HER-2/neu Assessment in Breast Cancer Using the Original FDA and New ASCO/CAP Guideline Recommendations

Impact on Selecting Patients for Herceptin Therapy

Matteo Brunelli, MD, Erminia Manfrin, MD, Guido Martignoni, MD, Samantha Bersani, BaD, Andrea Remo, MD, Daniela Reghellin, MD, Marco Chilosi, MD, and Franco Bonetti, MD

Key Words: HER-2/neu; Immunohistochemistry; Fluorescence in situ hybridization; FISH; FDA; ASCO/CAP

DOI: 10.1309/MD79CDXN1D01E862

Abstract

*We evaluated HER-2/neu status in 100 consecutive ductal breast carcinomas by using the Food and Drug Administration (FDA) and American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) scoring systems. With the FDA system, scores were 3+ in 23.0%, 2+ in 25.0%, and 0 or 1+ in 52.0% of cases. With the ASCO/CAP system, scores were 3+ in 16.0%, 2+ in 34.0%, and 0 or 1+ in 50.0%. With the FDA and ASCO/CAP systems, respectively, 3+ cases (n = 23 and 16, respectively) showed high-grade, granular HER-2/*neu *amplification in 15 (65%) and 14 (88%); low-grade, borderline amplification in 7 (30%) and 1 (6%); and chromosome 17 polysomy without amplification in 1 (4%) and 1 (6%).*

*Concordance between schemes was higher for cases with high-grade, granular HER-2/*neu *amplification (concordance coefficient, 0.74). Cases with low-grade, borderline HER-2/*neu *amplification showed poor concordance (concordance coefficient, 0.20).*

*The FDA and ASCO/CAP schemes for HER-2/neu evaluation select patients differently for trastuzumab therapy. Major discordance is present for low-grade, borderline HER-2/*neu *amplification. FDA low-grade, borderline tumors would be reclassified as without HER-2/*neu *amplification or as polysomic. The ASCO/CAP scheme has a great concordance coefficient between strong 3+ immunohistochemical cases and cases with high-grade, granular HER-2/*neu *amplification.*

HER-2/neu overexpression in immunohistochemical analysis and/or gene amplification by fluorescence in situ hybridization (FISH) have been found to be prognostic and predictive in patients with breast cancer, and positive results select patients for treatment with trastuzumab (Herceptin) immunotherapy, which has been shown to inhibit the proliferation of human breast cancer cells.[1-4] The US Food and Drug Administration (FDA) approved the immunotherapy only for patients whose breast cancer cells show strong 3+ immunohistochemical staining, defined as uniform, intense membrane staining of more than 10% of invasive tumor cells, and for cases with 2+ staining (partial or basolateral membrane staining of >10% of invasive tumor cells) showing gene amplification by FISH, expressed as a ratio of more than 2 when comparing HER-2/*neu* gene and chromosome 17 fluorescent signals.[5,6]

Recently, the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) convened an expert panel that developed recommendations for optimal HER-2 testing performance.[1] A positive 3+ HER-2 result was considered as uniform, intense membrane staining of more than 30% of invasive tumor cells, and HER-2/*neu* gene amplification was considered as a ratio of more than 2.2. Moreover, major molecular issues regarding HER-2/neu status pose significance to 3 cytogenetic abnormalities, such as chromosome 17 polysomy and low-grade, borderline vs high-grade, granular HER-2/*neu* amplification.[7]

Accurate assignment of the HER-2 status of invasive breast cancer is essential to clinical decision making in the treatment of breast cancer in adjuvant and metastatic settings. We selected a series of consecutive ductal breast carcinomas and evaluated the clinical impact of selecting

patients for trastuzumab treatment with different HER-2/neu cutoff values as defined by the original FDA system and the new ASCO/CAP guideline recommendations.

Materials and Methods

Samples

We selected 100 formalin-fixed, paraffin-embedded, consecutive samples of invasive ductal breast carcinoma.

Immunohistochemical Analysis

According to the recommendations from the manufacturer of the HercepTest kit (DAKO, Glostrup, Denmark), tissue sections mounted on slides and stored at room temperature were stained within 4 to 6 weeks from sectioning to maintain antigenicity. The guidelines for FDA scoring were as follows: 3+, strong, complete membrane staining in more than 10% of the malignant cells; 2+, weak to moderate complete membrane staining in more than 10% of the malignant cells; 0 or 1+, no or fewer than 10% of cells staining, respectively. These cases were regraded according to the ASCO/CAP guideline recommendations.[1] In this system, a positive 3+ HER-2 result was considered as uniform, intense membrane staining of more than 30% of invasive tumor cells. Interpretations by the FDA and ASCO/CAP systems were done independently and in a masked manner, one relative to the other, and separated by time.

