**Abstract**

**CONTEXT:** Overexpression of HER-2/neu oncogene in breast cancer patients is correlated with disease free survival (DFS) and overall survival (OS). The most commonly used methods for the detection of HER-2/neu status are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). However, there is a lot of controversy with regard to the best method. Most of the FISH studies chose arbitrary cut-off levels for positive results (10%) and had no validation. **AIM:** In order to address these issues, we designed a pilot study of 38 samples with known IHC status representing all 4 categories. **SETTINGS AND DESIGN:** FISH was performed using Vysis Pathvysion™ probe. For validation, 5 cases of reduction mammoplasty were analyzed using same protocols. **RESULTS:** Our results showed significant discordance between FISH and IHC. The rate of discordance was much higher in the 0, 1+, and 2+ categories compared to published literature. This could be due to the lower cut-off rates for positive amplification established by validation in our study (5.7% vs 10%). Our analysis showed that FISH positive and IHC negative patients have a poor prognosis in terms of DFS and OS compared to FISH negative and IHC negative patients. Further, our results also showed that IHC in comparison to FISH has a comparable specificity (98%), but has a very low sensitivity (46%). **CONCLUSION:** Based on these results, we consider FISH to be the gold standard for detecting HER-2/neu status in breast cancer.

**Key Words:** Breast cancer, HER-2/neu, Amplification, IHC, FISH, DCIS, Pathvysion™, Validation.

**Introduction**

The human epidermal growth factor receptor-2 (HER2) is one of the four members of type 1 growth factor receptor family, designated HER1 to HER4 (c-erbB-1 to c-erbB-4). The interaction between HER monomers and its various ligands (e.g. epidermal growth factor, transforming growth factor α) results in a diversity of signal transduction from the intracellular tyrosine kinase domain that regulate cell growth, survival and differentiation.

The HER2 (also known as c-erbB-2 or neu) proto-oncogene is mapped to chromosome 17q12-21.32 and is amplified and overexpressed in approximately 25% of invasive breast carcinomas. This alteration predicts shortened disease-free survival (DFS) and poor clinical outcome in cases of breast carcinoma. HER-2/neu overexpression has significant therapeutic implications because of availability of trastuzumab (Herceptin, Genentech, San Francisco, CA), a recombinant humanized mouse monoclonal antibody directed against the HER-2/neu protein for the management of metastatic breast cancer. Patients with HER-2/neu amplification have shown favorable clinical response with trastuzumab. Optimal use of this therapy, however, requires accurate determination of HER-2/neu status, the method for which has not been standardized.

There have been several approaches to accurately determine the HER-2/neu status. The most relevant and clinically practical assays are: immunohistochemical...
detection of HER-2/neu protein (IHC) and detection of HER-2/neu gene amplification using fluorescence in situ hybridization (FISH). While both these methods are widely used in clinical laboratories, each has several advantages and disadvantages. Various studies have been done to compare and correlate the results of IHC with FISH, but the results are ambiguous at best and are quite contrasting with some studies showing IHC being superior while others showing FISH being the best assay for determining the HER-2/neu status.9,10,14-19

Because of the contradictory and confusing data, we have designed a retrospective study to evaluate the efficacy of FISH method strictly adhering to American College of Medical Genetics (ACMG) guidelines20 for standardization of the method, and compared these results to IHC in breast cancer. This strictly validated FISH method with an objective cut-off value for scoring HER-2/neu amplification would in turn provide our patient population with reliable measure of HER2/neu status.

Materials and Methods

Experimental Design

Formalin-fixed, paraffin-embedded tissue blocks from 38 patients with breast cancer were obtained from the archives of the surgical pathology division from the year 1998 to 2001. The histopathological characteristics of these patients are listed in Table 1. Immunohistochemistry (IHC) for HER-2/neu had already been done at the time of initial diagnosis on all of these patients. Patients were selected in such a way that all the IHC categories of 0, 1+, 2+ and 3+ were represented in equal numbers. An institutional review board approval (IRB) was obtained before the paraffin blocks were selected. FISH was performed on sections from the same paraffin blocks on which IHC had been done.

