

Research article

# DAX-1 expression in human breast cancer: comparison with estrogen receptors ER- $\alpha$ , ER- $\beta$ and androgen receptor status

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## Abstract

**Background:** So far there have been no reports on the expression pattern of DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) in human breast cells and its relationship to the estrogen receptors, ER- $\alpha$  and ER- $\beta$ , and the androgen receptor (AR).

**Methods:** In this study we evaluated, by immunohistochemistry and Western blot analysis, the presence and distribution of DAX-1 in benign breast disease (BBD), *in situ* carcinoma (CIS), and ductal and lobular breast carcinomas.

**Results:** In BBD and breast carcinomas, DAX-1 was present in both the nuclei and the cytoplasm of epithelial cells, although in infiltrative carcinomas the percentage of nuclear immunoreaction was higher than in CIS. An important relation was observed between DAX-1 and AR expression and between this orphan receptor and nodal status.

**Conclusion:** DAX-1 might modify the AR and ER- $\beta$  intracellular location, and because a direct positive relation between the expression of these three receptors was found it could be assumed that the presence of DAX-1 in neoplastic cells might indicate a possible failure of endocrine therapies.

**Keywords:** androgen receptor, breast cancer, DAX-1, estrogen receptor- $\alpha$ , estrogen receptor- $\beta$

## Introduction

Estrogens and androgens have a crucial role in the proliferation and progression of breast cancer [1]. Estrogens are potent mitogens that mediate its proliferative action through the induction of cyclin D1, the major regulator of entry into the G1 stage of the cell cycle, and promote the secretion of positive or negative paracrine growth signals by breast stroma cells, stimulating epithelial cells to proliferate [2,3]. Androgens might indirectly enhance breast cancer progression, because they are transformed into estrogens by the P450 cytochrome aromatase complex [4].

The biological effects of estrogens are mediated by four different estrogen receptor pathways: (1) classical ligand-dependent [5], (2) ligand-independent [6], (3) DNA-binding-independent [7], and (4) cell surface non-genomic

signalling [8]. The first three pathways are mediated by two structurally related, but distinct, estrogen receptors (ERs): ER- $\alpha$  and ER- $\beta$ . The expression patterns of these two receptors suggest that the genes encoding ER- $\alpha$  and ER- $\beta$  are differentially expressed in various tissues. ER- $\alpha$  expression seems to predominate in the female reproductive organs, whereas ER- $\beta$  expression predominates in non-reproductive organs and the male reproductive tract [9]. Both receptors have distinct cellular distributions, regulate separate sets of genes, and can oppose each other's actions on some genes; these differences suggest that the two receptors could have different effects [1].

Most human breast cancers express ER- $\alpha$ , and this expression is generally considered a manifestation of their hormonal dependence. In breast cancer, the interruption

AR = androgen receptor; BBD = benign breast disease; CIS = *in situ* carcinoma; DAX = dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X; ER = estrogen receptor; NDS = normal donkey serum; TBS = Tris-buffered saline.

of ER- $\alpha$  function is an effective therapeutic strategy [10]. Treatment with antiestrogens (tamoxifen) is now the first-line therapy for metastatic disease, and also adjuvant therapy after mastectomy. Despite the initial benefits of tamoxifen, most patients eventually relapse with tumors that are not only tamoxifen-resistant but are also stimulated by this agent [11]. Although some reports have shown that the expression of ER- $\beta$  protein decreases in neoplastic breast cells, suggesting that ER- $\beta$  could be an inhibitor of tumorigenesis [1,12], other studies have suggested that ER- $\beta$  expression is maintained during breast cancer progression [3,13,14]. The function of ER- $\beta$  in breast pathobiology is therefore unclear, partly because most studies have focused on its mRNA levels rather than on the protein [15].

The functional status of androgen receptor (AR) might be related to the pathogenesis and biological behavior of female breast cancer [16]. The tissues that express the lowest levels of AR are the adult breast, the colon, the lung, and the adrenal gland [17], and a low expression of AR has also been shown in breast tumors [18]. The molecular mechanism for AR expression loss in breast cancer is attributed to the active loss of an allele of the gene encoding AR; this loss might be crucial in predicting and influencing the response of breast cancer to endocrine therapy [16]. In addition, the loss of expression or function of this receptor is related to the activity of regulator proteins such as DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), an unusual orphan member of the nuclear hormone receptor superfamily whose expression pattern is restricted to those tissues directly involved in steroid hormone production and reproductive function [19]. DAX-1 functions as a global negative regulator of steroid hormone production by repressing the expression of multiple genes involved in the steroidogenic pathway [20]. The function of DAX-1 in females is unknown [21], and its role in breast cancer has not yet been studied.