FISH Analysis

FISH was performed on all cases using the PathVysion HER-2/*neu* DNA probe kit from Vysis/Abbott, Downers Grove, IL.

From each tumor, 5-μm sections were cut from paraffin-embedded blocks. The paraffin was removed from the sections with two 10-minute washes in xylene. After hydrating in 100%, 85%, and 70% ethanol solutions (10 minutes) and rinsing in distilled water (10 minutes) and twice in phosphate buffer solution (pH 7; 10 minutes each), the slides were fixed in methanol-acetic acid, 3:1, for 10 minutes and air dried. Next, the sections were treated in a 2× standard saline citrate (SSC) solution for 15 minutes at 37°C; dehydrated in consecutive 70%, 85%, and 100% ethanol solutions for 1 minute each; and dried. Next, the sections were bathed in 0.1 mmol/L of citric acid (pH 6) solution at 85°C for 1 hour. Then they were again dehydrated in a series of ethanol solutions and dried. The tissue was digested by applying 0.75 mL of pepsin (Sigma, St Louis, MO) solution (4 mg/mL in 0.9% sodium chloride, pH 1.5) to each slide and incubating them in a humidified box for 30 minutes at 37°C. Next, the slides were rinsed with distilled water for a few seconds, dehydrated again in graded ethanol solutions, and dried. Centromeric probes for chromosomes 17 and locus-specific HER-2/*neu* gene were used (Abbott kit).

Next, 10 μL of diluted probe was applied to each slide, and coverslips were placed over the slides. Denaturation was achieved by incubating the slides at 80°C for 10 minutes in a humidified box; then hybridization was done at 37°C for 3 hours. The coverslips were removed and the slides immersed at room temperature in 0.5× SSC for 2 minutes, in 50% formamide/1× SSC for 5 minutes, and in 2× SSC for 2 minutes. The slides were air dried and counterstained with 10 μL of 4′,6-diamidino-2-phenylindole (DAPI in Fluorguard, 0.5 μg/mL, Insitus, Albuquerque, NM).

The slides were examined by using an Axioplan (Zeiss, Oberkochen, Germany) with appropriate filters for SpectrumOrange (locus-specific probe HER-2, Abbott), SpectrumGreen (centromeric probe 17, Abbott), and the UV filter for the DAPI nuclear counterstain. The signals were recorded with a CCD camera (Axiocam HRm, Zeiss). From 100 to 200 neoplastic nuclei were counted and scored according to different cutoff values.

Specimens were determined to be amplified if the ratio of HER-2/*neu* signals to chromosome 17 centromere signals was higher than 2.0 according to the FDA score. Gene amplification was reevaluated according to the ASCO/CAP guideline recommendations using the ratio of 2.2. Polysomy for chromosome 17 was defined when the ratio was 1 in tumors having 3 or more locus-specific HER-2/*neu* and centromeric 17 signals. High-grade, granular HER-2/*neu* amplification was scored when locus-specific signals were 4 or more per neoplastic nucleus.[7] The FDA and ASCO/CAP interpretations were done independently and in a masked manner, one relative to the other, and separated by time.

Statistical Analysis

The κ statistic, which is a measure of agreement between observers that corrects for chance agreement, was used to evaluate intraobserver and interobserver concordance between the FDA and ASCO/CAP systems. Concordance was considered fair when κ values ranged from 0.00 to 0.20, moderate with a range from 0.21 to 0.45, substantial with a range from 0.46 to 0.75, and almost perfect with a range from 0.76 to 0.99.

Results

The immunohistochemical results according to 2 cutoff values are summarized in **Table 1**. With the FDA system, scores were 3+ in 23.0%, 2+ in 25.0%, and 0 or 1+ in 52.0% of cases. With the ASCO/CAP grading scheme, scores were 3+ in 16.0%, 2+ in 34.0%, and 0 or 1+ in 50.0% of cases.

