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Estrogen and Progesterone Receptor Status Determined by the Ventana ES 320 Automated Immunohistochemical Stainer and the CAS 200 Image Analyzer in 236 Early-Stage Breast Carcinomas: Prognostic Significance

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The quantitation of estrogen and progesterone receptors (ER and PgR) has become the standard of care in the evaluation of patients with primary breast carcinoma. It has been demonstrated that ER and PgR detected by immunohistochemical methods in formalin-fixed paraffin-embedded tissue can be quantified by computerized image analysis. In this study, ER and PgR levels were determined by using an automated immunohistochemistry stainer (Ventana ES 320) and an image analyzer (CAS 200) in a series of 236 patients with stage I/II carcinoma of the breast. The degree of correlation of the ER and PgR levels determined by the dextran-coated charcoal method (DCC) with image analysis quantitation was high (r = 0.75). The agreement between both methods was 77% for ER and 73% for PgR. Hormone receptor levels were correlated with prognosis as determined by overall survival. An ER level of 30 fmol/mg as determined by image analysis was established to stratify the patient population most effectively into favorable and unfavorable prognostic groups ($P = 0.003$). An ER level of 20 fmol/mg for prognostic stratification reached statistical significance ($P = 0.03$). The DCC method was not able to stratify the patients into prognostic groups at the traditionally accepted cutpoint of 10 fmol/mg ($P = 0.52$). We conclude that when used in combination, automated immunohistochemistry and quantitative image analysis offer a favorable alternative to the DCC method in assessment of ER and PgR status in human mammary carcinoma. In addition, quantitative immunocytochemistry techniques may prove superior to the DCC method in specimens in which there is limited tumor volume (including fine-needle aspirates), stroma-rich tumors, and early-stage lesions including intraductal carcinoma.

KEY WORDS: ER, PgR, automated immunohistochemistry, image cytometry

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INTRODUCTION

Several clinicopathologic variables influence prognosis in breast cancer, including stage, histologic grade, nodal status, hormone receptor status, and tumor size [1–4]. Most women with node negative breast carcinoma have an excellent prognosis, but a subset of these patients will experience progression of disease with tumor-related death. Numerous studies have investigated morphologic and clinical features that may stratify these low stage patients into favorable and unfavorable prognostic groups [1,2]. Tumor size, histologic grade, nuclear grade, and tumor type have been known for some time to have prognostic significance [3,4]. Several studies have shown an independent value of proliferation index as a prognostic variable for the stratification of patients with breast cancer into favorable and unfavorable groups [5–7]. In each of these studies, the relationship between clinical outcome and proliferative activity was one in which the unfavorable groups had the higher proliferation indexes.

The measurement of estrogen and progesterone receptor status has become standard practice in the evaluation of patients with primary breast carcinoma [8]. Traditionally, estrogen and progesterone receptors (ER and PgR) have been quantitated by a biochemical ligand-binding assay, the dextran-coated charcoal (DCC) method. Documentation of the clinical relevance of ER and PgR levels is most often based on these DCC ligand-binding studies [9–13]. More recently, immunohistochemical techniques for the assessment of hormone receptor status have become popular, although standardization of these assays has been incomplete [11,12,14–37]. While there has been relatively good correlation between the immunohistochemical techniques (IHC) and the DCC ligand-binding assay, [11,12,15–36] several studies have investigated the clinical relevance of ER and PgR levels as determined by immunohistochemical methods [11,14,22–24,31–33,35,37]. While direct comparisons of new techniques to the DCC method are an important step in the validation process, an independent correlation of the immunocytochemical method with clinical outcome is essential. The final assessment of the clinical usefulness of these new techniques rests on this correlation with biologic behavior, and not on simple correlation with the DCC results. In the present study, we investigated the feasibility of using a Ventana ES 320 automated immunohistochemistry slide stainer followed by a CAS 200 computerized image analyzer to evaluate ER and PgR status and to correlate ER, PgR, levels with patient overall survival and other known prognostic indicators in a series of 236 patients with stage I and II carcinoma of the breast.

