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Receptor Heterogeneity of Human Breast Cancer as Measured by Multiple Intratumoral Assays of Estrogen and Progesterone Receptor

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Abstract—*Multiple intratumoral tissue samples from the primary mass of 30 consecutive invasive breast cancer patients were assayed for estrogen receptor (ER) and progesterone receptor (PR) by the dextran-coated charcoal method following frozen section histopathological examination. Steroid receptor status of each sample was classified as positive (R+) or negative (R−), based only upon quantitative guide lines from the ER/PR results. Four out of 32 (12.5%) of the invasive cancers had an intratumoral sample classified as R+ and one sample as R−. R+ invasive ductal carcinomas has a highly significant degree of tubule formation (P = 0.005) when compared with R− invasive ductal cancers. While the quantitative ER content (r = 0.18) and the degree of quantitative variation in ER content (P = 0.04) did not correlate with the tumor cellularity of the individual samples, tumor cellularity (P = 0.005) and ER content (P = 0.005) were lower in the samples from the tumor border than from the central tumor samples. Variations in ER and PR content may be found on a regional basis within a breast tumor mass resulting from heterogeneity of tumor subpopulations and/or differences in tumor cellularity.*

INTRODUCTION

ESTROGEN receptor (ER) status has been identified as an important therapeutic and prognostic factor in breast cancer [1]. Response to hormonal therapy, duration of response, disease-free survival after adjuvant treatment and overall survival [1,2] have been shown to be better in patients with ER-positive (ER+) than in those with ER-negative (ER−) tumors when based on assays of the primary tumor. Furthermore, the response to endocrine therapy has been shown to correlate not only with the qualitative presence of ER but also with its quantitative value [2, 3]. Likewise, the natural history, as measured by tumor growth, has a relationship to ER [4]. Recent reports have attempted to refine this discriminant by demonstrating the added strength of using quantitative receptor values, i.e. patients may benefit from prognostic considerations and therapeutic regimens based upon quantitation of ER into low, medium and high ranges [4, 5]. It is therefore essential to have an insight into the qualitative and quantitative variations, i.e. heterogeneity, in receptor content which may be encountered in a primary breast tumor. This property of heterogeneity may account for the fact that 40% of ER+ patients do not respond to endocrine therapy while 10% of ER− patients show an objective response.

Intersite heterogeneity in tumor receptor content has been demonstrated in patients when metastatic sites have been compared to the primary tumor [6–11]. In selected cases multiple assays of individual tumors have yielded intratumoral qualitative and quantitative variation [7, 12–14]. Correlations between histological features and estrogen receptor status have been found by numerous investigators [14–18]. When based upon a single ER assay of the primary tumor, ER+ tumors are more commonly associated with low histological grade, while R− tumors exhibit increased nuclear dysplasia,

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tumor necrosis and peritumoral lymphoid infiltrate.

Furthermore, some investigators have stressed that tumor cellularity influences the results of the biochemical assay for receptor determination [19–22], whereas others have not observed this relationship [15, 18]. These previous investigations have not attempted to define the role of tumor cellularity and histological features in variations of hormone receptor content within primary breast cancers. The purpose of this investigation was to determine the incidence of intratumoral variation in receptor content of human breast cancer and to correlate this variation with morphological features of the tumor samples.

MATERIALS AND METHODS

Thirty consecutive cases of primary breast cancer in females were examined to detect variation of ER and PR content in multiple sites from the primary tumor. The protocol consisted of taking tissue samples from different regions of primary tumor specimen, performing a frozen section for histological analysis of each sample and then biochemically assaying the same sample for ER and PR content.

Tissue collection

Biopsy or mastectomy specimens were transported immediately from the operating room (within 1 hr) to the pathology laboratory for processing. Tumor sampling was consistently performed by one pathologist (G.W.L.). A minimum of three samples were taken from each tumor for frozen section and receptor analysis. Samples were removed from the center (T) of the primary mass, the border (B) of primary and surrounding breast tissue, and surrounding (S) breast intimately associated with but not containing macroscopically recognizable tumor. At least two cryostat sections from each sample were fixed immediately and stained with hematoxylin and eosin. The remaining tissue was separated from the cryogen and delivered on solid CO_2 to the hormone receptor laboratory. Tissues ranged in size from 50 to 700 mg. Tumor was stored at -70°C for 1–10 days before it was assayed for receptor content.