The 23 breast carcinomas scored as 3+ immunohistochemically **Image 1** showed high-grade, granular HER-2/*neu* amplification in 15 cases (65%); low-grade, borderline amplification in 7 (30%); and polysomy of chromosome 17

DOI: 10.1309/MD79CDXN1D01E862

Table 1
HER-2/neu Immunohistochemical Expression Values According to the Original FDA and New ASCO/CAP Grading Schemes*

	Immunohistochemical Value		
Grading Scheme	**0/1+**	**2+**	**3+**
FDA	52	25	23
ASCO/CAP	50	34	16

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; FDA, US Food and Drug Administration.
* Data are given as number of cases.

without HER-2/*neu* gene amplification in 1 (4%) when scoring was performed according to the FDA scheme **Table 2**. In contrast, 16 cases scored as 3+ immunohistochemically showed high-grade, granular HER-2/*neu* amplification **Image 2** in 14 cases (88%); low-grade, borderline amplification in 1 (6%); and polysomy of chromosome 17 without HER-2/*neu* gene amplification in 1 (6%) when scoring was performed according to the ASCO/CAP scheme (Table 2).

Concordance between the grading schemes was higher for cases with high-grade, granular HER-2/*neu* amplification (concordance coefficient, 0.74). Cases with low-grade, borderline HER-2/*neu* amplification showed poor concordance (concordance coefficient, 0.20). Polysomy of chromosome 17 showed intermediate concordance (concordance coefficient, 0.63).

Immunohistochemical evaluation revealed fair intraobserver concordance ($\kappa = 0.15$) for 3+ immunohistochemical cases with the FDA system and substantial concordance ($\kappa = 0.50$) with the ASCO/CAP systems. Intraobserver concordance was fair ($\kappa = 0.20$) for amplification by FISH with the FDA system and almost perfect ($\kappa = 0.80$) with the ASCO/CAP system.

Interobserver concordance was fair ($\kappa = 0.16$) for 3+ immunohistochemical cases with the FDA scoring system and moderate ($\kappa = 0.40$) for amplification by FISH with the FDA scoring system. Interobserver concordance was substantial ($\kappa = 0.71$) for amplification by FISH with the ASCO/CAP system when scoring low-grade amplification and almost perfect ($\kappa = 0.80$) when scoring high-grade amplification.

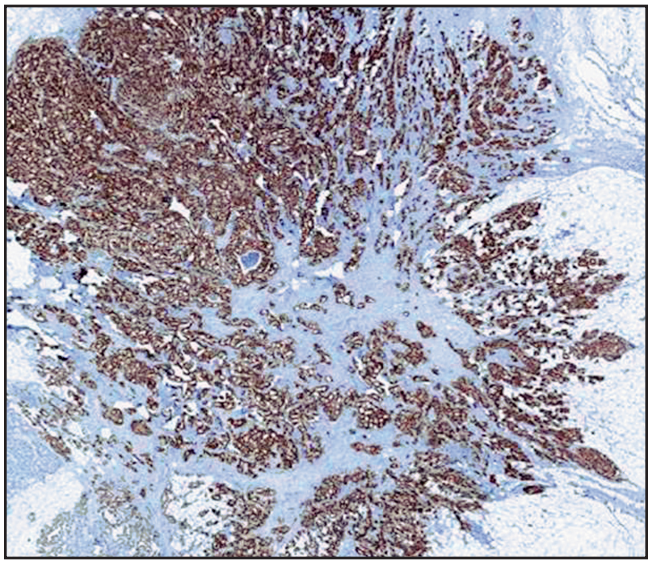

Image 1 Breast carcinoma showing strong 3+ HER-2 immunohistochemical expression according to the American Society of Clinical Oncology/College of American Pathologists grading scheme (×10).

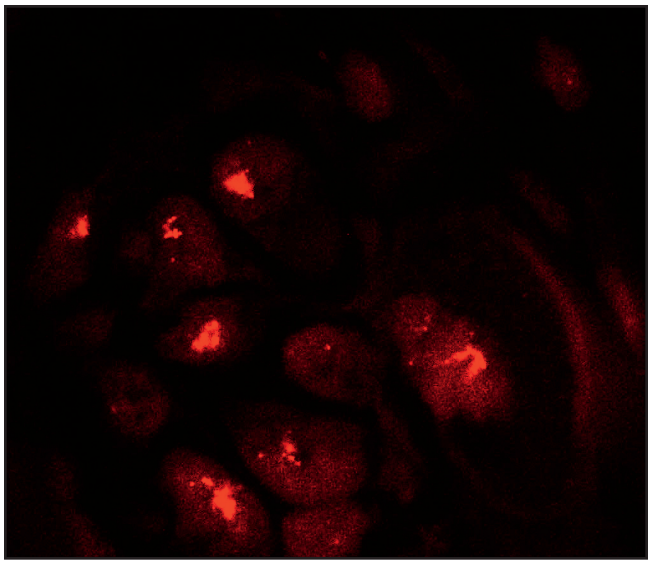

Image 2 High-grade, granular HER-2/*neu* gene amplification showing clusters of SpectrumOrange fluorescent signals (×63).

