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Multiple Steroid Receptors in Human Breast Cancer

II. Estrogen and Progestin Receptors in 672 Primary Tumors

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Summary. *Estrogen (E) and progestin (P) receptors (R) were investigated in 672 human primary breast tumors (664 from women and 8 from men). Premenopausal patients have both a lower frequency of ER positive tumors (64%) and a lower ER content (80 ± 63 fmol/mg protein) than postmenopausal women (74% and 249 ± 351 fmol/mg protein). All eight cases of male breast cancer were ER-positive. PR was found in 45% of the tumors. In contrast to ER, the presence of PR and the absolute PR level are not related to patient age. Except for the month of March, the mean monthly ER values do not fluctuate in postmenopausal patients: A peak is observed in the ER content of breast cancer from patients who underwent surgery during the month of March. Absolute ER content appears to be statistically unrelated to the tumorous cellular density (TCD); expression of the results as femtomoles per milligram of protein gives an underestimate of the actual receptor concentration. TCD must thus be considered in interpretation of the ER content. The resuls are in agreement with the idea that the steroid receptors are a characteristic of a population of cancer cells within a breast tumor and that ovarian factors are implicated in the steroid receptor content.*

Introduction

During neoplastic transformation, some breast cancers retain hormone dependence of normal mammary tissue and may regress with appropriate endocrine therapy [11, 28, 44, 45]. Steroid receptor sites seem to be a common denominator and the most useful marker for hormone dependence or responsiveness [11, 18, 29, 30, 37, 46]. The presence of estradiol receptor (ER) and more recently that of progesterone receptor (PR) have been shown to be helpful guides to the therapy of breast cancer [13, 17, 20, 29, 36].

Within the last three years, more than 800 primary and metastatic breast cancers have been investigated for ER and PR contents in this laboratory. The technical features of the assay have already been published [25].

In this paper, we will examine the correlations between receceptor status and certain other parameters such as patient age, menopausal status, and tumor histology.

The abbreviations used are SR, steroid receptor; ER, estrogen receptor; PR, progestin receptor ; TCD, tumorous cellular density; HPG, histoprognostic grade; HT, histological type.

Materials and Methods

Estrogen (E) and Progestin (P) Receptors (R) were investigated in 672 human primary breast tumors. The specimens were obtained from 664 women and 8 men. For each patient the following clinical observations were recorded: age, date of operation, endocrine status, and clinical staging. At the time of surgery tumors were divided into two representative parts, one for pathologic examination and the other for R analysis. The tumors for R analysis were stored in liquid nitrogen until processing. The simultaneous determination of tumor ER and PR content in cytoplasmic extracts has already been described [25].

Protein in the cytosol was measured according to Lowry et al. [23]. DNA was determined in the pellet obtained after high-speed centrifugation by the method of Burton [3]. The correlation between cytoplasmic protein and DNA was calculated as related to the weight of frozen tissue and total cytosol volume obtained after centrifugation.

Pathologic Features of the primary tumors based on examination of routine slides were reviewed by one of the authors [39]. To estimate the cellular density in tumors we have established an arbitrary classification of carcinomas according to the tumorous cellular density (TCD) on a scale from I to III. TCD I includes cases where the tumor epithelial all component is low in comparison to the stromal component. TCD III is the opposite, i.e., the stromal component

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is low relative to the epithelial component. Tumors that are intermediate in cell composition are categorized as TCD II.

The Statistical Analysis was based on analysis of variances, Student's t-test, and the χ^2 method.

Results

Estrogen Receptor

In the present study, estrogen receptor (ER) content was investigated in 672 cases of human primary breast cancers detected in the Marseilles (France) area.

The Incidence of Breast Cancer (white + black areas of the histogram) and the presence of estrogen receptors (black areas) as a function of patient age are shown in Fig. 1. The highest incidence of breast cancer occurs in patients in the 50- to 70-year age groups. The median age was 64 years. Thus in this series the higest incidence of breast cancer was in the postmenopausal population. This is in agreement with previous findings in other areas [34].

