

An immunocytochemical method for assaying oestrogen receptors in breast cancers. A comparison with the steroid binding assay

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Summary. The presence of oestrogen receptors was studied in 105 human breast carcinomas using monoclonal antibodies (Abbott ER-ICA kit). The oestrogen receptors of neoplastic cells were semiquantitatively measured and correlations were made to receptor values determined by a dextran-coated charcoal (DCC) steroid binding assay and to histological grade.

Immunoreactive cells were found in about 2/3 of the tumours. Usually only a fraction of the cells within each tumour were immunoreactive, and the staining intensity varied among different cells. In general, well differentiated tumours had a greater proportion of immunoreactive cells than poorly differentiated ones. In most cases (65/98) a good agreement was found between the ER-ICA method and the DCC assay. However, in 33 cases discrepancies were demonstrated.

Key words: Oestrogen receptors, Breast carcinomas, Immunocytochemistry

Introduction

Endocrine therapy is a major treatment modality in the management of breast cancer patients. About 35% of the patients respond to various kinds of hormone therapy, of which antioestrogen today is the most widely used. The steroid binding assay for oestrogen receptors provides valuable information for selecting patients for endocrine treatment (De Sombre et al., 1979). About 55% of patients with oestrogen receptor positive (ER+) tumours respond to endocrine treatment, whereas patients with receptor negative (ER-) tumours show a very low response rate (5-10%). Thus, although steroid binding assays are of considerable usefulness in clinical practice, there is a need to improve the

prediction of endocrine responses.

The steroid binding assays of oestrogen receptors have several inherent limitations. They are carried out on cytosols obtained by homogenizing the tissue and thus the identity of the cells of the analyzed tissues is lost. Therefore, the contribution of the various cell populations (both malignant and normal) to the receptor value cannot be determined. Moreover, although standardization of the steroid binding assay has been carried out (EORTC Breast Cancer Cooperative Group, 1980) unresolved problems in the quality control procedures remain. The lability of the receptors is another serious problem. The binding activity of the receptors is influenced by the level of endogenous and exogenous hormones and antihormones, as well as temperature and pH. Heterogeneity with respect to oestrogen receptor expression is to be expected, but demonstration of this phenomenon is not possible using the steroid binding assay (Osborne, 1985).

Several methods to visualize oestrogen receptors in histological sections have been attempted. Thus, various authors have used antibodies to oestradiol (Nenci et al., 1976; Pentschuk et al., 1978; Kurzon et al., 1978; Walker et al., 1980), radioactively labelled oestradiol ligand conjugate, and fluorescence oestrogen (Lee et al., 1978; Pentschuk et al., 1979; Rao et al., 1980). However, a critical analysis shows that none of these methods is likely to visualize true oestrogen receptors (Chamness et al., 1980).

Green and co-workers (1980) have obtained monoclonal antibodies to the oestrogen receptor protein purified from the human breast cancer line MCF-7. Their use in immunocytochemical staining of histological sections has been reported (King et al. 1985). To further investigate the usefulness of this approach, we have applied these antibodies using the technique recommended by Abbott Laboratories, and correlated the results to the level of oestrogen receptors determined by DCC assay as well as to morphological parameters.

Materials and methods

Tumour material

Biopsy specimens were obtained from 105 breast cancer patients admitted to the Norwegian Radium Hospital. Samples of the surgically removed tumours were processed for light microscopy and for immunocytochemical and biochemical analyses of ER. For ER analysis the tumour specimens were frozen and stored in liquid nitrogen before further processing.

Immunocytochemistry

The immunocytochemical determinations were carried out essentially as described in the instructions from Abbott Laboratories. Briefly, 5 µm, thick frozen sections were cut and placed on slides prepared with a tissue adhesive. The sections were then immediately put in a 3.7% formaldehyde-PBS (phosphate buffered saline) solution for 10-15 minutes, transferred to cold methanol (-10 to -25°C) for 5 minutes, placed in cold acetone (-10 to -25°C) for 1 to 3 minutes and finally rinsed for 5 minutes in PBS at room temperature. Excess goat serum was removed prior to incubation of the sections with primary antibody for 30 minutes at room temperature, followed by secondary antibody for 30 minutes and peroxidase-antiperoxidase complex for 30 minutes. Staining was performed with diaminobenzidine and H₂O₂ for 6 minutes. Each of the steps described was followed by rinsing in PBS for 5 minutes. Special care was taken to avoid drying of the sections.

