Evaluation of the United States Food and Drug Administration-approved Scoring and Test System of HER-2 Protein Expression in Breast Cancer

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ABSTRACT

Purpose: **The purpose of this study was to investigate the prognostic significance of assessment of human epidermal growth factor receptor (HER)-2 oncogene protein overexpression of breast cancer tissue by the United States Food and Drug Administration (FDA)-approved HercepTest and grading system (negative, 0 or 1+; weakly positive, 2+; strongly positive, 3+). Furthermore, results of the HercepTest were correlated with immunohistochemical results obtained using different antibodies and protocols and with HER-2 oncogene gene amplification assessed by fluorescence *in situ* hybridization (FISH).**

Experimental Design: **HER-2 status in 303 patients with lymph node-positive breast cancer was investigated by using a rabbit polyclonal antibody (DAKO) by conventional immunohistochemistry and by applying the HercepTest. Furthermore, the monoclonal antibody CB-11 was used in conventional immunohistochemistry and with the NexES automatic stainer, which is also under consideration for FDA approval for determination of eligibility for Herceptin therapy. Results were compared with FISH analysis performed in all 2+ and 3+ specimens (103 cases) and 104 HER-2-negative specimens.**

Results: **3+ positive carcinomas were found in 8.9–15.7% of specimens. FISH revealed that almost exclusively 3+ positive cases were amplified, with the HercepTest and the NexES automatic stainer giving the best results. In univariate analysis, staining with the HercepTest revealed a significantly worse prognosis in 3+ cases. Also, 3+ cases were significantly associated with lower estrogen receptor levels and histological grade III tumors.**

Conclusions: **This study shows that the results of the FDA-approved HER-2 grading and test system correlated strongly with findings in FISH. Furthermore, HercepTest proved to be of prognostic relevance. Strict adherence to the given protocols is critical.**

INTRODUCTION

It is well known that in approximately 20–30% of patients with breast cancer, tumor cells show an amplification and/or overexpression of the tyrosine kinase receptor HER-2[3] (c-erbB2; Refs. 1–3). This M_r 185,000 transmembrane protein (p185) is a member of the epithelial HER family, which also includes the epidermal growth factor receptor (HER-1/c-erbB1), HER-3, and HER-4. Overexpression of HER-2 has been shown to be associated with a dismal prognosis in patients with node-

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positive breast cancer and possibly also in patients with node-negative breast cancer (1–3). The determination of HER-2 protein overexpression is also considered a predictive factor in the therapy of breast cancer. It was shown to signify resistance to cyclophosphamide/methothrexate/5-fluorouracil therapy (4) and tamoxifen therapy (5, 6). On the other hand, an increased sensitivity to anthracycline-containing regimens, particularly when given in high doses, was observed (7, 8). More importantly, the monoclonal antibody trastuzumab (Herceptin; Genentech, Inc., San Francisco, CA), directed against the HER-2 protein has resulted in inhibition of tumor growth in both preclinical and clinical studies. This effect can be increased when used in combination with several chemotherapeutic agents (9–12). This effect was particularly apparent in patients with a strong (3+) expression of the HER-2 antigen on tumor cells.

These facts clearly show that exact determination of HER-2 status will be of central importance in the treatment of breast cancer. A number of techniques have been used to assess protein or mRNA overexpression and DNA amplification. Due to technical and financial constraints and also due to the requirement for prospective collection of fresh tissues for most of the assays available, many techniques are beyond the scope of most pathology laboratories (3). Clearly, IHC appears to be best suited for this purpose, followed by FISH. Both are performed on sections cut from formalin-fixed, paraffin-embedded specimens, allowing analysis on a cell-by-cell basis. With regard to IHC, however, it has not yet become clear which of the antibodies used is most appropriate. Comparison of a large panel of antibodies revealed a percentage of HER-2 positivity in the range of 2–30% (13). Furthermore, the method of grading protein expression differed in the various studies, impeding evaluation of data (3). Recently, a standardized IHC kit for the evaluation of HER-2/neu protein expression (HercepTest for Immunoenzymatic Staining; DAKO, Glostrup, Denmark) has been approved by the FDA. According to the FDA-approved manufacturer's instructions, membranous staining should be graded as negative (0 or 1+), weakly positive (2+), and strongly positive (3+). The prognostic significance of such a grading system has, to our best knowledge, not yet been proven. Furthermore, concern has been raised by some authors that the DAKO system might not perform well enough (14, 15). Others have considered the assay to be reliable (16).

