Detection and Distribution of Estrogen Receptor in Human Breast Cancer by a Combined Biochemical/Immunohistochemical Multiple Microsample Assay

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ABSTRACT

The relationship between high estrogen receptor (ER) levels and the regression of tumor growth following estrogen ablative therapy is well-established in human breast cancer. Generally however, even with treatment, tumors may become ER-negative and unresponsive to further hormonal therapy. This has traditionally been explained as a clonal expansion of ER-negative cells that have a selective growth advantage over ER-positive ones in the absence of estrogen. According to this hypothesis, once such a clone is established the tumor's growth can proceed unimpeded, after which reversal to a hormone-controlled state is improbable.

Commonly, the measurement of ER for clinical screening of patients for hormone therapy is done by steroid-binding assay (SBA) which measures the uptake of radiolabelled estradiol by a single sample of tumor. However, this method measures only previously-unoccupied receptor sites. Furthermore, if the sample does not consist entirely of tumor cells and has a high proportion of connective tissue, fluid, or serum

contaminants, the quantitation of ER may be invalidated and tumors having a small proportion of highly ER-positive cells susceptible to endocrine response could remain undetected.

In order to further understanding of ER distribution in breast cancer a comparative multiple microsample assay was performed on 21 tumors. Four 13 X 1.5 X 1.5 mm (approx. 40 mg) microsamples from each tumor were divided equally and longitudinally, one half used for SBA by cold-agar gel electrophoresis (CAGE), and the other half for an estrogen receptor immunochemical assay (ER-ICA) employing monoclonal antibodies against ER.

For SBA, cytosols of microsamples were prepared by sequential homogenization and centrifugation, then incubated with tritiated 17β-estradiol. CAGE was used to separate receptor-bound hormone from unbound hormone. Uptake of estradiol was quantitated by scintillometry.

Albumin, which binds estradiol nonspecifically, and in high concentrations interferes with ER estimation, was measured by radial immunodiffusion assay.

For ER-ICA 6 μm frozen sections were incubated with monoclonal anti-human ER antibody. Following this, a peroxidase: anti-peroxidase (PAP) immunohistochemical

procedure was applied to all sections in order to localize ER-bound antibody. Each stained section was examined by four independent observers to determine per cent carcinoma (PCC) and per cent specific ER staining (PSS). SBA-derived ER concentrations were then corrected for PCC.

The proportion of tumor cells stained specifically for ER in each microsample was estimated and adjustment made for differences in staining intensity (SI). The resulting histoscore is an arbitrary value that allows these data to be ranked and compared with SBA-derived data.

Qualitatively, 68 % of all microsamples were ER-positive, and 28.5 % were negative by both methods, with an overall correlation of 0.97.

Quantitatively, there was a correlation of 0.75 between SBA-derived corrected ER and ER-ICA derived histoscores for corresponding microsamples.

The high correlation between SBA and ER-ICA data demonstrates the potential value of combined biochemical and immunohistochemical methods for screening breast cancer patients most likely to benefit from endocrine

therapy, and thus avoiding the complications associated with other forms of therapy, such as radiation and chemotherapy.

The distribution of ER within the 21 tumors studied revealed a variety of staining patterns, both homogeneous and heterogeneous, the latter demonstrating various degrees of intermixing of ER-positive and ER-negative tumor cells.

Within individual tumors heterogeneous, as opposed to homogeneous, ER distribution patterns were seen in 12 of the 15 (80 %) ER-positive tumors. The checkerboard staining pattern observed in 9 of these might represent a variant type of tumor in which ER-negative status is attained randomly throughout the tumor, rather than by means of a focal, clonal expansion of ER-negative cells. If so, it may be possible that appropriate manipulation of the cell environment could reverse the tendency for breast tumor cells to become hormone-independent.

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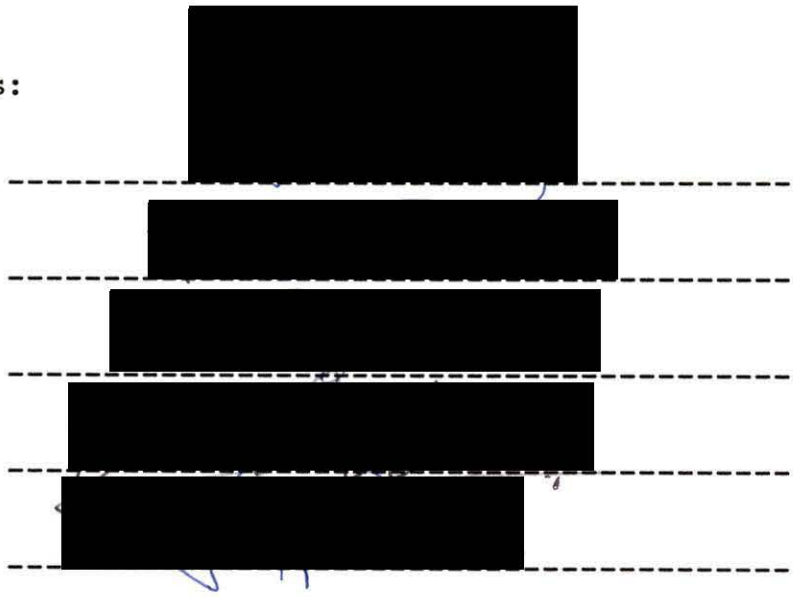

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This work is dedicated to the late Dr. F.T. (Tom) Algard, my teacher and friend, for providing me with the initial opportunity to pursue this interesting project.

I. INTRODUCTION

1. Development of the estrogen receptor model

The Scottish physician George Beatson was the first to recognize the remission of breast cancer following removal of the ovaries of some, but not all, premenopausal patients, and following removal of the adrenal glands of postmenopausal patients (Beatson, 1896). Extensive clinical experience since that time has indicated that little or no remission is seen in the majority (75-80 %) of postmenopausal patients following ovariectomy (Baker, 1977).

These findings suggested that some breast tumors grow under hormonal control, and removal of endogenous sources of estradiol deprive them of the conditions necessary to sustain growth. Therefore, a method of predicting which patients are most likely to benefit from endocrine therapy would eliminate subjecting them to unnecessary major surgery, or to therapeutic measures that have a high probability of failure.

Some normal mammalian tissues, such as uterus, ovaries and testes, require steroid hormones for their functional integrity and growth, while some do not, and thus could be regarded as the respective counterparts of hormone-dependent and hormone-independent breast tumors.

The studies of Gorski et al. (1968) and Jensen et al. (1968) indicated that steroid hormones interact with target tissues in a two-step mechanism in which the circulating hormone diffuses into the target cells from the plasma and binds first to a cytoplasmic ER. The resulting hormone-receptor complex is translocated to the nucleus where its interaction with nuclear acceptors activates RNA polymerase, and one of the resulting proteins may be progesterone receptor (PgR) (see Figure 1, page 21).

That PgR is a gene product stimulated by estrogen also has predictive value for determining the response of primary breast tumors to endocrine therapy, defined in terms of objective remissions in which the tumor decreases in size by at least 50 % (Mirecki and Jordan, 1985).

By this criterion, the average response rates are : 77 % for ER-positive, PgR-positive; 27 % for ER-positive, PgR-negative; and less than 10 % for ER-negative, PgR-negative, tumors (deSombre et al. 1979 ; Osborne et al. 1980 ; Saez et al. 1984).

The failure of some ER-positive, PgR-positive tumors to respond to endocrine therapy could be due to

false-negative PgR assays that result from the occupation of PgR by endogenous progesterone, especially during the luteal phase of the menstrual cycle of pre-menopausal patients. Correspondingly, the clinical responsiveness of apparently receptor-negative tumors has been explained by the deterioration of receptors during their preparation for assay. These limitations have, nonetheless, had no major impact on the overall predictive value of receptor assays for endocrine therapy responsiveness (Mirecki and Jordan, 1985).

Another effect of hormone-receptor binding is to initiate DNA synthesis, culminating in cell division. Thus, translocation of the hormone-receptor complex appears to convert ER to an active form that directly or indirectly initiates cell division (Jensen et al. 1968). Consistent with this hypothesis is the ability of ER to bind to uterine nuclei in vitro and to activate their RNA polymerase (deSombre et al. 1974).

Elucidation of the mechanism of specific binding of hormone to receptor began with the synthesis of radio-active estrogens, including ^{3}H-hexestrol by Glascock and Hoekstra (1959) and ^{3}H-estradiol by Jensen and Jacobson (1960). Subsequently, labelled estradiol became a useful tool for localization of ER in estrogen target tissues.

In 1961 Folca et al. reported that uptake of radiolabelled hexestrol injected into breast cancer patients prior to adrenalectomy was greater in those whose tumors later underwent remission. Tumors that did not bind labelled hormone were unresponsive to adrenalectomy (Jensen et al. 1973).

The first convincing evidence of sex steroid receptors in human breast carcinomas was reported by Jensen et al. in 1962. These findings supported the hypothesis that the ability of tumors to bind estrogen indicated hormone dependency, and therefore amenability to anti-hormone therapy, whereas those unable to bind hormone had a much less favourable outlook, since they had presumably escaped hormonal control (i.e., their growth was autonomous).

2. Problems of ER status of tumors and predictive value for response to endocrine therapy

Numerous studies have concluded that approximately one-half of malignant breast biopsies contain ER, and that 55 to 60 % of these respond to additive or ablative hormone therapy (Thompson and Lippmann,1979). The latter could be oophorectomy, adrenalectomy, or treatment with estrogen competitors such as tamoxifen.

Knight et al. (1977) summarized the use of steroid hormone receptors as a prognostic factor in primary breast cancer. In most investigations, absence of ER in tumors is associated with earlier recurrence of disease and poorer survival than in those with ER present. However, a few show no predictive value for ER content (McGuire et al. 1975). The failure of ER levels to be truly predictive may be due to a number of reasons.

First, the quantity of functional ER may be insufficient to initiate a response. This is especially true where tumors consist of heterogeneous assemblies of hormone-dependent and -independent cells. Thus, although detectable ER may be present, enough tumor cells lacking ER exist as to dominate the response to hormone-ablation therapy (Wittliff and Savlov, 1978; Lee, 1980; Walker et al. 1980). Such functional heterogeneity may account for the lethality of these cancers.

This biologic diversity is an important obstacle to treatment, since rapid selection of resistant variants occurs. In fact, chemotherapeutic agents may enhance the selection process by eliminating sensitive variants and leaving resistant ones to proliferate (Fidler, 1984). This decreases the opportunity for subsequent chemotherapy to be effective.

Secondly, single large-sample assays for ER may not always reflect the actual amount of carcinoma present in the sample. Van Netten et al. (1982) report little consistent relationship between percent carcinoma content (PCC) and measured protein values, possibly because of variations in noncancerous cellular constituents, tissue fluid, the quantity and character of the connective tissue stroma, and serum contamination. In their study, 24 of 90 (27 %) ER-positive tumors changed from a low to high ER category after correction had been made for PCC. There was a tendency for uncorrected ER values to be higher in samples exhibiting a large PCC, however high ER levels in tumor samples containing little carcinoma may remain undetected unless PCC is incorporated into ER measurements.

Thirdly, there is the possibility that the molecular integrity, and therefore the functional integrity, of ER is eventually impaired because of a deficit in the pathway that normally follows the interaction of estradiol and ER in target cells (Klute et al. 1978). Such impairment could be due to presently unknown genetic or epigenetic factors (Henderson and Canellos, 1980; Wittliff and Savlov, 1978; Wittliff et al. 1976). Bypassing this endocrine constraint, therefore, may be a

means by which such cells survive this impairment.

Shafie (1980) reported that MCF-7 breast cancer cell lines were unresponsive to estradiol in cell culture, but when transplanted into mice that genetically lack a thymus, both the growth of the cells and their capacity to form tumors were dependent on estradiol. It appears, therefore, that mammary cancer cell growth *in vivo* is subject to inhibition that can be overcome by estradiol and other estrogens. Shafie suggests a specific estradiol-ER complex may control induction or stimulation of gene product synthesis by the target cell, such as PgR or a de-inhibitor of cell growth. Such a product would, perhaps, explain endocrine resistance in malignant cells that continue to express ER.