The aim of this study was to elucidate the expression pattern of DAX-1, ER- $\alpha$ , ER- $\beta$ , and AR in different types of breast carcinoma by immunohistochemical techniques and Western blot analysis, to improve our knowledge of the influence of these receptors in the development and progression of breast cancer and their possible influence in endocrine therapies.

## Materials and methods

### Materials

Breast samples used in this study were obtained by total or partial mastectomy and included a total of 67 cases: 14 cases of *in situ* carcinoma (CIS), 24 of infiltrative ductal carcinoma, 15 of infiltrative lobular carcinoma, and 14 of benign proliferative diseases including ductal and lobular hyperplasia, apocrine metaplasia, fibroadenoma, and fibro-

cystic changes. Benign proliferative diseases and CIS were observed in perimenopausal women (average  $\pm$  SEM age  $45.81 \pm 13.46$  years), whereas infiltrative and invasive tumors belonged to postmenopausal women of higher average age ( $59.93 \pm 14.89$  years). All infiltrative tumor samples were classified by the TNM system [22]. After surgery, the hormonal status of each invasive carcinomatous lesion was evaluated, and antiestrogen treatment (tamoxifen) was applied in the positive cases for its cognate receptors. Removal of tissues and the study of archive samples were performed with the consent of the patients' relatives and permission of the Ethics Committee of the Príncipe de Asturias Hospital, Alcalá de Henares, Spain.

Each specimen was divided into two approximately equal portions: one was processed for immunohistochemistry (fixed with 10% formalin and embedded in paraffin), and the other was frozen in liquid nitrogen and maintained at  $-80^{\circ}\text{C}$  for Western blot analysis.

### Immunohistochemistry

Sections 5  $\mu\text{m}$  thick were processed by using the avidin-biotin-peroxidase complex (ABC) method. After the removal of paraffin, sections were hydrated and incubated for 20 min in 0.3%  $\text{H}_2\text{O}_2$  to inhibit endogenous peroxidase activity; to retrieve the antigen the sections were incubated with 0.1 M citrate buffer (pH 6) for 30 seconds in a conventional pressure cooker [23]. After being rinsed in Tris-buffered saline (TBS), the slides were incubated with 3% normal donkey serum (NDS) in TBS for 30 min to prevent non-specific binding of the first antibody. The sections were then incubated with mouse monoclonal anti-ER- $\alpha$  (Novocastra Laboratories Ltd, UK) (1:60 dilution), rabbit polyclonal anti-ER- $\beta$  (Chemicon, Temecula, CA, USA) (1:500 dilution), rabbit polyclonal anti-AR (Novocastra) (1:50 dilution), and rabbit polyclonal anti-DAX-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:20 dilution) primary antibodies, each diluted in TBS containing 0.3% NDS. Sections were incubated overnight with primary antibodies: at  $37^{\circ}\text{C}$  for ER- $\alpha$ , ER- $\beta$ , and DAX-1, and at  $20^{\circ}\text{C}$  for AR.

The sections were then washed in TBS and incubated for 1 hour with either pig anti-rabbit (for ER- $\beta$ , AR, and DAX-1) or rabbit anti-mouse (for ER- $\alpha$ ) biotinylated immunoglobulins (Dako, Barcelona, Spain), diluted 1:400 in TBS plus 0.3% NDS. After being washed in TBS, the sections were incubated with avidin-biotin-peroxidase complex (Dako) for 1 hour and developed with 3,3'-diaminobenzidine (DAB), using the glucose oxidase-DAB-nickel intensification method [24]. Sections were then dehydrated and mounted in DePex (Probus, Badalona, Spain).

To assess the specificity of the immunoreaction, negative and positive controls were used. For negative controls, adjacent sections of each pathology (benign proliferative

diseases, and ductal and lobular carcinomas) were incubated with preimmune serum depending on the first antibody (mouse serum [for ER- $\alpha$ ] and rabbit serum [for ER- $\beta$ , AR, and DAX-1]), or using the antibody preabsorbed with an excess of purified antigens, or omitting the primary antibody. As positive controls, sections of rat ovary for ER- $\alpha$ , human testes for ER- $\beta$ , and human prostate for the AR and DAX-1 were processed with the same antibody.

