Prognostic Value of Estrogen and Progesterone Receptors Measured by Enzyme Immunoassays in Human Breast Tumor Cytosols


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ABSTRACT

Clinically significant cut-off values to discriminate between receptor-positive and -negative, and the prognostic value of estrogen receptors (ER) and progesterone receptors (PgR) measured by enzyme immunoassay (EIA) have not yet been established. We have therefore measured ER and PgR by EIA in cytosols from 205 primary breast cancer biopsies. Clinically significant cut-off values (30 fmol/mg protein for ER; 27 fmol/mg protein for PgR), as related to tumor recurrence (median follow-up, 47 months), have been established by isotonic regression analysis. These data were compared to those obtained by simultaneously performed dextran-coated charcoal (DCC) assays (cut-off values: 18 fmol/mg protein for ER, and 26 fmol/mg protein for PgR) on the same cytosols, and to DCC assays performed previously (up to 10 years ago) on cytosols prepared from other parts of the tissue biopsies (cut-off values: 18 fmol/mg protein for ER, and 23 fmol/mg protein for PgR). Using the cut-off values for the EIA and the DCC assays performed on the same cytosols, the discrepancies between receptor status appeared less than 10% both for ER and for PgR. Furthermore, the concentrations of ER or PgR detected with the EIA or DCC assay were highly and significantly correlated (Spearman rank correlations: for ER, Rs = 0.94; for PgR, Rs = 0.88; P < 0.0001). After classification in different phenotypes with respect to ER/PgR status (+/+, +/-, –/–, and –/+), analysis for relapse-free survival and overall survival showed equal prognostic power in the comparable groups in the order, from favorable to unfavorable, of +/+ > +/- (-/+), > –/– (x²: P < 0.0001), irrespective of the assay which has been used for quantification of the receptor. It is concluded that both the conventionally used DCC and the newly available EIA methods are equally useful for assessing ER and PgR status.

INTRODUCTION

The quantitative assessment of estrogen and progesterone receptors in cytosolic extracts of human primary breast tumor biopsies is well recognized as an aid for predicting prognosis and choice of therapy (for reviews: see Refs. 1–3). Until recently, the assays routinely used to measure these receptors involved the use of radioactive steroids or their stable analogues. The most widely used method to separate free from bound ligand is the DCC technique, introduced by Korenman and Dukes in 1970 (4). Disadvantages of this assay are that: (a) the assay is very laborious; (b) expensive equipment is needed; (c) radioactive waste is produced; and (d) relatively large amounts of tissue (±400 mg) are required to prepare sufficient volume of cytosols to perform multiple-point Scatchard analysis (5). All these problems could be circumvented by use of the recently developed EIAs for ER and PgR. These assays are based upon the recognition of antigenic sites on the receptor molecule, and are therefore not influenced by endogenous hormones and do not detect other proteins which nonspecifically bind estrogens or progestins. A major advantage of these EIAs is that they can be performed on very small amounts of tissue because of their superior sensitivity compared to the DCC method (6), and the requirement of minor amounts of cytosol. Especially this requirement for minor amounts of tissue makes these EIAs very attractive because in recent years smaller amounts of tissue were, and will become, available as a result of the breast screening programs and the rapidly increasing wish to measure also other (possible) cell biological prognostic factors, such as growth factor receptors, oncogene (over)expression, DNA-ploidy, and mitotic index. The urge for more cell biological information counts in particular for the small primary tumor biopsies of node negative patients. In this patient group 25–30% of the tumors will likely recur within 5 years, and specific additional cell biological information as mentioned above may enable identification of a subgroup of high risk patients liable for adjuvant chemotherapy.

So far, many studies, including a European and an American multicenter study (7, 8), have shown that ER values obtained with ER-EIA are strongly correlated to those obtained with the conventionally used DCC assay. ER-EIA is also an excellent method for the measurement of nuclear receptors (9). Limited data are currently available for PgR-EIA, but this assay also has been shown to give comparable results to those obtained by the DCC method (10–12). The slopes of the regression curves generally indicate that more receptors are being detected with the ER-EIA, especially at low receptor concentrations. This is probably due to the higher sensitivity of the assay and the detection of “occupied” receptors which are not detected by the DCC method which only measures “free” receptors under the routinely used standard conditions. Despite the very strong overall correlations observed between the EIA and DCC methods, the use of the same cut-off level to distinguish between receptor positivity and negativity generally results in discrepancies in the classification of the receptor status of approximately 10%. Thorpe has recently reported that the ER-EIA yielded the “more biologically correct” result (6), suggesting more frequent false-negative results with the DCC method. All 10 biopsies which scored positive for ER with the EIA and negative with the DCC assay were either progesterone receptor positive or had nuclear ER (6).

