HER-2/*neu* Testing in Breast Carcinoma: A Combined Immunohistochemical and Fluorescence *In Situ* Hybridization Approach

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We evaluated 750 consecutive invasive breast carcinomas for HER-2/*neu* utilizing a combination of immunohistochemical (IHC) and fluorescence *in situ* hybridization (FISH) methodologies. IHC reactions of 3+ were considered HER-2/*neu* positive and 0 and 1+ IHC reactions were considered HER-2/*neu* negative. IHC reactions of 2+ were considered inconclusive and reflexed to FISH analysis. In addition, a 10% sampling and validation FISH analysis was performed on the positive and negative IHC tests. One hundred thirty-eight cases (18.4%) were HER-2/*neu* positive by IHC and/or FISH. One hundred twenty-three of the positive cases (89%) were 3+ IHC reactions and 14 positive cases were inconclusive by IHC and amplified by FISH. There was concordance with FISH in 77 of 78 (98.7%) of the positive or negative IHC cases that were tested (95% confidence interval [CI] = 93.1 to 100%). A single IHC-negative case showed HER-2/*neu* amplification by FISH. Thirty-nine cases were 2+ IHC (5.2%); 14 (36%) were amplified, 24 (62%) were not amplified, and one was not interpretable. HER-2/*neu* positivity was observed in 34% of grade 3 ductal carcinomas, 11.4% of grade 2 ductal carcinomas, 3.2% of grade 1 ductal carcinomas, and 3.2% of lobular carcinomas. Occasional cases with discordant IHC expression of HER-2/*neu* within the *in situ* and invasive carcinoma elements were also identified. IHC reliably characterized HER-2/*neu* in approximately 95% of the cases studied (95% CI = 93.0 to 96.2%) and was effective as a primary method for evaluating HER-2/*neu* status. In this study, 2+ IHC reactions were a heterogeneous group best regarded as indeterminate or inconclusive; in this series, only 36% were amplified by FISH analysis. Our findings suggest that a combination of IHC and FISH testing with FISH analysis performed reflexly on all 2+ IHC cases can optimize HER-2/*neu* testing.

KEY WORDS: Breast carcinoma, FISH, HER-2/*neu*, Immunohistochemistry.

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At this time, the ideal method for accurately determining HER-2/*neu* status in breast carcinoma has yet to be determined (1, 2). Both immunohistochemical (IHC) and fluorescence *in situ* hybridization (FISH) methodologies have been approved for clinical usage by the FDA. There are advantages and disadvantages to each methodology. IHC is accurate, relatively inexpensive, and can be performed quickly with little technical difficulty (1, 2). It is, however, not an optimal quantitative assay, and there is significant variation between different antibodies available for use (3–5). FISH testing is highly accurate and relatively easy to quantitate, but more expensive, time consuming, and technically difficult (1, 2). It has been suggested that perhaps a combination approach using both methodologies for HER-2/*neu* testing might be advantageous (6).

Currently the DAKO HercepTest (DAKO, Carpinteria, CA) is the only IHC test approved by the FDA for evaluating HER-2/*neu* status in breast carcinoma (2). Although its overall reported performance has been good, there have been questions raised concerning its accuracy, particularly related to 2+ positive reactions (7). Such reactions were originally recommended to be interpreted as "indeterminate" instead of "weakly positive" and did not correlate well with the clinical trial assay (CTA) investigational IHC test (6, 8). Because of these uncertainties (as well as cost considerations), we elected to use the DAKO polyclonal antibody (as used in the HercepTest) utilizing a standard HIER IHC technique to evaluate 750 breast carcinomas

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consecutively submitted for HER-2/*neu* evaluation. The IHC assay was calibrated with the HercepTest and validated against an FDA-approved FISH test. IHC results were scored as with the HercepTest, but 2+ IHC reactions were considered inconclusive instead of weakly positive and reflexed for further FISH testing. All 3+ IHC positive and 0 to 1+ negative IHC reactions were interpreted as such with validation FISH analysis performed on a subset of each group.