### Immunoblotting

For Western blot analysis, tissues were homogenized in extraction buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 1 mM 1,4-dithiothreitol, and 0.2 M EDTA, pH 8) in addition to a cocktail of protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10  $\mu$ g/ml leupeptine, and 1  $\mu$ g/ml aprotinin) and phosphatase inhibitors (200  $\mu$ g/ml sodium fluoride and 50  $\mu$ g/ml sodium orthovanadate) in the presence of 0.5% Triton X-100 and 0.1% SDS. Homogenates were centrifuged for 10 min at 10,000 rpm at 4°C. Supernatants were mixed with an equivalent volume of SDS buffer (10% SDS in Tris/HCl, pH 8, containing 50% glycerol, 0.1 mM 2-mercaptoethanol, and 0.1% bromophenol blue). The mixture was then denatured for 4 min at 96°C, and 70  $\mu$ g aliquots of protein were subjected to SDS-polyacrylamide-gel electrophoresis (SDS-PAGE; 9% polyacrylamide). After SDS-PAGE, proteins were transferred to nitrocellulose membranes (0.2  $\mu$ m pore size) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, and 20% methanol) for 4 hours at 250 mA constant current. In brief, the blots for ERs were blocked in 5% Blotto (Santa Cruz) for 50 min; the sheets for AR and DAX-1 were blocked in 5% Blotto in addition to 1% NDS for 30 min. After being blocked, blots were incubated overnight with primary antibodies diluted 1:400 (ER- $\alpha$ ), 1:4000 (ER- $\beta$ ), or 1:200 (AR and DAX-1) in 1:9 blocking solution at room temperature (for ER- $\alpha$ , ER- $\beta$ , and AR) or at 37°C (for DAX-1).

After extensive washing with TBS/Tween 20, the membranes were incubated with the following peroxidase-conjugated secondary antibodies, diluted in 1:9 blocking solution: goat anti-mouse for ER- $\alpha$ , or goat anti-rabbit for ER- $\beta$  and DAX-1 (Chemicon) at 1:4000. For AR the sheets were incubated with the pig anti-rabbit biotinylated immunoglobulins (Dako) at 1:2000 and afterwards an extensive wash with streptavidin-peroxidase (Zymed Laboratories Inc, South San Francisco, CA, USA) at 1:8000 dilution in the same 1:9 blocking solution. Finally, the membranes were developed with an enhanced chemiluminescence kit, in accordance with the procedure described by the manufacturer (Amersham, Little Chalfont, Buckinghamshire, UK).

### Statistical analysis

The possible correlation between the principal parameters recorded in the 39 cases that showed infiltrative carcinoma

(patient age, axillary lymph node status, disease-free duration, and DAX-1, ER- $\alpha$ , ER- $\beta$ , and AR expression), were evaluated by means of a multivariate analysis of the principal components. The purpose was to obtain a small number of linear combinations from the seven variables. These combinations account for most of the variability in the data with minimum loss of real data.

The results of principal components analysis were confirmed by determination of the correlation of DAX-1 expression and the status of ER- $\alpha$ , ER- $\beta$ , AR, and several clinicopathological factors, by using Fisher's exact test or the  $\chi^2$  test (two-tailed).

Correlation of the DAX-1 expression between the different pathologies studied was calculated with the non-parametric Spearman correlation coefficient. Statistical analysis was performed with a StatGraphics computer program.

## Results

### Western blot analysis

The results of the Western blot analysis are shown in Fig. 1. For each antibody, only a single band was found at the corresponding molecular mass: 65 kDa for ER- $\alpha$ , 59 kDa for ER- $\beta$ , 130 kDa for AR, and 55 kDa for DAX-1. For ER- $\alpha$ , a similar expression was observed in benign diseases (Fig. 1a) and in CIS (Fig. 1a). Immunoreaction was more intense in cases presenting infiltrative ductal carcinoma (Fig. 1a) and infiltrative lobular carcinoma (Fig. 1a). The band corresponding to ER- $\beta$  was only observed in infiltrative lobular carcinoma (Fig. 1b). AR and DAX-1 immunoreactions were observed in benign diseases as well as in both cancer types (Fig. 1c,d).

