*Original Article*

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# Assessment of c-erbB2 Expression by IHC and FISH in Invasive Breast Cancer— A Comparative Study: Experience from a Single Institute

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**Abstract**

*Introduction:* An accurate assessment of c-erbB2 expression in invasive breast cancer (IBC) has become crucial to precisely recognize the candidates to be treated with Trastuzumab. Presently, fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) are most commonly employed methods for evaluating c-erbB2 status. Recent literature has documented a strong correlation between the two c-erbB2 diagnostic analyzes. However, discordance between both the assays has been rarely reported. Therefore, we aimed to compare and correlate FISH and IHC results for c-erbB2 expression in Indian breast cancer patients. *Material and methods:* A total of 388 formalin fixed, paraffin embedded blocks of invasive breast cancer were retrospectively evaluated for c-erbB2 status by IHC (DAKO) and FISH (PathVysion dual-probe system) and results were compared. *Results:* 92.5% cases with IHC 3+ score showed significant concordance with the FISH results; while c-erbB2 gene amplification was noted in 48.3% of IHC 2+ cases. A large number of referral cases in the study group and variation in pre-analytical and analytical factors have attributed in escalating the number of indeterminate cases expressing c-erbB2 gene amplification. Additionally, an inverse correlation was revealed between ER/PR expression and c-erbB2 status. *Conclusion:* The results of the current study established a high degree of concordance between IHC and FISH in Indian breast cancer patients with 3+ immunoreactivity. However, reflex testing by FISH is recommended for IHC equivocal cases in order to avoid false results related to technical and interpretation errors, usually encountered while performing an immunohistochemical assessment.

**Keywords:** Breast cancer; c-erbB2; Concordance; Fluorescent in situ hybridization (FISH); Immunohistochemistry (IHC).

## Introduction

Human epidermal growth factor-2 (HER-2) oncogene, also known as c-erbB2 or HER-2/neu and it's close relatives HER-1, HER-3 and HER-4 belong to the HER family of tyrosine kinase receptors.[1] It modulates cell growth, survival, and differentiation through multiple signal transduction pathways.[2] Acquired genetic defects lead to the aberrant functioning of the c-erbB2 gene and consequently protein overexpression in the cell membrane, which facilitates the acquisition of advantageous properties of a malignant cell. Therefore, it has been implicated in the pathogenesis of various human malignancies such as breast carcinoma, gastric carcinoma, esophageal carcinoma, ovarian carcinoma and others.[3] Amplification of c-erbB2 DNA has been stated in around 15–30% of invasive breast cancers (IBCs) and possesses both prognostic and predictive significance. Aforementioned alterations in the c-erbB2 oncogene are associated with an aggressive tumor phenotype, increased lymph node metastasis and reduction in disease-free and overall survival in breast carcinoma.[2,4]

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However, this endured a turnaround with the arrival of targeted therapy against HER-2 gene in the form of the humanized mouse anti-HER-2 monoclonal antibody Trastuzumab (Herceptin) and Lapatinib, dual receptor tyrosine kinase inhibitor. This breakthrough has significantly improved clinical outcome in c-erbB2 positive breast cancer patients. Unfortunately, Trastuzumab therapy is expensive and imports certain serious adverse effects like cardiac toxicity especially when used in combination with anthracyclines. Furthermore, Herceptin and Lapatinib have been found to be effective only in tumors possessing true c-erbB2 gene amplification.[5,6] Hence, it becomes a prerequisite to accurately identify the subset of patients who would benefit from this novel mode of therapy.

