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DNA/Protein Ratio as an Index of Oestrogen Receptor Content in Human Breast Cancer

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Abstract—*The nuclear DNA/cytoplasmic protein ratio was related to the oestradiol receptor content in both cytoplasmic (ERc) and nuclear (ERn) fractions in 165 cases of breast cancer. The patients under study were divided into groups according to whether their biopsies contained functional (ERc/ERn = +/+), abnormal (+/0) or non-detectable receptor (0/0). The mean μg DNA/mg protein ratio was significantly decreased in both the abnormal and receptor-negative groups, with means of 30.4 and 30.2 respectively, when compared to the mean of 52.8 for the receptor-positive group. When the DNA/protein ratio for each patient was related to age, a discriminant line which clearly differentiated the ER +/+ group from the other two groups was obtained. It may be concluded that an increased DNA/protein ratio is related to the presence of ERn. Furthermore, since the DNA content per cell is higher in ER-negative tumours, an increase in cellularity must exist in ER-positive tumours.*

INTRODUCTION

ONE OF the questions which even today has not been answered satisfactorily concerns the lack of efficacy of antioestrogen therapy in patients with breast cancers containing oestradiol receptors (ER) [1–5]. Arguments raised to explain this problem maintain that it might be a question of abnormal oestradiol receptors or due to failures in the translocation of the cytosolic receptor to the interior of the nucleus which would both cause deprivations in the biological functional character of the receptors [1–3]. To overcome this problem, determinations on breast cancers have been proposed which might be markers of oestrogen action in breast tumour cells. Two determinations which can be mentioned are the simultaneous determination of progesterone receptors together with ER and the simultaneous determination of ER in the soluble or cytoplasmic fraction (ERc) and in the nuclear pellet (ERn), which was proposed by various authors and followed in this study [2–4].

Experiments *in vitro* have shown that the addition of different quantities of oestrogenic hormones to MCF-7 cell lines containing ER [6] has a stimulating effect on DNA and protein synthesis as a consequence of cellular growth [7, 8]. On the other hand, studies of flow cytometry [9] made on human breast cancers showed that a high ploidy level is associated with ER-negative tumours. Consequently, cellular or tissue DNA might be interpreted as a marker of the presence of ER in breast cancer and perhaps of its biological functional character.

The above-mentioned findings prompted us to verify if the modifications in the DNA of breast cancers depend on their ER content and functional character. In this study 165 cases of breast cancer are reviewed; their nuclear DNA/cytoplasmic protein ratios are related to the presence of ER in the soluble and nuclear fractions. The possible effect of the plasmic oestradiol levels on the DNA/protein ratios of breast cancers which contain ER in both the cytoplasmic and nuclear fractions is also studied.

MATERIALS AND METHODS

Patients and specimens

The studies were performed on primary breast tumours of 165 patients, aged 28–91 yr, before they had undergone chemotherapy, radiological or hormonal treatment. In every case, the breast biopsy specimens were obtained at the time of surgical mastectomy. They were divided into two portions; one portion was sent for histopatho-

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logical examination and the other for the ER study. The tumorous tissue retained for the ER determination was kept at -25°C with a buffer of 0.5 M sucrose, 10 mM HEPES and 1.5 mM $MgCl_2$ mixed in equal parts (v/v) with glycerol [10]. The ER determination was performed in a period of less than 1 week.

Venous blood samples were taken from the patients 2 hr before surgical mastectomy. All the samples were anticoagulated with heparin and the resulting plasmas were kept frozen at -20°C until the moment of estradiol determination.

