

## CerbB2 Status in Breast Cancer: Pathologic Issues

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### Abstract

**Introduction:** *CerbB2 status determination in breast cancer is currently performed for predictive and prognostic information. However, much has yet to be learned about the accuracy, reproducibility and clinical correlation of the different testing methods available today. This is a review of the testing methods commonly in use and their applications in the management of breast cancer.* **Methods:** *A Medline search for review articles with keywords cerbB2, Her2 testing, immunohistochemistry (IHC), fluorescence in situ hybridisation (FISH) and breast cancer was done. Manual cross-referencing from the afore-mentioned articles and perusal of the package inserts, recommendations and manuals from the manufacturers of various commercially available test kits were also performed.* **Results:** *The 2 main methods of testing in use in most routine laboratories are IHC and FISH on formalin fixed, paraffin embedded, breast cancer tissue. There is no single, universally accepted method and the plethora of testing and interpretative protocols has confounded the issue of clinical correlation of cerbB2 status with outcome and patient selection for therapy. The current consensus in the literature is leaning towards FISH; however, as pointed out in this paper, the use of FISH as a routine test in most diagnostic laboratories is not yet a reality mainly due to cost, technical and manpower constraints. In this regard, IHC is by default the preferred choice in many routine laboratories.* **Conclusion:** *Universal standardisation of testing methods for cerbB2 status determination, together with further clinical studies on the correlation of each method with outcome and response to therapy, will aid in the identification of the ideal method for stratifying patients with breast cancer into prognostic and optimal therapeutic groups.*

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**Key words:** *Fluorescence in situ hybridisation (FISH), Her2, Herceptest, Herceptin, Immunohistochemistry (IHC)*

### Introduction

The cerbB2 gene, also known as Her2/neu, encodes a transmembrane cell receptor with tyrosine kinase activity and epidermal growth factor receptor homology.<sup>1</sup> All normal and the majority of breast cancer cells contain 2 copies of the cerbB2 gene and produce low levels of the corresponding protein. Encoded on chromosome 17q21, cerbB2 is overexpressed in 20% to 30% of breast cancers.<sup>2,3</sup>

Breast cancers with cerbB2 overexpression have increased sensitivity and response to anthracycline-based chemotherapy.<sup>4-7</sup> A novel treatment modality using humanised anti-cerbB2 antibodies, trastuzumab (Herceptin), that targets the tumour cells with protein overexpression in patients with advanced breast cancers,<sup>8,9</sup> has also contributed to an increasing demand for accurate and reliable cerbB2 testing. However, there is currently no widely accepted and universally standardised test for cerbB2 status.

Quantitative assay methods, such as Southern blotting (which measures gene copy number), Northern blotting (mRNA values) and Western blotting (protein

concentrations), have been used.<sup>10</sup> Limitations of these molecular techniques are insensitivity to low levels of amplification owing to the dilutional effect of non-neoplastic tissue in the sample and an inability to distinguish overexpression of an in situ from an infiltrating carcinoma.<sup>11</sup> Moreover, these methods require fairly large amounts of fresh tissue and are not practical for use in the routine diagnostic surgical pathology laboratory. In this context, methods that use formalin fixed, paraffin embedded tissue derived from routine surgical biopsy material are ideal. The fact that, in one study, cerbB2 status has been shown to be unchanged after chemotherapy and in metastatic sites compared to the primary tumour,<sup>12</sup> further enhances the utility of methods that can be performed on archival biopsy material. The 2 main types of assays currently in use are immunohistochemistry (IHC), which detects the oncoprotein overexpression in cells, and fluorescence in situ hybridisation (FISH), which measures gene amplification/copy numbers within the tumour cells.