Fourthly, there is the known ability of nonreceptor proteins, e.g., albumin and sex hormone-binding globulin SHBG), to bind estradiol (Thompson and Lippmann, 1979). Contamination by proteins may account for up to 50 %, on average, of this type of binding and as much as 60 % of this is albumin (Teulings *et al.* 1975). Therefore, the measurement of ER concentration in terms of tumor protein must include assaying the albumin content of the sample and subtracting it from the measured total protein.

In summary, the predictive value of ER assays of breast tumors may be limited by:

1) heterogeneous distribution of ER among tumor cells;

2) varying proportions of non-tumor constituents throughout the tumor;

3) possible impairment of the functional ER system, but with an additional mechanism by which tumor cells can by-pass the normal pathways that are responsible for hormone dependency;

4) the ability of certain nonreceptor proteins to bind estradiol and thus compete with the receptor.

3. Methods of measuring ER: an overview

3.1 Tissue-slice method

Initially, thin (0.5 mm) fresh tumor slices were incubated with solutions of tritiated hormone in the presence and absence of an inhibitor of hormone binding, such as nafoxidine (Jensen et al. 1973).

This procedure suffered from the limitation that only fresh tumor specimens could be used, since freezing disrupted the nuclear membrane, releasing receptor into the incubation medium and making it unavailable for nuclear translocation.

3.2 Sucrose density centrifugation (SDC)

SDC of small (200 mg) samples of tumor cytosols proved useful for estimation of extranuclear receptor for both fresh or frozen tissue. Toft and Gorski (1966) first directly demonstrated estrogen-binding protein in rat uterus in vitro, and characterized hormone-receptor complexes by SDC.

In this method tumor cytosol is incubated with ^{3}H-estradiol, then layered on a prepared 5-20 % or 10-30 % sucrose gradient. A parallel gradient is layered with cytosol incubated with an excess of an unlabelled competitor and ^{3}H-estradiol to determine nonspecific binding of steroid to nontumor constituents. After several hours of centrifugation, fractions are collected and counted to determine peaks of bound radiolabelled estradiol (Thompson and Lippmann, 1979).

3.3 Cold agar-gel electrophoresis (CAGE)

The cold-agar gel electrophoresis (CAGE) method developed by Wagner (1972), and modified by others (e.g., van Netten et al. 1977), separates ER from other cytosol components according to its different electrophoretic mobility.

Following electrophoresis, receptor-bound radiolabelled estradiol is found on the anodal (+) side of the starting well. Free steroid and nonreceptor binding components are carried to the cathodal (-) side. Although albumin moves beyond the receptor to the anode, complete dissociation of hormone from albumin occurs during electrophoresis (van Netten et al. 1977).

Because some radioactive hormone may be left in the receptor region of the gel by this dissociation, control cytosols in which the receptor, but not plasma-binding components, has been destroyed by heating or by other means, are used to measure this effect. The amount of ER is determined by scintillometry, and expressed as femtomol (10^{-15} moles) per mg tumor protein. Tumor protein is measured by first determining total protein by the method of Lowry et al. (1951), and albumin concentration by radial immunodiffusion. The difference

between these is the tumor protein concentration.

As with all SBAs, this method measures previously unoccupied ER sites only, so is not a true measure of the total ER content of the tumor cells.

In premenopausal patients generally, receptors are more likely to be at least partially occupied by endogenous hormone, and therefore be unavailable to radiolabelled hormone.

Using an ion-exchange procedure based on the hydroxylapatite technique (Erdos et al. 1970), Thorsen (1980) demonstrated significant levels of occupied receptor in samples that were negative by SBA. Thus, under conditions of occupancy of steroid binding sites by endogenous estrogens, SBA could underestimate the true ER level, in premenopausal patients, especially.

3.4 Other biochemical methods for ER assay

Other methods that have been used to separate and measure ER include dextran-coated charcoal (Korenman, 1968), gel filtration, (Godefroi and Brooks, 1973), isoelectric focussing (Coffer and King, 1974), protamine sulphate (Lippmann and Huff, 1976), and DEAE ion-exchange (Santi et al. 1973). The disadvantages of

many of these are the cost of equipment required to detect and record radio-labelled bound receptor, and the risks associated with using radioactive substances. These methods are further discussed in Thompson and Lippmann (1979).

3.5 Enzyme immunoassay; polyclonal antibodies to estradiol

A number of investigators have attempted to use anti-estradiol antibody to characterize receptor, generally using the indirect peroxidase:antiperoxidase (PAP) staining technique developed by Sternberger et al. (1970). This and other immunoperoxidase techniques are reviewed by Falini and Taylor, 1983; Burns, 1983; and Vandesande, 1983.

Briefly, histological sections of tumor are incubated with a conjugated-estradiol solution (for example, bovine serum albumin-polyestradiol) which is expected to bind to unoccupied receptor, followed by anti-estradiol antibody (primary antibody). A second, or "link", antibody against the primary antibody is added in excess so as to leave a free antigenic binding site on the second antibody. Finally, a peroxidase: antiperoxidase complex against the second antibody is

applied. Incubation of the resulting antibody "sandwich" with an appropriate substrate for peroxidase and a chromogen, allows visualization of the target antigenic sites.

A frequent problem in this procedure was the high level of nonspecific staining associated with the low affinity, high capacity, so-called type II binding sites initially found by Clark et al. (1978) in rat uterus. Few of these are occupied below 10^{-9} M, but progressively more of them are filled by estradiol at higher concentrations; most of them are occupied when 10^{-7} M is reached.

Chamness et al. (1980) suggest that another order of non-specific binding of estradiol (type III) is associated with soluble molecules such as albumin, and, although their individual affinity for estradiol is very low their collective capacity is substantial, and allows them to bind large amounts of estradiol above 10^{-7} M. (ER is designated as type I binder, and has a K_D = 10^{-10} M).

In most of the histochemical studies for ER detection reviewed by Chamness the concentrations of estradiol added to tumor samples ranged from 10^{-8} to as

high as 10^{-4} M, suggesting that these methods could have been detecting primarily type II and III binding rather than type I.

Nenci (1981) presents similar objections to the application of immunoperoxidase histochemical methods as the sole means for predicting patient response to anti-estrogen treatment.

3.6 Direct determination of receptor by monoclonal antibody (MCA) to receptor

Problems of nonspecificity of antibodies were greatly ameliorated by the development of a method for producing monoclonal antibodies (Köhler and Milstein, 1975).

Briefly, this involves injecting an animal, commonly a mouse or a rabbit, with pure antigen against which the desired antibody is to be produced. After sufficient time has elapsed for the animal's immune system to form antibodies to the antigen, the animal is sacrificed, its spleen is removed and the immunocytes are separated. These cells are fused <u>in vitro</u>, induced by polyethylene glycol, with myeloma cells to produce so-called hybridoma cell lines (Köhler and Milstein, 1975).

These have the advantages of rapid reproduction, immortality, and the ability to manufacture large quantities of immunoglobulins of high specificity (Cuello et al. 1983). The desired clone of such hybridomas can be identified selectively and reinjected intraperitoneally into recipient animals in which they grow as a tumor. Monoclonal antibodies can be harvested as needed from the ascitic fluid produced by the tumor.

The application of this technique in enzyme immunoassay detection of ER in frozen tumor sections was developed by King and Greene (1984). Subsequently a kit for detection of ER using monoclonal antibody against ER was produced commercially as ER-ICA (Estrogen Receptor Immunochemical Assay) by Abbott Laboratories (see Materials and Methods).

With the exception of using monoclonal, instead of polyclonal antibodies for the primary antibody, the ER-ICA procedure is very similar to the PAP method of Sternberger et al. (Figure 2, page 23). The antibody recognizes ER that contains pre-bound endogenous estradiol as well as unoccupied ER, the former being undetectable by conventional methods that measure uptake of hormone.

The ability to directly examine the distribution of ER is of potential use in explaining why some tumors that are found to be ER-positive by SBA fail to respond to hormone therapy.

Since estradiol exerts its effects at the cellular level the intracellular concentration of ER in these tumors may be too far below the threshold at which hormone-induced effects are initiated. Hence, this method of directly visualizing ER distribution circumvents many of the difficulties associated with procedures that indirectly measure ER.

In 1984, participants in an international symposium summarized multicenter studies of the efficacy of detection of ER by ER-ICA. The consensus among many researchers was that it was both an effective and reproducible means of evaluating heterogeneity and intratumor distribution of ER, even though quantitation was more subjective than in SBA methods (Jensen 1984).

The original paper by King and Greene (1984), concerning detection of ER by means of monoclonal rat antibodies to human ER, reported that specific staining was confined to the nucleus in frozen, fixed sections of human breast tumors and uterus, rabbit uterus, other

mammalian reproductive tissues, and fixed MCF-7 breast cancer cells.

No cytoplasmic staining in any of the ER positive tissues or tumor cells was observed, and it was suggested that ER commonly isolated in cytosols of homogenates could represent receptor that is loosely associated with the nucleus.

Binding of estradiol to receptor results in a tighter association with the nucleus, and therefore may not be the result, as earlier interpreted, of translocation to the nucleus of hormone-cytoplasmic ER complexes (King and Greene, 1984).

In support of King and Greene, Welshons et al. (1984), using cytochalasin B-induced enucleated rat pituitary GH3 cells that normally bind estradiol with high specificity, observed that cytosols had little estrogen-binding activity, while nuclei contained significantly more unoccupied ER.

Welshons et al. conclude that in the absence of hormone, the loose association of ER with the nucleus causes it to diffuse into the cytosol during the cell's disruption, and the cytosolic ER fraction is really an extraction artefact.

The response to steroid binding may not be translocation, therefore, but an increase in affinity of hormone-ER complexes for the nucleus.

More recently, Marchetti et al. (1987) report both cytoplasmic and nuclear staining for ER in breast cancers using a different monoclonal antibody, against calf uterus rather than human ER (this anti-calf uterus antibody was previously shown to interact with human ER). An increase in sensitivity of the procedure is achieved by application of an avidin-biotin immunocytochemical procedure. This utilizes the high affinity of avidin for biotin by employing a biotinylated second antibody and an avidin-linked PAP complex.

These investigators suggested that the cytoplasmic localization of ER, which seems to contradict the findings of King and Greene, might be due to different sources of antibody and antigen, variations in the purification procedure of the antigen,or to cytoplasmic redistribution of receptor that is not hormone-bound and therefore only weakly attached to the nucleus.

4. The purposes of this study are:

1) to determine if the presence of ER in corresponding microsamples of breast tumors can be detected by SBA that measures uptake of radiolabelled estradiol by ER, and by ER-ICA that uses monoclonal antibodies against ER directly;

2) to determine, by semiquantitative means, whether ER levels in corresponding microsamples for the two methods are comparable, after corrections for per cent carcinoma, per cent specific staining and staining intensity are made;

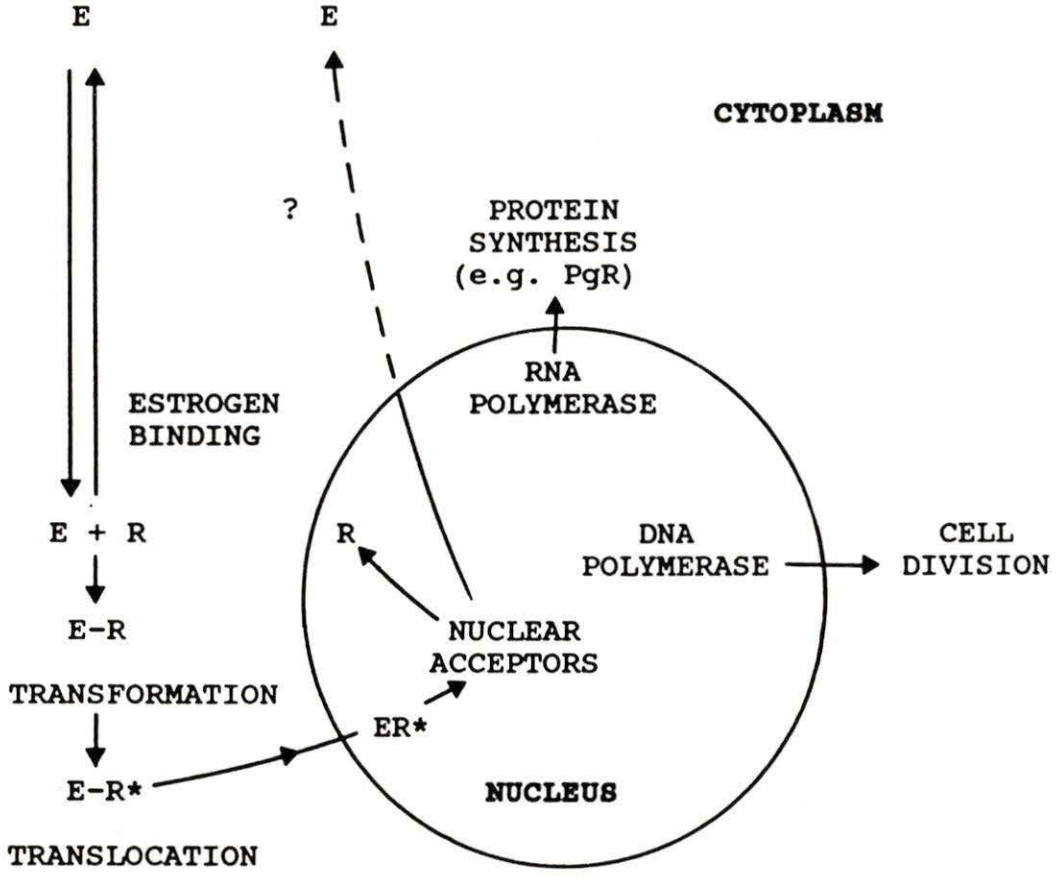
3) to assess the heterogeneity of ER distribution between four microsamples taken from widely separate areas of each tumor, and within individual microsamples;

4) to relate differences in ER distribution in breast tumors to their possible significance in effective treatment.