The Presence of ER is Related to the Age of the Patients. When premenopausal patients (30–50 years) are compared with postmenopausal women (50–90 years), χ^2-analysis of the frequency of positive ER tumors in relation to the age of patients reveals a significant correlation ($P < 0.001$). Premenopausal patients (64%) have a lower frequency of positive ER than postmenopausal women (74%) ($P < 0.05$). When the incidence of ER-positive tumors is examined according to the age groups of patients, we find that 40% of tumors in the 20–30 age group were positive for ER. In contrast, 64% in the 30–50 age group, 74% in the 50–90, group and 100% in the over-90 group were ER-positive. All eight cases of male breast cancer were ER-positive. This is in agreement with other studies [2, 22, 35, 42].

ER Content in Relation to the Age of Patients was studied in 494 patients with ER-positive tumors. The age of menopause was arbitrarily set at 50 years of age (the mean of the general population). The results are shown in Fig. 2. ER content varied widely from quite undetectable levels to over 1,800 fmol/mg protein. However, in patients less than 50 years of age (except artificially menopausal women) the average ER content was significantly lower than in patients over 50 ($P < 0.001$). The average ($\pm$ SD) ER content was 80 $\pm$ 63 fmol/mg protein in premenopausal women ($n = 82$ of 131 mammary primary tumors) and 249 $\pm$ 351 fmol/mg protein in postmenopausal women ($n = 412$ out of 541 cases). The high standard deviation in patients above 50 years of age is due to the tumors with very high ER contents

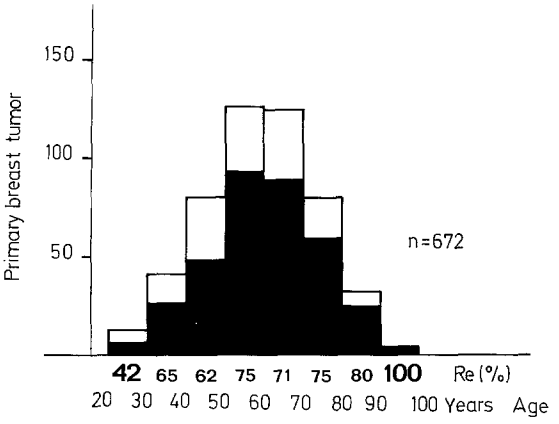

Fig. 1. Distribution of estrogen receptor by patients' age in 672 cases of breast cancer in relation to the age of patients (10-year age groups). The black areas represent positive ER tumors. $n = 672$

