Prognostic significance of CD24 and claudin-7 immunoexpression in ductal invasive breast cancer

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Abstract. This study aimed to identify the CD24 and CD44 immunophenotypes within invasive ductal breast carcinoma (IDC) subgroups defined by immunohistochesmistry markers and to determine its influence on prognosis as well as its association with the expression of Ki-67, cytokeratins (CK5 and CK18) and claudin-7. Immunohistochemical expression of CD44 and CD24 alone or in combination was investigated in 95 IDC cases arranged in a tissue microarray (TMA). The association with subgroups defined as luminal A and B; HER2 rich and triple negative, or with the other markers and prognosis was analyzed. $CD44^+/CD24^-$ and $CD44^-/CD24^+$ were respectively present in 8.4% and 16.8% of the tumors, a lack of both proteins was detected in 6.3%, while $CD44^+/CD24^+$ was observed in 45.3% of the tumors. Although there was no significant correlation between subgroups and different phenotypes, the $CD44^+/CD24^-$ phenotype was more common in the basal subgroups but absent in HER2 tumors, whereas luminal tumors are enriched in $CD44^-/CD24^+$ and $CD44^+/CD24^+$ cells. The frequency of $CD44^+/CD24^-$ or $CD44^-/CD24^+$ was not associated with clinical characteristics or biological markers. There was also no significant association of these phenotypes with the event free (DFS) and overall survival (OS). Single $CD44^+$ was evident in 57.9% of the tumors and was marginally associated to grading and not to any other tumor characteristics as well as OS and DFS. $CD24^+$ was positive in 74.7% of the tumors, showing a significant association with estrogen receptor, progesterone receptor and Ki-67 and a marginal association with CK18 and claudin-7. Expression of claudin-7 and Ki-67 did not associate with the cancer subgroups, while a positive association between CK18 and the luminal subgroups was found (P=0.03). CK5, CK18 and Ki-67 expression had no influence in OS or DFS. Single $CD24^+$ (P=0.07) and claudin-7 positivity (P=0.05) were associated with reduced time of recurrence, suggesting a contribution of these markers to aggressiveness of breast cancer.

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Introduction

Breast carcinoma is a disease which is known for its striking histopathological heterogeneity recognized for a long time and which is classified into several histological subtypes (1).

In recent years, molecular profiling technologies have provided further evidence that breast cancer is a heterogeneous disease at the molecular level. The major breast cancer biological subtypes have been defined by gene expression profiles (2) or histochemical biomarkers (3) and have led to a working model for breast cancer molecular taxonomy (4). According to this classification, breast cancers can be subdivided into luminal tumors expressing estrogen receptor (ER) and the progesterone receptor (PR) (ER^+/PR^+) which may be further subclassified into luminal A and luminal B tumors. The latter subtype exhibits either HER2 positivity or high expression of the proliferation marker Ki-67 (5). There are two ER/PR negative subtypes: HER2 rich ($ER^-/PR^-/HER2^+$), and triple negative (negative for the three markers). These subtypes of breast cancer show distinct behaviors related to prognosis, survival and response to specific therapies (1). Within the triple negative disease tumors two subtypes were identified: basal like and a new molecular subtype referred as $claudin^{low}$ characterized by the low gene expression of claudins (claudin-4, claudin-7 and claudin-3) (6). It is possible that this heterogeneity is derived in part from the transformation of different subsets of stem/progenitor cells in each biological subtype as proposed by Dontu *et al* (7).

Cancer stem cells have been defined as a subset of tumor cells that display stem cell properties including self-renewal and differentiation, giving rise to several cell types in the tumor, contributing to its heterogeneity (8).

In breast cancer, a subpopulation of cells characterized by the surface markers $CD44^+/CD24^{low}$ has been reported to be highly tumorigenic when injected into immunocompromised mice (9). Shipitsin *et al* (10) reported that gene expression

of $CD44^+$ and $CD24^+$ cells isolated from breast carcinomas exhibited distinct profiles and that the $CD44^+$ profile showed genes consistent with a stem-cell like profile.

