SUPPLEMENT

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HER2 Testing in Breast Cancer: NCCN Task Force Report and Recommendations

Robert W. Carlson, MD; Susan J. Moench, PhD; M. Elizabeth H. Hammond, MD; Edith A. Perez, MD; Harold J. Burstein, MD, PhD; D. Craig Allred, MD; Charles L. Vogel, MD; Lori J. Goldstein, MD; George Somlo, MD; William J. Gradishar, MD; Clifford A. Hudis, MD; Mohammad Jahanzeb, MD; Azadeh Stark, PhD, MBA; Antonio C. Wolff, MD; Michael F. Press, MD, PhD; Eric P. Winer, MD; Soonmyung Paik, MD; and Britt-Marie Ljung, MD; for the NCCN HER2 Testing in Breast Cancer Task Force

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NCCN
500 Old York Road
Suite 250
Jenkintown, PA 19046
215–690–0300
www.nccn.org

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- Discuss the prevalence of invasive breast cancer characterized as HER2 positive
- Recognize the molecular characteristics of the HER2 biomarker in both healthy breast epithelial cells and some invasive breast tumors
- Understand the clinical evidence leading to the incorporation of the HER2 biomarker as both a prognostic and predictive factor in the most recent version of the NCCN Breast Cancer Clinical Practice Guidelines in Oncology
- Explain the characteristics, advantages, and disadvantages of the different testing methods used for measuring the level of HER2 protein expression and/or gene amplification in samples of invasive breast tumors
- Understand the sources of variability associated with the different HER2 testing methods and the recommendations of the HER2 Testing Task Force to limit such variability
- Discuss the roles played by both the pathologist and the oncologist in assuring the appropriate applications of targeted therapy in breast cancer

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HER2 Testing in Breast Cancer: NCCN Task Force Members

*[P]Robert W. Carlson, MD/Chair§†
Stanford Hospital and Clinics
*[P]D. Craig Allred, MD≠
Baylor College of Medicine
*Harold J. Burstein, MD, PhD§†
Dana-Farber/Partners CancerCare
W. Bradford Carter, MD§¶
H. Lee Moffitt Cancer Center & Research Institute at the University of South Florida
William B. Farrar, MD§¶
Arthur G. James Cancer Hospital & Richard J. Solove Research Institute at The Ohio State University
*[P]Lori J. Goldstein, MD§†
Fox Chase Cancer Center
*[P]William J. Gradishar, MD§‡
Robert H. Lurie Comprehensive Cancer Center of Northwestern University
*[P]M. Elizabeth H. Hammond, MD≠
LDS Hospital/ Intermountain Health Care
*[P]Clifford A. Hudis, MD§†Þ
Memorial Sloan-Kettering Cancer Center
*Mohammad Jahanzeb, MD§‡
St. Jude Children's Research Hospital/University of Tennessee Cancer Institute
Seema Khan, MD¶
Robert H. Lurie Comprehensive Cancer Center of Northwestern University
*Britt-Marie Ljung, MD§≠
UCSF Comprehensive Cancer Center
Lisle M. Nabell, MD§‡†
University of Alabama at Birmingham Comprehensive Cancer Center
[P]Soonmyung Paik, MD≠
National Surgical Adjuvant Breast and Bowel Project Foundation
*[P]Edith A. Perez, MD‡Þ
Mayo Clinic Jacksonville
*[P]Michael F. Press, MD, PhD≠
University of Southern California
Mary Lou Smith, JD, MBA§¥
Research Advocacy Network
*George Somlo, MD§‡ξ
City of Hope Cancer Center
*[P]Azadeh Stark, PhD, MBA≠
Henry Ford Hospital Main Campus
Richard L. Theriault, DO, MBA§†
University of Texas M. D. Anderson Cancer Center
*[P]Charles L. Vogel, MD†Þ
Cancer Research Center, Inc.
John H. Ward, MD§‡
Huntsman Cancer Institute at the University of Utah
[P]Eric P. Winer, MD§†
Dana-Farber/Partners CancerCare
*[P]Antonio C. Wolff, MD§†Þ
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins

Key:

* Writing Committee member;
[P] Presenter; § Breast Cancer Panel member

Specialties: † Medical Oncology; ¶ Surgical Oncology; ‡ Hematology/Oncology; ≠ Pathology; ξ Bone Marrow Transplantation; ¥ Patient Advocacy; Þ Internal Medicine.

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Supplement

HER2 Testing in Breast Cancer: NCCN Task Force Report and Recommendations

Robert W. Carlson, MD; Susan J. Moench, PhD; M. Elizabeth H. Hammond, MD; Edith A. Perez, MD; Harold J. Burstein, MD, PhD; D. Craig Allred, MD; Charles L. Vogel, MD; Lori J. Goldstein, MD; George Somlo, MD; William J. Gradishar, MD; Clifford A. Hudis, MD; Mohammad Jahanzeb, MD; Azadeh Stark, PhD, MBA; Antonio C. Wolff, MD; Michael F. Press, MD, PhD; Eric P. Winer, MD; Soonmyung Paik, MD; and Britt-Marie Ljung, MD; for the NCCN HER2 Testing in Breast Cancer Task Force

Key Words

Breast cancer, HER2 testing, fluorescence in situ hybridization, immunohistochemistry, NCCN Clinical Practice Guidelines in Oncology, trastuzumab, chemotherapy

Abstract

The NCCN HER2 Testing in Breast Cancer Task Force was convened to critically evaluate the ability of the level of HER2 expression or gene amplification in breast cancer tumors to serve as a prognostic and a predictive factor in the metastatic and adjuvant settings, to assess the reliability of the methods of measuring HER2 expression or gene amplification in the laboratory, and to make recommendations regarding the interpretation of test results. The Task Force is a multidisciplinary panel of 24 experts in breast cancer representing the disciplines of medical oncology, pathology, radiation oncology, surgical oncology, epidemiology, and patient advocacy. Invited members included members of the NCCN Breast Cancer Panel and other needed experts selected solely by the NCCN. During a 2-day meeting, individual task force members provided didactic presentations critically evaluating important aspects of HER2 biology and epidemiology: HER2 as a prognostic and predictive factor; results from clinical trials in which trastuzumab was used as a targeted therapy against HER2 in the adjuvant and metastatic settings; the available testing methodologies for HER2, including sensitivity, specificity, and ability to provide prognostic and predictive information; and the principles on which HER2 testing should be based. Each task force member was charged with identifying evidence relevant to their specific expertise and presentation. Following the presentations, an evidence-based consensus approach was used to formulate recommendations relating to the pathologic and clinical application of the evidence to breast cancer patient evaluation and care. In areas of controversy, this process extended beyond the meeting to achieve consensus. The Task Force concluded that accurate assignment of the HER2 status of invasive breast cancer is essential to clinical decision making in the treatment of breast cancer in both adjuvant and metastatic settings. Formal validation and concordance testing should be performed and reported by laboratories performing HER2 testing for clinical purposes. If appropriate quality control/assurance procedures are in place, either immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) methods may be used. A tumor with an IHC score of 0 or1+, an average *HER2* gene/chromosome 17 ratio of less than 1.8, or an average number of *HER2* gene copies/cell of 4 or less as determined by FISH is considered to be HER2 negative. A tumor with an IHC score of 3+, an average *HER2* gene/chromosome 17 ratio of greater than 2.2 by FISH, or an average number of *HER2* gene copies/cell of 6 or greater is considered HER2 positive. A tumor with an IHC score of 2+ should be further tested using FISH, with HER2 status determined by the FISH result. Tumor samples with an average *HER2* gene/chromosome ratio of 1.8 to 2.2 or average number of *HER2* gene copies/cell in the range of greater than 4 to less than 6 are considered to be borderline, and strategies to assign the HER2 status of such samples are proposed. (*JNCCN* 2006;4(Suppl 3):S1–S22)

HER2-Testing Task Force Meeting: Rationale

The human epidermal growth factor receptor 2 (HER2) is a type of transmembrane protein receptor tyrosine kinase (RTK) known as ErbB that is important in initiating signal transduction pathways in normal and abnormal cells. The HER2 protein is overexpressed and/or its gene is amplified in 15% to 20% of invasive breast cancers. HER2 overexpression or gene amplification is associated with an aggressive phenotype of breast cancer, predicts for benefit from trastuzumab therapy (Herceptin; a recombinant humanized monoclonal antibody specific for the external region of HER2), and may predict breast cancer sensitivity to combinations of trastuzumab and selected chemotherapeutic agents, such as the anthracyclines.[1–8] The identification of individual patients with breast cancers that overexpress the HER2 protein or amplify the *HER2* gene is dependent on the determination of the HER2 status of invasive breast cancer cells. This single assessment of the presence or absence of the trastuzumab target is the central criterion used to direct critical decisions concerning patient eligibility for trastuzumab therapy (Figure 1).[9]

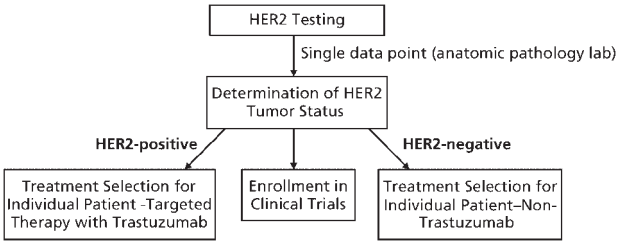

Figure 1 Pivotal role of the HER2 test result in selecting therapy in breast cancer.

The most recent version of the NCCN Breast Cancer Clinical Practice Guidelines in Oncology incorporates tumor hormonal receptor (e.g., estrogen receptor and/or progesterone receptor) and HER2 receptor expression or gene amplification as both prognostic factors for outcome and predictive factors for responsiveness to adjuvant systemic therapy.[10,11] The importance of these factors is emphasized by the stratification of patients by hormonal receptor status and HER2 status before assessment of anatomic prognostic factors such as tumor size, lymph node involvement, tumor grade, angiolymphatic invasion, or mitotic rate. A false-negative HER2 test result denies the patient access to trastuzumab therapy and its potential for substantial clinical benefit, whereas a false-positive result exposes the patient to the risks (e.g., cardiotoxicity) and expense of costly trastuzumab therapy without likelihood of therapeutic benefit.