Histopathology

Histological examination of each frozen section and of the remaining paraffin-processed slides of each case was performed without knowledge of the ER/PR results. Each case was examined for the following histologic parameters: classification of tumor type [23], histological grade [24] and size of primary tumor. Mucin content by alcian blue staining, lymphocytic infiltrate, tumor cell size, tumor necrosis and stromal fibrosis were graded in a semiquantitative fashion based on a 0–4 scale, with 0 representing absence of the feature and 4 representing relative maximum possible detection of the feature in each slide. Sample cellularity was recorded as the percentage of the frozen section area occupied by tumor cells.

Estrogen receptor and progesterone receptor assays

Frozen tissue was finely pulverized under liquid nitrogen. Ice-cold Tris buffer (10 mM Tris–HCL, pH 7.4, at 0°C, 1.5 mM EDTA, 10% glycerol, 1 mM monothioglycerol) was added to the tumor powder (0.5 g/3 ml Tris) and the suspension was homogenized with a Polytron PT-ST (Brinkman Instruments, Inc.) at a speed setting of 3.5 for 3–10-sec intervals with 30-sec cooling periods. The homogenate was centrifuged at 105,000 ***g*** for 30 min. The supernatants (cytosol) were diluted with Tris buffer according to the tumor weight: approximately 1 ml Tris buffer/100 mg tumor. This dilution scheme yielded soluble protein values in the range of 1–3 mg/ml. Protein was subsequently determined by the method of Lowry *et al.* [25] with BSA as standard.

ER and PR of sample cytosols were determined by incubating 100 μl cytosol (in duplicate) with 100 μl of 6 μM [^{3}H]-estradiol (115 Ci/mmol) or with 100 μl of 10 nM [^{3}H]-promegestone (R5020, 87 Ci/mmol) in ice water for 3 hr. Nonspecific binding was assessed by incubating a parallel series of tubes (in duplicate) containing a 100-fold excess of diethylstilbestrol (ER assay) or R5020 (PR assay).

Receptor-bound and free ligands were separated by adding a 500 μl suspension of dextran-coated charcoal (DCC) (2.5 mg Norit A activated charcoal, 25 μg dextran in 1 ml Tris buffer, pH 8.0, 4°C) to each tube. After resuspending the charcoal periodically for 30 min the charcoal-adsorbed free ligands were sedimented by centrifugation at 0°C for 5 min at 2000 rpm.

The charcoal-resistant receptor–ligand supernatant (700 μl) was transferred into a 5-ml scintillation vial containing scintillation fluid. Radioactivity was determined in a Beckman Model LS8100 liquid scintillation counter with a counting efficiency of about 30%. ER and PR are expressed as fmol receptor/mg cytosol protein.

Hormone receptor assay interpretation

The steriod hormone receptor status of each sample was independently interpreted on the basis of quantitative guidelines for both ER and PR results. Steroid receptor status was classified as

positive or as negative on the basis of the following parameters:

Receptor-positive		Receptor-negative	
ER	PR	ER	PR
⩾10	<20	<10	<20
<10	⩾20		

The classical convention of ⩾10 fmol ER/mg cytosol was chosen to designate an ER+ tumor [1]. For a tumor sample to be considered PR+ the binding values must be ⩾20 fmol/mg protein. This value was raised from 10 to 20 fmol/mg since PR generally exhibits higher background values and greater assay variability [26, 27], especially in the low range of receptor binding. Using these guidelines for ER and PR positivity, tumors can theoretically be classified into four categories: ER+PR+, ER+PR−, ER−PR+ and ER−PR−. In this study the first three categories were combined and designated steroid receptor-positive (R+), while the last category was designated steroid receptor-negative (R−). Our justification for including ER−PR+ tumors in an R+ category is based on several lines of evidence. First, such tumors occur more frequently in premenopausal than in postmenopausal patients, in whom the elevated levels of endogenous estrogens occupy and sequester the estrogen receptor in the tumor cell nuclei, rendering receptor unavailable [28] for the conventional cytoplasmic ER assay. Secondly, since PR is a product of estrogen action, its presence in the absence of estrogen receptor would indicate an active but sequestered estrogen receptor system.

Statistical evaluation

Student's *t* test and the chi-square test with Yates correction were used to evaluate the significance of the variables.

