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Nuclear and Cytoplasmic Estrogen Receptors and Progesterone Receptors in Breast Cancer

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Abstract—*Tissues from 83 primary human breast carcinomas were investigated for cytoplasmic estrogen receptors, cytoplasmic progesterone receptors, and free and occupied nuclear estrogen receptors, and the relationship between the various receptor fractions was determined. The two most frequently observed receptor distributions were cytoplasmic estrogen receptors with free nuclear estrogen receptors, with or without occupied nuclear estrogen receptors, in the absence of progesterone receptors. Carcinomas with all receptors either negative or positive were found in about equal numbers and were the next most frequently encountered distribution. Another common combination was the presence of cytoplasmic estrogen receptors only, with none of the other measured receptors.*

INTRODUCTION

THE QUESTION why not all estrogen receptor positive breast carcinomas respond to endocrine manipulation has not been answered satisfactorily. Various explanations have been offered [1], but experimental data to confirm, reject or modify current hypotheses are still urgently required. We have estimated cytoplasmic estrogen receptors, occupied and free nuclear estrogen receptors and cytoplasmic progesterone receptors in a series of 83 primary breast carcinomas. In this paper we wish to report on the relationship between the various receptors in the tissues.

MATERIALS AND METHODS

Tissues

The tissues were primary breast carcinomas from patients aged between 30 and 86 yr. The average age was 59.7 and the median age 61 yr. The large majority (83%) were postmenopausal (>50 yr).

The tissues were snap-frozen and stored at −20°C if they could not be worked up immediately. The preparation of the cytosol and of the nuclear fraction was as follows: A 1-in-6 homogenate of the tissue in ice-cold 10 mM Tris–HCl buffer, pH 8.0 containing 0.5 mM dithiothreitol was prepared using a Sorvall omnimixer (four bursts at maximum speed for 15 sec, with 45 sec intervals). The homogenate was centrifuged for 10 min at 800 ***g***, and the supernatant decanted and spun again for 40 min at 100,000 ***g*** to obtain the *cytosol*. The pellet obtained from the low speed centrifugation was resuspended in 1 vol of Tris buffer and centrifuged for 10 min at 800 ***g***. The supernatant was discarded and the washed pellet weighed. Five volumes of Tris buffer containing in addition 0.4 M KCl and 0.558 g EDTA-Na_2 per litre were added to the pellet which was left at 4°C for 60 min with occasional vortexing. Subsequently the suspension was centrifuged for 40 min at 100,000 ***g*** to obtain the *nuclear extract*.

Four different extraction procedures for the nuclear estradiol receptor were compared:

1. A low speed pellet, washed once and homogenized with KCl and centrifuged.
2. A high speed pellet, washed once and homogenized with KCl and centrifuged.
3. A low speed pellet, washed once, extracted for 1 hr with KCl at 4°C and centrifuged.

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4. A high speed pellet, washed once, extracted for 1 hr with KCl at 4°C and centrifuged.

Unpublished results show that there is little difference between using a high or a low speed pellet. However, 1 hr/4°C extraction with KCl solution appears much more suitable than homogenizing the pellet with KCl. A further experiment showed that there is little difference in contamination with cytosol after washing the pellet once or twice before the extraction step. The procedure adopted follows closely the one described by Zava [2].

Estimation of cytoplasmic estrogen receptor (ER_c)

Cytoplasmic estrogen receptors were measured as described [3] by incubating cytosol with ^{3}H-estradiol and increasing amounts of nonradioactive estradiol at 25°C for 60 min. The sensitivity of this assay was determined by serially diluting a very high binding cytosol solution with a non-binding cytosol solution of the same protein concentration so as to obtain a series of dilutions of the estrogen receptor at a constant protein concentration and then performing a single point assay to determine the specific binding of the various receptor dilutions. The limit of the sensitivity of the assay is taken as the value for specifically bound estrogen at which the plot of bound estradiol vs dilution factor plateaus. The cut off value thus obtained was 4 fmole /mg protein. However it was decided that the values usually reported as borderline cases i.e., 4–8 fmole/mg protein would also be considered as receptor negative.

