ORIGINAL ARTICLE

Amplification of the HER2 gene in breast cancers testing 2+ weak positive by HercepTest immunohistochemistry: false-positive or false-negative immunohistochemistry?

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Background: The majority of cases of breast cancer scoring HER2 weak positive (2+) on immunohistochemistry (IHC) using the HercepTest are not associated with amplification of the HER2/neu gene.
Aim: To examine the reproducibility of IHC in cases scoring 2+ subsequently shown to have gene amplification by fluorescence in-situ hybridisation (FISH).
Methods: A retrospective analysis of 153 cases referred for FISH confirmation of a weak positive HercepTest (2+) result was performed. Repeat IHC was undertaken in cases with weak positive (2+) referral IHC and amplification of the HER2 gene by FISH.
Results: Amplification of the HER2 gene was confirmed in 29/153 cases (19%) scoring 2+ on IHC. Repeat IHC was carried out on 25 IHC 2+ cases: 7 (28%) scored 2+ on repeat IHC, 18 (72%) scored 3+ and were reclassified as strong positive. A heterogeneous expression pattern was present in 3/17 cases scoring 3+.
Conclusions: The majority of HercepTest 2+ results are not accompanied by gene amplification and represent ''false positive'' IHC in terms of prognostic or therapeutic relevance. A small proportion of HercepTest 2+ scores represent true 2+ IHC positive cases accompanied by gene amplification: a category probably biologically related to 3+ IHC cases. The remainder of cases of HercepTest™ 2+ accompanied by gene amplification represent a category of referral IHC 2+ weak positive, FISH amplified, repeat IHC 3+ strong positive, best described as ''false negative 2+ IHC''. This has implications for selection of cases for FISH analysis where weak positive (2+) IHC score is used as a triage for FISH testing, and for testing strategies in referral laboratories undertaking FISH analysis.

The HER2/neu protein, a 185-kDa membrane-associated cytoplasmic tyrosine kinase, is overexpressed in 20–30% of invasive breast cancers.[1 2] In the majority of cases, protein overexpression reflects underlying amplification of the HER2/neu gene, encoded on chromosome 17 (17q12)[3], although overexpression can also occur in the absence of gene amplification. This is a phenomenon reported in 3–7% of cases, possibly reflecting polysomy of chromosome 17.[3–5] Overexpression of the HER2 protein has been shown to be an independent, adverse prognostic variable in invasive breast cancer, and has also been reported to be a predictive variable in response to chemotherapy.[6–10] With the advent of specific targeted therapy in advanced breast cancer, overexpression of the HER2 protein has become a marker of eligibility for treatment with the novel humanised anti-HER2 monoclonal antibody trastuzumab (Herceptin),[11 12] and recent data indicate that treatment with Herceptin is also effective in the adjuvant setting.[13 14]

Analysis of HER2 status has become routine in the pathological reporting of invasive breast cancer. Most laboratories use either immunohistochemistry (IHC) or fluorescent in-situ hybridisation (FISH) for assessment of protein expression or gene amplification, respectively. There is evidence to suggest that FISH provides a more accurate measure of HER2 status than IHC,[15] and that testing based on FISH alone may be most cost-effective in the selection of patients for treatment with Herceptin[16] when the cost of treatment is factored. Nevertheless, many centres recommend the initial assessment of cases of invasive breast cancer by IHC, supplemented by FISH when IHC results are equivocal.[17–19] It has been suggested that laboratories should have a minimum throughput of cases, so as to ensure adequate technical and interpretative standards in testing for HER2 status.[20]

A diagnostic FISH service for HER2 in invasive breast cancer was established at The Adelaide and Meath Hospital, Tallaght, Dublin, Ireland (AMNCH) in 2002 to supplement the existing IHC service, and over time this has evolved to function as an informal reference facility for a number of hospitals in the Republic of Ireland. This study reports on our experience of cases with amplification of the HER2 gene confirmed by FISH undertaken on the basis of a 2+/weak positive HercepTest IHC score.

MATERIALS AND METHODS

Case selection

The records of the diagnostic HER2 service at the Applied Molecular Pathology Laboratory at AMNCH were reviewed for the period 2002–4. Cases of invasive breast carcinoma with 2+ HercepTest IHC and evidence of gene amplification by FISH were selected for study.

Tumour pathological parameters were obtained from the histopathology report from the referring institution, if available.

Immunohistochemistry

Review of referral HercepTest IHC was not undertaken, as HercepTest IHC slides were not available for review in cases referred from external sources. In each case, HER2 IHC was repeated using the HercepTest. In cases from AMNCH, IHC was repeated on the same tissue block used for original IHC and for

Abbreviations: AMNCH, The Adelaide and Meath Hospital; FISH, fluorescence in-situ hybridisation; IHC, immunohistochemistry

FISH. One additional block was tested in one case. In cases referred from external sources, the block used for FISH was used for repeat IHC, and was assumed to represent the block used for original IHC.

