

ORIGINAL ARTICLE

IHC for Her2 with CBE356 antibody is a more accurate predictor of Her2 gene amplification by FISH than HercepTest™ in breast carcinoma

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Background: Her2 (c-erbB-2/neu) overexpression in breast carcinoma predicts response to the anti-Her2 monoclonal antibody, trastuzumab, and is associated with a poor prognosis. When considering patients for trastuzumab treatment, Her2 protein expression is measured by immunohistochemistry (IHC) and, where staining is equivocal, by fluorescence in situ hybridisation (FISH) detection of Her2 gene amplification.

Aims: To compare IHC using CBE356 with IHC using the Food and Drug Administration approved HercepTest™.

Methods: CBE356 and HercepTest were analysed using 167 FISH characterised breast carcinomas. Immunohistochemical expression of Her2 was measured semiquantitatively. Sensitivity, specificity, predictive values, and overall accuracy were calculated for both IHC methods using gene amplification by FISH as the end point, and IHC and FISH assays were tested in Kaplan–Meier survival analysis.

Results: The accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of CBE356 positive (2+ and 3+) cases were 94%, 89%, 95%, 84%, and 97%, respectively, and of HercepTest positive (2+ and 3+) cases were 91%, 66%, 98%, 92%, and 91%, respectively. A positive result with CBE356, HercepTest, or FISH was associated with significantly decreased overall survival (log rank $p=0.005$, $p=0.0017$, and $p=0.0005$, respectively).

Conclusions: Positive IHC staining for Her2 using CBE356 is 3% more accurate and 23% more sensitive at predicting Her2 gene amplification by FISH than positive staining with HercepTest. Negative IHC using CBE356 antibody is 6% more likely to represent a truly negative result than negative staining with HercepTest. Overall, CBE356 was a more accurate predictor of Her2 gene amplification by FISH than HercepTest.

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The c-erbB-2/neu (hereafter Her2) gene on chromosome 17 encodes a growth factor receptor that is overexpressed in 20–30% of breast carcinomas as a result of gene amplification. Her2 amplification in breast carcinoma confers a poor prognosis in both axillary node positive^{1–3} and node negative breast cancer,^{3–9} and may also predict responsiveness to chemotherapy^{8, 10–13} and resistance to hormonal treatment.^{14–16}

“Accurate testing for Her2 aims to ensure that costly and potentially toxic trastuzumab treatment is not given to patients with non-overexpressing tumours and that every patient who might benefit from trastuzumab receives appropriate treatment”

Determination of Her2 overexpression in breast carcinoma is now of major clinical importance with the advent of the humanised anti-Her2 monoclonal antibody trastuzumab, which is beneficial in patients with metastatic breast carcinoma.^{17, 18} Demonstration of Her2 overexpression is a prerequisite for treatment with trastuzumab because it acts by specifically targeting Her2 overexpressing carcinoma cells. Accurate testing for Her2 aims to ensure that costly and potentially toxic trastuzumab treatment is not given to patients with non-overexpressing tumours and that every patient who might benefit from trastuzumab receives appropriate treatment.

Immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH) are established methods for detecting

Her2 overexpression in formalin fixed, paraffin wax embedded tissue in diagnostic laboratories. IHC for Her2 is a robust screening test when supported by rigorous quality control measures,¹⁹ and has a continuing role in diagnostic Her2 testing. There is a wide array of IHC antibodies with varying sensitivities available for Her2 testing, but the Food and Drug Administration (FDA) approved HercepTest IHC kit has achieved wide usage. The use of FISH to detect Her2 gene amplification in diagnostic laboratories is usually reserved for cases with equivocal staining by IHC. The FDA approved PathVysion FISH kit is one of the most widely used FISH assay systems.