MATERIALS AND METHODS

Case Selection and Specimen Processing

All IHC and FISH testing was performed in Southern California Permanente Medical Group Regional Laboratories on breast carcinomas consecutively submitted for HER-2/*neu* status from all 11 Kaiser Medical Centers in southern California. These tissues were all fixed in a standard fashion at each medical center using 10% buffered formalin. All tissues were processed and paraffin-embedded at a single regional histology laboratory, except for one medical center, which processed tissues on site using a comparable technique. Length of fixation varied to some degree, but was generally between 12 and 24 hours.

Immunohistochemistry Methodology

Testing was performed on a Techmate 1000 (Ventana, Tucson, AZ). Tissue sectioned at 4 μm was mounted on silanized capillary gap slides (Ventana), baked at 60°C for a minimum of 60 min, deparaffinized with xylene, and rehydrated through graded alcohols to distilled water. Sections were then placed in 0.01-M citrate buffer at pH 6.0 and heated in a steamer (Black & Decker, Model H390) for 20 min at 90°C. Anti-HER-2/*neu* polyclonal antibody (DAKO) was used at a dilution of 1:2000. A secondary biotinylated antibody was used and reactivity detected by an avidin-biotin immunoperoxidase system employing 3′-diaminobenzidine tetrahydrochloride as the chromogen.

The IHC test was initially calibrated against cell button control slides from the DAKO HercepTest to yield negative, weak, and strong membrane staining on the appropriate cells. It was then parallel tested with the ONCOR HER-2/*neu* Gene Detection System (Ventana) on 20 cases yielding complete concordance with 0 to 1+ (negative) and 3+ (strongly positive) reactions and a single discordance in a 2+ IHC case.

Immunohistochemistry Interpretation

Membrane staining was scored 0 to 3+, corresponding to results as described with the DAKO HercepTest, with at least 10% of the cells staining for 1 to 3+ reactions. Results were interpreted as negative with 0 and 1+ reactions, positive with 3+ reactions, and inconclusive with 2+ reactions. Positive and negative controls were run with each case. The positive control always included a 3+ reacting tumor and normal nonreacting ducts. All cases were interpreted by one of two pathologists (RR, JA), usually independently.

Cases interpreted as positive or negative for HER-2/*neu* overexpression by IHC methodology were reported as such. Cases that were inconclusive by IHC methodology were further tested by FISH methodology and reported based on the FISH findings (amplified or not amplified). In addition, approximately every 10th negative or positive case was also studied by FISH methodology as a validation procedure. All sections submitted for FISH analysis were screened by a pathologist to insure they contained appropriate tissue.

FISH Methodology and Interpretation

Four-μm thick sections of formalin-fixed paraffin-embedded tissue mounted on silanized slides were processed in the genetics laboratory for FISH study. An accompanying scored hematoxylin and eosin stained slide was received with each case. The slides were pretreated chemically and enzymatically to remove proteins that block DNA access. After denaturation, a hybridization solution containing labeled DNA probe complementary to the HER-2/*neu* gene sequence was applied to the tissue section, which was then incubated under conditions favorable for annealing of probe DNA and the genomic DNA sequence. After hybridization, the unannealed probe was washed off and the hybridized probe was detected using a fluorescently tagged ligand and a counterstain. An epifluorescence microscope (Zeiss Axioscope) equipped with an appropriate filter set was used to score the number of signals per nucleus according to the guidelines described by the manufacturer.

In 102 of a total of 117 cases, the ONCOR HER-2/*neu* Gene Detection System from Ventana Medical System, Inc. was used. In 15 cases, the PathVysion HER-2/*neu* DNA Probe Kit from Vysis, Inc. was used. Scoring of signals was performed according to the manufacturer's guidelines. With the ONCOR probe, signals from 40 randomly selected cancer nuclei from two distinct areas were enumerated. A mean signal of greater than four indicated that HER-2/*neu* amplification was identified, whereas a mean signal of less than or equal to four indicated that HER-2/*neu* gene amplification was not identified. With the Vysis probe, signals from 60 cancer nuclei from two distinct areas of the tumor were enumerated and a ratio of the total number of

HER-2/*neu* signals to the total number of chromosome 17 signals (CEP 17) was calculated. A ratio equal to or greater than two indicated HER-2/*neu* gene amplification, whereas a ratio of less than two indicated HER-2/*neu* gene amplification was not identified.

