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# Multiple Microsample Analysis of Intratumor Estrogen Receptor Distribution in Breast Cancers by a Combined Biochemical/Immunohistochemical Method*

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**Abstract**—*A multiple microsample analysis of estrogen receptors (ER) was performed on 81 samples obtained from 21 human breast cancers using a biochemical (BC) and immunohistochemical (IHC) method. Qualitative agreement occurred in 96% of the cases. Using both the percentage of cells staining specifically for ER as well as the intensity of staining (Histoscore) a semiquantitative correlation between the two analyses was obtained. Large variations in cellularity and the percentage of cells that stained specifically for ER existed within individual tumors. In most cases the variation in intensity of staining was minimal. Both the level and distribution of ER in different areas of individual tumors were calculated using a correction for differences in cellularity amongst samples. Heterogeneity in ER level as well as distribution was found in some tumors. In addition, a "checkerboard" type of staining with intermixed ER positive and ER negative cells was observed. This combined BC/IHC method may provide specific information about intratumor ER heterogeneity not available from either method alone.*

## INTRODUCTION

With the development of monoclonal antibodies directed against ER an IHC approach to receptor detection has become commercially available (Abbott Laboratories, International). Several studies have indicated that a useful correlation can be obtained between biochemical (BC-ER) levels obtained from a large sample of a tumor and the ER staining patterns of sections of the same tumor [1–9]. Others, however, have not been able to demonstrate such a correlation [10, 11]. One reason for this discrepancy may be that some tumors are heterogeneous entities with variations in cellularity, ER level and distribution in different areas [12, 13].

In a previous publication [13] we have shown, using a biochemical microsample technique, that 40% of breast tumors showed extreme heterogeneity in ER levels from different areas even when corrected for differences in carcinoma content between samples. Thus, in heterogeneous tumors, a single ER determination with a large piece of tumor may not provide a meaningful picture of the ER status.

The purpose of this study was two-fold: (1) to determine the correlation between BC-ER and IHC-ER using microsamples in such a way as to eliminate variations in cellularity and (2) to use this information to determine ER level and distribution in different areas of individual tumors. Such data allow the comparison of ER heterogeneity within individual tumors by two methods. The biochemical method measures estrogen binding to unoccupied receptors, the immunohistochemical method measures the presence of the receptor itself. To make this comparison possible we obtained four microsamples from each of 21 tumors and analyzed each microsample for ER by both the BC and the IHC method.

## MATERIALS AND METHODS

Surgical breast cancer specimens were obtained from the operating room immediately after excision

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and frozen at −70°C. Whenever possible, four microsamples (13 × 1.5 × 1.5 mm) were cut from each frozen tumor specimen as far apart as possible. While frozen, each microsample was divided longitudinally into two "sisterhalves" so that comparative analyses were performed on tissues no more than 750 μm apart. One half was biochemically analyzed for ER using the micromethod [13, 14], while the other half was mounted longitudinally on a cryostat specimen block and kept at −70°C. This latter sample was analyzed for the percentage of carcinoma per sample (PCS) and also used for the IHC analysis of ER.

*Biochemical ER analysis*

The BC-ER method has been described elsewhere in detail [13–15]. Briefly, each strip to be analyzed for ER was cut into small pieces and homogenized in 500 μl of TED buffer [tris(hydroxy methyl)-methylamine], 1,4-dithiothreitol, ethylenediaminetetraacetate (10/0.5/1.0 mmol/l., pH 7.5) using a Potter–Elvehjem glass–Teflon tissue grinder. The resulting homogenate was centrifuged in a Sorvall RC2-B refrigerated centrifuge at 50,000 ***g*** for 0.5 h at 4°C. Each sample yielded approx. 300 μl of clear supernatant. Two 50 μl aliquots were incubated for 0.5 h with 2,4,6,7-[$^3$H](N) estradiol (New England Nuclear, Boston, sp. act. 85–105 kCi/mol) in TED buffer to give a final concentration of $3 \times 10^{-10}$ mol/l, a concentration that is saturating ER levels at the lower end of the scale, but becomes progressively less saturating as the ER level increases [14]. One tube was warmed to 56° C for 30 min prior to incubation with tritiated estradiol to serve as a control. Each aliquot was then electrophoresed using the CAGE method [14]. One hundred μl of each supernatant was used for protein determination according to the method of Lowry *et al.* [16].