### FISH

FISH assays use fluorescein labelled deoxyribonucleic

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acid (DNA) probes that hybridise to the target DNA within the cell genome. It requires a high-grade fluorescence photomicroscope with digital image recording, the necessary software for reading and processing of the results, and the skills of an experienced technologist to carry out the time-consuming, expensive assays under strictly controlled conditions. Longer interpretation times and the services of a pathologist who has experience in interpreting FISH material is also a prerequisite. This makes the use of FISH assays beyond the scope of most basic clinical and diagnostic laboratories. However, FISH assays are more reproducible and consistent in results compared to IHC methods, as DNA is very stable and less susceptible to variations in tissue fixation and processing. Furthermore, because the definition of positivity is based on distinct signals in a cell, which represents chromosomal regions containing the amplified *cerbB2* gene, it is less arbitrary and subjective, compared to an IHC grading scale.

The 2 United States Food and Drug Administration (FDA)-approved FISH assays, INFORM and PathVysion, were shown in one study to have a 98% concordance rate with identical results in 51 of 52 cases, and disagreement in only 1 case (2%). The PathVysion kit has 2 distinct advantages over INFORM. One is that the use of direct probe labelling in PathVysion reduces non-specific cellular staining, which is commonly seen as a result of indirect labelling as used in the INFORM assay. Furthermore, the PathVysion *cerbB2* DNA probe kit utilises two labelled DNA probes. The first is a *cerbB2* probe that spans the entire *cerbB2* gene, whilst the second CEP 17 probe hybridises to the alpha satellite DNA located at the centromere of the chromosome 17. The inclusion of a chromosome 17 control allows for correction of the *cerbB2* signal number in cases of chromosome 17 aneuploidy (seen in over 50% of cases).<sup>13</sup>

#### *Transcriptional upregulation of mRNA*

The possibility of transcriptional upregulation of messenger ribonucleic acid (mRNA) in the cells with normal gene copy numbers as the cause for protein overexpression has not been adequately investigated to date. It may well account for the small number of cases categorised as “false positive”, where there is a discrepancy in findings between IHC (overexpression of protein) and FISH (normal gene copy numbers).

#### *IHC*

IHC assays for *cerbB2* introduced a quantitative aspect to IHC interpretation, which hitherto had been mainly qualitative in nature; for example, whether a tumour shows lymphoid or epithelial differentiation with lymphoid and cytokeratin markers.<sup>14</sup> The target antigen, the *cerbB2* protein, is expressed in the cytoplasmic membranes of

normal as well as malignant breast epithelium.<sup>15</sup> Radio-immunohistochemistry studies on frozen sections of breast cancers show a bimodal distribution of *cerbB2* expression, with levels over 10-fold above normal breast tissue associated with gene amplification and shorter disease-free and overall survival than patients with normal or minimally elevated *cerbB2* expression.<sup>16</sup> The ability of an IHC assay to discriminate between “normal” background levels of protein expression in the benign epithelium or non-overexpressing malignant cells, and levels in overexpressing malignant cells requires a delicate balance of sensitivity and specificity in the assay. The *cerbB2* protein is labile and antigenicity can be lost during tissue fixation, processing and with prolonged storage. Different methods of antigen retrieval that are used in IHC assays can also affect the results, as excessive antigen retrieval may result in high rates of positivity where even benign cells are highlighted. Conversely, inadequate antigen retrieval will give false negative results. A variety of IHC assays are currently in use, and these employ an array of different antibodies; examples of commonly used antibodies are the polyclonal Dako A0485 and the monoclonal Ventana CB11 antibodies, each of which have different levels of sensitivity and specificity.

Discrepancies in results between different laboratories can be somewhat ameliorated by first standardising tissue fixation and processing methods as well as immunoassaying protocols across laboratories, and many attempts have been made towards these ends.<sup>17</sup> The use of appropriate positive and negative tissue controls, with known and calibrated values of target protein, should be included in each staining run, thus allowing for monitoring of inter and intra laboratory reproducibility. Participation in external quality assurance programmes is one way in which laboratories can compare and standardise their IHC assays with that of other laboratories.