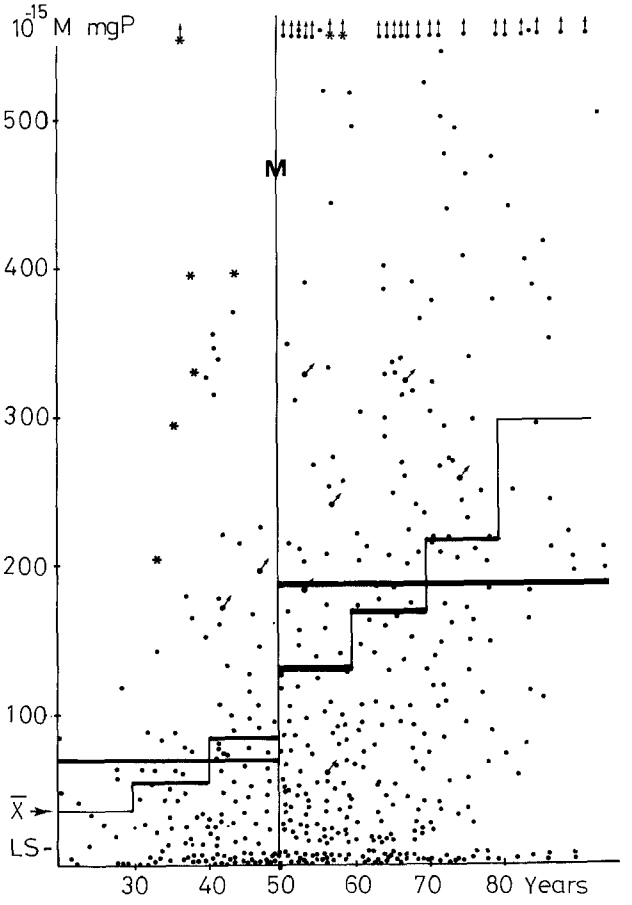

Fig. 2. Estradiol receptor (ER) content in 672 primary breast cancers in relation to the age of patients. Eight male breast tumors are included (↗). Stars represent the artificially menopausal women. ER values above 600 fmol are shown as vertical arrows. The mean ER content is shown for before and after the menopause (M) and for each age group (10 years)

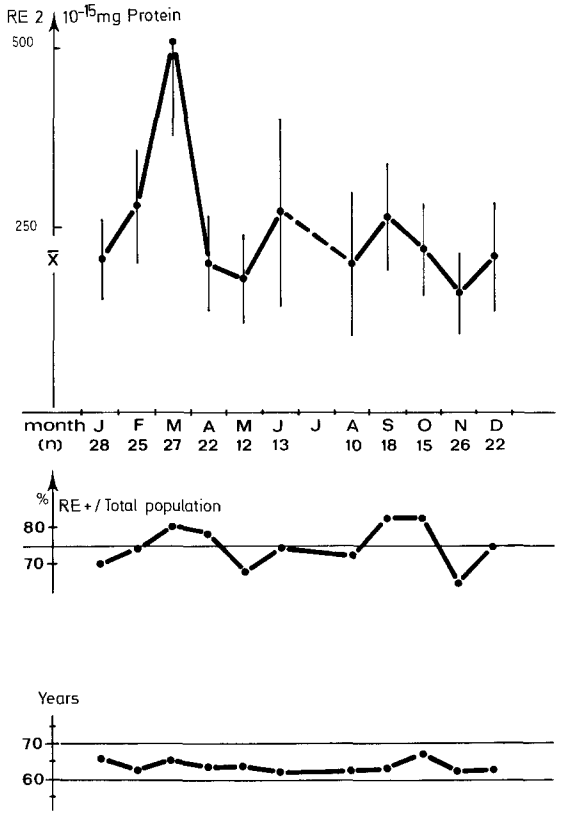

Fig. 3. Estradiol receptor (ER) content in 186 primary cancers from postmenopausal patients who had received no cancer therapy. The results are expressed in relation to the date (month) of surgery. (*n*) is the number of patients. The upper part represents the fluctuations of the mean monthly values (± SD) of ER content. The middle part shows the frequency of the presence of ER. The lower part gives the mean ages of patients

(over 1,000 fmol). The results confirm the previous findings of several other studies on relatively small numbers of patients [11, 22, 30].

Fluctuations in the Mean Monthly ER Values were examined in 186 primary mammary tumors from postmenopausal women who had not been subjected to any cancer therapy. As shown in Fig. 3, both the frequency of ER presence and the age of the patients (mean = 64.4 years) were similar to those in the breast cancer population as a whole. It is apparent that the ER content was two to three times higher in patients who underwent surgery in March than in the others ($P < 0.03$). This higher ER content observed in tumors resected in March was not due to a predominance of a different histological type of tumor (data not shown). The data are in agreement with those of Hughes et al. [16].

Estrogen and Progestin Receptors

Since both ER frequency and content increase with the age of patients and because of the suggestion by Horwitz et al. [13, 14] of a possible association of PR to ER, we examined the PR distribution and correlation of ER and PR with reference to the age of patients. The incidences of ER and PR in 498 primary breast tumors are depicted in Table 1.