Estimation of cytoplasmic progesterone receptors (PR_c)

Progesterone receptors were determined according to Pichon and Milgrom [4]. This method uses progesterone as the ligand and cortisol to distinguish receptor binding from binding to CBG (corticosteroid-binding globulin). The sensitivity of this assay was determined using the same technique as for the cytoplasmic estrogen receptor assay. The cut-off value thus obtained was 10 fmole/mg protein. All values below this point were taken as being receptor negative and all values greater than 10 fmole/mg protein as being receptor positive.

Estimation of nuclear estrogen receptors

Results of unpublished preliminary experiments have shown that at 30°C the specific binding increased steadily up to 5 hr and then started to decrease over longer periods of incubation (20 hr) as the effect of any protease activity became predominant. It was, therefore, decided to adopt the following procedures:

For the measurement of the *free nuclear estrogen receptors* (R_N) 200 µl aliquots of the nuclear extract were incubated in triplicate with 20,000 dis/min of tritiated estradiol (spec. act. 42 Ci/mmole) without or with 50, 100, 200, 1000 or 5000 pg of nonradioactive estradiol, respectively, for 20 hr at 4°C. For the estimation of *total nuclear estrogen receptors* the incubation was done at 30°C for 4 hr and the steroid concentrations were increased to 100,000 dis/min tritiated estradiol and 250, 500, 1000, 5000, 25000 pg of nonradioactive estradiol, respectively. At the end of the incubation the incubation mixture was cooled to 4°C (if necessary), and 1 ml of dextran coated charcoal suspension in buffer was added (10 mM Tris–HCl buffer pH 8.0 containing 0.5% Norit A, 0.05% dextran T70 and 0.037% EDTA-Na_2). The solution was vortexed and left for 20 min in an ice bath. The tubes were then centrifuged for 10 min at 800 ***g*** and the supernatant was decanted into counting vials. Eight ml of Bray's scintillator was added to each tube for liquid scintillation counting. *Occupied nuclear estrogen receptors* (ER_N) were calculated as the difference between total and free receptors [2]. The sensitivity of this assay was determined as described above and a plateau value of 8 fmole /mg protein was obtained in the graph of bound estradiol versus dilution factor. A comparison between our adopted method and the hydroxyapatite method for nuclear estrogen receptor assay described by Garola and McGuire [5] is at present under investigation.

Other methods

Protein (in the cytosol and nuclear extracts) was measured by the Folin method and DNA (in the nuclear pellet) by the indol reaction as before [6].

RESULTS

The concentration of the various receptors in the breast cancer tissues is shown in Table 1. In this table the results are arranged in increasing order of the estrogen receptor levels in the cytosol (ER_c). It should be noted that mainly ER_c+ tumors (binding > 8 fmole/mg) were selected for this study since it is in this

Table 1. Cytoplasmic estrogen and cytoplasmic progesterone receptors, nuclear bound and free estrogen receptors in human breast carcinoma

