Standardized Assessment of the HER2 Status in Breast Cancer by Immunohistochemistry

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Abstract
Immunohistochemistry (IHC) is widely used in surgical pathology, but it has been plagued by problems with reproducibility and lack of standardization resulting in poor concordance between laboratories. In particular, inaccuracy of routine human epidermal growth factor receptor 2 (HER2) testing in breast cancer patients has been a major issue. In 2006 this led the American Society of Clinical Oncologists (ASCO) and College of American Pathologists (CAP) to charge an expert panel with developing recommendations for HER2 testing. After subsequent publication and adoption of these guideline recommendations through dissemination of best practices, variation in clinical practice is expected to diminish and result in improved accuracy.

In this article, we review the role of genomic HER2 alterations in the development and treatment of breast cancer, highlight the importance of accurate and reproducible HER2 testing, and discuss practical approaches to standardize HER2 testing by IHC. Pre-analytic and analytic variables are addressed, and a practical algorithm for test interpretation is introduced.

Keywords: Breast cancer, HER2, immunohistochemistry, standardization, test interpretation

Published data from clinical trials suggest that only those breast cancer patients whose tumors demonstrate HER2 protein over-expression and/or gene amplification are likely to benefit from targeted therapy with trastuzumab. Since breast cancer is a disease with significant clinical diversity, an individualized approach to management and treatment decisions for each patient is necessary. A major clinical challenge is correctly identifying patients who would potentially benefit from adjuvant treatments, which would include chemotherapy, endocrine therapy, and also targeted therapy for patients with human epidermal growth factor receptor 2 (HER2)-positive disease. In current clinical practice, breast cancer biomarkers, including the estrogen receptor (ER) and HER2, are routinely assessed to help select patients who are appropriate candidates for treatment regimens that target these major molecular pathways of disease progression. However, the identification of patients who are likely to benefit from these targeted approaches remains challenging and requires that the assays for these biomarkers be as accurate as possible, given their role in determining optimal treatment. This review will focus on standardizing the immunohistochemical evaluation of HER2 protein expression in order to help ensure accurate and reproducible results.

Breast Cancer and the HER2 Genomic Alteration
Among new breast cancer patients, 15% to 20% will develop tumors that harbor a genomic alteration involving the HER2 gene locus. This alteration results in amplification of the region on chromosome 17 containing this proto-oncogene.

The protein product of the HER2 gene is a member of the HER-family of growth factor receptors involved in the complex regulation of proliferation, cell growth, and survival. Gene amplification is an early event in tumor development for this subset of breast cancers, drives HER2 gene and protein expression, and results in a marked increase in the number of HER2 receptor molecules at the membrane of tumor cells. The profuse over-expression of HER2 promotes dimerization of members of the HER-receptor family and activation of intracellular signaling cascades that drive cellular proliferation, promote angiogenesis, and enhance cell survival pathways, directly contributing to a more aggressive tumor biology and clinical behavior.

HER2 as a Target for Therapy in Breast Cancer
HER2 over-expression in breast cancer represents an ideal target for therapy, given the location of the receptor on the surface of tumor cells and its role in driving the clinical course of the disease. Trastuzumab was developed as a targeted biologic therapeutic against the HER2 receptor protein. Trastuzumab is a humanized monoclonal antibody that combines the mouse recognition sequence of a monoclonal antibody (clone 4D5) against an extracellular epitope of the receptor with a human IgG1. Trastuzumab demonstrates a high affinity and specificity for the HER2 receptor and in preclinical studies was shown to be effective at inhibiting the growth of HER2 over-expressing breast cancer cells.

In numerous clinical trials, HER2-targeted therapy has been shown to be remarkably effective against HER2-positive breast cancer in both the metastatic and the adjuvant settings, particularly in combination with cytotoxic chemotherapy. Recent clinical trials data have demonstrated that adjuvant trastuzumab in combination with or following chemotherapy can reduce the relative risk of recurrence by up to 50% in early stage HER2 positive breast cancer. The clinical efficacy of HER2 targeted therapy in selected patients with HER2-positive breast cancer provides the rationale for testing all newly diagnosed breast cancer patients for the HER2 genomic alteration and/or over-expression of the receptor protein.