The frequency of PR in tumors is lower than that of ER ($P < 0.01$) and in contrast to ER, is not related to age (averaged to 45%) except in the oldest age group, where ER content was the highest and all patients had

Table 1. Estrogen (R) and progestin (P) receptors (R) in 498 primary breast tumors in relation to the age of patients (10-year age groups). The frequency of the presence of ER (ER+) and PR (RP+) and the four different populations of receptors are expressed as percentages of the total population. The ER contents (± SD) of the tumors are expressed in relation to the age of patients (10-year age groups), before and after menopause (50 years). ER contents of tumors from menopausal patients are shown before ($n = 372$) and after ($n = 357$) the withdrawal of the highest ER values (over 1,000 fmol)

	Age (years)							
	20/30	30/40	40/50	50/60	60/70	70/80	80/90	> 90
Sample (*n*)	10	38	69	133	124	80	35	9
RE+	40%	60%	65%	73%	70%	69%	73%	100%
RP+	40%	50%	40%	41%	56%	54%	54%	100%
Receptor population								
RE+ RP+	40%	42%	34%	36%	50%	46%	54%	100%
RE+ RP−	—	18%	31%	37%	20%	23%	19%	—
RE− RP+	—	8%	6%	5%	6%	8%	—	—
RE− RP−	60%	32%	29%	22%	24%	23%	27%	—
ER content fmol/mg protein	22 ± 20	43 ± 42 71 ± 57	83 ± 63	131 ± 176	170 ± 211	213 ± 179 269 ± 347 (*n* = 372) 185 ± 199 (*n* = 357)	295 ± 194	425 ± 245

ER and PR. Table 1 also shows the four different populations of receptors in relation to the age of patients. Few tumors (less than 8%) contained PR only. In contrast, 40%–50% of the lesions examined contained both ER and PR. A smaller number had ER but not PR, except in the 40–60 age groups (perimenopausal), where about one-third had ER without PR. The possible significance of this will be discussed later.

Cellular Density in Tumors

Presumably, in a given breast tumor the R concentration has a representative value for the epithelial cancer cells; but it is not known whether R values correspond to an integrated constant value for each cell of the whole population or whether the concentration of R varies from cell to cell. With this in mind, we examined the ER concentration in relation to the tumorous cellular density (TCD) in 290 randomly chosen breast tumors (Table 2). The ER content of tumors within the same TCD is lower in patients below 50 years of age than in patients over 50. Despite an apparent increase in ER content, there is no significant difference in ER content with respect to TCD, because of the ER values.

The results are in agreement with the idea that a given breast tumor contains a mixture of cells that contain ER and cells that do not. Furthermore, both ER content and proportion of ER-containing cells can vary from zero to a very high amount. Several *reference parameters* are commonly used to express the receptor protein content in a tumor, but none of these has ever been related to the epithelial cancer cell population [1, 4, 8, 9, 44]. To study this problem, we compared the ER content expressed per milligram of protein and per milligram of DNA in 120 primary breast tumors. As seen in Fig. 4, the results have been considered with respect to the TCD. A linear regression analysis has been performed in each case: TCD I ($n = 27$): $y = 0.0313x + 1.5979$, $r = 0.8727$; TCD II ($n = 49$): $y = 0.0487x + 8.833$, $r = 0.7657$; TCD III ($n = 44$): $y = 0.0557x + 4.605$, $r = 0.7989$. As expected from the relatively heterogeneous population, the correlation coefficient is lower in TCD II than in the two other groups. The comparison of the slopes reveals a significant difference ($P < 0.01$) between the TCD I and TCD III lines but also between the TCD I and TCD II lines. The difference between TCD II and TCD III is not statistically significant ($P = 4.018$, $F_1 = 1.97$). The results suggest that expression of ER content in tumors with a low density of epithelial cells should be carefully considered, since ER determination by biochemical assay alone may give a false-negative result.

