Comparison of Immunohistochemical and Fluorescence In Situ Hybridization Assessment of HER-2 Status in Routine Practice

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Key Words: Her-2/neu; Fluorescence in situ hybridization; Immunohistochemistry

DOI: 10.1309/Q0DGL26RUCK1K5EV

Abstract

Because HER-2 expression in invasive carcinoma of the breast has well-documented ramifications for treatment and prognosis, accurate assessment of HER-2 status is critical. Comparative studies have shown high concordance rates between immunohistochemical analysis and fluorescence in situ hybridization (FISH) in cases with immunohistochemical scores of 0 or 1+ (negative) and 3+ (strongly positive) and low concordance rates among cases with immunohistochemical scores of 2+. The present study was performed to determine concordance rates in a setting more representative of routine clinical practice, in which multiple pathologists submit specimens to a single cytogenetics referral laboratory. We found a higher rate of discordance between immunohistochemical analysis and FISH (approximately 92%) in the groups with immunohistochemical scores of 2+ than reported in other studies. These results strongly support the practice of performing FISH in all cases with immunohistochemical scores of 2+, particularly in routine practice, in which interobserver variability in immunohistochemical scoring among multiple pathologists is likely to be high.

Since the first descriptions by Slamon et al[1,2] of HER-2/*neu* (*HER-2*) gene amplification in 20% to 30% of invasive carcinomas of the breast, basic and clinical research have not only elucidated its molecular structure, normal distribution and function, and potential role in oncogenesis (for review, see Rubin and Yarden[3]), but also have led to the development of targeted immunotherapy. This therapy, trastuzumab (Herceptin, Genentech, South San Francisco, CA), is a monoclonal antibody shown in clinical trials to be effective in combination with chemotherapy in patients with metastatic breast cancer that overexpressed the HER-2/neu protein (HER-2).[4,5] Patients were eligible for entry into the pivotal trials if overexpression of HER-2 in invasive carcinoma cells was documented by the Clinical Trials Assay, an immunohistochemical assay that included 2 mouse monoclonal antibodies, 4E5 and CB11.[6]

In 1998, the US Food and Drug Administration approved an immunohistochemical assay, HercepTest (DakoCytomation, Carpinteria, CA), based in part on its concordance with the Clinical Trials Assay; a variety of other antibodies also has been used to assess HER-2 status. Despite ease of use, uniform interpretation of immunohistochemical assays such as the HercepTest has proved elusive, with reports showing false-positive rates ranging from 6% to more than 50% with the HercepTest.[7-9] To decrease the interobserver variability inherent in HER-2 immunohistochemical assays, some investigators turned to fluorescence in situ hybridization (FISH) assays as an alternative, less subjective method of *HER-2* assessment.[10] Like immunohistochemical assays, FISH can be performed on formalin-fixed, paraffin-embedded tissue samples and thus can be performed at diagnosis or at relapse.

As laboratories gained experience with immunohistochemical analysis and FISH in the assessment of HER-2 status, a high degree of concordance between immunohistochemical and FISH results in cases scored immunohistochemically as 0 to 1+ (negative) and 3+ (positive) was noted, with cases scored immunohistochemically as 2+ forming a heterogeneous group thought by some to be better classified as "indeterminate" rather than "weakly positive."[11] In the light of this highly variable 2+ group, an algorithm for HER-2 testing developed: the use of immunohistochemical analysis as a screening tool, with confirmation by FISH in cases with 2+ immunohistochemical staining and/or in 1+ cases with high-grade histologic features.[12] Studies in which this algorithm was used have reported concordance rates of up to 98%.[13,14]

We performed the present study to determine the factors contributing to the lower concordance rates between immunohistochemical analysis and FISH seen in our laboratory compared with those in published comparative studies.[15-20] Thus, this study is not intended to be a comparison between the sensitivity and specificity of immunohistochemical analysis and FISH because reports of numerous excellent comprehensive studies on that issue have been published.[7,11-18,21-26] Rather, as a reference laboratory performing FISH on cases referred from a number of different pathologists, our experience might better reflect the day-to-day situation seen in a general practice setting.

Materials and Methods

Samples

Between October 2001 and March 2004, the cytogenetics laboratory at Fairview-University Medical Center, Minneapolis, MN, received 275 cases for *HER-2* FISH testing. Of these, 129 (46.9%) had immunohistochemical data available and were included in the study. The samples sent for FISH testing do not represent all of the breast cancer cases examined by the referring surgical pathology laboratories and, therefore, do not represent an accurate assessment of the *HER-2*–negative and *HER-2*–positive rates in breast carcinoma cases in our referral system.