However, the main area of discrepancy between IHC results for *cerbB2* status is in the interpretation of the assay. This is contributed to largely by the lack of definition of what constitutes a positive result and the lack of subsequent clinical validation of the designated positive result. Early IHC studies of *cerbB2* included as positive, cases in which cytoplasmic staining was present. This is now recognised as non-specific and probably a staining artefact. Only cell membrane staining is associated with amplification of the gene as determined by molecular methods of detection. In some studies, a subtraction method whereby the staining of normal epithelium is “subtracted” from the intensity of staining in the tumour cells was used, to correct for variability in background staining of normal epithelium.<sup>18</sup>

#### *Herceptest*

The Herceptest is the FDA-approved commercially

available kit system that uses an arbitrary scoring scale of 0 to 3+. No membrane staining or staining in less than 10% of tumour cells is given a score of 0. A faint, barely perceptible membrane staining in more than 10% of the tumour cells with the cells stained in only part of their membranes is given a score of 1+. Weak to moderate staining of the entire membrane in more than 10% of the tumour cells is given a score of 2+; and a strong, entire membrane staining of more than 10% of the tumour cells is given a score of 3+ (Fig. 1). The reporting guidelines<sup>19</sup> given by the manufacturers recommend that a score of 0 and 1+ be reported as negative, 2+ as weakly positive and 3+ as strongly positive for *cerbB2* overexpression, which is then used by the clinicians as a means for selection of patients for therapy.

Current recommendation from the United Kingdom reference laboratory group that runs an External Quality Assurance (EQA) scheme for *cerbB2* testing is that the HercepTest scoring method be used even for tumours assessed using other IHC methods.<sup>13</sup> Reassuringly, a study by Jacobs et al<sup>18</sup> showed a high level (97%) of interlaboratory agreement in assessing *cerbB2* status based on IHC assays using the same primary antibody as in the Hercep Test but different detection systems, assay methods and scoring schemes.

There still remains the question of how well HercepTest scores correlate with FISH assays for gene amplification, and the clinical significance of a positive result in terms of patient response to various forms of therapy. The HercepTest, in spite of its similarity in name to the therapeutic agent, Herceptin, does not utilise the same antibody that was used in the Herceptin clinical trial assay (CTA), performed for the selection of patients for the Herceptin clinical trials.<sup>20</sup> A study of more than 520 breast

cancers,<sup>21</sup> none of which were entered into the Herceptin clinical trials, compared the HercepTest with the same IHC assay that was used in the Herceptin trials. It showed a 79% overall concordance of staining result. It was largely on this basis that the US FDA approved the test kit. However, there has been no clinical correlation of outcome of therapy with the HercepTest results, although there are currently on-going trials that compare the HercepTest and other IHC assays in assessing *cerbB2* in various clinical settings, including the use of adjuvant Herceptin. A more detailed analysis of the HercepTest results indicate that a score of 3+ is highly likely to correspond with a positive result using the CTA and would have met the entry criteria for the Herceptin trials. But 42% of HercepTest 2+ results were negative by CTA (0-1+) and would not have been allowed entry into the trial. One other study also suggests a problem with sensitivity where there were high rates (57%) of *cerbB2* overexpression in one series of more than 1,000 breast cancers evaluated using the same antibody as used in the HercepTest,<sup>22</sup> although this was in a subpopulation of mainly recurrent tumours.<sup>23</sup> Another IHC assay which has just been approved by the US FDA is the Pathway kit.<sup>24</sup>

#### *IMH versus FISH*

Wang et al<sup>10</sup> compared IHC assays for *cerbB2* against 2 FISH assays, and they found that 26 of 52 cases (50%) showed medium to high IHC positivity (which is significantly higher than the 25% to 30% of *cerbB2* overexpression in many published reports) contrasting with only 14 cases (27%) that showed gene amplification by both the FISH assays. As the IHC and FISH assays measure related but distinct targets — protein concentrations versus gene copy numbers — it would be expected that they might show disagreement particularly in cases with high concentrations of protein without concurrent gene amplification, possibly via other mechanisms than gene amplification as mentioned earlier. However in most published reports, this disagreement is less than 10% of specimens or in as few as 3% of the *cerbB2* overexpressing cases.<sup>25</sup> (Furthermore, in the study by Wang et al, the main subgroup contributing to the disagreement was from the IHC medium positive but FISH negative cases.) Although it could not be ruled out that these cases were truly overexpressing *cerbB2* protein without gene amplification, the authors felt that the other variables in IHC, such as tissue fixation and processing, as well as antigen retrieval methods, contributed to the discrepancy. Another possibility may be that because the FISH assays use 4 signals in each cell nucleus as positive for gene amplification, or a ratio of 2, some of the cases with low gene amplification might not have been scored as amplified, thus underestimating gene amplification. Polysomy 17, with a corresponding increase in *cerbB2* gene copies, does not qualify for *cerbB2*