MATERIALS AND METHODS

Patient Population

The 236 patients in this study underwent biopsy at Duke University Medical Center, Durham, North Carolina, between 1984 and 1987 for the diagnosis of carcinoma of the breast. Patients' stage at the time of diagnosis was based on the TNM classification [38,39]. Seventy-six patients were classified as having stage I carcinomas and 160 were classified as stage II (100 stage IIA and 60 stage IIB) following definitive surgical resection. Eighty of the stage II tumors had positive nodes. On the basis of biologic outcome, patients were divided into two groups: the good-prognosis group included those patients alive and the poor-prognosis group included those patients who had died by October 1994.

Control/Calibration Tissues

The paraffin blocks from 67 cases of stage III/IV breast carcinoma were morphologically selected as potential calibration and control tissues on the basis of high tumor volume, low normal cellular elements, and minimal necrosis. These cases were pulled and analyzed for their ER and PgR status by image analysis, and DCC results were obtained from the charts. From these cases, negative, borderline, and positive cases were selected to control for the staining and imaging assay and several cases were selected to serve as calibrators.

Primary Antibodies

The primary antibody for the ER assay used in this study was a rabbit polyclonal antibody directed against sites present on the human ER antigen. The antibody is known to bind to the receptor in formalin-fixed, paraffin-embedded tissue. The primary antibody for the progesterone receptor (PgR) assay was a mouse monoclonal antibody (clone 1A6). The antibody is known to bind to receptors in formalin-fixed, paraffin-embedded tissue. Both the ER and PgR antibodies were supplied prediluted by Ventana Medical Systems (Tucson, AZ). Previously quantitated positive and negative ER and PgR tissue controls accompanied each assay run to ensure that the antibodies were applied and optimally active and that the instrumentation functioned properly. Appropriate species control serums (normal rabbit serum and IgG1 isotype controls, Ventana) were used as a negative control cocktail. A mouse monoclonal antivimentin (Ventana) antibody was used to assess the degree of antigenicity.

Sample Preparation

The tumors were sectioned, and representative portions were fixed in 10% neutral buffered formalin (10% NBF) for routine surgical pathology evaluation. After fixation, the specimens were customarily processed and paraffin embedded; 5-μm sections were cut and stained with hematoxylin and eosin (H&E) for routine histology, and the excess material was archived for future investigational studies. In all cases, formalin-fixed, paraffin-embedded tissue was retrieved for this study.

Study tissues were cut at a thickness of 5 μm and placed on +Plus+ slides (Baxter, Charlotte, NC). The

slides were oven-dried overnight at 65°C and then cooled for 30 min. The slides were deparaffinized in three cycles of xylene and then rehydrated in three changes of ethanol (ETOH). The slides were brought to water and immersed in a microwaveable pressure cooker with 1,500 ml of 10 mM citrate buffer (pH 6.0). The lid of the pressure cooker was tightly sealed and the unit was heated in a 700-W microwave (Quasar, model MQ7677BW, Elk Grove, IL) on high for 30 min. It is critical that boiling occurs during the last 10–12 min of the 30-min microwaving process, as can be demonstrated through the movement of the rubberized weight of the pressure cooker as it releases steam. Upon completion of the microwaving process the rubberized weight is removed to allow the complete release of steam. This is followed by a 30-min cooling period (15 min with the lid on and 15 min with the lid off).

Automated Immunohistochemistry Assay

Upon completion of the cooling process, slides were washed in two changes of Ventana wash buffer solution (Ventana) and the appropriate bar code label applied to each slide. The slides were then attached horizontally by metal clips on a level carousel within the temperature-equilibrated reaction chamber. The Ventana 320 was activated by loading the preprogrammed ER/PgR IHC recipe file. Each recipe file consisted of a specific sequence of buffer rinses, enzyme inhibitors, blocking serums, antibodies, detection complexes, chromagens, and counterstains that were used according to the manufacturer's instructions.