In this study, we show the results obtained with different antibodies and test systems including the DAKO system. The different grades of protein overexpression were compared with results obtained by FISH. The impact of the three grades of HER-2 staining intensity on survival was determined.

PATIENTS AND METHODS

Study Population. The study population consisted of 303 unselected cases of invasive breast cancer, which were part of two prospective multicenter studies of the Austrian Breast Cancer Study Group (studies 2 and 4, Refs. 17 and 18). The specimens were fixed in neutral-buffered formalin (4–10%) and embedded in paraffin. For each case, 4-μm-thick tissue sections were cut from representative blocks. Estrogen receptor density was determined using the dextran-coated charcoal method on snap-frozen tumor samples. All patients had node-positive

breast cancer with at least 10 lymph nodes isolated from the axillary fatty tissue.

FISH for HER-2/neu amplification was performed using the PathVysion Gene Detection System (Vysis, Inc., Downers Grove, IL). IHC for HER-2/neu protein expression was performed using the HercepTest (DAKO), a polyclonal rabbit anti-HER-2/neu antibody (DAKO) that is the same one used in the HercepTest kit, and monoclonal antibody CB-11 [from either BioGenex (San Ramon, CA) or Ventana (Frankfurt, Germany)]. For the latter, the NexES automatic stainer (Ventana) was used. All cases with 2+ and 3+ staining by any antibody used were analyzed by FISH. Furthermore, 104 randomly selected negative cases were analyzed by FISH.

FISH Assay. Formalin-fixed paraffin-embedded specimens were cut into 6-μm-thick sections, mounted on coated glass slides, and baked overnight at 56°C. Slides were deparaffinized in xylene, rehydrated through alcohols, and air dried. The sections were pretreated by immersion in HCl/Triton X-100 for 20 min, followed by immersion in purified water for 3 min and immersion in 2× SSC for 5 min. Subsequently, slides were placed in sodium thiocyanate for 10 min at 80°C and rinsed in purified water for 1 min and twice in 2× SSC for 5 min. Excess water was removed by blotting the edges with a paper towel. After incubation in a 0.4% pepsin solution (adjusted to pH 1.5 with 2 N HCl) at 37°C for 15 min, the enzymatic reaction was stopped by placing slides in two changes of 2× SSC for 5 min each. After the slides were air dried, they were immersed in 4% buffered formalin for 10 min, followed by two changes of 2× SSC; dehydrated by soaking in 70%, 80%, and 100% ethanol for 2 min each; and air dried for 1–3 min on a 45°C slide warmer.

A total of 10 μl of PathVysion HER-2 DNA probe mixture (Vysis, Inc.) containing a HER-2 DNA probe (190-kb Spectrum-Orange directly labeled DNA probe) and the CEP 17 DNA probe (5.4-kb SpectrumGreen directly labeled fluorescent DNA probe specific for the α satellite DNA sequence) was applied to the target area. After coverslipping, the edges of the coverslip were sealed, and the slides were placed in the HYBrite (Vysis, Inc.) unit. Denaturation was performed at 85°C for 5 min and hybridization at 37°C for 16–20 h.

To remove nonspecifically bound probe, slides were placed in three changes of 50% formamide/2× SSC for 7 min each and placed in 2× SSC, 2× SSC/0, and 1% NP40 for 4 min each, all at 46°C and under agitation. The slides were air dried in the dark, and 10 μl of DAPI in phenylenediamine dihydrochloride/Antifade counterstain were applied. Thereafter, sections were coverslipped and sealed. Before signal enumeration, slides were stored in the dark at 5°C.

Scoring of FISH Signals. Slides were assessed for HER-2/neu copy number by one observer (J. S.) by using an Olympus BX 60 fluorescence microscope. In cases of doubt, the slides were assessed by a second reviewer (G. O.) to reach a final diagnosis. Each slide was analyzed using DAPI, single (orange and green) bandpass, and triple bandpass filters. Slides were scanned at low power (×10 objective) using the DAPI filter to identify areas with optimal tissue digestion and areas with tumors. At least 100 and up to 200 cells were counted from all tumor areas in accordance with the standardized counting guide included in the assay kit. All cases with more than a mean number of four fluorescence signals per two signals of the

centromere of chromosome 17 were considered amplified (19). Specimens were considered negative when less than 10% of tumor cells showed amplification of HER-2/neu, paralleling the situation in IHC.