Immunohistochemical staining for HER2/neu was performed on 4 μm sections of formalin-fixed, paraffin-wax-embedded tissue. Sections were stained using the DakoCytomation HercepTest (DakoCytomation, Glostrup, Denmark), strictly following the manufacturer's guidelines. HercepTest immunohistochemistry was scored according to the standard scoring system recommended by the manufacturer: 0, no staining at all, or membrane staining in <10% of cells; 1+, weak or barely perceptible staining in >10% of cells, the cells stained in only part of the membrane; 2+, weak to moderate staining in the whole membrane in >10% of cells; 3+, strong staining in the whole membrane in >10% of cells. In all cases, interpretation was limited to invasive tumour.

HercepTest IHC, original (where available) and repeat, was scored by two observers (CB, MJ).

Fluorescence in-situ hybridisation

Following pretreatment using the VP2000 processor (Vysis (UK) Ltd, Richmond, UK), 4 μm sections of formalin-fixed, paraffin-wax-embedded tissue were denatured. Hybridisation with the Vysis LSI HER2/neu (Sprectrum Orange)/CEP 17 (Sprectrum Green) DNA probe (Abbott Laboratories, Des Plaines, Illinois, USA) was then performed for a minimum of 18 h. Following stringency washes, the slides were counterstained with 4′,6-diamidino-2-phenylindole. Signal enumeration was carried out using a Leica DMLB fluorescent microscope (Leica Microsystems, Wetzler, Germany) equipped with appropriate filters. Orange and green signals were counted in a minimum of 60 tumour cell nuclei from each section, and a signal ratio was obtained. Cases were scored independently by two observers. All observers had undergone specific training and validation in FISH analysis at the Pathology laboratories at Glasgow University, Glasgow, UK. A ratio score of <2.0 was classified as unamplified and a score of >2.0 as amplified.

RESULTS

A total of 208 cases of invasive breast cancer were referred to the Applied Molecular Pathology Laboratory at AMNCH for HER2 FISH during 2002–4. Two cases were not studied, as material available in the referred tissue block was insufficient.

Table 1 shows the IHC scores and FISH results of the 206 cases.

Out of 206 cases, 53 (26%) cases demonstrated amplification of the HER2 gene on FISH.

Table 1 shows the correlation between results of referral IHC and FISH.

In cases referred for FISH on the basis of a weak positive (2+) IHC score, amplification of the HER2 gene was demonstrated in 29/153 cases (19%).

IHC was repeated in 25 cases amplified by FISH and referred on the basis of a 2+ IHC score. In all, 18 (72%) cases were categorised as 3+ (strong positive) on repeat HercepTest IHC at the AMNCH laboratory. Table 2 shows the results of original and repeat IHC and of HER2/C17 ratio, together with pathological parameters in these cases. Significant staining heterogeneity was present in three cases, but in each case the criteria for a 3+ score were satisfied.

Poor fixation was noted in one case and the result of IHC may not be reliable.

Seven cases (18%) scored 2+ on repeat IHC.

The mean HER2/C17 ratio was 3.8 in the 29 cases with amplification and 2+ referral IHC, 4.2 in cases scoring 2+ on repeat IHC and 3.2 in cases scoring 3+ on repeat IHC.

DISCUSSION

Evaluation of HER2 status has become part of the core dataset in pathological reporting of invasive breast cancer, and has become more widely relevant in light of positive data from trials using Herceptin in the adjuvant setting. Accurate reporting of HER2 status is a prerequisite for the correct selection of patients for specific targeted therapy.

Despite some evidence that FISH testing predicts the therapeutically significant HER2 status more accurately, the approach of a primary IHC screen with supplementary FISH molecular confirmation is widely used, and recently updated national guidelines in the UK endorse this approach.[17] With one recent exception,[21] most reports suggest that IHC is strongly predictive of gene amplification status at the extremes of the IHC scoring scale (0/1+/3+), but that a weak positive (2+) result is less predictive[22–24], supporting the rationale for restricting FISH testing to this subset of cases.

Variations in fixative type, fixation time and processing conditions can lead to variations in the intensity of specific staining for HER2 in tumour cells and of staining of non-neoplastic epithelium.[19 25] Use of antigen retrieval methods to reverse the effects of tissue fixation and processing can enhance immunostaining for HER2, but this can result in a shift towards ''false'' positive staining (a positive result on IHC in the absence of gene amplification). A high degree of ''false positive'' IHC has been reported with the HercepTest kit (which uses heat-induced epitope retrieval), particularly in cases scoring 2+/weak positive.[22 24 26] Studies comparing results of HER2 testing in community hospital-based laboratories, with testing carried out in central reference laboratories, have shown significant discrepancies between the referring laboratory and the central laboratory results, with overestimation of IHC HER2 status in the referring laboratories, which is not substantiated by rigorous IHC and FISH testing in central laboratories.[27–29] To our knowledge, significant underscoring or false-negative IHC has not been reported.

