RAPID PUBLICATION

Specificity of HercepTest in Determining *HER-2/neu* Status of Breast Cancers Using the United States Food and Drug Administration–Approved Scoring System

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Purpose: To evaluate the specificity of the HercepTest for Immunoenzymatic Staining (Dako Corp, Carpinteria, CA) for determining *HER-2/neu* protein expression in breast cancer.

Materials and Methods: Forty-eight invasive breast cancers previously found to be *HER-2/neu*–negative by two different immunohistochemical (IHC) assays and not amplified for the *HER-2/neu* gene by fluorescence in situ hybridization were studied using the HercepTest kit. HercepTest was performed according to the manufacturer's guidelines, and the results were scored on a 0 to 3+ scale using the United States Food and Drug Administration (FDA)–approved grading system. In this system, cases scored as 2+ or 3+ are considered *HER-2/neu*–positive.

Results: Among these 48 cases, the IHC score using the FDA-approved scoring system was 0 in four cases (8.3%), 1+ in 16 (33.3%), 2+ in 21 (43.8%), and 3+ in seven (14.6%). Therefore, 58.4% of these cases were categorized as *HER-2/neu*–positive, and the specificity of the HercepTest kit for *HER-2/neu* expression was 41.6%. However, with the use of a modified scoring system that took into account the level of staining of nonneoplastic epithelium, the specificity increased to 93.2%.

Conclusion: Our results indicate that the HercepTest kit, when used in accordance with the manufacturer's guidelines and the FDA-approved scoring system, results in a large proportion of breast cancers being categorized as positive for *HER-2/neu* protein expression and that many of these seem to be false-positives. Consideration of the level of staining of nonneoplastic epithelium resulted in improved specificity. The current FDA-approved scoring system for HercepTest results should be reevaluated before its widespread use in clinical practice.

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THE *HER-2/neu* (c-*erb*B-2) oncogene encodes a 185-kda transmembrane protein (p185), which is overexpressed in 20% to 30% of invasive breast carcinomas.[1,2] Since 1987, when Slamon et al[1] first reported a significant relationship between amplification of the *HER-2/neu* oncogene and poor clinical outcome in breast cancer patients, numerous studies have examined the utility of *HER-2/neu* as a prognostic factor. Recent evidence also supports a role for *HER-2/neu* status of breast cancers as predictive of their sensitivity or resistance to various forms of systemic therapy. Most recently, *HER-2/neu* protein expression has been used to select patients for treatment with trastuzumab (Herceptin, Genentech, Inc, South San Francisco, CA), a monoclonal antibody to the *HER-2/neu* protein. Initial clinical trials have indicated that this therapy may be useful in prolonging the survival of patients with advanced, metastatic breast carcinoma.[3-5] Several studies have also indicated that tumors that overexpress *HER-2/neu* may show resistance to certain forms of chemotherapy (such as cyclophosphamide/methotrexate)[6-11] and sensitivity to others (such as doxorubicin).[12-15] Furthermore, some clinical studies have suggested that *HER-2/neu* overexpression is predictive of resistance to tamoxifen.[11,16-19]

Therefore, analysis of the *HER-2/neu* status of breast cancer specimens is assuming increasing clinical relevance. Immunohistochemistry (IHC) is commonly used for evaluating *HER-2/neu* protein expression on formalin-fixed, paraffin-embedded samples of breast cancer.[20-22] However, given that various assay protocols, *HER-2/neu* antibodies, and scoring systems are currently in use, variability in *HER-2/neu* IHC results has become a matter of legitimate concern.[23-27] A standardized IHC kit for the evaluation of *HER-2/neu* protein expression (HercepTest for Immunoenzymatic Staining, Dako Corp, Carpinteria, CA) has recently been approved by the United States Food and Drug Administration (FDA). Of note, this release coincided with the FDA's approval of trastuzumab.[28] As a result of these developments, there is now great interest among both clinicians and pathologists in evaluating the ability of the HercepTest assay to accurately determine the *HER-2/neu* status of breast

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cancers. The specificity of the assay is of particular concern, because low specificity, manifested as a large number of false-positive results, could result in inappropriate use of trastuzumab.