Table 2
Three Major Chromosomal Abnormalities of HER-2/neu Status Among Breast Carcinomas With 3+ Immunohistochemical Expression*

		HER-2/*neu* Amplification	
	Polysomy of Chromosome 17	**Low Grade, Borderline**	**High Grade, Granular**
FDA (n = 23)	1 (4)	7 (30)	15 (65)
ASCO/CAP (n = 16)	1 (6)	1 (6)	14 (88)

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; FDA, US Food and Drug Administration.
* Data are given as number (percentage).

DOI: 10.1309/MD79CDXN1D01E862

Discussion

Our findings indicate the following: (1) The FDA and ASCO/CAP grading schemes for HER-2/neu evaluation differently select patients for trastuzumab therapy. (2) Major discordance is present in the group with low-grade, borderline HER-2/*neu* amplification, and tumors classified in the FDA system as low-grade, borderline would be reclassified as without HER-2/*neu* amplification or as polysomic. (3) Polysomy of chromosome 17 and high-grade and low-grade, borderline HER-2/*neu* gene amplification are 3 major molecular patterns still significantly present in the group graded by the ASCO/CAP scheme; however, major concordance is present between strong 3+ immunohistochemical cases and high-grade, granular HER-2/*neu* amplification.

The FDA approved immunotherapy only for patients whose breast cancer cells show strong 3+ immunohistochemical staining.[1] Recently, ASCO and CAP convened an expert panel that developed recommendations for optimal HER-2 testing performance.[1] Major changes were as follows: a positive 3+ HER-2 result was considered as uniform, intense membrane staining of more than 30% of invasive tumor cells (previous FDA cutoff, 10% of tumor cells), and HER-2/*neu* gene amplification was considered as a ratio of more than 2.2 (previous FDA cutoff, ≥2).

An accurate assignment of HER-2/neu status of invasive breast cancer is essential to clinical decision making in the treatment of breast cancer in adjuvant and metastatic settings.[8,9] Occasional breast carcinomas with HER-2/neu 3+ immunostaining may be negative for gene amplification by FISH according to standard scoring criteria.[10-13]

The FISH method is technically more standardized and less affected by tissue variables than the immunohistochemical method and has emerged as the "gold standard" for assessment of HER-2/*neu* gene amplification status.[14,15] The finding of immunohistochemical 3+ staining without gene amplification has been generally attributed to 3 major factors: false-positive immunostaining, protein overexpression without gene amplification, and chromosome 17 aneuploidy or polysomy.[13] The standard FISH method not only analyzes whether the HER-2/*neu* gene is amplified but also assesses the degree of amplification, thus providing a more quantitative measurement. Previous clinical studies have shown that a significant proportion of tumors with even HER-2/neu immunohistochemical results of 3+ and gene amplification by FISH do not respond to trastuzumab therapy.[16,17] The gene dosage phenomenon could have a significant role in the spectrum of therapeutic responses and warrants further clinical investigation.

The FDA and ASCO/CAP grading schemes for HER-2/neu evaluation select patients differently for trastuzumab therapy, and major discordance is present in the group with low-grade, borderline HER-2/*neu* amplification in regard to cases with HER-2/*neu* gene amplification and a ratio of less than 4. Tumors classified as low-grade, borderline in the FDA system (ratio >2 but <4) would be reclassified as HER-2/*neu* not amplified or as polysomic cases by FISH. Patients who did not respond to immunotherapy may have cancerous cells with low-grade, borderline HER-2/*neu* amplification or chromosome 17 polysomy. To date, there is no strong evidence for the efficacy of trastuzumab in this group of patients. Clinical trials must consider these molecular phenotypes to study the usefulness of trastuzumab in the management of patients with breast cancer. However, a high level of concordance has been found between 3+ immunohistochemical cases and high-grade, granular HER-2/*neu* amplification (ratio >4) when considering the ASCO/CAP grading scheme (concordance coefficient, 0.71). These findings are similar to those described by Hameed et al.[18]

