Erythropoietin and Its Receptor in Breast Cancer: Correlation with Steroid Receptors and Outcome

Vassiliki Pelekanou,[1] Marilena Kampa,[1] Maria Kafousi,[2] Konstantina Dambaki,[2] Katerina Darivianaki,[2] Thomas Vrekoussis,[2] Elias Sanidas,[3] Dimitrios D. Tsiftsis,[3] Efstathios N. Stathopoulos,[2] and Elias Castanas[1]

[1]Laboratory of Experimental Endocrinology and Departments of [2]Pathology and [3]Surgical Oncology, University of Crete, School of Medicine, Heraklion, Greece

Abstract

Autocrine/paracrine erythropoietin (EPO) action, promoting cell survival and mediated by its receptor (EPOR) in various solid tumors, including breast carcinoma, questions about the prognostic and therapeutic interest of this system. The expression of EPO/EPOR is steroid dependent in some tissues; however, a clear relationship of EPO/EPOR and steroid receptors in breast cancer has not been established thus far. Recently, the field of steroid receptors has expanded, including rapid effects mediated by membrane-associated receptors, regulating cell survival or apoptosis. The aim of this study was to evaluate EPO/EPOR and membrane-associated steroid receptor expression in breast carcinoma, in view of their prognostic significance, compared with other established markers [estrogen receptor (ER)-progesterone receptor (PR) status and Her2 expression] and hypoxia-induced factor 1 nuclear localization in 61 breast cancer specimens followed for ≤90 months. We report that EPO-EPOR were expressed in 80% and 84% of samples, although 8% and 2% of nontumoral fields expressed EPO/EPOR too. Membrane-associated receptors for estrogen (mER), progesterone (mPR), and androgen (mAR) were expressed in 96%, 94%, and 93% of cases. Significant correlations between EPO-hypoxia-induced factor 1α, mER-ER, mER-EPO, mAR-EPOR, and mER-mPR-Her2 were found. Finally, EPO, EPOR, and mAR are inversely related to disease-free and overall survival. However, in view of the above correlations, we conclude that EPO/EPOR and membrane steroid receptors are not independent prognostic markers as they are closely related to other established markers. In contrast, they may represent possible new therapeutic targets. (Cancer Epidemiol Biomarkers Prev 2007; 16(10):2016–23)

Introduction

Breast cancer is the most common malignancy in Western societies, representing the second leading cause of women's cancer death. Death rate decreased on average 2.3% per year since 1990 (1). Although there has been substantial progress in prognosis, we are still in need of valuable prognostic and therapeutic markers in everyday's clinical practice. In this respect, the recent reports on the expression of erythropoietin (EPO) and its receptor (EPOR; refs. 2-5) in breast cancer, coupled with the established pleiotropic effects of the EPO/EPOR on tumor progression (6), raise the question of its possible prognostic significance.

EPO is a 30.4-kDa glycoprotein, produced by the kidney in response to hypoxia, acting on erythroid progenitors to stimulate erythrogenesis. EPO exerts its actions through binding to a specific membrane receptor (EPOR), a member of the cytokine receptor superfamily (7, 8). EPOR dimerizes up on EPO binding, triggering signaling cascades, which result in cell proliferation, differentiation, and survival (reviewed in ref. 6). EPO/EPOR have additionally been discovered in many extraerythroid tissues, including neural tissue and the developing heart and several normal cells or cancer cell lines (including breast cancer), suggesting a potential autocrine/paracrine function (discussed in ref. 9). The established proliferative, angiogenetic and antiapoptotic properties of EPO indicate novel functions of the molecule in tumor progression and invasion (10). These findings are of special concern about administration of recombinant human EPO in cancer patients with anemia. However, current knowledge on the subject is inconclusive (critically discussed in ref. 6).

EPO gene induction is mainly regulated by hypoxia-induced factor 1α (HIF1α), a subunit of HIF1, a heterodimeric (HIF1α-aryl hydrocarbon receptor nuclear translocator) hypoxia-related transcription factor that, in addition to EPO, induces the transcription of vascular endothelial growth factor, glucose transporter-1, inducible nitric oxide synthase, transferrin, and heme oxygenase complex (9). Aryl hydrocarbon receptor nuclear translocator, on the other hand, is also a common partner of the aryl hydrocarbon receptor, a nuclear transcription factor induced by xenobiotics and a reported partner of estrogen receptor (ER; see ref. 11 for a recent review). However, in addition to HIF1α, EPO secretion is tissue specific, additionally regulated by several factors, including thyroid hormones (12), nitric oxide (13), and steroids (reviewed in ref. 14). Indeed, in female reproductive

Received 12/6/06; revised 7/27/07; accepted 8/2/07.