Immunohistochemistry

For IHC studies, serial 3-4 mm tissue sections were prepared and rabbit polyclonal antibody C-erb 2 (cat # A 0485) from DAKO (Carpinteria, CA) was used. Pretreatment of the slides, optimal dilutions of the antibodies and optimal detection techniques were determined by use of conditions as described in the commercial information. In brief, sections were baked at 58°C for 30 minutes followed by deparaffinization in Xylene for 5 minutes, twice. The sections were then rehydrated in 100% and 95% ethanol for 3 minutes twice each and were rinsed in deionized water. Standard

heat induced epitope retrieval procedure with target retrieval solution was performed for 20 minutes. After cooling to room temperature, sections were rinsed in deionized water. Sections were placed in 3% hydrogen peroxide block for 30 minutes, washed in water and placed in phosphate-buffered saline for 5 minutes. Primary antibodies were diluted to a concentration of 1:3200 and the sections were incubated with the antibody for 30 minutes. After rinsing, immunodetection was performed with the DAKO LSAB®2 system. After rinsing the sections, they were counterstained with hematoxylin for 30 seconds.

Fluorescence In Situ Hybridization (FISH)

For FISH studies, serial 3-4 mm tissue sections were cut from the same blocks that were used for IHC studies. Vysis Pathvysion™ (Vysis Inc., Downers Grove, IL) probe was used and the specimen preparation, hybridization and post-hybridization washes were performed as per the manufacturer’s suggestions. Briefly, after deparaffinizing the unstained sections in Hemo-De (Fisher Scientific, Houston, TX) for 10 minutes three times, the sections were dehydrated in 100% ethanol for 5 minutes twice and air-dried. Subsequently, the sections were immersed in 0.2N HCl for 20 minutes, rinsed in purified water for 3 minutes and washed in 2xSSC for 3 minutes. Sections were then treated with sodium thiocyanate solution at 80°C for 30 minutes followed by rinsing in purified water for 1 minute and washing in 2xSSC for 5 minutes twice. The sections were then subjected to protease digestion (2 mg/ml) at 37°C for 30 minutes, washed in 2xSSC for 5 minutes twice and air-dried. The sections were then fixed in 10% buffered formalin for 10 minutes, again washed in 2xSSC for 5 minutes twice and air-dried. The sections were denatured in 70% formamide/2xSSC for 3 minutes at 75°C and dehydrated in grades of alcohol (2 minutes each in 70%, 85% and 100%). Hybridization with PathVysion™ (Vysis) probe was carried out overnight. Next morning, the sections were washed in 50% formamide/2xSSC for 1 minute at 42° C, followed by 2xSSC/0.1% NP-40 for 2 minutes at room temperature. The sections were counterstained with DAPI and stored at 4°C until analysis. All slides were analyzed using a Nikon E400 Eclipse microscope (Nikon, New York, NY) equipped with epifluorescence and triple band pass filters. Images were captured and analyzed using Applied Imaging Cytovision™ software (Applied Imaging, Santa Clara, CA).

Scoring and Analysis

Semi-quantitative scoring criteria were used based on
the intensity and percent of cells showing membrane staining according to the guidelines for HER-2/neu scoring. When no staining was observed or membrane staining was observed in less than 10% of the tumor cells, such specimens were classified as category 0 (negative). A score of 1+ (negative) represented a faint/barely perceptible membrane staining in more than 10% of the cells, however the staining was only seen in part

Table 1: Characteristics of our patient population

<table>
<thead>
<tr>
<th>#</th>
<th>IHC</th>
<th>Age</th>
<th>Race</th>
<th>Diagnosis</th>
<th>ER</th>
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*= discordant cases
of the membrane. A score of 2+ (positive) represented a weak to moderate complete membrane staining in more than 10% of the cells. A score of 3+ (positive) represented moderate to strong complete membrane staining in more than 10% of the cells. IHC scoring was previously done by a responsible pathologist and re-verified by one of the authors (SG).