Polysomy of chromosome 17 has been found to be a third major cytogenetic abnormality in HER-2/neu testing,[19] and we found it in 6% of 3+ immunohistochemical cases when using the ASCO/CAP grading scheme. Lal et al[20] suggested that polysomy 17 in these tumors would have an additive effect with genuine HER-2/*neu* amplification and contribute to the overall increase of HER-2/*neu* gene copies in the tumor cells, and, similarly, polysomy 17 in the absence of HER-2/*neu* gene amplification would result in a modest increase of HER-2/*neu* gene copies in the tumor cells. This modest increase in the HER-2/*neu* gene copies may result, in some cases, in increased HER-2/neu protein production to the level that could be demonstrated by immunohistochemical staining as strong as 3+. Among these issues there are, however, conflicting data. Downs-Kelly et al[21] suggested that chromosome 17 polysomy in the absence of HER-2/*neu* amplification does not have a significant biologic influence on HER-2 gene expression in breast carcinoma.

Low-grade, borderline HER-2/*neu* amplification tumors (ratio of HER-2 amplification <4) and those with chromosome 17 polysomy more often show intratumoral heterogeneity. This phenomenon may be due to nuclear truncation on tissue slides or in part to biologic reasons.[22]

Heterogeneity of HER-2/neu status in a tumor may be a rare event or underestimated.[23,24] Extensive analysis using tissue microarray cores from different breast cancer samples may contribute to estimation of the heterogeneity and to a better understanding of the variation in therapeutic responses and the conflicting data in studies about the prognostic and predictive role of HER-2/neu status in this subset of patients with breast cancer.[25]

The present study does not permit a determination of which FISH assay is better for clinical testing. The clinical significance of polysomy of chromosome 17 and low-grade, borderline HER-2/*neu* amplification remains to be addressed.

We showed that the FDA and ASCO/CAP grading schemes for HER-2/neu evaluation select patients differently for trastuzumab therapy and that major discordance is present

DOI: 10.1309/MD79CDXN1D01E862

in the group with low-grade, borderline HER-2/*neu* amplification. Further analyses using different techniques such as reverse transcription–polymerase chain reaction and clinical trials including these uncertain cases may shed light on the molecular biology of HER-2/neu status and its value as a marker of immunoresponsiveness, chemoresponsivness, or prognosis.[26,27] Moreover, the ASCO/CAP grading scheme has a good concordance coefficient between strong 3+ immunohistochemical cases and high-grade, granular HER-2/*neu* amplification.

From the Department of Pathology, Università di Verona, Verona, Italy.

Address reprint requests to Dr Bonetti: Anatomia Patologica, Dipartimento di Patologia, Università di Verona; Strada Le Grazie n. 8; 37134, Verona, Italy.

Acknowledgment: Financial support was received from Progetto Mammella Bando 2004 Fondazione Cassa di Risparmio di Verona Vicenza Belluno e Ancona MiUR, Verona, Italy.