IHC for HER-2/neu Protein Expression. Tissue sections (4 μm thick) were deparaffinized in two 5-min changes of xylene and rehydrated through alcohols to distilled water.

Diaminobenzidine was used as the chromogen in all cases, and the sections were counterstained with hematoxylin.

Positive controls were included in each run for all antibodies 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 or 1+ and 3+ (supplied in the HercepTest kit). According to the FDA, this control slide should allow standardization of staining results (15). All antibodies had been carefully established to give optimal, highly reproducible results on the positive controls, with an identical staining pattern of all antibodies.

Negative controls consisted of substituting normal rabbit serum or appropriate mouse IgG, respectively, for the primary antibodies.

In our hands, normal breast tissue was always negative (0 or 1+) for HER-2/neu overexpression and was taken as an additional negative control.

HercepTest IHC Assay. Staining was performed according to the protocol described in the manufacturer's guide accompanying the kit. Sections were subjected to heat-induced epitope retrieval by immersing slides in DAKO Epitope Retrieval Solution (0.01 M citrate buffer; pH 6) preheated to 95°C and then heating the slides in a water bath at 95°C for a total of 40 min, followed by a 20-min cooldown period at room temperature. Slides were incubated with the primary rabbit polyclonal antibody to the HER-2/neu oncoprotein for 30 min. Antibody was localized by incubation with the DAKO Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat antirabbit immunoglobulins) for 30 min.

Polyclonal Rabbit Antihuman c-erbB2 (Code A0485; DAKO). There was no antigen retrieval procedure because optimal results on the positive controls were obtained this way. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide for 10 min. After incubation with the primary antibody diluted 1:500 in 10% goat serum (in PBS) for 60 min, the antibody was localized by applying the DAKO ChemMate kit.

Monoclonal Antibody CB-11 (BioGenex). Sections were incubated for 40 min in 0.01 M citrate buffer (pH 6.0) at 95°C using a water bath, followed by a 20-min cooldown period at room temperature. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide for 10 min. After incubation with the primary antibody diluted 1:20 in 10% goat serum (in PBS) for 60 min, the antibody was localized by applying the DAKO ChemMate kit.

NexES Automatic Stainer (Ventana). After pretreatment as described for the CB-11 antibody, the slides were stained in the NexES automatic stainer with the "diaminobenzidine enhanced" protocol (reagents were from Ventana) with an incubation period of 32 min for the primary antibody clone CB-11 from Ventana. This automatic stainer, which is currently under consideration for approval by the FDA, is a computer-assisted device with standardized staining conditions due to a constant temperature of 37°C of the reaction chamber.

Grading of the Staining Intensity. Only membrane staining intensity and patterns were evaluated using the 0–3+ scale as illustrated by the HercepTest kit scoring guidelines. Scores of 0 or 1+ were considered negative for HER-2 overexpression; scores of 2+ were considered weakly positive; and scores of 3+ were considered strongly positive. To qualify for 2+ and 3+ scoring, complete membrane staining of more than 10% of tumor cells had to be observed.

All slides were read by two independent observers blinded to results obtained with the other antibodies and FISH. If differences between observers occurred, the slides were reinvestigated by both investigators using a multiheaded microscope.

Statistics. OS was defined as the time period from the day of surgery until the death of the patient. Data on patients who survived until the end of the observation period were censored at their last follow-up visit. Death from a cause other than breast cancer and survival until the end of the observation period were considered censoring events. DFS was defined as the time period from the end of primary therapy until first evidence of progression of disease, if the patient showed no evidence of disease after primary therapy.

Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (20). The Cox proportional hazards model was used for multivariate analysis. HER-2 staining intensity, patient age at time of diagnosis, menopausal status, histological grading, estrogen receptor density, and tumor stage were entered into Cox regression.

IHC tests were correlated with the Spearman's coefficient of correlation. ANOVA was used as appropriate.

For all tests, $P \leq 0.05$ was considered significant. All Ps given are the results of two-sided tests.