Grading

A modified Bloom-Richardson grading scheme was used for grading all breast carcinomas (9). Lobular carcinomas included nuclear grade 1 and 2 tumors but not pleomorphic (nuclear grade 3) variants that were included among other subtypes.

RESULTS

Utilizing a combination of IHC and FISH results, we found that a total of 138 cases (18.4%) of 750 exhibited HER-2/*neu* overexpression or amplification and considered them to be HER-2/*neu* positive (Table 1). Six hundred eleven cases were HER-2/*neu* negative and one case was inconclusive.

By IHC alone, 123 cases were 3+ positive (16.4%), 39 cases were 2+ inconclusive (5.2%), and 588 cases were 0 to 1+ negative (78.4%). Within the positive cases, membrane staining was fairly uniform throughout and present in the majority of invasive tumor cells (Figs. 1, 2, 3, and 4). Some cases also showed nonspecific cytoplasmic staining.

Inconclusive Cases (IHC)

The 39 inconclusive cases displayed 2+ membrane staining in at least 10% of the invasive tumor cells and usually had been repeated with similar results. Some cases were difficult to characterize as 2+ (either 1+ *versus* 2+ or 2+ *versus* 3+).

Ten of the inconclusive cases were needle biopsies with a minority component of neoplastic cells showing 2+ membrane staining, often associated with accentuated staining at the biopsy edges where the reaction appeared artifactual. These cases were interpreted as inconclusive in part because of limitations in confidently characterizing the reaction. Technical limitations such as uneven staining (probably related to suboptimal fixation) contributed to an inconclusive interpretation in some cases; however, these technical problems were also occasionally observed in 3+ positive and negative cases.

TABLE 1. Combined Immunohistochemical and FISH HER-2/*neu* Results

	IHC Only	Total (IHC and/or FISH)
Positive	123 (16.4%)	138 (18.4%)
Inconclusive	39 (5.2%)	1 (<1%)
Negative	588 (78.4%)	611 (81.5%)
Total	750	750

IHC, immunohistochemical; FISH, fluorescence *in situ* hybridization.

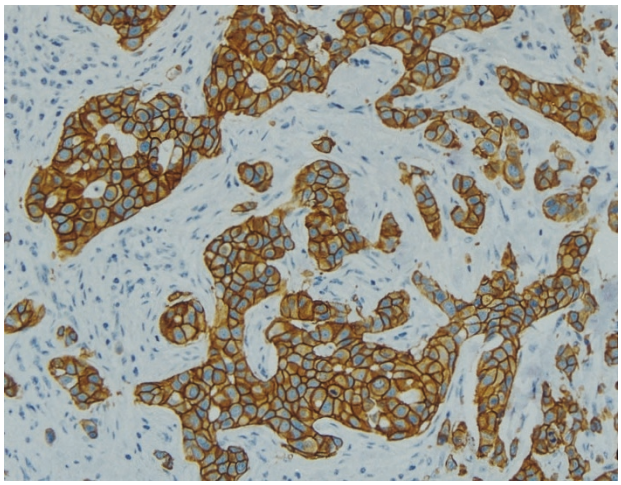

FIGURE 1. Invasive ductal carcinoma showing diffuse and uniform 3+ IHC HER-2/*neu* membrane staining (original magnification, 100×).

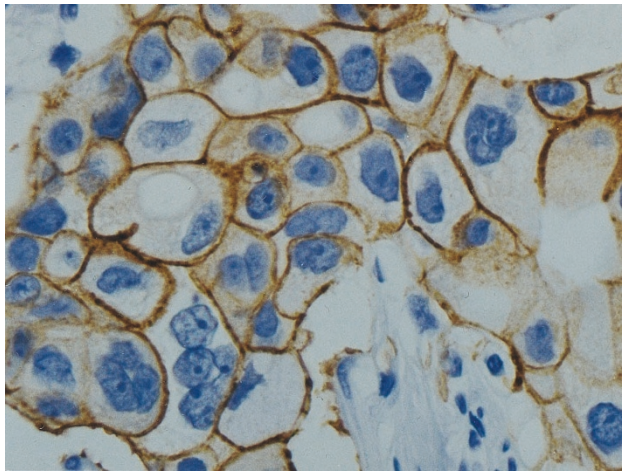

FIGURE 2. 3+ IHC HER-2/*neu* membrane staining (original magnification, 400×).