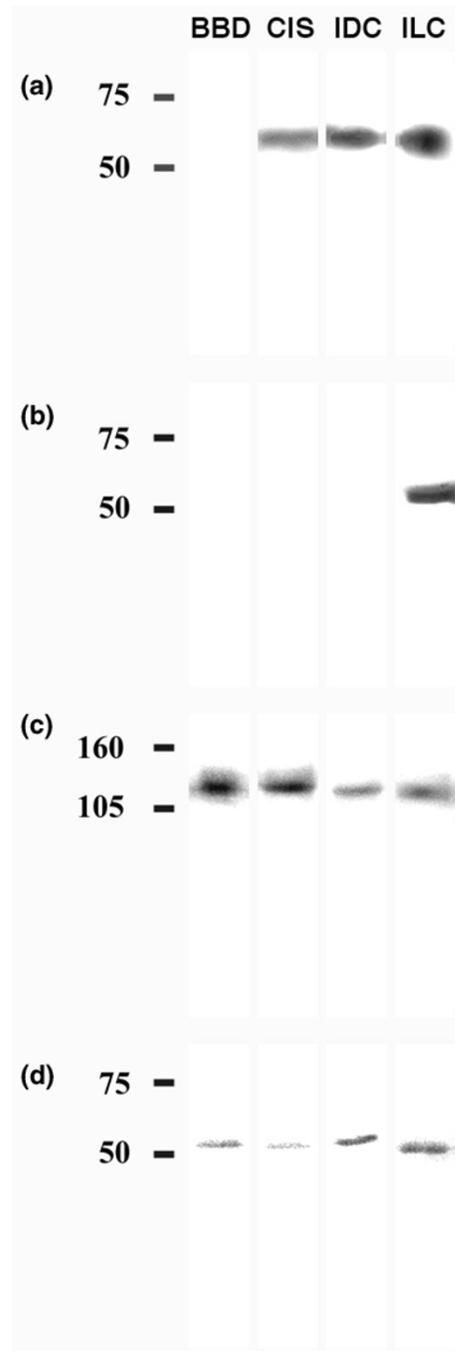
### Immunohistochemical study of control sections

The immunohistochemical study showed no reaction in the negative controls incubated with the preimmune serum, using the antibody preabsorbed with an excess of purified antigen, or omitting the primary antibody. Immunostaining of rat ovary, human testis, and human prostate sections was always positive.

### Immunohistochemical study of ER- $\alpha$

The results obtained by immunohistochemistry are summarized in Table 1.

In benign breast diseases no immunoreaction to ER- $\alpha$  was observed (Fig. 2a). In CIS, 42.86% of cases showed nuclear immunoreaction to this receptor (Fig. 2b), and 28.57% presented cytoplasmic reaction. In ductal infiltrative carcinomas (Fig. 2c) the percentage of cases immunostained in the nuclei increased (62.5%), but those cases showing cytoplasmic reaction decreased (12.5%). Infiltrative lobular carcinomas showed nuclear labelling in 60% of cases (Fig. 2d) and cytoplasmic immunoreaction in 40%.

**Figure 1**

Immunoblots of ER- $\alpha$  (a), ER- $\beta$  (b), AR (c), and DAX-1 (d) in human breast. Relative molecular masses are shown at the left. Each blot is representative of its respective group. For all panels: lane 1, benign breast disease; lane 2, *in situ* carcinoma; lane 3, infiltrative ductal carcinoma; lane 4, infiltrative lobular carcinoma.

#### Immunohistochemical study of ER- $\beta$

In benign breast diseases no ER- $\beta$  expression was found (Fig. 2e). In CIS, immunostaining was observed in 28.57% of cases, always in the cytoplasm (Fig. 2f).

In infiltrative ductal carcinoma the percentage of cases showing nuclear immunoreaction was similar to that observed in CIS. However, the percentage of cases showing cytoplasmic labelling (Fig. 2g) was increased (37.5%). In infiltrative lobular carcinoma (Fig. 2h) the percentage of cases showing cytoplasmic immunoreaction was even higher (60%).

#### Immunohistochemical study of AR

In the benign diseases no nuclear immunoreaction was observed (Fig. 2i), although cytoplasmic immunoreaction was observed in a small proportion of cases (21.4%). In the remaining pathologies only a few cases showed nuclear immunostaining, and the number of cases showing cytoplasmic immunoreaction increased from CIS (28.57%; Fig. 2j) and infiltrative ductal carcinomas (37.5%; Fig. 2k) to infiltrative lobular carcinomas (60%; Fig. 2l).

#### Immunohistochemical study of DAX-1

All pathologies studied, including benign breast diseases, showed either nuclear or cytoplasmic immunoreaction to DAX-1 (Fig. 2m–p). The cytoplasmic immunoreaction was granular and appeared in epithelial cells (Fig. 2n).

