erb-b2 Amplification by Fluorescence In Situ Hybridization in Breast Cancer Specimens Read as 2+ in Immunohistochemical Analysis

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Abstract

We conducted this study to ascertain the prevalence of erb-b2 gene amplification in breast cancer specimens read as 2+ in immunohistochemical analysis. Slides from patients with metastatic or recurrent breast cancer were eligible for fluorescent in situ hybridization (FISH) study if they were read as 2+ immunohistochemically for erb-b2 by a certified pathologist. The PathVysion kit (Vysis, Downers Grove, IL) was used for FISH studies. Amplification of the erb-b2 gene was defined as an erb-b2/CEP17 (chromosome 17 centromere) ratio of 2 or more in 30 tumor cells counted. From May 2003 to June 2004, 221 slides were submitted from 24 hospitals around the island. Of 216 successful hybridizations, 96 (44.4%) were determined to be erb-b2 amplified. In addition, the topoisomerase IIα (T2α) gene was coamplified in 11 (21%) of 53 and deleted in 8 (15%) of 53 erb-b2 amplified cases. The erb-b2 gene amplification rate was very high in cases determined to be 2+ by immunohistochemical analysis; therefore, determination of erb-b2 status by FISH in cases scored 2+ immunohistochemically is strongly recommended.

Breast cancer ranks as the second most common cancer for women in Taiwan. The crude incidence and mortality rates are 42.7 and 10.6 per 100,000 population, respectively, with 4,642 new cases and 1,149 deaths reported in the year 2000.1 In the same time period, the incidence and mortality rates for whites, African Americans, and Asian/Pacific islanders in the United States were 150 and 26.7, 119 and 35.4, and 97 and 12.6 per 100,000 population, respectively.2 The local breast cancer incidence peaks between the ages of 40 and 50 years, with a mean age at occurrence of 47 years.1 Taiwanese women have a lower incidence of breast cancer but a higher probability of dying of it once a diagnosis has been made,1 and this cancer occurs more commonly in midlife, with significant financial and social implications.

Since the first report of erb-b2 as a poor prognostic factor for breast cancer in 1987,3 the significance of this oncogene as an adverse prognostic factor has been noted in many other cancers, such as ovary, lung, stomach, and pancreas.3-6 Amplification of the erb-b2 gene or overexpression of the erb-b2 protein has been detected in 10% to 30% of breast cancers3,7-16 and, together with the associated topoisomerase IIα (T2α) gene aberrations, shows no change over time,7,9 after treatment,7 and no difference between primary and metastatic lesions.8,9 Although no difference in erb-b2 status has been noted between males (15% overexpression in immunohistochemical analysis, 11% amplification in fluorescence in situ hybridization [FISH]) and females10 or between whites and African Americans,11 its expression in young Koreans (≤45 years; 47.5%)17 and in a small study of Chinese women in Taiwan (43.2%)18 has been somewhat higher.

Analysis of the Cancer and Leukemia Group B 8541 protocol concluded that erb-b2 overexpression identified patients...
most likely to benefit from high doses of adjuvant doxorubicin, suggesting it to be a predictive marker for anthracycline response. The molecular basis of this phenomenon has been attributed to concurrent amplification of the T2α oncogene, with increased expression of T2α enzymes, a target for the anthracyclines, and this has been confirmed to be the significant target for doxorubicin response in a prospective clinical trial. Molecular genetic studies in erb-b2–amplified primary breast cancers demonstrated coamplification of the T2α gene in 44% and deletion in 42% of cases, with no change in gene copy number in all non–erb-b2–amplified tumors.

The US Food and Drug Administration has approved the HercepTest (DAKO, Carpinteria, CA) and Ventana Pathway (Ventana, Tucson, AZ) as immunohistochemical assays for detection of erb-b2 protein expression in breast cancer and PathVysion (Vysis, Downers Grove, IL) as a FISH method to detect erb-b2 gene amplification. Many studies have since reported more than 90% concordance between the immunohistochemistry and FISH tests, but both tests usually were performed in the same laboratory with a large volume of samples processed annually. In May 2003, the National Comprehensive Cancer Network (Jenkintown, PA) updated its oncology practice guidelines, recommending FISH over immunohistochemical analysis to determine erb-b2 status, based on studies documenting comparable trastuzumab (Herceptin) response and survival duration for FISH-amplified cases, regardless of immunohistochemical status.