FIGURE 1. **Two-step mechanism for interaction of estrogen (17β-estradiol) with cytoplasmic estrogen receptor**

Hormone (E) bound to carrier protein in plasma becomes dissociated from the carrier and enters target cell to bind to specific cytoplasmic receptor (R).

Formation of hormone-receptor complex (E-R) transforms R to active form. E-R is then translocated to the nucleus and binds to acceptor sites on DNA. This interaction stimulates cell division and RNA polymerase activity. One of the resulting gene products is progesterone receptor(PgR).



(After Mirecki & Jordan,1985)

FIGURE 2. **Immunohistochemical (PAP) method for detection of estrogen receptor in breast tumor frozen sections**

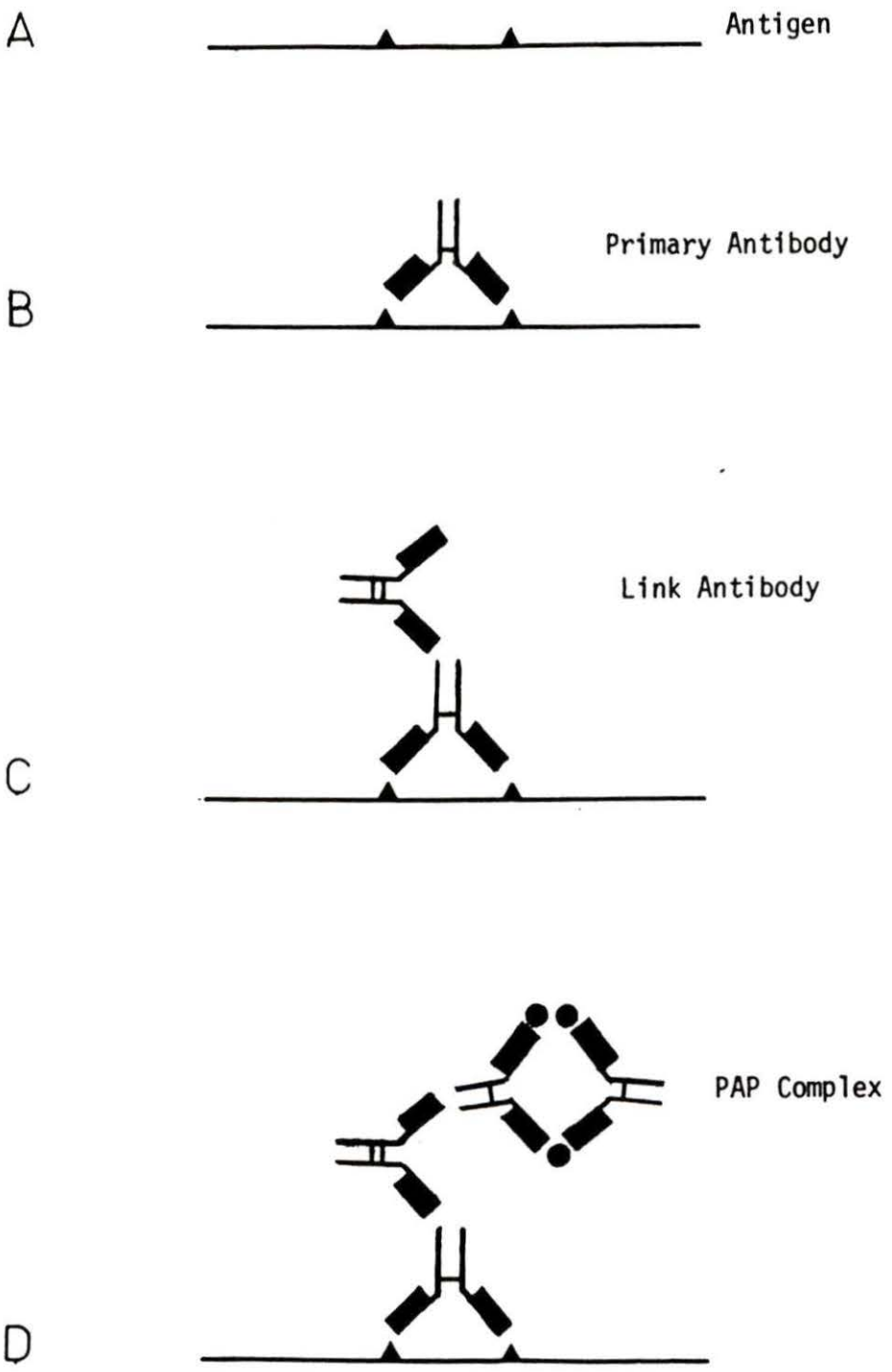
A - Antigen (estrogen receptor) represented by dark triangles on tumor microsample section.

B - Primary antibody added (rat monoclonal anti-estrogen receptor) attaches by Fab portions to antigen.

C - Second, or link, antibody (goat anti-rat IgG) in excess attaches to primary antibody, leaving one free Fab fragment.

D - PAP complex added (rat peroxidase: anti-peroxidase) attaches to second antibody at free Fab portion of second antibody. Dark circles represent peroxidase molecules.

Final visualization of receptor-bound IHC complexes results from incubation with hydrogen peroxide and a chromogen, diaminobenzidine tetrahydrochloride. The reaction product is a stable, yellow-brown, precipitate.


A
Antigen
B
Primary Antibody
C
Link Antibody
D
PAP Complex

II. METHODS AND MATERIALS

1. Preparation of tumor microsamples for assay (Figure 3, page 34)

For each of the 21 tumors assayed four 1.5 X 1.5 X 13mm microsamples (approx. 40 mg each) were cut under a dry ice downdraught from previously frozen (-70C) fresh surgical specimens. Each microsample was divided equally and longitudinally with a scalpel; one half was used for SBA and the sister half for ER-ICA. Thus the sister halves were no more than 750 µm apart.

For SBA, each microsample was homogenized by hand in a Potter-Elvehjem glass/teflon homogenizer for 30sec in 500 µl TED (tris[hydroxymethyl] methylamine, ethylene diamine tetra-acetate, dithiothreitol) buffer, (10 / 0.5 / 1 mmol/liter, pH 7.5) then centrifuged at 50000g (16000 rpm) for 30 min at 0-4C in a refrigerated Sorvall RC2-B centrifuge (SM24 head).

1.1 Preparation of microsamples for SBA

A fifty-microliter aliquot of cytosol for each microsample was incubated with 12.5 µl 2,4,6,7-^{3}H(N) estradiol (New England Nuclear, Boston; spec.acy. 85-105 kCi/mol) in TED buffer for 30 min at 4C.

As a positive control 50 µl of calf endometrium (prepared cytosol from fresh slaughterhouse material) was similarly incubated.

As negative controls 50 µl of each tumor cytosol and 50 µl of calf endometrium were incubated in a 56C water bath for 30 min to destroy ER.

1.2 Determination of tumor protein concentration

Aliquots of all cytosols were also used to determine total protein concentration by the Lowry method (Lowry et al. 1951). Serum albumin concentration was assayed by radial immunodiffusion using a commercial kit (Meloy Laboratories). The difference between the total protein and albumin concentrations represents the concentration of tumor protein, in terms of which measured ER is expressed.

1.3 Cold-agar gel electrophoresis to separate ER

To separate ER-bound from unbound estradiol, 50 µl aliquots of all cytosols previously incubated with radiolabelled estradiol were subjected to CAGE, 200 mA, for 2.5 h at 2-4C.

Following CAGE, the anodal portions of the gels were sliced with clean scalpel blades and, together with the

remaining contents of the starting wells, were placed in glass scintillation vials containing Biofluor scintillation cocktail. Specific counts were measured on a Beckman scintillation counter and converted to ER concentration in femtomol per mg tumor protein (FMTP).

1.4 Assignment of ER level ranges for SBA

Arbitrarily, the cutoff point for intermediate vs. high receptor level was set at 100 FMTP, and a level <1 FMTP was considered negative as previously utilized (van Netten et al. 1982; 1985).

2. Immunohistochemistry (ER-ICA)

2.1 Preparation of microsamples for ER-ICA : sectioning and fixation

The sister half of each frozen microsample was mounted intact on a brass cutting block, immediately embedded in Tissue-Tek cryostat medium and left at -20C for 15 min to set before being sectioned.

Two frozen 6 µm sections were cut from each microsample and placed on glass slides pre-coated with tissue adhesive. These were immediately fixed with 3.7% freshly-prepared formalin in phosphate-buffered saline

(PBS), pH 7.4, for 15 min, then cold (-20C) methanol for min, followed by cold acetone for 2 min. After two successive 5 min baths in PBS the ER-ICA procedure was immediately commenced, or the slides were immediately stored at 20C in a storage medium (2.8 g sucrose, 0.33 g anhydrous magnesium chloride, 250 ml glycerol, made up to 500 ml with PBS).

As positive controls, either smears of fixed, frozen MCF-7 breast cancer tissue culture cells, or fixed sections of tumors previously determined to have high ER concentration by routine SBA on large tumor samples were used (MCF-7 cells typically express high concentration of ER). Negative controls are described below.

2.2 Characterization of ER by ER-ICA (Figure 2, page 23)

All subsequent ER-ICA reagents were supplied in kit form by Abbott Laboratories, Ltd (Chicago). Sections were first incubated in a humidified chamber at room temperature (RT) with normal goat serum for 15 min to block nonspecific binding of subsequent reagents. Excess serum was gently wiped off the slides with a clean paper tissue before application of the next reagent.

For tumor microsample sections and positive control slides, rat anti-human ER monoclonal (primary) antibody as it was supplied in the ER-ICA kit, was added dropwise and incubated for 30 min at RT. Following this and subsequent incubations, sections were gently jet-washed for 10 sec with PBS, placed in 2 successive PBS baths for 5 min each, then gently wiped with a clean tissue to remove excess buffer. Care was taken not to allow slides to dry out.

As a negative control, one of the two sections from each microsample and a sample of MCF-7 cells were incubated with normal rat serum instead of anti-ER antibody in order to eliminate the possibility, however slight, of false positives resulting from any naturally-occurring antibodies binding to non-receptor sites in the ER-negative tumors being tested.

Sequentially the following antibodies were added, as provided in the ER-ICA kit, to all sections and incubated for 30 min at RT: goat anti-rat second antibody; rat horseradish-peroxidase: anti-horseradish peroxidase (PAP). The result is a greatly amplified signal represented by a high concentration of PAP complex bound to receptor sites via the second antibody.

2.3 Visualization of ER by chromogen-substrate reaction

A reaction mixture of 4 mg of a chromogen, 1.7mM diaminobenzidine.4HCl (DAB), and a substrate for peroxidase, 0.06 % hydrogen peroxide in PBS, were mixed immediately prior to use, then added to all sections for 6 min at RT under low light intensity, since both peroxide and DAB are photolabile. The reaction causes DAB to be converted to its oxidized form, which is an insoluble golden-brown precipitate attached to the PAP complexes, bound to the sequence of antibodies.