RESULTS

Ninety-seven samples of breast tissue from 30 female patients contained 39 breast cancers (32 primary invasive cancers and seven carcinomas *in situ*). The 30 patients ranged in age from 35 to 84 yr (mean = 63 yr). The 32 cancers ranged in largest diameter from 0.7 to 3.0 cm (mean = 1.6 cm). Nineteen (63%) patients were postmenopausal and 11 (38%) were pre/perimenopausal. Twenty-four (75%) of the 32 invasive carcinomas were found to be R+ by receptor assay (9 pre/peri-, 15 postmenopausal), while 8 (40%) were R− (3 pre/peri-, 5 postmenopausal). The distribution of menopausal and receptor status was within the range expected from a larger population [2].

Seventy-four of the 97 samples contained carcinoma as determined by histological examination of the samples prior to receptor assay. Of these, seven samples were found to contain carcinoma *in situ* without invasive disease. The remaining 67 samples represented multiple assays of the 32 invasive primary carcinomas. The distribution of steroid receptor status by tumor classification is presented in Table 1.

Table 1. Distribution of hormone status by tumor type classification

Tumor type	*n*	Receptor status R+	R−
IDC	20	16	4
ILC	8	6	2
LCIS alone	5	3	2
DCIS alone	2	0	2
Other	4	2	2
Total	39	27	12

IDC = invasive ductal carcinoma; ILC = invasave lobular carcinoma; LCIS = lobular carcinoma *in situ*; DCIS = ductal carcinoma *in situ*; other = 2 R+ papillary ca. and 1 R− mucinous ca. and 1 R− medullary ca.

Tumor grade

From 67 samples containing invasive carcinoma,* R+ tumors had a significantly lower grade ($P = 0.005$) than R− tumors (Table 2). By using this grading system, the significance of each component with reference to steroid receptor status could be determined. The degree of tubule formation was found to be significantly more ($P = 0.05$) in the presence of steroid receptors than in R− tumors when the entire group was considered, whereas differences in nuclear pleomorphism and mitotic activity were not

Table 2. Comparison of receptor positive (R+) tumors with receptor-negative (R−) tumors

	R+	R−	*P*
Tumor grade (1–3)	1.8	2.4	0.005
tubule formation (IDC only) (1–3)	2.1	2.9	0.005
nuclear pleomorphism (1–3)	2.0	2.0	N.S.
mitotic frequency (1–3)	2.2	2.4	N.S.
Tumor necrosis (0–4)	0.04	1.1	0.01
Lymphoid infiltrate (0–4)	1.6	2.5	0.01
Tumor cellularity	28%	21%	N.S.
Fibrosis (0–4)	1.1	1.1	N.S.
Mucin content (0–4)	0.5	0.7	N.S.

*The Bloom and Richardson grading system utilizes the invasive component only in determining the result.

statistically significant. Furthermore, within the group of invasive ductal carcinomas the association between tubule formation and steroid receptors was of even greater significance ($P = 0.005$).

Lymphoid infiltrate

R– tumors had a significantly higher ($P = 0.01$) degree of lymphoid infiltration in the peritumoral region than R+ in 67 samples containing invasive carcinoma (Table 2). The results of this analysis did not change when the seven samples containing carcinoma *in situ* were included.

Tumor necrosis

The amount of tumor necrosis, invasive and/or *in situ*, was also found to be associated with the absence of steroid receptors ($P = 0.01$) (Table 2). In the 74 samples containing carcinoma, a highly significant positive association was found between the presence of any necrosis and the protein yield per unit of tumor weight ($P = 0.001$), i.e. protein yield increases in the presence of necrosis.

Tumor cellularity

The range of tumor cellularity for the samples was <5–80%. Most sample cellularities were relatively low (<50%), a finding in keeping with previous estimates [17–20]. A statistically significant difference in tumor cellularity between R+ and R– samples was not detected in 74 samples containing carcinoma (Table 2). Likewise, there was no difference between pre/perimenopausal tumor cellularity. The four samples with carcinoma and less than 5% cellularity were R– and were excluded from further evaluations. Twenty-three samples taken from surrounding tissue contained normal breast and fat tissue. Twenty-one were classified as R–. Two samples without carcinoma on histological examination were classified as R+ based upon the elevated ER and/or PR.