Recent reports on very large comparative studies between immunohistochemical analysis and FISH for detection of erb-b2 give a more balanced view on the relationship between the 2 assays. Researchers at the Memorial Sloan-Kettering Cancer Center, New York, NY, found a very high concordance rate between immunohistochemical scores of 0, 1+, and 3+ and FISH data in 2,279 cases studied; however, 25% of cases scored as 2+ immunohistochemically demonstrated gene amplification in FISH studies. Investigators at Impath (Los Angeles, CA) reported 20% of immunohistochemical scores as 3+ in 116,736 specimens and 22.7% FISH amplification in 16,092 specimens, and in 6,556 specimens with both tests done, gene amplification was found in 4.1% of specimens with an immunohistochemical score of 0, 7.4% with a score of 1+, 23% with a score of 2+, and 92% with a score of 3+. An Australian study reporting on 1,536 cancers found immunohistochemical scores of 3+ in 12% of cases, of which 98% were amplified by FISH, and 2+ in 13% of the cases, of which 23% were amplified by FISH. Large-scale studies recommend a FISH assay for specimens with an immunohistochemical score of 2+ because interlaboratory reproducibility of HER-2/neu status is poor by immunohistochemical analysis without standardization of the procedure.

Five years ago, the Taiwan Co-operative Oncology Group (TCOG) launched a correlative study for erb-b2 through its pathology subcommittee. The detection of erb-b2 at member hospitals performed by immunohistochemical analysis was correlated with HercepTest immunohistochemical analysis and PathVysion FISH performed at the molecular genetics laboratory of the National Health Research Institutes (Taipei, Taiwan). Although the data were not published, in 88 specimens (negative, 53; positive, 35) from 11 hospitals, the concordance rate was 100% for all specimens with immunohistochemical scores of 0 (FISH–) and 3+ (FISH+). It was decided to launch a nationwide FISH confirmation study for any case of metastatic breast cancer with erb-b2 with an immunohistochemical score of 2+ determined by a certified pathologist, with the addition of T2α gene evaluation in the latter part of the 1-year study.

**Materials and Methods**

**Patients**

All patients entered into this laboratory study had metastatic or recurrent breast cancer and an immunohistochemical score of 2+ for erb-b2 and would not be eligible for insurance-covered trastuzumab therapy unless gene amplification was detected by the FISH study. Specimen slides with an immunohistochemical score of 2+ (specimens with scores 0, 1+, and 3+ excluded) as determined by in-house pathologists from any region in Taiwan could be submitted to the FISH laboratory for evaluation on request from the patient’s attending physician and after obtaining informed consent from the patient. A FISH assay for HER-2/neu is very expensive and is not performed routinely because it is not covered by health insurance; it is performed only if the patient bears the cost. There were no restrictions placed on the antibodies selected for the previous immunohistochemical procedure. Standard criteria for a 2+ reading as established for the HercepTest were adhered to for interpretation. Blank specimen slides were labeled with the patient’s pathology number, but no other information was available to the laboratory performing the FISH studies.

**Fluorescence In Situ Hybridization**

The FISH study for erb-b2 and the T2α gene copy number was performed using the PathVysion and T2α/CEP 17 dual-color probes (Vysis) according to the manufacturer’s instructions. Hybridization occurred with the orange erb-b2/T2α probe and green CEP 17 (staining chromosome 17 centromere) probe. Specimens 3 to 4 μm thick were placed on silane-coated slides, baked at 56°C for 2 hours, deparaffinized, pretreated (1.0 mol/L of sodium thiocyanate in 1.0 mol/L of tris(hydroxymethyl)aminomethane hydrochloride, pH 8.0), and digested with pepsin (Sigma, St Louis, MO).
(4 mg/mL in 0.9% sodium chloride solution, pH 2.0) for 12 to 14 minutes. They then were fixed (in 10% buffered formalin) and denatured in solution (7 parts formamide, 1 part standard saline citrate, and 2 parts water; pH, 7.0-7.5) for 5 minutes. After dehydration with 70%, 85%, and 100% ethanol, they were hybridized overnight at 37°C.