Our experience confirms that the majority of 2+ IHC results were not accompanied by amplification of the HER2 gene. We did, however, identify a significant subset of cases in which

Table 1 Results of immunohistochemistry and fluorescence in-situ hybridisation in invasive breast cancer

Referral IHC FISH	0	1+	2+	3+	N/S	Total
Amplified	0	3*	29	15	6	53
Not amplified	5	10	123	1†	12	151
Unsatisfactory			1		1	2
Total	5	13	153	16	19	206

FISH, fluorescence in-situ hybridisation; IHC, immunohistochemistry; N/S, not specified.
*IHC repeated: in two cases, heterogenous staining was predominantly 1+, but with foci of 3+, one case was 1+ on repeat IHC.
†IHC repeated: 2+ on repeat.

Table 2 Pathology, immunohistochemistry and fluorescence in-situ hybridisation parameters in cases of breast carcinoma

Case	Type	Grade	ER	Node stage	Referral IHC	FISH ratio	Repeat IHC
1	IDC	N/A	N/A	N/A	2	5	3
2	IDC	2	POS	N2	2	2.3	2
3	PD	3	N/A	N/A	2	2.8	3
4	IDC	2	POS	N0	2	7.9	3
5	IDC	3	POS	N/A	2	6.2	3
6	IDC	2	N/A	N/A	2	2.05	3
7	IDC	3	NEG	N/A	2	8.2	3
8	IDC	N/A	POS	M1	2	2.2	3
9	IDC	3	N/A	N1	2	2	2
10	IDC	2	NEG	N1	2	6.8	3
11	IDC	2*	POS	N2	2	2.3	3
12	ILC	N/A	N/A	N/A	2	2.1	3
13	IDC	2	NEG	N0	2	4.9	3
14	IDC	N/A	N/A	N/A	2	2.6	3
15	IDC	N/A	N/A	N/A	2	4	2
16	IDC	2	POS	N2	2	6.4	2
17	IDC	2	POS	N1	2	2.1	2
18	IDC	2	POS	N1	2	3.4	2
19	IDC	3	N/A	N0	2	2.2	2
20	IDC	3	N/A	N/A	2	2.2	ND
21	IDC	2	N/A	N/A	2	5.2	3
22	IDC	2	NEG	N0	2	2.36	ND
23	IDC	3	POS	N1	2	4.5	3
24	IDC	3	N/A	N/A	2	4	3
25	ILC	2	POS	N2	2	2.6	3
26	IDC	3	N/A	N0	2	4.48	ND
27	ILC	2	POS	N/A	2	3.9	3
28	IDC	3	NEG	N/A	2	2.7	3
29	IDC	3	N/A	N0	2	2.12	ND

ER, oestrogen receptor; FISH, fluorescence in-situ hybridisations; IDC, invasive ductal carcinoma, no special type; IHC, immunohistochemistry; ILC, invasive lobular carcinoma; N/A, not available; ND, not done; NEG, negative; POS, positive; PD, poorly differentiated.

apparent discordance between IHC and FISH results reflects a potential problem with the IHC result reporting.

On the basis of our results, we suggest that the majority of 2+ HercepTest IHC scores are not accompanied by gene amplification and represent ''false positive'' IHC in terms of prognostic or therapeutic relevance of the result in the context of the pathology report. A small proportion (7/153 (5%)) of 2+ HercepTest IHC scores represent true positive IHC, reflecting underlying gene amplification: a category previously reported, and probably therapeutically and prognostically related to 3+ IHC/gene-amplified cases.[15]

The remainder of the cases in our series represent a different category in which referral IHC was 2+ weak positive, FISH for HER2 demonstrated gene amplification and repeat IHC in the central laboratory was 3+ strong positive. This could be best described as false-negative initial 2+ IHC.

No distinguishing features in terms of tumour type, grade or stage were identified in cases with gene amplification scoring 3+ or 2+ on repeat IHC. These findings raise important issues in terms of HER2 status evaluation in the pathological reporting of invasive breast cancer, particularly with respect to the algorithm for referral of cases for FISH testing. On the basis of our findings, at least some cases of breast cancer testing 2+ weak positive on HercepTest IHC actually represent false-negative IHC (ie, true 3+ positive on repeat), which represents a small but real risk of inappropriate treatment stratification if confirmatory molecular testing is not undertaken. If routine practice is to refer all 2+ IHC cases for FISH confirmation, it may be appropriate to consider validation of the IHC score either locally or in the referral laboratory before FISH, to avoid unnecessary expensive and labour-intensive FISH analysis in this small group of cases.

In summary, we have shown that weak positive (2+) HER2 IHC, in addition to representing a ''false positive'' in terms of concordance with amplification status, may also represent true false-negative IHC in a small proportion of cases. This may have potentially significant therapeutic implications if confirmatory molecular testing is not included in the routine evaluation protocol for HER2 in invasive breast cancer.

Take-home messages

- Weak positive (2+) HercepTest immunohistochemistry (IHC) correlates poorly with amplification status by fluorescence in-situ hybridisation (FISH): most cases are not associated with gene amplification.
- Significant discrepancies have been shown between IHC at referring laboratories and that in a central FISH testing laboratory.
- A small proportion of weak positive (2+) HercepTest IHC cases represent false-negative IHC.
- Repeat IHC could be considered before referral for FISH.

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