The highest percentages of cases showing positive immunoreaction (in the nucleus or in the cytoplasm) were found in cancer patients (ductal and lobular). Analysing the DAX-1 immunoreaction between the different pathologies studied by using the non-parametric Spearman correlation coefficient, we found that the only statistically significant difference was between benign diseases and infiltrative lobular carcinomas ( $P = 0.037$ ,  $P < 0.05$ ).

#### Association of DAX-1 expression with ER- $\alpha$ , ER- $\beta$ , and AR status and other clinicopathological factors

The results of the principal components analysis, presented as a two-dimensional component plot (Fig. 3), show the weights for the principal components analysed (patient age, axillary lymph node status, disease-free duration, and DAX-1, ER- $\alpha$ , ER- $\beta$ , and AR immunoreaction). Each variable is represented by a point and the reference lines are drawn at 0 for each dimension. A weight close to 0 therefore indicates that the variable contributes little to the component. All of these variables explain the 53.85% of variability in the original data. The variables that form an acute angle with respect to the origin are more correlated. Thus, the most related variables were AR and DAX-1 expression, although relations between DAX-1 and nodal status, AR and ER- $\beta$ , and between ER- $\alpha$  and both patient age and disease-free duration were also found. However, no relation was found between the nodal status and disease-free duration or ER- $\alpha$  because they were in opposite quadrants.

When the Fisher's exact or  $\chi^2$  test was used (Table 2), a statistically significant difference was observed between DAX-1 nuclear expression and nodal status ( $P < 0.05$ )

Table 1

Numbers of samples showing immunoreaction to ER- $\alpha$ , ER- $\beta$ , AR, and DAX-1

Protein	BBD ( <i>n</i> = 14)		CIS ( <i>n</i> = 14)		Infiltrative ductal carcinoma ( <i>n</i> = 24)		Infiltrative lobular carcinoma ( <i>n</i> = 15)	
	N	C	N	C	N	C	N	C
ER- $\alpha$	0 (0)	0 (0)	6 (42.86)	4 (28.57)	15 (62.5)	3 (12.5)	9 (60)	6 (40)
ER- $\beta$	0 (0)	0 (0)	0 (0)	4 (28.57)	0 (0)	9 (37.5)	0 (0)	9 (60)
AR	0 (0)	3 (21.4)	1 (7.15)	4 (28.57)	3 (12.5)	9 (37.5)	1 (6.66)	9 (60)
DAX-1	3 (21.42)	5 (35.72)	3 (21.43)	4 (28.57)	9 (37.5)	6 (25)	7 (46.66)	5 (33.33)

Results in parentheses are percentages. AR, androgen receptor; BBD, benign breast diseases; C, cytoplasmic immunoreaction; CIS, *in situ* carcinomas; ER, estrogen receptor; N, nuclear immunostaining.

and between DAX-1 nuclear and cytoplasmic expression and AR expression ( $P < 0.05$  and  $P < 0.001$ , respectively).

## Discussion

In previous reports, ER- $\alpha$  mRNA was detected in 92% of breast cancers, and ER- $\beta$  mRNA in 85%; however, after protein translation, the percentages of cases decreased to 72% and 32%, respectively [25]. This agrees with the observation of a decrease in ER- $\beta$  protein expression when the neoplastic cells begin to proliferate in proliferative preinvasive mammary tumors [12]. This decreased level of ER- $\beta$  protein, associated with cell proliferation, suggests a protective effect of ER- $\beta$  against the mitogenic activity of estrogens in mammary premalignant lesions [12]. In comparison, the decrease in ER- $\alpha$  protein expression after translation is lower, suggesting a higher receptivity to estrogens in these preinvasive tumor tissues, and an increased risk of tumorigenesis [26].

In this study we observed that a high proportion of infiltrative lobular carcinoma cases (60%) showed nuclear immunostaining for ER- $\alpha$ ; the percentage was similar for infiltrative ductal carcinomas (62.5%). This finding contrasts with the absence of immunoreaction in BBD samples. These results are in agreement with those observed by Palmieri and colleagues [3], who found low ER- $\alpha$  expression in fibrocystic disease and high ER- $\alpha$  expression in invasive ductal cancer.