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Requests for reprints: Elias Castanas, Laboratory of Experimental Endocrinology, University of Crete, School of Medicine, P. O. Box 2208, Heraklion 71003, Greece. Phone: 30-2810-394580; Fax: 30-2810-394581. E-mail: castanas@med.uoc.gr

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doi:10.1158/1055-9965.EPI-06-1023

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organs, EPO/EPOR expression is regulated by estrogen and/or progesterone (15-20). Nevertheless, no conclusive data exist on the correlation of EPO/EPOR with steroid receptors in normal mammary gland and breast carcinoma.

Breast cancer is a hormone-sensitive neoplasm, relying, at least at initial stages, on estrogen action, mediated by intracellular ERs. ER is the primary target for chemoprevention and endocrine therapy, providing prognostic and predictive information of tumor response to endocrine treatment (21). However, several reports indicate steroid action in cells lacking classic receptors (22, 23). These findings led to the identification of membrane binding elements, considered as new steroid hormone targets (reviewed in ref. 24). The nature of membrane steroid receptors remains elusive, as no conclusive biochemical characterization has been made until. It was proposed that they may represent the same molecule as intracellular receptors, docked to the plasma membrane via posttranslational modifications, or a completely new entity of steroid binding molecules (reviewed and discussed in ref. 24). Steroids, acting via membrane sites, exert short-term pharmacologic actions different from those of intracellular receptors, including ion mobilization, hormone or enzyme secretion, and cytoskeleton modifications (25-29), possibly involved in several physiologic or pathologic processes, including cancer. Indeed, androgen membrane receptors (mAR) are expressed in human prostate carcinomas (30) and correlate to the differentiation grade of the tumor, expressed by the Gleason score (31), although their activation inhibits the growth of experimental tumors in animals (32, 33). In breast cancer cells, estrogen membrane receptor (mER) and mAR exert opposing effects, promoting survival (mER) or apoptosis (mAR; ref. 34).

Data available on EPO/EPOR steroid receptor correlation in breast cancer are inconclusive (5). In the present study, we investigated the expression of EPO and EPOR by immunohistochemistry in 61 surgical breast cancer specimens and correlated them with (intracellular and membrane) steroid receptor expression. We report that EPO/EPOR relate to membrane receptors and that progression of cells from normal to neoplastic modifies the expression of these markers.

Materials and Methods

Patients. Sixty-one patients with operable invasive breast carcinoma, ages 26 to 77 years (mean, 56; median, 58), were included in the present study. All women were operated in the Department of Surgical Oncology, University Hospital of Heraklion, between 1998 and 2004. Forty-eight (79%) patients had a ductal carcinoma, 9 (15%) had lobular carcinoma, and the remaining 4 (6%) patients had a mixed-type carcinoma. Tumor-node-metastasis staging of tumors is presented in Table 1. Fifty-five patients (90%) have received preoperative chemotherapy. After the operation, all but one patient received chemotherapy and 60 had radiotherapy, and in 42 patients, antiestrogen was administered. The disease-free survival of patients ranged from 1 to 69 months (mean, 41.5), whereas the overall survival ranged from 2 to 69 months (mean, 44.1 months). The ethics and scientific committees of the University Hospital of Heraklion approved the present study.

Table 1. Clinicopathologic and biological characteristics of patients included in the present study

	Mean	Median	Minimum	Maximum	No. cases
Age	56.24	58	26	77	
Mean diameter	3.0	2.5	0.8	9.5	
Grade	2.18	2	1	3	
DFS (mo)	41.51	46	1	69	
OS (mo)	44.08	46	2	69	
TNM stage					
$T_1N_0M_0$					3
$T_1N_1M_0$					25
$T_1N_1M_1$					1
$T_1N_2M_0$					1
$T_2N_0M_0$					3
$T_2N_1M_0$					42
$T_2N_1M_1$					2
$T_2N_2M_0$					2
$T_3N_1M_0$					8
$T_4N_1M_0$					2
ER+					50 (82%)
PR+					54 (89%)
Her2+					17 (28%)
HIF1α					53 (87%)

Abbreviations: DFS, disease-free survival; OS, overall survival; TNM, tumor-node-metastasis.

Tumor Analysis. All tumor specimens were put on ice, immediately after surgery, transferred to the Pathology Department, and formalin fixed/paraffin embedded following a standardized protocol. Seven serial sections (3 μm) were cut from each tissue block. One was stained with H&E and two slides were used for the immunohistochemical identification of EPO and EPOR. The remaining four slides were stained for mER, progesterone membrane receptor (mPR), and mAR, whereas staining with bovine serum albumin (BSA) was used for the estimation of nonspecific membrane steroid receptor binding. Two independent investigators reviewed the slides in consensus and blindly to the patients' clinical data. Additional biological data were retrieved from the Pathology Department database, including size and grade of the tumor, ER and PR status, the expression of Her2/neu, and the nuclear localization of HIF1α (Table 1).