Use of the HER2 receptor and hormonal receptor biomarkers in breast cancer is an example of the successful application of genomic and proteomic technologies to cancer evaluation and treatment.[12,13] Biomarkers such as HER2 and estrogen receptors allow for targeted therapy because they are direct cellular targets of therapeutic interventions with trastuzumab or endocrine therapy, they can be measured in the laboratory, and they are correlated with clinical response to therapy.[8,12,14] Furthermore, the clinical benefits of targeted therapy in patients not selected for the presence of the target will probably be modest.[13] Thus, accurate identification of patients with HER2-positive (i.e., HER2 overexpression/gene amplification) versus -negative (i.e., normal/low, non-amplified levels of HER2 protein/gene) disease has implications not only for patients with HER2-positive disease, but also for the 80% to 85% of breast cancer patients with HER2-negative disease.[15] Clinically important effects of particular therapies in populations with HER2-negative disease may be better understood when the subset of patients with HER2-positive disease is accurately identified and selectively excluded from certain clinical studies.

The NCCN HER2 Testing in Breast Cancer Task Force was convened to critically evaluate the ability of the level of HER2 expression or gene amplification of breast cancer tumors to serve as a prognostic and a predictive factor in the metastatic and adjuvant settings, to assess the reliability of methods of measuring HER2 expression or gene amplification in the laboratory, and to make recommendations regarding the interpretation of test results. The need for this evaluation intensified after reports of HER2 testing problems encountered in clinical studies involving adjuvant breast cancer therapies targeted to the HER2 receptor.[16–20] The task force generated recommendations to guide the overall process of HER2 testing to limit test variability. The consensus of the task force was that the HER2 receptor is a biomarker used in the setting of breast cancer for which laboratory testing processes must undergo intense scrutiny and reevaluation. The important roles of both the clinician and the pathologist in assuring the accurate quantification of breast cancer biomarkers, the correct interpretation of biomarker test results, and the appropriate application of targeted therapy were overarching themes the task force acknowledged.

HER2-Testing Task Force Meeting: Process

Task Force members came from both NCCN and non-NCCN institutions, and include some members of the NCCN Breast Cancer Clinical Practice Guidelines Panel.[10] Additionally, breast cancer experts with special expertise in HER2 biology, testing, or trastuzumab were invited. In all, 24 Task Force members represented medical oncology, pathology, radiation oncology, surgical oncology, epidemiology, and patient advocacy. All Task Force members were identified and invited solely by NCCN.

Topics for discussion were elaborated in a formal agenda developed by the task force chair, and individual members were assigned topics for focused, didactic presentations based on high-level scientific evidence whenever possible. Substantial time was allowed for discussion after each scientific presentation. An evidence-based consensus approach[21] was used to formulate recommendations relating to the pathological and clinical application of the evidence to

breast cancer patient evaluation and care. In areas of controversy, this process extended beyond the meeting to achieve consensus. Draft versions of this report were circulated among all of the task force members for review and comment.

HER2 Biology

HER2 is a member of the epidermal growth factor receptor (EGFR) family of protein RTKs known as ErbB. Other names for HER2 are ErbB2 and *neu*; the latter relates to the initial isolation of a *HER2* oncogene from rat neuroglioblastoma cells.[22,23] The other known members of the ErbB family are HER1, also called ErbB1 and EGFR; HER3 (ErbB3); and HER4 (ErbB4).

The ErbB family of receptors is involved in cellular growth, differentiation, and survival through the process of signal transduction. Typically, the binding of a growth factor, or ligand, to the ErbB receptor initiates a complex series of sequential events beginning with receptor dimerization and its enzymatic phosphorylation, which in turn catalyzes the phosphorylation of the first in a series of intracellular proteins acting as signaling intermediates. Many of these intermediates propagate the signal through enzymatic phosphorylation or dephosphorylation of other molecules. The final targets of this process are regulatory molecules, such as transcription factors, which are modified in response to the signal so as to affect the transcription of specific genes.[24]

The ErbB RTKs are single-subunit glycoproteins which span the cell membrane and can be divided into several distinct regions (Figure 2).[25] At least 7 growth factors have been identified as activating ligands for ErbB RTKs.[26] Growth factors typically activate RTKs by inducing the RTK monomer units to dimerize, resulting in the formation of homodimers (e.g. dimers containing the same monomer units) or heterodimers (dimers of 2 different ErbB monomers, such as the HER2/HER3 dimer).[26,27]

With the exception of the HER3 receptor, which does not have intracellular tyrosine kinase activity, dimer formation typically brings the tyrosine kinase regions of the monomers into close proximity, allowing cross-phosphorylation of the tyrosine regulatory residues of each monomer unit, subsequently activating the RTK to phosphorylate tyrosine residues on

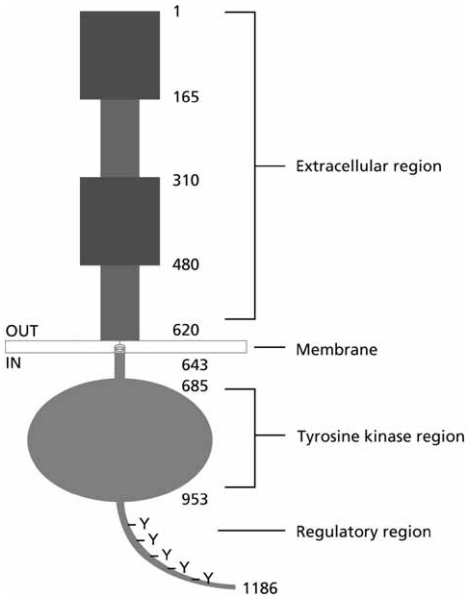

Figure 2 Schematic diagram of the structure of an ErbB receptor. Amino acid residue numbers are specific for the HER1 receptor. The letter Y identifies tyrosine residues in the intracellular regulatory region of the protein which undergo reversible phosphorylation/dephosphorylation. Adapted from Burgess et al.[25]

other signaling proteins. No ligand specific for HER2 has been identified to date, although the conformation of the extracellular region of HER2 without bound ligand is similar to the "activated" conformation of other RTKs with bound ligands.[28–30]

The ErbB RTKs are involved in the normal growth and development of a number of organs, including the heart, breast, and central nervous system.[27,31,32] Healthy, diploid breast epithelial cells typically contain 2 copies of the *HER2* gene, each located on 1 of 2 copies of chromosome 17.[33] However, during certain phases of the cell cycle, up to 4 *HER2* gene copies and more than 2 copies of chromosome 17 can be present in a normal cell.[34] The *HER2* gene is a proto-oncogene in that it is a normal gene with the potential to become an oncogene upon molecular alterations, such as mutation, amplification of its wild-type form, or overexpression of its protein product.

A number of other genes have been identified as putatively involved in breast cancer development. *C-myc* gene amplification is associated with HER2 amplification, increased proliferative activity and poor prognosis.[35,36] In addition, a high frequency of *topoisomerase II-alpha* and *HER2* gene co-amplification has also been reported.[37–39]

Finally, some of the downstream intermediates and transcriptional targets of the signaling pathways involving ErbB receptors may be affected by the activation of signal transduction processes involving other types of receptors, such as those which bind estrogen. Such "cross-talk" between receptor types would be expected to be bi-directional and could have implications for the administration of therapies targeted to the HER2 receptor, the estrogen receptor, or both.[40]

Trastuzumab: Putative Mechanisms of Action

Currently, trastuzumab is the only U.S. Food and Drug Administration (FDA)-approved therapy targeted to the HER2 receptor, although other HER2-targeted agents are under study. The precise mechanisms of action of trastuzumab are unknown,[41] but very recent studies have provided insight into one way in which trastuzumab, in combination with chemotherapy, may act as a cytotoxic agent. Clinical data show that co-amplification of the *c-myc* and *HER2* genes is associated with either a decreased or increased breast cancer recurrence rate, depending on whether trastuzumab is added to a chemotherapeutic regimen of doxorubicin plus cyclophosphamide followed by paclitaxel.[35] These results support the hypothesis that the pro-proliferative/pro-angiogenic/pro-apoptotic/invasive signals characteristic of dysregulated *c-myc* genes[42] acting in concert with the anti-apoptotic signals associated with dysregulated *HER2* genes result in increased proliferation and survival of breast tumor cells; suppression of HER2 activity by trastuzumab facilitates apoptotic processes occurring with chemotherapeutic treatment.[35] Because all patients with HER2-positive breast cancer do not respond to trastuzumab therapy, the identification of other co-amplified genes can potentially provide a means of identifying patients with HER2-positive breast cancer that is most likely to respond or to be resistant to treatment with trastuzumab.

Methods of Detecting HER2

The most frequently used tests to determine HER2 expression or gene amplification are immunochemistry (IHC) tests, which evaluate the level of HER2 protein in invasive breast cancer cells, and fluorescence in situ hybridization (FISH) tests, which assess whether *HER2* gene amplification has occurred in invasive breast cancer cells (Tables 1 and 2). Currently, 2 IHC assays and 2 FISH assays are approved by the FDA for the determination of HER2 status of breast cancer. IHC and FISH assays are the focus of most of the subsequent discussion on HER2 testing, with particular emphasis on the IHC test known as HercepTest and the FISH test known as PathVysion.

The HercepTest (Figure 3; Table 1), like all IHC tests for HER2, is based on the selective staining of cells that overexpress (i.e., exhibit abnormally high concentrations of) the membrane-bound HER2 protein. The semiquantitative HercepTest scoring criteria used to evaluate the extent and intensity of cell staining is described in Figure 4.[43]

The PathVysion FISH method (Figure 5; Table 1) relies on 2 fluorescently labeled probes that are complimentary to either the *HER2* gene or the centromere of chromosome 17 on which the *HER2* gene resides. The number of each type of fluorescent signal per cell is then used to determine whether amplification of the *HER2* gene has occurred. The probe for chromosome 17 serves as an internal control as well as a marker of aneusomy, a phenomenon characterized by additions (polysomy) or deletions (monosomy) of copies of chromosome 17. *HER2* gene amplification is defined by the PathVysion method as an average ratio of *HER2* gene copy number to chromosome 17 copy number per cell of greater than or equal to 2.0. Interpretation is based on the fact that significant amplification of the *HER2* gene should be seen independent of the increase in chromosome 17 copies.[44] *HER2* gene to chromosome 17 ratios of 1.8 to 2.2 are considered "borderline" between HER2 amplification and non-amplification according to the Pathvysion assay. Examples of breast tissue showing the presence and absence of *HER2* gene amplification by the PathVysion method are shown in Figure 6, which also provides information on the relative frequencies of different *HER2* gene/chromosome ratios of invasive breast cancer cells measured in one large study.[20] FISH testing in all clinical trials to date has been performed using the PathVysion method.