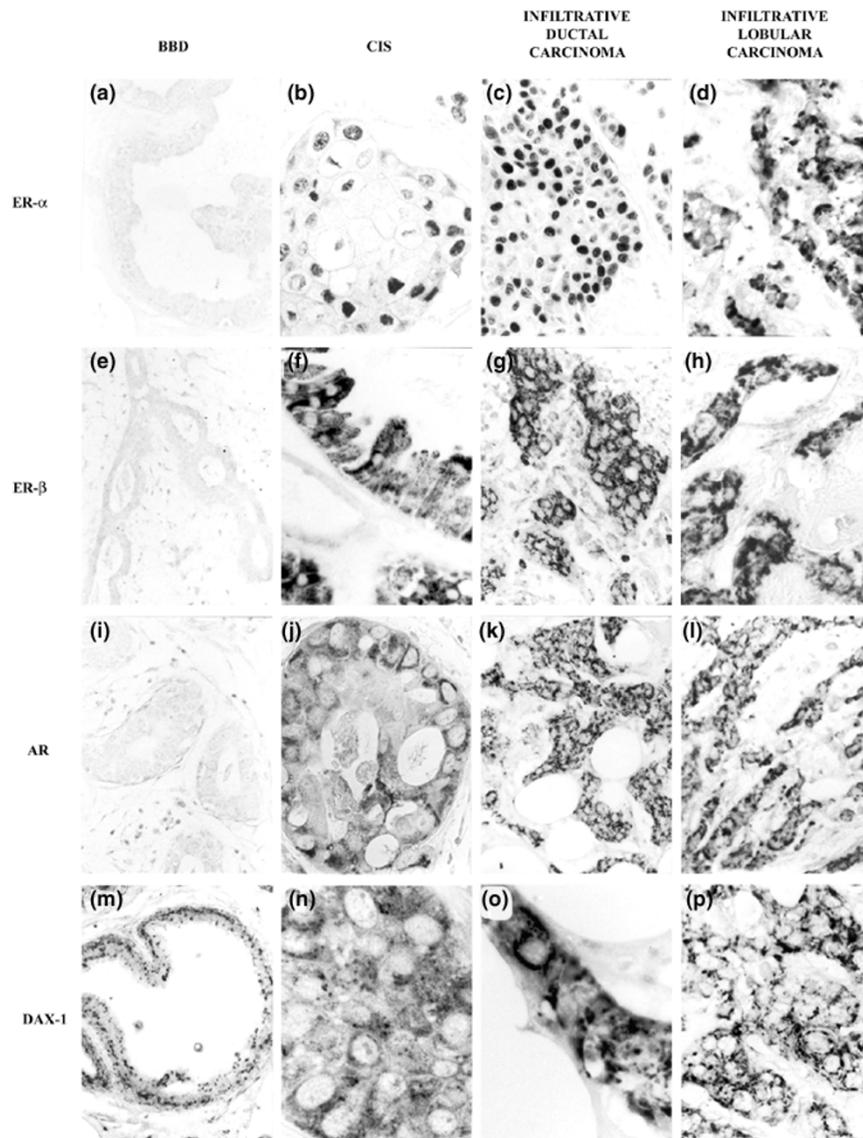
Our data revealed no correlation between the expression of ER- $\alpha$  and that of ER- $\beta$ ; thus, all breast carcinomas analysed gave a negative nuclear reaction to ER- $\beta$ , although cytoplasmic expression was found in some cases. This cytoplasmic location suggests that ER- $\beta$  is inactive because of the absence of a ligand, because these receptors can escape rapidly from the nucleus and shuttle repeatedly between nucleus and cytoplasm [27]. It has also been reported that, in endothelial cells, ER- $\beta$  can act via the cytoplasm through a novel pathway [28]. This signal transduction pathway functions through 17 $\beta$ -estra-

diol-activated ER- $\alpha$  and ER- $\beta$  receptors and induces the transcriptional activation of Stat-regulated promoters; the pathway involves an agonist-bound ER-activated phosphorylation cascade, resulting in the nuclear transcriptional activation of target transcription factors [28].

Jarvinen and colleagues [13] found that 60% of breast tumors were ER- $\beta$ -positive and that this expression was significantly associated with negative axillary node status and low grade of malignancy. Mann and colleagues [29] reported that the expression of ER- $\beta$  in more than 10% of cancer cells was associated with better survival. However, Dotzlaw and colleagues [30] showed that tumors co-expressing ER- $\alpha$  and ER- $\beta$  were node-positive and tended to have higher grade of malignancy. These data suggest that ER- $\beta$  might have a role in breast cancer cells and that this role depends on the presence or absence of ER- $\alpha$  expression.

