A Formalin-Fixed, Paraffin-Processed Cell Line Standard for Quality Control of Immunohistochemical Assay of HER-2/neu Expression in Breast Cancer

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Key Words: HER-2/neu; Cell line standard; Immunohistochemistry; Fluorescence in situ hybridization; Quality control

Abstract

To ensure the accuracy and reproducibility of immunohistochemical assays for determining HER-2/neu status of patients with breast cancer, a reliable standard for monitoring assay sensitivity is necessary. We optimally fixed and paraffin processed human ovarian and breast carcinoma cell lines SKOV-3, MDA-MB-453, BT-20, and MCF-7 in quantities sufficient to meet the needs of a laboratory for the foreseeable future. The material was tested, alongside HercepTest kit cell lines (DAKO, Carpinteria, CA), by 7 breast cancer centers in the United Kingdom and France with different immunohistochemical assays and markers. The cell lines also were analyzed by fluorescence in situ hybridization (FISH) by 2 centers using HER-2/neu kits. FISH produced 100% agreement between the 2 centers: SKOV-3 and MDA-MB-453 showed HER-2/neu amplification and BT-20 and MCF-7 did not. Immunohistochemical analysis and a common evaluation method produced 100% agreement that SKOV-3 and MCF-7 showed 3+ and zero HER-2/neu overexpression, respectively. For MDA-MB-453, there was 71% (5/7) concordance of 2+ immunohistochemical staining and 86% (6/7) concordance of zero or 1+ staining for BT-20.

The cell lines provide a valuable standard for gauging HER-2/neu assay sensitivity irrespective of the antibody, antigen retrieval system, detection system, or method of evaluation used.
lie in the variation in the sensitivity of assays between different laboratories and the different methods of evaluation frequently used to interpret the results.13-16 This is less of a problem for some of the routine immunohistochemical markers that are used merely as adjuncts to histopathologic interpretation (US Food and Drug Administration Class I devices) and the results of which require a qualitative rather than a quantitative assessment.9,17,18 In contrast, for markers such as those to HER-2/neu and estrogen receptors, the results of which are analyzed semiquantitatively and suggest a specific line of treatment to the oncologist or clinician treating the patient (US Food and Drug Administration Class II devices), it is vital that the reported index of expression closely reflect the biological expression of the tumor and is not influenced adversely by inappropriate assay sensitivity or the method by which the results are evaluated.

To ensure the accuracy and reproducibility of the results for HER-2/neu obtained by immunohistochemical analysis, a standard control is necessary by which day-to-day variation in the sensitivity of the assay can be accurately monitored. In this respect, the use of composite tissue blocks representative of several tumors with varying levels of overexpression for the HER-2/neu protein is not ideal. Tumor material frequently is difficult to acquire, and the quantity available for use for quality control is likely to be limited. Consequently, even during a relatively short time, a laboratory may need to use several different cases as control specimens, each with individual variations in tumor expression and fixation. Obviously this is not ideal for a standard by which to stringently gauge assay sensitivity for HER-2/neu overexpression, as an unpredicted fall or rise in a laboratory’s assay sensitivity may not be readily detectable when using tumors of differing expression and fixation as control specimens.

One way around this problem is to use cell lines fixed and processed in a similar way to histopathologic specimens.19 Although the characteristics of a cell line are liable to change with different treatments or cell passages, large-scale cell production could permit a single harvest of a sufficient quantity of cells with a specific level of expression.20,21 Such a harvest of cells of the same phenotype all fixed and processed at the same time would permit a large and long-lasting bank of standard control material. Thus, it would have the potential to provide a consistency over a relatively long period that is not possible with tissue-based control samples.

The aim of the present study was to examine the feasibility of developing a cell line control for HER-2/neu analysis using 4 cell lines with differing levels of overexpression for HER-2/neu, ranging from unequivocally negative to unequivocally positive. These cell lines were characterized in terms of their HER-2/neu gene amplification and protein overexpression by FISH and immunohistochemical analysis, respectively, in several breast cancer centers using different antibodies and methods. The selected cell lines were optimally fixed and paraffin processed into composite blocks in sufficient amounts to provide for the needs of a routine laboratory for a protracted period.