Patient code	ER_c	R_N	ER_N	PR_c
WY-1	0.0	0.8	0.0	—
KL-2	0.4	—	—	—
PA-8	1.7	0.8	4.9	—
FE-5	2.6	0.6	1.7	0.4
HY-1	3.6	2.7	1.3	0.0
PO-8	4.8	10.2	11.0	0.0
SA-6	6.2	0.0	1.9	3.3
JO-7	6.5	2.3	5.2	0.0
SM-14	6.6	6.4	4.5	12.9
TH-8	6.6	4.8	0.0	0.0
RE-5	6.8	9.4	4.1	2.8
JA-6	6.9	4.5	3.8	0.0
JE-3	7.1	2.8	4.3	—
TO-6	7.2	20.0	3.3	0.0
CL-5	7.4	0.6	0.0	5.8
BR-13	7.6	11.5	4.5	8.5
SI-3	8.3	14.0	14.8	0.0
CO-3	8.3	0.8	3.9	3.7
HA-11	8.5	42.4	11.0	3.8
FE-6	8.5	2.0	0.0	1.5
KE-7	8.8	28.1	86.5	11.5
MT-1	8.9	8.3	1.8	—
DO-14	9.2	5.0	0.5	2.1
MB-1	9.7	79.1	33.2	4.4
CR-9	9.8	25.0	5.4	0.0
VE-2	10.3	13.5	7.0	2.5
MO-16	10.6	19.1	2.3	0.0
EL-3	10.6	11.8	41.9	22.0
HA-26	11.0	5.2	0.0	1.84
GR-11	11.5	28.0	—	—
SP-2	11.7	3.3	2.6	0.0
CO-10	12.0	7.3	3.2	0.0
SL-1	12.2	10.3	—	0.0
BA-19	12.5	4.1	27.8	6.9
SI-6	12.5	3.1	0.0	0.3
FE-4	12.6	11.3	0.0	9.5
KE-8	14.0	10.3	20.2	10.4
JO-10	15.4	10.7	0.0	0.6
RO-11	15.7	15.5	48.8	0.0
ST-13	16.1	28.3	0.0	0.0
WA-14	16.5	59.9	14.7	35.4
CA-13	17.2	20.2	7.8	4.3
MA-19	18.0	11.9	3.3	0.0
BO-8	18.0	11.7	—	44.8
BE-7	18.0	19.2	—	15.5
MA-21	19.0	8.1	3.1	—
WE-10	23.0	11.0	2.2	0.0
BO-12	26.7	28.2	22.8	21.9
VI-2	27.0	9.8	0.0	—
CO-13	28.2	30.3	9.2	0.0
FR-4	29.5	19.8	14.6	6.7
SN-3	29.6	3.4	0.0	0.0
GO-6	32.6	15.6	12.4	17.0
HO-7	32.7	32.5	0.0	—
DA-15	33.3	31.0	48.7	6.9
WA-10	36.6	0.0	21.9	5.5
MK-2	37.6	30.3	16.6	—
NI-7	40.0	19.4	1.1	7.3
WI-14	40.0	32.0	1.5	—
NE-4	40.0	39.0	78.8	0.0
UR-1	43.7	28..9	6.5	115.6
SH-7	44.1	122.1	16.9	0.0
RY-4	44.2	35.7	0.0	1.8
GR-10	58.7	27.3	0.0	0.1
MM-3	59.8	24.0	—	—
SZ-1	61.9	57.0	0.0	2.0
MI-10	64.8	7.4	0.0	—
BR-17	65.3	51.6	0.0	0.0
JO-8	67.0	110.9	0.0	0.0
CA-15	69.0	40.4	0.0	6.2
RE-14	74.3	128.5	0.0	3.4
VI-4	76.0	82.9	27.1	55.5
BU-10	79.5	41.0	—	7.2
JA-8	83.8	8.2	—	6.4
SM-16	99.2	67.5	0.0	5.2
DU-6	106.0	25.7	0.0	—
MY-1	107.9	43.0	42.3	68.7
BA-15	108.5	12.5	—	8.7
BR-19	113.8	4.6	—	0.9
TR-1	119.0	12.4	0.0	3.3
SM-16	126.7	36.4	7.6	4.4
HA-29	129.3	70.9	0.0	0.3
DU-7	210.9	41.7	0.0	0.2

All results are given in fmole steroid bound per mg soluble protein, (−) Not done (insufficient tissue); ER_c cytoplasmic estrogen receptor; PR_c, cytoplasmic progesterone receptor; ER_N, occupied nuclear estrogen receptor; R_N, free nuclear estrogen receptor.

group that further discrimination is required for prediction of response to endocrine treatment. Since the lack of tissue precluded full details of saturation analyses for some tissues, the results in Table 1 represent single-point assays, i.e., data obtained from the difference in binding of tritiated steroid (1 nM) in the presence and absence of 100-fold excess non-radioactive steroid.

The average estrogen receptor concentration in the cytosol was 35.3 fmole/mg protein with an S.D. of 39.1 and it ranged from 0.0 to 210.9. The median result (50% percentile) was 17.2. Cytoplasmic progesterone receptors ranged from 0.0 to 115.6 fmole/mg protein and the mean concentration ± S.D. was 8.3 ± 18.2 The median was 2.5. The statistics of the soluble nuclear estrogen receptors were as follows: Free nuclear estrogen receptor, mean ± S.D. 24.1 ± 26.7, range 0.0–128.5 fmole/mg protein, median 13.8; occupied nuclear estrogen receptor, mean ± S.D. 9.9 ± 17.1, median 3.2, range 0.0–86.5 fmole /mg protein. These data are summarized in Table 2. Linear correlations between the various receptors were calculated. A significant positive coefficient of correlation (r) was obtained only for the pair: cytoplasmic estrogen receptor vs free nuclear estrogen receptor ($n=82$, $r=0.42$, confidence limit $>99.9\%$). There was no correlation between progesterone and estrogen receptors in our series if all results were compared regardless of re-