For biochemical analysis the correction of measured ER values on the basis of PCS was performed according to the equation:

$$\text{Corrected ER} = \frac{\text{Measured ER} \times 100}{\text{PCS}}.$$

The PCS was estimated by four observers (one of whom was a pathologist) working independently. PCS was expressed as the percentage of the microsample that would be occupied if all viable carcinoma could be consolidated into a single homogeneous area of the longitudinal section. When disagreement occurred between observers slides were re-evaluated and a value assigned by mutual agreement.

Arbitrarily, the BC-ER cutoff point for intermediate vs. high receptor level was set at 100 fmol/mg of tumor protein and a level < 1 fmol/mg of tumor protein was considered negative as previously utilized [13, 15].

*Immunohistochemical analysis of ER*

The IHC detection of ER was performed as described in the Abbott ER-ICA monoclonal antibody kit (Abbott Laboratories International North Chicago, IL 60064).

The frozen "sisterhalf" of each biochemical portion was cut on the cryostat into 6 μm sections for the estimation of the PCS and for the IHC-ER detection. Care was taken to cut the sections longitudinally so that the full section (13 × 1.5 mm) could be analyzed. Sections were mounted on glass slides coated with tissue adhesive fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by cold methanol and acetone (−10°C) for 4 and 2 min, respectively. When not processed immediately sections were stored in a special storage medium (42.8 g sucrose, 0.33 g anhydrous $MgCl_2$, 250 ml glycerol in 250 ml of PBS at −10°C to −20°C).

All the following incubations were performed at room temperature (RT) in a humidified chamber. Sections were first incubated for 15 min with normal goat serum in order to reduce nonspecific binding of subsequent reagents. Sections were then incubated successively with monoclonal rat anti-ER antibodies, goat anti-rat IgG serum and rat horseradish peroxidase : anti-horseradish peroxidase (PAP) complexes. Each antibody incubation was for 30 min and was followed by two successive 5 min washes in PBS. After the final wash sections were incubated for 6 min with 1.7 mmol/l. of diaminobenzidene (DAB) and 0.06% $H_2O_2$ in PBS. Next the sections were rinsed in distilled water for 5 min, stained lightly with hematoxylin, washed for 5 min in running tap water and dehydrated through graded ethanols and xylene and finally mounted. Sections that were incubated with normal rat IgG in place of monoclonal anti-ER antibodies were used as controls.

*Histological evaluation*

The percentage of carcinoma cells that stained specifically for ER was analyzed by two observers working independently. This assessment was made by estimating the average percentage of tumor cell nuclei that stained specifically in each section when scanned lengthwise from one end to the other.

Like the biochemical ER evaluations, the values for the percentage of cells staining were estimated as if the whole microsample consisted entirely of carcinoma cells. For example, 20% cells staining meant that 20% of the whole microsample was stained. This was done to optimize a comparison between the BC-ER and IHC-ER methods.

The IHC staining was evaluated using a double grading system [10] in which the percentage of stained cells as well as the intensity of specific staining was assigned a value. For the percentage of

*Table 1. Comparison of qualitative ER detection using the BC and IHC methods*

| | Number of cases (%) | | |
|---|---|---|---|
| | BC-ER positive | BC-ER negative | Total |
| IHC-ER positive | 55 (68) | 2 (2) | 57 (70) |
| IHC-ER negative | 1 (1) | 23 (29) | 24 (30) |
| Total | 56 (69) | 25 (31) | 81 (100) |

staining 1 = 1–10%, 2 = 11–20%, 3 = 21–30%, 4 = 31–40%, 5 = 41–50%, 6 = 51–60%, 7 = 61–70%, 8 = 71–80%, 9 = 81–90%, 10 = 91–100%. For the intensity of staining the numbers 0, 1, 2, 3 were used to define absent, low, moderate and high staining, respectively. For each microsample the number derived from staining intensity was added to the number obtained for the percentage of specific staining [10]. The sum of these two values was called the "Histoscore", which was then compared to the corrected ER level obtained from the sisterhalf.