All diagnostic laboratories will require access to accurate, reproducible Her2 testing and part of that process will be to put in place tests that are financially viable and have been validated against FDA approved tests and against survival. Our aim was to test the accuracy of IHC staining with the CBE356 antibody for the detection of Her2 overexpression in a series of breast cancers. We compared the sensitivity and specificity and overall accuracy of this antibody with HercepTest IHC at predicting gene amplification by PathVysion FISH and we examined the prognostic value of each test in terms of overall survival.

Abbreviations: CI, confidence interval; FDA, Food and Drug Administration; FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry

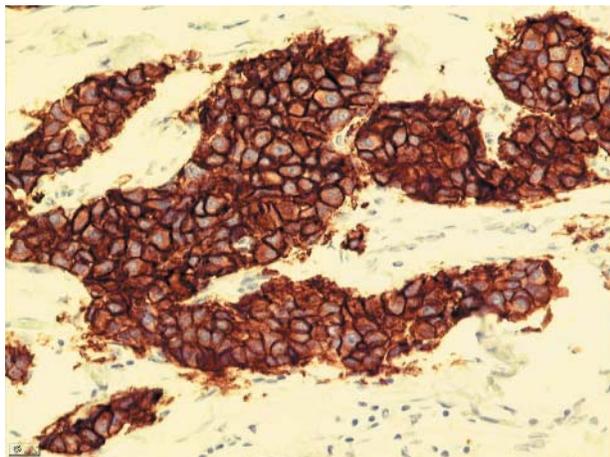


Figure 1 Her2 immunohistochemistry using CBE356 showing 3+ staining; original magnification, $\times 200$.

MATERIALS AND METHODS

Tissue samples

Formalin fixed, paraffin wax embedded blocks of 167 breast carcinomas, resected between 1984 and 1998, which had prospective follow up, were retrieved from the files of the Glasgow Royal Infirmary, UK. All patients were female and the mean age was 70 years (range, 30–87). There were 152 invasive ductal carcinomas, 12 invasive lobular carcinomas, two medullary carcinomas, and one primary squamous cell carcinoma. Twenty two tumours were grade 1, 63 were grade 2, and 82 were grade 3. The mean tumour size was 34 mm (range, 6–130). Seventy six tumours were axillary lymph node positive, 68 were node negative, and 23 cases did not have axillary lymph node dissection.

Immunohistochemistry

HerceptTest

IHC using HerceptTest™ (anti-Her2 polyclonal antibody; Dako, Ely, UK) on a Techmate automated staining system (Dako) was performed on 165 cases according to the kit instructions, with epitope retrieval in 10 mmol/litre citrate buffer in a water bath at 95° C for 40 minutes. Semiquantitative scoring (by JJG) using the standard protocol (with reference to images published by Dako) was

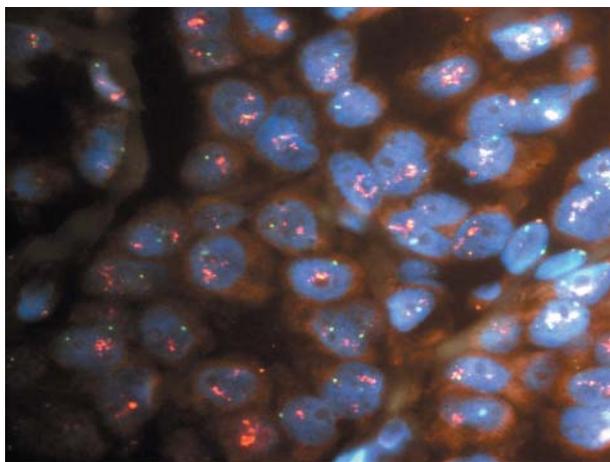


Figure 2 PathVysion fluorescence in situ hybridisation showing Her2 gene amplification as a red signal. The green signal represents chromosome 17.

performed on all cases where scores of 0, 1+, 2+, and 3+ were allocated if there was no staining or if 10% or more of carcinoma cells showed weak, moderate, and intense membrane staining, respectively.