The FISH assay method known as INFORM uses a single probe for the *HER2* gene. Assay results are reported as the average number of gene copies per cell. A non-amplified result is considered to be an average *HER2* copy number per cell of less than or equal to 4. This method does not have a way to identify whether an increase in *HER2* gene copy number is accompanied by a corresponding increase in the number of

Table 1 Methods for Determining HER2 Status of Breast Cancer Cells

Method	Immunohistochemistry (IHC)		Fluorescence In Situ Hybridization (FISH)		Detection of Extracellular Domain of HER2 (ECD)	Chromogenic In Situ Hybridization (CISH)
Brand name of assay	HercepTest®[43,44]	Pathway®[45]	PathVysion®[33,44,46]	INFORM®[44,47]	Immuno 1®/ ADVIA Centaur®[48,49]	CISH [50–52]
Manufacturer	DAKO	Ventana	Abbott	Ventana	Bayer	Zymed
Sample used in assay	Tissue-invasive cancer cells	Tissue-invasive cancer cells	Tissue-invasive cancer cells	Tissue-invasive cancer cells	Serum	Tissue-invasive cancer cells
Assay target	HER2 receptor protein	HER2 receptor protein	HER2 gene and chromosome 17	HER2 gene	Extracellular fragment of HER2 receptor	HER2 gene
Methodology	Primary polyclonal A0485 antibody targeted to intracellular region of HER2 receptor; detection via binding of secondary antibody coupled to dextran peroxidase	Primary monoclonal CB11 antibody targeted to intracellular region of HER2 receptor; detection via binding of a biotin-conjugated secondary antibody followed by binding of avidin/ streptavidin conjugated to enzyme	Hybridization of two DNA probes: red fluore attached to probe specific for HER2 gene; green fluore attached to probe specific for chromosome 17. 60 cells analyzed	Hybridization of biotin-labeled oligonucleotide specific for HER2 gene; detection through binding of fluorescently-labeled avidin. 40 cells analyzed	ELISA; Primary monoclonal antibodies NB-3 and TA-1 (one is labeled with fluorescein and other is either linked to enzyme or a chemiluminogenic molecule) specific for the ECD of HER2 added to sera; detection via binding of immunocomplex to anti-fluorescein antibodies in the solid phase,followed by addition of substrate in case of Immuno 1 assay	Hybridization of digoxigenin-labeled DNA probe specific for HER2 gene; detection via binding of antidigoxigenin antibody labeled with fluorescein, followed by anti-fluorescein antibody coupled to peroxidase
Evaluation of HER2 status	HER2 level graded from 0/1+ (normal amount HER2 protein/trace negative); 2+ (weakly positive) to 3+ (strongly positive) for HER2 protein	HER2 level graded from 0/1+ (normal amount HER2 protein/trace negative); 2+ (weakly positive) to 3+ (strongly positive) for HER2 protein) [HercepTest scoring]	Average HER2 gene/chromosome 17 ratio of greater than or equal to 2.0 is classified as gene amplification; gene amplification is noted as FISH positive; absence of gene amplification is noted as FISH negative; polysomy is detectable	An average of greater than 4 copies of HER2 gene/cell is classified as gene amplification; gene amplification is noted as FISH positive; absence of gene amplification is noted as FISH negative. polysomy cannot be detected.	Elevated ECD concentrations often defined as >15 ng/mL	Gene amplification is noted as none (1-5 copies), low-level (6-10 copies), or high-level (>10 copies)
FDA approval to define eligibility to receive trastuzumab	Yes	Yes	Yes	Yes	FDA Approval - for follow-up and monitoring of patients with metastatic breast cancer, only	No - in development

Table 2 Advantages and Disadvantages of Methods Used for Determining HER2 Status in Breast Cancer

Method	IHC (Hercep Test®)	IHC (Pathway®)	FISH (PathVysion®)	FISH (INFORM®)	ECD	CISH
Advantages	Widely available; less expensive and shorter assay time than FISH; potentially capable of detecting HER2 overexpression by single gene; can detect invasive cancer cells in tissue more easily than FISH; permanent staining	Widely available; less expensive and shorter assay time than FISH; potentially capable of detecting HER2 overexpression by single gene; can detect invasive cancer cells in tissue more easily than FISH; permanent staining	Semiquantitative scoring method-less subjective than IHC scoring method; capable of distinguishing between polysomy and HER2 gene amplification; probe to chromosome 17 acts as an internal control	Semiquantitative scoring method-less subjective than IHC scoring method	Noninvasive; allows for "real-time" assessment; capable of detecting HER2 protein after surgical resection of breast; some evidence to support correlations between ECD levels in serum and HER2 levels in tumor, and between changes in ECD levels and response to trastuzumab	Fluorescence equipment is not required; less expensive than FISH; can be used in conjunction with IHC; can detect invasive cancer cells in tissue more easily than FISH; permanent staining; high level of concordance between FISH and CISH
Disadvantages	Results may be affected by tissue fixation and processing methods; subjective scoring method frequently used	Results may be affected by tissue fixation and processing methods; subjective scoring method frequently used	Possible HER2 overexpression by single gene not detectable; more expensive than IHC; difficulties may be associated with identification of invasive tissue; difficult interpretation of borderline amplification; staining is not permanent	Possible HER2 overexpression resulting from single gene not detectable; not able to detect polysomy; more expensive than IHC; difficulties may be associated with identification of invasive tissue; interpretation of borderline amplification not well defined; staining is not permanent	Detects only circulating levels of ECD; dependent on cleavage of ECD fragment from membrane-bound receptor; most studies do not support correlation between baseline ECD levels and response to trastuzumab; clinical relevance is unknown	Possible HER2 overexpression resulting from single gene not detectable; difficult interpretation of borderline amplification
Studies	Ross et al.[24]; Wang et al.[44]; Hanna[53]; Perez et al.[54]	Ross et al.[24]; Perez et al.[54]	Ross et al.[24]; Wang et al.[44]; Perez et al.[54]; Persons et al.[55]	Ross et al.[24]; Wang et al.[44]; Perez et al.[54]; Vera-Roman and Rubio-Martinez[56]	Esteva et al.[49]; Fornier et al.[57]; Baselga et al.[58]; Carney et al.[59]	Ross et al.[24]; Tanner et al.[50]; Madrid and Lo[51]; Hanna and Kwok[52]; Vera-Roman and Rubio-Martinez[56]

copies of chromosome 17. Borderline levels of *HER2* gene amplification have not been specifically defined with the INFORM assay, although it has been proposed that average *HER2* gene copy numbers/cell falling into the range of greater than 4 to less than 6 should be considered borderline.[56] This range is based on the assumption that most cells exhibiting polysomy are characterized by 3 to 5 copies of chromosome 17.[51]

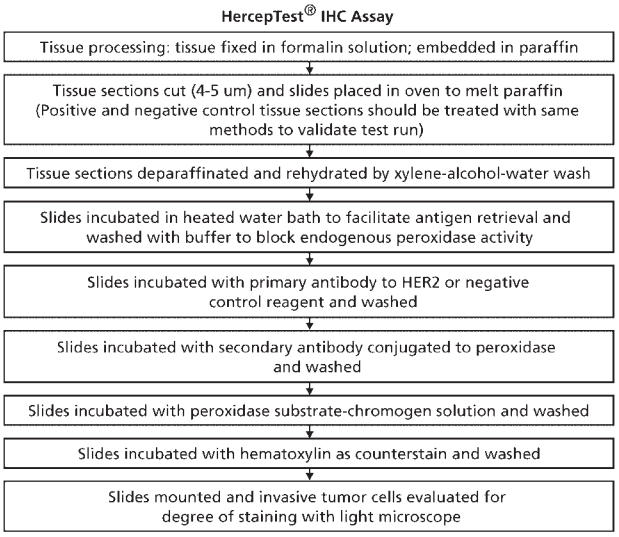

Figure 3 Methodology used in the HercepTest IHC assay. From DAKO HercepTest Package Insert.[43]

Another method of determining HER2 status, called chromogenic in situ hybridization (CISH), incorporates some of the advantages of both the FISH and IHC methods (Tables 1 and 2).[50–52] Permanent staining and ready identification of invasive tissue using a light microscope as characterized by IHC is combined in the CISH method with the selective staining of the *HER2* gene as characterized by FISH. Further, the CISH method for assessing HER2 tumor status was recently shown to be 97% concordant with FISH.[52] In addition, measurements of circulating levels of the extracellular fragment of the HER2 receptor have also been used in studies involving assessments of HER2 tumor status (Tables 1 and 2).[48,49]

Questions relating to the application of HER2 testing in the clinical setting are ultimately of paramount importance and include, "What prognostic and predictive information can be obtained through determination of HER2 tumor status? (Can the level of HER2 overexpression/gene amplification in breast cancer tumors be correlated with clinical outcome in the absence and presence of therapy?)

Some of the questions relating to the association of HER2 test results with molecular events involving HER2 and its role in cellular transformation include, "Do FISH and IHC results correlate with the functionality of the HER2 gene and its protein product, respectively?" and, "Are discordances between the different test methods a reflection of real biologic differences?"

A key methodologic question relating to HER2 testing is which variables can affect the performance of IHC and FISH assays?