Receptor assay

According to the procedure adopted [10], ER was determined in the cytoplasmic or soluble fraction and in the nuclear pellet. A minimum of 150 mg of the breast biopsy specimen, trimmed of fat and coagula, was homogenized in buffer HEPES-EDTA-dithiothreitol (20 mM HEPES, 1.5 mM EDTA and 0.125 mM dithiothreitol) at 1 ml for each 50 mg of tissue. After the tissue was homogenized (Ultra-Turrax followed by further homogenization in a glass tissue grinder) it was centrifuged at 7000 *g* for 15 min at 4°C to yield a cytosol supernatant or soluble fraction and a crude nuclear pellet. One hundred and fifty-microliter aliquots of cytosol or nuclear suspension were added to 50 μl of [^{3}H]-estradiol in 7 increasing molar concentrations in the 10^{-10}–10^{-9} M range. Two additional tubes were also prepared containing 10^{-9} M [^{3}H]-estradiol with and without diethylstilboestrol (DES) in order to determine the percentage of non-specific binding. All the tubes were then incubated at 4°C for 18 hr. After incubation the [^{3}H]-oestradiol–receptor complex was separated from the free [^{3}H]-oestradiol using the dextran-coated charcoal method for the cytoplasmic fraction and by filtering the nuclear pellet. The Scatchard plot was followed in order to calculate the quantity of ER; the results were expressed in fmol/mg protein for the cytoplasmic fraction and fmol/mg DNA for the nuclear pellet.

Protein and DNA assays

The cytoplasmic protein concentration was determined following Lowry *et al.*'s method [11]. The DNA from the nuclear pellet was determined by Burton's method [12].

Plasmic oestradiol assay

In every case the determination of plasmic oestradiol levels was carried out from an extraction of the plasmic steroids with diethylether. The final quantification was made by a radioimmunoassay procedure using tritiated oestradiol and dextran-coated charcoal to separate the bound from the free fractions. This method does not require purification since the antibody used is highly specific; the cross-reaction with oestrone was 8% and less than 4% for other related steroids. The results were expressed in pg/ml plasma. The intra-assay variation coefficient was 9% and the inter-assay 12%.

Criteria for ER-positivity

The criteria used to classify a breast cancer as ER-positive in both tissue fractions were based on the K_d obtained from the Scatchard plot. This K_d should lie between 0.5×10^{-10} and 5×10^{-10} M, and the percentage of non-specific binding should be less than 30%.

Groups established

The 165 cases studied were divided into groups according to whether their biopsies contained a functional (ERc/ERn = +/+), abnormal (+/0) or non-detectable (negative) receptor (0/0).

Statistical analyses

In order to establish a comparison between the means, Student's *t* test was used considering the levels of significance in agreement with the number of repetitions [13]. The Chi-square (χ^2) test with Yate's correction was used to compare the proportions. The regression linear coefficient was estimated following the least-squares method.

The parameters of age and the DNA/protein ratio of each patient were used to calculate the 'discriminant functions' used to distinguish the patients of the ER +/+ group from those of the other two (+/0 and 0/0). The statistical-mathematical criteria applied [14] yielded as many discriminant functions as there were groups. The straight line which differentiated these two groups was obtained by equalizing the two discriminant functions.

The Wilks' test was used to compare profile of mean values between the ER +/+ group and the other two groups (+/0 and 0/0). The statistical significance of such a test was determined by its transformation in *F*.

RESULTS

Duct carcinoma was the most frequent histologic type, accounting for 88% of the cases (Table 1), followed by lobular carcinoma, which represented 6%. The remaining cases of breast cancers were distributed among the other histologic types as shown in Table 1.

Of the 165 breast cancer biopsies studied, 38 had ER in both the cytoplasmic fraction and nuclear pellet (ERc/ERn = +/+), 36 had ER only in the cytoplasmic fraction (+/0) and the remaining 91 did not have ER (0/0) (Table 2). No case was

Table 1. *Histologic type of breast carcinoma and receptor status*

Histologic type	Receptor status ER +/+	ER +/0	ER 0/0
Duct carcinoma	34	33	78
Lobular carcinoma	2	2	6
Tubular carcinoma	1	1	1
Solid carcinoma	1	—	1
Papillary carcinoma	—	—	2
Medullary carcinoma	—	—	2
Colloid carcinoma	—	—	1

found in which ER was present in the nuclear pellet but not in the cytoplasmic fraction (0/+).