The proportion of positively stained cells was semiquantitatively evaluated and graded according to the following scale:

- : no immunoreactive cells;
- +
 : less than 1/3 of the neoplastic cells immunoreactive;
- ++
 : 1/3-2/3 of the neoplastic cells immunoreactive;
- +++
 : more than 2/3 of the neoplastic cells immunoreactive.

The staining intensity of individual cells was subjectively graded from + to +++.

DCC Steroid Binding Assay of Oestrogen Receptors

ER was measured by a standard dextran-charcoal method with ³H-oestradiol as the ligand, essentially as recommended by European Organization for Research on Treatment of Cancer (EORTC Breast Cancer Cooperative Group, 1980). The quality of the receptor results was monitored by the inclusion of quality control samples in each run and by participation in the running EORTC European Quality Assessment of Steroid Receptor Assays.

Results of the ER assay are given as pmol binding capacity per gram cytosol protein (pmol/g, numerically equivalent to fmol/mg). In breast cancer, values > 10 pmol/g protein are regarded positive, 3-9 pmol/g

borderline, and < 3 pmol/g negative (McGuire et al., 1973).

Light microscopy

Fresh tumour tissue was fixed in buffered formalin and later dehydrated and embedded in paraffin. Haematoxylin and eosin-stained sections from the paraffin blocks were used for light microscopical examination. The tumours were classified and graded according to the WHO recommendations.

Results

Clinical data

Specimens from 102 women and 3 men were included in the study. Thirty-eight of the women were premenopausal and 64 postmenopausal. Seven of the patients had been treated with tamoxifen at the time of biopsy, whereas all the others had had no endocrine treatment. Of the tumours studied, 72 were primaries, 4 regional lymph node metastases removed at the primary operation, 26 were locoregional recurrences, and 3 were distant metastases. The follow up period (from 2 to 6 months) was too short to permit evaluation of post-biopsy treatment results. Therefore, no correlations to clinical responses to hormone treatment are reported in this study.

Light microscopical classification

Of the 72 primary tumours 55 were infiltrating ductal carcinomas. Three of the female ductal carcinomas were WHO grade 1, 43 were WHO grade 2 and 7 were WHO grade 3. Ten carcinomas were variants of infiltrating lobular type, and 5 were more rare types (apocrine carcinoma, medullary carcinoma and tubular carcinoma). In 2 cases we were unable to classify the tumours neither as ductal nor lobular.

Immunocytochemical results (ER-ICA)

In 72 cases, cells binding the anti-ER antibodies were detected, whereas 30 tumours (12 from premenopausal women and 18 from postmenopausal women) were not stained at all with the antibody (Fig. 1). The immunoreactivity was located over the nuclei and not in the cytoplasm. The tumours showed considerable heterogeneity with respect to ER content. Thus, the proportion of immunoreactive neoplastic cells varied widely among the different ER positive tumours. In some cases only a small percentage of the tumor cells were immunostained, while in others almost all cells were stained. Also, the staining intensity of positive cells varied from cell to cell even within the same tumour. Well differentiated carcinomas contained, in general, a greater proportion of immunoreactive cells than poorly differentiated ones.