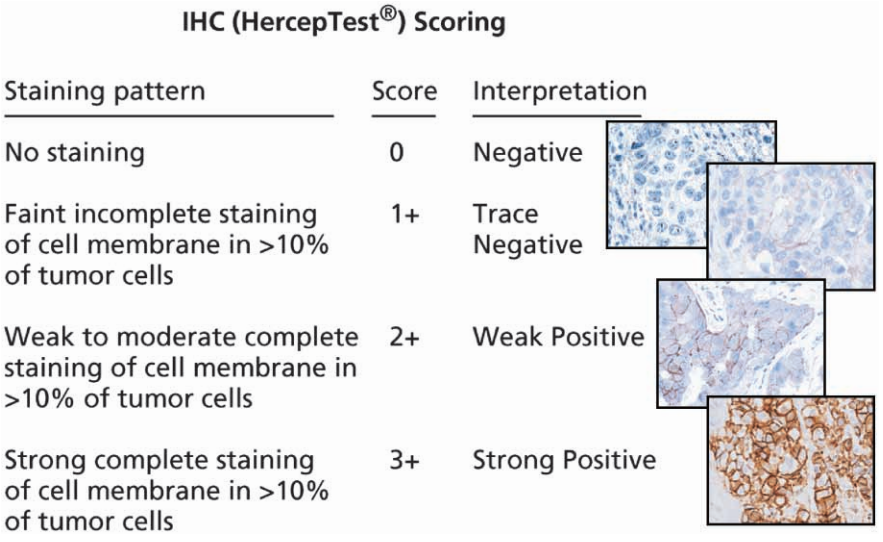

Figure 4 Scoring method used in the HercepTest IHC assay.[43] Figure courtesy of Kenneth Bloom, MD.

HER2 Testing: Clinical Issues

HER2 Status as a Prognostic Factor

HER2 overexpression or gene amplification in tumor samples has been identified as an indicator of poor prognosis for overall survival in trastuzumab-naïve patients with breast cancer.[60,61] This observation has been supported by a number of studies that have also identified a correlation between HER2 overexpression or gene amplification, ER- and PR-negative tumors, and a more aggressive tumor natural history.[62–64] However, not all studies evaluating the relationship between HER2 tumor status and clinical outcome have identified HER2 as a reliable indicator of poor prognosis in breast cancer.[24] Furthermore, some researchers have suggested that the type of HER2 testing method may influence measured associations between HER2 tumor status and clinical outcome (Figure 7).[24,34,65,66]

HER2 Status as a Predictive Factor

A number of retrospective studies suggest that HER2 positivity of

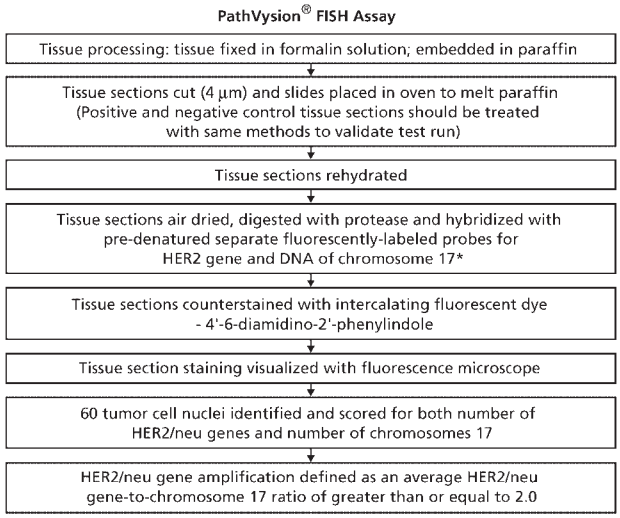

Figure 5 Methodology used in the PathVysion FISH assay.[46]

breast cancer tumors is a marker of benefit from doxorubicin-containing chemotherapeutic regimens.[2–6,67] Evidence also suggests that this may be a consequence of *topoisomerase II-alpha* gene amplification in the setting of *HER2* gene amplification rather than a direct effect of doxorubicin on the HER2 gene or its protein product.[4,37,39,68,69]

Clinical trials have shown that trastuzumab substantially increases the likelihood of an objective response and overall survival for patients with metastatic HER2-positive breast cancer.[1,70,71] In addition, the relative risk of recurrence is decreased by about 50% when trastuzumab is added to adjuvant cytotoxic chemotherapy in patients with HER2-positive breast cancer.[19,72–74] However, many questions remain concerning the ability of HER2 assays to predict benefit from trastuzumab, the optimal means of selecting patients to receive such treatment, and the optimal schedule/duration of trastuzumab administration. The following sections represent a brief summary of selected clinical studies highlighting some of the issues associated with HER2 testing and response to trastuzumab therapy.

Trastuzumab monotherapy in the metastatic setting Table 3 shows results from a re-analysis of tissue samples from a clinical trial on the use of first-line trastuzumab monotherapy in patients with metastatic breast cancer.[8,71] Inclusion criteria for patient enrollment included breast cancer tumors with HER2 scores of either 2+ or 3+ as determined by IHC using the Clinical Trials Assay, which used 2 different antibody systems. It is not currently available but was used as a comparison assay to achieve FDA approval for the HercepTest.[24,75]

Retrospective FISH analyses were also performed on available tumor samples. A response rate of 35% (95% confidence interval [CI], 25%–47%) was seen in the patients with tumors that were either IHC 2+ or 3+ and FISH-positive (amplified), whereas a much lower response rate of 3% (95% CI, 0%–20%) was seen for patients with tumors scored as IHC 2+ or 3+ and FISH-negative (non-amplified). These results indicate that most patients exhibiting a beneficial clinical response to trastuzumab have HER2-positive tumors by both IHC and FISH methods. However, in one patient, a tumor characterized as IHC 2+ or 3+ and FISH negative responded to trastuzumab monotherapy (Table 3).[8]

In the study by Cobleigh et al.,[70] which included patients who had received previous chemotherapy, however, no tumors that were either IHC 2+ or 3+ and FISH-negative responded to trastuzumab monotherapy, whereas 19% of patients with IHC 2+ or 3+ and FISH-positive tumors experienced an objective response.[8,70] Although these studies show substantially higher response rates in patients with breast tumors characterized as HER2 positive by FISH than in those with HER2 FISH-negative tumors, results from a study by Vogel et al.[71] indicate that only half of patients with FISH-positive tumors experienced clinical benefit after trastuzumab monotherapy (Table 3). These results suggest that a substantial number of these patients have tumors not responsive to single-agent trastuzumab.

Trastuzumab with chemotherapy in the metastatic setting The pivotal randomized phase III study evaluated the efficacy and safety of trastuzumab in combination with chemotherapy (anthracycline plus cyclophosphamide or single-agent paclitaxel) versus chemotherapy alone in patients with metastatic breast cancer that was HER2 positive by the Clinical Trials Assay. Only patients with tumors characterized by IHC scores of 2+ or 3+ were included in the study, and most of the analyses were performed on the IHC 2+ or 3+ group as a whole.[1] Subsequent analyses of these data included a retrospective determination of HER2 tumor status by FISH.[8,76] Interestingly, although an analysis of Slamon et al.'s[1] data showed an increased response rate

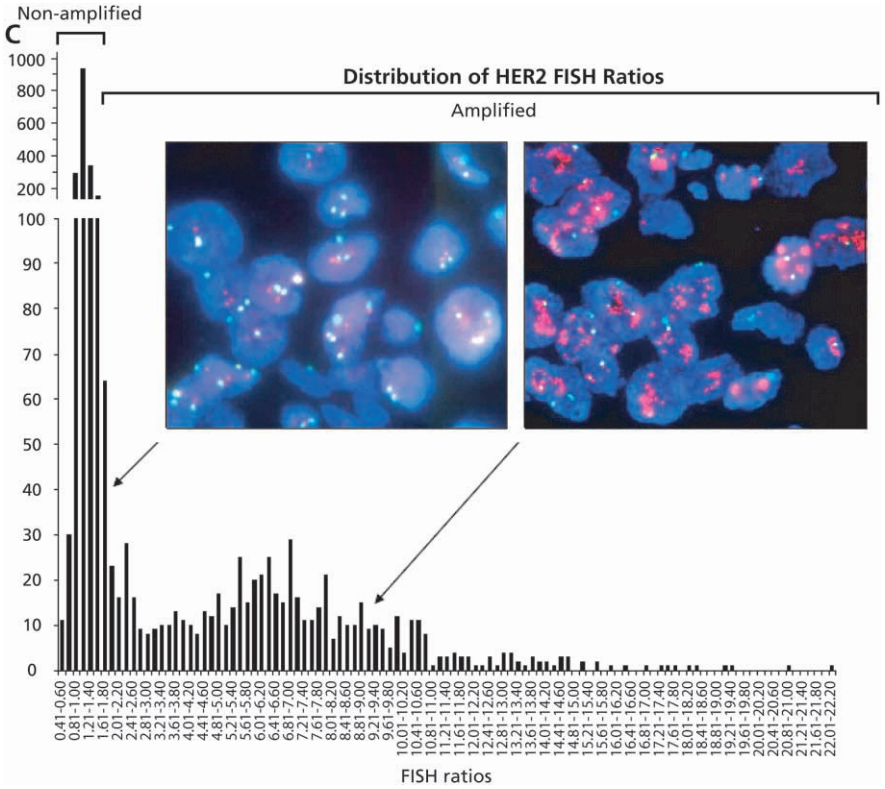

Figure 6 Distribution of *HER2* gene/chromosome 17 ratios in 2,502 breast cancer tumor samples analyzed using the PathVysion FISH method. A non-amplified result is defined as an average *HER2* gene to chromosome ratio of less than 2.0; an amplified result is defined as an average *HER2* gene to chromosome ratio of greater than or equal to 2.0. Inset: Demonstrations of a HER2 non-amplified and a HER2 amplified result in samples of invasive breast tumors. The probes to the HER2 gene and chromosome 17 are shown as red and green colors, respectively. Adapted from Press et al.[20]; with permission.

for trastuzumab treatment in the group with IHC 2+ or 3+ FISH-amplified tumors versus in the group with IHC 2+ or 3+ FISH non-amplified tumors, the response rate in the group with IHC 3+ FISH-amplified tumors was nearly identical to that in the group with IHC3+ FISH non-amplified tumors.[76] These results suggest that patients with tumors characterized as IHC 3+ and FISH-negative may have also received some benefit from trastuzumab, although only a small number of patients were included in this subset.[76]

However, analyses of time to disease progression and overall survival according to HER2 tumor status indicated that trastuzumab therapy was associated with a significant increase in time to progression in patients with FISH-negative HER2 tumors (Table 4). A relative risk of 0.66 (CI, 0.45–0.99) was determined for patients with FISH-negative tumors undergoing chemotherapy plus trastuzumab compared with patients with FISH-negative tumors undergoing chemotherapy only. This effect on time to disease progression, however, was more pronounced for patients with tumors characterized as FISH-positive (relative risk [RR]=0.44; CI, 0.34–0.57). Unlike the former group, the latter group also showed an increase in overall survival with trastuzumab therapy (RR=0.69; CI, 0.53–0.91). Survival differences in the patients with FISH-negative tumors did not reach statistical significance (RR=1.07; CI, 0.70–1.63).