CBE356

IHC was performed on all 167 cases on a Techmate automated staining system (Dako) using CBE356 mouse monoclonal antibody, clone 10A7 (Novocastra, Newcastle Upon Tyne, UK) to the external domain of Her2. The primary antibody concentration and antigen retrieval methods were first calibrated against FISH characterised tumours using dilutions of 1/40, 1/80, and 1/200, with and without microwave pressure cooker antigen retrieval. A 1/200 primary antibody dilution (60 minutes of incubation with primary antibody at 25°C) was shown to be the most sensitive and specific, with antigen retrieval in a microwave pressure cooker for 12 minutes in EDTA buffer. Semiquantitative scoring (by BD) using the standard protocol (with reference to images published by Dako) was performed on all cases where scores of 0, 1+, 2+, and 3+ were allocated if there was no staining or if 10% or more of carcinoma cells showed weak, moderate, and intense membrane staining, respectively.

FISH

FISH analysis using a PathVysion detection kit was performed on all 167 cases after pretreatment with the VP2000 tissue processor (Vysis UK Ltd, Richmond, UK). Hybridisation was performed on a Misha hybridisation station (Shandon, Runcorn, Cheshire, UK). After posthybridisation washes, sealed slides mounted in 0.5 µg/ml DAPI in “Vectashield” were analysed by epifluorescence microscopy. FISH stained sections were scanned at $\times 1000$ magnification and three separate carcinoma areas were identified. Twenty nuclei were assessed in each area, the chromosome 17 copy number was counted for each cell, and the ratio of Her2 signals to chromosome 17 signals was calculated. The normal mean Her2 to chromosome 17 ratio was defined as less than two, and a ratio greater than two was interpreted as gene amplification.

Statistics

Statistical analysis was performed using SPSS statistical package (version 6.1.3). Using FISH data as the end point, overall accuracy, sensitivity, specificity, and positive and negative predictive values were calculated for CBE356 positive staining (2+ and 3+ cases combined) and for HerceptTest positive staining (2+ and 3+ cases combined). Follow up information was available on 165 patients. Kaplan–Meier disease specific survival curves were calculated for FISH amplified and FISH non-amplified cases, for HerceptTest 0/1+, 2+, and 3+ cases, and for CBE356 0/1+, 2+, and 3+ cases; 95% confidence intervals were calculated for the median survival in each case. The log rank test was used to compare survival curves. Patients who died of other causes were censored.

RESULTS

Staining with the CBE356 antibody showed a clear membrane pattern (fig 1) characteristic of the Her2 protein. IHC scores for both antibodies are shown for both FISH amplified and non-amplified cases (table 1). The overall accuracy of the CBE356 antibody was greater than that of the HerceptTest (94% and 91%, respectively) when PathVysion FISH amplification (fig 2) was taken as the end point (table 2). The CBE356 and HerceptTest results were comparable in terms of overall specificity (95% and 98%, respectively; table 2), but the CBE356 antibody showed a much greater overall

Table 1 Semiquantitative CBE356 and HercepTest IHC scores against amplification of Her2 gene by FISH

	CBE356 IHC score				HercepTest score			
	0	1+	2+	3+	0	1+	2+	3+
Her2 FISH not amplified	107	18	4	2	125	3	1	1
Her2 FISH amplified	2	2	6	26	9	3	12	11
Total	109	20	10	28	134	6	13	12
% Of total	65	12	6	17	81	4	8	7

FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry.

sensitivity than the HercepTest (89% and 66% respectively) and a greater negative predictive value (97% and 91%, respectively), whereas the HercepTest showed a greater positive predictive value than CBE356 (92% and 84%, respectively).