For FISH analyses a minimum of 100 nuclei were scored for each of the cases by two experienced investigators independently (SG and GV). Signal enumeration was performed with a triple-band pass filter in a blinded fashion such that both the scorers did not know the IHC scores. The slides were also coded randomly by a person not involved in this study. FISH scores were expressed as ratio of HER-2/neu signals (spectrum orange) per chromosome 17 signals (spectrum green). If the ratio of spectrum orange to spectrum green was ≥ 2, then the sample was considered to have HER-2/neu amplification and if the ratio was <2, then the sample was considered as normal. The slides were first scanned at 100x magnification to ensure objective evaluation of signals. Precise signal enumeration of high level amplification (>20x ratio) was not possible because of coalescing fluorescence of signal clusters.

Validation for FISH

For validation purposes as recommended by the ACMG guidelines, 5 cases of mammoplasty from a normal cohort without histological abnormality and with no known history of cancer were used. FISH with Pathvysion™ probe was carried out using the experimental protocol including scoring and data analysis that were identical to breast cancer cases. Analytical sensitivity was calculated based on the percentage of cells showing a ratio of orange to green of ≥2.

Results

FISH analysis was successful in all the cases attempted. In mammoplasty samples, HER-2/neu interphase analysis showed 98.42% ± 2.07 (range 95.00 – 100.00%) of the cells with orange to green ratio of less than 2, thus indicating no amplification, while 1.58% ± 2.07 (range 0.00 – 5.00%) of the cells showed orange to green ratio of equal to or more than 2 thus indicating HER-2/neu amplification (Table 2). To distinguish true amplification from background noise, cut off levels were set at 2 SD above the mean percentage of control cells with orange to green ratio of equal to or more than 2 thus giving a cut off level of 5.72%.

Correlation of IHC to FISH

The results of IHC correlation to FISH are presented in Tables 3-6. Our analysis showed that based on our analytical sensitivity, there is a poor correlation between IHC and FISH with the discordance rates ranging from 10 to 80%. The only exception was the IHC score of 3+ where except for 1 false positive result with IHC, there is strong concordance with FISH (Table 6).

In those cases that were scored as 0 on IHC, FISH showed three cases with amplification (3/8 = 38% discordance) (Table 3). In those cases that were scored as 1+ on IHC, FISH showed eight cases with amplification (8/10 = 80% discordance) (Table 4). In those patients with IHC score of 2+, FISH showed amplification in only 5 cases (5/10 = 50% discordance) (Table 5). With IHC score of 3+, FISH showed only one false positive case (1/10 = 10% discordance) (Table 6). Assuming FISH is the gold standard for accurate determination of HER-2/neu status, our results showed that IHC has a sensitivity of 43% in comparison to FISH while the specificity is 94%.

Since FISH assays have been known to show inter
Discussion

Our results show a poor concordance between the IHC and FISH results, for the IHC 0, 1+ and 2+ categories. Published reports have documented that the 1+ and 2+ categories are the most difficult to assess and FISH should be considered in these cases.\(^\text{10,18,22-26}\)

Our results with a very high discordance of 50-80% (for IHC 2+ and 1+) certainly strengthen this argument. Several reasons could account for the low sensitivity with the IHC assay. Since the assay is directed towards the detection of protein, the technical considerations such as pre-analytical tissue processing, reagent variability, antigen retrieval and very subjective scoring might adversely affect the result. A FISH assay directed at the gene itself might overcome all these drawbacks. FISH has several advantages over IHC such as ease of use, reproducibility and very objective scoring criteria. As shown by several studies, FISH is highly reproducible and reliable with very limited reagent variation.\(^\text{27-29}\) FISH assay has a very high specificity of 100% and sensitivity of 96-98% compared to IHC which has a very low sensitivity of 42.8% as seen in our results. Unlike IHC, FISH offers a unique advantage of quantifying inter-observer variation. IHC being very subjective, is prone to wide variation in interpretation, thus further complicating the results. FISH on the other hand being quantitative, has high inter-observer correlation as shown in our results.

