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Original Paper

Is the Negative Prognostic Value of High Oestrogen Receptor (ER) Levels in Postmenopausal Breast Cancer Patients Due to a Modified ER Gene Product?

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Recently, it was found that, among post menopausal breast cancer patients receiving no adjuvant therapy, the highest oestrogen receptor (ER) levels (ER^{++}) as opposed to the intermediate ER levels (ER^{+}) indicated a poorer prognosis in terms of recurrence-free survival (Thorpe *et al. Eur J Cancer* 1993, 29A, 971–977). In the present study, we confirm, in a series of 218 node negative, postmenopausal patients in whom ER was determined using a one-dose saturating method, that ER^{+} tumours have a more negative effect on disease-free survival (DFS) than ER^{+} tumours ($P = 0.02$). In another series of 87 ER positive, postmenopausal patients, we found a significant correlation ($P = 0.04$) between the ER level and $ER^{+}R$ ratio (ER protein/ER-specific mRNA): the higher the ER level, the more numerous the high $ER^{+}R$ ratio cases ($ER^{+}R > 1.5$), reflecting an imbalance between the ER protein level and ER-specific mRNA. From these results, we hypothesise that high ER levels related to a high $ER^{+}R$ ratio suggest the presence of a modified ER gene product.

Key words: breast cancer, oestrogen receptor, prognostic value
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INTRODUCTION

WITH THE increasing diversity of breast cancer treatments, prognostic factors are of crucial importance so that patients likely to benefit from adjuvant therapy can be identified and overtreatment avoided in subjects with a favourable prognosis. Lymph node involvement is currently one of the best prognostic factors. However, nodal status alone is not a sufficiently accurate yardstick to predict the clinical course of the disease since a significant proportion (20–30%) of patients with node negative disease will ultimately develop a recurrence [1]. Hence the reason why intensive investigations have been conducted in search of sensitive and reliable biological markers of prognosis.

Knight and colleagues [2] were the first to demonstrate that the absence of oestrogen receptors (ER) was linked to more rapid and more frequent metastatic dissemination. Despite numerous investigations, the role of ER status remains to be clarified. Some authors confirm that the presence of ER is a favourable prognostic factor [3–6], while others contend that ER is of limited, if any, prognostic value [7–10]. Such diametrically opposed opinions in the literature have several explanations: differences in the populations studied, in the length of follow-up, in the cut-offs used to separate positive and negative ER, and finally the possible interaction between the variables introduced in the statistical analysis.

It is generally accepted that there are interactions between menopausal status, adjuvant treatment and ER levels. In order to evaluate the prognostic value of ER separately, it seemed appropriate to study a single group, e.g. postmenopausal women without adjuvant treatment. Recently, Thorpe and associates [11] reported that among untreated postmenopausal patients, the prognosis was as poor in those with the highest ER levels as in ER negative cases. The aim of our study on a cohort of node negative, postmenopausal patients receiving no adjuvant treatment, was to confirm the higher risk of relapse in patients with tumours exhibiting a high ER level.

In previous studies, we reported the prognostic value of the $ER^{+}R$ ratio which corresponds to the ER protein level over the ER-specific mRNA level [12, 13]. These studies were based on

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the assumption that mutations or post-translational modifications of the ER gene product could generate protein variants with a different half-life from that of the wild-type ER protein. Evidence in support of such a modification was provided by comparing the ER protein level to that of ER-specific mRNA. It was then possible to separate the ER positive population into two groups, the first had a low ratio (ER^+R_1) and the second, a high ratio (ER^+R_2). The group with a low ratio was found to have a low risk of relapse. Here, we verify that high ER levels are indeed related to a high ER^+R ratio, and propose that this finding suggests the presence of a modified ER gene product in these cases.

PATIENTS AND METHODS

Patients

Two groups of patients were included in this study. The first included 218 node negative, postmenopausal women without any adjuvant treatment, and enabled us to confirm the higher risk of relapse for patients with the highest ER levels. This group came from a series of 728 patients aged 26–80 years, treated at the Institut Gustave-Roussy, France by primary surgery between January 1980 and December 1983 (105 patients had a mastectomy and 113 had conservative treatment with radiotherapy). The median observation period was 134 months (98–180), during the course of which 120 patients had disease progression (local recurrence, contralateralisation of the carcinoma and/or distant metastasis).

Because ER^+R ratios were not available in this first group, we selected a second sample of 87 ER positive, postmenopausal patients to demonstrate the correlation between ER level and ER^+R ratio. This group, in which the prognostic value of the ER^+R ratio had been previously demonstrated [14], came from a cohort of 171 patients treated at the Institut Gustave-Roussy, France for an operable non-metastatic breast carcinoma (41 patients had a mastectomy and 46 had conservative treatment with radiotherapy). 57 of the 87 patients in this group received adjuvant treatment (hormonotherapy). Women were classified as postmenopausal when amenorrhea had persisted for at least 2 years.