Clinical assays to assess the HER2 status include immunohistochemistry (IHC), which detects protein over-expression, or fluorescence in situ hybridization (FISH), which detects gene amplification. Both assays have been clinically validated in...
prospective randomized clinical trials and have received Food & Drug Administration (FDA) approval for predicting a clinical response and patient benefit from HER2-targeted treatment. Since published data suggest that only those breast cancer patients whose tumors demonstrate protein over-expression and/or gene amplification by the above assays are likely to benefit from therapy with trastuzumab, the results of HER2 assays stand alone in determining which breast cancer patients will be the most appropriate for HER2-targeted therapy. Therefore, accurate, reliable, and reproducible results are a high priority for ensuring optimal patient treatment.

**IHC vs FISH for HER2 Evaluation: Complementary Approaches**

The IHC and FISH methodologies for evaluating the HER2 status in breast cancer should be considered complementary in nature.

Fluorescence in situ hybridization evaluates the status of the HER2 gene in the nucleus, while IHC evaluates over-expression of the receptor protein at the surface of the cell. In the majority of HER2-positive cancers, HER2 protein over-expression is the result of gene amplification, thus HER2 gene/protein status should be highly correlated in most cases. Consequently, HER2 gene/protein discordant results are most commonly due to technical issues. However, unusual HER2 genotypes, such as polysomy for chromosome 17 and genomic heterogeneity, can lead to discrepant non-correlating cases that may be clinically important.

For such cases, the assessment of both the gene and the protein may be necessary for ensuring optimal patient treatment. The ASCO/CAP task force, in developing recommendations for HER2 testing, concluded that both tests were equally efficient in identifying patients who are candidates for HER2-targeted therapy. In addition, the assays must be properly validated, and all aspects of the test are performed in a highly standardized fashion with good quality control.

**Standardizing HER2 IHC Testing: Pre-analytic Variables**

**Tissue Handling**

The growing importance of molecular pathology and the use of biomarkers in clinical medicine have led to an increasing emphasis on optimal tissue preparation for these assays.

An important variable in the analysis of macromolecules, including proteins, RNA, and DNA, is the time interval between the surgical interruption of blood flow by the surgeon to the initiation of tissue fixation. This interval has been termed the ischemic time. Numerous studies have documented tissue ischemia, acidosis, and enzymatic degradation during this interval, leading to the progressive loss of these macromolecules. These deleterious effects terminate when the tissue sample is placed into formalin and chemical fixation begins. Thus, an excessive time delay before tissue fixation is begun has the potential to reduce the efficacy of analysis of clinically important protein and nucleic acid target molecules in these tissue samples.

The emerging data for breast cancer specimens suggest that delays as short as 1 to 2 hours can begin to compromise assay validity for ER, progesterone receptor (PR), and HER2.

Every effort should be made to transport breast excision samples from a patient having a documented or suspected cancer from the operating room to the pathology laboratory as soon as possible for an immediate gross assessment. The success of such efforts will require better communication and cooperation between surgeons, the operating room staff, and the pathology laboratory.

Upon receipt in the pathology laboratory, the specimens must be oriented, carefully inked for surgical margin assessment, sliced into sections at 2 to 5 mm intervals, and placed into formalin. If gross tumor is easily identifiable, a small portion of tumor and fibrous normal breast tissue can be placed together in the same cassette and put immediately into fixative at the time of the initial gross examination. This helps to initiate good tissue fixation and also ensures that normal breast elements are available as an internal positive control and have been handled and fixed identically to the tumor tissue.

**Tissue Fixation**

The only fixative for breast tissue samples that has been clinically validated for ER and HER2 testing is 10% phosphate buffered formalin. This is based on both the collective body of knowledge in the published literature and the outcomes of numerous clinical trials that have correlated outcomes with the expected or characteristic immunoreactivity of ER and HER2.

In addition, the FDA approval for assay kits analyzing ER, PR, and HER2 explicitly state that 10% phosphate buffered formalin fixation should be used. The FDA approval for the kits is invalid if an alternative fixative is used.

**Fixation Time**

Breast tissue samples must be fixed in 10% neutral buffered formalin for no less than 6-8 hours and for not more than 72 hours before processing, according to published testing guidelines. Formalin is aqueous, completely dissolved formaldehyde. Because formalin penetrates tissue at an approximate rate of 1 mm/hour, breast excision samples must be cut into slices in a timely fashion to initiate formalin infiltration and uniform chemical fixation throughout the tissue. Tissue placed into formalin without cutting into sections will not be exposed to formalin in a timely fashion and will remain ischemic until penetration by the fixative. The chemical reaction of fixation involves protein cross-linking by formaldehyde, which is a time-dependent reaction. The amount of time the tissue is exposed to formalin affects the degree of protein cross-linking, and this in turn will affect the amount of antigen retrieval necessary for proper assay performance.