Table 2. Summary of statistical data of receptors in breast cancer tissue

	Age of patients (yr)	ER_c	R_N	ER_N	PR_c
Number	79	83	82	73	69
Mean	59.7	35.3	24.1	9.9	8.3
S.D.	12.6	39.1	26.7	17.1	18.2
S.E.M.	1.4	4.3	3.0	2.0	2.2
Median	61	17.2	13.75	3.2	2.5
Range	30–86	0–210.9	0–128.5	0–86.5	0–115.6

Table 3. Cytoplasmic (ER_c) and free nuclear estrogen receptors (R_N) in breast cancer tissues

	ER_c+	ER_c-	Total
R_N+	55	4	59
R_N-	12	11	23
Total	67	15	82

$\chi^2=18.6$. $\chi^2_{corr.}=16.0$ (>99.9%).
ER+ is defined as binding of 8 or more fmole/mg protein, ER− is binding of less than 8 fmole/mg protein.
$\chi^2_{corr.}$ Yates correction.

ceptor status. However, if the comparison was restricted to PR_c+, ER_c+ tissues, a significant positive correlation was obtained for the two cytosol receptors ($n=11$, $r=0.58$, $p<0.05$) and between the free nuclear estrogen receptors and the progesterone receptor (PR_c+, R_N+; $n=10$, $r=0.61$, $P<0.05$) while there was no correlation between PR_c+ and occupied nuclear estrogen receptors (ER_N). By defining steroid binding values greater than 8 fmole/mg protein for estrogen receptors and greater than 10 fmole/mg protein for progesterone receptor as receptor positive and those below these values as receptor negative, the following frequencies were obtained for the various receptor combinations in the cancer tissues (Tables 3–8). The tables were evaluated by Chi-square tests with Yates correction. In the majority of cases (55 of 67) tissues with cytoplasmic estrogen receptors also contained free nuclear estrogen receptors; only four of 15 ER_c- tissues contained free nuclear estrogen receptors. This difference is statistically significant (Table 3.) A similar distribution was obtained if cytoplasmic and total nuclear estrogen receptors were compared. In contrast, less than half (20 of 58) of the specimens with cytoplasmic estrogen receptors had estrogen occupied nuclear receptor sites (Table 4). Only one of 15 tissues without cytoplasmic estrogen receptors contained occupied nuclear estrogen receptors (Table 4). The relation between free and occupied nuclear estrogen receptors in the tissues is shown in Table 5. Most of the tissues with occupied nuclear estrogen receptors also contain free nuclear estrogen receptors (19 of 21), and over half of the tissues without appreciable amounts of occupied nuclear estrogen receptors do contain free nuclear estrogen receptors. Only about 20% of ER_c positive tissues contained cytoplasmic progesterone receptors as well (Table 6).

Table 4. Cytoplasmic and occupied nuclear estrogen receptors in breast cancer tissues

	ER_c+	ER_c-	Total
ER_N+	20	1	21
ER_N-	38	14	52
Total	58	15	73

$\chi^2=4.5$. $\chi^2_{corr.}=3.2$ (n.s.).

Table 5. Free and occupied nuclear estrogen receptors

	ER_N+	ER_N-	Total
R_N+	19	32	51
R_N-	2	20	22
Total	21	52	73

$\chi^2=5.9$. $\chi^2_{corr.}=4.6$ (>95%).

It is worth noting, on the other hand, that 11 of 12 progesterone receptor positive tissues contained free nuclear estrogen receptors (Table 7). In the progesterone receptor negative group about two thirds contained free nuclear estrogen receptors. The proportion of tissues with occupied nuclear estrogen receptors was significantly higher in the progresterone receptor positive group than in the progesterone receptor negative group (Table 8).

With the limited number of tissues studied so far, and the considerable number of possible receptor combinations, only preliminary observations can be made on the frequency of certain configurations. A summary of the observed receptor combinations is given in Table

Table 6. *Cytoplasmic estrogen and progesterone receptors*

	ER_c+	ER_c-	Total
PR_c+	11	1	12
PR_c-	46	11	57
Total	57	12	69

$\chi^2=0.8$. $\chi^2_{corr.}=0.2$ (n.s.)

