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Review

Increased Use of Immunohistochemistry for Oestrogen Receptor Measurement in Mammary Carcinoma: the Need for Quality Assurance

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This paper outlines the changes which have occurred over the last 25 years in the methods employed for the measurement of oestrogen receptors to aid the management of women with breast cancer. Immunohistochemistry is now the method of choice and knowledge of oestrogen receptor status is being used with increasing frequency for the selection of adjuvant treatment as well as for the treatment of metastatic disease. It is essential that good quality assurance procedures are established so that results are reproducible and can be used with confidence in individual centres as well as being comparable with those produced elsewhere. A retrospective study of 170 women with metastatic breast cancer provides the basis for a discussion on the advantages and pitfalls of the immunohistochemical assay. Particular emphasis is paid to the choice of cut-off and how the results may be applied in patient management. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

FOR OVER 100 years it has been known that some breast cancers are hormone sensitive and that tumour growth can be slowed by removal of circulating oestrogens. Beatson was the first to demonstrate this in 1896 [1] when he achieved regression of metastatic breast cancer in a proportion of premenopausal women following oophorectomy. Other surgical procedures which modulate hormone levels such as adrenalectomy and hypophysectomy followed and remission of disease was seen in approximately one-third of women [2]. There was great interest in finding a way of selecting the women who would benefit from this form of hormone treatment so that the rest could be spared from ineffective surgery.

In 1971, Jensen and colleagues [3] demonstrated that the presence of a high affinity oestrogen binding protein in excised primary or metastatic mammary carcinoma predicted the likelihood of a favourable response to adrenalectomy. Since then, these binding proteins, now known as oestrogen receptors (ER), have assumed an increasingly important role in the management of breast cancer. Initially, the amount of ER was measured on tumour cytosols prepared from metastatic lesions and the information obtained was used to predict response to endocrine treatment for recurrent disease. It soon became practice to carry out assays on primary tumours and record the ER status which was then available to aid treatment decisions in the event of a recurrence in an inaccessible site. It was later found that these data could be used to predict the likelihood of early relapse, thus ER became a prognostic, as well as a predictive, indicator [4]. Initially, assay results were reported as positive or negative, with a cut-off point determined by clinical response. Later, numerical values were given as it became apparent that tumours with high receptor levels were more likely to be hormone responsive. Coincidental with the development of ER cytosol assays was the introduction of the anti-oestrogen, tamoxifen, in the treatment of metastatic breast cancer [5] and this drug, together with aromatase inhibitors and antiprogestins rapidly replaced ablative surgery of endocrine organs as the hormone treatment of choice. Tamoxifen is also now widely used as an adjuvant following surgery and ER status has assumed an

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important role in identifying patients likely to benefit from such treatment [6]. Therefore, given the current clinical importance of ER, it is vital that the methods used to measure it are accurate and reproducible.

HISTORY OF THE ASSAY

The use of ER in the management of breast cancer is one of the first examples of the transition of a biological marker from a promising area of research into a routine prognostic indicator, now measured and used in laboratories and clinics worldwide.

How has this change come about? One of the main reasons must be the current availability of antibodies which recognise the ER protein in formalin-fixed, paraffin-embedded tissue sections. The first ER assays were carried out on pieces of tumour tissue which had to be frozen immediately after removal from the patient and stored under special conditions. The assay method was usually a dextran-coated charcoal, radioactive ligand binding assay (LBA) followed by Scatchard analysis [7]. This was carried out on cytosols prepared from the frozen tissue and took 2 days to complete. Considerable technical expertise was required to achieve consistently reliable results which led to the setting up of external quality assurance schemes. Despite these drawbacks, the value of the clinical information provided by the assay was well established by the work carried out and reported in the late 1970s and early 1980s.

The next development was the introduction of antibodies to ER. These took a long time to produce as the ER protein is very labile and was difficult to purify by the methods available at the time. In the mid-1980s, Jensen and colleagues described an enzyme immunoassay (EIA) for tumour cytosols [4] and an immunocytochemical assay (ICA) for frozen tissue sections [8]. The antibodies were made available commercially in kit form (Abbott Laboratories, Illinois, U.S.A.). Once results with the more convenient EIA had been validated against the LBA, the advantage of this method, which is simpler and avoids the use of radioactive material, was quickly realised and more people started to measure ER. There were great expectations that results with the EIA would be more reproducible than those obtained with the LBA and initially, when specialist laboratories were doing the assay, this was so. However, this improvement was lost as the number of assay laboratories increased.