There was a significantly greater proportion ($\chi^2 = 28.7$; $P < 0.0005$) of tumours with ER concentrations higher than 80 fmol/mg in the cytosolic fraction in the ER +/+ group as compared to the ER +/0 group (Table 3).

The ER +/+ tumours had a mean of 52.7 for the DNA/protein ratio (Table 2 and Fig. 1); this was significantly higher than those corresponding to the ER +/0 ($t = 3.5$; $P < 0.001$) and the ER 0/0 ($t = 4.4$; $P < 0.001$) groups. Nevertheless, the means for the DNA/protein ratios of these last two groups had no significant statistical difference ($t = 0.05$; $P > 0.05$).

The mean DNA value for the ER +/+ breast cancers (Table 2) was also significantly higher than those corresponding to ER +/0 ($t = 2.42$; $P < 0.05$) and ER 0/0 tumours ($t = 4.1$; $P < 0.001$). However, the t values were not as high as those obtained from the DNA/protein ratio. The small variations in the protein averages of the groups (Table 2) showed no statistical significance between the ER +/+ and the other two groups; t values were 1.04 and 1.18 for the ER +/0 and ER 0/0 groups respectively.

No significant correlation ($r = 0.25$; $P > 0.05$) was found between the plasmic oestradiol levels and the DNA/protein ratio in patients with ER +/+ tumours (Fig. 2).

When the DNA/protein ratio was related to age, a significant discriminant straight line (Wilks = 0.79; $P > 0.001$) was obtained which clearly differentiated the ER +/+ group from the other two (Fig. 3). The DNA/protein ratio was clearly higher in young ER +/+ patients when compared to the ratios of the other two groups for patients of the same age (Fig. 3). However, this difference decreased as age increased; thus the groups could not be differentiated on the basis of the ratio in patients over the age of 80 yr.

DISCUSSION

In this study the proportion of breast cancers containing measurable quantities of ER in both cellular fractions (Table 2), or at least in the cytoplasmic one, is similar to that found by Laing *et al.* [2], who determined ER in both cellular fractions. Our results also agree with those of Laing *et al.* [2] in the sense that a high concentration of cytoplasmic receptors (>80 fmol/mg protein) is more likely to be associated with the presence of nuclear receptors (Table 3).

We do not think that any kind of plasmic contamination could affect our results in terms of false ER-positive, since the interference by 'sex-steroid-binding globulin' or 'alpha-fetoprotein' is decreased by using DES in the evaluation of the percentage of non-specific binding.

Table 2. *Oestrogen receptor status of breast-biopsy specimens and means ± standard deviations of DNA, protein, DNA/protein ratio and age of the patients*

Receptor status	No. of cases	Age (yr)	DNA μg/50 mg tissue	Protein mg/50 mg tissue	DNA/protein
ER +/+	38	62.24 ± 10.06	94.39 ± 77.67	1.88 ± 0.83	52.76 ± 35.88
ER +/0	36	56.19 ± 13.25	60.72 ± 27.40	2.08 ± 0.80	30.41 ± 11.91
ER 0/0	91	52.42 ± 12.95	50.68 ± 41.25	1.75 ± 0.69	30.21 ± 20.94

Table 3. *Concentrations of cytoplasmic and nuclear receptors and oestrogen receptor status in the breast-biopsy specimens*

Receptor status ERc/ERn	Cytosolic fraction Receptor content	No. of cases	Nuclear fraction Receptor content	No. of cases
+/+	>80 fmol/mg protein	32	>1000 fmol/mg DNA	24
	5–80 fmol/mg protein	6	25–1000 fmol/mg DNA	14
+/0	>80 fmol/mg protein	8	—	—
	5–80 fmol/mg protein	28	—	—