Table 7. *Cytoplasmic progesterone receptor and free nuclear estrogen receptor*

	PR_c+	PR_c-	Total
R_N+	11	39	50
R_N-	1	18	19
Total	12	57	69

$\chi^2=2.7$. $\chi^2_{corr.}=1.6$ (n.s.).

Table 8. *Cytoplasmic progesterone receptor and occupied nuclear estrogen receptor*

	PR_c+	PR_c-	Total
ER_N+	8	12	20
ER_N-	2	40	42
Total	10	52	62

$\chi^2=12.4$. $\chi^2_{corr.}=10.0$ ($>99.7\%$)

9. The two most frequently encountered receptor distributions were ER_c+, R_N+, ER_N-, PR_c-, (22 tissues), followed by ER_c+, R_N+, ER_N+, PR_c-, (nine tissues). Carcinomas with all receptors either negative or positive were found in approximately equal numbers (15 tissues total). Another more frequent combination was the presence of only cytoplasmic estrogen receptors and no other receptors (eight cases).

In about two thirds of cases the free nuclear estrogen receptors were in excess of the occupied nuclear estrogen receptors; the average ratio of free to occupied nuclear estrogen receptors of all cases was 3.1. The average ratio of R_N/ER_N in the tissues where this value was greater than 1, was 4.4; in those where it was less than 1, the average ratio was 0.4.

Total nuclear estrogen receptors were higher than cytoplasmic estrogen receptors in slightly less than half the tissues (46%). The average ratio ER_N total/ER_c in this group was 3.8, while it was 0.4 in the remainder. The mean ratio of all tissues was 2.0.

In about one quarter of tissues the ratio of free nuclear estrogen receptors to cytoplasmic estrogen receptors was greater than 1. The average value for the ratio in this group was 2.3. In the majority the receptor concentration was greater in the cytoplasm (mean ratio 0.5). The mean ratio for all tissues was 1.0. An even smaller proportion of tissues contained more occupied nuclear estrogen receptors than cytoplasmic receptors (17%). The average ratio ER_N/ER_c in this group was 3.0 while in the other it was 0.2. The overall mean ratio was 0.7. The results are summarized in Table 10.

Table 9. *Frequency of various steroid receptor combinations in breast cancer tissues*

ER_c	R_N	ER_N	PR_c	No. of tissues	Percentage of total
+	+	−	−	22	35.5
+	+	+	−	9	14.5
+	+	+	+	8	12.9
−	−	−	−	7	11.3
+	−	−	−	8	12.9
+	+	−	+	1	1.6
−	+	−	−	3	4.8
+	−	+	−	2	3.2
−	+	+	−	1	1.6
−	−	−	+	1	1.6
				62	100%

Binding of more than 8 fmole/mg protein was considered receptor positive for estrogen receptors; the cut-off point for the progesterone receptor was 10 fmole/mg.

DISCUSSION

The results presented in this paper could explain why not all cancer tissues with measurable cytoplasmic estrogen receptors respond to endocrine manipulation. It is generally accepted that binding to the cytoplasmic receptor is only one, probably the first step, in the mechanism of estrogen hormone action. Subsequent events include the transfer of the estrogen-receptor complex to the nucleus and binding to nuclear acceptor sites. If there is a fault anywhere in this sequence, hormonal regulation is impaired, even in the presence of normal amounts of cytoplasmic hormone receptors.

Less than half of the cancers with significant amounts of cytoplasmic receptors also contained estradiol bound to nuclear receptors. Garola *et al.* [5] observed a greater proportion of cancer tissues that contained both cytoplasmic estrogen receptors and occupied nuclear estrogen receptors: of 21 tissues with measurable cytoplasmic estrogen recep-