After the initial series of buffer rinses and normal serum pre-incubations, the ER and PgR primary antibodies are dispensed using a 100-μL metered dose per slide volume mechanical plunger. The specific antibodies are localized by a universal antirabbit and antimouse secondary IgG-biotinylated antibody cocktail (Ventana). This step is followed by a strepavidin–enzyme conjugate and visualized as a brown stain with diaminobenzidine (DAB) chromogen with a copper sulfate enhancement. Each step is incubated for a precise amount of time and at a 42°C standardized temperature. At the end of each incubation step, the instrument rinses the sections to stop the reaction and remove unbound material that could potentially cause background. Following the automated staining process, the slides were rinsed in tap water, followed by sodium acetate incubation and then counterstained with 1.5% methyl green for 5 min. The nuclear counterstain methyl green was chosen, as it provides the best spectral separation from the brown DAB chromagen [40]. After counterstaining, the slides were dehydrated in acetone and cleared in xylene. The slides were then coverslipped and labeled.

Image Receptor Quantitation

Quantitation was achieved by measurement with a CAS 200 computerized image analyzer, using the Quantitative ER/PgR Beta-Software Package Version 2.51 (Becton-Dickinson/Cell Analysis Systems, Chicago, IL). The instrument and its operation have been previously described [41]. A light microscope is linked to an interactive computer, and measurements are acquired through the use of specific software applications [42]. Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with methyl green (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and green (methyl green) [40]. The ER/PgR software application is used to measure the percentage of cell nuclei in the tissue section that contain ER or PgR, and the density, or concentration, of receptors in those nuclei. A determination of heterogenity of staining from field to field is also provided.

The image measurement takes into account not only the amount of nuclear area covered by DAB chromagen, but also how intense the chromagen is staining by determining the percentage of light transmission (%T) through the nuclear area. The less light transmitted through the nuclear area, the more DAB chromagen or staining is present in that nuclear area, thus measuring the level or receptor present.

Both nuclear and antibody thresholds were set with the negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide [35,40].

Standardization between immunohistochemical assays of the fmol/mg values was established through the use of a calibrator and two control tumor tissues in each assay. Additionally, the calibrator and control tissues were treated in the same manner as the sample tissue. This calibration and control tissue originated from tumor blocks with adequate tumor volume and distribution and a known ER and PgR value as predetermined by the DCC ligand-binding assay. A one point linear calibration is used during the assay of sample tissue. Quantitation was performed on 10 fields that contained DAB staining for each sample tissue.

STATISTICS

Comparison of immunohistochemical results with ER and PgR levels determined by the DCC ligand-binding assays was performed by the McNemar's test for paired proportions. Survival was defined to be the interval from initial diagnosis of the breast cancer to death or last follow-up time, where patients still alive at last follow-up were right censored in the survival analysis. Survival probabilities were estimated by the Kaplan–Meier method [43]. Differences in survival between the groups were tested with the log-rank statistic, adjusted for multiple comparisons where appropriate [44,45]. A test for trend in survival was based on the method of Tarone [46].

TABLE I. Summary of Clinical, Histologic, and Therapeutic Data Compared to Survival in Patients With Stage I/II Breast Carcinoma

Variable	Categories	Chi-squared	P
Age	<49, 50–59, 60–69, 70+	5.49	0.14
Vascular invasion	No, yes	1.96	0.16
Histologic grade	1–2, 3	0.36	0.55
Nuclear grade	1–2, 3	2.73	0.10
TNM stage	I, II	7.55	0.006
Nodal status	N0, N1	14.9	0.0001
Tumor size	<2 cm, ≥2 cm	9.25	0.002

TABLE II. Estrogen DCC and Immunohistochemistry/Image Analysis (IHC/IA) Comparison in Patients With Stage I/II Breast Carcinoma

	DCC-ER positive	DCC-ER negative
IHC/IA-ER positive	74	50
IHC/IA-ER negative	18	94
IHC/IA positive ≥20 fmol/mg		
IHC/IA negative <20 fmol/mg		
DCC positive ≥10 fmol/mg		
DCC negative <10 fmol/mg		

DCC, dextran-coated charcoal.