Of the premenopausal women, about 1/3 had strongly

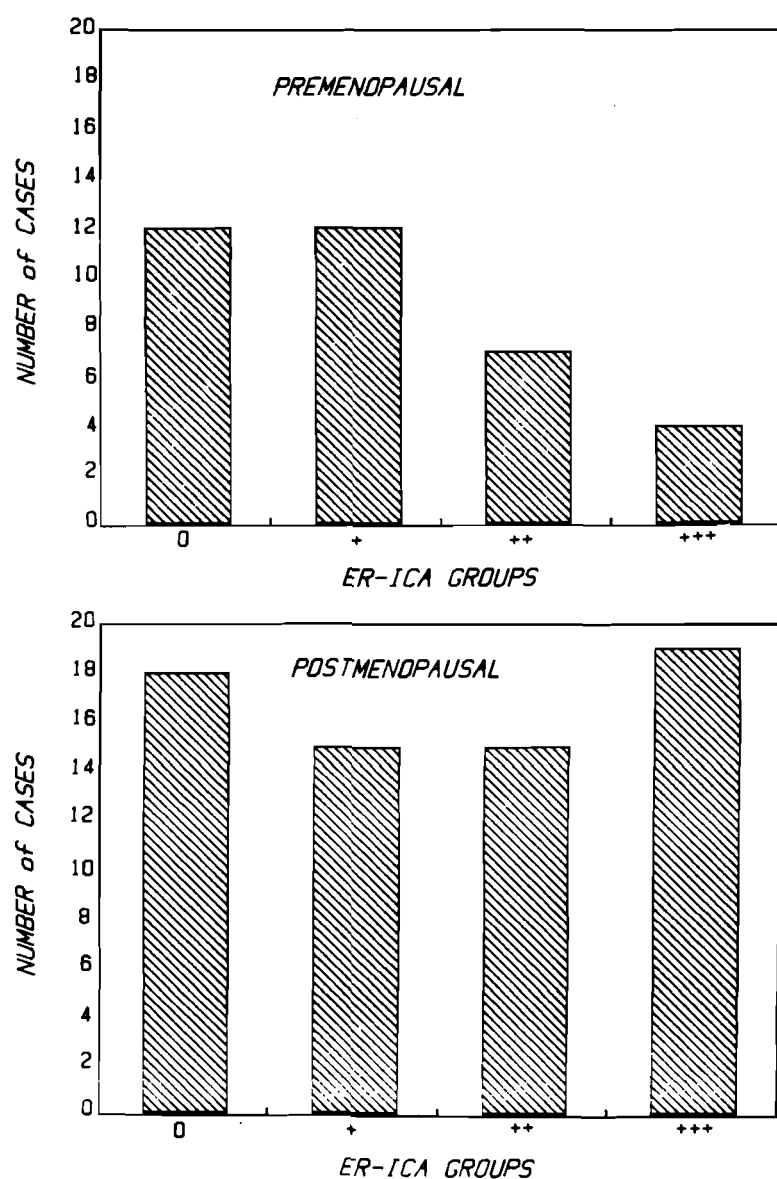
Table 1. Relationship in primary ductal carcinomas between ER-ICA content and histological grade.

ER-ICA-content*	Histological grade (WHO)			All
	1	2	3	
Premenopausal				
0 (-)	0	5	1	6
1 (+)	0	8	1	9
2 (++)	0	5	0	5
3 (++++)	0	0	0	0
All	0	18	2	20
Postmenopausal				
0	0	5	4	9
1 (+)	1	7	0	8
2 (++)	1	5	1	7
3 (++++)	1	8	0	9
All	3	25	5	33

* Notation see text

Table 2. ER content measured by the immunocytochemical method and the steroid binding assay.

ER-ICA-content	ER content in the DCC assay (pmol/g cytosol protein)			All
	0 - $\leq$ 10	10 - $\leq$ 100	>100	
0 (-)	21	5	2	28
1 (+)	17	9	2	28
2 (++)	6	13	2	21
3 (+++)	3	6	12	21
All	47	33	18	98