The ICA did not live up to initial expectations either. It was thought that knowledge of the distribution of ER-positive tumour cells would improve clinical relevance and that heterogeneity of expression would explain the lack of hormone responsiveness in some tumours, which were found to be positive by the cytosol assay. However, this theory was not borne out in practice. Another major problem with the ICA was that it could only be carried out on frozen tissue and so, given this and the above reasons, it was not widely adopted. Many attempts were made using a variety of antigen retrieval methods to make the ICA work on formalin-fixed, paraffin-embedded tissue sections. However, none of these proved entirely satisfactory until the advent of heat-mediated antigen retrieval by microwave [9] or pressure cooker [10]. This methodology and the subsequent arrival of antibodies such as ID5 [11], which do work well on fixed tissue [12], have dramatically changed the situation and more and more histology laboratories are now measuring ER using immunohistochemistry. Results obtained by the cytosol assays and ICA on fixed tissue have been compared and there is good agreement between them [13–16]. There are certain advantages and disadvantages associated with each method, as shown in Table 1.

RECEPTOR VALUES

When the LBA was first developed, Scatchard analysis of the data produced a numerical result which was reported in terms of femtomoles of oestradiol bound per mg cytosol protein (fmol/mg protein). The ability to construct a Scatchard plot from the ligand binding data divided the 'positive' cases from the 'negative' cases. In practice, this gave a clinical cut-off value of 10 fmol/mg protein. Later, the clinical cut-off for the EIA was found to be 20 fmol/mg protein. Thus, in practical terms for both of these assays it was recognised that tumours with low levels of ER activity were unlikely to be hormone responsive; a fact that has to be remembered when determining a cut-off value for ICA.

QUALITY ASSURANCE

Any assay which is used in a clinical setting should have good quality assurance procedures. Excellent assurance schemes for the ER cytosol assay were organised in Europe by the EORTC [17, 18]. At the moment there is no such international scheme for immunohistochemical methods, although a number of initiatives have been undertaken. There are two aspects to quality assurance for immunohistochemistry. One concerns the quality of the staining technique and the other consistency in assessment of staining.

Immunohistochemical staining

In the U.K. the first is addressed by the National External Quality Assessment Scheme (U.K. NEQAS-ICC) which now includes ER in the list of proteins for which they offer an assessment service. The number of laboratories subscribing to this scheme has increased over the last 3 years. There has been a general improvement in the quality of staining, such that now over 70% of laboratories produce acceptable staining of ER on slides provided by NEQAS. The scheme requires each laboratory to stain circulated sections cut from three tumours on which the ER content has previously been determined by the cytosol assay. The stained sections are returned for assessment by four NEQAS assessors. The participants also send a stained example from a tumour diagnosed in their own laboratory. Interestingly, 97% of laboratories produce acceptable staining of their own sections, clearly showing that each has adapted suitable protocols for their local conditions.

If the results of any assay are to be used for clinical decision making, control over the method is extremely important. For immunohistochemistry, it is important to realise that variability in staining can occur for a number of technical reasons. These include the method of antigen retrieval, concentration of primary antibody and sensitivity of the detection system. All have to be carefully controlled and the use of positive and negative controls is therefore essential. The positive controls should include different samples previously shown to contain different levels of ER. The use of tissue with low ER status is particularly important to check the sensitivity of the methodology.

Immunohistochemical assessment

Assessment of staining is a more difficult area in which to seek concordance of results. Unlike the cytosol assay, where a

Table 1. Comparison of immunocytochemical and cytosol assays

Immunohistochemistry	
Advantages	Disadvantages
Works on routine, formalin-fixed, paraffin-embedded tissue	Subjective, semi-quantitative assessment
No extra tissue required	All receptors demonstrated may not be functional
Can be used on archival material for retrospective analysis	Limited standardisation and quality control of the assay
Possible to assay very small tumours routinely	No agreement on the method of evaluation of staining
Possible to do the assay on FNAs and needle core biopsies so it is possible to monitor receptor status during therapy	No agreement on the correct cut-off value between positive and negative
Possible to ensure that the tissue section contains tumour	
Possible to relate receptor content to morphology	
Increased specificity because (a) positive cells can be recognised in tumours of low cellularity; and (b) false positive results due to ER in adjacent normal tissue can be avoided	
Knowledge of intra-tumour heterogeneity	
Internal quality control for ER-negative tumours if some normal tissue (which stains positively) is included in the section	
Cytosol assays	
Advantages	Disadvantages
Objective assessment with numerical results and good reproducibility	Relatively large amounts of tissue required
Technically and clinically validated	Care must be taken over the conditions of storage and assay
Good quality control schemes available	LBA is labour intensive and time consuming and uses radioactivity
LBA measures functional receptors	EIA kits are expensive
	May underestimate ER content if there is a large number of receptors occupied by oestradiol or if the tumour is sparsely cellular
	May overestimate ER content if there is a large amount of ER in surrounding normal tissue
	No exact control of quality of sample so there is the possibility of false negative results due to assay of non-tumour tissue