Median follow-up time was estimated from the times by reversing the role of the censoring indicator in Kaplan–Meier analysis.

RESULTS

In the present study, 236 early-stage breast carcinoma patients were evaluated for the presence of ER and PgR status in their tumors. Fifty-nine died of disease within 10 years. Table I lists the traditional prognostic parameters evaluated: TNM stage, nodal status, tumor size, age, vascular invasion, histological grade, and nuclear grade. Of these seven parameters, only three were statistically significant in terms of predicting overall survival: TNM stage ($P = 0.006$), nodal status ($P = 0.0001$), and tumor size ($P = 0.002$). Patients were categorized by TNM stage as follows: stage I contained 76 patients with 11 deaths (14%) and stage II contained 160 patients with 48 deaths (30%). Within the node status categories, there were 31 deaths in 156 node-negative patients (20%) and 28 deaths in 80 node-positive patients (30%). Patients were categorized into two groups by tumor size of <2 cm (91 patients with 13 deaths, 14%) and ≥2 cm (145 patients with 46 deaths, 32%). Age, vascular invasion, histological grade, and nuclear grade failed to stratify patients based on survival.

The ER levels as determined by a Ventana ES 320 automated immunohistochemical slide stainer (IHC) and CAS 200 image analyzer (IA) were generally similar to the levels obtained by the DCC ligand-binding assay (Table II). For this comparison, ER was considered positive by the DCC method at the traditionally accepted cutpoint of ≥10 fmol/mg and by IHC/IA methods as described in this communication at the cutpoint of ≥20 fmol/mg. No evidence of unequal distribution between the two data sets was seen ($P = 0.89$, McNemar's test for paired proportions). Similarly, PgR levels determined by the IHC/IA methods were generally in agreement with the DCC ligand-binding results (Table III), and there was no evidence that the ligand-binding and immunohistochemical results had unequal proportions ($P = 0.69$, McNemar's test for paired proportions). For this comparison, PgR was considered positive by the DCC method at the traditionally accepted cutpoint of ≥10 fmol/mg and by IHC/IA methods as described in this communication at the cutpoint of ≥10 fmol/mg. Representative examples of ER and PgR with formalin-fixed, paraffin-embedded tissue stained by Ventana immunohistochemical technique are seen in Figure 1A and B.

TABLE III. Progesterone DCC and Immunohistochemistry/Image Analysis (IHC/IA) Comparison in Patients With Stage I/II Breast Carcinoma

	DCC-PgR positive	DCC-PgR negative
IHC/IA-PgR positive	56	49
IHC/IA-PgR negative	14	117
IHC/IA positive ≥10 fmol/mg		
IHC/IA negative <10 fmol/mg		
DCC positive ≥10 fmol/mg		
DCC negative <10 fmol/mg		

DCC, dextran-coated charcoal.

Discordant results where DCC were positive and IHC/IA was negative occurred for ER in 18 patients and for PgR in 14 patients. DCC was negative and IHC/IA positive for ER in 50 patients and for PgR in 49 patients. Where DCC was positive and IHC/IA negative for either ER or PgR, morphologic assessment, as is only possible with the IHC/IA method, indicated that in 12 out of 18 ER results and 12 out of 14 PgR results, the tumor samples showed no receptor reactivity in regions of carcinoma. Receptor reactivity was present but only in regions of normal glandular elements. With image analysis, only areas of carcinoma were chosen for quantitation, thus avoiding some of the false-positive results obtained by DCC.