References

1. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol.* 2007;25:118-145.
2. Ross JS, Gray GS. Targeted therapy for cancer: the HER-2/neu and Herceptin story. *Clin Leadersh Manag Rev.* 2003;17:333-340.
3. Ridolfi RL, Jamehdor MR, Arber JM. HER-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence in situ hybridization approach. *Mod Pathol.* 2000;13:866-873.
4. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science.* 1987;235:177-182.
5. Birner P, Oberhuber G, Stani J, et al. Evaluation of the United States Food and Drug Administration–approved scoring and test system of HER-2 protein expression in breast cancer. *Clin Cancer Res.* 2001;7:1669-1675.
6. Jacobs TW, Gown AM, Yaziji H, et al. Specificity of HercepTest in determining HER-2/neu status of breast cancers using the United States Food and Drug Administration–approved scoring system. *J Clin Oncol.* 1999;17:1983-1987.
7. Lal P, Salazar PA, Hudis CA, et al. HER-2 testing in breast cancer using immunohistochemical analysis and fluorescence in situ hybridization: a single-institution experience of 2,279 cases and comparison of dual-color and single-color scoring. *Am J Clin Pathol.* 2004;121:631-636.
8. Pauletti G, Dandekar S, Rong H, et al. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J Clin Oncol.* 2000;18:3651-3664.
9. Jacobs TW, Gown AM, Yaziji H, et al. Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. *J Clin Oncol.* 1999;17:1974-1982.
10. Rossi E, Ubiali A, Cadei M, et al. HER-2/neu in breast cancer: a comparative study between histology, immunohistochemistry, and molecular technique (FISH). *Appl Immunohistochem Mol Morphol.* 2006;14:127-131.
11. Hammock L, Lewis M, Phillips C, et al. Strong HER-2/neu protein overexpression by immunohistochemistry often does not predict oncogene amplification by fluorescence in situ hybridization. *Hum Pathol.* 2003;34:1043-1047.
12. Roche PC, Suman VJ, Jenkins RB, et al. Concordance between local and central laboratory HER2 testing in the Breast Intergroup Trial N9831. *J Natl Cancer Inst.* 2002;94:855-857.
13. Tubbs RR, Pettay JD, Roche PC, et al. Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. *J Clin Oncol.* 2001;19:2714-2721.
14. Bartlett JM, Forsyth A. Detection of *HER2* gene amplification by fluorescence in situ hybridization in breast cancer. *Methods Mol Med.* 2006;120:309-322.
15. Bartlett J, Mallon E, Cooke T. The clinical evaluation of HER-2 status: which test to use? *J Pathol.* 2003;199:411-417.
16. Menard S, Valagussa P, Pilotti S, et al. Response to cyclophosphamide, methotrexate, and fluorouracil in lymph node–positive breast cancer according to HER2 overexpression and other tumor biologic variables. *J Clin Oncol.* 2001;19:329-335.
17. Pegram MD, Pauletti G, Slamon DJ. HER-2/neu as a predictive marker of response to breast cancer therapy. *Breast Cancer Res Treat.* 1998;52:65-77.
18. Hameed O, Chhieng DC, Adams AL. Does using a higher cutoff for the percentage of positive cells improve the specificity of HER-2 immunohistochemical analysis in breast carcinoma? *Am J Clin Pathol.* 2007;128:825-829.
19. Salido M, Tusquets I, Corominas JM, et al. Polysomy of chromosome 17 in breast cancer tumors showing an overexpression of ERBB2: a study of 175 cases using fluorescence in situ hybridization and immunohistochemistry. *Breast Cancer Res.* 2005;7:R267-R273.
20. Lal P, Salazar PA, Ladanyi M, et al. Impact of polysomy 17 on HER-2/neu immunohistochemistry in breast carcinomas without HER-2/*neu* gene amplification. *J Mol Diagn.* 2003;5:155-159.
21. Downs-Kelly E, Yoder BJ, Stoler M, et al. The influence of polysomy 17 on HER2 gene and protein expression in adenocarcinoma of the breast: a fluorescent in situ hybridization, immunohistochemical, and isotopic mRNA in situ hybridization study. *Am J Surg Pathol.* 2005;29:1221-1227.
22. Wang S, Hossein Saboorian M, Frenkel EP, et al. Aneusomy 17 in breast cancer: its role in HER-2/neu protein expression and implication for clinical assessment of HER-2/neu status. *Mod Pathol.* 2002;15:137-145.
23. Hanna W, Nofech-Mozes S, Kahn HJ. Intratumoral heterogeneity of HER2/neu in breast cancer: a rare event. *Breast J.* 2007;13:122-129.
24. Shin SJ, Hyjek E, Early E, et al. Intratumoral heterogeneity of HER-2/neu in invasive mammary carcinomas using fluorescence in-situ hybridization and tissue microarray. *Int J Surg Pathol.* 2006;14:279-284.
25. Sapino A, Marchio C, Senetta R, et al. Routine assessment of prognostic factors in breast cancer using a multicore tissue microarray procedure. *Virchows Arch.* 2006;449:288-296.
26. Tubbs R, Pettay J, Skacel M, et al. Gold-facilitated in situ hybridization: a bright-field autometallographic alternative to fluorescence in situ hybridization for detection of HER-2/*neu* gene amplification. *Am J Pathol.* 2002;160:1589-1595.
27. Tubbs R, Pettay J, Hicks D, et al. Novel bright field molecular morphology methods for detection of *HER2* gene amplification. *J Mol Histol.* 2004;35:589-594.

DOI: 10.1309/MD79CDXN1D01E862

HOLOGIC®

First and Only FDA Cleared
Digital Cytology System

Genius™ Cervical AI

Genius™ Review Station

Genius™ Digital Imager

Empower Your Genius With Ours

Make a Greater Impact on Cervical Cancer
with the Advanced Technology of the
Genius™ Digital Diagnostics System

Click or Scan
to discover more

genius™
DIGITAL DIAGNOSTICS