This study by the Austrian Breast Cancer Study Group was performed after receiving the approval of a local human investigations committee.

RESULTS

Study Population. The mean age of the patients was 51.8 ± 10.5 years; 150 (49.5%) patients were postmenopausal, 137 (45.2%) patients were premenopausal, and the menopausal status of 16 patients was not known (5.3%). A lumpectomy was performed on 66 (22%) patients, and a mastectomy was performed on 237 (78%) patients. According to the International Union against Cancer criteria, stage 1 (tumor $\leq$ 2 cm) disease was found in 163 (53.8%) specimens, and stage 2 (tumor $>$ 2 cm but $\leq$ 5 cm) disease was found in 124 specimens (40.9%); in 16 cases (5.3%), the stage was unknown. Histological tumor grade, as determined by a modified Bloom and Richardson score (21), was I in 20 (6.6%), II in 176 (58.1%), and III in 107 (35.3%) specimens (as determined by G. O.).

A combined adjuvant chemotherapy (on day 1, 20 mg/m^2 doxorubicin and 1 mg/m^2 vincristine; on days 29 and 36, 300 mg/m^2 cyclophosphamide, 25 mg/m^2 methotrexate, and 600 mg/m^2 fluorouracil) in combination with tamoxifen was applied to 135 (44.6%) patients. Tamoxifen alone was administered to 129 (42.5%) patients, and 39 (12.9%) patients did not receive

Table 1 HER-2 protein status determined by IHC (*n* = 303)

| Test | No. of cases scored as | | |
	0	2+	3+
HercepTest	222 (73.3%)	39 (12.9%)	42 (13.9%)
Polyclonal antibody (DAKO; *n* = 300)[a]	205 (67.7%)	45 (14.9%)	50 (16.5%)
NexES	223 (73.6%)	43 (14.2%)	37 (12.2%)
CB-11	264 (87.1%)	12 (4%)	27 (8.9%)

[a] Insufficient material was left to investigate three cases with the polyclonal DAKO antibody.

Table 2 Correlation between HercepTest and other tests for HER-2 protein expression

| Test | No. of cases scored as | | |
	0/1+	2+	3+
HER-2 3+ positive (HercepTest; *n* = 42)			
Polyclonal antibody (DAKO)	0	4 (9.5%)	38 (90.5%)
NexES	1 (2.4%)	5 (11.9%)	36 (85.7%)
CB-11	10 (23.8%)	7 (16.7%)	25 (59.5%)
HER-2 2+ positive (HercepTest; *n* = 39)			
Polyclonal antibody (DAKO; *n* = 38)[a]	6 (15.8%)	23 (60.5%)	9 (23.7%)
NexES	11 (28.2%)	27 (69.2%)	1 (2.6%)
CB-11	33 (84.6%)	4 (10.3%)	2 (5.1%)
HER-2 negative (HercepTest; *n* = 222)			
Polyclonal antibody (DAKO; *n* = 220)[a]	199 (90.5%)	18 (8.2%)	3 (1.3%)
NexES	211 (95%)	11 (5%)	0
CB-11	221 (99.5%)	1 (0.5%)	0

[a] Insufficient material was left to investigate three cases with the polyclonal DAKO antibody.

Table 3 Correlation between FISH and IHC

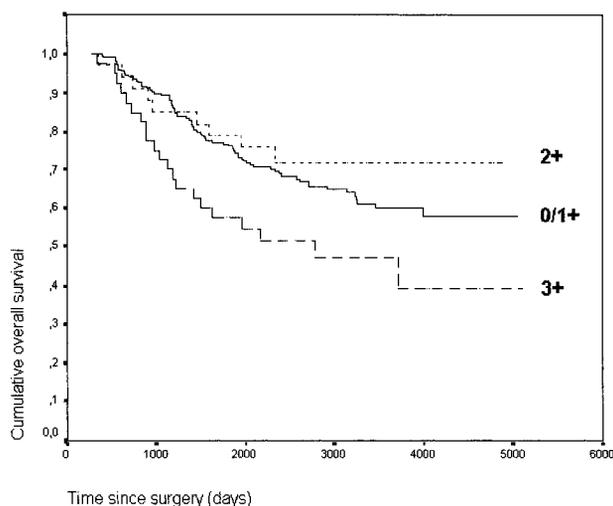| Test | No. of cases scored as | | |
	0/1+	2+	3+
Amplification in FISH (*n* = 35)			
HercepTest	1 (2.9%)	0	34 (97.1%)
Polyclonal antibody (DAKO)	0	2 (5.7%)	33 (94.3%)
NexES	2 (5.7%)	2 (5.7%)	31 (88.6%)
CB-11	7 (20%)	6 (17.1%)	22 (62.9%)
No amplification in FISH (*n* = 172)			
HercepTest	131 (76.2%)	37 (21.5%)	4 (2.3%)
Polyclonal antibody (DAKO; *n* = 169)[a]	116 (68.6%)	40 (23.7%)	13 (7.7%)
NexES	131 (76.2%)	38 (22.1%)	3 (1.7%)
CB-11	165 (95.9%)	4 (2.3%)	3 (1.7%)