Figure 1: Graph showing interobserver variation for FISH analysis

The discordance rate seen in our results is higher than the published literature, for the 0, 1+ and 2+ categories. This could be because we have designed our assay in a way to validate and determine the sensitivity so that objective cut-off levels can be established for accurate interpretation. We believe that just as validation is essential for any other FISH assay, as recommended by ACMG, FISH for Her-2/neu should also be validated by the laboratory performing the test.

Most published reports set an arbitrarily chosen cut-off for observer variability, we have analyzed our data using Pearson-r Correlation Coefficient analysis for such variability. This analysis showed excellent correlation (r = 0.9545) (Figure 1) between the two scorers for the FISH data, indicating that the high rate of discordance is not due to inter observer variation.

\[\begin{array}{cccccc}
\text{Case} & \text{IHC score} & \text{HER2/neu: Chromosome 17 signal ratio} \\
\hline
& \leq 2 & 2 & 3 & 4 & \geq 5 \\
1^* & 1+ & 21.80 & 32.40 & 35.10 & 9.70 & 1.00 \\
2^* & 1+ & 75.50 & 10.50 & 10.50 & 2.50 & 1.00 \\
3^* & 1+ & 92.50 & 7.50 & 0.00 & 0.00 & 0.00 \\
4^* & 1+ & 79.00 & 12.50 & 3.00 & 2.50 & 3.00 \\
5 & 1+ & 98.50 & 0.50 & 1.00 & 0.00 & 0.00 \\
6^* & 1+ & 2.00 & 28.20 & 27.80 & 29.40 & 12.60 \\
7 & 1+ & 99.00 & 0.00 & 1.00 & 0.00 & 0.00 \\
8^* & 1+ & 93.00 & 6.00 & 1.00 & 0.00 & 0.00 \\
9^* & 1+ & 92.00 & 7.50 & 0.50 & 0.00 & 0.00 \\
10^* & 1+ & 76.00 & 8.40 & 15.10 & 0.00 & 0.50 \\
\end{array}\]

\(\text{Table 4: Comparison of FISH with IHC 1+}\)

\[\begin{array}{cccccc}
\text{Case} & \text{IHC score} & \text{HER2/neu: Chromosome 17 signal ratio} \\
\hline
& \leq 2 & 2 & 3 & 4 & \geq 5 \\
1^* & 2+ & 59.00 & 27.00 & 8.50 & 2.00 & 3.50 \\
2 & 2+ & 90.50 & 6.00 & 3.50 & 0.00 & 0.00 \\
3^* & 2+ & 95.50 & 3.50 & 1.00 & 0.00 & 0.00 \\
4 & 2+ & 92.50 & 6.50 & 0.00 & 0.00 & 1.00 \\
5^* & 2+ & 96.00 & 4.00 & 0.00 & 0.00 & 0.00 \\
6^* & 2+ & 96.50 & 3.50 & 0.00 & 0.00 & 0.00 \\
7^* & 2+ & 97.00 & 2.00 & 0.00 & 0.00 & 1.00 \\
8^* & 2+ & 96.00 & 2.00 & 1.00 & 0.50 & 0.50 \\
9 & 2+ & 0.00 & 1.00 & 17.50 & 27.50 & 54.00 \\
10 & 2+ & 93.50 & 3.50 & 1.50 & 1.50 & 0.00 \\
\end{array}\]

\(\text{Table 5: Comparison of FISH with IHC 2+}\)

\[\begin{array}{cccccc}
\text{Case} & \text{IHC score} & \text{HER2/neu: Chromosome 17 signal ratio} \\
\hline
& \leq 2 & 2 & 3 & 4 & \geq 5 \\
1^* & 3+ & 0.00 & 0.00 & 0.00 & 0.00 & 100.00 \\
2^* & 3+ & 0.00 & 0.00 & 0.00 & 0.00 & 100.00 \\
3 & 3+ & 0.50 & 0.00 & 0.00 & 0.00 & 99.50 \\
4 & 3+ & 0.00 & 0.00 & 0.00 & 0.00 & 100.00 \\
5 & 3+ & 2.60 & 49.00 & 23.80 & 8.80 & 1 5.80 \\
6 & 3+ & 72.70 & 9.10 & 1 3.50 & 3.70 & 1.00 \\
7 & 3+ & 99.00 & 0.00 & 1.00 & 0.00 & 0.00 \\
8^* & 3+ & 93.00 & 4.50 & 0.00 & 0.00 & 0.00 \\
9^* & 3+ & 95.50 & 4.50 & 0.00 & 0.00 & 0.00 \\
10 & 3+ & 2.00 & 4.50 & 13.50 & 2.00 & 78.00 \\
\end{array}\]