Survival analysis

The maximum follow up was 17.2 years (median, 5; n = 165). Patients without Her2 amplification survived significantly longer than those with amplification: median survival was 11.7 years (95% confidence interval (CI), 7.4 to 15.9) and 2.6 years (95% CI, 1.6 to 3.7), respectively (log rank: p = 0.0005; fig 3). Patients with tumours scoring 0/1+ with the CBE356 antibody survived significantly longer than those scoring 2+ or 3+, with a median survival of 11.7 years (95% CI, 7.9 to 15.5), 2.9 years (95% CI, 0 to 6.8), and 2.3 years (95% CI, 0.3 to 4.3), respectively (log rank: p = 0.005; fig 4). Similarly, survival was significantly longer in patients with tumours scoring 0 or 1+ with the HercepTest than in those scoring 2+ or 3+, with a median survival of 11.7 years (95% CI, 7.7 to 15.7), 2.3 years (95% CI, 1.7 to 2.9), and 1.4 years (95% CI, 0 to 5.8), respectively (log rank: p = 0.0017; fig 5).

DISCUSSION

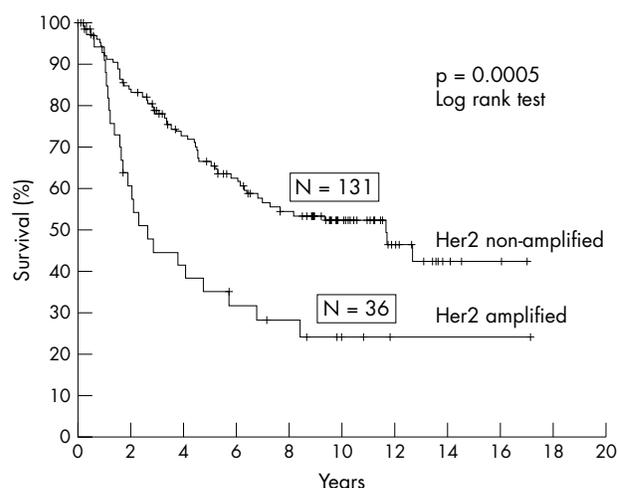
There has been much debate recently regarding interlaboratory variability in Her2 testing that is centred predominantly on inconsistencies in IHC assays. Because selection of patients for trastuzumab treatment and for entry into trials investigating the benefit of this treatment in earlier disease is based on these assays, and because the cost of 12 weeks of monotherapy is at least £5300/patient, accurate testing on formalin fixed, paraffin wax embedded tissue is of the utmost importance.

Inconsistencies in the sensitivity of IHC Her2 testing have been attributed to the use of different antibodies, differences in tissue fixation and IHC reagents, and the inclusion (or not) of antigen retrieval methods.²⁰⁻²³ The HercepTest kit has overcome some of these problems by using standardised methodology and reagents and by the inclusion of cell line controls. Nonetheless, the reported sensitivity of the HercepTest varies between different centres and, although it

Table 2 Comparison of CBE356 and HercepTest IHC positive cases according to sensitivity, specificity, accuracy and predictive values

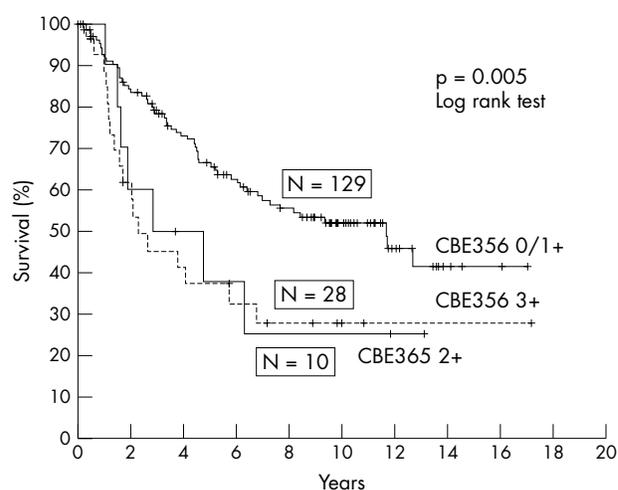
	CBE356 2+/3+	HT 2+/3+
Sensitivity	89%	66%
Specificity	95%	98%
PPV	84%	92%
NPV	97%	91%
Accuracy	94%	91%

HT, HercepTest; IHC, immunohistochemistry; NPV, negative predictive value; PPV, positive predictive value.