Table 10. *Ratios of various concentrations in cytoplasm and nuclei*

	R_N/ER_N			$(ER_N+R_N)/ER_c$			R_N/ER_c			ER_N/ER_c		
	All cases	Ratio >1	Ratio <1	All cases	Ratio >1	Ratio <1	All cases	Ratio >1	Ratio <1	All cases	Ratio >1	Ratio <1
Number	48	32	16	72	33	39	81	23	58	72	12	60
Mean	3.1	4.4	0.4	2.0	3.8	0.4	1.0	2.3	0.5	0.7	3.0	0.2
S.D.	4.1	4.5	0.3	3.5	4.6	0.3	1.2	1.6	0.3	1.4	2.3	0.3
S.E.M.	0.6	0.8	0.1	0.4	0.8	0.05	0.1	0.3	0.04	0.2	0.7	0.03
Range	0.0– 21.3	1.02– 21.3	0.0– 0.9	0.0– 24.1	1.1– 24.1	0.0– 0.9	0.0– 8.1	1.06– 8.1	0.0– 0.99	0.0– 9.8	1.3– 9.8	0.0– 0.9
Median	1.8	2.8	0.3	0.9	2.4	0.5	0.7	1.7	0.5	0.3	2.3	0.2

tors, 17 had nuclear receptor sites occupied by estrogen. This difference could be due to some loss of nuclear receptors due to protease [7] in our assay. If one introduces to the data of Garola cut-off points for ER_c+ and ER_c-, similar to the ones used by us, the results become much more similar to ours.

Most of our tissues with occupied nuclear estrogen receptor had also significant levels of free nuclear estrogen receptors. Garola found a similar proportion. About 60% of tissues without occupied nuclear estrogen receptors were found to contain free nuclear estrogen receptors in our series, while Garola observed only one case (of 11) in which measurable amounts of free nuclear estrogen receptors were present in the absence of measurable amounts of occupied nuclear estrogen receptors.

Panko and MacLeod [8] reported on free nuclear and cytoplasmic estrogen receptors in 139 human breast cancers. Their mean values for cytoplasmic estrogen receptors were similar to ours while our average free nuclear estrogen receptors were higher than theirs (24.1 compared with 10.3). They also found a small proportion of cases with positive nuclear receptors and negative cytoplasmic receptors (3%); in our series the proportion was 6%. While our largest group (64%) consisted of tissues with both cytoplasmic and free nuclear estrogen receptors, this group comprised only 27% in Panko's series. The group with neither cytoplasmic nor free nuclear estrogen receptors was smaller in our series (13%) than in Panko's (34%). This is probably due to the fact that we selected mainly ER_c+ tissues for this study.

Since the biosynthesis of progesterone receptors is controlled by estrogen, Horwitz *et al.* [9] have proposed the measurement of progesterone receptors as a means of testing the sequence following the initial receptor binding and thus improving the selection of breast cancer patients for endocrine therapy. Evidence for the usefulness of this proposal has been presented [10]. It was, therefore, of interest to correlate progesterone receptors with both cytoplasmic and nuclear estrogen receptors in the cancer tissues. If only receptor positive tissues were selected a significant correlation was obtained between the two cytoplasmic receptors (ER_c+, PR_c+). In a much larger series by McGuire *et al.* [11] it was similarly noted that the likelihood of finding progesterone receptor in a breast cancer increases with increasing cytoplasmic estrogen receptor concentration. The distribution of cytoplasmic progesterone and estrogen receptors in the tissues in the tissues we studied was similar to that reported by McGuire [10] for ER_c- tissues (ER_c-, PR_c-, 19%; ER_c-, PR_c+, 1%), but different for the ER_c+ carcinomas, in that we found a smaller proportion of ER_c+, PR_c+ specimens (20 of 73, or 27%, as compared to 55% in McGuire's series).

The average ratio between free and occupied nuclear estrogen receptor in the breast cancer tissues of this series (3.1) is somewhat higher than the ratio calculated from Garola's data (2.6, ref. [5]), but the range of results and the proportion of ratios above and below 1 are very similar. For the ratios between the nuclear estrogen receptors and the cytoplasmic estrogen receptor we found about 2–3

fold higher average values than those one obtains from Garola's or Panko's [8] data, and the percentages of ratios above 1 were considerably higher in our series. This is most likely due to different methodologies employed for the assays of cytoplasmic and nuclear receptors.

Which clinical significance can be attached to the various receptors and receptor fractions will only become apparent after prolonged clinical follow-up of the patients.

Addendum—In a recent paper [A. GEIER, R. GINZBURG, M. STAUBER and B. LUNENFELD, Unoccupied binding sites for estradiol in nuclei from human breast carcinomatous tissue. *J. Endocr.* **80,** 281 (1979)] the presence of unoccupied binding sites for estradiol in extracts from cell nuclei from human breast carcinoma has been confirmed.

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