Immunohistochemical slides were not available for cross-reference with the FISH slides, and we were entirely dependent on the pathologist at the hospital of origin for adequate and suitable slide selection. The entire slide had to be scanned to detect cells with gene amplification. Amplification of erb-b2 was determined if the ratio of copies of the erb-b2 gene (spectrum orange) and copies of the chromosome 17 centromere (spectrum green) was 2.0 or more in at least 30 tumor cells counted. A ratio of 5.0 or more was scored as high amplification and a ratio of 2.0 or more but less than 5.0 as low amplification. Normal specimens showed a ratio of less than 2.0, and closer to 1.0 [Image 1].

Personnel performing the FISH (C.L. and J.M.L.) had been trained and certified at Vysis headquarters in the United States. Our molecular genetics laboratory has performed 200 to 400 FISH assays annually since 1999.

**FISH Report**

To ensure objective interpretation of the FISH assay, all FISH reports are given with 2 to 3 microscopic images to include at least 30 cells, so that the pathologist or clinician responsible for the case can check the validity of the FISH interpretation. The images were taken by using a Nikon Coolpix 990 (Nikon, Tokyo, Japan) camera attached to an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan). The FISH interpretation is recorded as amplified or not amplified.

**Results**

Samples were submitted during a period of 13 months, May 2003 to June 2004. The specimens were submitted from 24 hospitals around the island of Taiwan. Many of the hospitals are TCOG affiliated and had submitted slides for the correlation study 5 years ago. Slide submission per hospital ranged from 1 to 54 slides; 10 hospitals submitted fewer than 3 slides.

Of the 221 specimens on which FISH was performed for detection of the erb-b2 gene, 5 were not placed on coated slides and slipped off during processing, resulting in inclusion of 216 slides in the final report. Amplification was found in 96 (44.4%) of 216 specimens, and in the last 53 cases analyzed, 11 (21%) showed coamplification of the T2α gene and 8 (15%) showed deletion of the T2α gene [Table I]. In all, 19 (36%) of 53 specimens demonstrated T2α gene abnormalities; 34 (64%) had normal copies of the T2α gene. The FISH+ rate for the 24 hospitals ranged from 0% to 80%. When hospitals were grouped into 4 geographic regions for Taiwan, the FISH+ rates for north, central, south, and eastern Taiwan were 39% (35/89), 33% (13/40), 49.5% (55/111), and 38% (5/13), respectively.

FISH assays were performed on 20 occasions during the 13-month period, processing between 1 and 30 slides manually on each occasion. Two working days are required for processing each batch of specimens.

Low amplification was demonstrated in 39% of the cases (37/96), leaving 61% (59/96) with gene amplification that was increased 5-fold or more. Aside from the 5 specimens lost during processing, our hybridization success rate was 100%, with no need to repeat the analysis of any slides. False-positive results would not be possible unless the specimen on the slide was not that of the patient’s breast cancer. There is the possibility of false-negative data, which would arise if the recut slides were devoid of tumor, so the judgment of the in-house pathologist for slide selection was all-important for providing adequate and representative slides.

**Discussion**

This was a prospective study to ascertain erb-b2 gene amplification status by FISH in cases scored immunohistochemically as 2+. The 221 specimens were submitted by 24 hospitals around the island of Taiwan. Immunohistochemical studies were performed with various antibodies (including HercepTest) to erb-b2 in the pathology departments of the 24 hospitals and read as 2+ by the in-house pathologist. Patient selection was limited to those with recurrent or metastatic disease, because should there be confirmation of erb-b2 gene amplification, the patient would be eligible for trastuzumab therapy.