Immunohistochemical Analysis

Immunohistochemical testing was performed in a central laboratory on formalin-fixed, paraffin-embedded tissue samples using the HercepTest (DakoCytomation, Carpinteria, CA), following the manufacturer's instructions. Briefly, this procedure includes deparaffinization and rehydration steps, followed by an epitope retrieval step in which the tissue sample is incubated in a citrate buffer solution at 90°C to 95°C for 20 minutes. The slide then is subjected to a series of alternating washes in tris(hydroxymethyl)aminomethane hydrochloride buffer and incubation steps with first a peroxidase-blocking reagent for 5 minutes and then with HER-2 primary antibody followed by a visualization reagent (dextran polymer conjugated with horseradish peroxidase and goat antirabbit immunoglobulins) for 30 minutes each, and finally with a 3,3'-diaminobenzidine chromogen solution. After a final wash, the slide is counterstained with hematoxylin.

Scoring was performed according to the manufacturer's recommendations by pathologists in a number of different practice groups, each with at least 5 years of experience in clinical practice; several of the referring pathologists had particular experience in breast pathology and immunohistochemical interpretation of HER-2 testing.

FISH Analysis

FISH was performed on formalin-fixed, paraffin-embedded tissue specimens from each of the 129 patients using the PathVysion kit (Vysis, Downers Grove, IL). Included in this kit are probes to the *HER-2* gene locus at 17q11.2-12 (labeled in SpectrumOrange) and to the centromeric region of chromosome 17 (CEP 17; labeled in SpectrumGreen). FISH was performed according to the manufacturer's instructions (after obtaining proficiency certification on completion of training by Vysis), with minor modifications as described subsequently.

Briefly, unstained 3- to 5-μm-thick paraffin sections were cut from blocks chosen by the referring pathologists and placed on positively charged slides. On receipt, the slides were placed in an oven at 90°C for approximately 5 hours, deparaffinized in xylene, and dehydrated in a series of ethanol washes. After pretreatment in 0.2N hydrochloric acid and sodium thiocyanate solutions, digestion in a protease solution for 16 minutes, and fixation in 10% neutral buffered formalin, the slides were subjected to denaturation and hybridization with 10 μL of the PathVysion probe/buffer mixture.

In some cases, an alternative pretreatment method was used, in which the hydrochloric acid and sodium thiocyanate steps were replaced by immersion in 2× saline sodium citrate at 75°C for approximately 17 minutes and digestion in 0.25 mg/mL of Proteinase K (Roche Diagnostics, Indianapolis, IN) for 15 to 17 minutes. In our experience, this latter method yields results of equal or greater consistency in a shorter period. After overnight hybridization, slides were washed, dehydrated in an ethanol series, and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride.

We analyzed 60 randomly selected nuclei, each with at least 1 *HER-2* and 1 CEP 17 signal, from different areas of invasive carcinoma; we analyzed fewer cells in cases in which a limited number of invasive malignant cells were present (eg, core biopsy specimens). The total numbers of *HER-2* and CEP 17 signals were expressed as a ratio of *HER-2*/CEP 17. Ratios of less than 2.0 were interpreted as normal and ratios of 2.0 or more as amplified.

DOI: 10.1309/Q0DGL26RUCK1K5EV

Results

Of the 129 specimens submitted to our laboratory for *HER-2* FISH testing for which immunohistochemical results were available, 23 cases (17.8%) were classified by immunohistochemical analysis as negative (0, 2 cases; 1+, 21 cases), 93 (72.1%) were classified as weakly positive (2+), and 13 (10.1%) as positive (3+). To reflect routine practice more accurately, the immunohistochemical scores were accepted without further review. Concordance between the immunohistochemical and FISH scores (defined as cases that were immunohistochemically negative/FISH nonamplified or immunohistochemically positive/FISH amplified) was found in 35 cases (27.1%) and discordance in 94 cases (72.9%) **Table 1**.

Concordance varied slightly when cases were classified by type of specimen (core biopsy vs excision or excisional biopsy) **Table 2**. Rates of discordance ranged from 62% (45/73) for excisional biopsy specimens to 79% (33/42) for core biopsy specimens. Tumor type (ie, invasive ductal or invasive lobular carcinoma) had no statistically significant effect on concordance rates. Of the 78 invasive ductal carcinomas, 25 (32%) were concordant and 53 (68%) were discordant. Of the 11 lobular carcinomas, 4 (36%) were concordant and 7 (64%) were discordant. Discordance rates varied slightly when based on tumor grade **Table 3**, although these differences were not statistically significant when cases scored immunohistochemically as 2+ were isolated. The institutions that referred more than 5 cases for FISH had concordance rates ranging from 21.9% to 37.9%.