Immunohistochemical Staining of EPO and EPOR. After deparaffinization and hydration, slides were subjected to three cycles (5 min) of citrate buffer (0.01 mol/L, pH 6.0) incubation in a microwave oven (500 W) and treated with 3% hydrogen peroxide for 15 min. They were incubated with primary antibodies for EPO (1:100) and EPOR (1:150; both from Santa Cruz Biotechnology). UltraVision LP Detection System and Fast Red chromogen (Lab Vision Co.) were used for detection. The dilutions used were obtained experimentally, to maximize signal to noise ratio, on tissues of known positivity for EPO and EPOR (renal and placental tissue, respectively). Counterstaining was done using Mayer's hematoxylin. Known positive and negative controls (omission of the primary antibody) were used in every run.

The following regions of a slide were constantly examined: the tumor mass (excluding necrotic regions), tumor growing edge, areas of *in situ* carcinoma (DCIS), other characteristic juxtatumoral areas (ex hyperplastic

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ducts), and noncancerous peritumoral tissue. Slides were estimated for the presence and the intensity of staining (expressed in a scale of 1-3 and the percentage of positive cells). The H-score (35) was used for the analysis of results, calculating the intensity and the percentage of staining by the formula (%*1 + %*2 + %*3). Cases with an H-score <25 were considered negative.

Fluorescence Detection of mER, mPR, and mAR. For the detection of membrane steroid receptors, a (partial) regeneration of membrane proteins was done as described previously (31). Briefly, after a mild melting of the embedding medium at 42.5°C for 20 min, slides were dewaxed and rehydrated, incubated (37°C, 2 h) in citrate buffer (0.01 mol/L, pH 6.2), and washed in TBS (10 mmol/L, NaCl 150 mmol/L, pH 7.4). To minimize nonspecific absorption of albumin to membrane structures, we preincubated all slides with 3% BSA for 40 min. Then, the slides were washed in TBS and incubated for 1 h in the dark with 10^{-6} mol/L estradiol-BSA-FITC, progesterone-BSA-FITC, or testosterone-BSA-FITC (4 molecules FITC and 8-12 molecules steroid/molecule BSA) or 10^{-6} mol/L BSA-FITC (nonspecific binding, 12 molecules FITC/molecule of BSA, all from Sigma Hellas) in TBS. To eliminate binding of conjugate steroid to intracellular ER, PR, or AR (as microscopic tissue slides contain sectioned cells), 10^{-4} mol/L of ICI-182780 (Tocris), RU-486, or cyproterone acetate (Sigma Hellas) were included in the incubation buffer, respectively. Slides were then rinsed with TBS, coverslipped using polyvinyl alcohol mounting medium with Dabco antifading (Fluka Biochemika), and examined in a fluorescence confocal laser scanning microscope (Leica TCS SP). Photographs were taken from each slide (at least five different fields). Our previous data, both in breast cancer cells (34) as well as on cells of different origin (PC12 pheochromocytoma cells; ref. 36), brain tissue (37), and prostate cancer (26, 28, 31, 32), indicate that (*a*) steroid-specific antagonists do not modify the membrane binding characteristics of steroids, suggesting a different primary structure of the molecules (28, 30, 31); (*b*) saturation with high concentrations of steroids decreases the binding (37); and (*c*) BSA saturation decreases nonspecific binding of the protein-steroid conjugate on membranes (30). Based on these considerations, we advanced the above method for the tracing of membrane steroid receptors.

To detect objectively positive membrane staining, we applied a random 25-point spot grid (Chalkley array; ref. 38) on pictures obtained under identical conditions of exposure and laser intensity. The number of spot positively stained membrane cross-sections is a first indicator of membrane steroid receptor expression. The same grid, applied on BSA-FITC–stained samples, measures nonspecific association of ligands (see Fig. 2D for a representative image). Finally, the subtraction of these two numbers yields an estimate of positively stained cancer cells for membrane steroid receptors. We have preferred not to refer to stain intensity, as each steroid-BSA conjugate or BSA is labeled with a different number of FITC molecules.

Statistical Analysis. Statistical analysis was done by the use of appropriate parametric and nonparametric tests, as described in the Results section, by the use of Statistical Package for the Social Sciences version 14 and AMOS version 6 (SPSS, Inc.). Results attaining a statistical value of 0.05 were considered significant.

Results

Detection of EPO and EPOR. Twelve of the examined cases were negative for EPO, whereas 10 cases were negative for EPOR (H-score ≤25). Six of these tumors were negative for both EPO and EPOR, whereas the remaining cases were positive in one variable. EPO immunostaining was intracellular in all cases. In 47 cases (77%), EPO was marking more intensely at the growing edge of the tumor (Fig. 1D) than the tumor mass, whereas 3 cases were positive for EPO immunohistochemistry only in the tumor mass itself (Fig. 1C). Additionally, in 14 cases, *in situ* intraductal carcinoma (DCIS) was found as well. In all cases, DCIS was positive for EPO (Fig. 1B). Finally, positive staining was observed in five peritumoral nontumoral tissue, although usually hyperplastic ducts were moderately positive (Fig. 1A).