Sections were washed in distilled water for 10 min prior to counterstaining with 1 % Harris hematoxylin for 10 sec, then were dehydrated in an alcohol-to-xylene series and mounted with Permount medium.

2.4. Evaluation of percent carcinoma (PCC)

The estimation of PCC in each microsample section was conducted independently by four observers, one of whom was a qualified pathologist, by examining stained sections under 100X of a compound microscope.

PCC was expressed as that percentage of the area of the microsample section that would be occupied if all

the viable tumor were consolidated into a single area of the section. Where disagreement between observers occurred, sections were re-evaluated and a consensus reached.

A correction was then made to the biochemically-determined ER value as follows in order to minimize the effect of tumor content on ER evaluation (van Netten et al. 1982):

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

2.5 Evaluation of staining

Two parameters were considered:

1) per cent tumor cells specifically stained for ER;

2) intensity of specific staining

As with PCC determination, these were ranked by three independent observers, and where disagreement occurred, a consensus was reached following re-evaluation of the slides.

2.5.1 Determination of per cent specific staining

The percentage of cells specifically stained in each section (PSS) was determined by estimating the

proportion of the area of the section that would be occupied by specifically-stained cells if they were consolidated into one contiguous area. The percentages were then ranked as follows:

0% = 0	31 - 40 % = 4	71 - 80 % = 8
1 - 10 % = 1	41 - 50 % = 5	81 - 90 % = 9
11 - 20% = 2	51 - 60 % = 6	91 - 100 % = 10
21 - 30% = 3	61 - 70 % = 7	

2.5.2 Determination of staining intensity (SI) and histoscores

SI was determined by assigning a value to the average staining intensity for all tumor cells specifically stained for ER, as follows:

negative = 0	low = 1
moderate = 2	high = 3

Intermediates between these values were not considered due to the subjective limitations inherent in evaluating subtle colour intensity differences.

For each ER-ICA microsample the values for PSS and SI as defined above were summated and the resulting value, the histoscore, is an arbitrary one that has no units and is used only to compare the two methods.

A histoscore value of 1 to 8 was considered intermediate, and a value of 9 or more high, for ER so that an equal number of tumors would be categorized as high for ER by both methods. When no ER-specific staining was observed in the microsample, it was considered negative.

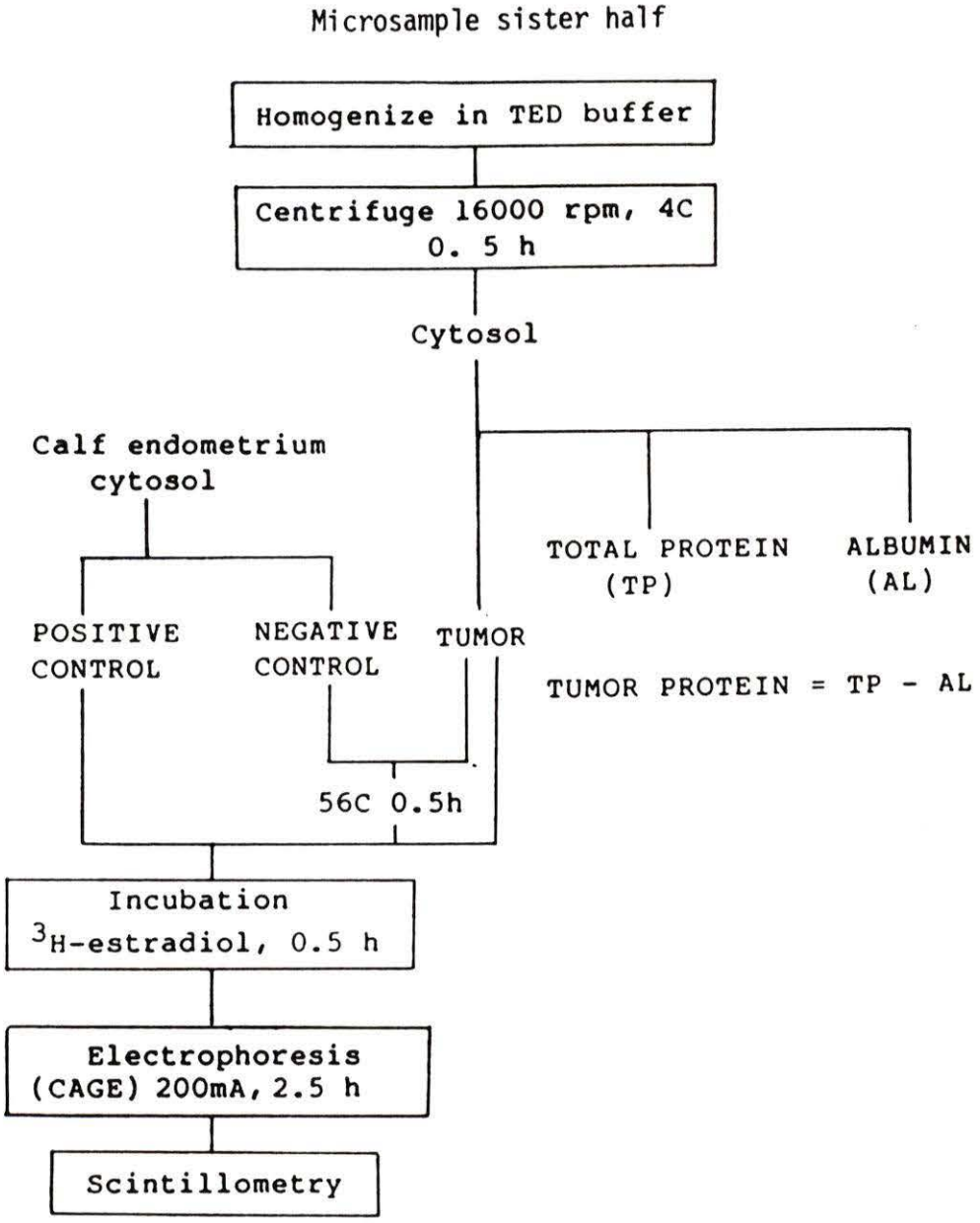
FIGURE 3. **Flow chart for steroid-binding assay (SBA) for estrogen receptor content of breast tumor microsamples**

Four microsamples of approximately 20 mg each from widely-separate areas of each tumor are used. The microsamples and their cytosols are maintained at 2-4C throughout the procedure.

Negative control cytosols of tumor are heated to destroy estrogen receptor. Calf endometrium cytosol is used as a positive control.

Tumor protein concentration is the difference between total protein and albumin concentrations, and estrogen receptor concentration is expressed in terms of the amount of tumor protein.

STEROID-BINDING ASSAY (SBA) FOR ESTROGEN RECEPTOR



$$[ER]\ (\text{femtomol per mg tumor protein}) = \frac{\text{net counts}}{\text{tumor protein}} \cdot k$$

k is a factor incorporating dilution, incubation concentration, and specific radioactivity of labelled estradiol solution.

III. RESULTS

1. Semiquantitative comparison of SBA and ER-ICA methods

Of the 21 tumors studied, 19 were classified as ductal carcinomas, and 2 (TB and DJ) as lobular carcinomas. A total of 81 microsamples was analyzed for ER by both SBA and IHC methods. Three microsamples were not available for analysis due to insufficient carcinoma content.

Table 1, page 44 indicates that there was complete qualitative agreement in 78 of the 81 microsamples (96.5 %). Fifty-five (68 %) of the microsamples measured ER-positive, and 23 (28.5 %) ER-negative, by both methods.

Figure 4, page 46 indicates that only a weak correlation between the two methods exists when the SBA-ER level is 1-10 and >100 FMTP. In the range of 10 - 100 FMTP the correlation is noticeably higher. Quantitatively, the correlation coefficient for all 81 microsamples was 0.75.

1.1 Intratumor variations in PCC, PSS and SI

Figures 5 a, b and c, page 48, summarize the characterizations of intratumor variations in PCC, PSS, and SI between the microsamples of the 21 breast tumors.

Figure 5a shows that more than half of the tumors had variations in PCC of 20 % or greater with microsamples taken from different areas of the same tumor. In one patient (TD) the large variation was due to trace amounts of carcinoma in one microsample, but an average amount (50 %) in the other three.

Figure 5b indicates that PSS can vary considerably between microsamples from widely-separated areas of the same tumor. Seven of the 21 tumors show a 20 % or greater variation between microsamples. Approximately one half of the tumors, however, had no obvious variation in PSS.

Figure 5c demonstrates that variation in the SI between microsamples of each tumor was minimal in most cases. Since the total range of this measurement is less than that for PCC and PSS, this is not surprising. As stated previously, subtle differences in stain intensity are not easily evaluated by simple visual examination.

1.2 Assessment of ER-specific staining for ER-ICA method

Specific staining for ER was confined to the nucleus of all ER-positive tumors studied as well as in MCF-7 cells used as positive controls.

For controls in which normal rat serum was substituted for monoclonal anti-ER, specific staining was totally absent, indicating that ER-specific staining is not attributable to any component of rat serum. Nonspecific 'background' staining, where it occurred rarely, was barely perceptible to all observers.

2. Heterogeneity of ER distribution in ER-ICA method

2.1 Inter-microsample distribution of ER-specific staining (low-order heterogeneity)

Figure 6, page 50, represents graphically the four microsamples analyzed from each of the 21 tumors. Each tumor is represented by a square within which each quadrant is a microsample. Quadrants in corresponding positions within the squares for individual tumors represent sister-halves of each microsample. The white, shaded, and black quadrants represent absent, moderate, and high ranges for corrected ER and histoscores, respectively, as defined in Methods and Materials.

Qualitatively, of the 21 tumors analyzed, 13 (62 %) were ER-positive in all quadrants, and 6 tumors (28.5 %) were ER-negative by both methods. In the remaining 2 tumors (patients CE and MD) both positive and negative microsamples occurred within the same tumor by both methods. Thus, 15 of the tumors had detectable ER by one or the other method.

Using three categories of ER concentration for the SBA-ER method, 6 tumors were considered homogeneous-positive for ER (all microsample were within the same ER range in each tumor: patients FM, KA, DE, MD, DJ, BM); and 8 were heterogeneous-positive (patients TD, TB, EM, BR, CF, DP, MC, and NA), having both high and moderate ER-positive microsamples. Only one tumor (CE) had both ER-positive and ER-negative microsamples with this method.

The corresponding values for the ER-ICA method were: 11 homogeneous ER-positive (patients TD, EM, FM, KA, DE, CE, CF, DJ, BM, MC and NA) and 3 heterogeneous ER-positive (patients TB, BR and DP); only one patient (MD) had both positive and negative microsamples with this method.

In summary, 12 (57 %) of the 21 tumors had equivalent ER ranges in all microsamples by both methods, 4 (19 %) differed by one microsample each, and 5 (24 %) differed by two or more microsamples each.

It is important to realize that "heterogeneous" and "homogeneous", in the sense used above, refer to an intermicrosample comparison for each of the tumors, and do not take into account variations of ER distribution within individual microsamples, which is considered below.

2.2. Intra-microsample distribution of ER-specific staining (high-order heterogeneity)

Figures 7 to 11, page 52, illustrate patterns of ER distribution within individual ER-ICA treated microsamples.

Figure 7 illustrates an example of a tumor in which virtually all the cells are specifically stained for ER to a high degree. Three of the 15 ER-positive tumors in this study contained this pattern (patients BM, MC and NA), and all were positive for ER following routine and microsample SBAs. These were designated 'solid-positive'.

Figure 8 shows a tumor that is ER-negative, indicating no specific staining, designated "solid-negative". Six of the 21 tumors in this study demonstrated the same staining pattern (patients LG, RS, DB, SM, RL and NG). All these were ER-negative also for routine and microsample SBAs.

Additionally, one tumor (MD) that was otherwise ER-positive had one microsample that was measured as solid-negative.

As represented by figure 9, ER-specific staining of very few isolated cells was seen in 3 of the tumors analyzed (patients KA, CE and MD). Unstained cells clearly predominate in these tumors, and routine and microsample SB assays of the same tumors confirmed that their ER levels were intermediate as previously defined.

Figure 10 represents a pattern observed in which specific staining is arranged in small, solid-positive tumor cell clusters interspersed with areas of a more heterogeneous 'checkerboard' stain distribution, so called because nuclei within this category are variously unstained, highly-, or moderately-stained, in a random array. Two of the 15 ER-positive tumors (patients TD, DJ) showed this 'cluster-checkerboard' pattern.