Currently, c-erbB2 expression in IBCs can be determined either by testing for gene amplification by polymerase chain reaction (PCR), Southern blot and fluorescence in situ hybridization (FISH); messanger RNA (mRNA) using Northern blot or protein overexpression via immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA) and Western blot on cytosols. In addition, recently described techniques for c-erbB2 detection include Chromogenic in situ hybridization (CISH) and Silver enhanced in situ hybridization (SISH). Among all the aforestated techniques, SISH is the most sensitive for recognizing c-erbB2 DNA amplification.[7,8] Nonetheless, the most commonly employed Food and Drug Administration (FDA) approved procedures for determining the c-erbB2 status include IHC and FISH.[9] Immunohistochemistry (IHC) is a semi-quantitative assay, which is economical, less labor intensive and more commonly available but is prone to exhibit reproducibility issues and disparity in test results due to technical differences and interobserver variability. On the other hand, FISH outweighs the drawbacks of IHC owing to its quantitative nature.[10,11] American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommends evaluation of patients with borderline IHC result by FISH to ascertain c-erbB2 status.[12] Several studies have attempted to draw a comparison between the results of these two techniques, so as to determine the "gold standard" for c-erbB2 testing, but, were unable to arrive at a conclusion.[13-16] Although the controversy continues over the most appropriate methodology to evaluate c-erbB2 status, there is clear consensus that a very strong correlation exists between IHC and FISH. However, discordance between both the assays have been rarely reported.[16,17]

Further, the status of steroid hormone receptors, primarily estrogen (ER) and progesterone (PR), is prognostically important as it plays a crucial role in the management of IBC. The development of anti-hormonal therapies such as Tamoxifen, have drastically improved the disease-free survival in women with ER/PR positive breast cancers. Apart from c-erbB2, ASCO/CAP has also approved IHC for determining steroid hormone receptor immunoreactivity. Despite the profound correlation, there exists a minor degree of disparity among the two hormonal receptors.[13,16,18]

To the best of our knowledge, there are not too many Indian studies correlating the IHC profiles of ER, PR, and c-erbB2 with each other and comparing the two c-erbB2 diagnostic analyses. With this impetus, the present study was undertaken to understand the relationship among the two hormone receptors and the results of IHC and FISH for c-erbB2 gene expression in patients with IBC at our institution.

## Materials and Methods

The present retrospective cross-sectional study conducted on 775 patients with a histomorphologically confirmed diagnosis of invasive breast cancer (including both in-hospital and referral cases) registered during the period of January 2011 till December 2013 in the Department of Pathology, Kasturba Medical College, Manipal. The patients' information was retrieved from the pathology and hospital records according to the prepared checklist which included age, ER and PR status, c-erbB2 protein overexpression and c-erbB2 gene amplification by FISH. This study was approved by the Manipal Institutional Ethical committee (IEC no 15.49).

### *Case selection*

All formalin fixed, paraffin embedded blocks from the abovementioned cases, which were scored as equivocal (2+)/positive (3+) by immunohistochemistry (IHC) were eligible for the study. Therefore, among the 775 patients, 388 were included and the remaining 387 cases were omitted from the study, as they were scored as negative (0/1+).

### *Immunohistochemical analysis*

All the cases were immunohistochemically evaluated for ER and PR, and c-erbB2 protein overexpression. Sections of four micrometer

thickness were obtained from blocks with adequate and well preserved invasive breast carcinoma, carefully mounted on pre-treated poly-L-lysine-coated slides and incubated overnight at 37°C. Deparaffinization of the sections was done via two changes of xylene, then dehydrated using absolute alcohol followed by rehydration through a series of decreasing alcohol concentrations. Thereafter, endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol and finally epitope retrieved by heating the slides at 125–127°C for 30 seconds, pressure 21–25 psi in 10 mmol/L citrate buffer (pH 6) using a water bath. Polyclonal anti-rabbit c-erbB2 primary antibody (DAKO, Glostrup, Denmark) was applied. The Envision Kit (DAKO) was employed for introduction of the secondary antibody and the reaction signals were recognized with 3,3'-diaminobenzidine (DAB) followed by light nuclear counter staining with Mayer's hematoxylin. The 2013 guidelines of the ASCO/CAP were used for the interpretation of staining and c-erbB2 protein overexpression was scored as 0 (negative), no stain or faint and incomplete membrane staining in ≤10% of the tumor cells; 1+ (negative), barely perceptible and incomplete membrane staining in >10% of the tumor cells; 2+ (equivocal/indeterminate/weakly positive), weak to moderate incomplete circumferential membrane staining observed in >10% of the tumor cells or intense and complete circumferential membrane staining in ≤10% of the tumor cells; 3+ (positive), strong and complete circumferential membrane staining in >10% of the tumor cells.[19,20] The application of this scoring system has varying interpretations that depend on the quality and quantity of reaction, the type of antibody used, and the observer evaluation.[8]