Of 4,500 new breast cancer cases reported each year,1 if around 20% to 40% have metastatic disease at diagnosis, around 25% were erb-b2+, and 12% of the total cases had immunohistochemical scores of 2+ (from the Australian
**Image II** Fluorescence in situ hybridization (FISH) studies of the HER-2/neu and topoisomerase IIα genes. The green dots represent the chromosome 17 centromere and the orange dots, HER-2/neu or topoisomerase IIα genes. **A**, **B**, and **C**, HER-2/neu FISH showing high amplification (**A**), low amplification (**B**), and no amplification (**C**) of the signals. **D**, **E**, and **F**, Topoisomerase IIα FISH showing 2 copies of topoisomerase IIα/EP17 (**D**), amplification as indicated by multiple orange signals (**E**), and deletion (**F**); orange signals are less than green signals.
Amplification of the \( \text{erb-b2} \) gene was startlingly high (44.4\%) for cases with immunohistochemical scores of 2+ compared with the 10\% to 25\% reported in the literature.\(^\text{12,16,25,26}\) Amplification of the \( \text{erb-b2} \) gene was 5-fold or more in 61\% of cases (56/96) and less than 5-fold but 2-fold or more in 39\% (37/96), so that low amplification was not the reason for a negative immunohistochemical interpretation. False-positive gene amplification would be unlikely unless there was slide mislabeling by in-house pathology department staff or slides with an immunohistochemical score of 3+ were submitted for FISH confirmation, which would be a redundant act for the clinician because an immunohistochemical score of 3+ signed off by a certified pathologist already enables a patient with metastatic breast cancer to receive trastuzumab, and that was why the onus was on the clinician to initiate and request a FISH assay. A clinician could be biased clinically in selecting cases from the pool of immunohistochemical scores of 2+ that he or she would consider to be more likely to have \( \text{erb-b2} \) gene amplification and send only those specimens for further study. In fact, there would be a higher probability of false-negative gene amplification interpretation should there be no tumor present on the submitted unstained slide.

An interpretation of immunohistochemical results of 2+ for \( \text{erb-b2} \) can be affected by various factors—specimen factors, antibody and methods factors, and human factors. The targeted patient population for this study consisted of newly diagnosed patients with metastatic disease and patients whose disease recurred after a disease-free interval, so that fixation methods could not be standardized in advance.

A small yet significant discrepancy has been reported for immunohistochemical results obtained from formalin- and Bouin-fixed specimens.\(^\text{13}\) The antibody chosen for immunohistochemical study can affect the final interpretation as a result of differential sensitivities.\(^\text{13}\) An immunohistochemical score of 3+ was recorded for 48 (12.2\%) of 394 cases in one laboratory but increased to 109 (27.7\%) of 394 in another laboratory with antigen retrieval,\(^\text{13}\) but that manipulation potentially could increase false-positive readings.\(^\text{13}\)

Complete agreement among 5 pathologists in interpretation of HER-2/neu protein overexpression using the DAKO HercepTest was only 48\% in a study done in a medical center, and distinguishing weakly (2+) from strongly (3+) positive results showed agreement in 59\% of positive cases. Although interpretation definitely can improve with experience accumulated from the large volume of specimens processed in a medical center,\(^\text{29}\) it may be much more difficult in smaller pathology laboratories staffed by 1 or 2 pathologists to acquire the finesse in interpretation of immunohistochemical studies. Therefore, it is not difficult to understand why concordance in \( \text{erb-b2} \) interpretation between local and central laboratories for the Intergroup N9831 study was only 74\%.\(^\text{23}\) In the first 104 patients enrolled in the National Surgical Adjuvant Breast and Bowel Project B-31 ascertained to have \( \text{erb-b2} \) tumors, 18\% of cases could not be confirmed in a central laboratory.\(^\text{22}\) This can only lead to the conclusion that when precise determination of \( \text{erb-b2} \) status is needed, it should be done in a centralized laboratory.

During the latter part of the study, \( T2\alpha \) gene status also was evaluated and found to be amplified or deleted in 19 (36\%) of the 53 cases, which is much lower than the 40\% to 90\% \( T2\alpha \) gene abnormalities reported for \( \text{erb-b2} \) 3+ cases.\(^\text{22}\)

The determination of \( \text{erb-b2} \) status in breast cancer is of the utmost importance, not only in metastatic disease to determine eligibility for trastuzumab therapy but also in the adjuvant setting for selection of appropriate adjuvant chemotherapy.\(^\text{19,20,30}\) Based on our findings, we recommend FISH over immunohistochemical analysis for \( \text{erb-b2} \) evaluation. However, in view of cost, facility, and personnel constraints, at least all cases with immunohistochemical scores of 2+ should be confirmed by a FISH assay, preferably in a centralized laboratory. Up to 50\% of cases with an immunohistochemical score of 3+ were reported as FISH– in 1 study,\(^\text{31}\) and FISH was recommended for all such cases, but this scenario is rather unusual. In view of the high false-negative findings for \( \text{erb-b2} \) from this study, the FISH assay for cases with \( \text{erb-b2} \) immunohistochemical scores of 2+ is offered as a free service to all patients with breast cancer in Taiwan with recurrent disease.

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