[a] Insufficient material was left to investigate three cases with the polyclonal DAKO antibody.

parison with the results in the NexES automatic stainer showed that four amplified cases were negative or 2+ in IHC, whereas three cases with 3+ staining did not show amplification. The remainder of the antibodies showed a worse correlation with FISH results.

Estrogen Receptor Density and IHC. The mean estrogen receptor level was 85.2 ± 115.2 fm/liter; the median value was 48 fm/liter (range, 0–752 fm/liter). There was a significant decrease in the density of estrogen receptors in patients with a 3+ staining compared with those with 2+ staining and negative cases (*P* = 0.035, ANOVA). The HercepTest revealed that negative cases had a mean estrogen receptor level of 85.2 ± 112.8 fm/liter, 2+ cases had a mean estrogen receptor level of 117.3 ± 138.7 fm/liter, and 3+ cases had a mean estrogen receptor level of 55.8 ± 96.7 fm/liter. The respective figures obtained with the Ventana system were 86.6 ± 114.7 (negative cases), 112.1 ± 142.4 (2+ cases), and 45.6 ± 62.5 fm/liter (3+ cases; *P* = 0.01). The respective figures obtained with the polyclonal DAKO antibody were 82 ± 105 (negative cases), 125.1 ± 161.1 (2+ cases), and 62.1 ± 97.3 fm/liter (3+ cases; *P* = 0.04). The respective figures obtained with the CB-11 antibody were 93 ± 121.5 (negative cases), 42 ± 35.2 (2+ cases), and 40 ± 54.8 fm/liter (3+ cases; *P* = 0.005).

Grading and IHC. There was a highly significant association between cases with 3+ staining at HercepTest and grade III tumors (*P* = 0.0001, Spearman's coefficient of correlation).

Survival Analysis. Only HER-2 status determined with the HercepTest showed a significant impact on OS (*P* = 0.0221; Fig. 1). Pairwise comparison using a log-rank test revealed that patients with 3+ staining had a significantly shorter OS than patients with negative staining (*P* = 0.0142) and those with 2+ staining (*P* = 0.0246; Table 4). No significant difference in OS was found between patients with negative and 2+ staining (*P* = 0.38), although a trend toward longer OS of 2+ cases was detected (Fig. 1). Patients with negative HER-2 status had a median survival time of 96.2 ± 3 months, patients with 2+ staining had a median survival time of 104.3 ± 9.1 months, and, finally, those with 3+ staining had a median survival time of 90.3 ± 10.4 months. HER-2 status determined with the NexES automatic stainer (*P* = 0.44), the polyclonal antibody from DAKO (*P* = 0.299), and monoclonal antibody CB-11 (*P* =

any treatment. Tamoxifen was administered at a dose of 2 × 10 mg p.o. daily for 2 years. The mean follow-up time was 81.7 ± 40.6 months. The mean DFS was 67.3 ± 43.7 months. The 5-year survival rate of all patients was 55%.

IHC. There was a highly significant correlation of the results of the various IHC tests (*P* < 0.0001, Spearman's coefficient of correlation; Tables 1 and 2). However, the number of 3+ specimens ranged from 8.9–15.7%, which signifies an important difference in test performance.

FISH. Two cases that demonstrated 2+ or 3+ staining with one of the antibodies used had to be excluded from FISH analysis due to lack of material.

A total of 212 specimens were investigated by FISH, but in 5 specimens (2.4%), it was not possible to obtain any results, despite several trials. A total of 14 (6.6%) specimens delivered no results at the first trial but performed well at the second trial.