The expression of ER- $\alpha$  and ER- $\beta$  in breast cancer cells is crucial in determining whether antiestrogen therapy would be efficient, because some antiestrogenic drugs such as 4-OH-tamoxifen are more competitive than other estrogenic substance antagonists of ER- $\beta$  [31], and do not display agonist activity when the receptor concentration increases. ER- $\beta$  therefore seems to suppress the partial agonist activity of tamoxifen on ER- $\alpha$  [32], so the response to this treatment will evidently depend on the distribution of these receptors. In our study, we found that those patients that showed ER- $\alpha$  expression were usually not the same as those who showed ER- $\beta$  expression, and most ER- $\alpha^+$ /ER- $\beta^-$  patients had a poor prognosis and a worse response to hormonal therapy than ER- $\alpha^+$ /ER- $\beta^+$  patients.

In postmenopausal women, only about 25% of circulating testosterone arises from that secreted by the ovary. The remaining 75% comes from circulating precursors derived from either the adrenal cortex or the ovary [33]. Circulating levels of these androgenic steroids might be important in the maintenance of local estrogen synthesis. Adrenal

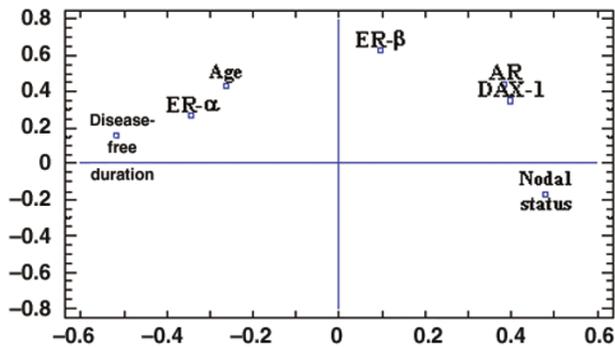
**Figure 2**

Expression of estrogen receptors ER- $\alpha$ , ER- $\beta$ , androgen receptor (AR) and DAX-1 in human breast. **(a–d)** Immunohistochemistry of ER- $\alpha$ . **(a)** Negative immunoreaction in ductal hyperplasia (magnification  $\times 300$ ). **(b)** Sample of ductal carcinoma *in situ* (CIS) showing a strong immunolabelling of ER- $\alpha$  in the nuclei of neoplastic cells (magnification  $\times 500$ ). **(c)** A strong nuclear reactivity to ER- $\alpha$  antibody was observed in samples of infiltrative ductal carcinoma (IDC; magnification  $\times 250$ ). **(d)** ER- $\alpha$  was observed in the cytoplasm of some cells in infiltrative lobular carcinoma (ILC; magnification  $\times 300$ ). **(e–h)** Immunohistochemistry of ER- $\beta$ . **(e)** Fibroadenoma was always negative to ER- $\beta$  (magnification  $\times 300$ ). **(f)** Ductal CIS showing an intense cytoplasmic immunoreaction to ER- $\beta$  (magnification  $\times 400$ ). **(g)** Positive reaction to ER- $\beta$  in the cytoplasm of IDC cells (magnification  $\times 250$ ). **(h)** ILC showing cytoplasmic reaction to ER- $\beta$  (magnification  $\times 450$ ). **(i–l)** Immunohistochemistry of androgen receptor (AR). **(i)** Negative reaction to AR antibody in hyperplasia (magnification  $\times 300$ ). In CIS **(j)** (magnification  $\times 400$ ) and IDC **(k)** (magnification  $\times 200$ ), cytoplasmic immunolabelling of AR was observed. **(l)** ILC showing cytoplasmic immunostaining of AR (magnification  $\times 200$ ). **(m–p)** Immunohistochemistry of DAX-1. An intense cytoplasmic reaction with granular pattern was observed in cases from ductal hyperplasia **(m)** (magnification  $\times 200$ ), CIS **(n)** (magnification  $\times 600$ ), IDC **(o)** (magnification  $\times 600$ ), and ILC **(p)** (magnification  $\times 350$ ). BBD, benign breast diseases.

androgens might therefore promote breast cancer, although higher levels of these hormones might prevent it [34]. Androgens have two primary effects on mammary tumor cells: (1) in the absence of estrogens they stimulate the growth of breast cancer cells by binding to ER- $\alpha$ , and

this effect can be blocked by treatment with antiestrogen; (2) in the presence of estrogens they act as antiestrogens to inhibit the stimulation of the growth of breast cancer cells by estrogen (this effect is exerted through AR and can be blocked by antiandrogens) [4].