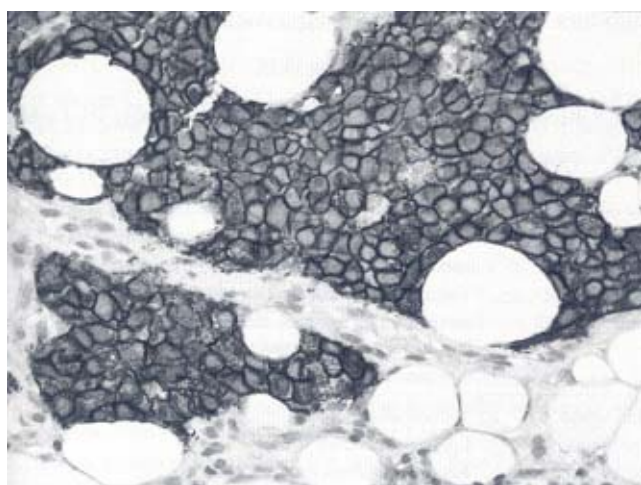


Fig. 1. Example of strongly positive, complete cytoplasmic membrane staining for *cerbB2* by immunohistochemistry (3+). (Immunohistochemistry, original magnification  $\times 200$ )

amplification based on FISH criteria but it could still potentially cause *cerbB2* protein overexpression. Another study by Field et al<sup>26</sup> compared the Dako A485 antibody, the Herceptest and FISH analysis in the evaluation of the *cerbB2* status of 122 breast carcinomas. They found no evidence of *cerbB2* gene amplification in any of the cases they graded as 2+ using the HercepTest, suggesting that a 2+ result should probably be redefined as “equivocal” rather than “positive”.

The 4 US FDA-approved assay methods were approved for specific purposes. 1) The HercepTest and Pathway for identification of patients with *cerbB2* overexpression who may benefit from trastuzumab, 2) the INFORM kit for identifying node-negative patients with a poor prognosis, and 3) the PathVysion kit for identifying patients likely to benefit from different dose levels of cyclophosphamide / doxorubicin / fluorouracil (CAF). Whether these kits are interchangeable for these purposes might be presumed but has not yet been demonstrated.<sup>27</sup>

Table I compares and contrasts some of the advantages and disadvantages of these 2 main methods of *cerbB2* status evaluation in tissue.<sup>28</sup>

### New Frontiers

Many different therapeutic strategies have been developed to target *cerbB2* overexpressing cancer cells, the most widely tested in the clinical setting being the monoclonal antibodies — i.e., trastuzumab. Other approaches are tyrosine kinase inhibitors, *cerbB2* antisense oligonucleotides that can inhibit *cerbB2* expression and cell proliferation, and a ribozyme approach to reducing *cerbB2* expression. All these methods are being studied *in vitro*, in clinical trials or in both.<sup>29</sup> Yet another approach is attempts to decrease the transcription of *cerbB2* genes using gene therapy. Transcription factors, like PEA3<sup>30</sup> and adenovirus type 5 E1A protein,<sup>31</sup> have been found to repress *cerbB2*

promoter function. A phase 1 trial of E1A gene transfer for advanced breast and ovarian cancers has been completed.<sup>32</sup>

Clinical trials to study the value of Herceptin (trastuzumab) in an adjuvant setting are on-going.

*CerbB2* is the prototype for a molecular discovery that has been translated into a clinically valuable marker and an effective therapeutic product,<sup>29</sup> and this has only heightened the urgency to standardise methods for *cerbB2* status evaluation; thus, enabling better correlation of *cerbB2* expression with outcome in the many current and future investigations.