**Figure 3** Kaplan-Meier survival curve showing the effect of Her2 amplification by fluorescence in situ hybridisation on disease specific survival. Vertical lines represent censored patients.

has achieved high sensitivity in some reports,²⁴⁻²⁸ it is costly and therefore its widespread use has budgetary implications for laboratories wishing to set up mandatory testing.

FISH is the gold standard for detecting Her2 amplification,²⁹⁻³¹ but is dependent on adequately skilled personnel, and the assay is relatively expensive to set up and to perform when compared with in house immunohistochemical assays. Her2 amplification is usually associated with protein over-expression,^{1 29 32} and the current recommendation is to use

**Figure 4** Kaplan-Meier survival curve showing the effect of CBE356 immunohistochemical staining for Her2 on disease specific survival. Vertical lines represent censored patients.

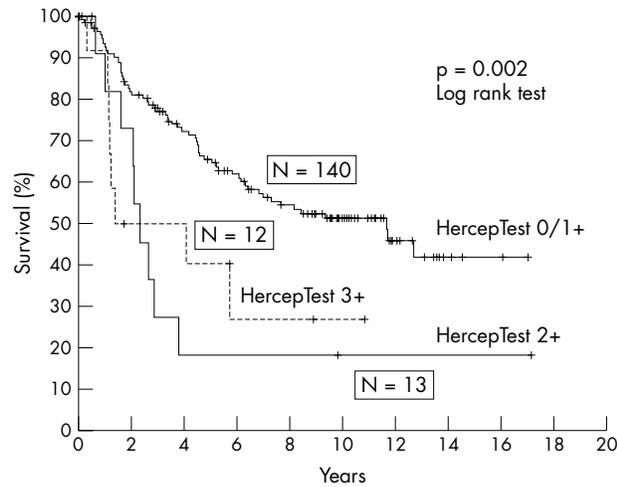


Figure 5 Kaplan–Meier survival curve showing the effect of HercepTest immunohistochemical staining on disease specific survival. Vertical lines represent censored patients.

IHC as a primary screening test and to select only cases that stain equivocally by IHC for FISH analysis.^{33–35}

There is a wide variety of IHC antibodies for Her2 now available. We tested CBE356 and CB11 antibodies on a small pilot series of breast carcinomas and found CBE356 staining to be comparable to HercepTest staining in pattern and intensity. In our hands, background cytoplasmic staining was high with the CB11 antibody (Novocastra, Newcastle upon Tyne, UK), making interpretation difficult, so we did not include this antibody in the formal study. To optimise staining with CBE356, we calibrated the primary antibody concentration against amplification of Her2 with FISH in a training set before staining the large series. CBE356 has the advantage of being considerably less expensive than the HercepTest kit (costing less than £5/test and approximately £30/test, respectively). We tested the sensitivity and specificity of CBE356 IHC compared with PathVysion FISH analysis on a large series of breast carcinomas and we validated the IHC results in terms of survival.

In our study, positive IHC with CBE356 for Her2 was 89% sensitive at predicting gene amplification by FISH, compared with a 66% sensitivity rate only using HercepTest IHC. Specificity using the two IHC methods is roughly similar, at 95% and 98%, respectively (table 2). The main difference in sensitivity is accounted for by the fact that 35% of the total number of cases showing Her2 amplification by FISH had a score of zero or 1+ using HercepTest, compared with only 12% with CBE356. According to the HercepTest manufacturer's