In general, patients with tumors characterized as either IHC 3+ independent of FISH status or FISH positive independent of IHC status exhibited similar times to progression and overall survival after administration of trastuzumab-containing therapy. However, these analyses did not permit direct comparison of the 2 types of HER2 testing methods with respect to benefit from trastuzumab therapy (Table 4).[76]

Results from a multicenter phase II study designed to assess the efficacy and safety of trastuzumab and vinorelbine in patients with metastatic breast cancer showed that similar high overall response rates were observed in patients with HER2-positive tumors regardless of whether HER2 tumor status was determined as IHC 3+ or FISH positive.[77] In an analysis of 2 phase II studies involving administration of trastuzumab with docetaxel and either cisplatin or carboplatin in advanced breast cancer, inclusion criteria included HER2-positive tumor status by either IHC 2+ or 3+ test result or positive FISH test result. Retrospective FISH testing of tumors from most patients enrolled in these studies revealed an increased overall response in patients with FISH-positive disease (relative to those with FISH-negative disease) in one study, and similar response rates in patients with FISH-positive and FISH-negative tumors in the other study.[78] These results may be related to the small number of patients in these studies and to the efficacy of combination docetaxel and cisplatin therapy in advanced breast cancer. However,

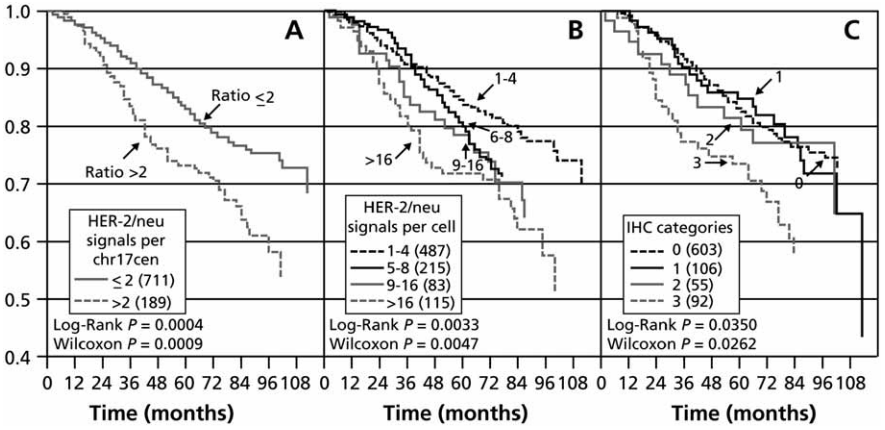

Figure 7 Effect of HER2 tumor status on survival of patients with breast cancer. Kaplan-Meier estimates of the relationship between HER2 tumor status as a function of HER2 testing method and survival of patients with breast cancer. Panel a shows ≤2 and >2 HER2 signals per chromosome 17 centromere signal; panel b shows increasing levels of HER2 signals/cell (patients stratified by maximum signals/cell within each specimen, 4 arbitrary strata); panel c shows increasing HER2 receptor protein expression levels. (N) = number of patients. Patients included in this study had stage I to III breast cancers that were either node-negative or node-positive, underwent either total (75%) or partial (25%) mastectomy, and received adjuvant postoperative radiotherapy and/or chemotherapy. Adapted from Pauletti et al.[34]; with permission.

both studies showed a substantially prolonged progression-free period in patients with FISH-positive disease relative to those with FISH-negative disease.

Trastuzumab with Hormonal Therapy Clinical trials evaluating the combined effects of trastuzumab with endocrine therapies in patients with HER2-positive, hormonal receptor–positive invasive breast cancer are underway, but results are not yet available. Results from recent clinical trials involving trastuzumab therapy in adjuvant and metastatic breast cancers suggest that hormonal receptor status itself does not appear to be associated with benefit from trastuzumab.[19,73,79]

The potential for "cross-talk" between intermediates in overexpressed growth factor and hormonal signaling pathways in breast cancer has been investigated in a number of clinical studies evaluating the effect of HER2 status on clinical response to hormonal therapy. Many of these studies involved small numbers of patients and a variety of HER2 testing methods. For example, serum levels of the extracellular fragment of HER2 were measured in some,[80–83] and different IHC assay methods were used in others.[84,85] These studies show conflicting results concerning the effect of HER2 status on clinical hormone sensitivity. For example, several studies were interpreted as providing evidence for tamoxifen resistance[81,86,87] or resistance to other types of hormonal therapies[82,83,87] in patients with HER2-positive tumors, whereas other study results did not support an association between HER2 overexpression or gene amplification and response to either tamoxifen or aromatase inhibitors.[85,88–91] Despite inconsistent data, however, HER2 status has been, and may continue to be, considered in clinical decision-making involving hormonal therapies.[92]

Trastuzumab with Chemotherapy in the Adjuvant Setting Trastuzumab added to adjuvant chemotherapy has been shown to substantially increase disease-free survival and decrease risk of disease recurrence by about 50% for patients with early-stage, HER2-overexpressed or gene-amplified invasive breast cancer (Figure 8).[19,72–74] In these studies, patients were treated with trastuzumab for periods of 9 weeks to 2 years.[19,72–74] The HER2 tumor status of patients enrolled in these trials was IHC 3+ and/or FISH positive,[19,73] IHC 2+ and FISH positive,[73] FISH positive alone,[72] or IHC 2+ or 3+ and positive by chromogenic in situ hybridization (CISH).[74] Depending on specific eligibility requirements, confirmatory HER2 testing at a central testing facility or reference laboratory was required in some of the studies. For example, original eligibility for enrollment in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 trial included an IHC 3+ score using the HercepTest assay, strong membrane staining of 33% of tumor cells by any other IHC assay, or a FISH-positive result from any laboratory accredited to perform such testing. However, quality assurance testing of tissue samples at a central testing facility revealed a high rate of false-positive IHC test results from the accredited laboratories.[16] As a result, inclusion criteria were modified to specify that measurement of HER2 status by IHC testing had to be performed or confirmed at an approved laboratory. Only patients with tissues samples with IHC scores of 3+ from an approved laboratory or judged to be either FISH positive using an FDA-approved FISH test performed at any laboratory were subsequently eligible for study

Table 3 Trastuzumab Monotherapy in Metastatic Breast Cancer Patients: Analysis of Relationships of Clinical Outcome to HER2 Tumor Status as Determined by FISH Testing*

	Number of Patients	
	FISH positive	FISH negative
Total Patients Evaluable	82	29
CR	7	0
PR	22	1
CR + PR	29 (35%) (95% CI, 25%–47%)	1 (3%) (95% CI, 0%–20%)
CR + PR +SD > 6 months	41 (50%)	1 (3%)

*HER2 status of samples was either IHC 2+ or IHC 3+

Original data from Vogel et al.[71]; reanalysis of FISH-negative samples from patients showing a clinical response performed by Dr M. F. Press and described in Mass et al.[8] Statistical evaluation of reanalysis previously unpublished.

enrollment.[16] Eligibility criteria for the phase III trial (N9831) of adjuvant chemotherapy in the presence and absence of trastuzumab conducted by the North Central Cancer Treatment Group (NCCTG) were also modified after reports of high levels of discordance when HER2 tumor status was evaluated locally versus centrally.[17,18] In this study, central testing was then required for trial eligibility (see "Methodologic Issues").

HER2 Testing: Summary of Clinical Issues

The use of HER2 tumor biomarker status in clinical decision-making (Figure 1) has been limited by several factors including the reliability of test results; difficulties in interpreting results from clinical trials in which populations with differing HER2-status tumors were evaluated as a single group (e.g., IHC 2+ and 3+ scores); and, in some cases, problems associated with retrospective testing and analyses. In addition, no clinical information is available on the benefit of HER2-targeted therapy in patients with breast cancer with a HER2 status of 0 or 1+ by IHC and positive by FISH.[76] Further, very little information is available regarding the benefit of trastuzumab in patients with breast cancer that is characterized as having a HER2 tumor status of IHC 3+ and FISH non-amplified.

HER2 Testing: Biological Issues

HER2 Tests: Surrogates of Biologic Processes

The most important purpose of evaluating the HER2 status of an individual patient's tumor is to predict whether a clinically important benefit from a particular therapy is likely. Therefore, assessment of the functionality of the HER2 gene and its protein product (i.e., effect on activated pathways downstream of the HER2 receptor) is a goal of HER2 testing,[24] even though an association between the biomarker and a biologic end point does not guarantee that the biomarker will be clinically useful.[93] Currently, however, assessments of potential for clinical benefit are made using only the determination of the presence or absence of *HER2* gene amplification or overexpression of HER2 protein. For example, although clinical studies involving trastuzumab have typically enrolled only patients with HER2-positive tumors, whether the level of HER2 protein overexpression or *HER2* gene amplification (increased numbers of gene copies or higher gene/chromosome ratios) is associated with increased clinical benefit from trastuzumab remains unclear.

HER2 Tumor Status: Fixed or Dynamic?

A difference in the HER2 status of primary and metastatic breast cancer tumors has been proposed as a possible explanation for trastuzumab resistance.[94] Results from several studies have identified discordance between the HER2 status of primary tumors and metastatic cells in some patients with metastatic breast cancer,[94–97] although this phenomenon was considered to be relatively uncommon in most of the studies.

HER2 Status as Determined by IHC versus FISH: Cases of True Biologic Discordance?