Materials and Methods

Cell Lines

Approximately 1 × 10^8 cells from each of the human breast carcinoma cell lines, MDA-MB-453, BT-20, and MCF-7, and the ovarian carcinoma cell line SKOV-3 were received frozen from the European Collection for Cell Cultures (CAMR, Salisbury, Wiltshire, England). Samples of each cell line (3 × 10^6 cells) for analysis by FISH were thawed and centrifuged at 1,800 rpm (Sanyo Centaur 2 centrifuge, Sanyo Gallenkamp, Loughborough, England) for 10 minutes. The supernatant was aspirated and the pellet resuspended in 10 mL of hypotonic solution (0.075-mol/L concentration of potassium chloride prewarmed to 37°C). Following incubation for 10 minutes at 37°C, the cells were centrifuged at 1,800 rpm for 10 minutes, the supernatant aspirated, and the pellet resuspended in acetic methanol (methanol/glacial acetic acid, 3:1), with the sequence of centrifugation and fixation repeated 2 more times. The slide preparations for FISH analysis were made by placing a drop of the fixed cell suspension on a clean glass microscope slide and allowing it to air dry and age overnight on the bench.

One drop of the fixed cell suspension was fixed overnight in 10% phosphate-buffered formalin (pH 7.0) for approximately 20 hours, embedded in agar, and processed to paraffin wax, using a method described previously.22 Briefly, with the fixed cells permitted to sediment in the base of a 25-mL polyethylene container with a conical base, the bulk of the formalin was removed, and the cell suspension removed to a 1.5-mL microcentrifuge tube and allowed to sediment again for approximately 30 minutes. The supernatant was removed, leaving the cells in 10 to 50 µL of fixative, in which the cells were resuspended. The microcentrifuge tube containing the cell suspension was heated in a 60°C water bath to which was added prewarmed and equilibrated 4% agar. The agar-cell mix then was transferred to 5-mm diameter × 5-mm deep polyethylene molds from which small agar-cell mix cylinders were extruded carefully. The agar-cell mix cylinders were fixed in formalin for another 2 hours and then placed in a standard tissue-processing cassette and hand processed to paraffin wax. Cylinders, one from each cell line, were embedded together in paraffin wax to make several composite blocks.
Fluorescence In Situ Hybridization

FISH analysis was performed and evaluated at 2 centers, the University Hospital of Wales, Cardiff (M.J.M.), and the Institut Curie, Paris, France (J.C.). In both centers, a ratio of more than 2 HER-2/neu/17 centromere probe signals by FISH was considered to correspond to gene amplification. The mean number of HER-2/neu signals and chromosome 17 signals per nucleus were calculated by dividing the total number of signals by the number of nuclei examined.

University Hospital of Wales

The cell preparations were denatured according to the recommendations in the Vysis PathVysion HER-2 DNA probe kit (Vysis, Downers Grove, IL), after which they were hybridized with the Vysis kits HER-2 locus-specific and 17 centromere-specific probes. Subsequent FISH analysis was performed using 4,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), and Texas Red filters on the Vysis Smart Capture system. The cells were tested and screened on 2 separate occasions. Approximately 60 interphase nuclei were screened for each cell line.

Institut Curie

FISH analysis was performed using the Oncor Inform HER-2/neu (ERBB2) gene amplification detection system (Ventana Medical Systems, Tucson, AZ) and the instructions given by the supplier, with slight modification. Briefly, the preparations were denatured in 70% formamide/2× standard saline citrate (SSC), pH 7, at 72°C, for 4 minutes. Ten microliters of digoxigenin-labeled HER-2/neu DNA probe were mixed with 0.5 µL of denatured biotin-labeled chromosome 17 alpha-satellite DNA probe (Oncor, Gaithersburg, MD) and were applied to the denatured preparations. Slides were incubated for 15 hours at 37°C; washed in 50% formamide/2× SSC, pH 7, at 43°C for 15 minutes; and washed in 2 baths of 2× SSC, pH 7, at 37°C for 4 minutes each. Detection was achieved using fluorescein-labeled avidin/antidigoxigenin rhodamine (Oncor). The nuclei were counterstained using a DAPI/antifade solution. Slides were viewed with an epifluorescence Leica DMRB microscope (Leica, Wetzlar, Germany) fitted with a triple band DAPI/FITC/Texas Red filter. Images were captured using a Quantsics digital camera (Photometrics, Tucson, AZ) and Quips FISH imaging software (Vysis). A total of 40 interphase nuclei were screened for each cell line.

Immunohistochemical Analysis

Sections were cut at a thickness of 3 µm and mounted onto slides coated with the adhesive 3-aminopropyltriethoxysilane and dried in a 60°C oven for 4 hours to ensure maximum adhesion. The position of the cell lines on the microscope slides, after cutting, is shown in Figure 1. Each circular area of cell line was 4 mm in diameter, and the areas were 2 mm apart. The approximate surface density for the cell lines was 800 to 1,100 cells per mm².