There are numerous reports on clinically significant cut-off points to define the ER and PgR status as positive or negative from assays performed with the DCC method (range, 3–30 fmol/mg protein). However, the superior sensitivity of the EIA and the different principles of both the EIA (antigenic recognition site) and the DCC method (based on ligand binding) make the establishment of a clinically relevant cut-off level to distinguish between receptor positivity and negativity for the EIAs imperative. This has not been established yet. The purpose of the present investigation has been to assess the prognostic value of ER and PgR measured by EIAs, and to determine cut-
off levels. In this report we present the results of EIAs for ER and PgR in 205 primary breast cancer biopsies. We have correlated these findings with tumor recurrence and overall survival. Moreover, these data have been compared with those obtained with simultaneously performed DCC assays on the same newly prepared cytosols, and with those of previously performed DCC assays (up to 10 years ago).

PATIENTS AND METHODS

Patients

This study was performed on a group of 205 patients (mean age, 58.6; range, 29–87 years) with operable breast cancer who underwent breast conserving surgery (63) or modified mastectomy (142) with axillary lymph node dissection in the years 1978 through 1984. Of these patients, 54 were pre-, 133 were post-, and 12 were perimenopausal, and for six patients the menopausal status was unknown. In those years patients with medially or centrally located (T1/T2) tumors, or T3/T4 tumors, were irradiated on the parasternal lymph nodes. Ultimately, nearly all patients (19 excepted, including six M1 patients who already had distant metastasis at time of primary surgery) received some form of irradiation, on the breast/thoracic wall and/or on one or more lymph node areas. Women under 56 years of age and with positive lymph nodes generally received adjuvant chemotherapy (cyclophosphamide, methotrexate, 5-flourouracil). Of the tumors with specified differentiation grade, 12 were classified as well, 52 as moderately, and 115 as poorly differentiated. Lymph nodes were dissected from 92% (188/205) of the patients, and histological examination was used to confirm the number of lymph nodes with tumor involvement (N+: 124; No: 77; Nx: 4).

Methods

Tumors. Primary breast tumors were placed on ice immediately after excision, and transported to the laboratory at temperatures below −60°C, and stored at −80°C until processing for routine biochemical measurements for ER and PgR (Assay 1: ER1 and PgR1) during 1978 through 1984. The remainder of the biopsies were stored in liquid nitrogen and were used for new ER and PgR measurement by both the biochemical method (Assay 2: ER2 and PgR2) and immunological method (Assay 3: ER3 and PgR3).

Receptor Assays. For routine biochemical steroid binding assays of ER and PgR (Assays 1 and 2) with the DCC method procedures were used exactly as recommended by the EORTC (5). The ER-EIA and PgR-EIA assays (Assay 3) were performed as recommended by the manufacturers (Abbott Laboratories, Illinois) on the same cytosols as used for the DCC assay (Assay 2). The buffer used for preparing cytosols for the DCC assays, which differs from the buffer as recommended by the manufacturer for the EIAs, was shown to have no effect on the performance of EIA (10). All assays have been performed on cytosols which have been stored at −80°C for up to 2 weeks. In EIAs, diluted cytosols with protein concentrations between 1 and 2 mg/ml were used. From tumors of 187/205 patients ER values have been obtained by all three assays, and for PgR from 171 of the tumors. Data from all three assays were not available for every tumor biopsy sample because for Assay 1 some PgR data were missing in the records, and for Assay 2 in some instances the remaining tumor biopsies were too small to prepare enough cytosol to reliably perform two complete multiple-point Scatchard analysis (ER2 and PgR2). For Assay 3, data of all tumor biopsies are available because only minor amounts of cytosol is required for the EIA assay.

Protein Analysis. Protein assay was performed with the Bio-Rad method (Coomassie brilliant blue) with human serum albumin (Kabi Diagnostica) as a standard.