Arbitrarily a Histoscore range from 1 to 8 was considered intermediate, and ≥ 9 was considered high for ER, so that an equal number of cases would be categorized as high by both the BC-ER and IHC-ER method. When no specific staining was observed in the sample it was classifed as negative.

*Specific staining patterns*

The pattern of stain distribution was expressed as all positive, all negative or intermediate. This last category was used when mixtures of positive and negative cells occurred within the same tumor or when all cells stained with only a low/moderate intensity. The intermediate staining was subdivided into three types: Type I (focal staining), Type II (field staining) and Type III (checkerboard staining) (Fig. 4).

## RESULTS

In the series of 21 tumors, 19 were classified as ductal carcinomas while two were identified as lobular carcinomas. A total of 81 microsamples was obtained and analyzed for ER by both BC and IHC methods. Three microsamples were not available for analysis.

Table 1 shows that there was complete qualitative agreement in 78 of the 81 microsamples, with 55 (68%) of the samples measuring positive and 23 (29%) measuring negative by both methods. In Fig. 1 the data are presented with three categories of ER level: absent, intermediate and high. Twenty-two (27%) of the samples measured high, 19 (23%) measured intermediate and 23 (28%) were absent by both methods. In 14 of the remaining 17 samples

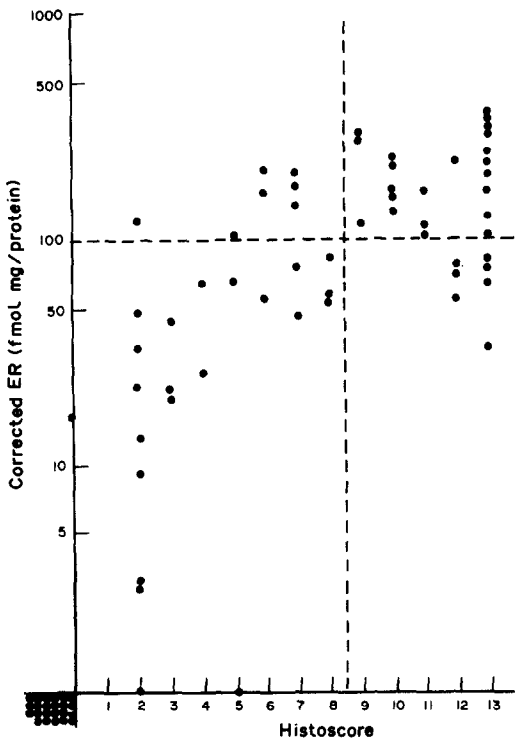


*Fig. 1. Comparison of ER levels in 81 microsamples obtained from 21 tumors using the BC and IHC method on "sisterhalves". The dotted lines mark the arbitrary cutoff point between intermediate and high levels. Points plotted outside the graph represent 0 values.*

there was only some degree of quantitative but not qualitative disagreement between the two methods. The overall correlation coefficient was 0.75. Figure 1 also indicates that there was only a weak correlation between the two methods when the BC-ER level was between 1 and 10 fmol/mg tumor protein.

Figures 2a, b and c summarize the results of assessments which characterize intratumor variations in PCS, percentage specific ER staining and the intensity of staining between microsamples for 21 breast tumors.

Figure 2a shows that more than half of the tumors had variations in carcinoma content of 20% or larger with microsamples taken from different regions of the tumor. In one patient (TD) the large variation was due to the presence of trace amounts of carcinoma in one microsample and an average amount (50%) of carcinoma in the other samples.

Figure 2b indicates that the percentage of specific ER staining can vary significantly between microsamples taken from different areas of individual tumors. Seven of the 21 tumors used in this study show a 20% or greater variation between microsamples. Approximately one half of the tumors, however, did not show any obvious variation.

Figure 2c demonstrates that the variation in intensity of specific staining between microsamples of individual tumors was minimal in most cases.