For immunohistochemical evaluation of estrogen/progesterone receptor, pre-staining and endogenous enzyme blocking processes were performed identical to HER-2 staining. Primary antibody clones utilized in our institute for ER and PR were DAKO EP1 and DAKO PgR636 respectively. The best-preserved and best-stained areas of the sections were assessed. Interpretation of nuclear intensity and proportion of invasive cancer cells that displayed staining was done as per Quick score.[21] For the current study, ER/PR positivity was defined as nuclear staining in >10% of tumor cells and all other results, i.e. nuclear staining observed in <10% of tumor cell nuclei was regarded as negative.[22] All the tests were interpreted in conjunction with positive and negative controls and if required, were repeated. The controls were previously tested positive and negative test samples.

***FISH for c-erbB2 gene amplification***

Fluorescence in situ hybridization was performed on 335 cases, which comprised of weakly positive (score 2+) and positive cases (score 3+) as 53 cases did not consent to undergo assessment for c-erbB2 gene amplification. The sample was outsourced to Oncoquest Laboratories Limited, Bangalore, India. FDA approved PathVysion HER-2 DNA probe test kits (Abbott Laboratories, Abbott Park, IL, USA) were employed for FISH. This kit comprises of a dual colored probe: the locus-specific identifier (LSI) HER-2 DNA probe, specific for c-erbB2 gene locus (17q11.2-q12) labeled in Spectrum Orange and chromosome enumeration probe (CEP) 17 DNA probe labeled in Spectrum Green, specific for the alpha satellite DNA sequence at the centromere of chromosome 17 (17p11.1-q11.1). FISH analysis was conducted on formalin fixed, paraffin embedded sections placed on acid treated, and double poly-L-lysine covered glass slides. Then, the cellular double-stranded DNA was denaturated into single strands, which were later hybridized with the PathVysion probes. The unbound probes were removed by multiple washes and the nuclei were counterstained with DAPI (4,6 diamidino-2-phenylindole). Sections were scored instantaneously using an upright fluorescence microscope prepped with suitable excitation and emission filters to allow visualization of the signals. The interpretation was performed on interphase cells of the specimen. The determination of the presence of c-erbB2 gene amplification was based on the counting of immunofluorescent signals for HER-2 and CEP17 within the nuclei of the tumor cells. Latest 2018 ASCO/CAP recommendations for scoring HER-2 gene amplification by dual-colour FISH are as follows: a positive test result is indicated by HER2/CEP17 ratio ≥ 2.0 and an average of more than or equal to 4.0 HER-2 copy number signals per cell and the criteria for negativity is an average of less than 4.0 HER-2 signals per cell with an HER2/CEP17 ratio of < 2.0. The recent guidelines also propose that for all other results addition workup is required before rendering a definitive diagnosis.[20] In the current study, at least 20 interphase nuclei from cells in a homogeneous and contiguous malignant population; showing a minimum of one green and one orange signal, were enumerated for each of the cases. Results were scored as positive/amplified (HER2:CEP17 ratio ≥2.0 with an average HER2 copy number of either <4.0 or ≥4.0 signals/cell; HER2:CEP17 ratio < 2.0 with an average HER2 copy number ≥6.0 signals/cell), equivocal (HER2:CEP17 <2.0 with an average HER2 copy number ≥4.0 and <6.0 signals/cell) and

negative/non-amplified (HER2/CEP17 ratio <2.0 with an average HER2 copy number <4.0 signals/cell) according to ASCO/CAP 2013 guidelines.[19]

### *Statistical evaluation*

All statistical analyses were performed using Statistical package for social science (SPSS) version 24 for Windows (IBM Inc, NY). The concordance and correlation between the immunohistochemical silhouette of ER, PR & c-erbB2 and the two c-erbB2 diagnostic techniques (IHC & FISH) were evaluated by calculating percent agreements. In addition, contingency tables were also analyzed using the Pearson's chi-square test to identify significant associations between different variables. All statistical tests were two-sided and a *p*-value of <0.05 was considered significant.

## Results

### *Clinical parameters*

The analysis of 388 IBCs revealed that the patients belonged to the 27–82 age group with a median of 49 years and a standard deviation of ±10.5 years. All except two (2/388; 0.5%) were female (386/388; 99.5%) patients. 224 (57.73%) patients were ≤50 years while 164 (42.26%) were >50 years of age.