The use of standard IHC assays and antigen retrieval protocols with under- or over-fixed tissue may lead to technical problems in performing the assay and have the potential for yielding false-negative or even false-positive test results, which could alter adjuvant treatment decisions and adversely affect outcome.

**Needle Core Biopsies vs Breast Excision Specimens**

There is a misconception that smaller biopsy samples will fix more quickly than larger resection specimens and therefore require less time in formalin. Formalin will penetrate more quickly into these smaller samples; however, small biopsy samples require the same amount of fixation time as larger resection samples, because chemical fixation requires adequate
time for protein cross-linking to take place.\textsuperscript{18} Similarly, larger resection specimens need to be incised in a timely manner to ensure adequate penetration of formalin so the chemical reaction of fixation can be completed.

A number of studies comparing ER/PR and HER2 IHC assays on needle core biopsies and resection specimens from the same patient have suggested that the needle core may be a better tissue sample for testing by IHC because these tissues are usually placed in formalin in a more timely fashion, will infiltrate more quickly because of their size, and therefore may receive more consistent tissue fixation.\textsuperscript{18} This conclusion assumes these tissues have all received an adequate time in formalin fixative and the lesion has been adequately sampled and is representative of the patient’s tumor. Needle core biopsies can be problematic for HER2 evaluation as they may be prone to certain artifacts that can make interpretation difficult. These include problems such as edge effect (concentration of staining at the edge of the tissue profile) and crush artifacts. An awareness of these artifacts is important so aberrant staining is not over-interpreted. Furthermore, patients with a negative HER2 assay result on a needle core biopsy should have the HER2 assessment of their tumor repeated on the excision tissue sample if there is a clinical profile suspicious for HER2 positive disease.

**Standardizing HER2 IHC Testing: Analytic Variables**

**HER2 Assay Selection**

Many laboratories use a HER2 testing algorithm in which breast tumor samples are screened by IHC, and FISH testing is reflexively performed only on cases scored as equivocal or 2+.\textsuperscript{19} Other laboratories utilize primary FISH testing for HER2. Either approach is acceptable, provided the tests are properly validated, performed, and interpreted. The HER2 testing algorithm assumes that IHC assays are standardized, have rigorous quality control, good concordance with FISH results, and good inter-laboratory agreement. The use of FDA-approved test kits containing well-standardized, high-quality reagents of known specificity and sensitivity are recommended to help ensure accurate and consistent results.

**Technical Validation**

The ASCO/CAP guidelines\textsuperscript{11} require a HER2 IHC test be validated before it can be offered clinically by a laboratory. Validation is performed by testing 25-100 samples that have been fixed in formalin using the standardized operating procedure for the laboratory, and then tested in parallel by an alternative method (FISH if validating IHC) using a previously validated assay in either the same laboratory or in another laboratory. The concordance between the 2 methods or laboratories must be at least 95% for both HER2 positive and HER2 negative test results.

**Routine Use of Controls for IHC Testing**

Positive and negative controls should be run with every assay to help ensure proper assay performance. Daily evaluation of control material including known negative, equivocal and positive clinical samples, or cell line controls can be used to monitor for assay drift and inadequate assay performance. Commercially available HER2 IHC test kits contain formalin-fixed paraffin embedded breast cancer cell lines expressing

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**Figure 1** Algorithm of a standardized critical approach for the evaluation of a HER2 IHC assay.
different levels of HER2 that can be used as a batch control (Image 1). These batch controls help to ensure proper assay performance and to calibrate the appropriate assay sensitivity and dynamic range for each staining run. In addition, the inclusion of a sample of tumor known to be HER2 positive on the same slide as the test sample will help to ensure that all reagents were dispensed onto the slide and the assay is performing properly for the test sample (Image 2).

**Test Interpretation for HER2 IHC**

The pathologist’s critical evaluation of a HER2 assay plays an important role in determining the selection of the most appropriate adjuvant treatment regimen for the breast cancer patient. This evaluation requires proper training and experience in HER2 IHC analysis, strict criterion for interpreting the results of the test, and a broad equivocal category for cases that are not clearly positive or negative. A diagnostic algorithm for the evaluation of HER2 IHC assay results is shown in Figure 1. The critical evaluation of a HER2 IHC assay begins with a review of the batch controls and the on-slide positive control to help ensure proper assay performance. The slide of the patient sample of tissue should be scanned to help ensure an invasive tumor is easily identifiable and there is no significant HER2 staining present within normal breast epithelial cells. Staining of adjacent normal breast tissue elements (with the exception of apocrine metaplasia) suggests the assay is too sensitive and the results for the invasive tumor should be considered inadequate for interpretation. The test should be repeated on another block of the patient’s tumor if possible.
Image 3. HER2-positive Breast Cancer by IHC (3+). (A): Poorly differentiated invasive carcinoma arising in a 40-year-old female is suspicious for HER2-positive disease given the patient's age and histologic grade (hematoxylin and eosin stained [H&E] section, ×200 original magnification). (B): The HER2 IHC assay shows a diffuse intense circumferential membrane “chicken-wire” staining pattern throughout the tumor (scored as 3+), consistent with HER2-positive breast cancer by IHC (DAKO Hercep Test, ×200 original magnification).