Follow-up. All patients were routinely examined every 3–6 months during the first 5 years, and once a year thereafter. Of the 205 patients included in this study, 71 have died (nine of which without evidence of relapse). They all count as failures in the OS analysis. Eighty patients showed evidence of disease during follow-up. These patients together
and respective standard deviations after logarithmic transformation of the data obtained by the three assays are listed. Statistically significant differences (WSR test: \( P < 0.0001 \)) were observed between the mean ER1 or PgR1 values obtained from Assay 1 with both those of ER2 or PgR2 from Assay 2, and ER3 or PgR3 from Assay 3, respectively. ER was observed to be higher in the newly prepared cytosols (obtained from different parts of the tissue as has been used for the initial Assay 1), and for PgR the opposite was observed (Table 1). Fig. 1A–D show the scatterplots of logarithmically transformed data. Receptor values from Assay 2 were plotted against those of Assays 1 and 3 (for ER: Fig. 1, A and B, and for PgR: Fig. 1, C and D, respectively). As could be expected, because these analyses were performed on the same cytosols, the strongest associations were observed between the data obtained with Assays 2 and 3 (for ER: \( R_s = 0.94, n = 197 \), Fig. 1B; and for PgR: \( R_s = 0.88, n = 196 \), Fig. 1D). However, despite the highly significant \( (P < 0.0001) \) correlations between the data obtained with the three assays, the scatterplots show a considerable spread in individual values obtained by Assay 1 and Assay 2, performed recently on cytosols prepared from other parts of the stored tumor biopsies (Fig. 1A for ER, and 1C for PgR). The solid lines drawn in the scatterplots represent the clinically significant cut-off value, of data obtained with the respective receptor assay, to distinguish between receptor-positive and receptor-negative. The assessment of these cut-off values and the concordance between the different assays will be discussed below.

The correlations between ER and PgR did not differ significantly in either assay (for all three assays: \( P < 0.0001 \)), with Spearman rank correlations varying from 0.56 to 0.60 (scatterplots not shown).

Cut-off Points to Define ER and PgR Status. For the assessment of the cut-off values for ER and PgR measured by Assays 1, 2, and 3, the method of IRA has been used (see: "Materials and Methods" section). Analyses have been performed with respect to relapse-free survival (RFS). To enable direct comparison of the three assays only patients have been included for whom ER or PgR values were known from all three assays (for ER: \( n = 187 \), and for PgR: \( n = 171 \)). The IRA results (Table 2) show that for all three assays low levels were associated with a short mean RFS (≤50 months), and high levels with a long RFS (>100 months). For ER2 and ER3 the choice of the cut-off level seems to be obvious at 18 and 30 fmol/mg protein, respectively. For ER1 this is less obvious, as there was a gradual increase of the RFS with increasing levels of ER1. A choice was made for 18 fmol/mg protein as cut-off level for ER1.

For the PgR assays we observed more or less the same pattern, and cut-off levels were chosen at 23 (PgR1), 26 (PgR2), and 27 fmol/mg protein (PgR3). Note, that among the PgR-positives according to Assays 2 and 3, there was still a continuing trend for a longer RFS with increasing PgR.

Classification of ER and PgR Status, and Concordance between Assays. Employing the cut-off levels that have been established above by IRA for ER and PgR for the respective assays, the classification of ER and PgR status obtained from the three different assays is shown in Table 3. Despite the wide spread in individual values of ER when comparing the scatterplots from ER1 against ER2 (Fig. 1A), and ER3 (scatterplot not shown), the amount of tumors scoring positive for ER is about the same for the three assays (Table 3; 66.8% for ER1, compared to 73.8% for ER2 and 67.3% for ER3, respectively). For PgR less tumors scored positive by Assays 2 and 3, when compared to Assay 1 (Table 3; 47.4% for PgR2 and 47.8% for PgR3, compared to 62.0% for PgR1).

Using the cut-off levels for ER and PgR for the respective assay, the concordance in the receptor status (both positive or both negative) between assays can be derived from the data listed in Table 3. For Assays 2 and 3, performed on the same cytosols, the concordance was very high (92.5% for ER and 90.9% for PgR, respectively). Despite possible tissue heterogeneity with respect to receptor distribution, also the concordance in receptor status between assays which have been performed.
Table 3 Classification of ER and PgR status using the EIA and both the DCC methods

classification of ER and PgR status (percentage of total), using the cut-off points which have been established by isotonic regression analyses (IRA; in Table 2). Data obtained with Assay 2 have been compared with those of Assays 1 and 3. For ER, the concordance found in receptor status between ER1 and ER2 was 83.4% (62.0%: +/+ and 21.4%: -/-), and between ER2 and ER3 was 92.5% (66.8% + 24.7%). For PgR, the concordances were 78.4% and 90.9%, respectively.