### *FISH evaluation*

Of the 388 cases, FISH for detecting c-erbB2 gene amplification was performed on 335 cases as the remaining 53 cases did not approve for the investigation due to various reasons. C-erbB2 was amplified by FISH in more than half of the cases (214/335, 63.9%) whereas it was unamplified in 35.2% cases (118/335). Three cases (0.9%) were reported as inconclusive/equivocal and were excluded from statistical analysis. Therefore, the study group evaluated for correlating of c-erbB2 status with other parameters comprised of 332 patients.

### *IHC evaluation*

All the 388 cases were immunohistochemically assessed for hormone receptor status and c-erbB2 protein overexpression. Fifty percent (194/388) cases expressed positivity for ER receptor while less than one-third (120/388, 30.92%) cases were PR-positive. Statistical evaluation disclosed a direct association ($p < 0.05$) between estrogen and progesterone receptors.

Furthermore, immunohistochemical staining for c-erbB2 revealed a substantial number of cases (255/388, 65.72%) exhibiting 2+ (equivocal/indeterminate) reactivity and 34.37% (133/388) cases showed 3+ (positive) reactivity.

### *Association of c-erbB2 expression with age and hormonal state*

Statistical evaluation of the study group revealed 64.8% (127/196) patients with age ≤50 years were amplified with c-erbB2 gene and 63.9% (87/136) patients categorized above the age of 50 were also FISH-amplified. Although, no significant correlation ($p > 0.05$) between the two age groups and c-erbB2 gene amplification was noted.

With regard to the association of hormonal state with c-erbB2 status, a large subset of ER-negative cases (84.5%, 153/181) were FISH-amplified compared to 40.3% (61/151) among ER-positive cases with $p < 0.001$. 78.6% (191/243) of PR-negative cases were also amplified with c-erbB2 gene whereas a significant number of PR-positive (74.1%, 66/89) cases were non-amplified with a compelling statistical difference ($p < 0.001$). Additionally, 19.2% (64/332) cases were ER+ PR+ and FISH-negative whereas a greater part of the study population (151/332, 45.5%) was ER-PR- and FISH-positive. Thereby, signifying an inverse correlation ($p < 0.05$) between the hormonal receptors and c-erbB2 geneamplification. However, our study also unveiled a small number of cases (21/332, 6.32%) amplified for c-erbB2 DNA and expressing immuno-positivity for both ER and PR receptors and 7.8% (26/332) were triple negative (ER- PR- and FISH-negative) (Table 1).

### *Comparison between c-erbB2 expression by IHC and FISH*

ASCO/CAP recommends FISH as the standardized method for detecting c-erbB2 DNA amplification. Hence, it was considered as a gold standard in our study while correlating both the diagnostic assays. The comparison between IHC scores and the FISH results have been tabulated (Table 2). Analysis of 332 cases revealed 121 with an IHC score of 3+ and 211 with an IHC score of 2+. Amongst the 121 positive IHC cases, 112 (92.5%) were FISH-amplified for c-erbB2 DNA and very few cases (9/121, 7.5%) were non-amplified. While the assessment of 211 indeterminate IHC cases, showed amplification in 102 (48.3%) cases whereas more than half of the cases (109/211, 51.6%) did not demonstrate c-erbB2 gene amplification (Table 2). Further, the

**Table 1:** Correlation of c-erbB2 expression with age, ER and PR status

| Parameter | | C-erbB2 status by FISH – number (%) | |
|---|---|---|---|
| | | Amplified | Non-amplified |
| Age (in years) | <50 (*n* = 196) | 127 (64.8%) | 69 (35.2%) |
| | >50 (*n* = 136) | 87 (63.9%) | 49 (36.0%) |
| ER status | Positive (*n* = 151) | 61 (40.4%) | 90 (59.6%) |
| | Negative (*n* = 181) | 153 (84.5%) | 28 (15.4%) |
| PR status | Positive (*n* = 89) | 23 (25.8%) | 66 (74.1%) |
| | Negative (*n* = 243) | 191 (78.6%) | 52 (21.3%) |