EPOR staining is expressed as discrete membrane dots, compatible with the membrane localization of the receptor (Fig. 1K), although in some cases a diffuse intracellular pattern was also detected. However, only membrane staining was taken into account. In 50 cases (82%), the tumor mass was positive for EPOR, with staining being more intense at the growing edge of the tumor (Fig. 1H) than the tumor mass (Fig. 1G). Noncancerous peritumoral tissue, hyperplastic or not, was negative (Fig. 1E) in all but one case. In eight cases, EPOR-positive ducts were observed (Fig. 1E) and seven additional DCIS foci were also positive (Fig. 1F and J). Vessels were constantly positive for EPOR (Fig. 1H, *arrowheads*). EPOR stained tumors more intensely than EPO, with a median H-score of ∼225 compared with ∼150 for EPO.

An interesting debate in the current bibliography challenges the validity of studies dealing with EPOR detection (ref. 39 and discussion by various groups, for example, refs. 40-46). Indeed, some authors debated the specificity of commercial EPOR antibodies. Especially for the antibody used in this study (EPOR or C-20, raised against the intracellular COOH terminus of the full-length EPOR), it was reported that it recognizes several non–EPOR-related proteins and especially variants of the heat shock protein 70 family (39). This lack of complete specificity (expected in a polyclonal antiserum) is responsible for the results present also in the present study. Indeed, as shown in Fig. 1K, EPOR immunostaining of cells shows two different components: a diffuse intracellular staining, which might be nonspecific, and a membrane staining, present as distinct dots, which might represent EPOR staining, based on the following criteria: (*a*) EPOR is a transmembrane protein. As presented in Fig. 1K, a dotted image was revealed in our samples, compatible with the detection of a membrane protein. Although heat shock protein 70 variants are presumably stained too, the latter might result in a diffuse staining pattern, compatible to the role of heat shock proteins as folding stabilizers of intracellular proteins. Additionally, although heat shock protein could be up-regulated or even secreted in cancer (47), the lack of staining in the pericellular space (Fig. 1K) ensures that the membrane spots might be

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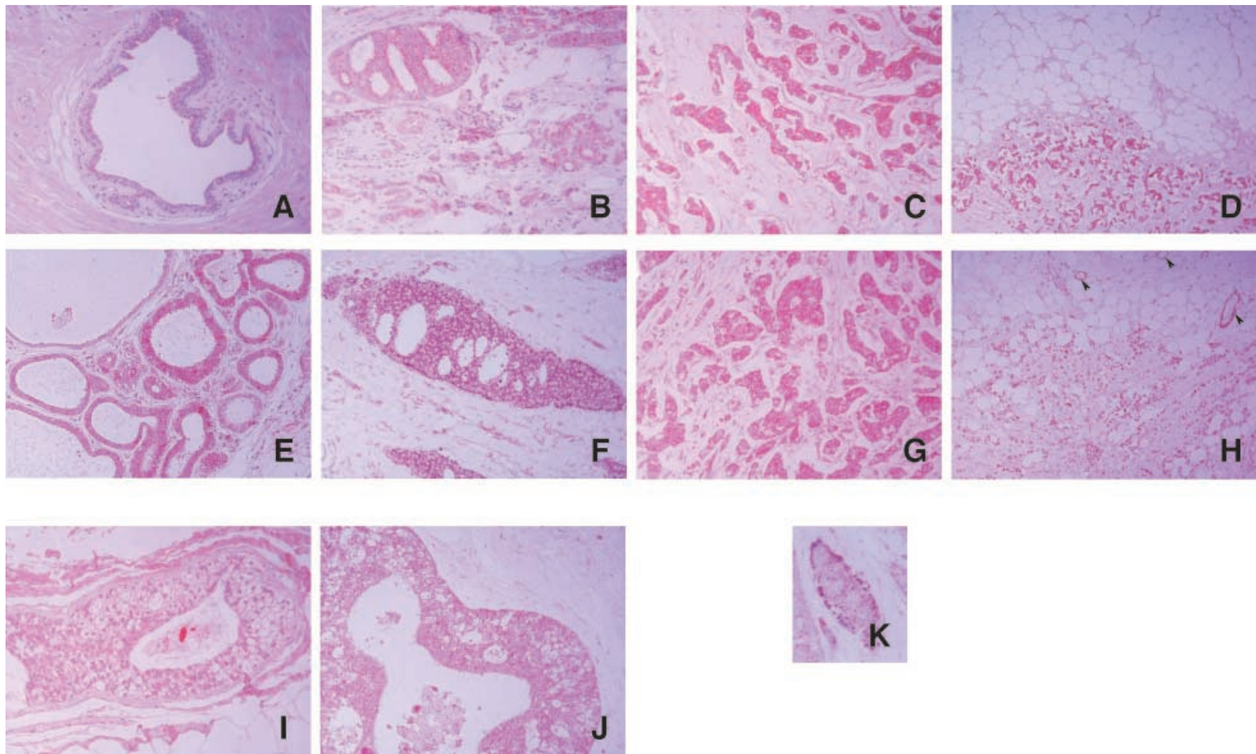