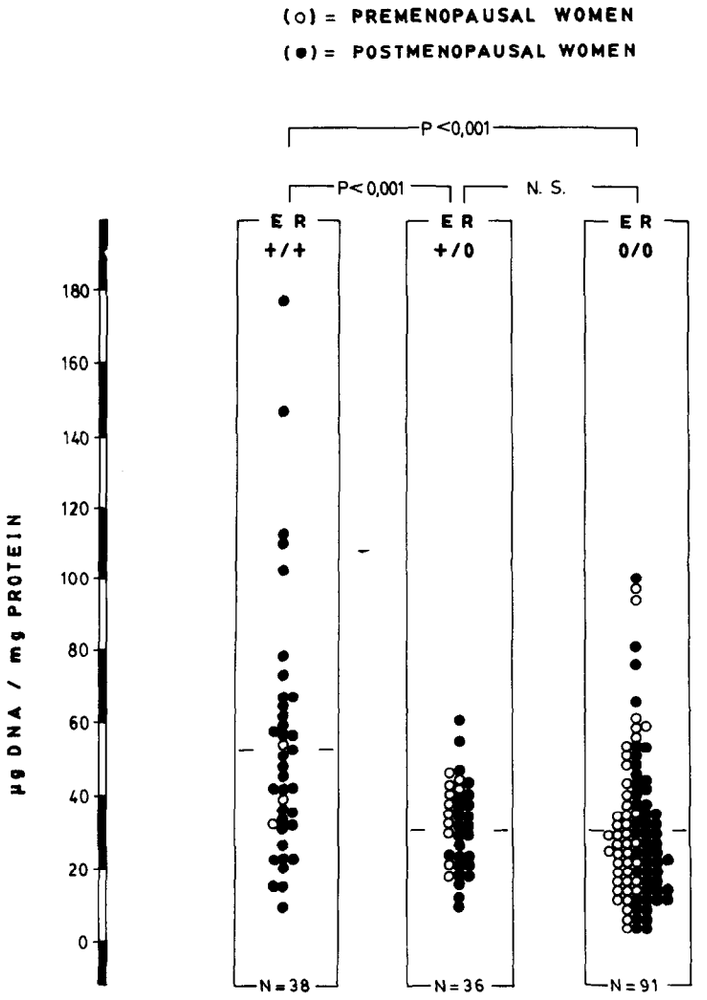

Fig. 1. Values of the µg DNA/mg protein ratio in groups of patients with breast cancer: those with ER in the cytoplasmic fraction and nuclear pellet (+/+); those with ER only in the cytoplasmic fraction (+/0); those without ER in either fraction (0/0). N.S. = not significant.

The fact that only significant differences in the DNA/protein ratio are found among the patients with ER +/+ breast cancers when compared to the other two groups suggests that the increase in DNA involves the presence of ER in the nuclear fraction. This strengthens the importance of determining the ER in the nuclear pellet, above all if ER are determined in only one of the cellular fractions, in agreement with authors that have determined ER in both fractions [2, 3], as we have done in the present study.

The absence of a relationship between the DNA/protein ratios and their corresponding plasmic levels of oestradiol in the ER +/+ group may be due to the fact that the majority of patients in this group were premenopausal and, therefore, had low levels of plasmic oestradiol.

The DNA/protein ratio together with the age of the patient are useful criteria that permit the inference of the presence of ER in the breast cancer. This is especially valid in young patients, where a high figure for this ratio is a faithful indication of the presence of ER in both cellular fractions.

Finally, since the DNA content per cell is higher in ER-negative tumours [9] and the protein concentrations do not experience significant variations, then the increase found in the DNA/protein ratio in ER-positive tumours must be interpreted as an increase in the cellularity of such tumours.