The purpose of this study was to evaluate the HercepTest kit in a series of breast cancers previously shown to be negative for *HER-2/neu* protein expression by two other IHC assays and nonamplified for the *HER-2/neu* gene by fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

Study Design

We previously studied 100 consecutive cases of invasive breast cancer for *HER-2/neu* protein overexpression using two IHC methods and for *HER-2/neu* gene amplification by FISH.[29,30] Of note, the two prior IHC assays employed different methodology and scoring systems but used the same primary antibody (Dako rabbit antihuman polyclonal antibody to c-*erb*B-2 oncoprotein, code number A0485; Dako Corp),[29,30] which is a concentrate of the same anti–*HER-2/neu* antibody provided in prediluted form in the HercepTest kit. In these prior analyses, 22.9% of the cases showed *HER-2/neu* protein overexpression by one of the IHC methods, 23.7% showed *HER-2/neu* overexpression by the second IHC method, and 25.8% showed *HER-2/neu* gene amplification by FISH. Among these 100 cases, there was sufficient tissue remaining in the paraffin block for further IHC analysis in 48 cases that lacked *HER-2/neu* overexpression by both IHC assays and lacked *HER-2/neu* gene amplification by FISH. These 48 cases constitute the population for this study. All cases had been accessioned at Beth Israel Deaconess Medical Center (BIDMC), Boston, MA, between July 24, 1997, and February 18, 1998. The tissue from these cases was fixed initially in alcoholic formalin (Anatech, Ltd, Battle Creek, MI) followed by fixation in 10% neutral buffered formalin. For each case, 4-μm thick tissue sections were cut from a representative paraffin block and applied to positively charged slides.

In the first of the prior IHC assays, performed at PhenoPath Laboratories (PPL), Seattle, WA, tissue sections were subjected to heat-induced epitope retrieval (HIER) by immersion in citrate buffer (pH 6.0) preheated to greater than 90°C and heating in a Black & Decker vegetable steamer (Black & Decker Corp, Towson, MD) for 20 minutes before incubation with the anti–*HER-2/neu* antibody on a Dako Autostainer. Primary antibody was localized using the LSAB+ Detection System (labeled streptavidin biotin immunoperoxidase; Dako Corp) according to the manufacturer's instructions using the Dako Autostainer. Membrane staining intensity and pattern were evaluated using a 0 to 4+ scale (0, completely negative; 1+, faint membranous positivity; 2+, moderate membranous positivity; 3+, strong, circumferential membranous positivity; and 4+, extremely strong, circumferential membranous positivity). For a score of 2+ to 4+, membrane staining in the majority of the tumor cells was required to be present. Cytoplasmic immunostaining was noted but not incorporated into the final scoring. For each case, infiltrating carcinoma and adjacent normal epithelium (if available) were separately scored. A final subtracted score of the tumor minus normal epithelium was used to correct for variability in background staining of normal epithelium (which should not overexpress the *HER-2/neu* protein). Either a final subtracted score of ≥ 2 or tumor cell staining of 3+ or greater was required to categorize a case as *HER-2/neu*–positive.

In the second of the previous IHC assays, performed at BIDMC, tissue sections were subjected to HIER by heating in a microwave oven in citrate buffer (pH = 6) for a total of 10 minutes before immunostaining using the Ventana 320 automated immunostainer (Ventana Medical Systems, Tucson, AZ). The primary antibody to the *HER-2/neu* oncoprotein was used at a 1:500 dilution, and diaminobenzidine (DAB, Sigma Chemicals, St. Louis, MO) was used as the chromogen. *HER-2/neu* staining was considered positive when the tumor cells showed intense circumferential cell membrane staining, easily identified with a 10× objective. In all of these cases, staining was observed in the majority (> 50%) of the tumor cells. Tumors in which there was cytoplasmic staining without distinct cell membrane staining were scored as negative.