Figure 1. Detection of EPO and EPOR in surgical specimens of breast cancer. **A** to **D** and **I.** Immunochemical staining of EPO. **E** to **H** and **J.** Immunohistochemical staining of EPOR. **A** and **E.** A typical case of a hyperplastic duct, slightly positive for EPO (**A**) and EPOR (**E**). Remark that surrounding nonneoplastic tissue is negative for either marker. **B** and **F.** A case of DCIS heavily stained for EPO (**B**) and EPOR (**F**). Tumor mass is stained positive for EPO (**C**) and EPOR (**G**), whereas the growing edge of the tumors infiltrating the negative stroma is stained more intensely than the tumor mass itself for both antigens (**D** and **H**). *Arrowheads* point out blood vessels. A case of comedo *in situ* carcinoma is presented in **I** (EPO) and **J** (EPOR). Note that the tumor area is heavily stained for both markers. **K.** A higher magnification of EPOR-positive cells reveals a granular membrane staining, typical for EPOR.

full-length EPOR. (*b*) mRNA isolation from a few of these samples followed by reverse transcription-PCR for EPOR confirmed the presence of EPOR transcripts in the same samples (data not shown). (*c*) Finally, vessels in our study are, as expected, constantly positive for EPOR immunoreactivity (Fig. 1H).

As presented in Fig. 1, EPO and EPOR expression differs during the evolution of breast cancer: Nontumoral tissue is negative, hyperplastic ducts are moderately positive, whereas DCIS is constantly heavily stained for both variables. This result could reflect the increasing need of oxygen/nutrient supply of proliferating tumor cells or, conversely, an action of the EPO/EPOR system on tumor angiogenesis leading to an increased oxygen/nutrient supply to the tumor. This is further supported by the fact that the growing edges of tumors are more intensely stained than the tumor mass.

Six of our cases were untreated before the operation, whereas the rest 55 had been administered a preoperative therapy. All untreated cases were EPO and EPOR positive. EPO staining was slightly lower and EPOR intensity of staining was slightly higher in cases in which preoperative chemotherapy had been administered (H-scores: 200 $\pm$ 14 and 182 $\pm$ 6 for EPO and 194 $\pm$ 12 and 219 $\pm$ 6 for EPOR in patients who had not or had received preoperative chemotherapy, respectively).

Detection of Membrane Steroid Receptors. We detected mER, mPR, and mAR in 58, 57, and 57 of 61 cases, respectively. DCIS foci were always positive. Typical cases for mER, mPR, mAR, and nonspecific BSA-FITC staining are presented in Fig. 2A to D, respectively. Nontumoral breast tissue was negative for membrane steroid receptors, as was previously reported in prostate cancer (30, 31). mER was slightly more abundant than mPR and mAR in cases in which preoperative chemotherapy had been administered (7.3 $\pm$ 1.7 and 9.3 $\pm$ 0.5 positive counts for mER, 10.0 $\pm$ 2.0 and 8.0 $\pm$ 0.5 for mPR, and 10.6 $\pm$ 1.6 and 8.8 $\pm$ 0.5 for mAR, in patients who had not or had received preoperative chemotherapy, respectively).

Comparison of EPO/EPOR and Membrane Steroid Receptors with Clinical and Biological Variables. We have further explored whether the expression of EPO/EPOR and membrane steroid receptor expression correlates with several biological variables, considered as established prognostic factors in breast cancer (ER, PR, and Her2 expression) and HIF1α nuclear localization. Data from the above variables were extracted from the Pathology and Surgical Oncology Departments' databases. Results of significant correlations are graphically presented in Fig. 3A. It is to note that no correlation of either of the examined variables with tumor grade was

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found. In addition, as patients included in the present study had a variable therapeutic regimen before surgery, and had a variable tumor-node-metastasis status (see Table 1), we have done a correlation analysis in the whole population as well as in more homogeneous patient subgroups. As shown, tumors with a higher tumor-node-metastasis score express complex interactions, indicative of a more complex interplay of biological factors in the evolution of breast cancer. In general, a positive correlation was found among couples of membrane steroid receptors (mER, mPR, and mAR), suggesting a possible common regulation of the three-membrane steroid binding proteins in breast cancer. Furthermore, they correlate with intracellular ER and/or PR, suggesting a possible common regulation of membrane and intracellular receptors. A constant finding in all subset of data is the negative correlation between Her2 and EPOR on one hand and HIF1α nuclear localization on the other, suggesting a possible inverse role of the expression of either receptor on the cell membrane. Finally, EPO correlates with the nuclear localization of HIF1α, its transcriptional regulator, as well as with EPOR. Hierarchical clustering (Fig. 3B) revealed the existence of three independent clusters: cluster one (divided in two further subclusters: ER-PR-Her2 and mER-mPR-mAR), a second involving HIF1α, and a third grouping EPO and EPOR. It is interesting that HIF1α joins the clusters of steroid receptors/Her2 at a shorter distance than that of EPO/EPOR, indicating that the latter might reflect a different biological property of the tumor, perhaps related to its oxygen and nutrient supply, or a regulation of the EPO/EPOR system in breast cancer by other factors, in addition to HIF1α.