Discussion

Despite more than a decade of research into the role of *HER-2* gene amplification in breast cancer, there has not been developed a testing modality that maximizes reproducibility and ease of use and retains diagnostic accuracy. Immunohistochemical analysis and FISH remain the tests of choice for many practicing surgical pathologists and often are used together as a 2-tiered analysis: immunohistochemical analysis alone for obviously negative (0-1+) and strongly positive (3+) cases and FISH as a confirmatory test in indeterminate or weakly positive (2+) cases. This algorithm is designed to limit the number of false-positive cases (and, therefore, limit the number of patients who would otherwise be exposed to the potential toxic effects of trastuzumab therapy) and detect the cases that might overexpress the HER-2 protein despite showing no gene amplification, a situation that has been reported in approximately 3% of cases.[10] When such an algorithm is followed, however, the laboratory performing FISH is exposed to a skewed population of cases (ie, predominantly 2+ immunohistochemical scores). It follows that the concordance rates

Table 2
Rates of Discordance Between Immunohistochemical Analysis and FISH Based on Specimen Type*

Specimen Type	Discordant
Core biopsy (n = 42)	33 (79)
Excision/excisional biopsy (n = 73)	45 (62)
Cell pellet (n = 3)	1 (33)

FISH, fluorescence in situ hybridization.
* *Discordance* was defined as cases that were immunohistochemically negative/FISH amplified or immunohistochemically positive/FISH nonamplified. Data are given as number (percentage) of discordant results. $P < .06$.

Table 3
Rates of Discordance Between Immunohistochemical Analysis and FISH Based on Tumor Grade*

Tumor Grade	Discordant
1 (n = 13)	11 (85)
2 (n = 40)	30 (75)
3 (n = 36)	19 (53)

FISH, fluorescence in situ hybridization.
* *Discordance* was defined as cases that were immunohistochemically negative/FISH amplified or immunohistochemically positive/FISH nonamplified. Data are given as number (percentage) of discordant results. $P < .05$.

Table 1
Concordance Between Immunohistochemical and FISH Results*

HercepTest Score	FISH Nonamplified (Ratio <2.0)†	FISH Amplified (Ratio ≥2.0)†	Concordance by Immunohistochemical Score‡
0 (negative)	2	0	2/2 (100)
1+ (negative)	21	0	21/21 (100)
2+ (weakly positive)	86	7	7/93 (8)
3+ (positive)	8	5	5/13 (38)
Concordance by FISH score	23/117 (19.7)	12/12 (100)	—

FISH, fluorescence in situ hybridization.
* *Concordance* was defined as cases that were immunohistochemically negative/FISH nonamplified or immunohistochemically positive/FISH amplified. For proprietary information, see the text. $P < .001$.
† FISH results are expressed as a ratio of the number of HER-2 signals to the number of centromere 17 signals. Results in the "Concordance" row are given as number concordant with immunohistochemical results/number tested (percentage).
‡ Immunohistochemical results are given as number concordant with FISH results/number tested (percentage).

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between immunohistochemical analysis and FISH obtained by such a referral laboratory might not reflect those obtained in other studies. For example, we do not routinely examine all breast cancer cases by FISH regardless of immunohistochemical score. Similarly, our concordance rates represent routine clinical practice, in which a number of different surgical pathologists from different practice groups have scored the immunohistochemical stain. Therefore, interobserver variability is likely to be higher than in studies in which consensus immunohistochemical scores are obtained before performing FISH.[15,16,18,23]

The discordance between immunohistochemical analysis and FISH seen in our laboratory highlights the differences in sensitivity and specificity of these 2 testing methods and, as noted by Paik et al,[19] might reflect the volume of the immunohistochemical assays performed in the referring surgical pathology laboratories. The discordance found in the present study might call into question the efficacy of the current testing algorithm. Our results underscore the importance of FISH testing in cases scored immunohistochemically as 2+, as the algorithm states, and because a majority of 3+ cases in the present study were not amplified by FISH, support reflex testing for cases scored immunohistochemically as 3+. Indeed, the significant discordance rate seen in the present study (approximately 86% among cases scored immunohistochemically as 2+ and 54% among 3+ cases) suggests that the use of FISH alone, as some authors have advocated,[8] would streamline testing by obviating the need for confirmatory FISH testing.