ER assay

After histological analysis, the tumour specimens selected by the pathologist were carefully dissected on a refrigerated plate and immediately (less than 15 min) stored in liquid nitrogen until required. ER levels were determined by a one-dose saturating method (ER-LBA) previously described [15]. In some cases, in the second sample of 87 ER positive, postmenopausal patients, an enzyme immunoassay (ER-EIA) was used [14]. A cut-off level of 10 fmol/mg cytosol protein was used to separate negative and positive tumours. A cut-off level of 100 fmol/mg cytosol protein was chosen to distinguish between moderate (+) and high (++) ER levels. This cut-off is close to the level of Thorpe and colleagues [11] as well as others [17–19] in other contexts. The laboratory performed continuous quality control studies in collaboration with other European Laboratories in the EORTC receptor group.

ER^+R determination

Total cellular RNA was isolated from frozen tumour by the guanidinium cesium chloride method, as previously described [12]. The steady state levels of ER mRNA were determined by Northern blot analysis of total RNA. Sequential hybridisations were performed after ^{32}P labelling by the "random primed" DNA labelling method (Boehringer kit, Mannheim, Germany) of the c-DNA probe (the 300 bp *StuI/HindIII* fragment of human ER cDNA isolated from pOR3, a gift from P. Chambon). Evaluation of ER mRNA was performed as previously described [12]. Steady state levels of ER mRNA were estimated by scanning the autoradiograms. Membranes were exposed to Kodak X-Omat AR films, without an intensifying screen, for various periods. For each sample, the level of ER mRNA was normalised according to the steady state level of β-actin mRNA. Two aliquots of RNA extracted from a pool of tumours were run on each gel and used as an internal standard. The amount of ER mRNA present in the reference RNA was determined by comparing the intensity of the 6.3 kb band with that of bands obtained with varying amounts of single stranded M13 recombinant containing the 300 nt ER cDNA fragment used as probe. At least two independent determinations were performed for each sample. ER^+R values were determined by calculating the ratio between the ER protein (in fmol/mg of cytosol protein) and ER mRNA (in pg per 4 μg of total RNA). The variations of this ratio were not related to tumour heterogeneity since we previously showed that a high stroma cell concentration (+++), found in only 12% of the tumours, was equally distributed among the two groups, ER^+R_1 and ER^+R_2 [12]. A cut-off of 1.5 was chosen to separate ER^+R_1 (low risk of relapse) and ER^+R_2 (high risk of relapse) groups [12, 13]. This cut-off value of 1.5 was replaced by a cut-off value of 3 when ER-EIA was used, as previously explained [14].

Statistical analysis

Cox's multiple regression model [20] was used for censored survival data. Several variables can be taken into account simultaneously with this method, and those exerting a major effect on recurrences can be identified. Disease-free survival (DFS) was calculated as the time from the first day of initial treatment to the day on which local disease or distant metastasis was diagnosed or to when death due to cancer occurred, whichever came first. The occurrence of the first of any one of these three events determined the recurrence rate (DRR), the complementary value being the DFS rate (DFS = 1 − DDR).

Comparisons of characteristics between groups of patients were performed using standard χ^2 tests in the relevant contingency tables. Two-sided P values <0.05 were considered significant.

RESULTS

Disease-free survival of postmenopausal patients

In order to confirm the results obtained by Thorpe and associates [11], we compared the negative effect of ER^+ and ER^{++} levels on DFS in the first series of 218 node negative, untreated postmenopausal patients. Using the actuarial method, a univariate analysis demonstrated a significant difference ($P = 0.02$) between ER^+ and ER^{++} patients whereas no significant difference was observed between ER^+ and ER^- tumours (Figure 1). A multivariate analysis, which included clinical tumour size, histological grade, age and ER levels, showed that the most powerful prognostic factors were histological grade and clinical tumour size. Only a trend ($P = 0.15$) was found for ER status: the risk of relapse was 1.4-fold greater for the highest ER levels (Table 1).