Image 4. HER2-negative Breast Cancer by IHC (1+). (A): Classic invasive lobular carcinoma arising in a 77-year-old female has a characteristic “single-file” growth pattern and would unlikely be HER2 positive. (B): The HER2 IHC assay shows faint, partial membrane staining (scored as 1+), consistent with HER2-negative breast cancer by IHC (DAKO Hercep Test, ×200 original magnification). The pattern and intensity of the staining is best appreciated at lower magnifications.
When the controls are in order and the normal breast elements are negative, then the invasive portion of the patient’s breast tumor sample can be further evaluated for HER2 expression. The scoring of HER2 results assessed by IHC must be semi-quantitatively evaluated to be clinically relevant. Human epidermal growth factor receptor 2 IHC tests are all interpreted as positive (3+), negative (0-1+), equivocal (2+), or inadequate for accurate interpretation.

**Positive Result for HER2 IHC**

Only cases with a diffuse intense circumferential membrane “chicken-wire” staining pattern in >30% of the tumor (scored as 3+) should be considered HER2 positive by IHC. For the typical HER2-positive case, the chicken-wire pattern of staining is readily identified at low power (×10) and is seen diffusely throughout the areas of an invasive tumor (Image 3). Tumors with this staining pattern show a good concordance with gene amplification by FISH in the vast majority of cases, and these patients will be the most likely to benefit from HER2-targeted therapy. Higher grade poorly differentiated breast cancers, which tend to occur in younger patients, are more likely to be HER2 positive.

**Negative Result for HER2 IHC**

Breast tumors with absent or weak membrane staining (scored as 0 or 1+, Image 4) typically demonstrate a normal HER2 gene status and are regarded as negative. Tumors with this staining pattern show a good concordance with an absence of amplification by FISH in the vast majority of cases, and these patients will be unlikely to benefit from HER2-targeted therapy. Lower grade well differentiated breast cancers are more likely to be HER2 negative. Less than 1% of grade 1 breast tumors will show evidence of HER2 gene amplification and receptor over-expression.

**Equivocal Result for HER2 IHC**

Breast tumors with circumferential membrane staining showing a thin pattern of staining and/or heterogeneity in staining distribution (<30% of tumor cells) should be scored as equivocal (scored as 2+, Image 5). In correlative studies, equivocal HER2 IHC staining has shown poor agreement with HER2 FISH and is considered inconclusive. Breast tumors with an equivocal HER2 IHC result need to be reflexively tested by FISH to assess for HER2 gene amplification, in an attempt to resolve the HER2 status of the tumor for clinical decisions on adjuvant therapy. If the tumor shows isolated groups, clusters, or single cells with strong HER2 membrane staining, this may represent genomic heterogeneity for HER2 gene locus resulting in a composite of HER2-positive and HER2-negative tumor cells. Use of the IHC stained slide to target the cells with protein over-expression for HER2 FISH analysis is a good way to confirm HER2 genomic heterogeneity and ensure an accurate assessment of the HER2 status. At present, the clinical significance of this finding in terms of the potential benefit from trastuzumab therapy is unclear, but the report should contain a comment describing these findings.

*Image 5. HER2-equivocal Breast Cancer by IHC (2+). (A): Poorly differentiated invasive carcinoma arising in a 49-year-old female is also suspicious for HER2-positive disease given the patient’s age and histologic grade (H&E stained section, ×200 original magnification). (B): The HER2 IHC assay shows areas of circumferential membrane staining (arrow) with varying intensity that is not seen throughout the tumor (scored as 2+ or equivocal) (DAKO Hercep Test, ×200 original magnification). A reflex FISH assay was ordered, and it showed that the HER2 gene was amplified (HER2/CEP17=5.2).*
Inadequate for Interpretation and Exclusion Criterion