All 48 cases had also been previously analyzed for *HER-2/neu* gene amplification using the Oncor/Ventana INFORM *HER-2/neu* Gene Detection System (Ventana Medical Systems; formerly sold by Oncor, Inc, Gaithersburg, MD) at BIDMC in a laboratory certified by Oncor as proficient in the procedure. The methodology and interpretation were in accordance with the guide accompanying the kit[31] as previously described.[29] Briefly, tissue sections were digested with proteinase, denatured, and hybridized with Oncor biotinylated *HER-2/neu* DNA probe. Oncor Fluorescein-Labeled Avidin Detection Reagent and Oncor Anti-Avidin Antibody were used for probe detection. Nuclei were counterstained with 4′-6′-diamidino-2′-phenylindole (DAPI)/Antifade. Slides were examined using a fluorescence microscope. Twenty randomly selected invasive tumor cell nuclei in each of two separate, distinct microscopic areas were evaluated for *HER-2/neu* gene copy number (ie, a total of 40 nuclei per case). Cases were scored as amplified by FISH when the mean number of fluorescent signals per nucleus was greater than four.

HercepTest IHC Assay

In this study, *HER-2/neu* protein expression was evaluated using the HercepTest for Immunoenzymatic Staining at PPL according to the protocol described in the manufacturer's guide accompanying the kit. Tissue sections were deparaffinized in two 5-minute changes of xylene and were rehydrated through alcohols to distilled water. Subsequently, sections were subjected to HIER by immersing the slides in Dako Epitope Retrieval Solution (0.01 mol/L citrate buffer; pH = 6) preheated to 95°C, and then heated in waterbath at 95°C for a total of 40 minutes, followed by a 20-minute cooldown period at room temperature. Slides were incubated with the primary rabbit polyclonal antibody to the *HER-2/neu* oncoprotein (as supplied prediluted in the HercepTest kit) on a Dako Autostainer for 30 minutes at room temperature. Antibody was localized by incubating slides with the Dako Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat antirabbit immunoglobulins) for 30 minutes using the Dako Autostainer. Diaminobenzidine (DAB) was used as the chromogen, and the sections were counterstained with hematoxylin. Positive controls were included in each staining run and consisted of freshly cut breast cancer cases known to express *HER-2/neu* and a control slide consisting of three pelleted, formalin-fixed, paraffin-embedded human breast cell lines with staining intensity scores of 0, 1+, and 3+ (supplied in the HercepTest kit). Negative controls consisted of substituting normal rabbit serum (Dako Negative Control Reagent) for the *HER-2/neu* primary antibody. Only membrane staining intensity and pattern were evaluated using the 0 to 3+ scale as illustrated in the HercepTest kit scoring guidelines. As defined in the HercepTest kit guide, scores of 0 or 1+ were considered negative for *HER-2/neu* overexpression, 2+ was weak positive, and 3+ was strong positive. To qualify for 2+ and 3+ scoring (ie, positive), complete membrane staining of more than 10% of tumor cells had to be observed.

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We also used a modification of this scoring system that took into consideration the level of staining of nonneoplastic epithelium present on the same slide as the cancer. In this system, nonneoplastic epithelium was also graded on a 0 to 3+ scale using the same criteria used for assessment of tumor cell staining. Cases were considered *HER-2/neu* positive only when the difference between the tumor cell staining score and the nonneoplastic epithelial cell staining score was ≥ 2.

Calculation of HercepTest Assay Specificity

Specificity of the HercepTest was defined as the number of true-negative cases (ie, cases that were negative for *HER-2/neu* protein expression by HercepTest that were also negative for *HER-2/neu* protein expression by both prior IHC assays and negative for *HER-2/neu* gene amplification by the FISH assay) divided by the total number cases that were *HER-2/neu*–negative by both prior IHC assays and by the FISH assay (ie, true-negatives and false-positives by HercepTest). Specificity was expressed as a percentage.