**Table 2:** Comparison of IHC and FISH results for detection of c-erbB2 expression

| C-erbB2 protein overexpression by IHC | C-erbB2 FISH amplified | C-erbB2 FISH non-amplified | Concordance by IHC | Discordance by IHC |
|---|---|---|---|---|
| Positive (3+) (*n* = 121) | 112 | 9 | 112/121 (92.5%) | 9/121 (7.5%) |
| Equivocal (2+) (*n* = 211) | 102 | 109 | 102/211 (48.3%) | 109/211 (51.6%) |

concordance and discordance between IHC and FISH results were evaluated. The concordance rate was defined as the number of FISH-amplified cases with an immunostaining score of 3+ or 2+ divided by the sum of immunohistochemically positive (3+) and equivocal (2+) cases. In addition, the discordance rate was the ratio of the number of immunohistochemically discrepant 3+ or 2+ cases (IHC positive or equivocal but non-amplified) and the sum of cases with immunostaining score 3+ and 2+.[23] In our study, the concordance between FISH results and IHC for scores of 3+ and 2+ was 92.5% and 48.3% respectively, while the discordance noted between the two assays for immunohistochemically positive (3+) and equivocal (2+) cases were 7.5% and 51.6% respectively ($p < 0.001$). We also analyzed the data by merging the samples with immunostaining score 2+ and 3+ and the rate of concordance and discordance observed were 64.4% (214/332) and 35.5% (118/332) respectively.

## Discussion

Assessment of c-erbB2 gene status has become crucial while reporting of invasive breast cancer. IHC and FISH are the two FDA-approved methods commonly employed in clinical practice for testing of c-erbB2 expression. However, there exists a small degree of disparity between both the assays. Therefore, this study was undertaken to recognize and elucidate the agreement and disagreement among assays of c-erbB2 protein overexpression and c-erbB2 gene amplification (i.e. IHC and FISH).

In the present study, more than half of the sample population (63.9%) was found to express amplification for c-erbB2 DNA. This outcome was in concordance with the results of Payandeh et al.[23] However, the percentage of FISH-amplified patients reported in our study was indeed greater with regard to other Indian and western studies.[6,14,24-26.] This could be considered a plausible referral bias as our institution is a tertiary care center catering to numerous cancer patients. Also, the omission of IHC negative (1+) cases from our study group, leads to a relative increase in the proportion of patients expressing c-erbB2 gene amplification. Very few cases (0.9%) were observed to be FISH-equivocal, which was within the range specified by ASCO/CAP guidelines.[14]

Notably, in our study a large fraction (64.8%) of younger patients were revealed to possess amplified c-erbB2 gene, which was also substantiated by other investigators.[6,24,28] In addition, a considerable number (63.9%) of patients with age >50 years were also FISH-positive. Nonetheless, a significant association was not established between the two age groups and c-erbB2 gene expression.

Regarding the relationship between hormonal receptors and c-erbB2 status, an inverse correlation was noted. These observations were consonant with those reported by Panjwani et al., Eswarachary et al., Mostafa et al., and Prati et al.[6,24,27,29] The rationale behind these observations may be attributed to an intricate network of cross-talk between estrogen and growth factor receptor tyrosine kinase, i.e. c-erbB2.[30] On the contrary, 18.3% cases did express co-positivity for ER and c-erbB2 receptors in our study. Massarweh et al., Shou et al., and Osborne et al. postulated that IBCs with amplified c-erbB2 gene are more likely to be exhibit de novo resistance to Tamoxifen, due a surge in ER co-activator AIB1 triggered by c-erbB2

cross-talk with ER signaling pathways. AIB1 boosts the estrogen agonistic activity of the selective ER modulator, thus facilitating proliferation and survival of tumor cells.[30–32] Consequently, the subset of breast cancer harboring immuno-positivity for ER and high-level gene amplification are certain to display an unfavourable tumor phenotype and less liable to benefit from endocrine therapy.[33] We also identified a small percentage (7.8%) of triple negative patients. As per the existing literature, such patients are known to be associated with an aggressive clinical course, rapid metastatic spread and poor response to targeted therapies.[33]