Needle core biopsies with limited invasive tumor, edge artifact, or significant crush artifact should be approached with caution and may not be able to be accurately interpreted. For instance, focal strong staining is sometimes seen along tissue edges or in regions where tissue is disrupted due to an unequal distribution of assay reagents. Also, crush artifact can be produced when thin gauge vacuum extracted needles are used for sampling. These artifacts and regions should be ignored during assay interpretation to avoid false-positive results and inappropriate therapy with trastuzumab. In addition, assays with strong membrane staining of normal ducts and lobules should be excluded from interpretation, since this may represent either inadequate fixation or a technical error, such as an inappropriate antibody dilution. Results should always be reported in the context of appropriate positive, negative, equivocal, internal, and external controls run with each assay. A list of potential exclusion criteria for the interpretation of a HER2 IHC result is shown in Table 1. For each of these situations, the results of the HER2 IHC test should be interpreted with caution, and consideration should be given to repeating the test on a different sample or sending the block for HER2 FISH testing.

Critical Evaluation of HER2 Assay Results

Before a final HER2 determination is reported, the interpreting pathologist should critically review all aspects of the test to ensure the information is as accurate as possible. Clinical breast tissue samples that do not meet the pre-analytical requirements for the test should be interpreted with caution, and consideration should be given to further testing on a different sample, a different block, or by an alternative methodology. In addition, the patient’s HER2 test result should fit or correlate with the clinical profile of the tumor. In a retrospective review of more than 1000 consecutive HER2 assays, HER2-positive disease correlated with younger age at diagnosis, ER negative results, axillary lymph node metastasis, lymphatic invasion, and high-grade histology. In multivariate analysis, high histologic grade and younger patient age were found to be significant independent predictors of HER2 positivity. Patients with a clinical profile of HER2-positive breast cancer may benefit from FISH testing when their IHC results are negative.

Table 1 Exclusion Criterion for the Interpretation of HER2 IHC

- Excessive time delays from tissue sample collection to the initiation of formalin fixation (time from tissue collection to fixation ideally should be approximately 1 h or less; see reference 5. A tracking mechanism may need to be put into place to ensure proper tissue handling).
- Tissue samples fixed for less than a minimum of 6-8 h in neutral buffered formalin.
- Tissue samples fixed for greater than 72 h in neutral buffered formalin.
- Tissue samples fixed in an alternative fixative other than formalin, unless that process has been specifically validated by the laboratory.
- Tissue samples with only ductal carcinoma in situ and no invasive carcinoma.
- Tissue samples on unstained slides stored for >6 weeks prior to testing.
- Tissue samples, particularly needle biopsies, with significant crush artifact and/or edge artifact.
- Tissue samples with significant staining of normal breast elements within the tissue.

Conclusions

The assessment of HER2 status in breast cancer is critical for the management of disease and therefore a priority for pathological standardization. The selection of the most appropriate adjuvant treatment regimen, including whether the patient is a candidate for HER2-targeted therapy, is heavily dependent on reliable and accurate laboratory results assessing the HER2 status as part of their diagnostic evaluation.

The IHC and FISH methodologies are acceptable for determining the HER2 status of newly diagnosed breast cancer as long as the assays have been properly validated through a multi-step process involving selection and acquisition of equipment and reagents, training, and parallel testing of 25-100 cases to achieve at least 95% concordance with another validated assay offered in the same lab or another lab. Quality controls must be in place and continuously monitored. Immunohistochemistry and FISH both have technical and interpretive challenges that require experience and training to help ensure accurate results. In addition, these tests are complementary; examining different aspects of the biology underlying HER2-driven breast cancer, and in some cases, both may be needed to help ensure the most accurate result for determining treatment.

Regardless of the methodology, the ancillary testing of newly diagnosed breast cancer for predictive biomarkers such as ER and HER2 should reflect a consensus between the clinicians caring for these patients and the pathology laboratory. Among the most important lessons learned from using HER2 testing is the need for standardization of all parameters of testing, and this applies equally to IHC and FISH assays. This standardization includes all aspects of pre-analytical tissue-sample handling, the type and duration of fixation, tissue processing, assay performance, interpretation, and reporting. Rigorous standardization may be a challenge for many institutions, but it will be necessary to ensure optimum patient care.

It follows that a commitment to reliable and reproducible test results is essential for any laboratory performing HER2 determinations. Rigorous standardization will in all likelihood help improve the accuracy, reliability, and reproducibility of these assays as well as concordance rates between IHC and FISH results. These steps will help to provide the best possible information to treating clinicians and will ultimately result in selecting the most appropriate treatment regimen for patients diagnosed with breast cancer.