A variation of this was observed in 3 other tumors (patients FM, CF, and to a lesser extent, DJ) in which the tumor sections were predominantly solid-positive, but had a relatively small proportion of checkerboard staining. These were designated as being 'solid-checkerboard.'

All these tumors were similarly ER-positive in both routine and microsample SBAs.

Figure 11, represents the uniformly random checker-board staining pattern that typified all microsamples in 4 of the ER-positive tumors studied (patients TB, EM, BR, DE). The random juxtaposition of highly-stained, moderately-stained, and unstained tumor cells is visibly different from the other staining patterns described.

Thus, a total of 9 of the 15 ER-ICA determined ER-positive tumors (60 %) demonstrated some degree of the checkerboard staining pattern.

2.3. Variations of ER distribution in ER-positive tumors

Figure 12, page 54, illustrates intermediate ER-level staining patterns of ER-positive tumors excluding

solid-positive cases. Four tumors each expressed both focal and checkerboard patterns (pathways A and C); these were the patterns previously described as cluster-checkerboard and solid-checkerboard.

One tumor expressed a field pattern (pathway B) in which most of the tumor cells are specifically stained to some degree, but no intense staining is evident.

Three tumors were mainly of a checkerboard pattern (pathway C). Another three tumors presented mainly relatively few isolated, specifically-stained cells. In these cases the corresponding ER values were predominantly in the intermediate category as defined in Materials and Methods.

TABLE 1. **Qualitative comparison of ER-positive and negative results of steroid-binding and immunohistochemical assays on 21 breast tumors**

The number (unparenthesized) and percentage (parenthesized) of microsamples for steroid-binding assay (Corrected ER) and immunohistochemical method (histoscore) are shown.

Number (%) of microsamples

	CORRECTED ER		
	POSITIVE	NEGATIVE	TOTAL
HISTOSCORE POSITIVE	55 (68)	2 (2.5)	57 (70)
HISTOSCORE NEGATIVE	1 (1)	23 (28.5)	24 (30)
TOTAL	56 (69)	25 (31)	81 (100)

Number (%) of microsamples

FIGURE 4. **Comparison of ER-positive microsamples from 21 breast tumors assayed for ER by steroid-binding assay (Corrected ER) and immuno-histochemical method (Histoscore)**

The horizontal and vertical dashed lines indicate cutoff points for high and low ER levels, and high and low histoscores, respectively. The curved line represents the second-order correlation for the two methods.

The closest correlation is in the range 10 - 100 femtomol per mg tumor protein, and the range 3 - 7 for histoscore values. The greater scatter from the line of correlation at the extreme histoscore ranges is indicative of the problem of discerning differences in staining intensity at low and high extremes.

Zero values for corrected ER are not shown so as not to bias the comparison of ER-positive assays by both methods.

The correlation for ER-negative tumors was 0.88 (26 microsamples were ER-negative by one or the other method; 23 were ER-negative by both methods).

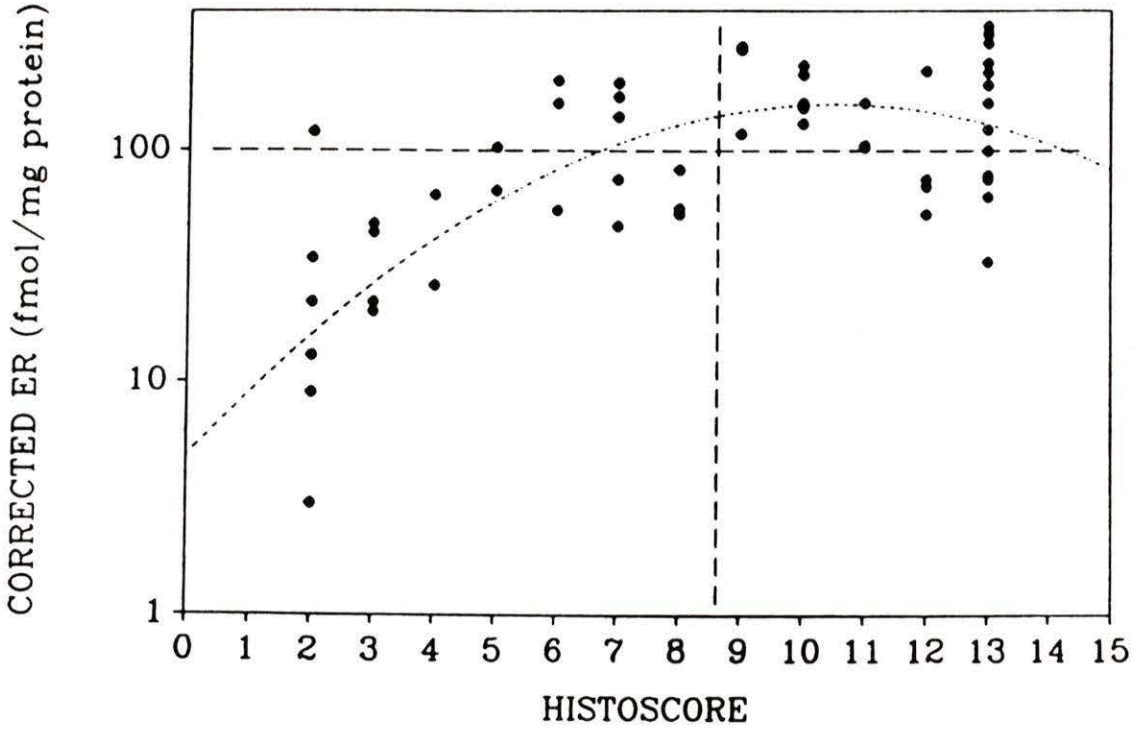

CORRECTED ER (fmol/mg protein)
100
10
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
HISTOSCORE

FIGURE 5. **Ranges of carcinoma content, specific staining, and stain intensity between 4 microsamples in each of 21 breast tumors**

Patients' initials are indicated on left vertical axis. Asterisks indicate cases in which only 3 microsamples were available for analysis.

a - variation in carcinoma content, from 1 to 45 %.

b - variation in ER-specific staining, from 1 to 30 %.

c - variation in intensity of ER-specific staining (SI). Integer values are: 0 - negative staining; 1 - low SI; 2 - moderate SI; 3 - high SI.

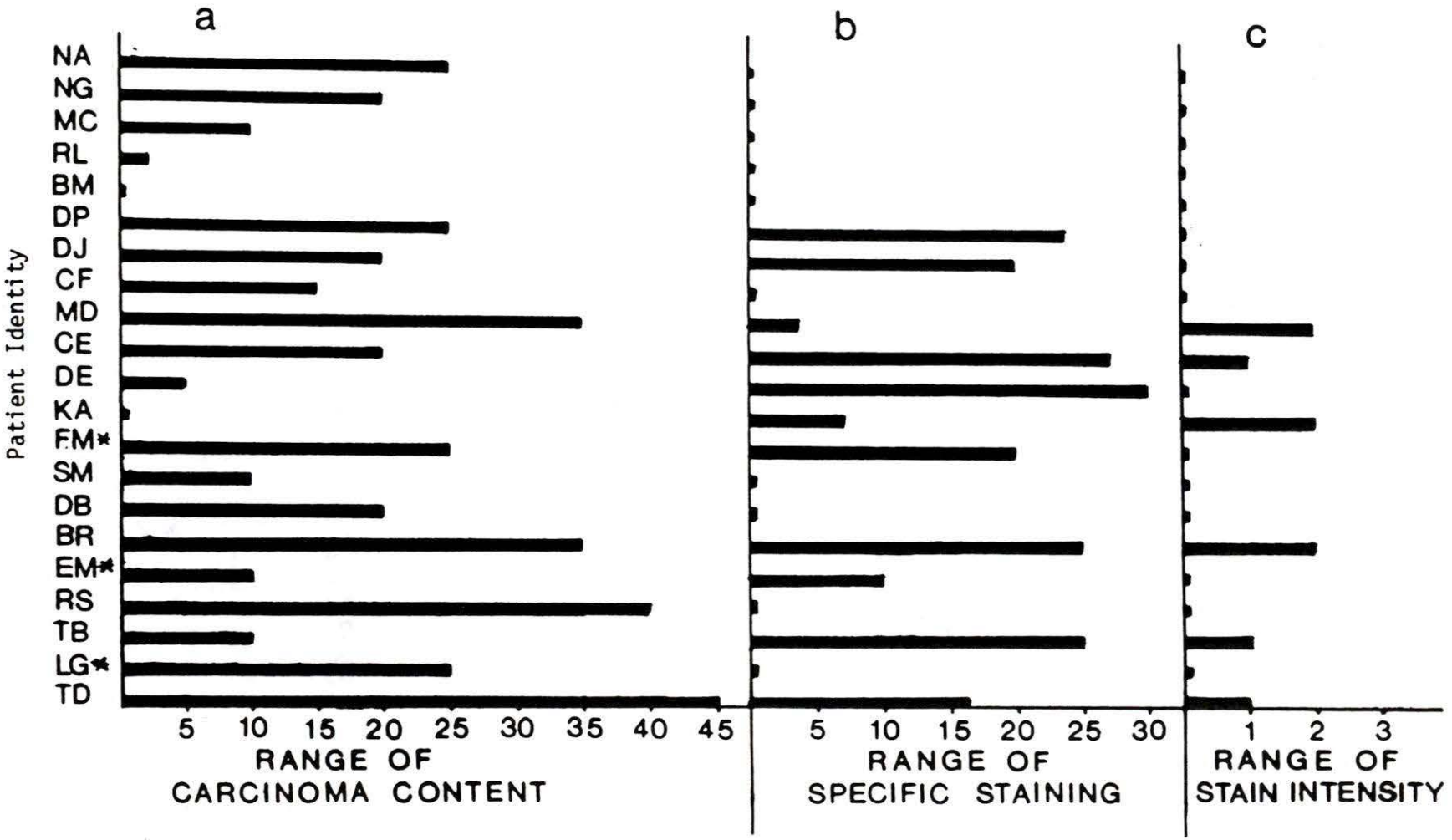
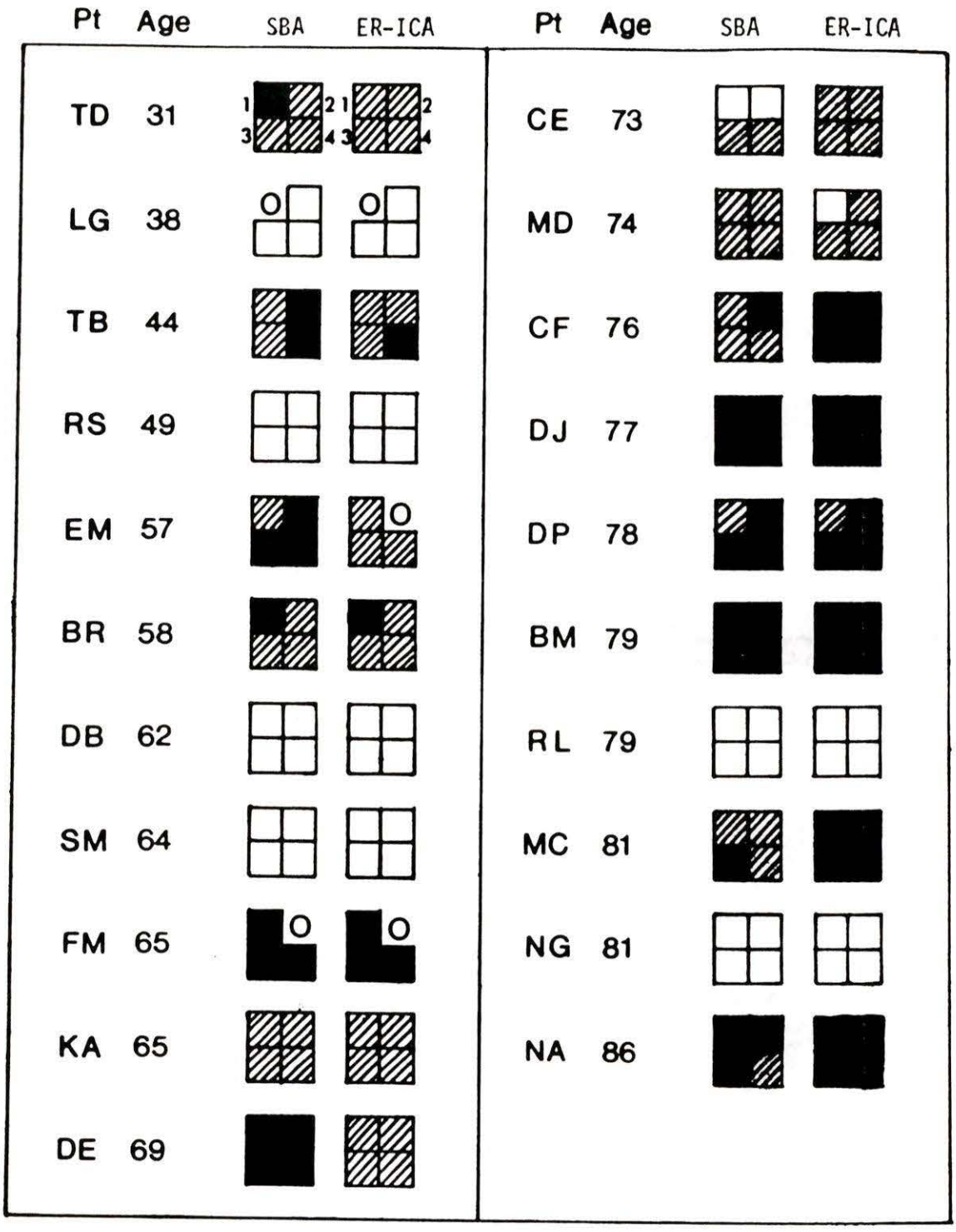