Four sections, along with 2 DAKO cell line controls (DAKO A/S, Glostrup, Denmark) were sent to collaborating United Kingdom breast cancer centers in Cardiff, Liverpool, Manchester, and London and to 1 French center in Bievres, where they were immunostained with the antibody and method that each center routinely uses to determine the HER-2/neu status of patients with breast carcinoma. This consisted of a total of 7 immunocytochemical protocols and 4 monoclonal or polyclonal antibodies. The DAKO cell line controls were immunostained at the same time using the same antibody and method as used on the new cell line standard.

The immunohistochemical results were evaluated by 3 assessors (A.R., B.J., A.R.D.) scoring independently using a method of evaluation described previously. Briefly, the scoring method was as follows: no staining or membrane staining in less than 10% of tumor cells, 0; faint, barely perceptible membrane staining in more than 10% of tumor cells, 1+; weak to moderate complete membrane staining observed in more than 10% of tumor cells, 2+; and strong complete membrane staining in more than 10% of tumor cells, 3+. The median score from the 3 assessors was used to record the level of expression for each cell line.

Time Trial to Assess Antigen Decay in Cut Sections

An ongoing time trial to assess the extent of Her-2/neu antigen decay in the cut sections was implemented with testing to take place in 2 centers using the CB11 clone and the DAKO polyclonal antisera at the following times: 0, 2, 4, 8, 12, 24, 36, and 48 weeks. The immunostaining was conducted in duplicate, with freshly cut sections acting as the external control samples.

Results

Fluorescence In Situ Hybridization

In both collaborating laboratories using the FISH analysis and 2 different assays, there was 100% agreement that the cell lines SKOV-3 and MDA-MB-453 showed HER-2/neu gene...
amplification and that the cell lines BT-20 and MCF-7 did not have HER-2/neu gene amplification.

### Immunohistochemical Analysis

The morphologic features and the HER-2/neu expression of the cell lines are shown in **Image 2**. The results show that when the expected level of sensitivity was achieved on the DAKO cell line control (3+, 1+, 0), the sensitivity on the new cell line control was 3+, 2+, 2+, and 0 with the CB11 clone and 3+, 2+, 1+, and 0 with the HercepTest or the DAKO polyclonal antibody.

### Correlation of Protein Overexpression With Gene Amplification

For the cell line SKOV-3, there was 100% (7/7) laboratory assay concordance between 3+ HER-2/neu protein overexpression as determined by immunohistochemical analysis and HER-2/neu gene amplification as determined by FISH. Similarly, there was 100% concordance between “0” protein overexpression and no gene amplification for the cell line MCF-7. For the cell line MDA-MB-453, the level of concordance between 2+ overexpression by immunohistochemical analysis and gene amplification by FISH was 71% (5/7), while for BT-20 there was 86% (6/7) concordance between a “0” or 1+ score by immunohistochemical analysis and no amplification by FISH.

### Time Trial to Assess Antigen Decay in Cut Sections

There was no obvious deterioration of the HER-2/neu antigen at 2, 4, and 8 weeks when sections of this age were stained using the Novocastra CB11 clone (Novocastra Laboratories, Newcastle upon Tyne, England; Menarini Diagnostics, Finchampstead, Berkshire, England; Novocastra Laboratories, Newcastle upon Tyne, England; Oncogene Research Products, CN Biosciences UK, Nottingham, England; Vector Laboratories, Peterborough, England).

### Discussion

Some authors have questioned the reliability of the immunohistochemical assay for accurately predicting the likely response of patients to trastuzumab therapy, while others have emphasized its reliability, providing that standardized methods with rigorous control of sensitivity are used.
used. Such stringent control is not possible without a graded standard against which laboratory results can be monitored on a day-to-day basis. Our present study sought to provide a solution to this problem by developing such a standard using formalin-fixed, paraffin-processed cell lines with differing levels of overexpression for HER-2/neu.

The use of cell lines to control assay sensitivity for HER-2/neu is by no means a novel idea, as it is an essential component of the DAKO HercepTest kit. However, this kit is relatively expensive, and in the standard kit, only 5 cell line–based control slides and sufficient reagents to perform 35 tests are provided. Some workers buy the constituent reagents separately, in which case they are left without the availability of the valuable control slides, which currently are not purchasable separately. In addition, other workers prefer to use different antibodies from the one used in the HercepTest. The present study provides a solution to this problem by describing a cell line–based standard by which assay sensitivity may be accurately gauged, regardless of the choice of antibody.