ER, oestrogen receptor(s); LBA, ligand-binding assay; EIA, enzyme imunnoassay; FNA, fine needle aspirates.

numerical result is produced, evaluation of staining is subjective. While some people advocate the use of image analysis, others consider that this has not yet reached the stage of development where it can be used quickly, reliably and efficiently.

There is no agreement so far as to the 'best' way to assess the staining, nor what the cut-off should be. There are two main components to the appearance of the stained tumour cells: the intensity of the staining and the proportion of cells stained. An additional feature is the uniformity of staining within the positive cells. All these features can be used individually or in combination. In a study of 170 women with metastatic breast cancer treated with tamoxifen, in the Guy's Hospital Breast Unit (GHBU), we found a good correlation between immunostaining and response to treatment, irrespective of the actual method of assessment [13]. It is important that the method which is finally adopted should have the least inter-observer variability [19]. Many people favour reporting the proportion of positively stained cells irrespective of the intensity of staining but there is much to be said for a combination of proportion and intensity, as both appear to provide information. Whatever the method used, however, there is no doubt that the more ER present in the tumour cells, the greater is the likelihood of a favourable response to endocrine treatment for metastatic disease [3]. Figure 1, derived from our own data described above, shows the relationship between the duration of clinical response and ER values measured by both the cytosol assay and ICA evaluated by the 'category score' which takes into account the overall appearance of the staining pattern. ER-'positive' tumours had moderate or strong staining and the ER-'negative' tumours had weak (not visible under the low power of the microscope) or no staining [20]. Response was assessed by UICC criteria; responders being defined as those patients who showed a complete or partial response and non-responders as those with either static or progressive disease. The women with the highest levels of ER (by LBA or ICA) were the most likely to be in remission a year after the start of the treatment.

CUT-OFF POINT

There is much debate about the correct cut-off point to distinguish ER-'positive' from ER-'negative' tumours. One of the problems is achieving a balance between sensitivity and specificity. If 'any staining' is considered to be positive then the sensitivity will be very high and very few responders will be included in the negative group. However, some non-responders will be included in the positive group, thus reducing the specificity. Conversely, if only tumours with staining in almost all of the cells are called positive, the specificity will be high at the expense of sensitivity and if this is used to select women for endocrine treatment it will not identify all of the patients who could benefit. Clearly, a balance has to be struck between the extremes. Table 2 shows the relationship between the two parameters in an example from our own data, based on the proportion of tumour cells staining and derived from the 170 patients treated in the GHBU described above.

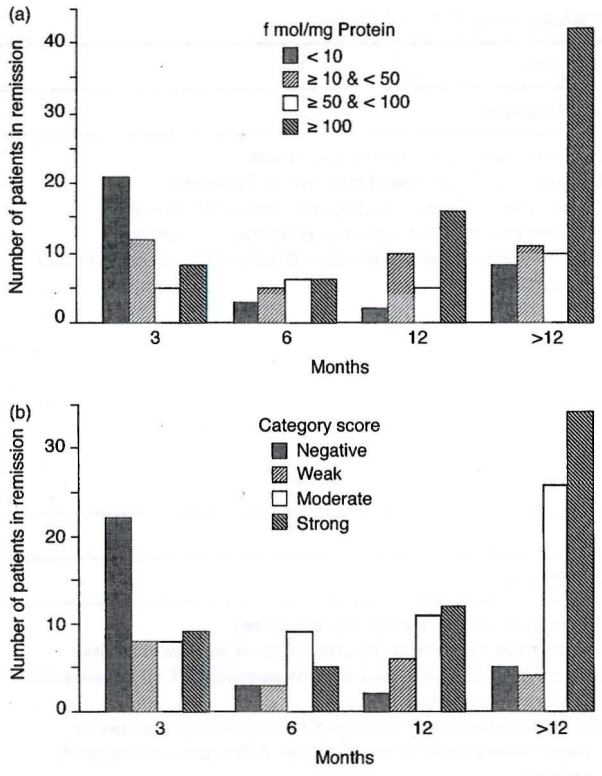

Figure 1. (a) Relationship between duration of clinical response and oestrogen receptors (ER) determined by enzyme immunoassay (EIA). (b) Relationship between duration of clinical response and ER determined by immunocytochemical assay evaluated by the category score.