The coefficients of correlation (r) between the estrogen receptor content of each tumor sample as measured by DCC assay and the percentage of tumor cells in 51 samples containing carcinoma and ER $\geqslant$10 fmol/mg protein were 0.12 for the pre/perimenopausal patients, 0.11 for the postmenopausal patients and 0.18 for both groups of patients combined (Fig. 1). These low r values indicate that estrogen receptor content did not correlate with tumor cellularity of the sample when a consecutive population of patients is considered. Likewise, the protein yield from each sample did not correlate ($r = 0.09$) with the tumor cellularity (data not shown).

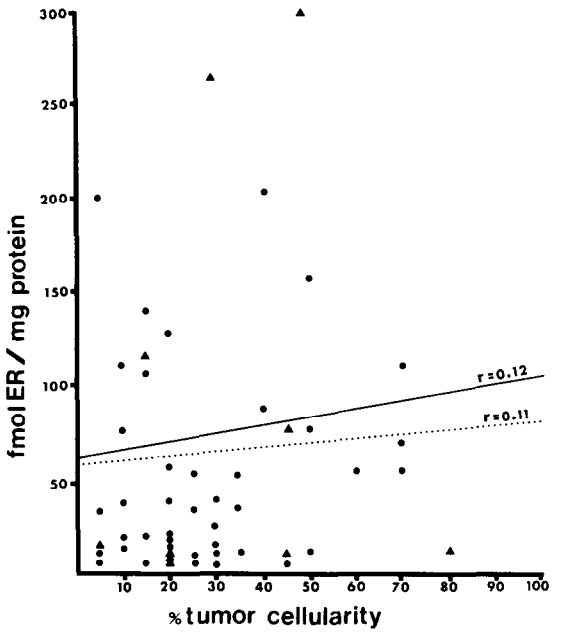

Fig. 1. This scatter diagram shows the estrogen receptor (ER) content as a function of the percentage of tumor cells in 51 samples of primary breast cancer from pre/perimenopausal (△) and postmenopausal (●) patients. Linear regression analysis demonstrates that ER content does not correlate with the amount of tumor cells in the sample when a population of consecutive pre/perimenopausal (——) (r = 0.12), postmenopausal (·····) (r = 0.11) or both pre/peri- and postmenopausal (r = 0.18) patient tumors are considered (r = coefficient of correlation).

Intratumoral intersample receptor variation

For each tumor, steroid receptor status of the central tumor (T) sample was compared with the tumor border (B) sample (Table 3). Multiple assays from 28 (87.5%) of the 32 tumors showed concordance between the T and B samples. In the remaining four (12.5%) cases (two pre/perimenopausal, two postmenopausal), a T sample was classified as R+ and a B sample as R–. All four tumors were $\leqslant$2 cm in diameter. Differences in lymphoid infiltrate, tumor necrosis, tumor grade and tubule formation were not significant. Furthermore, the difference in mean diameter of the tumors with multiple assay receptor status concurrence and those with qualitative variation was below the level of statistical significance.

The differences between the means of ER content in the paired samples from the central portion of the tumor (T) and tumor border (B) were found to be highly significant ($P = 0.005$) in the 32 invasive carcinomas (Table 4). Furthermore, the mean tumor cellularity of the samples taken from the central portion of the tumor (T) was significantly higher ($P = 0.005$) than the mean cellularity of the border samples (B). In two of the 24 R+ invasive tumors the ER content of the border samples was 2-fold and 4-fold greater, respectively, than that of the central tumor

sample. In both cases the tumor cellularity of the central tumor sample was 20% higher than that of the border sample. In the remaining 22 tumors the central portion had a consistently higher content of ER and tumor cellularity.

Evaluation of the quantitative variation between tumor samples was done for R+ tumors. Greater than 2-fold (range 3–34-fold) differences in the ER content between samples were seen in 8/15 (53%) tumors from the postmenopausal and 3/9 (33%) tumors from the pre/perimenopausal patients. When R+ tumors with >2-fold differences in ER between the two paired samples were compared with those with ≤2-fold difference, the observed distributions of menopausal status ($P = 0.4$) and tumor cellularity ($P = 0.4$) were not significantly different from that expected (Table 5).