**Fig. 1.** Distribution in various ER-ICA groups of 102 breast cancers from pre- and postmenopausal women.

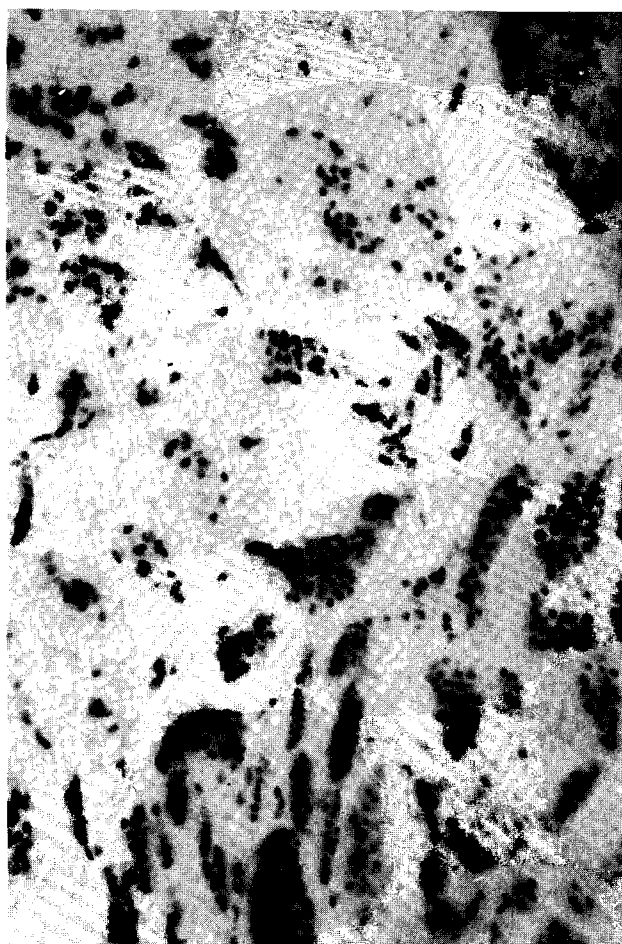


Fig. 2. *Infiltrating ductal carcinoma:* Abundant ER-ICA positive tumour cells are arranged in trabeculae and small groups. $\times 280$

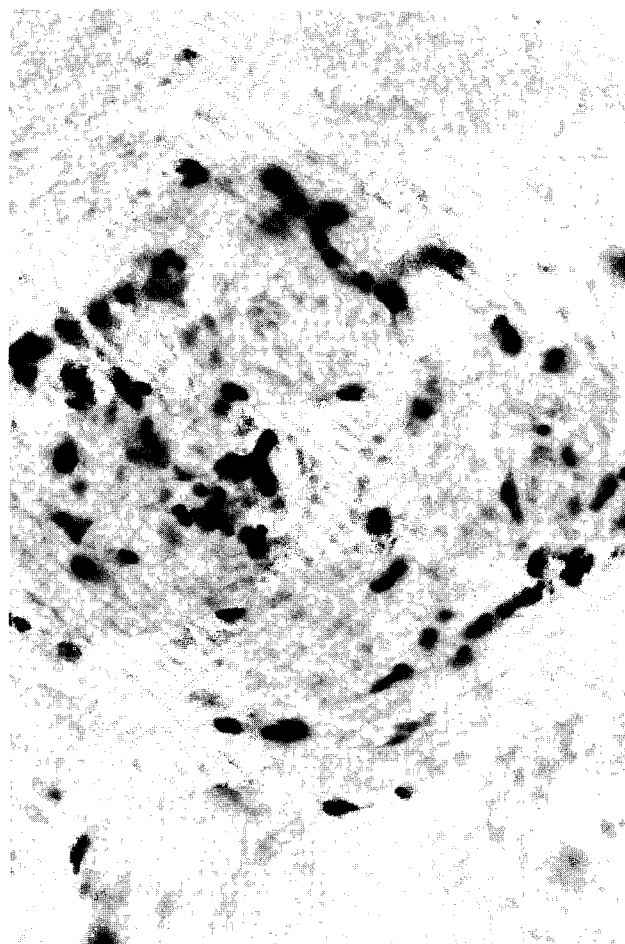


Fig. 3. *Infiltrating lobular carcinoma:* Tumour cells with uneven ER-ICA staining intensity. $\times 560$

positive tumours, 1/3 had weakly positive tumours and 1/3 had ER-ICA negative tumours (Fig. 1). Of the postmenopausal women approximately 1/2 had strongly positive tumours, 1/4 had weakly positive tumours and 1/4 had ER-ICA negative tumours.

The 3 male breast carcinomas were all oestrogen receptor positive (2 weakly positive and 1 strongly positive).

In the positive tumors, the proportion of stained tumour cells varied considerably between individual cells (Figs. 2, 3). In a few cases an additional weak diffuse cytoplasmic staining was present in scattered cells.

Correlation to WHO grade

The majority of the tumours examined were ductal carcinomas. For this group the results of the ER-ICA tests were correlated with the WHO grading (Table 1). WHO grade 2 tumours were predominant, both in pre- and postmenopausal women. No grade 1 tumours were seen in the premenopausal group. Most of the cases in the premenopausal carcinomas of grades 2 and 3 were weakly stained (15 of 20 were in ER-ICA groups 0 and 1).

In the postmenopausal group three tumours were of WHO grade 1 and 25 of grade 2 (Table 1). These tumours were more evenly distributed with respect to ER content. Four of the 5 postmenopausal women with WHO grade 3 tumours had ER- tumours.