To obtain large quantities of cell lines at a standard level of protein expression, we adopted the approach used by Ruby and McNally, using a very large volume of cells from a single harvest. However, we further extended the quantity of sections that can be subsequently obtained from the formalin-fixed, paraffin-processed material by producing agar cylinders of cells with uniform density instead of the cell pellets used by Ruby and McNally. We calculated that 700 to 1,000 sections, of a 3-µm thickness, could be obtained from one of the blocks that we prepared containing the 4 cell lines. Thus, just 1 block would provide control of a minimum of 700 HER-2/neu assays. Even in the unlikely scenario of the HER-2/neu assay being performed by a laboratory daily, 5 days a week, this 1 block would provide sufficient control material for at least 3 years. As it is, in the present study, 7 composite blocks were produced, each containing the 4 cell lines with a standard and known level of HER-2/neu expression, enough to provide even the busiest of laboratories with sufficient material to last a minimum of 21 years. Obviously such quantities are unlikely to be required by 1 laboratory, and, therefore, the process lends itself to mass-scale production of control blocks by 1 institution for a number of others.

While there is little evidence to suggest that the HER-2/neu antigens will decay with time in the formalin-fixed, paraffin-processed blocks, it is likely that this will occur in the cut sections. To study this, we undertook a time trial to assess the extent of antigen decay in cut sections stored at room temperature. Although this time trial is ongoing, 8 weeks after cutting the sections and with testing at 2 centers using the CB11 clone and the DAKO polyclonal Her-2/neu antibody, there seemed to be no obvious deterioration in the antigen.

By purchasing the cell lines in the desired volume, it is possible for a routine histopathology laboratory without cell culture facilities to devise its own cell line control using the technology described. At the College of American Pathologists Conference in June 1999 on solid tumor prognostic factors, it was proposed that “if cell lines are used as standards for HER-2/neu testing, ways to promote production of cell line controls must be disseminated to practicing pathologists.” We hope that the present article assists in this dissemination by providing details of a relatively simple way of producing a cell line standard for HER-2/neu.

If reliable, standardized reagent controls are readily available, the long-standing debate about which antibody, method, or reagent to use in order to standardize immunohistochemical assays becomes obsolete, because if all assays are adjusted to achieve an agreed-on end point on an agreed-on biological standard, then the method by which this end point is achieved becomes largely irrelevant.

While the main aim of the present study was to provide a suitable standard so that assay sensitivity can be gauged

<table>
<thead>
<tr>
<th>Study Center/Cell Line</th>
<th>No. of Nuclei Counted</th>
<th>HER-2/neu</th>
<th>CEP</th>
<th>HER-2/CEP 17 Ratio</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>University of Wales Hospital, Cardiff</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SKOV-3</td>
<td>59</td>
<td>571 (9.6)</td>
<td>212 (3.6)</td>
<td>2.7</td>
<td>Amplified</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>60</td>
<td>504 (8.4)</td>
<td>237 (4.0)</td>
<td>2.1</td>
<td>Amplified</td>
</tr>
<tr>
<td>BT-20</td>
<td>58</td>
<td>123 (2.1)</td>
<td>182 (3.1)</td>
<td>0.7</td>
<td>Not amplified</td>
</tr>
<tr>
<td>MCF-7</td>
<td>59</td>
<td>123 (2.1)</td>
<td>165 (2.8)</td>
<td>0.8</td>
<td>Not amplified</td>
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<tr>
<td>Institut Curie, Paris, France</td>
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<tr>
<td>SKOV-3</td>
<td>40</td>
<td>488 (12.2)</td>
<td>116 (2.9)</td>
<td>4.2</td>
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<tr>
<td>MDA-MB-453</td>
<td>40</td>
<td>388 (9.7)</td>
<td>128 (3.2)</td>
<td>3.0</td>
<td>Amplified</td>
</tr>
<tr>
<td>BT-20</td>
<td>40</td>
<td>76 (1.9)</td>
<td>88 (2.2)</td>
<td>0.9</td>
<td>Not amplified</td>
</tr>
<tr>
<td>MCF-7</td>
<td>40</td>
<td>108 (2.7)</td>
<td>112 (2.8)</td>
<td>1.0</td>
<td>Not amplified</td>
</tr>
</tbody>
</table>

CEP 17, centromere of chromosome 17 probe.
accurately for an important predictive marker, there is the potential for such an approach to provide an almost limitless supply of suitable control material for other antibodies. Problems surrounding the use of surgically removed tissues for quality control, the acquisition of which has become increasingly difficult in the United Kingdom owing to adverse publicity regarding issues relating to organ retention, also are partly circumvented if cell lines are used instead.