Table 3. Receptor status variation in primary breast cancers

T/B receptor status	No. of paired samples	%
T+/B+	20	62.5
T+/B-	4	12.5
T-/B+	0	0
T-/B-	8	25.0

Paired samples from the central tumor (T) and tumor border (B) of 32 invasive breast cancers were assayed for ER and PR by the DCC method. Receptor status for each sample was classified as receptor-positive (+) or negative (-) based upon the quantitative results using the guidelines described in Materials and Methods.

Table 4. Comparison of the intratumoral samples

	T	B	*P*
ER (fmol/mg protein)	70.0	19.0	0.005
Tumor cellularity	30.5%	13.3%	0.005
Lymphoid infiltrate (0–4)	2.4	2.4	N.S.
Necrosis (0–4)	0.2	0.3	N.S.

Table 5. Quantitative variation of ER content within receptor-positive breast cancers

	Variation in ER content		
	>2-fold	≤2-fold	*P*
Menopausal status			
pre/peri-	3	6	0.4
post-	8*	7	
Difference in sample cellularities			
≤10%	3	6	0.4
≥20%	8*	7	

*In two of these cases the samples with less tumor cellularity had the larger ER content.

DISCUSSION

When multiple intratumoral samples from the primary tumor mass were compared, intratumoral qualitative variations in steroid receptor content, i.e. R+ vs R-, were found in 12.5% (4/32) of invasive breast cancers. Heterogeneity in the estrogen receptor status of breast cancer has been demonstrated by other authors when comparing multiple assays (Table 6). The incidence of qualitative variation has ranged from 17 to 32% within the primary tumor and 12–34% between multiple sites. In these studies, the estrogen receptor content *only* was considered in primary tumors selected for their relatively large size [7, 12, 13]. In contrast, we studied consecutive patients without regard to tumor size and combined the ER and PR content to determine the steroid receptor status of each sample. Had ER content *only* been considered, this incidence in our patients would have increased to 20%. Furthermore, this variation was not limited to larger tumors, as all of the four tumors (from four patients) with steroid receptor status discordance had a largest diameter of ≤2 cm.

If the relative amount of tumor cells within a tumor sample influences the hormone receptor content, the estrogen receptor content should correlate with the amount of tumor in the tumor sample. Previous investigations by others have demonstrated that the presence or absence of ER does not have a relationship with the number of tumor cells in a corresponding tumor sample [8, 15, 18]. Likewise, the differences in sample cellularities between R+ and R- tumors in this study were not statistically significant. The quantitated ER content in R+ tumors did not correlate with the tumor cellularity of the same sample from pre/perimenopausal patients ($r = 0.12$), postmenopausal patients ($r = 0.11$) or both groups combined ($r = 0.18$).

Table 6. Estrogen receptor variation in human breast cancer when assays from multiple sites are compared—previous literature

Reference	*n*	% with heterogenous ER results
[6]	29	24
[12]	(7)	(28)
[13]	(12)	(17)
[7]	38 (25)	34 (32)
[9]	59	14
[10]	24	29
[11]	46	12

The numbers and percentages enclosed by parentheses represent the results of multiple assays of the primary tumor instead of comparison of the primary with a metastatic site or comparisons of metastatic sites.

Other authors have noted that the ER content of a tumor sample may be underestimated if low cellularity is present [21, 22]. The general lack of correlation observed between tumor cellularity of the sample and quantifiable estrogen receptor may not hold true in two important exceptions that emphasize the usefulness of concurrent histological examination of the receptor sample. Samples with less than 5% cellularity were all R–; therefore, samples with this low cellularity should be considered as 'indeterminate' if steroid receptor values are low. In addition, two samples which histologically revealed normal breast tissue without carcinoma were classified as having positive steroid receptor values when based on the biochemical assay results alone. Clearly, accuracy of the steroid receptor status interpretation depends in part upon a tumor sample with adequate cellularity.