Correlation between ER-ICA and DCC steroid binding

Material for steroid binding analysis was not available in 7 cases. The ER content of 98 tumours assayed by the immunocytochemical method and the relationship to the receptor-levels determined by the DCC steroid binding assay are shown in Table 2. It is seen that 28 specimens were ER-, 28 were weakly positive (+), 21 moderately positive (++) and 21 strongly positive (+++). By and large the results obtained with the ER-ICA test corresponded well with the DCC steroid binding assay (Table 2). However, of 47 tumours that were classified as negative by the steroid-binding assay ($0 < 10$ pmol/g cytosol protein), 26 showed varying degrees of immunoreactivity. In 5 of the 47 cases the progesterone receptor assay was positive (not shown). Conversely, of the 18 patients with biochemical values above 100 pmol/mg cytosol protein, 2 were ER-ICA negative and 2 were only weakly positive (+).

Discussion

The present study of oestrogen receptors in 105 breast carcinomas shows that immunoreactive cells (ER-ICA positive cells) were present in about 2/3 of the tumours analyzed. DeSombre and co-workers (1986) reported in 126 biopsies from patients with breast cancer an excellent agreement between the ER-ICA method and the conventional steroid binding assays. Also in our study there was by and large good agreement between the two assays. In most cases, tumours with immunoreactive cells were found to be ER+ also in the DCC steroid binding assay (44 cases), whereas tumours with no immunoreactive cells were ER- in the DCC steroid binding assay (21 cases). However, important discrepancies were found. This, in 7 tumours that were ER+ by the DCC method, no immunoreactive cells were found with the ER-ICA test. Conversely, in 26 cases immunoreactivity was found in spite of a negative DCC analysis.

There are several possible explanations of the observed discrepancies between the results obtained by the two methods for measuring oestrogen receptors. First *different parameters* are analyzed by the two methods. Thus, the steroid binding assay measures the ability of molecules in the cytosol to bind radioactive oestradiol, whereas the ER-ICA test measures the presence of the antigen in the nucleus as defined by the binding of the antibody. The binding of oestradiol to the cytosolic oestrogen receptor is unstable and sensitive to temperature and other assay conditions. Moreover, it is inhibited by anti-oestrogen (tamoxifen). Hence, when patients are on tamoxifen treatment, false negative receptor results may be obtained, a fact which limits the value of the steroid binding assay. In contrast, the ER-ICA test recognizes oestrogen receptor molecules whether or not they are blocked by oestrogens or anti-oestrogens (Heubner et al., 1986). The antibodies used here have been shown to be specific for the oestrogen receptor protein (Greene et al., 1984; King and Greene, 1984; King et al., 1985).

The *sampling* of the tumor material may explain part of the discrepancies between the ER-ICA test and the DCC steroid binding assay. In the DCC assay, all parts of the tumor tissue are homogenized and the assay measures the mean receptor content of the whole specimen. In breast carcinomas normal cells of different categories may contribute substantially to the receptor values measured. In contrast, in the ER-ICA method, as performed in the present study, it is possible to study the receptor content specifically in the malignant cells. Also, individual cells are studied, permitting evaluation of the receptor heterogeneity within each tumour.

In the present study, the intensity of nuclear staining varied within the same tumour. High DCC values for oestrogen receptor were found to be associated with either a moderate to high number of strongly immunoreactive tumour cells or with a large number of more weakly stained tumour cells (not shown). Other workers have also graded the staining intensity and correlated these values to steroid binding oestrogen

receptor values (Ozzello et al., 1976; Charpin et al., 1986; McCarty et al., 1986; Heubner et al., 1986) and concluded that the ER-ICA test provides information complementary to the standard steroid binding assay. Clearly, in addition to the proportion of positively stained cells a rough quantitation of the staining intensity of the cells may give useful information. However, in our experience immunocytochemical staining intensity should be interpreted with caution. Even if a standardized procedure is used, small variations in the individual steps in the procedure may influence the staining intensity.

The clinical value of the ER-ICA test is presently not clear. The predictivity with respect to clinical response will depend on the setting of the cut off point. Most likely, a small fraction of positively stained tumour cells is not sufficient to give an objective clinical response to endocrine treatment. If the cut of points is set to 33% positively stained cells, 43% of the tumours in the present study would be considered receptor positive, an incidence that corresponds well with the response rate to endocrine treatment. To correctly assess the clinical value of the ER-ICA it is necessary to directly correlate immunocytochemical results and clinical responses to endocrine treatment.

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