In 35 of the 207 (16.9%) tumor specimens analyzed, which included all cases with 2+ and 3+ staining in IHC for which FISH analysis was possible, an amplification was revealed by FISH, including 2 cases with low-grade amplification.

FISH and IHC. Correlation of the results of FISH and IHC revealed that essentially all cases with 3+ staining in IHC were amplified (Table 3). Comparison with the results of the HercepTest revealed that one amplified case was negative in IHC, whereas four 3+ cases did not show amplification. Com-

Fig. 1 This graph shows the cumulative OS of patients, depending on the result of HER-2 IHC obtained with the HercepTest. *0/1+,* negative specimen; *2+,* weakly positive specimen; *3+,* strongly positive specimen. The 3+ cases had a significantly shorter survival time compared with 0/1+ and 2+ cases (*P* = 0.022, log-rank test).

Table 4 5-year survival rates (percentage of patients)

A. 5-year OS rate				
	Immunohistochemical score of			
Test	0	2+	3+	
HercepTest[a]	70.5%	75.6%	51.5%	
Polyclonal antibody (DAKO)	70.8%	67.5%	57.9%	
NexES	70.5%	66.1%	59.3%	
CB-11	69.1%	81.8%	56.9%	
FISH				
No HER-2 amplification				70.3%
HER-2 amplification				57.3%
B. 5-year DFS rate				
	Immunohistochemical score of			
Test	0	2+	3+	
HercepTest[a]	57.7%	78.33%	45.4%	
Polyclonal antibody (DAKO)	58.2%	61.9%	53.9%	
NexES	58.2%	65.5%	51.9%	
CB-11[a]	58.2%	100%	44%	
FISH[a]				
No HER-2 amplification				62.7%
HER-2 amplification				48.8%

  [a] Significant results.

0.1499) were of no significant prognostic value. Analysis of the subgroup investigated with FISH analysis revealed no prognostic influence of amplification on OS (*P* = 0.1235).

In multivariate analysis, only two factors were found to exhibit prognostic significance on OS, namely, tumor stage (*P* = 0.021) and tumor grade (*P* = 0.002); HER-2 status, estrogen receptor density, menopausal status, and age showed no statistically significant influence on survival.

The therapy regimen applied did not influence survival. Stratification with the therapeutic regimens did not reveal any influence on the prognostic significance of HER-2 status in bivariate analysis (*P* > 0.05).

In univariate analysis, 3+ staining with the HercepTest (*P* = 0.0016) and the CB-11 antibody (*P* = 0.005), as well as amplification determined with FISH (*P* = 0.0093), showed a significantly decreased DFS.

At HercepTest, 3+ cases had a significantly shorter DFS than 2+ cases (*P* = 0.0013) or negative cases (*P* = 0.0083). Most interestingly, 2+ cases had a significantly longer DFS than cases with negative immunostaining (*P* = 0.0414).

In multivariate analysis, only tumor grade (*P* = 0.0001) showed a significant influence on DFS.

The 5-year rates of OS and DFS are compiled in Table 4.

DISCUSSION

This study revealed two important findings: (*a*) the level of correlation between FISH and IHC, and in particular the FDA-approved HercepTest, but also the NexES automatic stainer in the evaluation of HER-2 status in invasive breast cancer was high; and (*b*) the three-grade system of reporting findings in IHC was shown to be of prognostic significance. Analyses with the HercepTest revealed that 3+ HER-2 positivity signified a significantly worse course of the disease. This finding is corroborated by the fact that in this study, almost exclusively 3+

cases were found to be amplified in the FISH assay, which is known to signify a dismal prognosis. In comparison, a 2+ HER-2 status was of no negative prognostic value. Interestingly enough, 2+ cases had a trend toward a longer OS and higher estrogen receptor levels and also had a significantly longer DFS when compared with HER-2-negative cases. We are the first to observe this trend, but at present, we have no explanation for this finding.

Results obtained with the HercepTest proved to be of prognostic significance in univariate analysis, whereas only minor differences in the results given by the other antibodies/test systems prevented the demonstration of the prognostic value of HER-2 status. This finding explains in part (besides the various grading methods used for assessment of HER-2 protein overexpression) the differences in the prognostic value of HER-2 found in various studies.

