

# Demonstration of progesterone receptors in paraffin wax sections of breast carcinoma

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## Abstract

Several immunohistological methods for the demonstration of progesterone receptors were tried on routinely processed paraffin wax sections of breast carcinoma, using Abbott's PgR-ICA monoclonal antibody. The best results were obtained with the avidin-biotin-immunoperoxidase complex method with no prior trypsinisation or DNase digestion, and with imidazole added to the final diaminobenzidine developing solution. A simple semiquantitative scoring system was used to assess the staining results which were then compared with the results obtained by a standard dextran-coated charcoal biochemical assay. Of 31 cases examined, the results of the two methods were concordant in 25 (81%) of cases. This is near the higher end of the concordance range obtained by several other authors using frozen sections. The discordance encountered in a few cases was possibly the result of sampling errors which are more likely to occur with the chemical rather than the histological method.

It is concluded that the method described here is fairly reliable and would greatly simplify the process of assessment of progesterone receptors in breast, and possibly other tumours.

A monoclonal antibody for the demonstration of progesterone receptors in frozen tissue sections has recently become available in this country. Using this antibody and sections of breast carcinoma with known progesterone receptor concentrations, we experimented with several immunohistological techniques in an attempt to find a method that could clearly show the receptors in routinely processed paraffin wax sections.

## Methods

The study was carried out on cases of breast carcinoma with known progesterone receptor concentrations, as estimated by a standard dextran-coated charcoal biochemical assay.<sup>1</sup> The specific antibody used for immunohistology was the Abbott PgR-ICA monoclonal (Abbott laboratories, Chicago, Illinois). A representative section was chosen from each case, and new sections, 5 µm thick, were cut from the corresponding formalin fixed, paraffin wax embedded block of tumour tis-

sue. Sections were then kept overnight in an incubator at 37°C. In some cases tumour tissue stored at -70°C was available. Frozen sections, 8 µm thick, were cut from these tumours and used for comparison.

In the preliminary part of the study, paraffin wax sections of two progesterone receptor strongly positive tumours and one progesterone receptor negative tumour were used. Six sections from each case were investigated. Two were pretreated with trypsin, two with DNase,<sup>2</sup> and two were not pretreated. One section from each group was used for the demonstration of progesterone receptors by the peroxidase-antiperoxidase (PAP) method, and the other section was used for the demonstration of the receptor by the avidin-biotin-peroxidase complex (ABC) technique. The ABC technique, with no pretreatment, was the only method that gave good results that could be clearly visualised and was adopted for the rest of the investigation.

In the next phase of the investigation 10 cases of breast carcinoma were studied. These included five progesterone receptor positive and five negative cases, assayed biochemically. Three paraffin wax sections from each case were studied. The receptors were investigated in two sections by the ABC technique, but the final staining result was developed in one by diaminobenzidine (DAB) and in the other by a mixture of DAB and imidazole.<sup>3</sup> The third section was used as a negative control.

Briefly, the sections were dewaxed by placing the slides in two changes of xylene, for two minutes each, and then hydrated through graded alcohols. Endogenous peroxidase activity was blocked by applying 3% hydrogen peroxide in methanol for 30 minutes. After rinsing in three changes for five minutes each of 0.2M TRIS-buffered-saline (TBS), pH 7.6, the sections were covered with normal goat serum for 30 minutes. This was then tipped off and the sections incubated with a few drops of PgR-ICA monoclonal antibody for 16-20 hours at 4°C. After rinsing in three changes of TBS for five minutes each sections were covered for two hours by biotinylated anti-rat IgG (Vector Laboratories), diluted 1/100 in TBS. Sections were then rinsed in TBS as before and incubated for two hours with avidin-biotin complex (Dakopatts). After rinsing in TBS, the sites of peroxidase activity were visualised by incubating the sections in 0.05% DAB (Sigma) and 0.01% hydrogen peroxide, either with or without added 0.05% imidazole, for six and 15 minutes, respec-

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tively. Sections were then washed in tap water for two minutes, lightly counterstained with Cole's haematoxylin, dehydrated in alcohol and xylene, and mounted with Permount. The control slides were treated in the same way except for replacement of the specific antibody with the negative control antibody supplied with the PgR-ICA kit.