a
b
c
Patient Identity
NA
NG
MC
RL
BM
DP
DJ
CF
MD
CE
DE
KA
FM*
SM
DB
BR
EM*
RS
TB
LG*
TD
5 10 15 20 25 30 35 40 45
RANGE OF
CARCINOMA CONTENT
5 10 15 20 25 30
RANGE OF
SPECIFIC STAINING
1 2 3
RANGE OF
STAIN INTENSITY

FIGURE 6. **Semiquantitative graphic comparison of results of steroid-binding assay (SBA) and immunohistochemical assay (ER-ICA) for estrogen receptor in 21 breast tumors**

Pt: patients' initials. Each large square represents the whole tumor sample and each quadrant, numbered 1 to 4 for the first patient only, indicates corresponding microsamples for both methods. Circles indicate microsamples that were not available for analysis.

White quadrants indicate microsamples that are negative for estrogen receptor (ER); hatched quadrants indicate moderate levels of ER; black quadrants indicate high levels of ER. Cutoff points for each ER range are defined in Materials and Methods.


Pt
Age
SBA
ER-ICA
TD 31
LG 38
TB 44
RS 49
EM 57
BR 58
DB 62
SM 64
FM 65
KA 65
DE 69
Pt
Age
SBA
ER-ICA
CE 73
MD 74
CF 76
DJ 77
DP 78
BM 79
RL 79
MC 81
NG 81
NA 86

All magnifications are 240 X

Figure 7. **Solid-positive specific staining pattern for estrogen receptor (ER, patient BM)**

All tumor cells are densely-stained using the ER-ICA immunohistochemical method that employs monoclonal antibodies against human ER. This tumor was similarly highly ER-positive by steroid-binding assay.

Figure 8. **Solid-negative staining pattern for ER (patient SM)**

All tumor cells are negatively-stained with regard to ER. The purple colour is the hematoxylin counterstain for nuclei. This tumor was also negative by steroid-binding assay.

Figure 9. **Isolated specific staining pattern (patient KA)**

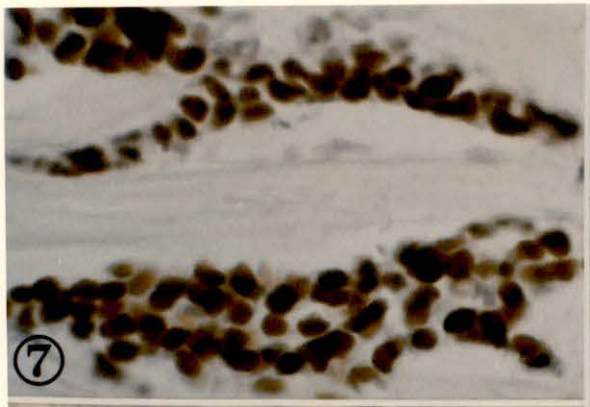
Only a relatively small proportion of tumor cells (arrows) was stained specifically for ER. In the steroid-binding assay, this tumor was categorized as intermediate for ER level.

Figure 10. **Cluster-checkerboard specific staining pattern (patient FM)**

Regions of uniformly positive ER-specific staining are seen, especially near the rounded tips of the areas of tumor. The remaining areas of tumor show a less homogeneous arrangement, with some negatively-stained cells appearing intermixed with tumor cells staining with different intensities. This tumor was homogeneously highly-positive for ER by steroid-binding assay.

Figure 11. **Checkerboard specific staining pattern (patient TB)**

The highly characteristic random intermixing of unstained and specifically-stained cells is demonstrated. Furthermore, the specifically-stained cells have a variety of staining intensities. This tumor was also heterogeneous for ER by steroid-binding assay, with half of the microsamples having intermediate, and half high, levels of ER.



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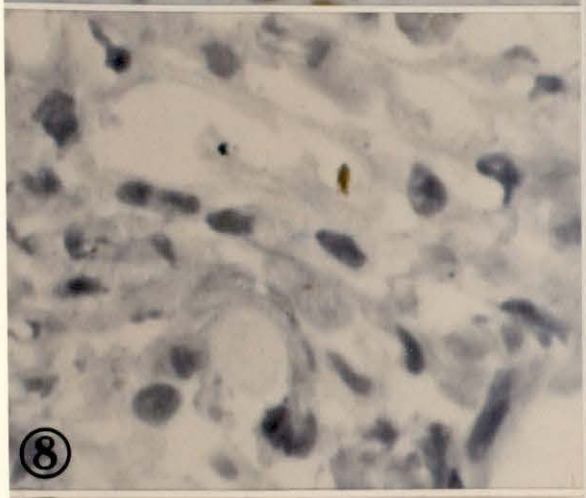

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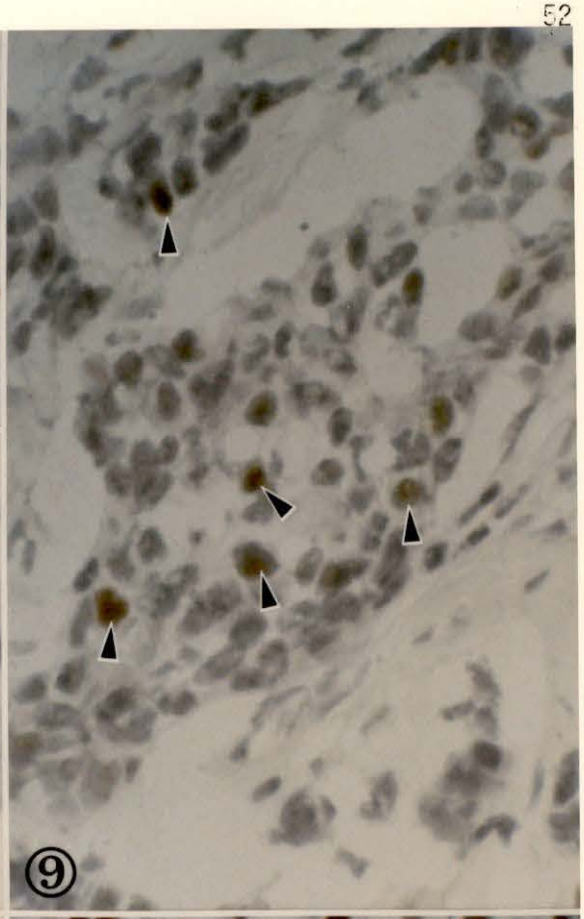

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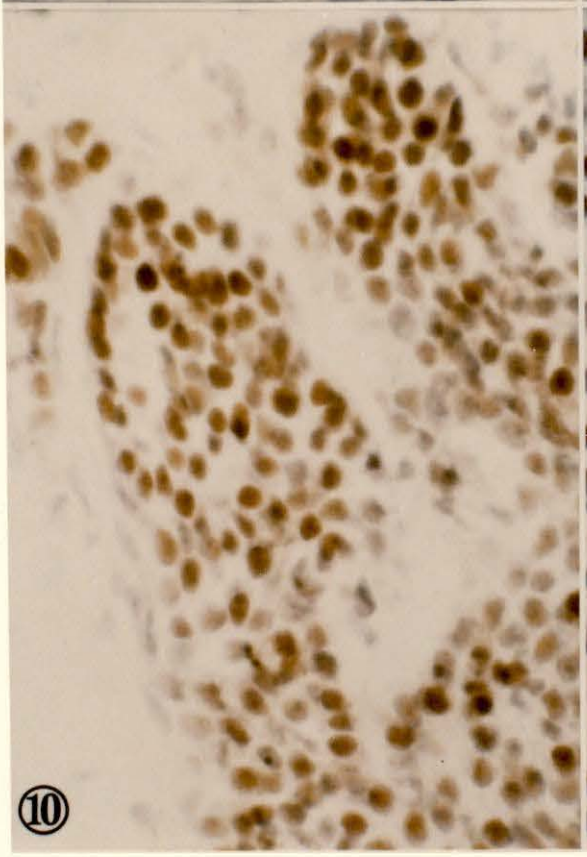

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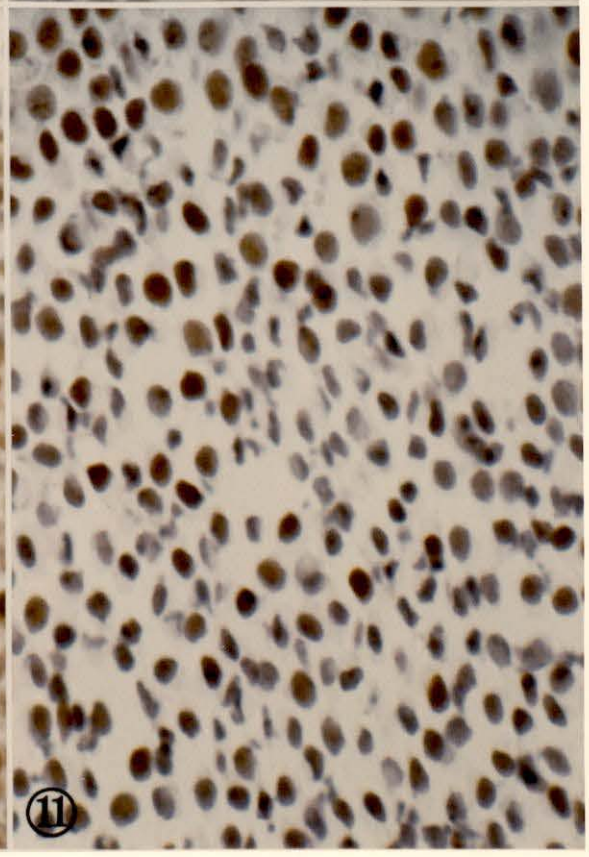