guidelines, the expected proportions of 0/1+, 2+, and 3+ cases are 70%, 10%, and 20%, respectively. The negative rate using HercepTest in our series was 85%, the 2+ rate was 8%, and the 3+ rate was 7%. The reason for the high number of 0/1+ cases, apparently at the expense of the 3+ cases, is not clear. One possible explanation is that the HercepTest assay had only recently been introduced to our laboratory at the time of staining, potentially resulting in technical or interpretative inexperience. The distribution of cases using CBE356 was closer to the guidelines, with a negative rate of 77%, an equivocal rate of 6%, and a positive rate of 17%; 72% of the total amplified cases scored 3+ with CBE356 and 17% scored 2+ (table 1). Using the HercepTest, 34% of all amplified cases scored +2 and only 31% of cases scored +3. Sixty percent of the CBE356 2+ cases were amplified, whereas almost all (92%) of HercepTest 2+ cases showed FISH amplification of Her2, explaining the higher positive predictive value of the HercepTest (92%) compared with 84% for CBE356 (tables 1, 2). Other studies have shown a much lower level of FISH amplification of IHC 2+ cases,^{19 25 36 37} suggesting that a large proportion of the HercepTest 2+ cases should have shown 3+ staining. The increased likelihood of CBE356 rather than HercepTest IHC giving a 3+ staining pattern could reduce the number of cases requiring FISH analysis, at a cost of at least £80/test, representing a cost saving in addition to that afforded by the considerably cheaper CBE356 IHC assay.

“CBE356 has the advantage of being considerably less expensive than the HercepTest kit (costing less than £5/test and approximately £30/test, respectively)”

No other published study has tested the accuracy of CBE356 at predicting gene amplification. The 89% sensitivity of CBE356 coupled with 95% specificity compares very favourably not only with the HercepTest in our series, but with other IHC studies. Press *et al* described the sensitivity of HercepTest to be 70% (based on cases showing moderate or high intensity staining) and that of CB11 to be 72% in a study of 117 molecularly characterised tumours, although their in house antibodies R60 (polyclonal) and 10H8 (monoclonal) were more sensitive at 91% and 88%, respectively.²³ Earlier reports using the HercepTest showed oversensitive staining compared with other antibodies,^{27 28} but these results were based on expected rate of overexpression rather than comparison with gene amplification.

Therefore, we have shown that CBE356 IHC is both a more accurate and a more sensitive predictor of Her2 gene amplification by FISH than the FDA approved HercepTest in our series. Importantly, a negative result with CBE356 is more reassuring than a negative result with HercepTest. Truly positive cases are more likely to have strongly positive 3+ staining with CBE356 than with the HercepTest, reducing the need for FISH analysis. We have validated CBE356 IHC staining in terms of survival and shown that positive CBE356 IHC staining for Her2 is associated with a significantly shorter time to death from breast cancer ($p = 0.005$). As expected, patients with FISH amplification had a significantly shorter survival than those without amplification ($p = 0.0005$). For HercepTest staining, time to death from breast cancer was also significantly decreased in patients with 2+ and 3+ tumours compared with those whose tumours showed 0/1+ staining ($p = 0.0017$).

In an era in which IHC is considered an effective screening tool for the detection of Her2 amplification in breast carcinoma, we propose that the use of CBE356 antibody should be considered by laboratories that wish to set up accurate and cost effective Her2 testing. Further studies with larger numbers are needed to corroborate our data, and

Take home messages

- We compared immunohistochemical (IHC) staining for Her2 using the CBE356 antibody with the HercepTest and found that CBE356 is 3% more accurate and 23% more sensitive at predicting Her2 gene amplification by fluorescence in situ hybridisation (FISH) than positive staining with the HercepTest
- Negative IHC using the CBE356 antibody is 6% more likely to represent a truly negative result than negative staining with HercepTest
- Thus, CBE356 provides a cheaper, more accurate predictor of Her2 overexpression than the HercepTest

further validation of IHC staining against response to trastuzumab treatment is required to confirm the robustness of this antibody.

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