Results from studies of breast cancer cell lines indicate that a complex, nearly exponential relationship exists between the density of HER2 receptor on the cell membrane and the average *HER2* gene copy number to chromosome 17 ratio.[98,99] However, a few reports of tumors that exhibited true biologic discordance of HER2 status as assessed by IHC and FISH methods have been confirmed. For example, confirmed cases of tumors exhibiting HER2 overexpression as characterized by an IHC 3+ score without gene amplification have been reported, and these cases have typically been considered to be the result of single gene overexpression.[8,71,100] Another explanation for tumors with a score of 3+ by IHC without *HER2* gene

Table 4 Effect of Trastuzumab Plus Chemotherapy vs. Chemotherapy Alone on Time to Disease Progression and Overall Survival as a Function of HER2 Tumor Status* in Patients with Metastatic Breast Cancer

	FDA Analysis of Data from Slamon et al.[1]		Analysis of Mass et al.[8](Based on study of Slamon et al.[1])	
HER2 Tumor Status	Time to Disease Progression, RR (95% CI)	Overall Survival, RR (95% CI)	Time to Disease Progression, RR (95% CI)	Overall Survival, RR (95% CI)
IHC 3+ (independent of FISH)	0.42 (0.33–0.55)	0.70 (0.54–0.92)		
IHC 2+ (independent of FISH)	0.82 (0.54–1.24)	1.09 (0.71–1.58)		
FISH positive (independent of IHC)	0.44 (0.34–0.57)	0.69 (0.53–0.91)	0.45 (0.35–0.57)	0.71 (0.55–0.92)
FISH negative (independent of IHC)	0.66 (0.45–0.99)	1.07 (0.70–1.63)	0.61 (0.39–0.95)	1.10 (0.69–1.73)

*All tumors were IHC 2+ or 3+ for HER2 by the Clinical Trials Assay.

amplification by FISH involves polysomy of chromosome 17; a *HER2* gene/chromosome ratio of less than 2 with polysomy is not an indicator of *HER2* gene amplification by FISH testing but may still result in overexpression of the HER2 protein.[44,101] In addition, cases of tumors scored as 0 or 1+ by IHC and positive by FISH have also been reported[20] and may represent cells in the early stages of protein overexpression. Another possible explanation for tumors with IHC 0 or 1+ results that are FISH positive may be loss of a copy of chromosome 17 (i.e., monosomy), resulting in a HER2 gene/chromosome ratio of 2 or greater using the PathVysion FISH assay but fewer than 4 copies of the *HER2* gene.[102]

A number of studies have evaluated the degree of concordance between HER2 results obtained using various assay methods (different types of IHC or IHC vs. FISH), and between HER2 test results obtained at different laboratories.[16–18,20,56,103–105] Criteria for comparing IHC and FISH assay results include a definition of concordance as IHC 0, 1+, and 2+ scores in agreement with a FISH-negative result and an IHC 3+ score in agreement with a FISH positive result.[16–18]

Reports from a number of studies document a substantial percentage of false-positive[16–18,103,104] and false-negative HER2 test results.[20,104] Comparisons of HER2 overexpression as measured by IHC at local laboratories versus *HER2* gene amplification as measured by FISH testing performed at a reference laboratory revealed relatively low concordance rates (66%–87%).[16–18,20,106] Reported concordance rates between local and reference FISH test results were generally somewhat higher (87% and 92%),[18,20] although concordance between local and central FISH testing was only 67% in one study with a low number of tumor samples scored by the FISH method.[17] In most cases, discordances associated with the IHC method were attributed to variability in testing methodology rather than problems inherent to a particular method or true biologic discordances. These were found to be greatest in settings in which HER2 testing processes were not well controlled.[16–18,103,104] The results of several studies have indicated that IHC assays performed by a qualified laboratory can accurately determine the HER2 status of tumors. For example, a large reference laboratory using rigorous quality assurance measures found a positive predictive value for a IHC 3+ score of 91.6% and a negative predictive value for IHC 0 or 1+ scores of 97.2% using FISH testing as the gold standard.[107] Furthermore, a 20% overall false-positive rate for IHC testing found after early analyses of tumor samples from the NSABP B-31 study dropped to 5.6% when performed using the IHC HercepTest, 8.6% when performed using any other IHC method, and 8.3% when performed using the FISH method after implementation of a successful quality assurance program.

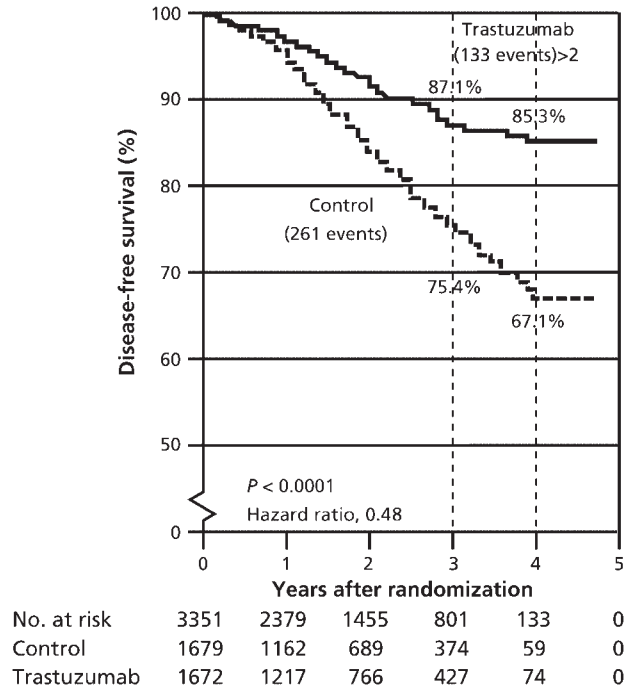

Figure 8 Kaplan-Meier estimates of disease-free survival for patients with HER2-positive breast cancer undergoing therapy with a doxorubicin/cyclophosphamide regimen followed by paclitaxel with or without trastuzumab. Data is from the combined analysis of the NSABP B-31 and NCCTG N9831 trials of adjuvant therapy in breast cancer. From Romond et al.[19]; with permission.

HER2 Testing: Methodologic Issues

Strict quality assurance measures had a dramatic effect on the accuracy of HER2 testing in the NSABP B-31 study of trastuzumab therapy combined with chemotherapy in the adjuvant treatment of breast cancer.[103] Initial high rates of discordance between local and central IHC testing were successfully reduced after a stringent quality assessment and assurance plan was implemented, which included restriction of IHC testing to reference laboratories that performed a high volume of HER2 tests or had a high concordance rate with FISH testing. Some of the sources of variability in HER2 testing methodology that must be accounted for in a quality assurance program are outlined:

Tissue Fixation

The type of issue fixative used is known to impact HER2 test results, particularly with IHC testing.[53,108–110] Furthermore, the time between tissue removal and initiation of fixation and duration of the fixation process may also affect results. These factors have been found to affect estrogen receptor results in samples of invasive breast carcinoma as evaluated by IHC methods.[111,112]

Assay Method

Although 4 FDA-approved assays specific for HER2 are available (2 IHC assays and 2 FISH assays) for which standardized protocols exist, many non-standardized, non-approved HER2 assay procedures are in use, particularly for HER2 status evaluated by IHC. One report estimated that at least 30 different HER2 antibodies, most of which are specific for the intracellular portion of HER2, have been used in HER2 IHC assays,[53] and variability in HER2 test results has been associated with the type of primary antibody used.[113–115]

Antigen retrieval methods, often used in IHC protocols to improve the ability of paraffin-embedded tissue to undergo immunostaining, can artificially increase stain intensity and dramatically affect the results of IHC assays.[20,116–118] Evidence exists to indicate that a substantial percentage of pathology laboratories use variable antigen retrieval methods (Hammond MEH; Unpublished results from survey of external proficiency testing participants, College of American Pathologists; 2004). For example, because of their ready availability, heating devices such as pressure cookers or microwaves are sometimes substituted for a water bath, which is the device specified by the HercepTest protocol to be used in the antigen retrieval process (Figure 3).

Perhaps most importantly, many of the HER2 testing methods in use have not undergone technical validation in that they have not been tested and proven reliable against another designated "gold standard" assay known to provide accurate results.[9,75,119,120]

Interpretation of HER2 Test Results

Scoring HER2 status using either IHC or FISH is also associated with a number of problematic issues. HER2 status defined as IHC 2+ highlights some of the problems associated with the HercepTest IHC scoring method (Figure 4). In one large study, 14% of the tumor samples were scored as IHC 2+ but only 12% of the tumors with this score were found to be FISH amplified.[54]

IHC scoring methods are often described as semi-quantitative or subjective. A non-continuous system, such as the HercepTest method (Figure 4), is generally considered inadequate to describe the continuum of protein expression represented in tissue samples.[44,53,75] FDA-approved scoring guidelines are vague and give no helpful guidance to pathologists. Adding other requirements to the IHC scoring guideline, such as

uniform staining processes and a "chicken-wire" pattern, has been proposed.[9] Image analysis methods, such as digital microscopy and the automated cellular imaging system (ACIS), in which HER2 scoring of tissue samples is performed by computer, are becoming more widely available in pathology laboratories and have been reported to dramatically increase the accuracy and precision of IHC test result scoring.[121–123]

Scoring problems associated with FISH testing have been reported to occur when samples with borderline gene/chromosome ratios (close to 2.0) were evaluated.[9,55] *HER2* gene/chromosome 17 ratios in the 1.8 to 2.2 range are considered "borderline amplified" in the PathVysion method[46] although no specific range of HER2 gene copies/cell has been defined as borderline amplified in the protocol for the INFORM method.[47] Currently, no high-level evidence or agreement is available on how results in the borderline range should be interpreted or confirmed. To some extent, the scoring difficulties associated with FISH testing are likely to be caused by, in part, difficulties associated with choosing specific cells to include in the determination. In addition, false-negative or false-positive FISH test results may be attributable to the length of enzymatic digestion steps during tissue processing.[9] Problems identifying regions of invasive tumor in samples stained with 4', 6-diamidino- 2-phenylindole have also been reported.[9,20,124]

NCCN Task Force Recommendations for HER2 Testing

The NCCN HER2 Testing in Breast Cancer Task Force recommends that all laboratories performing HER2 testing for clinical purposes use a methodology that has been validated by a documented high level of concordance with another validated test. Test reporting should be complete, including a description of the methodology used and the results of validation and concordance testing. An ongoing quality assurance program should be in place.

Assurance of the Quality and Accuracy of Laboratory Testing

Validation of HER2 Testing The procedure for validating any test offered by a laboratory involves several steps (Table 5). The laboratory must use appropriate equipment consistently, assure that laboratory personnel are trained in the use of the equipment, and develop a standard operating procedure for the test to be offered. Personnel must then be trained on this standard operating procedure using a standardized training plan. The new procedure must be tested on a group of clinical cases of the same type on which the test will be offered. This testing must be done in parallel with a validated clinical test for the same analyte (HER2). If the new test (e.g., HER2 receptor by IHC) is to be compared with a previously validated complementary test (e.g., *HER2* gene by FISH), the samples are tested by both methods and results compared. Alternatively, the test can be validated by having the test run in parallel by another laboratory in which a validated assay is already offered. The number of tests required for a successful validation is not well defined, but ranges from 50 to 100, depending on the variety of results possible and the amount of variation in results encountered in the test. A new test should show at least 95% concordance with the validated assay to which it is compared. Borderline cases should not be used to calculate this concordance.