When the expected level of expression was achieved on the DAKO cell line controls, the level of expression for the cell lines SKOV-3, MDA-MB-453, BT-20, and MCF-7 was found to be 3+, 2+, 1+, and 0, respectively, when using the HercepTest, or the DAKO polyclonal antisera. Achieving the expected result on the DAKO cell line–based control with the CB11 clone resulted in scores of 3+, 2+, 2+, and 0 on SKOV-3, MDA-MB-453, BT-20, and MCF-7, respectively.

In both of the collaborating laboratories using the FISH analysis and 2 different assays, there was 100% agreement that the cell lines SKOV-3 and MDA-MB-453 showed HER-2/neu gene amplification and that the cell lines BT-20 and MCF-7 did not. The discrepancy in the number of signals between the 2 analytes for the most amplified cell line (SKOV-3) is probably because the HER-2/neu signals form a cluster in the nuclei of these cell lines, often making it difficult to recognize them individually and to enumerate them precisely. Such clusters in interphase nuclei are related to homogeneously staining regions observed in metaphase chromosomes (Image 1).

The level of concordance between HER-2/neu gene amplification determined by FISH and protein overexpression determined by immunohistochemical analysis was excellent for the cell line showing amplification and high overexpression (3+; SKOV-3) and the cell lines showing no amplification and none (0) or little (1+) protein overexpression (MCF-7 and BT-20, respectively). The level of gene amplification as determined by FISH for the cell line MDA-MB-453

<table>
<thead>
<tr>
<th>Study Code</th>
<th>Antibody</th>
<th>SKOV-3</th>
<th>MDA-MB-453</th>
<th>BT-20</th>
<th>MCF-7</th>
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<tr>
<td>A</td>
<td>CB11</td>
<td>3</td>
<td>2</td>
<td>2</td>
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<tr>
<td>B</td>
<td>CB11</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>CB11</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>10A7</td>
<td>3</td>
<td>1</td>
<td>0</td>
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<td>HercepTest</td>
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<td>2</td>
<td>1</td>
<td>0</td>
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<tr>
<td>F</td>
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<td>2</td>
<td>0</td>
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</tr>
<tr>
<td>G</td>
<td>3B5</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

* See Table 1.
† Expected level of staining for a valid result on the DAKO cell line control slide, as given in the HercepTest protocol (DAKO, Ely, Cambridgeshire, England).
was judged to be less than that of SKOV-3, though still with a ratio of greater than 2 for the HER-2/neu/CEP 17 signals. In the present study, this level of amplification equated to a 2+ level of protein expression with 5 (71%) of 7 of the immunohistochemical assays used.

The current control has the advantage of providing a graded series of expression for each of the 3+, 2+, 1+, and 0 categories when using the HercepTest or the DAKO polyclonal antisera. It is appreciated that this method of evaluation may be justifiably criticized for being overly simplistic. However, we have used this system because of its use in previous major publications, its inclusion in the recommendations by Roche-sponsored United Kingdom referral centers, and because it was the most appropriate scoring system by which to compare the level of “expected” overexpression exhibited in the DAKO cell lines with that shown by the new cell lines. We chose the DAKO cell lines from the HercepTest kit as the point of reference because they are familiar to most workers in this field, because the HercepTest is itself undergoing extensive and ongoing validation, and because of the obvious similarities between the two cell line control systems. It was not the purpose of the present study to question methods of evaluation, or indeed which levels of overexpression most closely predict clinical outcome. Which level of sensitivity is ultimately found to be most appropriate and which method of evaluation is used to establish this is still a matter for debate. However, the value of the cell line standard control we have described will remain, as it provides a biological constant against which the variable of immunohistochemical assay sensitivity for HER-2/neu can be accurately gauged, regardless of which antibody, antigen retrieval system, detection system, or method of evaluation ultimately is used and considered to be appropriate.

**Table 4**

**Correlation of HER-2/neu Protein Overexpression by Immunohistochemical Analysis With HER-2/neu Gene Amplification by FISH**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Gene Amplification</th>
<th>Protein Overexpression</th>
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<tbody>
<tr>
<td>SKOV-3</td>
<td>Amplified</td>
<td>3+ 2+ 1+ 0</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Amplified</td>
<td>0 5 1 2</td>
</tr>
<tr>
<td>BT-20</td>
<td>Not amplified</td>
<td>1 2 4 7</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Not amplified</td>
<td>0 0 0 7</td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization.

References


From the 1United Kingdom National External Quality Assessment Scheme for Immunocytochemistry and the Department of Histopathology, University College London Medical School, London, England; 2Immunocytochemistry and Molecular Pathology Unit, University of Wales College of Medicine, Cardiff;