FISH Studies

All 21 3+ IHC positive cases on which validation FISH analysis was performed were amplified (0% negative FISH cases with a 95% CI of 0 to 16%; the overall CI is actually less because additional tests performed outside the study group have to date all been concordant with FISH results) (Table 2). Seventeen of the 21 3+ IHC cases exhibited more than 20 signals per nucleus (Oncor) or a ratio of more than 10 (Vysis) (Fig. 5). The remaining four cases showed amplification ranging from 8.2 to 19 signals per nucleus. Among 57 cases interpreted negative by IHC, all but one showed no amplification by FISH analysis (1.75% positive FISH cases with a 95% CI of 0.04 to 9.4%). A single case showing 1+ IHC membrane staining exhibited amplification by

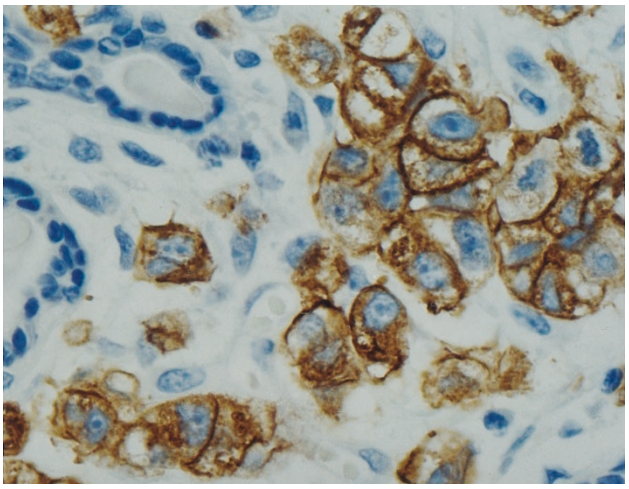

FIGURE 3. 3+ IHC HER-2/*neu* membrane and nonspecific cytoplasmic staining with negative internal control normal mammary ducts (original magnification, 400×).

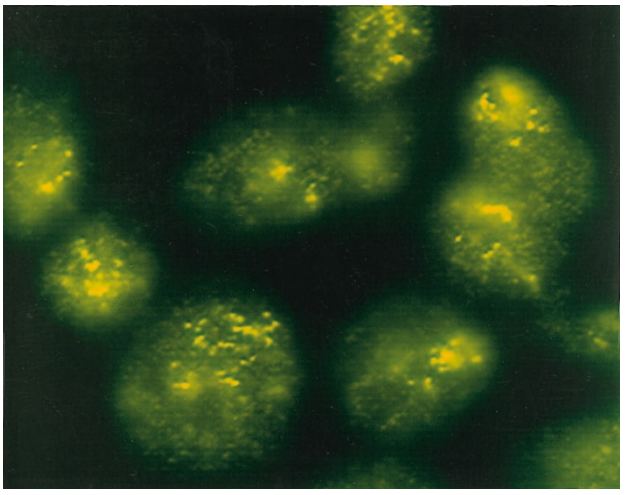

FIGURE 5. Invasive ductal carcinoma showing high HER-2/*neu* amplification by FISH (more than 20 signals per nucleus). Same case as seen in Figure 3.