As the addition of imidazole to the developing solution considerably enhanced the staining intensity without increasing the percentage of positive cells (see below), paraffin wax sections of 21 more cases and frozen sections of three cases were stained by the ABC technique and developed using the DAB/imidazole mixture. Two frozen sections were cut from each case at  $-20^{\circ}\text{C}$  and mounted on slides coated with poly-L-lysine to ensure good adhesion.<sup>4</sup> One slide was fixed in acetone and the other in buffered formalin for 15 minutes and then washed in TBS. All other steps were identical with those applied to paraffin wax sections.

The staining results were assessed semi-quantitatively according to the percentage of cells stained and the intensity of the staining, as described previously.<sup>2</sup> A scale of 1–3 was used for each of these components. The resulting two figures were multiplied by each other, and the final result was expressed as follows: negative (– no staining); weakly positive (+ total score of 1–3); moderately positive (++ score 4–6); and strongly positive (+++ score >6). Scoring was carried out by two independent observers without knowledge of the biochemical results. Differences between the two observers were solved by re-examining the case jointly with a multiheaded microscope in the presence of a third observer.

As is customary, a biochemical assay result was considered negative when the progester-

one receptor concentration was less than 10 fmol/mg cytosol protein, and positive when it was 10 fmol or more.

## Results

The deep brown positive staining for progesterone receptors was mostly seen in the nuclei of tumour cells. In some cases a weak cytoplasmic staining and a variable degree of stromal staining were also present. These were noted in treated and untreated imidazole sections, as well as, to a lesser extent, in the few frozen sections examined. The extranuclear staining did not interfere with the interpretation of the results which were assessed on the basis of the nuclear staining of neoplastic cells only. Many non-neoplastic ducts and acini contained a variable number of cells with positively stained nuclei, and staining was sometimes noted in secretions present in the lumina of normal ducts, but these were not included in the assessment procedure.

The percentages and distribution of stained cells were similar in treated and untreated paraffin wax sections, as well as in the corresponding frozen sections examined. The intensity of staining was higher in sections treated with imidazole than in those not treated, but was highest in frozen sections. Interpretation of the results, however, was easier in paraffin wax sections because of their better quality.

In many strongly positive cases the intensity of the staining and the number of stained cells were much higher at the invading peripheral part of the tumour than at its central, usually less cellular, and more fibrotic part. This increased positivity was not related to the site of the peripheral part of the tumour in the section. In other words it was not the artefactual heavy staining which is sometimes seen in the periphery of sections stained with immunoperoxidase techniques.

The detailed immunohistological and biochemical results are shown in the table, and examples of positively stained cases are shown in figs 1 and 2.

Of the 10 biochemically negative cases, five (50%) were immunohistologically negative, four weakly positive, and one moderately

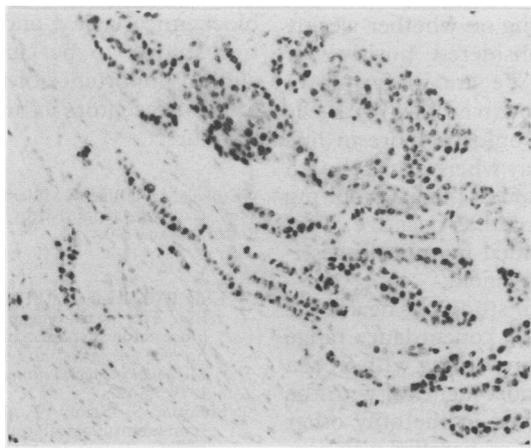
Details of biochemical and immunohistological results