Figure 3 is a graphic representation of four micro-

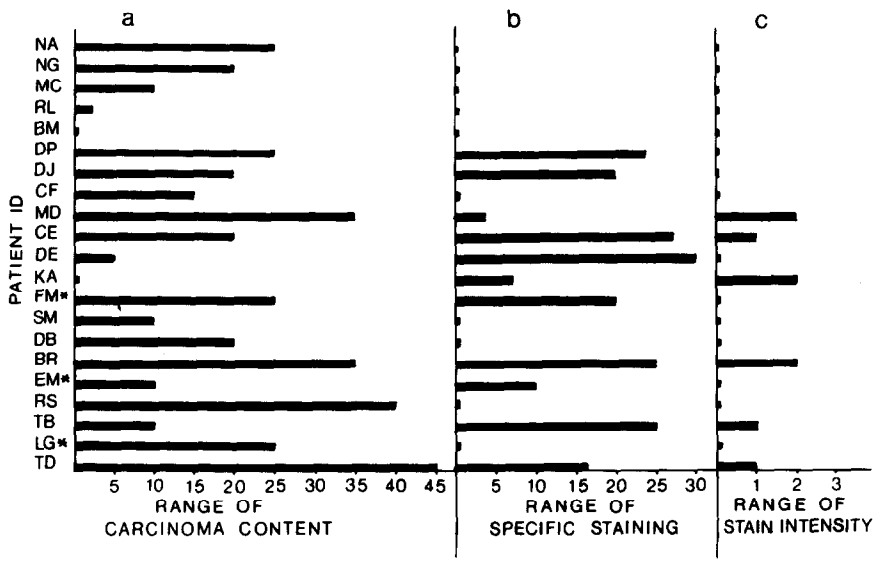


*Fig. 2. Tumors from 21 patients were analyzed for intratumor variations in: (a) carcinoma content; (b) specific staining for ER; (c) intensity of ER staining. For each tumor four microsamples were used for the analysis (only three microsamples were obtained from tumors marked with an asterisk). For a and b the range between microsamples was expressed as a percentage, for c it was expressed as a numerical value from 1 to 3, depending on the intensity of staining (see Materials and Methods section).*

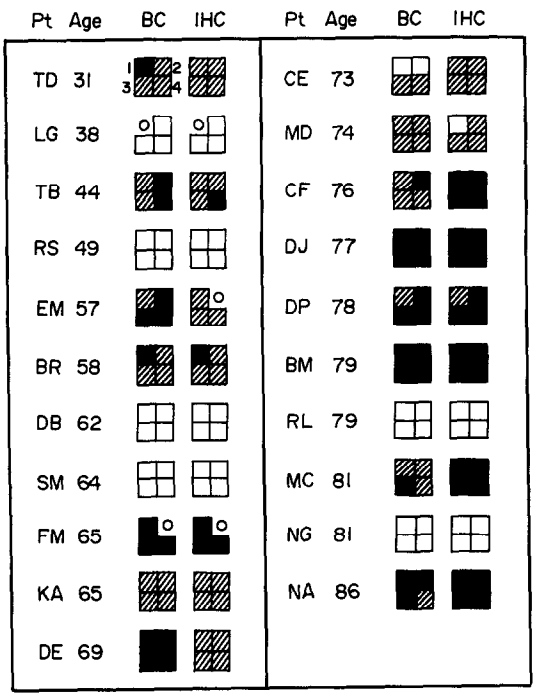


*Fig. 3. Graphic presentation of intratumor variations in ER levels and distribution of 21 breast tumors detected by the BC and IHC methods using "sisterhalves" of microsamples. Each quadrant represents a "sisterhalf" of one microsample. For each tumor the quadrants are numbered 1, 2, 3 and 4 as indicated for patient TD, where identical quadrant numbers are sisterhalves of the same microsample. ■ High level of receptors; □ intermediate level of receptors; ▨ absence of receptors; ○ sample was not analyzed.*

samples analyzed from each of 21 tumors. Each "sisterhalf" is represented by a quadrant. Qualitatively, of the 21 tumors analyzed 13 were ER positive in all four quadrants by both methods, six tumors were completely negative by both methods, and the remaining two tumors (CE and MD) expressed both positive and negative areas within the same tumor by either one or the other method.

Using three categories of the BC-ER method (absent, intermediate and high—Materials and Methods section) six tumors were judged homogeneously positive for ER and nine tumors were judged heterogeneous for ER by the BC-ER method.

The corresponding values for the IHC-ER method were 11 homogeneous positive and four heterogeneous. Three tumors (TB, BR and DP) were heterogeneous by both methods.