Table 2. Frequency of ER presence (ER+) and ER content in relation to the tumorous cellular density (TCD see Methods). Patients are classified as below and above age 50. ER+ is given as a percentage of the samples in each class of cellularity. ER content is expressed as femtomoles per milligram of protein (mean ± SD)

TCD	Age of patients					
	< 50 years			> 50 years		
	n	ER+	ER content	*n*	ER+	ER content
TCD I	13	69%	83 ± 102	60	85%	170 ± 250
TCD II	48	73%	125 ± 194	103	85%	235 ± 339
TCD III	18	56%	152 ± 149	48	79%	283 ± 314

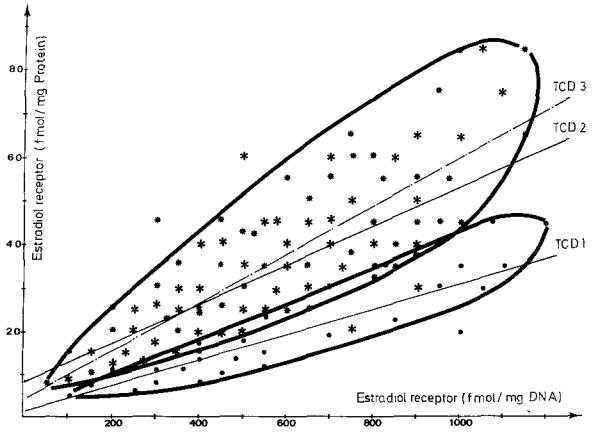

Fig. 4. Comparison of the ER content expressed per milligram of protein and per milligram of DNA in 120 primary breast cancers. According to the tumor cellular density (TCD), a linear regression analysis of ER content in samples is shown for each TCD. Two areas corresponding to the samples from TCD I and TCD II + TCD III samples are also shown

Discussion

The estrogen receptor (ER) data presented for a large population of human mammary carcinomas are in agreement with previous publications relating a higher frequency of ER-positive tumors in postmenopausal than in premenopausal patients [2, 22, 35, 42]. They further support the idea that a similar relationship exists for the absolute content. Due to the wide variances of the R levels within 10-year age groups, the significance of the results is reached only when the patients are divided into below and above 50 years of age (the menopause). This finding is emphasized in the oldest women, where ER content was high in all nine cases, and in the youngest patients (20–30 years), where only 42% of the lesions were positive.

The increase in ER content with increasing patient age could result from at least two factors: (1) a decrease in circulating estrogen, resulting in a decrease in translocation of cytoplasmic receptors to the nuclei, making them more available for assay, and (2) a decrease in circulating progesterone, which, in endometrium at least, partially inhibits the replenishment of estrogen receptors. Because of the use of labeled synthetic compounds with higher affinity constants than the natural hormones [25, 33], the low ER content at the cytosolic level could be better explained by nuclear translocation of R by endogenous hormones [14, 17, 19] than by an unavailability for assay of occupied cytoplasmic ER sites [2]. After menopause, the lower E blood levels would result in an accumulation of ER in the cytosol [40]. A role in ER depletion has been proposed for P, an ovarian factor that is also suppressed at menopause [41], but the ability of P to antagonize the replenishment of ER in the cytosol remains to be clearly established [14]. At the cellular level, P blocks the proliferation of endometrial cells induced by estrogen and induces these cells to enter the Go stage [7, 21]. This has never been shown in breast cancer cells. Whatever the site of action is, the ability of progesterone to antagonize the action of estrogen, presumably via ER, has to be considered [14]. Since hypophyseal factors have been far less thoroughly investigated, the results so far are more confusing [6, 15]; the endocrine status of the ovary, and thus the ovarian factors, could result in both situations hypothesized above. The presence of high levels of ER in male breast cancers supports the idea that the functioning ovary is an important regulator of ER in tumors in premenopausal patients, since E and P blood levels in man correspond to those typical of the postmenopausal status of women.

In postmenopausal women who have not received any anticancer therapy, the ER content is highest in the month of March. Moreover, this occurs in a representative population and independently of the frequency of positive ER tumors. In contrast to studies in animals [16], we have not been able to show a fluctuation cycle throughout the year. Occurring only in postmenopausal women, this phenomenon appears to be due to a nonovarian factor or factors not yet identified. Since the same observations were made in northern Germany 2 months later [16], a seasonal and/or nutritional origin of this factor could be hypothesized. A nutritional factor is an attractive idea in view of the concomitant rise in ER and spring growth of vegetable products, both of which occur 2 months later in northern countries. Phytoestrogens and fungus estrogens may play a role in this phenomenom, since we have shown that these compounds bind to ER in human breast cancer cells [26] and since short-term estrogen administration may increase ER content in certain tissues [15].