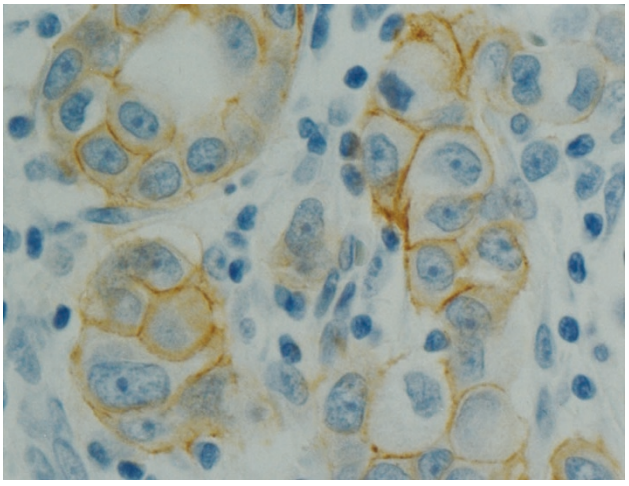

FIGURE 4. 2+ IHC HER-2/*neu* membrane staining completely encircling most tumor cells (original magnification, 400×).

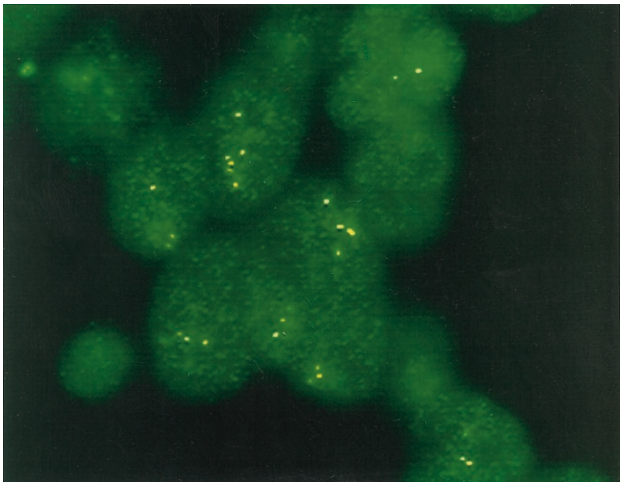

FIGURE 6. Invasive ductal carcinoma showing weak to moderate HER-2/*neu* amplification by FISH (7.2 signals per nucleus). Same case as seen in Figure 4.

TABLE 2. HER-2/*neu* FISH Studies

IHC	FISH Positive	FISH Negative	Total
3+	21	0	21
2+	14	24	39*
0–1+	1	56	57
			117

* A single case was not interpretable by FISH.
IHC, immunohistochemical; FISH, fluorescence *in situ* hybridization.

FISH (15.5 signals per nucleus). Thus, there was concordance in 77 of 78 validation FISH studies (98.7% with a 95% CI of 93.1 to 100%).

Among the 39 cases interpreted 2+ IHC inconclusive, 14 (36%) were amplified by FISH and 24 were not. One case could not be interpreted for technical reasons. The 2+ IHC cases that were amplified by FISH showed a range of positivity from 4.1 to more than 20 signals per nucleus (Fig. 6). Four cases displayed between 4 to 10 signals per nucleus, five cases between 10.1 to 15 signals per nucleus, and five cases more than 15.1 signals per nucleus.

In four additional cases 2 to 3+ IHC membrane staining was present in the ductal carcinoma *in situ* (DCIS) component of the tumor, whereas the invasive component was IHC negative (Fig. 7). We performed FISH analysis on these cases but did not include them with the 2+ IHC inconclusive cases. Two showed amplification by FISH within both the *in situ* and invasive components and two cases showed amplification within the DCIS elements but no amplification in the invasive elements. These were all interpreted as HER-2/*neu* negative in the final analysis. In all other cases with a mixture of DCIS and invasive carcinoma, the IHC reactions were similar or minimally different in both components.

