baselga J, Norton L, Albanell J, et al. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res.* 1998;16:2825-2831.
17. Slamon DJ, Leyland-Jones B, Shak S, et al. Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER-2 overexpressing metastatic breast cancer (HER2+/MBC) markedly increases anti-cancer activity: a randomized, multinational controlled phase III trial [abstract]. *Proc Am Soc Clin Oncol.* 1998;17:98A.
18. Wang S, Saboorian MH, Frenkel E, et al. Laboratory assessment of the status of Her-2/neu protein and oncogene in breast cancer specimens: comparison of immunohistochemistry assay with fluorescence in situ hybridisation assays. *J Clin Pathol.* 2000;53:374-381.
19. Hoang MP, Sahin AA, Ordonez NG, et al. HER-2/*neu* gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. *Am J Clin Pathol.* 2000;113:852-859.
20. Ridolfi RL, Jamehdor MR, Arber JM. HER-2/neu testing in breast carcinoma: a combined immunohistochemical fluorescence in situ hybridization approach. *Mod Pathol.* 2000;13:866-873.
21. Jacobs TW, Gown AM, Yaziji H, et al. Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. *J Clin Oncol.* 1999;17:1974-1982.
22. Vang R, Cooley LD, Harrison WR, et al. Immunohistochemical determination of HER-2/neu expression in invasive breast carcinoma. *Am J Clin Pathol.* 2000;113:669-674.
23. Couturier J, Vincent-Salomon A, Nicolas A, et al. Strong correlation between results of fluorescent in situ hybridization and immunohistochemistry for the assessment of the *ERBB2* (HER-2/*neu*) gene status in breast carcinoma. *Mod Pathol.* 2000;13:1238-1243.
24. Allred DC, Swanson PE. Testing for erbB-2 by immunohistochemistry in breast cancer. *Am J Clin Pathol.* 2000;113:171-175.
25. Kirk R. *Statistics: An Introduction.* 3rd ed. Fort Worth, TX: Holt Rinehart & Winston; 1990.
26. Cohen J, Cohen P. *Applied Multiple Regression/Correlation Analysis for the Behavioral Sciences.* 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associations; 1983.
27. Bast R, Ravdin P, Hayes DF, et al. 2000 Update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol.* 2001;19:1865-1878.
28. Pauletti G, Dandekar S, Rong H, et al. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J Clin Oncol.* 2000;18:3651-3664.
29. Mass RD, Sanders C, Charlene K, et al. The concordance between the clinical trials assay (CTA) and fluorescence in situ hybridization (FISH) in the Herceptin pivotal trials [abstract]. *Proc Am Soc Clin Oncol.* 2000;19:291A.
30. Buehler H, Bangemann N, Evers K, et al. Effective HER-2/neu diagnosis in breast cancer by a combination of immunohistochemistry and FISH [abstract]. *Proc Am Soc Clin Oncol.* 2000;19:294A.
31. Esteban JM, Ahn C, Battifora H, et al. Quantitative immunohistochemical assay for hormonal receptors: technical aspects and biological significance. *J Cell Biochem Suppl.* 1994;19:138-145.
32. Biesterfeld S, Kluppel D, Koch R, et al. Rapid and prognostically valid quantification of immunohistochemical reactions by immunohistometry of the most positive tumour focus: a prospective follow-up study on breast cancer using antibodies against MIB-1, PCNA, ER, and PR. *J Pathol.* 1998;185:25-31.