The high level of correlation between IHC 3+ staining and amplification detected by FISH makes it difficult to justify the routine use of FISH for determination of HER-2/neu status. The major disadvantages of FISH *versus* IHC are the high costs, the time-consuming tissue preparation and evaluation of slides, and the requirement of a fluorescence microscope. Also, in some cases, it is difficult to detect invasive carcinoma and identify areas with invasive cancer in cases with intraductal carcinomas. This requires a time-consuming rereview of the H&E-stained sections to definitely identify areas of invasive carcinoma.

In this study, only approximately 3% of cases showed 3+ staining without detectable amplification. These cases could represent a single copy overexpression of the *HER-2* gene at the mRNA transcriptional level or beyond. Low HER-2 DNA levels were found in cases with overexpression of the HER-2 protein in approximately 10% of cases by Southern blot analysis (2) and in 3–7% of cases by FISH (19, 22, 23).

HER-2 amplification with normal protein levels was observed in 0–20% of cases, depending on the assay used (in the two best performing assays, the results were 2.9% and 5.7%). One explanation for this finding is that particularly in cases with low-level gene amplification, gene transcription and posttranscriptional or -translational events could be down-regulated or abnormal, ultimately leading to low HER-2/neu protein levels or abnormal epitope production. Alternatively, tissue preservation could have been insufficient, leading to protein degradation.

One of the problems of IHC is the variation in the results obtained by different antibodies and staining procedures and, in our opinion, to a minor extent, the interobserver variability (an issue that is not addressed in this study). We have compared two different antibodies with two different staining procedures for each antibody. There was, in part, a considerable difference between results obtained with the same antibodies, depending on the staining procedure. In our hands, the antibody giving the most concordant results with FISH analysis was the polyclonal antibody used in the HercepTest kit, which performed better than the same polyclonal antibody applied with another protocol. Therefore, minor changes in the staining protocol may change results. This may explain why in some hands, the HercepTest kit does not perform so well. The influence of the staining protocol/system used was again demonstrated by the fact that the monoclonal antibody CB-11 applied with the NexES automatic stainer gave better results than the same antibody used with another protocol. However, different antibodies may give differing results; for example, Press *et al.* (13) reported that monoclonal antibodies 4D5 and CB-11 (antibodies used for the clinical trial assay) showed a lower percentage of breast cancers immunostained (21% and 19%, respectively) when compared with the polyclonal antibody from DAKO (26% of breast cancers stained).

Of course, variability in air humidity, temperature, the water used, and technician skills in various laboratories may also have an influence on the performance of the antibodies used. Clearly, independent of the antibody and test system used in routine practice, the method used should be thoroughly evaluated. Whenever possible, the results of IHC and FISH should be compared until the vast majority of HER-2-amplified specimens also demonstrate 3+ staining on IHC.

In conclusion, this study shows that the FDA-approved test and grading system (HercepTest) for the detection of HER-2 protein overexpression showed a very good correlation with findings in FISH. Furthermore, HercepTest results proved to be of prognostic relevance. Clearly, antibodies have to be evaluated extensively by laboratories to give optimal performance.

ACKNOWLEDGMENTS

REFERENCES

1. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science (Wash. DC), *235:* 177–182, 1987.

2. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ullrich, A. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science (Wash. DC), *244:* 707–712, 1989.

3. Hanna, W., Kahn, H. J., and Trudeau, M. Evaluation of the HER-2/neu (erbB-2) status in breast cancer: from bench to bedside. Mod. Pathol., *12:* 827–834, 1999.

4. Stal, O., Sullivan, S., Wingren, S., Skoog, L., Rutqvist, L. E., Carstensen, J. M., and Nordenskjold, B. C-erb-2 expression and benefit from adjuvant chemotherapy and radiotherapy of breast cancer. Eur. J. Cancer, *31A:* 2185–2190, 1995.

5. Borg, A., Baldetorp, B., Ferno, M., Killander, D., Olsson, H., Ryden, S., and Sigurdsson, H. ERBB2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. Cancer Lett., *81:* 137–144, 1994.

6. Elledge, R. M., Green, S., Ciocca, D., Pugh, R., Allred, D. C., Clark, G. M., Hill, J., Ravdin, P., O'Sullivan, J., Martino, S., and Osborne, C. K. HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. Clin. Cancer Res., *4:* 7–12, 1998.