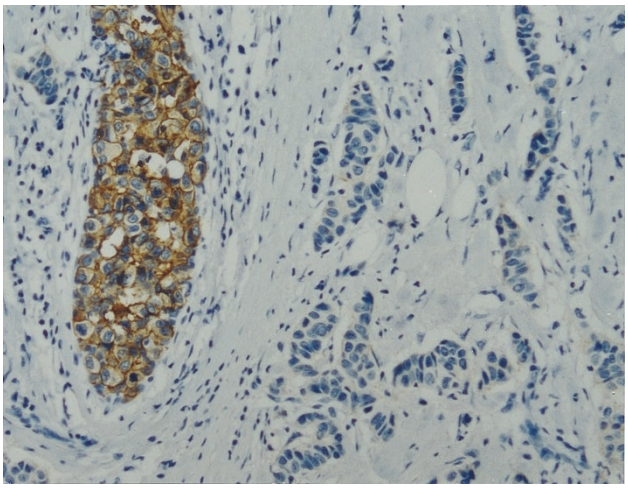

FIGURE 7. *In situ* and invasive ductal carcinoma showing discordant HER-2/neu protein expression. 3+ IHC membrane staining is noted within the DCIS (left) and negative staining within the invasive component to the right (original magnification, 100×).

Pathology

One hundred three of 304 (34%) grade 3 ductal carcinomas, 30 of 264 (11.4%) grade 2 ductal carcinomas, and three of 93 (3.2%) grade 1 ductal carcinomas were HER-2/*neu* positive (Table 3). Two lobular carcinomas among 63 studied (3.2%) were positive. All seven colloid carcinomas were HER-2/*neu* negative as were 19 additional special subtype carcinomas including nine mixed ductal/lobular carcinomas.

DISCUSSION

The currently evolving therapeutic and prognostic implications of HER-2/*neu* status in breast carcinoma have resulted in greater focus on HER-2/*neu* testing. IHC on paraffin embedded tissue and FISH are the two methodologies currently FDA-approved for use in HER-2/*neu* testing in selected circumstances and are generally available to most clinicians for such evaluation. Other methodologies, including protein blot analysis, IHC on frozen tissue, and ELISA are less practical for routine use (1, 2).

TABLE 3. HER-2/*neu* IHC/FISH: Pathology Correlations

	Total	Positive
Ductal-G1	93	3 (3.2%)
Ductal-G2	264	30 (11.4%)
Ductal-G3	304	103 (34%)
Lobular	63	2 (3.2%)
Colloid	7	0 (0%)
Other	19	0 (0%)

With χ^2 testing, the difference between G1, G2, and G3 are statistically significant ($P < .001$ overall with G1 *versus* G2, $P = .03$; G2 *versus* G3, $P < .001$; and G1 *versus* G3, $P < .001$).

IHC, immunohistochemical; FISH, fluorescence *in situ* hybridization.

IHC is by far the most popular and accessible testing modality. It directly detects HER-2/neu protein overexpression and provides very accurate results with advantages including relative ease of performance, rapid turn-around time, and relatively low cost. In large part because of unavoidable variations in tissue fixation and processing, as well as variations in testing methodologies and subjectivity in grading, it is not an ideal quantitative assay and cases occur that are difficult to interpret. FISH methodology is more accurate and quantitatively more precise, but time consuming, technically demanding, and more expensive. Because it measures HER-2/*neu* gene amplification and not protein overexpression, FISH methodology theoretically may not be completely concordant with IHC results. Although there is good correlation between HER-2/*neu* gene amplification and protein overexpression, approximately 3.5% of breast carcinomas overexpress HER-2/*neu* without amplification and a small undetermined percentage amplify HER-2/*neu* without overexpression (10–12). Direct comparisons between IHC on paraffin-embedded tissue and FISH HER-2/*neu* testing are limited, but generally show high concordance (13, 14). Concordance has been stated to be particular high between IHC on frozen tissue and FISH, probably because fixation problems are obviated (2, 10). In several studies, the discordance has been in the range of 5 to 10% of the cases, and it is not clear if performing FISH in lieu of IHC is necessary or cost effective (12, 13). In addition, despite its clear superiority as a quantitative assay, clinically validated FISH reference values have not been established (15–18).

In the present study, we used a combination testing approach to evaluate HER-2/*neu* status in breast carcinomas, utilizing IHC as a primary testing modality and FISH in IHC inconclusive cases. Although we did not use the HercepTest kit, we used the same polyclonal DAKO antibody as provided in the kit with standard heat induced epitope retrieval. This methodology was calibrated with the HercepTest and validated against the FDA-approved ONCOR HER-2/*neu* FISH assay. We scored the IHC reactions as recommended for the HercepTest, but interpreted 2+ membrane reactions as inconclusive instead of weakly positive.