Determination of Concordance Between Complementary HER2 Assays Acceptable performance for any validated HER2 assay is that it is concordant with the other form of HER2 testing on the same sample at least 95% of the time. This measurement of concordance can be accomplished during validation if the complementary test method is used for validation. If the same method (e.g., HER2 IHC compared with a validated HER2 IHC method by another laboratory) is used for validation, the concordance level can be indirectly inferred from the validating laboratory's level of concordance. For a laboratory to perform reflex testing to a complementary HER2 testing procedure, to evaluate HER2 status of samples with borderline scores, that laboratory must directly demonstrate that the complementary assay is concordant at least 95% of the time with a validated form of another type of HER2 assay performed on the same sample (Table 6). Otherwise, borderline cases must be sent to a reference laboratory that is qualified to perform the complementary assay procedure. If the concordance between complementary HER2 testing procedures performed in a laboratory falls below 95% for IHC 3+ and FISH-amplified samples or IHC 0/1+ and FISH non-amplified samples, complementary testing of the failing category must be done by another laboratory offering a validated complementary test. Borderline cases should not be included in concordance studies.

Table 5 Protocol for Validation of a HER2 Testing Procedure	
Step 1	Obtain 50-100 samples of the tumor type to be clinically run. This can be done with a tissue array* if desired. At least half of these cases should represent HER2 positive tumors (e.g., IHC 3+ or FISH amplified) if assay validation is performed using a complementary testing procedure.
Step 2	Run samples at least twice with standardized protocol using strict interpretation guidelines and reporting criteria.
Step 3	Provide sections of same tumors to another laboratory which has a validated laboratory HER2 testing procedure, preferably identical to the method you are using, and ask that interpretation and reporting criteria be identical to the ones you use (or use samples previously run by a reference laboratory). In-house validation of an assay can be done if the laboratory is already performing a validated HER2 testing procedure (i.e. validation of an assay can be performed in the process of determining the concordance between two complementary assays; See Table 6).
Step 4	Compare and record results.
Step 5	For assay validation, at least a 95% concordance rate with the validating laboratory should be achieved. Borderline cases should not be used to calculate this concordance. A validated assay should also demonstrate at least 95% concordance with a complementary assay either by direct testing (Table 6) or association with the levels of concordance between complementary testing achieved by the validating laboratory.

* 80 case tissue array designed by National Cancer Institute; Fitzgibbons et al.[125]

Table 6 Protocol for Evaluation of Concordance of a HER2 Testing Procedure with a Complementary HER2 Testing Method	
Step 1	Obtain 50-100 samples of the tumor type to be clinically run. This can be done with a tissue array* if desired. At least half of these cases should represent HER2 positive tumors (e.g., IHC 3+ or FISH amplified).
Step 2	After running samples with a validated testing procedure used in your laboratory, run samples with a complementary validated HER2 assay available in your laboratory or another laboratory. (Alternatively, validation of an unvalidated HER2 assay can be performed simultaneously with the determination of concordance between two complementary assays [See Table 5].
Step 3	Compare and record results.
Step 4	Concordance between IHC and FISH procedures is defined as at least 95% concordance between IHC 0,1+ and FISH non-amplified results, and IHC 3+ and FISH amplified results. Borderline cases should not be included in concordance studies.

* 80 case tissue array designed by National Cancer Institute; Fitzgibbons et al.[125]

Practical Application of Testing Methodologies

Tissue Fixation Breast tissue must be fixed in 10% buffered formalin. This recommendation is consistent with recent joint recommendations from the National Institute of Standards and Technology (NIST), the Cancer Diagnosis Program of the National Cancer Institute, the FDA, and the College of American Pathology (CAP), which specify that 10% buffered formalin must be used for samples that will undergo HER2 testing.[108]

Assay Method

IHC Assays

Validated IHC assays can be used to make an initial assessment of HER2 tumor status. Any IHC assay, whether FDA approved or not, must be validated by the laboratory providing the test before it offers the test and whenever the testing is modified. Validation can be performed using another validated method, either IHC or FISH.

All assay protocols must include positive and negative HER2 standard controls. Standardized, positive and negative HER2 controls are included with all commercial HER2 testing kits. An initiative to develop new standard controls, sponsored by NIST, is currently underway.[108]

The laboratory must maintain strict adherence to internal quality assurance procedures as mandated by Clinical Laboratory Improvement Act of 1988 (CLIA 88) legislation, which specifies that any test procedure must be validated, equipment must be calibrated and subject to routine quality control, procedures must be standardized, personnel must be trained in those procedures, and ongoing competency assessment must be performed.[117]

FISH Assays

FISH assays, including FDA- or non-FDA–approved assays, can be used for initial assessment of HER2 tumor status provided that the assays are validated by the laboratory providing the test before the test is

offered and whenever any modification of the testing is done. Validation can be done using another validated method.

The laboratory must maintain strict adherence to internal quality assurance procedures as mandated by the CLIA 88 regulations, which specify that any test procedure must be validated, equipment must be calibrated and subject to routine quality control, procedures must be standardized, personnel must be trained to those procedures, and ongoing competency assessment must be performed.[117]

All assay protocols must include positive and negative standard control tissues as described in the previous section. A validated FDA-approved version of the FISH assay is recommended as the "gold standard" for confirmatory testing, when necessary.

Oncologists must be aware of the different interpretations of *HER2* gene amplification, borderline *HER2* gene amplification, and *HER2* gene non-amplification associated with the numerical ranges specified by the 2 FISH assay types (PathVysion and INFORM).

CISH Assays

The task force reviewed the use of CISH as a means of determining HER2 tumor status. Although the task force acknowledged the existence of compelling evidence to indicate that CISH is potentially a very promising approach to HER2 testing, recommendations on the use of this test were not made, because no FDA-approved methodology for CISH testing is currently available.

Assays of Extracellular Domain (ECD) of HER2

The task force specifically reviewed the use of measurements of circulating levels of the extracellular domain of the HER2 receptor protein and found that the evidence did not allow for the use of such testing for prognostic or predictive purposes at the current time.

Test Result Reporting HER2 test reports must provide sufficient information for informed clinicians to effectively use the results in clinical decision making. HER2 test reports should include site of tumor; specimen type; histologic type; fixation method, fixation time; block examined; HER2 testing method and criteria, including information on standardization and validation of testing method, positive/negative controls, and details of reflex testing if performed; information on tissue staining and assay reagents; and laboratory quality assurance information.[9,117] Reports should also include a clear statement that HER2 testing was done on an invasive, not in situ, part of the tumor. Clinicians responsible for evaluating HER2 test results should be familiar with these criteria.

External Quality Assurance HER2 testing should be done only in laboratories accredited to perform such testing. Laboratory accreditation, offered by the Joint Commission on Accreditation in Healthcare Organizations (JCAHO), CAP, or by Centers for Medicare and Medicaid Services (CMS) trained state specific inspectors, is based on accreditation criteria included in CLIA 88 legislation. Ongoing proficiency testing is a necessary component of a laboratory's qualification for accreditation.

Currently, a proficiency testing exercise is offered by CAP as an 80-case array designed by National Cancer Institute statistical review to assure laboratory proficiency for HER2 testing.[125] This array-based exercise will continue to be offered by CAP for IHC HER2 testing competency, and a similar product will be available for proficiency testing for HER2 FISH testing, and for image analysis of either IHC or FISH HER2 testing. Such proficiency testing will probably become mandatory for laboratory accreditation in the future. Furthermore, laboratory accreditation guidelines for inspection of laboratories that perform HER2 testing will probably require documentation that the competency of pathologists performing such testing is monitored on an ongoing basis.

Assignment of HER2 Status

Recommendations for assignment of HER2 tumor status based on test results are summarized in Figure 9. Initially, the HER2 status of a patient can be determined by either IHC or FISH testing, provided that the test is performed in an accredited laboratory with a documented validated assay for HER2 status determination. If the initial testing is done using IHC, samples with borderline results (e.g., IHC 2+) must be subjected to reflex testing by a validated complementary HER2 testing procedure previously shown to be at least 95% concordant with the initial testing procedure (e.g, at least 95% concordant for both IHC 3+ and FISH-amplified results, and IHC 0,1+ and FISH non-amplified results on an ongoing basis). Laboratories not demonstrating 95% concordance between the complementary testing procedures must send borderline samples to a reference laboratory with a demonstrated concordance of at least 95% between the complementary testing procedures.