A huge number (92.5%) of our cases with IHC score of 3+ were FISH-amplified. Thereby, implying a high level of concordance between IHC and FISH. This was incoherent with prior national and international studies as well as ASCO/CAP guidelines.[6,14,23–26,34,35] Alternatively, 7.5% of the cases with immunostaining score of 3+ were unamplified by FISH. This rate of discordance was similar to those reported by Eswarachary et al. and Owens et al.[24,35] The putative reasons for IHC false positivity include excess antigen retrieval, increase in receptor expression without genetic alterations due to transcriptional or post-translational activation, artifactually elevated sensitivity of immunohistochemical assays, single copy over-expression of the c-erbB2 gene at the mRNA transcription level and/or beyond and gene amplification below the detection level of the FISH assay.[6,24,34,36,37] According to the references cited in the current study, the range of disagreement rate between IHC and FISH in immunohistochemically positive cases lies between 0–16%.[6,23-26,35,38] However, an Indian study authored by Makroo et al. reported a high degree of non-conformance of 29.5% among the two diagnostic analyses assessing c-erbB2 expression.[37]

Gene amplification was noted in 48.3% of IHC equivocal cases. This was disproportionate to the results of previous studies, that revealed an amplification range of 6–25%.[6,27,39-41] Further, ASCO/CAP guidelines reported c-erbB2 gene amplification in 23.9% of IHC 2+ cases.[14] The possible justification for incongruity in our results may be attributed to a large number of referral cases and variation in pre-analytical and analytical factors such as type of surgical specimen, duration of fixation (recommended cold ischemia time is less than one hour), quality of tissue fixative (ideally 10% neutral buffered formalin with pH 7.4 should be used), method of tissue processing, magnitude of antibody dilution and interobserver variability in IHC interpretation.[6,24,27,38,42,43] In addition, Lewis et al. stated that IBCs with 2+ immunoreactivity are likely to undergo clonal evolution, thereby exhibiting intratumoral heterogeneity; which also accounts for gene amplification in IHC equivocal cases.[44] Therefore, the latest 2018 ASCO/CAP recommendations mandates reflex testing by FISH in IHC equivocal cases.[20]

Again, the congruency between IHC and FISH was computed by combining the samples with 2+ and 3+ immunoreactivity and we recorded a drastic drop in the concordance rate from 92.5% to 64.4%. Our observation was in accordance with the findings of Payandeh et al., Tsuda et al. and Yaziji et al.[23,41,45] The significant fall in the concordance rate was due to the fact that, a substantial number of immunohistochemically equivocal cases (51.6%) were unamplified by FISH. In contrast, Panjwani et al. recorded a rise in the agreement rate from 80.1 to 87.7%, when IHC 2+ cases were included as most of their equivocal cases (66.6%) showed amplification for c-erbB2 gene.[6]

Lastly, one of the main limitations of our study was the exclusion of immunohistochemically negative cases. We do not recommend FISH testing for c-erBb2 status in IHC 0/1+ cases and firmly abide by the guidelines laid down by ASCO/CAP; which clearly mandates reflex testing by FISH in IHC 2+ cases. Also, considering the financial burden on Indian patients with invasive breast cancer, an additional expensive investigation (i.e. FISH) would further trample their livelihood. Another limitation is that the authors were blinded by the pre-analytical variables like fixation time and tissue processing, which could play havoc with interpretation of IHC and FISH results.

Timely diagnosis of breast cancer is not only important from the treatment and prognosis perspective, but also important from medicolegal point of view. Delay in diagnosis provide an opportunity to the patients and relatives to seek redress through the courts. According to Ward CJ et al.[46] breast carcinoma is a leading source of medicolegal litigations and failure or delay in diagnosis were the reasons for those litigations. Though the newer diagnostic techniques like FISH are available for the diagnosis of breast cancer, appropriate processing techniques, interpretation ability as well as timely diagnosis are essential to avoid potential legal litigation.

## Conclusion

To conclude, the results of the current study established a high degree of concordance between IHC and FISH in Indian breast cancer patients with 3+ immunoreactivity. However, reflex testing by FISH is recommended for IHC equivocal cases in order to avoid false results related to technical and interpretation errors, usually encountered while performing an immunohistochemical assessment. An accurate detection of c-erbB2 status would permit the patient to undergo appropriate treatment. Nonetheless, we advocate IHC as an economical and feasible initial step for HER-2 testing in patients with invasive breast cancer.

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