Ninety-five per cent of the cases were interpretable by IHC alone and 5% were considered inconclusive. IHC reliably identified all 3+ IHC HER-2/*neu* overexpressors and all IHC negative cases (0 to 1+) with concordance with FISH findings in all but one case. One hundred twenty-three cases (16.4%) were 3+ overexpressors by IHC and another 39 cases (5.2%) were characterized as 2+ overexpressors and designated inconclusive in our study. Thus, 162 cases (21.6%) were either 2+ or 3+ over-

expressors by IHC. FISH analysis revealed 14 of 39 inconclusive 2+ IHC cases (36%) to be amplified utilizing current suggested reference values. In this study, the significance of a 2+ IHC reaction was uncertain because the majority of such cases were not amplified by FISH. Although we suspect our results would be comparable using the HercepTest, we cannot state this with certainty and additional study of this issue would be of interest.

There is limited published information on 2+ IHC reactions using the HercepTest. Forty-two per cent of 2+ HercepTests were negative by the investigational IHC CTA used in the HERCEPTIN clinical trials (8). In a brief letter, Espinoza and Anguiano (19) reported 2+ IHC reactions in 11.1% of their cases (162 of 1453) using the HercepTest, but did not give detailed FISH results on this subgroup. Among 21 of their positive HercepTests (2+ and 3+ reactions), five (24%) were not amplified by FISH, which we believe is cause for concern and is not acceptable. In this study, the 2+ IHC cases were a heterogeneous group that was usually not amplified.

Nevertheless, we believe IHC may be reliably used as the primary methodology at this time for evaluating HER-2/*neu* status. It directly measures HER-2/neu protein overexpression and reliably characterized 95% of the cases in this series in a cost-effective, expeditious fashion. The cost of the IHC test as performed in our laboratory is approximately $20 *versus* $140 for FISH analysis. Performing the HercepTest would cost about $50 (excluding capital equipment expense for the DAKO Autostainer that is required to perform the test to FDA standards). The IHC methodology circumvents the potential small percentage of false negatives and false positives with FISH that are HER-2/*neu* amplified without overexpression or HER-2/*neu* overexpressors that are not amplified. The only case in this series that was interpreted negative by IHC but was amplified by FISH was a grade 3 primary ductal carcinoma that displayed 1+ membrane staining. Repeat IHC on the primary tumor and a lymph node metastasis showed similar 1+ and focally 2+ membrane staining. The significance of this isolated discordant result is not clear. It could represent a true false negative IHC or a HER-2/*neu* amplified breast carcinoma without protein overexpression.

We also noted several additional unusual cases in which FISH analysis might be potentially misleading, especially if appropriate areas of a given slide are not evaluated. In this study, almost all cases with mixed DCIS/invasive elements exhibited concordant HER-2/*neu* IHC expression; however, in four cases the DCIS component showed 3+ membrane staining and the invasive component was negative. In two cases, the FISH findings were concordant with IHC; however, in the other two both the DCIS and invasive elements were amplified by FISH. Because HER-2/*neu* status is based on the findings within the invasive component, these cases might be considered positive based on the FISH findings if interpreted out of context. We believe, however, that the latter two cases may represent "false positive" FISH findings (amplified but not overexpressed) for several reasons. In each case, the IHC reaction showed clear 3+ membrane staining within the DCIS component and no membrane reaction whatsoever in the invasive component. The discordance in these cases was unlikely due to fixation problems because vimentin staining in these areas confirmed the antigen integrity in both instances (20). It is also known that a higher percentage of DCIS cases (up to 55% of high grade DCIS) are HER-2/*neu* positive than invasive carcinomas (21). Furthermore, as noted above, a small percentage of invasive breast carcinomas demonstrate HER-2/*neu* amplification but no detectable overexpression (11).