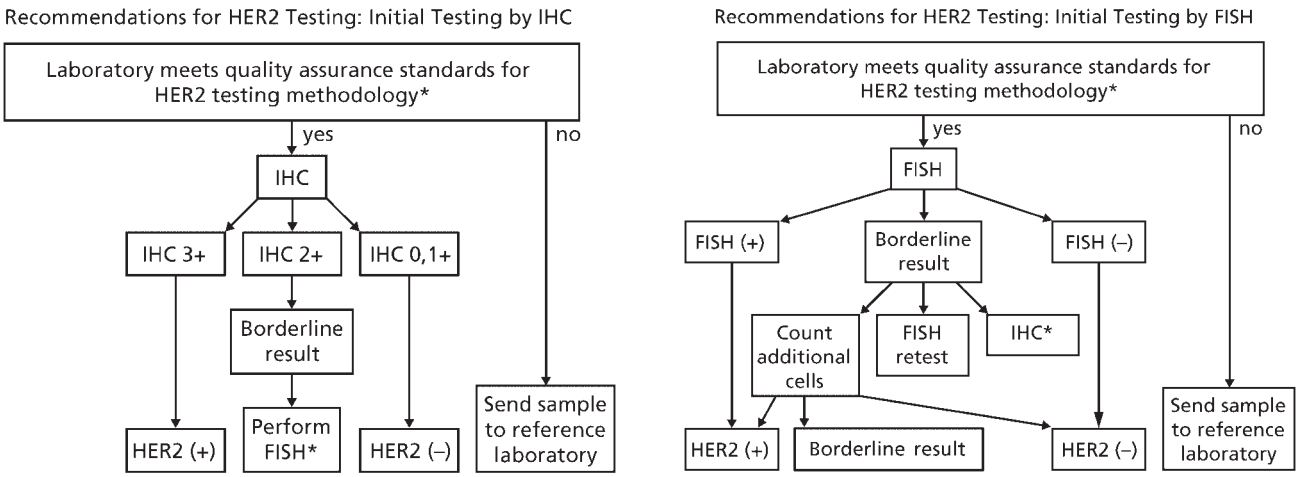

Figure 9 Recommendations for HER2 testing: *All HER2 tests must be validated. Validation of a HER2 test is defined as at least 95% concordance in 50 to 100 tumor samples when the testing method performed in a laboratory is compared with 1) another validated HER2 testing method performed in the same laboratory; 2) a validated HER2 testing method performed in another laboratory; or 3) validated reference laboratory test results. Borderline cases should not be included in the validation study. These algorithms are based on the assumption that all validated HER2 tests have been shown to be at least 95% concordant with the complementary form of HER2 test. Left panel: Borderline IHC cases (e.g. IHC 2+) are subjected to reflex testing by a validated complementary (e.g. FISH) HER2 testing method that has shown at least 95% concordance between 0 or 1+ IHC and FISH non-amplified results and IHC 3+ and FISH amplified results for 50 to 100 tumor samples. Right panel: Borderline FISH cases (an average HER2 gene/chromosome ratio of 1.8 to 2.2 or an average HER2 gene copy number of >4 to <6), should undergo 1) counting of additional cells; 2) retesting using FISH; or 3) reflex testing using a validated IHC method that is 95% concordant with FISH as described. A laboratory may perform only those tests which have been demonstrated to conform to these quality assurance standards. All other HER2 testing should be done in a qualified reference laboratory.

Image analysis methods for interpretation are strongly encouraged to assure consistency. If FISH is used for initial determination, amplified FISH results are considered positive; non-amplified results are considered negative. FISH results in the borderline range (average *HER2* gene/chromosome 17 ratio of 1.8 to 2.2 or average *HER2* gene copy number/cell of >4 to <6 gene copies/cell) are confirmed by one of the following methods: additional counting of cells, retesting with FISH, or reflex testing with a validated IHC method previously shown to be 95% concordant with the validated FISH method.

Conclusions

Studies have shown that both IHC and FISH testing methods can be used to successfully determine the HER2 status of breast tumor cells. However, regardless of whether IHC or FISH is used for initial testing, the use of strict quality control and assurance measures at each laboratory performing HER2 determinations of breast cancer tumors for clinical purposes is essential, including formal test validation and concordance studies. A recommended algorithm for assignment of HER2 tumor status that uses IHC or FISH test results is provided in Figure 9.

Clinical decision-making for the individual patient is becoming increasingly dependent on the results of genetic and biomarker tests. True evidence-based clinical validation of the prognostic and predictive utilities of biomarker tests is performed through prospective clinical studies in which rigorous quality control measures are systematically implemented and reported.[75,93,116,126–129] Ultimately, the results of such studies will enable the clinician to more effectively provide the patient with individualized therapeutic choices targeted to the characteristics specific to the patient's individual cancer. Application of targeted therapies in circumstances with the greatest likelihood of benefit should improve clinical outcomes while minimizing exposure of patients without an appropriate target to such therapies.

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Post-test Please circle the correct answer on the enclosed answer sheet.

1. The name HER2 refers to:
   A. A gene encoding for the HER2 protein
   B. A member of the epidermal growth factor family of receptors
   C. A protein involved in signal transduction processes associated with cellular growth, differentiation, and survival
   D. All of the above
   E. None of the above

2. Which of the following statements regarding HER2 is FALSE?
   A. The HER2 receptor is found in cells of healthy breast tissue.
   B. Some breast tumor cells exhibit amplification of the HER2 gene.
   C. There is no evidence of an association between HER2 gene amplification and the amplification of other genes.
   D. The HER2 receptor is found in cells of healthy heart tissue.
   E. Some breast tumor cells overexpress the HER2 protein.
   F. There are 2 copies of the HER2 gene, each located on one of 2 copies of chromosome 17 in healthy, resting, breast epithelial cells.

3. What percentage of breast cancer tumors exhibit HER2 protein overexpression or gene amplification?
   A. 5%–10%
   B. 10%–15%
   C. 15%–20%
   D. 25%–35%
   E. 80%–85%

4. TRUE or FALSE? The most recent version of the NCCN Breast Cancer Clinical Practice Guidelines in Oncology incorporates the HER2 biomarker only as a prognostic factor for outcome independent of therapeutic intervention.
   A. True
   B. False

5. Select the most accurate response. HER2 is associated with targeted therapy because:
   A. the HER2 receptor is the direct target of trastuzumab, which is a monoclonal antibody directed to the extracellular portion of the HER2 receptor protein.
   B. the HER2 receptor is the direct target of trastuzumab along with the estrogen receptor.
   C. clinical outcomes of patients receiving trastuzumab therapy have been correlated with overexpression of the HER2 protein or amplification of the HER2 gene.
   D. A and C
   E. B and C

6. Select the most accurate response. The types of testing methods approved by the FDA to determine the HER2 status of breast tumor cells for the purpose of selecting patients to receive trastuzumab therapy are:
   A. immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH).
   B. FISH and chromogenic in situ hybridization (CISH).
   C. IHC, FISH, and assays of the extracellular domain (ECD) of HER2.
   D. IHC and CISH.
   E. only IHC.
   F. only FISH.

7. Which of the following has/have NOT been associated with variability in results obtained using IHC methods to detect HER2?
   A. Use of different methods to fix tissue
   B. Use of different primary antibodies
   C. Use of positive and negative tissue controls
   D. Use of different types of antigen retrieval methods
   E. A subjective scoring system

8. Which of the following characteristics has/have NOT been associated with variability in the INFORM® or the PathVysion® FISH methods?
   A. Difficulties associated with the interpretation of borderline scores
   B. Use of non-standardized protocols in many laboratories
   C. Problems identifying areas of invasive breast cancer tissue
   D. A subjective scoring method using values of 0–3+
   E. Tissue staining that is not permanent
   F. Variable length of enzymatic digestion steps during tissue processing

9. TRUE or FALSE? Clinical decision-making regarding HER2 status in breast cancer has been limited by difficulties in the interpretation of clinical studies that have combined patients with HER2 tumor status defined as IHC 2+ and IHC 3+, problems associated with clinical studies using retrospective analyses of HER2 tumor status, and questions concerning the reliability of HER2 testing methods.
   A. True
   B. False

10. According to the NCCN HER2 Testing in Breast Cancer Task Force, which results signify that a breast cancer tumor sample should be considered HER2 positive?
    A. IHC 3+ or an average HER2 gene copy number/chromosome 17 copy number per cell of >2.2
    B. IHC 3+ or an average HER2 gene copy number per cell of ≥6

C. IHC 3+ only
D. IHC 3+ and an average HER2 gene copy number per cell of >4
E. FISH amplified only
F. A and B

11. According to the recommendations in this report, which of the scores below is NOT considered "borderline?"
A. IHC 2+
B. Between an average of 1.8 and 2.2 HER2 gene copies per cell by the FISH PathVysion® method
C. Between an average of >4 and <6 gene copies per cell by the FISH INFORM® method
D. None of the above
E. All of the above

12. Select the most accurate response. Breast tumor samples that have been evaluated by one method of determining HER2 status are subjected to testing using another HER2 detection method:
A. to determine the HER2 status of a sample with a borderline score by the original method.
B. to perform a concordance study between complementary (e.g., IHC and FISH) testing methods.
C. to validate the original testing method.
D. All of the above
E. None of the above

13. TRUE or FALSE? According to the NCCN Recommendations for HER2 Testing, both validated IHC and validated FISH procedures are acceptable methods for the initial determination of the HER2 status of breast tumor cells.
A. True
B. False

14. TRUE or FALSE? A laboratory that does NOT show at least 95% concordance between 2 complementary testing methods can use the FISH method to further evaluate the HER2 status of a sample determined by initial testing to be IHC 2+.
A. True
B. False

Post-Test Answer Sheet

Please circle one answer per question. A score of at least 70% on the post-test is required.

1.	a	b	c	d	e		8.	a	b	c	d	e	f
2.	a	b	c	d	e	f	9.	a	b				
3.	a	b	c	d	e		10.	a	b	c	d	e	f
4.	a	b					11.	a	b	c	d	e	
5.	a	b	c	d	e		12.	a	b	c	d	e	
6.	a	b	c	d	e	f	13.	a	b				
7.	a	b	c	d	e		14.	a	b				

Please evaluate the achievement of the learning objectives using a scale of 1 to 5.
(1 = Not met; 3 = Partially met; 5 = Completely met)

Discuss the prevalence of invasive breast cancer characterized as HER2 positive
1 2 3 4 5

Recognize the molecular characteristics of the HER2 biomarker in both healthy breast epithelial cells and some invasive breast tumors
1 2 3 4 5

Understand the clinical evidence leading to the incorporation of the HER2 biomarker as both a prognostic and predictive factor in the most recent version of the NCCN Breast Cancer Clinical Practice Guidelines in Oncology
1 2 3 4 5

Explain the characteristics, advantages, and disadvantages of the different testing methods used for measuring the level of HER2 protein expression and/or gene amplification in samples of invasive breast tumors
1 2 3 4 5

Understand the sources of variability associated with the different HER2 testing methods and the recommendations of the HER2 Testing Task Force to limit such variability
1 2 3 4 5

Discuss the roles played by both the pathologist and the oncologist in assuring the appropriate applications of targeted therapy in breast cancer
1 2 3 4 5

Please indicate the extent to which you agree or disagree with the following statements:
(1 = Strongly disagree; 3 = Not sure; 5 = Strongly agree)

The material was presented in a fair and balanced manner.
1 2 3 4 5

The information presented in this monograph was pertinent to my educational needs.
1 2 3 4 5

The information presented was scientifically rigorous and up-to-date.
1 2 3 4 5

The information presented in this monograph has motivated me to modify my practice.
1 2 3 4 5

I would recommend this monograph to my colleagues.
1 2 3 4 5

HER2 Testing in Breast Cancer: NCCN Task Force Report and Recommendations

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