Figure 2 illustrates the exploratory method by which an optimal ER level can be determined to best stratify the patient population into favorable and unfavorable survival groups. In Figure 2A, there was no statistical survival differences when the patients were stratified into three ER quantitation groups of <5 fmol/mg (negative), 5.1–10

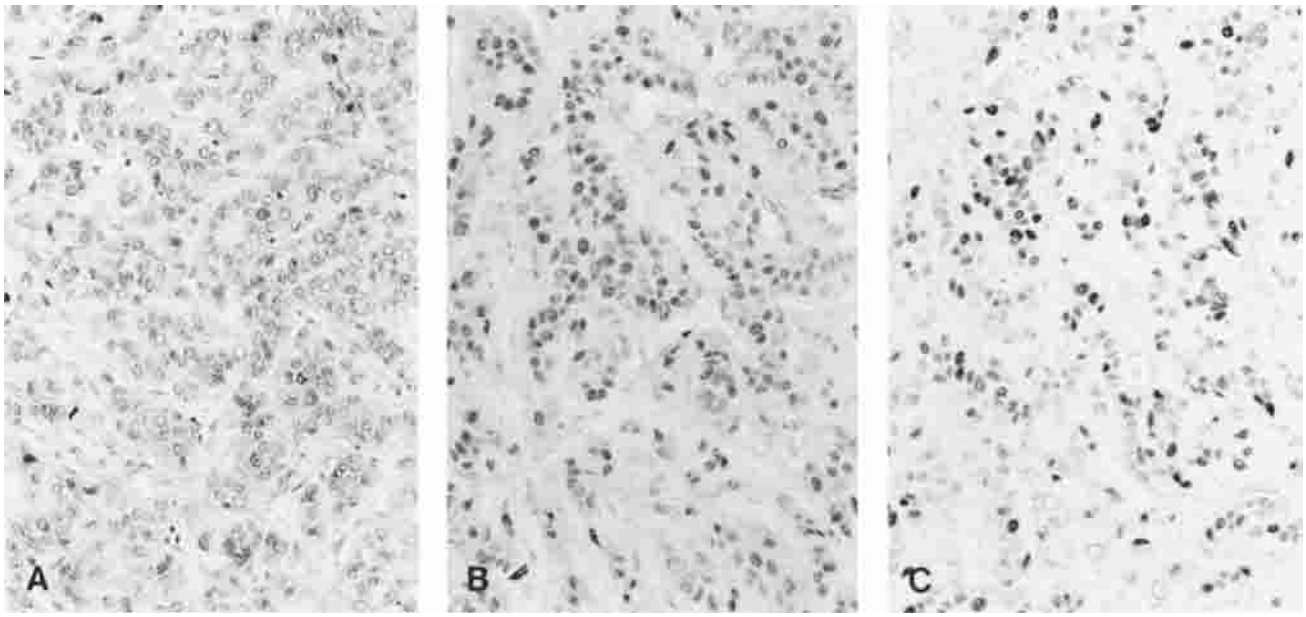

Fig. 1. Estrogen and progesterone receptor Ventana immunohistochemical staining in an early stage breast carcinoma. **A:** H&E, (×20). **B:** Estrogen receptor antibody immunoperoxidase-stained with a methyl green counterstain, (×20). **C:** Progesterone receptor antibody immunoperoxidase-stained with a methyl green counterstain, (×20).

fmol/mg (borderline), and ≥10 fmol/mg (positive). In a binary cutpoint system, ER level of 30 fmol/mg (determined by IHC/IA) was established to best divide the patient population into favorable and unfavorable survival groups (P= 0.003) (Fig. 2C), although statistical significance to a lesser extent was found with a binary cutoff of 20 fmol/mg (Fig. 2B).

Figure 3 illustrates the exploratory method by which an optimal PgR level can be determined to best stratify the patient population into favorable and unfavorable survival groups. In Figure 3A, there was no statistical survival differences though a trend was evident (P = 0.07) when the patients were stratified into three PgR quantitation groups of <5 fmol/mg (negative), 5.1–10 fmol/mg (borderline), and ≥10 fmol/mg (positive). In a binary cutpoint system, neither 20 (Figure 3B) nor 30 (Fig. 3C) fmol/mg (determined by IHC/IA) was found to divide the patient population into favorable and unfavorable survival groups (P = 0.33 and P = 0.44, respectively).