A correlation of the $CD44^+/CD24^{low}$ phenotype with specific breast cancer subtypes has been previously reported. This tumor phenotype appears to be common in basal like tumors (11,12) and in triple negative tumors (13). It has been suggested that tumor cells with a $CD44^+CD24^-$ subpopulation may have a worse clinical behavior (13,14). In contrast, other studies have not revealed a significant association of $CD44^+/CD24^-$ with a potential progress or recurrence (15,16). As tumorigenesis involves complex biological mechanisms, Park *et al* (12) recently highlighted the need for evaluation of CD44/CD24 in combination with other markers.

Our aim in the present study was to investigate the expression of CD44 and CD24 alone or in combination with a series of invasive ductal breast carcinomas arranged in a tissue microarray (TMA). Results were related to tumor subtypes defined by the immunohistochemical markers: ER and PR, HER2 and to the expression of Ki-67, basal and luminal cytokeratins (CK5, CK6 and CK18) with special focus on the tight junction protein claudin-7. In addition, we investigated the clinicopathological and prognostic significance with respect to overall survival (OS) and disease-free survival (DFS) of the different combinations of CD44 and CD24 and of the analyzed biological markers.

Materials and methods

Subjects. The cohort was assembled from patients with primary ductal invasive breast carcinoma (IDC) diagnosed at the Instituto do Cancer Arnaldo Vieira de Carvalho between 2001 and 2007 after approval by its Institutional Review Board. A total of 95 cases were retrieved from the medical files, and the following patient information was collected: age, histological classification, nuclear grade, tumor size, node status, tumor recurrences, regional and distant metastasis, treatment, disease-free survival (DFS), and overall survival (OS). Patients were enrolled according to the inclusion criteria consisting of suitable paraffin blocks for immunohistochemistry, adequate clinicopathological data and sufficient follow-up. The Nottingham system was used for assessing tumor nuclear grading. In all IDC cases the treatment involved mastectomy, radiotherapy, chemotherapy and axillary lymph node dissection. Cases with a positive immunohistochemical ER analysis received hormone therapy. Median follow-up of the analyzed IDC cohort was 4.8 years. At the final follow-up (4.9 years), 66 (69.5%) IDC patients were alive and 27 (28.4%) died of the disease. Characteristics of this retrospective cohort are detailed in Table I.

Table I. Clinicopathological properties of the breast carcinomas evaluated.

Parameters	Patients (%)
Age (years)	
Median (range)	58 (28-88)
Tumor size (cm)	
Median (range)	4.5 (0.4-12.0)
pTNM	
I	7 (7.4)
II	41 (43.2)
III	45 (47.4)
IV	2 (2.1)
Nuclear grading	
I	2 (2.1)
II	40 (42.1)
III	52 (54.7)
ND	1 (1.1)
Nodal status	
Positive	60 (63.2)
Negative	33 (34.7)
ND	2 (2.1)
Metastasis status	
M1	21 (22.1)
M0	60 (63.2)
ND	14 (14.7)
Condition	
Alive	66 (69.5)
Deceased	27 (28.4)
ND	2 (2.1)

ND, not known.

TMA construction. For each case, all available hematoxylin and eosin (H&E)-stained sections were reviewed to confirm the diagnosis of IDC and to select a representative tumor area for TMA construction and immunostaining.

For TMA construction, H&E-stained slides from each paraffin-embedded tumor block were checked to select a morphologically representative tumor area, which was then selected to construct the TMA paraffin block. Two tissue cylinders of each case with a diameter of 1 mm were punched from the marked tumor areas of each of the 95 donor paraffin blocks and distributed into 4 new recipient paraffin blocks using the Manual Tissue Arrayer I (Beecher Instruments, Silver Spring, MD, USA). Sections (3 μm) were cut from the TMA paraffin block using the paraffin tape-transfer system (Instrumedics, St. Louis, MO, USA). One section was stained with H&E to confirm the presence of the tumor by light microscopy, and standard slides were used for immunohistochemical analyses.