We have further evaluated the involvement of EPO/EPOR and membrane steroid receptor expression on the disease-free and the overall survival of patients. Cutoff points for each variable (mER, 6.5; mPR, 6; mAR, 7.5; EPO, 160.5; EPOR, 217.5) were determined as those maximizing the positive likelihood ratio after a receiver operating characteristic curve analysis (48). Kaplan-Meier survival analysis (Fig. 3C; Table 2) shows that EPO and EPOR expression correlate with a decreased disease-free and overall survival of patients. However, in a multivariate model, integrating all biological prognostic factors (ER, PR, and Her2; Table 3), mER, mPR, ER, and Her2 expression attained significance in disease-free survival, whereas ER, mER, mPR, and EPO expression are related to overall survival of patients. However, as we have presented above (Fig. 3), significant correlations of mER, mPR, mAR, and Her2 do not permit us to consider membrane steroid receptor expression as independent marker for prognosis. On the other hand, correlation of EPO with ER does not suggest an independent prognostic role of EPO expression, in breast cancer patients, although it could suggest a modulation of EPO/EPOR system by steroids, independent of HIF regulation.

Discussion

EPO is a hormone/growth factor acting on erythroid progenitors to stimulate erythropoiesis (see ref. 9 for a review). However, recent studies suggest that EPO is a pleiotropic cytokine, exerting broad tissue-protective effects in diverse nonhemopoietic organs, as well as in

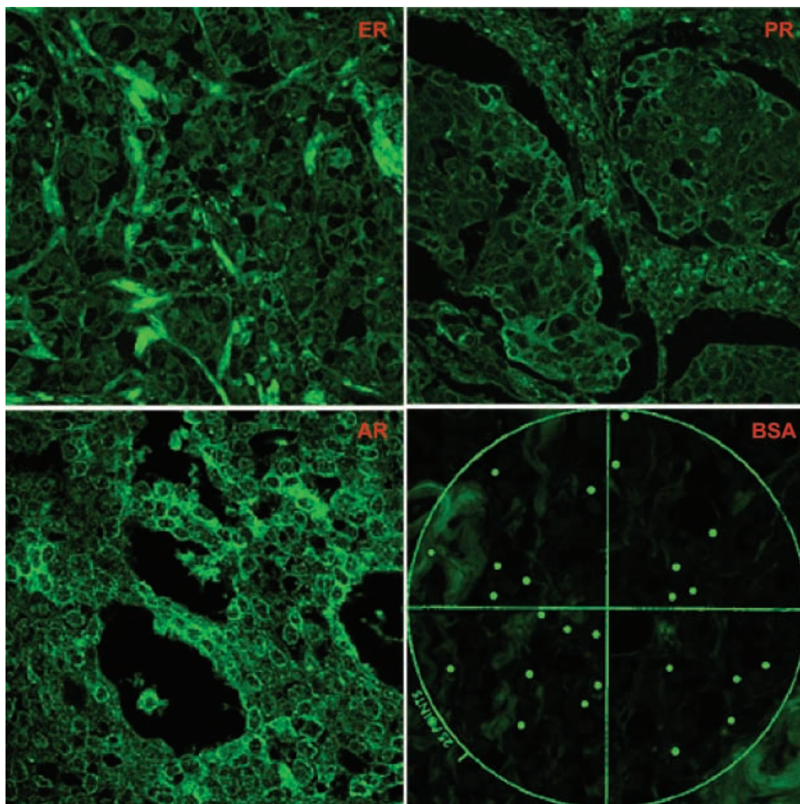

Figure 2. Detection of membrane steroid receptors in surgical specimens of breast cancer. Typical staining of mER, mPR, and mAR is presented. *Panel BSA,* nonspecific staining with BSA. In *panel BSA*, the Chalkley array (38) used for the quantification of membrane steroid receptors is also shown.