Stratification into favorable and unfavorable prognostic groups by ER status, as determined by IHC/IA, was independent of the statistically significant traditional prognostic parameters in this study: TNM stage, node status, and tumor size. ER status provided improved stratification compared to these traditional prognostic parameters. ER level, as determined by IHC/IA, appeared superior to tumor stage as a predictor of overall survival. Nodal status and tumor size were of equal or greater prognostic value than ER IHC/IA.

DISCUSSION

Measurement of estrogen and progesterone receptor levels has been shown to be of predictive value for both overall survival and response to endocrine therapy in patients with carcinoma of the breast [8–12]. ER and PgR analysis by ligand-binding assay has become standard practice in the management of advanced breast cancer [9–13]. Wittliff [10] demonstrated good correlation between ER level and response to hormone therapy; with 55% of women having ER-positive breast carcinomas responding to hormone therapy. When both ER and PgR were present 75–80% of patients responded to hormonal manipulation. Equally important was the finding that ER-negative neoplasms appeared to have an increased response rate to cytotoxic chemotherapy [10]. Allred [11] stated the primary reason for clinicians to order ER and PgR assays was to identify receptor-negative neoplasms that were more likely to relapse and not respond to tamoxifen therapy. Thus, accuracy of receptor determination (especially degree of test sensitivity) and relative bioactivity of the entity measured are extremely important issues for validating any technique measuring ER and PgR levels. Only biochemical ligand binding assays (especially the dextran-coated charcoal assay) have been extensively clinically validated [12,13].

In recent years, a variety of immunohistochemical techniques have been developed to measure ER and PgR levels in fresh frozen or in formalin-fixed, paraffin-