⑪

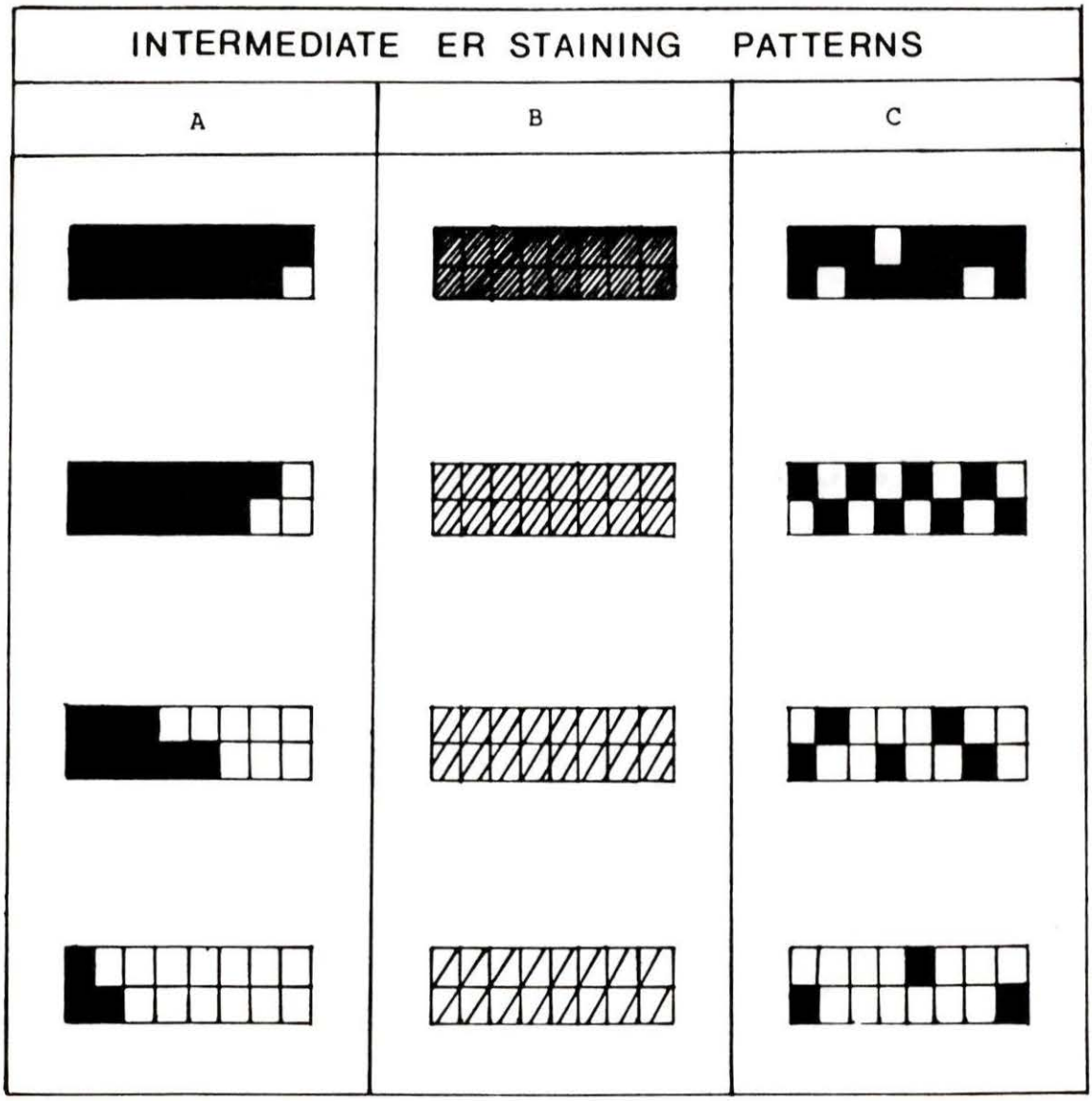
FIGURE 12. Intermediate variants of estrogen receptor staining observed in 21 human breast tumors

Each bar represents a group of adjacent tumor cells. Dark squares represent cells staining positive for ER with high intensity; hatched squares are cells with graded levels of intermediate staining intensity; white squares are cells that are unstained for ER.

Type A is a focal expression of ER-negative cells that gradually divide peripherally until they predominate.

Type B is a graded expression of ER in which all of the tumor cells gradually decrease their ER content over time.

Type C is a "checkerboard" type of ER expression. The loss of ER occurs randomly throughout the tumor so that the distribution of ER-positive and ER-negative tumor cells is noticeably heterogeneous.


INTERMEDIATE ER STAINING PATTERNS
A
B
C

IV. DISCUSSION

1. Qualitative and quantitative correlations between SBA and ER-ICA methods

From Table 1, page 44, and Figure 6, page 50, a high qualitative correlation between the SBA and ER-ICA methods was obtained, but the quantitative agreement was not as close, though significant. It is reasonable to expect that the hormone- and antigen-binding sites reside at different domains of the ER protein molecule, therefore these results are encouraging in assessing ER from two viewpoints. One limitation on improving the quantitative correlation between SBA and ER-ICA is the closed "window" represented by high intensities of specific staining in the ER-ICA method; the eye is unable to resolve increases in specific staining intensity beyond a certain point.

As is evident in figure 4, page 46, the correlation is better for histoscores corresponding to ER concentrations of 10 - 100, than for >100, FMTP. For very low histoscores (<3), the corrected ER values for the corresponding microsamples are widely spread, indicating that SBA is probably more sensitive than ER-ICA for detecting low ER levels because of poorer resolution

at progressively lower levels of ER-specific staining.

The correlation between negative ER and histoscore values was very high. From Table 1, page 44 only 2 of the 81 microsamples (~2 %) were negative for SBA, but positive for ER-ICA; only 1 of the 81 microsamples was positive for SBA and negative for ER-ICA.

Again, this is possibly due to inherent differences in sensitivity for extremely low ER levels between the two methods, as well as the fact that different entitities are being measured.

2. Intermediate ER-specific staining patterns in ER-ICA method

Upon closer scrutiny of ER-ICA sections corresponding to intermediate ER concentrations, three distinct staining patterns were identified: focal, graded, and checkerboard. Whether these truly represent intermediates in the transition from receptor-positive to receptor-negative status is not known; however, the data suggest the existence of hypothetical intermediates within three pathways by which breast tumors might progress from hormone dependence to autonomy.

These are illustrated in Figure 12, page 54.

Pathway A is a focal pathway in which clones of receptor- positive and negative cells may be located within a tumor in varying proportions depending on their at any given time. Clonal expansion results in clusters of cells that are either entirely receptor-positive or negative; within a cluster they may represent consecutive, adjacent generations of daughter cells. The overall appearance is of large, confluent regions of specific staining with foci of negatively-stained cells presumably reflecting clonal expansion of the newly-variant ER-negative cells. The cluster-checkerboard and solid-checkerboard staining observed were the closest representations of this pathway, seen in 33 % of the ER-positive tumors. In most of these cases, the checkerboard pattern was adjacent to regions of solid-positive, suggesting that the checkerboard pattern was initially derived from the latter.

Pathways B and C are considered field pathways, whereby changes in ER level occur throughout the whole tumor rather than focally. The 'graded' pathway (B) was seen in only 1 of the 15 ER-positive tumors; all tumor cells were stained to a slight extent. Such a pattern might represent a more-or-less uniform loss of

ER in all tumor cells as an intermediate stage to endocrine autonomy. Alternatively, it may simply be a technical problem, with loss of ER arising from errors in procedure.

The checkerboard staining pattern was seen to some extent in slightly more than half of the ER-positive tumors, suggesting that pathway C may represent the more usual transition from receptor-positivity to receptor-negativity.

These field pathways are difficult to explain, since focal expansion of clones that arise by random mutations and their subsequent selection by the host environment or by chemotherapy, for example, has been the traditional view of tumor evolution (Nowell, 1986).

Field effects might involve epigenetic and environmental events in which changes in primary DNA structure are of more limited importance than chemical and/or physical interactions between the tumor cells and the host environment, and between the cells themselves.

Epigenesis is one of the classical principles of modern developmental biology, referring to sequential changes that arise from interactions between developing cells and tissues and their extracellular environments.

Normal differentiation is thus driven partly by epigenetic changes superimposed on differential expression of the genome in different locations and at different times in the developing organism.

In tumor cell evolution, epigenetic changes could be due to DNA hypomethylation and/or re-methylation (Sutter and Doerfler, 1980). Gene activation is often associated with hypomethylation at specific locations and might therefore be an epigenetic regulatory mechanism that can cause reversible qualitative changes in tumor cell characteristics (Kerbel et al. 1985).

Sirbasku and Danielpour (1985) have indicated that mixed tumors of hormone-dependent (ER-positive) and hormone-independent cells (ER-negative) may survive for extended periods of time.

In spite of the fact that the present study investigates deployment of ER only, and the data can not definitively be extended to explain intermediates between hormone-dependent and autonomous tumors, the good correlation that is generally obtained between the presence of ER and the dependence of breast cancer on estrogens suggests that similar pathways may operate here.

Possible explanations for field pathways, if indeed they do occur in breast cancer, are:

1) presence of a variant type of breast tumor in which escape from endocrine control occurs as a random event throughout the tumor;

2) random disposal of an already-redundant ER system of a tumor undergoing progression is occurring;

3) ER is not expressed at the same level and at the same time for all tumor cells if their cell cycles are asynchronous;

4) cell migration could be occurring, especially if metastatic potential increases with loss of ER, as clinical experience indicates;

5) the checkerboard pattern, especially, may be indicative of epigenetic, rather than purely genetic, mechanisms that lead to endocrine autonomy. Thus each cell's spatial relationship to other cells, its differential exposure to blood supply, tumorigenic factors including hormones, or perhaps autocrine secretions produced by the cell itself, might influence its capacity to continue producing a functional ER system.

Ideas similar to the latter have been expressed by Rubin (1985) using examples from developmental biology and tissue culture, including Harris' (1982) observations of Chinese hamster ovary cells grown in tissue culture in the presence of bromo-deoxyuridine (BDU). These cells lose their capacity to grow in HAT medium as part of the adaptation to BDU. When treated with the dedifferentiating agent 5-azacytidine, this capacity is regained, indicating that the adaptation was not likely due to genetic mutation.

3. Significance of heterogeneity to assessment of tumor ER status

Heterogeneity may be of importance for the progression of breast tumors from hormone-dependency to the autonomous state (Webster et al. 1978; Isaaks, 1985). In breast cancer it is thought that this is paralleled by an overall reduction in ER level (van Netten et al. 1985; Allegra, 1984; Nenci,1981).

Although progression to autonomy in human breast cancer can be demonstrated clinically, there is surprisingly little evidence that this is associated with loss of ER (Osborne, 1985; Holdaway and Mason, 1984). In fact, there is a general tendency for ER levels to

increase with age, and repeat biochemical ER analyses on 83 breast tumors (0.5-8 y between biopsies) do not reveal a general trend toward lower ER levels as tumors progress (van Netten, unpublished observations).

Therefore, the intermediate staining patterns observed in this series may, or may not, represent transition stages between ER-positive and ER-negative tumors. At present there is not sufficient information to resolve this problem.

However, if the field pathway of tumor modulation from hormone-dependency to autonomy hypothesized above is valid, by analogy to the known plasticity of epigenetic phenomena in normal differentiation, it is conceivable that ER-heterogeneous tumors could be returned to hormone dependency. Following suitable manipulation of the extracellular environment, they could then be treated clinically by endocrine-ablative therapy.

The feasibility of this approach has been strengthened to a limited extent by the report of van den Berg et al. (1987) in which an increase in ER expression within a human breast cancer cell line was induced by recombinant human interferon. Interestingly, this

simultaneously sensitized the cells to the anti-proliferative effects of the antiestrogen compound tamoxifen.

While the investigations of other workers using different cell lines have not yielded similar results, van den Berg and co-workers suggest that a combination of low doses of interferon before tamoxifen therapy is commenced might have potential as an in vivo treatment. At the same time, they caution that, since a whole cell binding assay was used, their observations could be due to enhanced translocation of estradiol- or tamoxifen-ER complexes and/or retention within the nucleus, and not necessarily be the consequence of enhanced ER synthesis.

Thus it is still not clear whether their data represent an increase in the number of cells expressing ER, or in ER levels within the ER-positive population (van den Berg, pers. comm.).

For many tumors a single ER assessment based on SBA or ER-ICA alone may not truly represent the ER status of the whole tumor (e.g. tumors CE and MD). This is particularly true with the ER-ICA method since only a very thin section of the tumor is analyzed.

Thus multiple sections from different areas of tumors might provide more definitive information about their ER status, and also detect heterogeneity of ER distribution within each tumor.

Because heterogeneity of ER may be the major reason for ineffective, or partially effective, response to hormonal treatment of breast cancer, (Leith and Dexter, 1986; Osborne, 1985) as well as influencing the duration of successful response (Leith and Dexter, 1986; Fidler, 1984; Nowell,1986), the assessment of the degree of ER heterogeneity using SBA and ER-ICA in a combined multiple microsample assay may provide more clinically useful information over that of a single ER determination.

Thus if two tumors express the same concentration of ER after adjustment for PCC, PSS, and SI, but one is homogeneous, and the other heterogeneous ER-positive, the latter might require more aggressive therapy in order to ensure effective restraint of tumor cells that have escaped endocrine control, whereas the former has a much greater probability of responding to antiestrogen therapy alone.

Additionally, the distribution of ER could improve understanding of the mechanisms that generate ER heterogeneity and modulate the tumor's growth progressively towards hormone autonomy. Once such mechanisms are fully elucidated, autonomous tumors could possibly be reverted to hormonal regulation by manipulation of their extracellular environment. In this way, the necessity for more aggressive forms of treatment, such as chemotherapy or radiation, could be avoided.