RESULTS

Patient Data and Histologic Features of Carcinomas

The median age of the patients was 66 years (range, 36 to 89 years). Thirty-two of the 48 carcinomas (66.7%) were of infiltrating ductal type, seven (14.6%) were infiltrating lobular, four (8.3%) were invasive cancers with both ductal and lobular features, three (6.3%) were mucinous (colloid) carcinomas, and two (4.2%) were tubular carcinomas. The median size of the tumors was 15 mm (range, 6 to 90 mm). Histologic grading was performed using the Elston and Ellis[32] modification of the Bloom-Richardson grading system. Sixteen of the 48 carcinomas (33.3%) were grade 1, 18 (37.5%) were grade 2, and 14 (29.2%) were grade 3. Twenty of the 48 patients (41.7%) were axillary lymph node-negative and 13 (27.1%) were node-positive. Fifteen patients did not undergo axillary lymph node dissection. Forty-one of the 48 cases (85.4%) were estrogen receptor (ER)-positive and six (12.5%) were ER-negative. ER status was not determined in one case (Table 1).

Table 1. Clinical Data and Pathologic Features of Cases Analyzed for *HER-2/neu* Protein Overexpression by HercepTest (n = 48)

Characteristic	No.	%
Age, years		
Median	66	
Range	36-89	
Histologic type		
Infiltrating ductal	32	66.7
Infiltrating lobular	7	8.3
Mixed ductal and lobular	4	8.3
Mucinous (colloid)	3	6.3
Tubular	2	4.2
Size, mm		
Median	15	
Range	6-90	
Histologic grade		
1	16	33.3
2	18	37.5
3	14	29.2
Axillary nodal status		
Negative	20	41.7
Positive	13	27.1
No axillary dissection	15	31.2
ER status		
Positive	41	85.4
Negative	6	12.5
Not determined	1	2.1

HER-2/neu *Status*

All 48 cases were negative for *HER-2/neu* protein expression by previous IHC assays at both PPL and BIDMC, and none were amplified for the *HER-2/neu* gene by FISH.[29,30] However, using the HercepTest IHC kit and the FDA-approved scoring system, 28 of these cases (58.4%) were interpreted as positive (score of 2+ or 3+), and 20 (41.6%) were interpreted as negative (score of 0 or 1+) (Table 2). Therefore, if the results of the three previous *HER-2/neu* assays performed on these cases are considered true-negative results, then under these circumstances, the specificity of the HercepTest kit for *HER-2/neu* protein expression was 41.6%.

In 44 of these cases, nonneoplastic epithelium was present on the same tissue sections as the cancer. The HercepTest score in the benign epithelium was 0 in five cases (11.4%), 1+ in 15 (34.1%), 2+ in 21 (47.7%), and 3+ in three (6.8%). The difference between the tumor cell score and the nonneoplastic epithelial cell score was ≥ 2 in only three cases, and these three cases were considered *HER-2/neu*–positive. Therefore, when the level of staining of nonneoplastic epithelium was taken into consideration, the specificity of the HercepTest increased to 93.2%.

DISCUSSION

In this study, the HercepTest kit, when used in accordance with the manufacturer's guidelines and FDA-approved scoring system, categorized as *HER-2/neu*–positive almost 60%

Table 2. *HER-2/neu* Status by IHC Using the HercepTest Kit

Dako IHC Score*	Dako IHC Interpretation*	Cases No.	Cases %
0	Negative	4	8.3
1+	Negative	16	33.3
2+	Weak positive	21	43.8
3+	Strong positive	7	14.6

NOTE. HercepTest was performed on 48 cases that were all negative for *HER-2/neu* protein expression by two other IHC assays and nonamplified for the *HER-2/neu* gene by FISH.

*According to the manufacturer's FDA-approved guidelines.