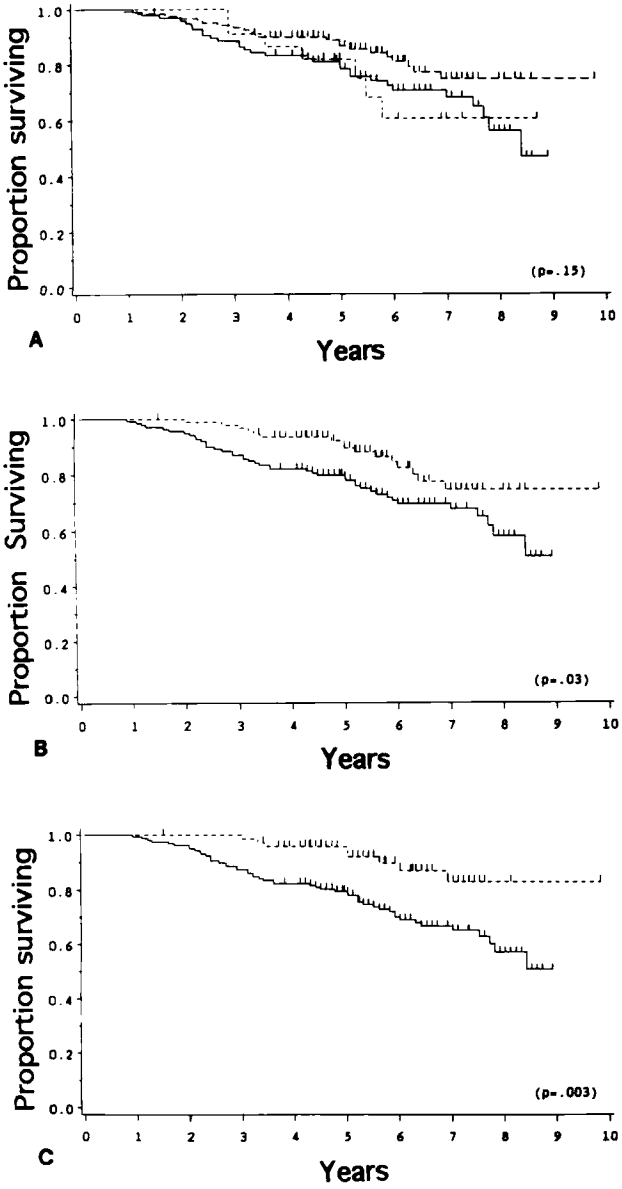

Fig. 2. Survival predicted by stratification of ER value using three different sets of cutpoints. **A:** Prognostic stratification using cutpoints of <5 fmol/mg (—— negative), 5.1–10 fmol/mg (- - - borderline), and >10 fmol/mg (— — positive) ($P = 0.15$) **B:** Prognostic stratification using 20 fmol/mg to divide patients into two groups: <20 fmol/mg (——) and those ≥20 fmol/mg (— —) ($P = 0.03$) **C:** Prognostic groups based on a cutpoint of 30 fmol/mg showed statistically significant stratification ($P = 0.003$). (——) <30 fmol/mg and (- - -) ≥30 fmol/mg.

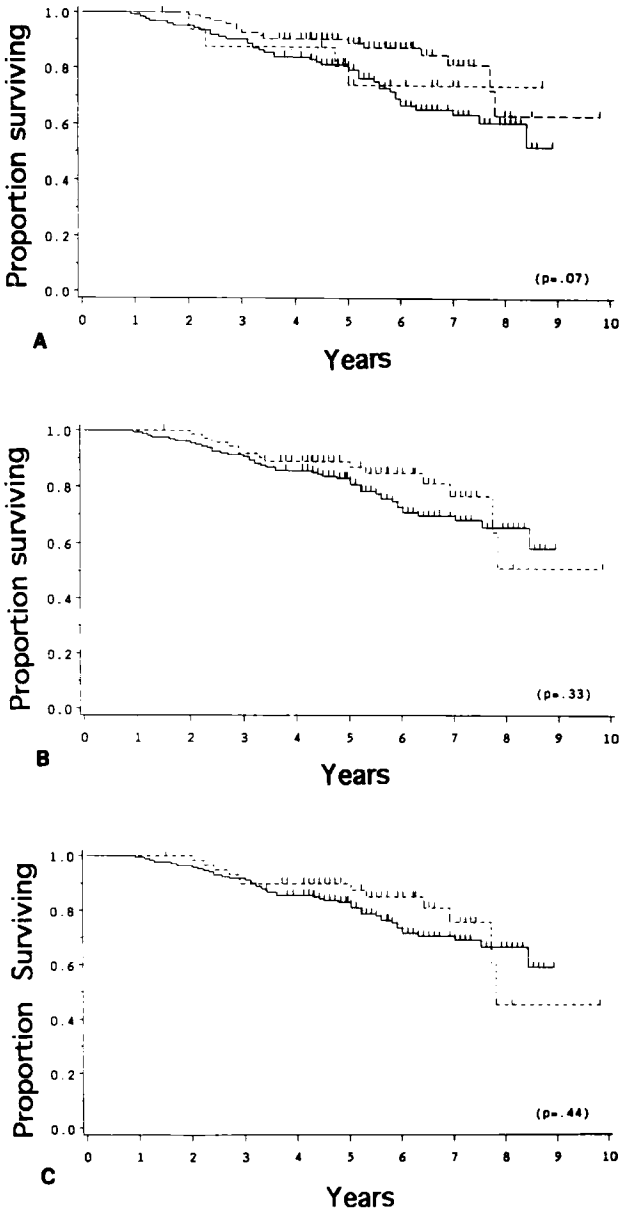

Fig. 3. Survival prediction based on PgR level using three different sets of cutpoints **A:** Prognostic group PgR values of <5 fmol/mg (—— negative), 5.1 to 10 fmol/mg (— — borderline), ≥10 fmol/mg (- - - positive) ($P = 0.7$). **B:** Prognostic groupings using 20 fmol/mg as a cutpoint: <20 fmol/mg (- - - negative) and ≥20 fmol/mg (—— positive) ($P = 0.33$) **C:** Prognostic groupings using 30 fmol/mg as a cutpoint. <30 fmol/mg (- - -) and ≥30 fmol/mg (——) ($P = 0.44$).