These two cases are unique in that a discordance in overexpression was apparent by IHC within the DCIS and invasive elements without a comparable discordance in amplification by FISH. We are not aware that this has been reported previously. IHC HER-2/*neu* discordance between the DCIS and invasive components has been noted by some authors in at least nine previously reported cases; however, all were studied by IHC and none by FISH (22–24). These cases and several similar cases evaluated apart from this study series will be reported in more detail separately. Although very unusual, these cases exemplify a potential source of error in establishing accurate HER-2/*neu* status in cases with mixed DCIS/invasive elements. Unless areas of the tissue block are preselected carefully, FISH interpretation might be misleading and possibly yield false positive results.

There was a clearcut relationship between tumor grade and HER-2/*neu* overexpression in this study (Table 3). HER-2/*neu* positivity was present in 34% of grade 3 ductal carcinomas, 11% of grade 2 ductal carcinomas, and only 3% of grade 1 ductal carcinomas. There were two positive lobular carcinomas among 63 tested (3%) and no HER-2/*neu* overexpressors among seven colloid carcinomas. The reported positivity rate in relation to tumor grade and histologic subtype is extremely variable, much like the reported overall positivity rate in breast carcinoma in general (1, 2). Other studies have reported results similar to ours, but some have not (25–30). These variations are, in part, probably related to inconsistencies in categorizing and grading breast carcinomas and to the fact that some studies that have small numbers and selected populations of breast carcinoma patients. The current study pop-

ulation includes an admixture of primary and recurrent breast carcinomas consecutively submitted for HER-2/*neu* study and thus is probably skewed with higher-grade tumors. The significant variation in HER-2/*neu* positivity among different grade ductal carcinomas and lobular carcinomas may explain some of the variation reported in HER-2/*neu* positivity between different series with variations in case mix.

Conclusion

The current study found that a combined approach using both IHC and FISH methodologies can optimize HER-2/*neu* testing on breast carcinomas. When properly calibrated, HER-2/*neu* testing by IHC on paraffin-embedded tissue is a very good first choice methodology that, in this study, reliably characterized 95% of the cases, including all 3+ positive and 0 to 1+ negative cases. Approximately 5% of the cases were interpreted 2+ by IHC (which we considered inconclusive) and were, with one exception, resolved by FISH analysis. The 2+ IHC group in this study was heterogeneous in nature with only 36% showing HER-2/*neu* gene amplification. Further studies of 2+ IHC reactions utilizing the HercepTest in parallel with FISH analysis might be of value. There is also a need for establishing a clinically validated cut-off value for HER-2/*neu* FISH amplification against which IHC may be further compared and calibrated. This would potentially allow for more accurate and clinically meaningful HER-2/*neu* testing in the future.

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Book Review

Mark HFL, editor: Medical Cytogenetics, 680 pp, New York, Marcel Dekker, 2000 ($195.00).

This compendium of cytogenetics is primarily aimed at the 550 or so physicians, Ph.D.s, clinical molecular biologists, and genetic counselors planning to take the certifying examination given by the 24th primary specialty board of the American Board of Medical Specialties. It goes without saying that I have picked up these facts from Dr Mark's book, which also contains everything else one might need if studying for the American Board of Medical Genetics. The book, however, will also be useful for residents in pathology, pediatrics, or internal medicine and for all others looking for a comprehensive coverage of this relatively young medical specialty.

The book consists of 20 chapters dealing with laboratory and clinical aspects of cytogenetics. Basic aspects of cytogenetics are systematically discussed, beginning with nomenclature, the nature of chromosomal and genetic abnormalities commonly encountered in practice, and current techniques used in the laboratories. Clinical problems that need cytogenetic work-up are presented in detail, and some are illustrated in black and white or color photographs. There are glossaries for readers less versed in cytogenetics. At the end, there is a chapter on standards for setting up a cytogenetics laboratory.

For those who do not know offhand the meaning of acronyms such as FISH, SKY, CGH, etc., this book is a godsend that should be on the reference shelf of their medical libraries. For those who want to use it as a textbook, the editor and his associates have prepared a set of study questions (included on the last 15 pages of the book). Professional cytogeneticists should have it handy for visitors or residents who are assigned to their laboratories for rotation. This is a truly multifunctional book that deserves to be made available to a wide range of readers.

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