Excluding those tumors in which nearly all carcinoma cells stained highly positive for ER (3 patients, NA, MC, BM), Fig. 4 represents an attempt to illustrate the intermediate staining patterns of the remaining positive tumors. Four tumors expressed both a Type I and Type III staining pattern, 1 expressed a Type II staining pattern and 7 were only of Type III.

## DISCUSSION

An assessment of ER variability in different regions from 21 breast tumors was made using the microsample method previously developed [13, 15]. One major modification was incorporated into the assay, namely the histological "sisterhalf" was used for both the estimation of PCS, and the analysis of ER by anti-ER antibodies. In this way, an optimal comparison can be made between biochemical and histochemical ER data.

Data from Table 1 and Fig. 3 indicate that, while a very high qualitative correlation existed between the two methods, the quantitative agreement was, as expected, not as close but was nevertheless significant ($r = 0.75$). Such agreement is encouraging

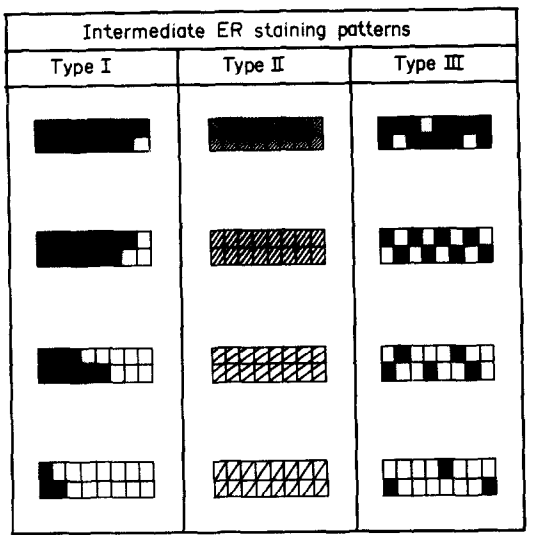


*Fig. 4. Intermediate variants of ER staining patterns observed in 21 human breast tumors. Each bar represents a graphic presentation of a group of adjacent tumor cells. ■ Cell staining positive for ER (high intensity); squares with progressively reduced shading, cells with graded intermediate staining intensity; □ cell without ER staining. Type I represents a "focal" expression of ER; Type II represents a "field" expression of ER and Type III represents a "checkerboard" expression of ER.*

in view of the fact that both methods probably measure different domains of the estrogen receptor protein.

For many tumors a single ER assessment either by the BC-ER or IHC-ER method may not give a true picture of the receptor status of the whole tumor (e.g. tumors CE and MD). This problem is particularly pronounced with the IHC-ER method since only a very thin section of the tumor is analyzed. Multiple sections taken from different areas of tumors not only would provide more definitive information about the ER status of the tumor, they may also detect ER heterogeneity within the tumor.

Heterogeneity of ER in breast tumors may be the major reason for ineffective, or partially effective, treatment [12, 17]. In addition heterogeneity may be an important factor influencing the duration of successful treatment response [12, 18, 19]. Therefore, the determination of the degree of heterogeneity may provide information that is clinically useful over and above the value of a single ER determination.

ER heterogeneity may be an important factor influencing the progression of breast cancer from the hormone-dependent to the autonomous state [20, 21]. Although such progression is evident in the clinic, there is surprisingly little evidence in human tumors that this event is associated with a loss of ER [17, 22]. In fact, there is a general tendency for ER levels to increase with age, thus clouding the picture [23]. Also, repeat biochemical ER analyses on 83 breast tumors (range 0.5–8 years between biopsies) do not indicate a general trend towards lower ER levels as tumors progress (unpublished observations). Therefore, we do not know whether the intermediate staining patterns we observed in this series (Fig. 4) in fact, represent transition stages between ER positive and ER negative tumors. Three distinct staining patterns were observed. Some degree of the Type III (checkerboard) staining was detected in 11 of the 21 breast tumors analyzed and it is possible that this type of staining reflects the random shedding of an already redundant ER system of a tumor in progression. Alternatively, it may identify some variant of breast cancer in which escape from endocrine control is not focal but occurs as a random event throughout the tumor [24].

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