embedded tissues [14–33,35,36]. The quantitative validity of these techniques has generally been established by comparison to ligand-binding assay results. Several studies [15,18–21,24,27,35–37] have shown good concordance between ligand-binding studies and immunohistochemical determinations with concordance rates ranging from 81% [26,27] to 97% [18]. Despite such levels of agreement, few studies have directly investigated the clinical relevance of immunohistochemical determinations of ER and PgR levels [14,22,23]. Andersen et al. [14] documented a significantly improved overall survival for patients with ER-positive as compared to ER-negative breast cancers when measured by immunohistochemical techniques. These investigators also demonstrated a higher response rate with endocrine therapy for ER-positive than ER-negative tumors. Pertschak et al. [22,23] found immunohistochemically determined ER levels to

have superior predictive value to those determined by the dextran-coated charcoal method.

While few studies have attempted to validate clinically the immunohistochemical methodology (IHC) for determination of ER and PgR levels in formalin-fixed, paraffin-embedded breast carcinomas, there are several technical and theoretical advantages to the use of the immunohistochemical technique. Staining of tissue sections allows direct visualization of neoplastic cell-staining characteristics and improves assessment of tumor receptor heterogeneity, location and nature of staining reaction [21,26]. The pathologist can precisely identify which cell populations are staining, the degree to which neoplastic cells stain, and the amount of neoplastic tissue within a given specimen. This information may more accurately reflect the ER and PgR levels within the malignant cells rather than giving crude averages of neoplastic cells, benign epithelium and stroma as obtained by ligand-binding assays. IHC can also be used to evaluate smaller volumes of tumor. Tesch has shown that immunohistochemical methods are considerably less expensive than ligand-binding assays [27].

No uniform method for scoring ER and PgR levels as determined by IHC has been accepted and a wide variety of scoring systems are being used. Techniques used have varied from qualitative visual estimates [17] to quantitation by image cytometry [15,16,30,35]. Investigators have used arbitrarily weighted and combined estimates of the proportion and intensity of positive-staining tumor cells [28–30], unweighted combined estimates of staining proportions and intensity [11,32], calculation of percentage of positive cells [34], and counting of any positive staining [14]. Because of the heterogeneity of quantitation techniques, no uniformly accepted cutpoints for negative, borderline, and positive categories exist for IHC assays [11].

Our data using image analysis quantitation of IHC ER assay yielded a cut point of 30 fmol/mg as the best level for stratification of low stage breast carcinoma patients into good and poor prognostic survival groups. This is higher than the 10 fmol/mg usually quoted for ER positivity by ligand-binding assays [10]. This difference may be due to the overall favorable prognosis of low-stage patients or may be the result of enriching the tumor cell population by not including stroma cells within the denominator. Regardless of the explanation, our data indicate a higher threshold should be used (30 fmol/mg) by IHC for the separation of patients into ER-positive and -negative groups for the assay to have validity in prediction of overall survival.

Unlike some prior IHC studies, performed on all stages of breast carcinoma [22], we were unable to document a PgR level by IHC/IA which had prognostic significance in early stage carcinomas. This may reflect the overall good prognosis of stage I and II breast cancer patients. Our data would indicate that ER levels (as determined by immunohistochemistry assay using a Ventana ER antibody/automated stainer and quantitated by a CAS 200 image analyzer) can stratify patients with stage I and II breast cancer into favorable and unfavorable survival groups. Similar findings have been reported by others [35]. ER assay by IHC/IA appears to have clinical value. This clinical validity and the lower cost, smaller sample size requirements, and direct visualization capability of IHC/IA make hormone receptor assay by this method a favorable alternative to ligand-binding assay. Further studies directly comparing clinical predictive value of IHC assays with ligand-binding assays in larger groups of patients and in patients at higher clinical stages are needed to evaluate fully the usefulness of ER and PgR assays using antibody techniques on formalin-fixed, paraffin-embedded tissue sections.

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