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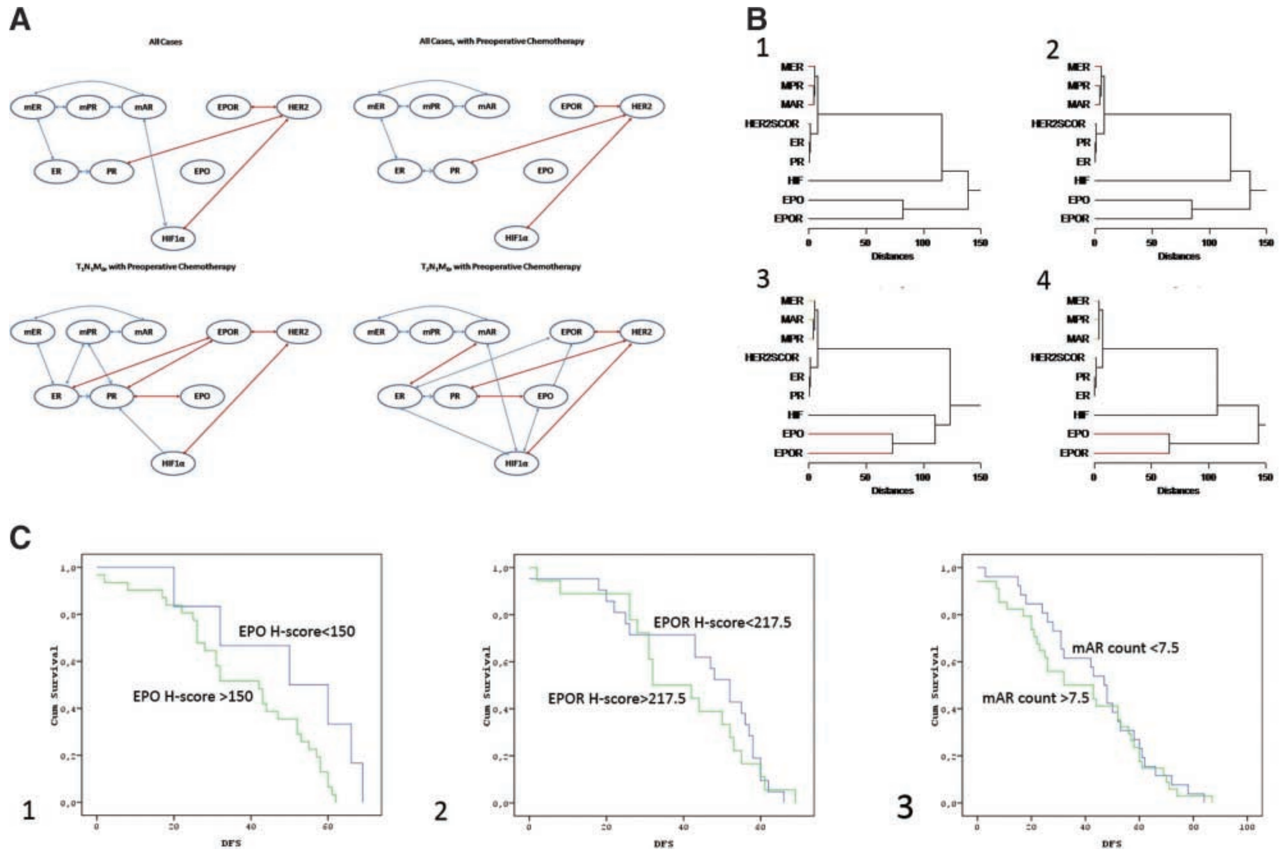

Figure 3. Statistical analysis of EPO/EPOR and membrane steroid receptors with other biological variables. **A.** Bivariate correlations among different biological data. Only significant ($P < 0.05$) positive (*blue*) and negative (*red*) correlations are presented. The four panels show the correlations in all patients (*1*), those who received preoperative chemotherapy (*2*), and those who received preoperative chemotherapy and were classified as $T_1N_1M_0$ (*3*) or $T_2N_1M_0$ (*4*). **B.** Classification tree analysis of biological data in our patients' population. *Numbers* represent the same categories as in **A**. **C.** Representative Kaplan-Meier survival curves for EPOR (*1*), EPO (*2*), and mAR (*3*). Disease-free survival is shown as the time variable. Cutoff points are indicated in the legend of Table 2.

malignancies (2, 49-52). Indeed, EPO binding to EPOR triggers several signaling cascades leading to cell growth–supporting ability (6). Nevertheless, current data about the interference of EPO in prognosis and its interrelation with other tumoral biological features remain inconclusive (reviewed in ref. 53), a result possibly attributed to the specificity of EPOR antibodies, discussed above (see Results). Previous studies in breast cancer specimens have reported positive immunohistochemical staining for EPO/EPOR (2, 5), although no clear relationship of EPO/EPOR expression with

Table 2. Kaplan-Meier analysis of EPO/EPOR and membrane steroid receptors in breast cancer

Variable	Disease-free survival		Overall survival	
	Log rank	*P*	Log rank	*P*
mER	24.3	0.143	26.6	0.085
mPR	8.5	0.933	16.1	0.444
mAR	**27.7**	0.023	**45.5**	0.0001
EPO	**97.4**	0.0001	**88.7**	0.0001
EPOR	**58.6**	0.0001	**13.1**	0.0001

NOTE: Log rank is presented, together with the statistical significance of each variable, for the disease-free and overall survival. Significant correlations are marked in **bold**. Analysis was done in the subset of patients who received a preoperative chemotherapy. Median values were used for the analysis of results. Cutoff values were calculated as those maximizing the positive likelihood ratio after a receiver operating characteristic curve analysis of patients' data (48). Cutoff values were as follows: mER, 6.5; mPR, 6; mAR, 7.5; EPO, 160.5; and EPOR, 217.5.