Correlation between ER level and ER^+R ratio

In order to explain this observation, we attempted to determine whether a correlation existed between ER level and ER^+R

Table 1. Factors predictive of disease-free survival in 218 postmenopausal breast cancers without adjuvant treatment using Cox's model

Factors	Number of patients	Multivariate analysis: Relative risk	Confidence intervals	*P* value
Clinical tumour size				
≤10 mm	20	1		
11–20 mm	71	1.7	0.8–3.7	N.S.
21–30 mm	86	2.3	1–4.7	0.03
>30 mm	41	3.2	1.4–7.2	0.001
Histological grading*				
I	41	1		
II	131	1.7	1.0–2.9	0.08
III	46	2.0	1.1–3.8	0.04
Age (years)				
≤60	93	1		
>60	125	0.8	0.6–1.2	N.S.
ER status†				
ER^+	96	1		
ER negative	45	1	0.43–1.51	N.S.
ER^{++}	77	1.4	0.9–2.1	0.15

*Determined by Bloom and Richardson's method [21]; †ER ≤ 10 fmol/mg = negative, 10 < ER ≤ 100 = ER^+, ER > 100 = ER^{++}.

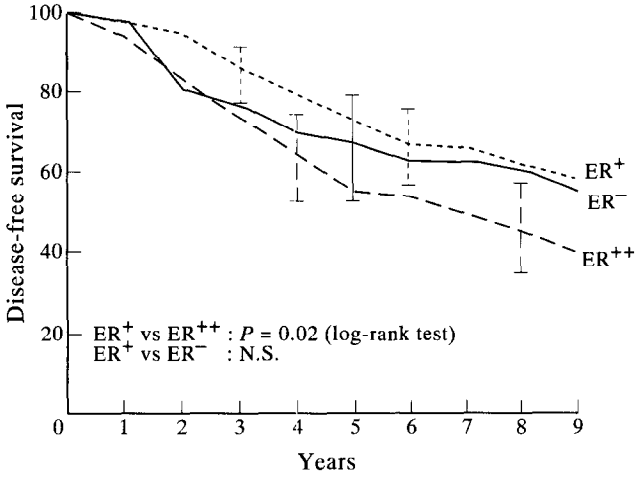

Figure 1. Disease-free survival according to ER status in 218 postmenopausal breast cancer patients without adjuvant treatment.

Table 2. Correlation between ER level and ER^+R ratio in ER positive, postmenopausal women with breast cancer

	ER level* 10–30	31–100	101–200	> 200
ER^+R Ratio†				
ER^+R_1 *n*	7	10	18	15
ER^+R_2 *n*(%)	1 (13%)	3 (23%)	14 (44%)	19 (56%)
		$P = 0.04$ (χ^2)		

n, number of cases.
* ER level is expressed in fmol/mg cytosol protein. ER positive >10 fmol/mg; †ER^+R ratio: ER protein/ER specific mRNA, ER^+R_1: ratio ≤ 1.5 (low risk of relapse) with ER-LBA [14] (ratio ≤ 3 with ER-EIA), ER^+R_2: ratio > 1.5 (high risk of relapse) with ER-LBA [14] (ratio > 3 with ER-EIA).

ratio in the second series of patients, since a high "abnormal" ER^+R ratio (ER^+R_2 group) had previously been shown to have a negative effect on DFS [14]. A significant relationship ($P = 0.04$) was observed between the ER^+R ratio and the ER level (Table 2): the higher the ER level, the more numerous were the ER^+R_2 cases. Moreover, the median ER level was significantly higher for ER^+R_2 than for ER^+R_1 (250 fmol/mg versus 157 fmol/mg respectively, $P = 0.004$). The ER^{++} level was not found to be of prognostic significance in this cohort (in which 57/87 patients received adjuvant treatment) (Figure 2).

DISCUSSION

It is now well established that ER levels in human breast tumours differ according to the menopausal status of the patients, with ER levels being significantly higher in post- compared to premenopausal women. Furthermore, the Danish Breast Cancer Cooperative Group (DBCG) trials showed that premenopausal receptor positive patients had a significantly longer DFS than receptor negative patients, while no difference was discernable between the two groups for the postmenopausal patients without adjuvant therapy [22]. In patients treated with adjuvant therapy which, by definition, affects the natural history of the disease, DFS is significantly longer for ER-positive than for ER-negative patients in both pre- and postmenopausal groups [23, 24]. The observation that ER positivity was associated with a longer DFS for all patient groups, except the postmenopausal group receiving no adjuvant therapy, prompted a more detailed study of the ER status in relation to DFS for this specific group of patients. Thus, Thorpe and associates, by using a three-point scale for the receptor status (low (−), intermediate (+) and high (++) receptor levels) instead of a subdivision into