V. LITERATURE CITED

Allegra, J. C. 1984. Mechanisms of hormone response and resistance: clinical studies. Reviews on Endocrine-related Cancer. 14: 53 - 54.

Baker, R. Robinson, (Ed.). 1977. Current trends in the management of breast cancer. Johns Hopkins University Press, Baltimore. 130.

Beatson, G.T. 1896. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of illustrative cases. Lancet. 2: 104 - 107.

Burns, J. 1983. The unlabelled antibody peroxidase-anti-peroxidase method (PAP) In Techniques in Immunocyto-chemistry. G.R. Bullock and P. Petrusz, (Eds.). Academic Press, N.Y. 91 - 105.

Chamness, G., Mercer, W.D., McGuire, W.L. 1980. Are histochemical methods for estrogen receptor valid? J. Histochem. Cytochem. 28(8): 792 - 798.

Clark, J.H., Hardin, J.W., Eriksson, H., Upchurch, S., Peck Jr., E. 1978. Heterogeneity of estrogen binding sites in the cytosol and nuclear fractions of the rat uterus. Biochem. Biophys. Res. Commun. 81(1): 1 - 7.

Coffer, A.I. and King, R.J.B. 1974. Isoelectric focusing of (^{3}H) - oestradiol-17β in flat beds of Sephadex. Biochem. Soc. Trans. 2: 1269 - 1272.

Cuello, A.C., Milstein, C., Galfré, G. 1983. Preparation and application of monoclonal antibodies for immunohistochemistry and immunocytochemistry In Immunochistochemistry. A.C. Cuello, (Ed.). Wiley, Chichester. 215 - 256.

deSombre, E.R., Smith, S., Block, G.E., Ferguson, D.J., Jensen, E.V. 1974. Prediction of breast cancer response to endocrine therapy. 1973 Intra-Science Symposium: New Ideas in Cancer Chemotherapy. Cancer Chemotherapy Reports, Pt. 1. 58(4): 513 - 519.

----------, Carbone, P.P., et al. 1979. Steroid receptors in breast cancer. N. Engl. J. Med. 301: 1011 - 1012.

Erdos, T., Best-Belpomme, M, Bessada, R. 1970. A rapid assay for binding estradiol to uterine receptor(s). Anal. Biochem. 37: 244 - 252.

Falini, B. and Taylor, C.R. 1983. New developments in immunoperoxidase techniques and their application. Arch. Pathol. Lab. Med. 107: 105 - 116.

Fidler, I.J. 1984. Recent concepts of cancer metastasis and their implication for therapy. Cancer Treat. Rep. 68: 193 - 197.

Folca, P.J., Glascock, R.F. Irvine, W.T. 1961. Studies with tritium-labelled hexoestrol in advanced breast cancer. Lancet. 2: 796.

Glascock, R.F.and Hoekstra, W.G. 1959. Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. Biochem. J. 72: 673 - 682.

Godefroi, V.C. and Brooks, S.C. 1973. Improved gel-filtration method for analysis of estrogen receptor binding. Anal. Biochem. 51: 335 - 344.

Gorski, J., Toft. D.O., Shyamala, G. et al. 1968. Hormone receptors: studies on the interaction of estrogen with the uterus. Rec. Progr. Horm. Res. 24: 45 - 80.

Harris, M. 1982. Induction of thymidine kinase in enzyme-deficient Chinese hamster cells. Cell. 29: 483 - 492.

Henderson, C. and Canellos, G.P. 1980. Cancer of the breast: the past decade. N. Engl. J. Med. 302: 78 - 90.

Holdaway, I.M. and Mason, B.H. 1984. Does receptor status in breast cancer vary with time, site and treatment? Rev. Endocr. Rel. Cancer 19: 17 - 21.

Isaaks, J.T. 1985. "Progression" in breast and prostatic cancers. Rev. Endocr. Rel. Cancer. 20: 13 - 18.

Jensen, E.V. and Jacobson, H.I. 1960. Fate of steroid estrogens in target tissue In Biological Activities of Steroids in Relation to Cancer. G.Pincus and E.P. Vollmer, (Eds). Academic Press, N.Y. 161.

----------. 1962. Basic guides to the mechanism of estrogen action. Recent Progr. Horm. Res. 18: 387.

----------,Suzuki, T., Kawashima, T., Stumpf, W.E. 1968. A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Nat. Acad. Sci.U.S.A. 59: 632 - 638.

----------,Block, G.E., Smith, S., deSombre, E.R. 1973. Hormonal dependency of breast cancer. In Breast Cancer: A Challenging Problem. M. Griem (Ed.). Recent Results in Cancer Research. Springer-Verlag, N.Y. 42: 55 - 62.

----------, (Ed.). 1984. Symposium on Estrogen Receptor Determination with Monoclonal Antibodies. Cancer Res. (suppl.) 46(8).

Kerbel, R.S., Frost, P., Litlepo, Fidler, I.J. 1985. Genetic and epigenetic mechanisms for the development of metastatic heterogeneity in neoplasms. Proceed. Amer.Assoc. Cancer Res. 26: 394 - 395.

King, W.J. and Greene, G.L. 1984. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature. 307: 745 - 747.

Klute, T.E., Heidemann, P., Wittliff, J.L. 1978. Molecular heterogeneity of cytosolic forms of estrogen receptors from human breast tumors. Cancer Res. 38: 4307 - 4313.

Knight, III, W.A., Livingston, R.B., Gregory, E.J., McGuire, W.L. 1977. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. Cancer Res. 37(12): 4669 - 4671.

Kŏhler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256: 495 - 497.

Korenman, S.G. 1968. Radio-ligand binding assay of specific estrogens using a soluble uterine macromolecule. J. Clin. Endocrinol. Metab. 28: 127.

Lee, S.H. 1980. Cellular estrogen and progesterone receptors in mammary carcinoma. Am. J. Clin. Pathol. 73: 323 - 329.

Leith, J.T. and Dexter, D.L. 1986. Mammalian Tumor Cell Heterogeneity. CRC Press, Boca Raton.

Lippmann, M. and Huff, K. 1976. A demonstration of androgen and estrogen receptors in human breast cancer using a new protamine sulphate assay. Cancer. 38: 868.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with phenol reagent.J. Biol. Chem. 19: 26.

Marchetti, E.,Querzoli, P.,Moncharmont, B., Parikh, I., Bagni, A., Marzola, A., Fabris, G., Nenci, I. 1987. Immunocytochemical demonstration of estrogen receptors by monoclonal antibodies in human breast cancer: correlation with estrogen receptor assay by dextran-coated charcoal method. Cancer Res. 47: 2508 - 2513.

McGuire, W.L., Carbone, P.P., Sears, M.E., Eschert, G.C. 1975. Estrogen receptors in human breast cancer: an overview. In Estrogen Receptors in Human Breast Cancer. W.L. McGuire et al. (Eds.). Raven Press, N.Y. 1 - 7.

Mirecki, D.M. and Jordan, V.C. 1985. Symposium on steroid hormone receptors and human breast cancer. Laboratory Medicine 16: 287 - 294.

Nenci, I. 1981. Estrogen receptor cytochemistry in human breast cancer: status and prospects. Cancer. 48: 2674 - 2686.

Nowell, P.C. 1986. Mechanisms of tumor progression. Cancer Res. 46: 2203 - 2207.

Osborne, C.K., Yochmowitz, M.G. et al. 1980. The value of estrogen and progesterone receptors in the treatment of breast cancer. Cancer. 46: 2884 - 2888.

----------. 1985. Heterogeneity in hormone receptor status in primary and metastatic breast cancer. Semin. Oncol 12: 317 - 326.

Rubin, H. 1985. Random mutation vs. heritable adaptations as the source of malignant transformation and its aftermath. Proceed. Amer. Assoc. Cancer Res. 26: 392 - 393.

Saez, S., Cheix, F. et al. 1984. Estrogen and progestereone receptors as prognostic factors in early breast cancer. Recent Results in Cancer Research. Springer-Verlag, N.Y. 91: 192 - 198.

Santi, D.V., Sibley, C.H., Perriand, E.R. 1973 . A filter assay for steroid hormone receptors. Biochemistry. 12: 2412 - 2416.

Shafie, S.M. 1980. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science. 209: 701 - 702.

Sirbasku, D.A. and Danielpour, D. 1985. Role of autocrine/ paracrine growth factors in progression of hormone-dependent mammary tumors of autonomy. Proceed. Amer. Assoc. Cancer Res. 26: 393 - 394.

Sternberger, L.A., Hardy Jr., P.H., Cuculis, J.J., Meyer, H.G. 1970. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase: anti-horseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. 18: 315 - 333.

Sutter, D. and Doerfler, W. 1980. Methylation of integrated adenovirus type 12 DNA sequences in transformed cells is inversely correlated with viral gene expression. Proc. Nat. Acad. Sci. U.S.A. 77: 253 - 256.

Teulings, F.A.G., Blonk-van der Wyst, J., Portengen, H. et al. 1975. Quantitation of estrogen receptors in human breast cancers by agargel electrophoresis. Clin. Chim. Acta. 64: 27.

Thompson, E.B. and Lippmann, M.E. 1979. Steroid Receptors and the Management of Cancer, I. CRC Press, Boca Raton. 8.

Thorsen, T. 1980. Occupied and unoccupied estradiol receptor in human breast tumor cytosol. J. Steroid Biochem. 13: 405 - 408.

Toft, D.O. and Gorski, J. 1966. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc. Natl. Acad. Sci. U.S.A. 55: 1574.

van den Berg, H.W., Leahy, W.J., Lynch, M. et al. 1987. Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. Br. J. Cancer. 55: 255 - 257.

Vandesande, F. 1983. Peroxidase-antiperoxidase techniques In Immunhistochemistry. A.C. Cuello. (Ed.). Wiley, Chichester. 59 - 77.

van Netten, J.P., Algard, F.T., Montessori, G., Weare, B. 1977. Electrophoretic assay of specific estrogen receptors: a contribution to methodology. Clin. Chem. 23: 2059.

----------, Coy, P., Carlyle, S.J., Brigden, M.L., Thornton, K.R., To, M.P. 1982. Estrogen receptor assay on breast cancer microsamples: implications of percent carcinoma estimation. Cancer. 49(11): 2383.

----------, Coy, P., Carlyle, S.J., Brigden, M.L., Thornton, K.R., Peter, S., Fraser, T., To, M.P. 1985. Heterogeneous estrogen receptor levels detected ia multiple microsamples from individual breast cancers. Cancer. 56(8): 2019 - 2024.

Wagner, R.K. 1972. Characterization and assay of steroid hormone receptors and steroid-binding serum proteins by agargel electrophoresis at low temperature. Hoppe Seylers Z. Physiol. Chem. 353: 1235.

Walker, R.A., Cove, D.H., Howell, A. 1980. Histological detection of oestrogen receptor in human breast carcinomas. Lancet. 1: 171 - 173.

Webster, D.J.T., Bronn, D.G., Minton, J.P. 1978. Estrogen receptor levels in multiple biopsies from patients with breast cancer. Am. J. Surg. 136: 337 - 338.

Welshons, W.V., Lieberman, M.E., Gorski, J. 1984. Nuclear localization of unoccupied oestrogen receptors. Nature. 307: 747 - 749.

Wittliff, J.L., Beatty, B.W., Savlov, E.D. et al. 1976. Estrogen receptors and hormone dependency in human breast cancer. In Recent Results in Cancer Research. G. St. Arneault et al. (Eds.). Springer-Verlag, N.Y. 57: 59 - 77.

----------, and Savlov, E.D. 1978. Biochemical basis for selection of hormonal manipulation in the patient with breast carcinoma. Int. J. Radiat. Oncol. Biol. Phys. 4: 463 - 467.

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----------, Thornton, I., Carlyle, S., Brigden, M.L., Coy, P., Goodchild, N.L., Gallagher, S., George, E.J. 1987. Multiple microsample analysis of intratumor estrogen receptor distribution in breast cancers by a combined biochemical/ immunohistochemical method. Eur. J. Cancer Clin. Oncol. 23(9): 1337 - 1342.

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Title of Thesis

Detection and distribution of estrogen receptor in human breast cancer by a combined biochemical-immunohistochemical multiple microsample assay

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