\(\text{Table 6: Comparison of FISH with IHC 3+}\)

\(\text{Table} = \text{discordant cases}\)
Carcinoma. Carcinoma than in lower-grade DCIS with invasive alone and also in higher-grade DCIS with invasive reported to be more frequent in higher-grade DCIS into consideration while evaluating the status of HER-2 strongly believe that DCIS component should be taken interpreted as 0 or 1+ with IHC. However, we cases with DCIS component would have been for scoring with IHC protocols. Thus, some of these Overexpression of HER-2/neu better clinical outcome. treatment with trastuzumab (Herceptin) might have a therapeutic usefulness of Herceptin in any given patient. or negative will have a negative impact on the categorizing the patients as being HER-2/neu positive, increasing the discordant rate from 50% to 80%. These rates are similar to those published in the literature. The clinical outcome in some of these discordant cases clearly showed that FISH is a superior prognostic factor, since majority of our cases where IHC is negative and FISH is positive had a poor outcome. Of the 11 cases that were false negative by IHC (IHC 0 and 1+; FISH positive for amplification) follow-up was available in 10 cases (Table 1). Of these 10 cases 3 patients developed metastatic disease (30%). Interestingly all these 3 cases were in the category of IHC 1+. There were 7 cases that were false positive by IHC (IHC 2+ and 3+; FISH negative for amplification), follow-up was available in 5 cases (Table 1). All the 5 patients were doing well with no evidence of disease after at least a minimum follow up of 2 years. Especially interesting is the false positive case of 3+ with IHC and no amplification by FISH. This patient was diagnosed to have lobular carcinoma which is unlikely to show HER-2/neu amplification. Falsely categorizing the patients as being HER-2/neu positive or negative will have a negative impact on the therapeutic usefulness of Herceptin in any given patient. Accurate assessment of HER-2/neu status in patients with false negative IHC assessment and subsequent treatment with trastuzumab (Herceptin) might have a better clinical outcome.

Overexpression of HER-2/neu within the ductal carcinoma in situ (DCIS) component is not considered for scoring with IHC protocols. Thus, some of these cases with DCIS component would have been interpreted as 0 or 1+ with IHC. However, we strongly believe that DCIS component should be taken into consideration while evaluating the status of HER-2/neu for prognostic purpose, since high grade DCIS has a very high probability of becoming invasive carcinoma. HER-2/neu gene amplification has been reported to be more frequent in higher-grade DCIS alone and also in higher-grade DCIS with invasive carcinoma than in lower-grade DCIS with invasive carcinoma. Thus, it is possible that HER-2/neu amplification in DCIS might serve as a good prognostic indicator to suggest progression to invasive metastatic carcinoma. Of the 8 cases with DCIS component in our study, 5 (63%) cases were discordant between IHC and FISH. Of these 5 cases, follow-up information is available in 4 cases. Of the 4 cases, two cases developed metastatic disease while the remaining two patients are doing well. The fact that 50% of patients with DCIS and HER-2/neu amplification in our study developed metastatic disease supports our contention that HER-2/neu amplification in DCIS might be a good marker for the potential development of metastatic disease.

Conclusion

In summary, based on our results, we consider FISH to be the gold standard for determination of HER-2/neu status in breast cancer. We also propose the validation of Her-2/neu by the laboratories doing the test. Even though the PathVysion™ assay is FDA approved, we strongly believe that each laboratory should validate and determine the sensitivity of each lot of the probe as recommended by the ACMG for other DNA probes. This would eliminate the reagent variability, offer an objective cut-off for a positive result and hence result in an accurate interpretation of the test. Even though our numbers are small, we feel that HER-2/neu amplification in DCIS component should also be considered in deciding treatment options.

References