7. Muss, H. B., Thor, A. D., Berry, D. A., Kute, T., Liu, E. T., Koerner, F., Cirrincione, C. T., Budman, D. R., Wood, W. C., Barcos, M., and Henderson, I. C. c-erb-2 expression and response to adjuvant chemotherapy in women with node-positive early breast cancer. N. Engl. J. Med., *330:* 1260–1266, 1994.

8. Paik, S., Hazan, R., Fisher, E. R., Sass, R. E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M. E., and King, C. R. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. J. Clin. Oncol., *8:* 103–112, 1990.

9. Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., Wolter, J. M., Paton, V., Shak, S., Lieberman, G., and Slamon, D. J. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER-2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J. Clin. Oncol., *17:* 2639–2648, 1999.

10. Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, D., Baly, D., Baughman, S. A., Twaddell, T., Glaspy, J. A., and Slamon, D. J. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with her2/neu-overexpression metastatic breast cancer refractory to chemotherapy treatment. J. Clin. Oncol., *16:* 2659–2671, 1998.

11. Gilewski, T., Seidman, A., Norton, L., and Hudis, C. An immunotherapeutic approach to treatment of breast cancer: focus on trastuzumab plus paclitaxel. Breast Cancer Medicine Service. Cancer Chemother. Pharmacol., *46:* 23–26, 2000.

12. Baselga, J. M., Tripathy, D., Mendelsohn, J., Baughman, S. A., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu overexpression metastatic breast cancer. Semin. Oncol., *26:* 78–83, 1999.

13. Press, M. F., Hung, G., Godolphin, W., and Slamon, D. J. Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res., *54:* 2771–2777, 1994.

14. Roche, P. C., and Ingle, J. N. Increased HER2 with U. S. Food and Drug Administration-approved antibody. J. Clin. Oncol., *17:* 434, 1999.

15. Jacobs, T. W., Gown, A. M., Yaziji, H., Barnes, M. J., and Schnitt, S. J. 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., *17:* 1983–1987, 1999.

16. Espinoza, F., and Anguiano, A. The HercepTest assay: another perspective. J. Clin. Oncol., *17:* 2293–2294, 1999.

17. Jakesz, R., Hausmaninger, H., Samonigg, H., Kubista, E., Haider, K., Mlineritsch, B., Schmid, M., Tausch, C., Reiner, G., Renner, K., Stierer, M., Jatzko, G., Hofbauer, F., Fridrik, M., Schennach, W., Sevelda, P., Dadak, C., Haid, A., Scholz, R., Lenzhofer, P., Steindorfer,

P., Berger, A., and Mischinger, H. J. Die Therapiestudien der Austrian Breast Cancer Group (ABC). Zentralbl. Chir., *123:* 28–32, 1998.

18. Jakesz, R., Hausmaninger, H., Haider, K., Kubista, E., Samonigg, H., Gnant, M., Manfreda, G., Tschurtschenthaler, G., Kolb, R., Stierer, M., Fridrik, M., Mlineritsch, B., Steindorfer, P., Mittlböck, M., and Steger, G. Randomized trial of low-dose chemotherapy added to tamoxifen in patients with receptor-positive and lymph node-positive breast cancer. J. Clin. Oncol., *17:* 1701–1709, 1999.

19. Persons, D. L., Borelli, K. A., and Hsu, P. H. Quantification of HER-2/neu amd c-myc gene amplification in breast carcinoma using fluorescence *in situ* hybridization. Mod. Pathol., *10:* 720–727, 1997.

20. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1985.

21. Elston, C. W., and Ellis, I. O. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology, *19:* 403–410, 1991.

22. Kallioniemi, O. P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L. C., Smith, H. S., Waldman, F. M., Pinkel, D., and Gray, J. W. ERBB2 amplification in breast cancer analyzed by fluorescence *in situ* hybridization. Proc. Natl. Acad. Sci. USA, *89:* 5321–5325, 2001.

23. Pauletti, G., Godolphin, W., Press, M. F., and Slamon, D. J. Detection and quantification of HER-2/neu gene amplification in human breast cancer archival material using fluorescence *in situ* hybridization. Oncogene, *13:* 63–72, 1996.

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Clin Cancer Res 2001;7:1669-1675.

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