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Fig. 2. Actuarial relapse-free survival curves stratified by ER or PgR status obtained with DCC or EIA methods. Actuarial relapse-free survival curves for patients whose tumors were receptor positive (+) or negative (−) were plotted. A, data obtained from ER1; B from ER2; C from ER3; D from PgR1; E from PgR2; and F from PgR3. Numbers in parentheses, failures/total amount of patients in each group.

on cytosols prepared from different parts of the tissue (Assays 1 and 2) was significant (83.4% for ER and 78.4% for PgR, respectively).

RFS Stratified by ER or PgR Status. Using the cut-off points determined by IRA for assessing ER and PgR status in each of the three assays, the prognostic value of ER and PgR with RFS as an endpoint was studied. The actuarial RFS curves constructed in Fig. 2 show a significantly longer RFS for patients with ER-positive or PgR-positive tumors (for all six: P < 0.002). By comparing the curves with each other no conclusions can be drawn with respect to a “clinically” better discrimination in a particular assay, for both ER (Fig. 2, A–C) and PgR (Fig. 2, D–F). For assessing this question, a comparison of the concordant and discordant cases would be more meaningful and will be discussed below.

Effect of Discordancy between ER or PgR Status on RFS. The significance of the level of discordancy of the receptor status obtained with the three assays was studied in an attempt to identify the most discriminating assay to predict RFS. For this, actuarial relapse-free survival curves, after subdivision of the patients four times in four groups (+/+, +/−, −/+ and −/−) for ER or PgR as shown in Table 3 for Assay 2 compared to Assays 1 and 3, have been constructed (Fig. 3, A–D). By comparing data obtained with Assays 1 and 2 it appeared that patients in the discordant groups, scoring positive for ER in one of the two assays, perform equally well in the RFS analysis as the patients in the double-positive group for ER, and significantly better than patients in the double-negative group (Fig. 3A). A comparable observation is made after analyses of combined ER2 and ER3 data (Fig. 3B). This suggests that repeated ER assays on different cytosols from the same tumor, or on the same cytosols by assays based on different principles, more accurately predict a high risk group of patients, namely if both assays score negative for ER.

With respect to PgR status, the curves of the discordant +/− groups fall in between those of the +/+ and −/− groups (Fig. 3, C and D). The number of patients in the −/+ discordant groups are too small to allow meaningful analyses.

Overall, by comparing the receptor status of Assay 1 with those of Assay 2 (Fig. 3, A and C), which both DCC assays have been performed on cytosols prepared from different parts of the tumor, one may conclude that due to tissue heterogeneity more frequently false negative than false positive results have been obtained. By comparing Assays 2 and 3 (Fig. 3, B and D), performed on identical cytosols but based on different principles, one can not make firm conclusions on which assay discriminates better due to too small numbers in the discordant groups.

RFS and OS Stratified by ER and PgR Status. Using the cut-off values of ER and PgR for DCC Assay 2 and EIA assay (Assay 3), as has been obtained by IRA (Table 2), actuarial RFS curves (Fig. 4, A and B) and OS curves (Fig. 4, C and D) have been constructed after classification in ER/PgR: +/+ and −/−. The group of patients whose tumors displayed, by either the DCC or the EIA method a receptor phenotype of ER−/PgR+ appeared negligible. In both the RFS and the OS analyses the patients in the ER+/PgR− group showed a better performance, irrespective of the assay which had been used for classification of the receptor status. Uniformly, the curves for patients with ER+/PgR− tumors fell in between those from patients with ER+/PgR+ and ER−/PgR− tumors. The RFS and OS analyses did not reveal significant differences between the DCC and EIA methods for receptor classification, both with respect to RFS (Fig. 4, A compared to B) and OS (Fig. 4, C compared to D). This implies that, when clinically significant cut-off values for the respective assays are established, both the conventionally used DCC method and the recently available EIA method are equally useful in analyses of the cytosolic content of ER and PgR.