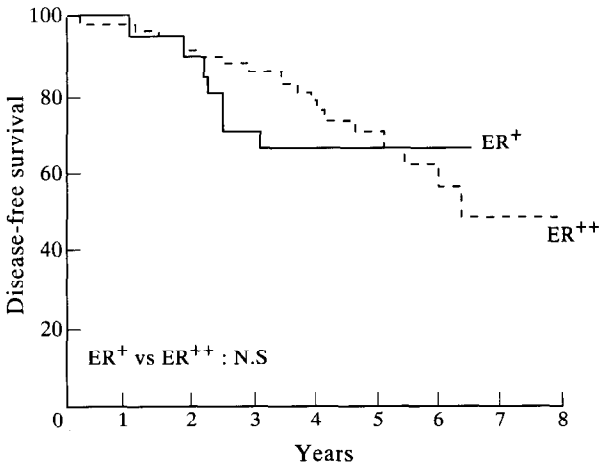

Figure 2. Disease-free survival according to ER status in the second population of 87 postmenopausal breast cancer patients, 57 of whom received adjuvant treatment.

two groups (positive versus negative) showed that, unexpectedly, the patients with the highest ER concentrations (ER^{++}) had the worst prognosis. Such a finding was first reported in 1983 by Black and associates [16].

Why higher ER levels are associated with a poorer prognosis in patients receiving no adjuvant therapy remains an enigma. Possible explanations are that the normal ER form is overexpressed or that an "abnormal" or variant form of ER has emerged (or both). Publications validating the existence of variant ER forms have appeared recently in the literature [25–31]. More specifically, two studies have indicated that an "abnormal" form of ER is more frequently found in the postmenopausal group [32, 33].

In the present study, we have confirmed that in postmenopausal women without adjuvant treatment, ER^{++} tumours have a worse DFS than ER^{+} tumours. In a multivariate analysis, the risk of relapse for the ER^{++} group of patients is 1.4-fold higher than that of the group with a moderate ER level (ER^{+}). These results are consistent with those obtained by Thorpe's group. Two reasons could explain the lack of statistical significance ($P = 0.15$) in the multivariate analysis compared to that observed by Thorpe and colleagues ($P = 0.04$): (i) the number of patients in our study is only a quarter of that in Thorpe's study (218 cases versus 952 cases, respectively); (ii) our median follow-up is 11 years (134 months) as compared to 4 years for Thorpe's group and the prognostic significance of ER status has been observed to decrease with time [34–36]. This second reason could also account for the lack of a significant difference between ER^{+} and ER^{-} tumours: at 4 years, as in the study by Thorpe and colleagues, we should probably have a significant difference between ER^{+} and ER^{-} tumours (see Figure 1), whereas there was no notable difference with a longer follow-up of 134 months.

In the second group of 87 ER positive patients, we did not find a negative effect of the ER^{++} tumours on prognosis. This is not surprising and concurs with the results of Thorpe, since many of the patients in this group (57/87) received adjuvant therapy, which was given to the more severe cases, mainly on the basis of lymph node involvement and independently of the ER status.

The significant relationship observed between the highest ER levels (ER^{++}) and the high $ER^{+}R$ ratio ($ER^{+}R_2$) is an argument in favour of the presence of an "abnormal" form of ER in this group of patients. Indeed, the determination of the $ER^{+}R$ ratio, which compares the level of ER protein to the level of ER-specific mRNA, is probably a good way of demonstrating a post-translational modification of the ER gene product [14]. As previously shown, a high $ER^{+}R$ ratio was categorised as a poor prognostic factor in a univariate as well as a multivariate analysis [14]. This high $ER^{+}R$ ratio, which reflects an imbalance between the ER protein and ER-specific mRNA, may provide evidence of molecular heterogeneity of the ER gene product in individual tumours. By using the $ER^{+}R$ ratio, it is possible to separate the ER^{++} population, which is largely postmenopausal, into two groups: those with a normal $ER^{+}R$ ratio ($ER^{+}R_1$), corresponding to a normal form of ER. This group has a prognosis which is as good as that of the ER^{+} group; and those with an abnormal $ER^{+}R$ ratio ($ER^{+}R_2$), probably corresponding to an "abnormal" form of ER. This group has a poorer prognosis than the ER^{+} patients as mentioned above.

Such results explain why the $ER^{+}R$ ratio appears to be a more powerful prognostic factor than ER status. It is easier to determine the prognostic value of ER^{++} status by using the $ER^{+}R$ ratio. As Thorpe and associates did not possess this ratio, they found that the prognostic value of ER^{++} was relatively modest since two populations are mixed: one with a normal $ER^{+}R$ ratio and a good prognosis and another with an "abnormal" $ER^{+}R$ ratio and a poor prognosis.

In conclusion we have confirmed the results obtained by Thorpe and colleagues showing that the ER^{++} have a negative effect on DFS, and have shown a relationship between ER^{+} status and the ratio $ER^{+}R$. These results strongly suggest functional heterogeneity of the ER protein in various tumours.

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