Case No	Biochemical assay (fmol/mg cytosol protein)	Immunohistology		Size (cm)
		Grade	Score	
1	0	–	0	
2	0	–	0	1.5
3	0	–	0	
4	0	–	0	
5	0	+	2 (1 × 2)	3.0
6	0	+	3 (1 × 3)	2.5
7	0	++	4 (2 × 2)	0.9
8	4	–	0	
9	6	+	2 (2 × 1)	1.3
10	9	+	1 (1 × 1)	2.0
11	11	+++	9 (3 × 3)	1.0
12	17	+	2 (1 × 2)	
13	25	+	2 (2 × 1)	1.5
14	27	++	4 (2 × 2)	2.5
15	35	++	4 (2 × 2)	2.5
16	35	++	6 (3 × 2)	3.5
17	35	+++	9 (3 × 3)	2.0
18	36	+++	9 (3 × 3)	
19	43	–	0	0.9
20	43	+++	9 (3 × 3)	
21	45	–	0	
22	48	+++	9 (3 × 3)	
23	51	++	4 (2 × 2)	
24	92	+	1 (1 × 1)	
25	138	+++	9 (3 × 3)	
26	153	+++	9 (3 × 3)	1.0
27	174	+++	9 (3 × 3)	2.0
28	298	++	6 (3 × 2)	2.5
29	324	+++	9 (3 × 3)	3.0
30	555	+++	9 (3 × 3)	1.5
31	6508	+++	9 (3 × 3)	



Figure 1 Progesterone receptor strongly positive intraduct cribriform carcinoma (ABC).

Figure 2 Progesterone receptor positive invasive ductal carcinoma (ABC).



positive; while of the 21 biochemically positive cases, 19 (90%) were immunohistologically positive (three weakly, five moderately, and 11 strongly) and two were negative. Thus there was an overall concordance of 24 out of 31 cases 77%.

If the immunohistologically weak positive cases are considered with the negative ones, the histological and biochemical results would agree in nine (90%) out of 10 negative cases, and in 16 (76%) out of 21 positive cases, with an overall concordance of 81% (25 out of 31 cases).

The only case which was biochemically progesterone receptor negative but showed more than weak immunohistological staining (case 7) was a small, 0.9 cm in maximum diameter, moderately differentiated invasive ductal tumour which had a biochemically estimated oestrogen receptor concentration of 21 fmol/mg cytosol protein (table).

The two biochemically positive but histologically negative cases (cases 19 and 21) had progesterone receptor concentrations of 43 and 45 fmol/mg cytosol protein, and oestrogen receptor concentrations of 29 and 0 fmol, respectively (table). They were both moderately differentiated invasive ductal carcinomas. One measured 0.9 cm in maximum dimension while the size of the other was not determined. The only immunohistological staining seen in the first case was a weak cytoplasmic staining, while the second case showed a moderate degree of staining in the fibrous tissue around the tumour as well as in luminal secretions within normal ducts.

The three biochemically positive but histologically only weakly positive cases (cases 12, 13 and 24) had progesterone receptor concentrations of 17, 25, and 92 fmol, and oestrogen receptor concentrations of 26, 50, and 12 fmol, respectively (table). They were all invasive ductal carcinomas which showed a moderate degree of differentiation.

Case 11, which had a progesterone receptor concentration of only 11 fmol/mg cytosol protein, had a central part which was almost completely negatively stained and a more peripheral part which was very strongly stained. The tumours in cases 17 and 18, which had relatively low concentrations of progesterone receptors also had weakly stained centres and strongly stained peripheral areas.