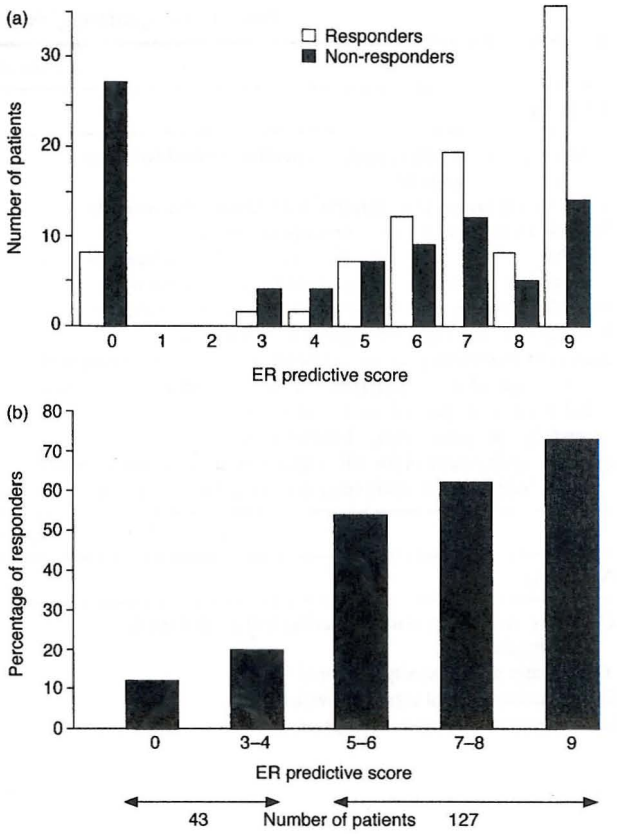

Figure 2. (a) Relationship between oestrogen receptors (ER) predictive score and response to tamoxifen treatment. (b) 'Likelihood of favourable response' to tamoxifen treatment based on ER predictive score.

A good balance between sensitivity and specificity can be achieved by amalgamating the scores obtained by separately estimating (a) the strength of staining on a scale of 0–3 (negative, weak, moderate, strong); (b) the proportion of cells staining grouped in quartiles (0–4); and (c) the uniformity of staining defined as negative, heterogeneous (either staining of variable intensity in all cells or mixed negative and positive areas within a tumour) or homogeneous (uniform depth of staining of all tumour cells) (0–2). This gives a range of 0–9 points and could be called a 'predictive score'. Figure 2(a) shows the 170 patients from our own study grouped in this way and Table 3 shows that a cut-off of ≥ 7 to distinguish 'responders' from 'non-responders' achieves a sensitivity of 71% and a specificity of 62%, a reasonable balance between the two. Using this predictive score, a sharp distinction can be made between the proportion of patients likely to benefit from hormone treatment and those who are unlikely to do so. Figure 2(b) shows that women with tumours with a score of fewer than 5 points have a less than 20% chance of a response and this could be useful as a practical clinical cut-off point.

When the cytosol assay was in use approximately 70% of tumours were found by Scatchard analysis to be ER-positive and about half of the women with these tumours benefited from endocrine treatment for metastatic disease. Another approach to the cut-off point would be for each laboratory to set its own threshold for the ICA in order to achieve an approximate two-thirds to one-third split. This would recreate the situation which pertained to the cytosol assay but would still have a low level of specificity. An important additional point to consider is the fact that lower levels of ER are found in younger women than in older women. Jensen has always held the view that different cut-offs should be used for pre- and postmenopausal women [3]. He believes that it might be better to classify tumours as ER-rich or ER-poor, using different criteria for pre- and postmenopausal women, a practice which could easily be applied to ICA.

Table 2. Examples of how different cut-off points affect sensitivity and specificity

Proportion of tumour cells staining	Patients *n*	Responders	Non-responders	Sensitivity (%)	Specificity (%)
0	32	4	28	95	34
Any cell + ve	138	83	55		
0–25%	55	13	42	85	51
> 25%	115	74	41		
0–50%	75	22	53	76	64
> 50%	95	65	30		
0–75%	101	32	69	63	83
> 75%	69	55	14		

CLINICAL USE

There is no point in doing the assay at all if it is not clinically useful. Experience with the cytosol assay was built upon the response to treatment in patients with metastatic disease.