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of invasive breast cancers that were previously shown to lack both *HER-2/neu* protein expression and *HER-2/neu* gene amplification in prior assays. These findings are subject to a number of different interpretations. First, it could be argued that the results using the HercepTest kit accurately reflect the *HER-2/neu* protein expression status of these cases and that our prior results, in which these cases were categorized as *HER-2/neu*–negative, were incorrect. However, this interpretation seems unlikely for several reasons. First, the *HER-2/neu* positivity rate by IHC using the HercepTest kit in this selected series of cases was substantially higher than the 20% to 30% rate of positivity noted in unselected series of breast cancers reported in other studies.[1,2] Second, all of the cases in this study that were scored as positive using the HercepTest kit lacked *HER-2/neu* gene amplification as determined by a FISH assay. Although prior studies have clearly shown that breast cancers may exhibit *HER-2/neu* protein expression in the absence of gene amplification, this phenomenon has been observed in only 3% to 7% of cases.[33-35] Third, the HercepTest assay was performed and the results were scored strictly in accordance with the manufacturer's recommendations and FDA-approved scoring system. Therefore, neither technical nor interpretive deviations from the proscribed method are likely to explain these results.

An alternative explanation for our findings is that the HercepTest assay, when used according to the manufacturer's FDA-approved guidelines, has low specificity for the detection of *HER-2/neu* protein expression. This interpretation is in agreement with the recent findings of Roche and Ingle.[36] These investigators noted a *HER-2/neu* positivity rate of 54% using the HercepTest kit in 59 cases that were found to be *HER-2/neu*–negative using another IHC assay. It could be argued that this comparison is not entirely valid, because these authors compared results of the HercepTest assay to an assay that uses a different antibody (monoclonal antibody CB11). However, our prior negative IHC results on the cases evaluated in the current study were obtained using the same primary anti–*HER-2/neu* antibody supplied in the HercepTest kit. Therefore, it seems unlikely that the observed discrepancies in IHC results between the HercepTest and other IHC assays is related to the nature of the primary antibody alone. It is possible that other methodologic aspects of the assay contributed to the low specificity observed in the study of Roche and Ingle[36] and in our study. However, our results strongly suggest that the low specificity is in large part related to the use of the recommended scoring system, because a dramatic improvement in specificity was noted when the level of staining of nonneoplastic epithelium was taken into account in our modified scoring system.

Previous studies have highlighted a number of potential problems in the use of IHC assays for *HER-2/neu*, including variability in tissue fixation and processing, variable sensitivity and specificity of commercially available antibodies, and differences in scoring criteria.[23-25] In our experience, variations in the type of fixative, length of tissue fixation, and details of tissue processing can result in differences in the intensity of specific staining for *HER-2/neu* in tumor cells as well as in variable levels of staining of nonneoplastic epithelium. In particular, fixatives that contain alcohol (including alcoholic formalin) can result in prominent staining of nonneoplastic epithelium in some cases. Any scoring system must, therefore, take into account the immutable fact that different fixation and processing protocols will be used in different laboratories. Our results suggest that consideration of the level of staining of nonneoplastic epithelium helps to ''normalize'' the level of *HER-2/neu* staining by serving as an internal control and may help to compensate for interlaboratory differences in tissue fixation and processing. However, one potential limitation of this approach is the lack of nonneoplastic epithelium in association with some primary tumors and in metastatic lesions.

The development of standardized methods for *HER-2/neu* IHC is clearly an important goal. However, our results suggest that the HercepTest kit, the first such proposed standardized assay, has low specificity for *HER-2/neu* protein expression when used in accordance with the manufacturer's guidelines and FDA-approved scoring system. Pathologists who perform assays for *HER-2/neu* and clinicians who use this information in formulating therapeutic recommendations need to be aware of these issues. In particular, the current FDA-approved scoring system for HercepTest should be re-evaluated before widespread use of the scoring system in clinical practice.

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