### Conclusion

In summary, IHC assay findings for *cerbB2* status should best be reported in explicit and specific terms, such as the percentage or proportion of positive tumour cells, their staining intensity and the assay method, especially the specific antibody, that was used. General interpretations of positive or negative can only be made against the background of known and standardised assay methods, in the context of a specific clinical setting and after correlation of the results with clinical outcome. For example, responsiveness to anthracyclines may be associated with lower levels of *cerbB2* overexpression than responsiveness to Herceptin; thus, requiring lower levels of “positive” results for selection of patients for anthracycline therapy compared to those for Herceptin therapy.

Some institutions have adopted a system where all cases of breast cancer for evaluation are tested by IHC analysis using a method that has been validated against FISH. For cases that are strongly positive or clearly negative by IHC, no further testing is performed in view of the excellent correlation of high levels of *cerbB2* expression (3+) by IHC with gene amplification by FISH. It is not thought that FISH would provide any further information on these tumours.<sup>33</sup> For cases that are equivocal or weakly positive

TABLE I: COMPARISON OF IMMUNOHISTOCHEMISTRY (IHC) AND FLUORESCENCE IN SITU HYBRIDISATION (FISH) IN *CerbB2* STATUS EVALUATION

| Method | Advantages                                                                                                                                                                                                                                                                                                                                   | Disadvantages                                                                                                                                                                                                                                                                                                                                  |
|--------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| IHC    | <ol style="list-style-type: none"> <li>1. Technically simple with short procedure time and achievable in most diagnostic laboratories.</li> <li>2. Can be used on routine paraffin embedded tissue.</li> <li>3. Needs light microscope for interpretation.</li> </ol>                                                                        | <ol style="list-style-type: none"> <li>1. Many antibodies with varying sensitivity and specificity.</li> <li>2. Target antigen affected by storage, tissue fixative, processing techniques and antigen retrieval methods.</li> <li>3. No universally accepted threshold for positivity.</li> <li>4. No standardised scoring system.</li> </ol> |
| FISH   | <ol style="list-style-type: none"> <li>1. Can be used on routine paraffin embedded tissue.</li> <li>2. Highly specific commercially available reagents.</li> <li>3. Target DNA is stable and not affected by storage and tissue processing methods.</li> <li>4. Standardised positive threshold.</li> <li>5. Qualitative results.</li> </ol> | <ol style="list-style-type: none"> <li>1. Technically more demanding than IHC.</li> <li>2. Longer procedure time.</li> <li>3. Requires fluorescence microscope for interpretation.</li> </ol>                                                                                                                                                  |

by IHC, further FISH is performed since many such cases seem to represent IHC false positive results.<sup>24,26</sup>

However, some immunohistochemically negative cases do show gene amplification and some strongly positive (3+ in the HercepTest scoring system) do lack gene amplification.<sup>34</sup> Moreover, preliminary data suggest that among cases that are strongly positive by IHC, only the cases that also show gene amplification by FISH respond to trastuzumab therapy.<sup>35</sup> Thus, limiting FISH testing to cases scored as 2+ by IHC will not eliminate all false-positive and false-negative IHC results, and precisely which subset of cases should be subjected to FISH following IHC analysis remains a matter of debate.<sup>24</sup>

Reliable, reproducible and clinically meaningful results in the assessment of cerbB2 status in breast cancer will go a long way in helping to successfully identify patients most likely to respond favourably to specific oncologic therapeutic intervention. Cost may be a long-term consideration but given the associated high cost of trastuzumab (Herceptin) and other related treatments, the potential clinical benefits as well as the known adverse effects especially the enhanced cardiotoxicity associated with trastuzumab,<sup>36</sup> the cost differential between cerbB2 assays should not be the over-riding factor in the choice of assay used. Bigger laboratories may consider it worthwhile investing in the initial set-up cost of training personnel and acquiring the additional equipment needed to perform FISH assays efficiently and reliably. Importantly, standardisation of IHC techniques and reporting methods, comparison of IHC results with FISH assays, and the correlation with treatment outcome of breast cancer cases will help unravel the perplexities associated with the reliable establishment of cerbB2 status in breast cancer.

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