Figure 3



Two-dimensional component plot showing the results of the principal components analysis obtained from the 39 patients with infiltrative carcinoma. A close correlation between DAX-1 immunoreaction, nodal status, and AR expression is observed. Component 1 is on the x-axis; component 2 is on the y-axis.

It has been reported that up to 80% of human breast tumors are AR-positive and that AR-negative status is correlated with the aggressive features of breast cancers [35], because patients with AR-negative tumors had a significantly poorer response rate to hormone therapy than those with positive tumors [16]. In contrast with these findings, in this study we observed nuclear immunostaining to AR in 7.15% of CIS, 12.5% of infiltrative ductal carcinomas, and only 6.66% of infiltrative lobular carcinomas. However, the percentage of cytoplasmic reaction to AR in these three pathologies was higher, suggesting that this receptor forms a multiprotein complex in the cytoplasm and, in the presence of ligand, AR becomes tightly bound in the nucleus [36]. This cytoplasmic location of AR could be explained by the presence of DAX-1. Recent studies have suggested that DAX-1 is not exclusively a nuclear orphan receptor but can be also detected mainly in the cytoplasm, functioning as a potent corepressor for estrogen receptors in mammalian cells, presumably by the

Table 2

**Association of DAX-1 with various clinicopathological factors and with estrogen receptor- $\alpha$ , estrogen receptor- $\beta$  and androgen receptor status**

Parameter	n	Negative	Nuclear location	P	Cytoplasmic location	P
Age (years)						
≤50	11	5	4		2	
>50	28	9	11	0.699	8	0.652
TNM class						
T <sub>1</sub>	19	10	7		2	
T <sub>2</sub>	16	6	6		4	
T <sub>3</sub>	2	1	1		0	
T <sub>4</sub>	2	0	0	0.8486	2	0.216
Nodal status (%)						
≤33	26	14	6		6	
33–90	10	2	7		1	
>90	3	0	1	<0.05	2	0.1439
Histological type						
Ductal	24	10	9		5	
Lobular	15	4	6	0.699	5	0.4028
Disease-free duration (months)						
5–20	3	0	1		2	
20–30	10	2	5		3	
>30	26	11	9	0.3081	6	0.1713
ER- $\alpha$						
–	10	5	3		2	
C	9	2	2		5	
N	20	7	10	0.6077	3	0.1649
ER- $\beta$						
–	23	9	7		7	
C	13	5	6		3	
N	3	1	2	0.7138	0	0.6766
AR						
–	20	12	6		2	
C	17	2	7		8	
N	2	0	2	<0.05	0	<0.001

direct occupation of the coactivator-binding surface and the subsequent recruitment of additional corepressor [37]. Moreover, Holter and colleagues [21] demonstrated that DAX-1 could sequester AR in the cytoplasm, inhibiting its activation, presumably by both transcriptional and nontranscriptional mechanisms, indicating a possible function of DAX-1 as a cytoplasmic retention factor. We have found an important relation between AR and DAX-1 expression in infiltrative carcinomas with the use of principal components analysis and Fisher's exact test, the significance being higher in the cytoplasmic location frequencies. This correspondence suggests that this orphan receptor might be a cytoplasmic retention factor and tethers AR.

DAX-1 is also able to sequester ER- $\beta$  in the cytoplasm in an agonist-dependent manner [37]. Like ER- $\beta$ , DAX-1 seems to be a candidate regulator of AR expression in tissues such as prostate and ovary [38]; it can be also assumed that this regulation occurs in breast tissues because we have observed a statistically significant correlation between ER- $\beta$  expression and that of AR.

Interestingly, the hormonal steroidogenic pathway seems to be specifically inhibited by DAX-1 in steroid-producing cells [21]. The modulation of DAX-1 expression by diverse bioactive substances and physiological conditions probably represents an important mechanism for controlling steroid hormone production [20].

## Conclusion

DAX-1 might modify the intracellular location of AR and ER- $\beta$  but not that of ER- $\alpha$ . Because we have observed a direct positive relation between nodal status and DAX-1 expression, it can be assumed that the presence of this receptor in neoplastic cells might indicate a possible failure of endocrine therapies; it would therefore be advisable to study DAX-1 expression in those patients presenting with breast cancer before hormonal therapy application, and to develop new therapeutic strategies that can modulate the action of this receptor to prevent the subsequent resistance that the neoplastic cells acquire.

## Competing interests

None declared.

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