Testing by FISH alone might well be more cost-effective than the dual approach, depending on the cost of the 2 methods and the relative frequency of 2+ and 3+ cases in the patient population for a given laboratory. By using a spectrum of prices obtained from a number of different laboratories, we calculated that performing FISH alone becomes more cost-effective than performing both immunohistochemistry and FISH when the number of cases scored immunohistochemically as 2+ is 1.6 to 2.6 times the number of cases scored as 0 or 1+ and 3+ combined.

Performing FISH alone might fail to identify cases (reported to be approximately 3%) in which the HER-2 protein is overexpressed in the absence of gene amplification.[10] However, the low immunohistochemical-FISH concordance rates seen in the present study suggest that a large proportion of cases scored immunohistochemically as 3+ might be being misclassified, making it difficult to determine in our population how many patients have overexpression without amplification. Because some of our referring surgical pathology laboratories recently have adopted the "FISH-alone" approach, we are currently working to determine the impact of this change in practice.

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References

1. Slamon DJ, Clark GC, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*. 1987;235:177-182.
2. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244:707-712.
3. Rubin I, Yarden Y. The basic biology of HER2. *Ann Oncol*. 2001;12(suppl 1):S3-S8.
4. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783-792.
5. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol*. 2002;20:719-726.
6. Mass R, Sanders C, Kasian C, 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;20:75a. Abstract 291.
7. Pauletti G, Dandekar S, Rong HM, 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.
8. Tubbs RR, Pettay JD, Roche PC, et al. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. *J Clin Oncol*. 2001;19:2714-2721.
9. Jacobs TW, Gown AM, Yaziji H, et al. Specificity of HercepTest in determining HER-2/neu status of breast cancers using the United States Food and Drug Administration–approved scoring system. *J Clin Oncol*. 1999;17:1983-1987.
10. Pauletti G, Godolphin W, Press MF, et al. Detection and quantitation of HER-2/*neu* gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene*. 1996;13:63-72.
11. Ridolfi RL, Jamehdor MR, Arber JM. HER-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence in situ hybridization approach. *Mod Pathol*. 2000;13:866-873.
12. Field AS, Chamberlain NL, Tran D, et al. Suggestions for HER-2/neu testing in breast carcinoma, based on a comparison of immunohistochemistry and fluorescence in situ hybridisation. *Pathology*. 2001;33:278-282.
13. Kobayashi M, Ooi A, Oda Y, et al. Protein overexpression and gene amplification of c-erbB-2 in breast carcinomas: a comparative study of immunohistochemistry and fluorescence in situ hybridization of formalin-fixed, paraffin-embedded tissues. *Hum Pathol*. 2002;33:21-28.
14. 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.

DOI: 10.1309/Q0DGL26RUCK1K5EV

15. 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.
16. 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.
17. 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.
18. McCormick SR, Lillemoe TJ, Beneke J, et al. HER2 assessment by immunohistochemical analysis and fluorescence in situ hybridization. *Am J Clin Pathol.* 2002;117:935-943.
19. Paik S, Bryant J, Tan-Chiu E, et al. Real-world performance of HER2 testing: National Surgical Adjuvant Breast and Bowel Project experience. *J Natl Cancer Inst.* 2002;94:852-854.
20. Roche PC, Suman VJ, Jenkins RB, et al. Concordance between local and central laboratory HER2 testing in the Breast Intergroup Trial N9831. *J Natl Cancer Inst.* 2002;94:855-857.
21. Gancberg D, Lespagnard L, Rouas G, et al. Sensitivity of HER-2/neu antibodies in archival tissue samples of invasive breast carcinomas. *Am J Clin Pathol.* 2000;113:675-682.
22. Bartlett JMS, Going JJ, Mallon EA, et al. Evaluating HER2 amplification and overexpression in breast cancer. *J Pathol.* 2001;195:422-428.
23. Birner P, Oberhuber G, Stani J, et al. Evaluation of the United States Food and Drug Administration–approved scoring and test system of HER-2 protein expression in breast cancer. *Clin Cancer Res.* 2001;7:1669-1675.
24. Lebeau A, Deimling D, Kaltz C, et al. HER-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. *J Clin Oncol.* 2001;19:354-363.
25. Thomson TA, Hayes MM, Spinelle JJ, et al. HER-2/neu in breast cancer: interobserver variability and performance of immunohistochemistry with 4 antibodies compared with fluorescent in situ hybridization. *Mod Pathol.* 2001;14:1079-1086.
26. Press MF, Slamon DJ, Flom KJ, et al. Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. *J Clin Oncol.* 2002;20:3095-3105.

DOI: 10.1309/Q0DGL26RUCK1K5EV