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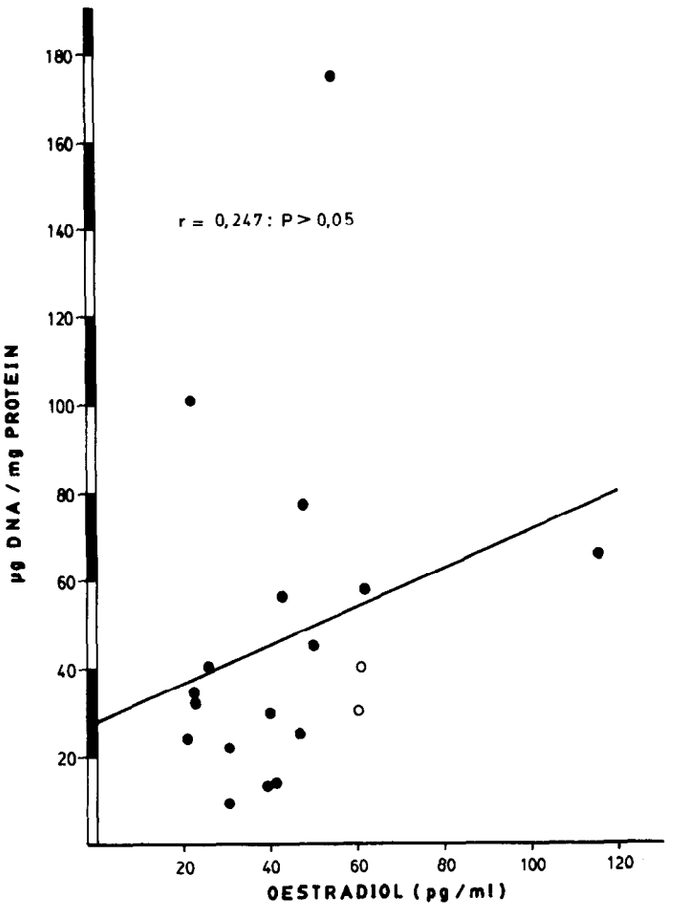

Fig. 2. Correlation between the plasmic oestradiol levels and the DNA/protein ratio in premenopausal (○) *and postmenopausal* (●) *patients with ER +/+ breast cancer.* r = *regression linear coefficient.*

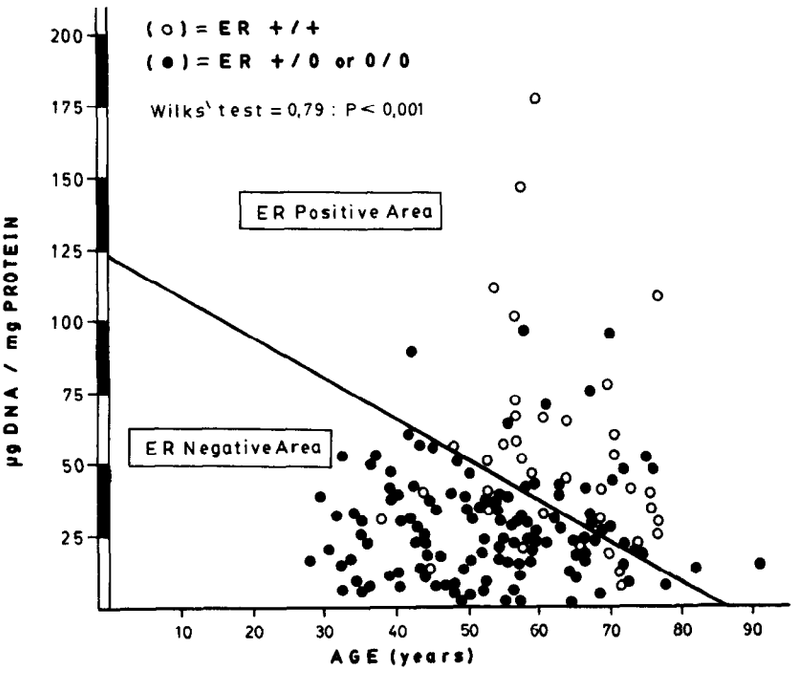

Fig. 3. Graphic figures of the discriminant straight line by which the ER +/+ (○) *group can be differentiated from the ER +/0 and ER 0/0* (●) *groups on the basis of the age of the patient and DNA/protein ratio.*

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