## Discussion

Although the clinical need for finding out the steroid receptor state of breast tumours has recently been questioned,<sup>5</sup> there are still many who believe in the importance of knowing this for planning the patients' management.<sup>6,7</sup> Even those who doubt the clinical usefulness of receptor estimation accept that it is important for studying the biology of breast cancer and for the evaluation of new therapeutic agents and assessing the results of clinical trials.<sup>5</sup>

Not only is there still a need for receptor estimation, but there is also a need for an alternative method that can be easily and more widely used on routinely processed tumour tissue sections. This would expand the range of the tumours examined and the centres in which such an examination is carried out, thus providing a more accurate picture that does not exclude small or unexpected tumours from which no fresh tissue would be available for a biochemical assay. It would also be more preferable to have a method that can work on paraffin wax sections, as frozen sections in many British centres are hardly carried out routinely now because of the advent of fine needle aspiration cytology and limited surgical intervention. Paraffin wax sections also have the added advantages of better quality and availability for retrospective studies.

To the best of our knowledge, there has been only one detailed published article dealing with the demonstration of progesterone receptors in paraffin wax sections of breast carcinoma, using a different antibody (LET 126, Transbio, Paris, France) and the PAP technique.<sup>8</sup> There has also been a short communication in which Abbott's PgR-ICA monoclonal and the ABC technique were used,<sup>9</sup> and a more recent article dealing with the demonstration of progesterone receptors in paraffin wax sections of endometrial adenocarcinoma and hyperplasia using the same monoclonal antibody and the PAP method.<sup>10</sup>

In this study we compared several immunohistological techniques and reached the conclusion that the best method, for the specific antibody used, is the avidin-biotin-immunoperoxidase complex method with no prior trypsinisation or DNase digestive step, and with imidazole added to the final DAB developing solution to enhance the staining result. Although some cases showed weak cytoplasmic or stromal staining, these were also observed in the sections which were not treated with imidazole and, to a lesser extent, in the few frozen sections studied. The clinical importance of this extra-nuclear and extra-epithelial staining is not obvious, but both have previously been noted by other authors using frozen sections and similar<sup>11</sup> or different progesterone receptor monoclonal antibodies.<sup>12</sup> In our study such staining was considered non-specific when the results were interpreted.

The simple scoring system which we used for interpreting the results was easily carried out and gave consistent results that correlated reasonably well with the results of the biochemical assay. The concordance between the biochemical and histological results varied be-

tween 77–81%, depending on whether weakly stained cases were considered positive or negative, respectively. We are in favour of including the weakly positive cases with the negative ones, as that would be more in line with the biochemical assay where all cases with receptor concentrations of less than 10 fmol/mg cytosol protein are regarded as negative. This has also been recommended by other investigators working in the same field.<sup>13–15</sup>

The concordance in our study was nearer the higher end of the 54–92% concordance range reported by other authors working with frozen sections,<sup>11–16</sup> and was almost identical with the 80% concordance reported in the only other published detailed study of paraffin wax sections in which a different antibody was used.<sup>8</sup> This consistency confirms the reliability of the method used in this study and also shows that there are always a few cases where the biochemical and histological methods give discordant results. The cause of this discordance is not easy to decide and may vary from one case to another. It seems that in some cases, however, such as very small and very large tumours, sampling may be the cause. In the former, inclusion of non-neoplastic tissue, which may be progesterone receptor positive or negative, might have an effect on the result of the biochemical assay; and in large tumours the receptor state may vary from one part of the lesion to another. In this respect we have frequently noted a stronger progesterone receptor staining at the peripheral part of some tumours compared with their central more fibrotic parts.

It is concluded that the immunohistological method described here is fairly reliable for the demonstration of progesterone receptors in routinely processed paraffin wax sections of breast tissue. There is a concordance of around 80% between the results obtained by this method and those obtained by the dextran-coated charcoal biochemical assay. In most of the few cases where discordant results are obtained the cause could be attributed to a sampling error which is more likely to affect the biochemical rather than the histological assay. The availability of such a histological method would simplify progesterone receptor estimation, obviate the necessity to remove a large part of the tumour in the fresh state for the

biochemical assay, and allow large retrospective studies to be carried out to assess the clinical importance of the presence or absence of these receptors in breast, and possibly other tumours.

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