DISCUSSION

The many advantages of the newly available EIAs for the measurement of ER and PgR make these assays very attractive to employ for routine practice. Encouraging in this respect are the excellent correlations which have been observed between receptor concentrations measured by the conventionally used DCC method and the EIA method, both for ER (7, 8) and for PgR (10–12). However, one should remain aware that the EIA assay and the DCC assay are based on an entirely different principle. Because the EIA assay also detects occupied receptors, possibly available occupied receptors in the cytosol preparations will be measured. Particularly at the clinically important low receptor concentrations the EIA assay detected more...
receptors than the DCC method (6). Therefore, higher cut-off levels might be expected for EIA.

The present study also shows excellent relationships between receptor concentrations measured by DCC and EIA. Despite these highly significant associations between the respective receptor data obtained by DCC and EIA assays, the mean receptor values obtained with ER-EIA are significantly higher ($P < 0.0001$) than those obtained with the DCC method. The mean PgR values between the two assays appeared to be similar ($P = 0.14$). Moreover, although the median ER values obtained in our laboratory with the routinely used DCC method was rather constant, i.e., 77 in 1985 ($n = 1138$), 67 in 1986 ($n = 1237$), and 77 fmol/mg protein in 1987 ($n = 1190$), the median value obtained in 1988 with the EIA assay was significantly higher (127 fmol/mg protein, $n = 1140$). With respect to median PgR values the same trend was observed, i.e., 45 ($n = 961$), 42 ($n = 1160$), and 36 fmol/mg protein ($n = 1225$) for the DCC assay, compared to 56 fmol/mg protein ($n = 891$) with the EIA assay. All together the above-mentioned differences suggest that, depending on the assay used, the cut-off values to discriminate between positivity or negativity may be different. Thus, for an adequate management of breast cancer by using receptor values obtained with EIA it is imperative to establish cut-off values from clinically significant parameters, such as (relapse-free) survival.

In the present study, attempts have been made to obtain an impression of receptor heterogeneity with respect to tissue distribution, and of receptor lability due to aging or to long-term storage of the tissue. Therefore, receptor values originally obtained by the DCC method (ERI and PgR1) during routine processing of the breast tumor biopsies, have been compared to the values obtained by the same DCC method (ER2 and PgR2) on cytosols prepared years later from different parts of the tumor tissue. The data presented in Table 1, showing that mean ER2 > mean ERI ($P < 0.0001$), and that mean PgR2 < mean PgR1 ($P < 0.0001$), suggest that these differences are not due
to random variations. In case of random differences, the mean receptor values should not have been different but should have averaged out resulting in comparable mean receptor values of the two consecutive assays. Therefore the differences are rather due to systematic variations, such as a better preservation of the intactness of ER during tissue processing (due to long-term experience in steroid receptor assays), and storage effects resulting in breakdown of the notoriously labile PgR. Overall, the real significance of the observed differences is not clear, since the search for cut-off levels related to duration of relapse-free survival by isotonic regression analyses (Table 2) revealed for both DCC assays comparable cut-off levels (18 fmol/mg protein for ER1 and ER2, and 23 and 26 fmol/mg protein for PgR1 and PgR2, respectively). Moreover, using the respective cut-off levels, the prognostic power with respect to recurrence of the tumor remained unchanged (Fig. 2).

The prognostic value of ER and PgR as measured by the newly available EIA (Assay 3) was evaluated by comparison with ER and PgR data obtained by the DCC Assay 2, because both assays have been performed on identical cytosols and thus allowed direct comparison. Based on the calculated cut-off levels, the concordance between the assays appeared high (Table 3). More important, irrespective of the assay used to measure the receptor, the prognostic power of ER and/or PgR, both by analysis of relapse-free survival (Figs. 2 and 4), and overall survival (Fig. 4), did not change. Analyses to choose which of the two assays discriminates better in predicting a relapse failed more or less because numbers in the discordant groups are too small (Table 3, Fig. 3). Patients that score negative on two assays show the poorest prognosis. Patients that score positive on one assay but negative on another show a better prognosis, closer to patients that score positive on both assays. This indicates that discordant observations might be due to false negative scores.

Although shown to be very useful for the purpose of the analyses described in this paper, one should keep in mind that the cut-off levels found should not be taken as definitive. Better choices may be suggested by more data, especially data on patients with intermediate receptor values between 0 and 40 fmol/mg protein.

In summary, the newly developed EIA assay for the measurement of cytosolic contents of ER and PgR appears to be equally suitable as the conventionally used DCC method. For a better definition of the clinically significant cut-off levels for receptor data obtained with the EIA assays large-scale prospective studies are necessary.

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