In contrast to ER, PR does not show an increase in frequency or content in relation to the age of patients. Moreover, PR content varies widely in the lesions and does not show any fluctuations throughout the year as does ER. A nonrandom distribution of PR with regard to ER suggests ER-PR interrelationships [31, 32]. In considering the distribution of PR and ER, we find that in 40%–50% of tumors both receptors are positive, and when ER is negative only 6%–8% of the tumors have PR. Thus the likelihood of positive PR increase to 70% in an ER-positive tumor. This is in agreement with other studies [13, 43, 44]. It should be noted that this percentage is in close agreement with that of tumors responsive to endocrine therapy [22, 29, 42]. Preliminary results of our group and from other laboratories indicate that the likelihood of a response is very much higher when both receptors are present in the tumor, suggesting that the presence of PR improves the selection of endocrine-responsive tumors. PR may be a marker of a functionally intact ER system [13, 14, 29].

Patients in the 40–60 age group have a higher incidence of negative PR in positive ER tumors. Nevertheless, the frequency of ER presence in these tumors remains at the same level. Since in this study the menopause has arbitrarily been considered to occur at the age of 50, it should be noted that these groups border on the menopause. It is well known that around the menopause this endocrine status shows appreciable variation in estrogen and progesterone blood levels, resulting from the frequent occurrence of prolonged menstrual irregularity and inadequate corpus luteum function. In addition these ovarian factors could perhaps directly affect their own receptors [5, 38, 40, 41]. Thus the absence of PR in a tumor that contains ER may not be due to dedifferentiation of the tumor but to a change in the host's hormonal status. This phenomenon is under active investigation in our laboratory.

From a histopathologic standpoint, we have marshalled the tumors into a scale of increasing density of the cancer cells. Since it has been suggested that only cancer epithelial cells contain the steroid receptor [10, 12], the density of these cells in the tumor is important in what concern the expression of the results in the biological significance of SR assay.

The ER content expressed per milligram of DNA and per milligram of protein was of similar magnitude provided they were of the same TCD. Moreover, in samples with a low density of cancer cells, the commonly used reference parameters appear to underestimate ER content. Thus, whatever reference parameter is used to express the ER content of a tumor sample, TCD is important and has to be taken into account, especially in samples with a low content of cancer cells [24]. Perhaps the SR content of a tumor sample should be correlated with the TCD for the selection of patients for en-

docrine therapy. Since the SR value does not result from an integration of a constant concentration in each cell, a low tumor SR content might reflect a low amount of receptor in a sample composed of many cancer cells, but could also reflect a high amount of SR in a tumor containing only a few malignant epithelial cells. This is in agreement with the concept proposed by McGuire et al. [31] of a given tumor as composed of a certain percentage (from zero to 100%) of receptor-containing cells, the clinical response to endocrine therapy correlating with this percentage. In TCD I samples, one could suppose that the percentage of SR-containing cells is close to 100% even if the SR content is relatively low. This may partially explain why certain ER-positive tumors fail to respond to endocrine therapy. If a tumor with a high cell density has a low ER content, perhaps the majority of cells have low or absent ER and the whole tumor will not respond to endocrine therapy despite a 'positive' ER assay. In other cases, the cellularity could not yield any information about the percentage of receptor-containing cells. Nevertheless, since it is well known that endocrine treatments do not cure advanced breast cancer patients [11, 22, 28], and that no direct cytotoxic effect of steroid hormones has been definitely demonstrated, it is possible that a combination of endocrine therapy with chemotherapy may be more effective than either one alone in endocrine-dependent tumors. The presence of ER and perhaps PR correlated with TCD could be used to select these patients.

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