Greater than 2-fold variation in the quantitated ER content between tumor samples was seen in 46% (11/24) of the R+ tumors (range 3–34-fold). Tilley *et al.* [12] found 5-fold differences in ER content within seven large breast cancers while Silfversward *et al.* [13] found 2–3-fold differences without reference to tumor cellularity. Previously, Hawkins *et al.* [14] had reported that cellularity influenced the differences in ER content found in 11 large breast cancers. Assuming that the receptor content of a primary breast cancer is distributed equally throughout the tumor mass, the variation in cellularity between samples should reflect a similar quantitative variation in receptor content. When all R+ samples were considered, the border samples did in fact have a lower mean ER content and tumor cellularity than did the central tumor samples. However, the degree of variation in ER content (>2- vs $\leqslant 2$-fold) was not influenced by menopausal status ($P = 0.4$) or the degree of difference (10 vs 20%) in sample tumor cellularity ($P = 0.4$). Furthermore, two of the four cases with qualitative discordant steroid receptor status and several R+ cases with large regional differences in estrogen receptor content had only slight differences in regional sample cellularity. While tumor cellularity is an important factor, it is also apparent that there are regional differences in steroid receptor content which are not completely explained by differences in sample cellularity.

A consistent regional variation in the steroid receptor content was found in 22 of 24 R+ breast cancers. In these cases the central portion of the tumor had a higher ER content than did the tumor periphery. While other authors have reported other conclusions [13, 19, 20], our results are not entirely at variance with their findings. Silfversward *et al.* [13] found progressively higher amounts of cytosol ER from the center to the periphery of 12 cancers selected for their large size; however, the highest values of intranuclear estrogen receptor were observed in the central portions of these cases. One would expect larger tumors to have more central sclerosis and/or necrosis and thus less steroid receptor than the unselected and smaller tumors in our study. In fact, Silfversward *et al.* [13] found central sclerosis in six of their 12 cancers. Other experimental data support our findings. The receptor content correlates with the growth fraction as determined by labelling index. As the labelling index increases in human breast cancers the ER content decreases [7]. Since the peripheral portion of mouse mammary tumor contains the largest growth fraction of tumor cell populations [29], in the absence of central sclerosis or necrosis the steroid receptor content of the central region of a breast tumor would be expected to be higher than in the peripheral region.

To evaluate the significance of each component of the Bloom and Richardson grading system [24], tubule formation, nuclear pleomorphism and mitotic frequency were considered separately. Tubule formation was found to be associated with R+ tumors ($P = 0.05$) independent of nuclear pleomorphism and mitotic activity. Since invasive lobular and medullary carcinomas are not classically considered to be tubule-forming neoplasms, we examined the invasive ductal carcinomas for the association of tubule formation with receptor and found a high level of significance ($P = 0.005$). Thus tubule formation may be a morphological sign of measureable estrogen receptor in invasive ductal cancers. This finding is not surprising since tubule formation has been found to be a histological discriminant of good prognosis [17] and a manifestation of morphological differentiation [24]; however, it must be confirmed by an analysis of a larger number of breast cancers. Furthermore, this correlation may not be present in invasive lobular carcinoma since none of the six ER+ ILCs in this study had tubules detected histologically.

The findings of this study support the concept that human breast cancer is composed of a heterogeneous population of neoplastic cells. These subpopulations may vary in functional properties of biological significance. The histological methods of assaying ER, both histochemical [20, 30] and immunocytochemical [20, 31], have found staining heterogeneity within primary tumor subpopulations. Unfortunately, the specificity of these methods for the ER is controversial at present [32, 33]. What is needed is a methodology which includes the sensitivity of histological examination with the specificity and

reproducibility of the biochemical assays [34]. This would allow a more discriminating investigation of the biological importance of functionally heterogenous tumor cell subpopulations in human breast cancer. In the clinical setting, such a methodology is necessary before the prospective subcategorization of patients based upon receptor quantitation can be done with accuracy.

In conclusion, primary human breast cancers possess a functional heterogeneity as measured by the ER and PR assays. This qualitative and quantitative variation may be found on a regional basis within a tumor mass resulting from heterogeneity of tumor subpopulations and/or differences in tumor cellularity. This impacts on the sampling of a primary tumor for steroid receptors to determine future therapy. Not only is sampling of an area of adequate tumor cellularity important but, in the absence of necrosis or sclerosis, the central portion of the tumor is more likely to be steroid receptor-positive and to have a higher receptor content than the more rapidly dividing subpopulations of the tumor periphery. While some histological features are associated with detectable receptor protein, i.e. tubule formation, and others with absence of receptor protein, i.e. necrosis and lymphoid infiltrate, in this study these features did not account for the intratumoral regional variation in estrogen and progesterone receptors.

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