Table 3. Response assessed according to the predictive score

Score	Patients (*n*)	Response (%)	Sensitivity (%)	Specificity (%)
9	49	35 (71)	40	83
8	13	8 (61)	49	77
7	31	19 (61)	71	62
6	21	12 (57)	85	52
5	14	7 (50)	93	43
4	5	1 (20)	94	38
3	5	1 (20)	95	33
2				
1				
0	32	4 (12)	96	14

The relationship between ER positivity and response to tamoxifen as adjuvant therapy was initially less clear. This was probably due to the difficulties experienced in obtaining ER status on patients from all the centres contributing to the trials investigating the efficacy of adjuvant endocrine treatment. However, overview analyses of the results, from the majority of adjuvant tamoxifen trials worldwide, have shown that the greatest benefit is in women with ER-positive tumours [6]. This has revitalised the interest in the use of ER in treatment selection. Most of the ER results in the overviews were obtained by the cytosol assay. Some studies have examined the relationship between the results obtained by ICA and outcome after adjuvant tamoxifen [21] but more work still needs to be done in this area. A recent editorial in the *British Medical Journal* [22] advocates a very low cut-off for selection of patients for adjuvant endocrine treatment, the San Antonio group having found a significant benefit in women with tumours containing only 1% of positive cells [23].

Perhaps different cut-offs should be used depending on the clinical situation, i.e. in the adjuvant or metastatic setting. Is the aim to identify patients who *will* respond to a particular regime, or those who will *not*? Unfortunately, in neither case will the results from an ER assay give a definitive answer but will only increase the likelihood of a correct prediction. Other factors must also be taken into consideration. These include time-related factors such as tumour size, lymph node involvement and distant metastatic spread and other biological factors such as histological grade and expression of proteins encoded by oncogenes. For example, in a woman who is node negative and has a small, low grade tumour, receptor status will have little impact. Similarly, a patient with widespread metastatic disease is unlikely to get a lasting benefit from tamoxifen, even if all tumour cells are strongly ER-positive.

IMMUNOHISTOCHEMICAL QUALITY ASSURANCE SCHEMES

There is no doubt that the advent of reliable immunohistochemical methods for the measurement of ER has increased interest in the assay which is now carried out in many pathology laboratories throughout the world. Concomitant with this is the awareness of the need for quality assurance schemes to ensure that staining is of high quality and comparable results are obtained in different laboratories. Several schemes are currently underway in different countries. The NEQAS programme in the U.K. is aimed at improving the quality of staining in individual laboratories, using their own methodology, although they do provide the details of a recommended method. In France, inter-laboratory studies have aimed at optimising the consistency of staining technique by concentrating on antigen retrieval methods [24].

There is an EORTC initiative in Europe, which has so far concentrated on finding the best way of assessing the staining. The European Community has also provided funding for pathologists from a number of different countries to try to improve consistency in the histological diagnosis of mammary carcinomas (J.P. Sloane, University of Liverpool). This group has started to include staining for ER in their evaluation. In the U.S., a recent editorial in the *Journal of Histotechnology* [25] summarised the findings of a survey on performance and methods of evaluation of ER on routine paraffin sections. A nationwide trend towards the use of ICA was revealed, with 49% of over 400 responding laboratories using this method in preference to the cytosol assay. Despite having detailed replies from only 23 groups, many others expressed an interest in further studies to achieve greater standardisation of the assay and all have been invited to participate [25].

It is likely that interest in ER ICA will continue to increase and with it will come a better understanding of the biology of breast cancer. A potentially more accurate way of identifying ER-positive tumours should do much to reduce the proportion of apparently ER-negative responders. On a more fundamental level, studies of the recently discovered second ER receptor may help to explain some of the paradoxical behaviour of ER [26]. This second ER (ERβ) is present in breast as well as some other tissues, including ovary and prostate, which do not contain the first receptor (now called ERα) [27,28]. Interesting data are already beginning to emerge relating to ERβ, which may explain the lack of response to hormone treatment in some ER-positive tumours. When separate antibodies against ERα and ERβ become available, immunohistochemistry will have a major part in investigating the possible role of ERβ in the management of breast cancer patients. It looks as if the interest in ER will be maintained well into the 21st century. Continued progress in quality assurance is therefore essential.

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