Table 3. Cox regression coefficients of biological variables

	Overall survival			Disease-free survival		
	B	SE	Significance	B	SE	Significance
mER	4.575	2.008	**0.023**	3.901	1.896	**0.040**
mPR	3.608	1.573	**0.022**	3.059	1.460	**0.036**
mAR	−1.578	0.787	**0.045**	−1.364	0.749	0.069
ER	−27.638	11.387	**0.015**	−23.395	10.517	**0.026**
PR	6.906	5.576	0.216	6.889	5.526	0.213
Her2	−3.734	2.018	0.064	−4.029	2.024	**0.047**
EPO	0.446	0.218	**0.041**	0.378	0.207	0.068
EPOR	0.087	0.045	0.055	0.076	0.043	0.081

NOTE: Significant correlations are shown in bold.

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steroid receptors has been established (52). In the present study, we assayed EPO/EPOR and steroid receptor expression in a series of 61 breast cancer specimens and relayed them to other biological markers and prognosis of the disease.

EPO gene induction is regulated mainly by HIF1α after hypoxia (9). In our samples, EPO expression correlates with the nuclear localization of HIF1α, suggesting that hypoxia is a major, albeit not the only, challenge for EPO production. In addition, the expression of EPO and EPOR during malignant transformation (negative in nontumoral tissue, slightly positive in hyperplastic ducts, and positive in DCIS, in the tumor mass, and especially at its growing edge) is indicative for a possible trophic role of EPO/EPOR system in the evolution of cancer or, alternatively, to its growing needs for oxygen and nutrient supply. In concordance, vessels are always positive in EPOR immunostaining. It is interesting that, in other types of breast hyperplasia (lactation), EPO is also expressed in breast epithelial cells and secreted in milk (54).

In extraerythroid tissues, EPO/EPOR system has been reported to be, in addition or in parallel to HIF1α, under the control of estrogen and/or progesterone (16, 17, 55, 56). As the breast is also under the control of steroids, we have assayed a possible relationship of EPO/EPOR and steroid receptor expression. We report that PR (positively related to ER) anticorrelates with EPO expression, suggesting that EPO is expressed in less differentiated carcinomas and might be related to worse prognosis, a result confirmed in Kaplan-Meier survival analysis. Alternatively and in view of steroid hormone-aryl hydrocarbon receptor heterodimerization (the latter receptor being a common partner of aryl hydrocarbon receptor nuclear translocator in addition to HIF1α), this inverse relationship could indicate sharing of the two systems for common resources (aryl hydrocarbon receptor nuclear translocator; see ref. 11 for a recent review).

In the present study, we have expanded the concept of steroid receptors to membrane ones, being responsible for the mediation of rapid, nongenomic actions of steroids (see ref. 24 for a recent review). We have previously shown that mAR is expressed in prostate cancer (31), being correlated with the Gleason score, although their activation induces regression of human prostate tumor xenografts (33) and potentiates the effect of cytoskeletal acting agents (34), suggesting a new potential cancer therapeutic target. Finally, in breast cancer cell lines and PC12 pheochromocytoma cells, mER and mAR exert opposing effects on growth, apoptosis, and secretion (34, 36). Here, we show for the first time that mER, mAR, and mAR are almost constantly coexpressed in tumor cells (but not in the surrounding nontumoral tissue), indicating a possible common regulation of all three proteins during the malignant transformation of breast tissue.

mER as well as EPOR have been related to the rescue of breast cancer cells subjected to several challenges (6, 34), although the activation of mAR promotes apoptosis (34) and induces tumor reduction in experimental animals (32, 33). Interestingly, expression of EPO, EPOR, and mAR related to a worse prognosis of patients. This result, taken together with reports indicating that EPO is a growth-regulating factor in several human malignancies, including breast cancer (57), debates the use of EPO in patients with breast neoplasia. In addition, they indicate a possible interconnection between membrane steroid and EPO/EPOR systems, a result currently under investigation. Such interactions have been previously reported between mER or mAR and the epidermal growth factor receptor (58, 59).

Based on our data, a question arises: Can EPO/EPOR and/or membrane steroid receptors represent new prognostic markers for breast cancer patients' outcome? In an inspired editorial, McGuire settled guidelines for the introduction of a new biological marker (60): A good biomarker might add independent information to currently applied variables, aiding in diagnostic, prognostic, predictive, and/or therapeutic decisions. Our results reveal significant correlation of both EPO/EPOR and membrane steroid receptors with other established biological variables, debating their value as independent prognostic indicators. However, in view of the administration of EPO in cancer patients and the results of the present study, we still are in the need of trials designed to assess the effect on survival, coupled with determination of expression and ligand affinity of EPOR on specific primary tumor types.

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