Immunohistochemistry. Monoclonal antibodies to CK5 (clone XM 26), CK6 (clone CHK6B), CK18 (clone DC10), PR (clone PgR636), Ki-67 (clone MIB1) and HER2 (polyclonal) were obtained from Dako (Dako, Denmark) and diluted 1:300, 1:100, 1:800, 1:500, 1:400, 1:2000, respectively. Each slide was also stained with 1:50 anti-ER (Neomarkers, clone 6F-11), claudin-7 diluted 1:500 (clone AB5487; Abcam, Cambridge, MA), CD44 (clone DF1485), raised against all forms of CD44, diluted 1:100 (Novocastra Laboratories Ltd., Newcastle, UK) and CD24 (clone C-20), diluted 1:100 (Santa Cruz Biotechnology, Palo Alto, CA, USA).

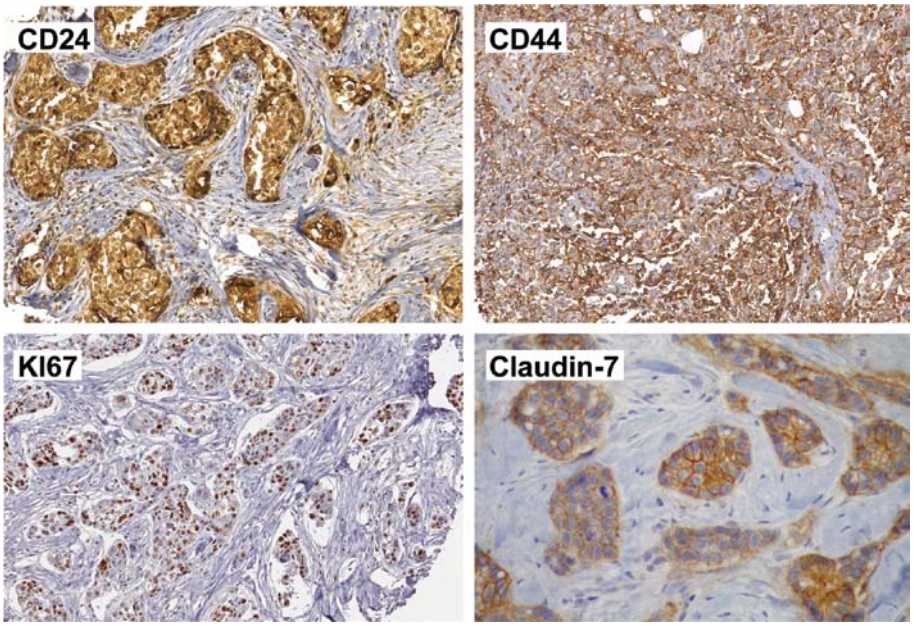

Figure 1. Immunopositivity of ductal invasive breast carcinomas for CD44, CD24, Ki-67 and claudin-7 (x200).

After deparaffinazation and rehydration of tissue microarray sections from formalin-fixed paraffin embedded samples, antigen retrieval was performed in a pressure cooker. After primary antibody incubation and secondary biotinylated streptavidin-peroxidase amplification, antigen detection was carried out by a solution containing 3,3' diaminobenzidine (Sigma, USA) and 6% H_2O_2. Counterstaining was performed with Harris hematoxylin. Positive controls were included in each staining reaction and consisted of breast cancer known to express each of the antigens of interest.

An Allred score of ER and PR nuclear immunoreactivity ≤2 was considered a negative result (17). For HER2 samples, lack of reactivity in <10% of the tumor cells was scored as 0. Barely perceptible focal membrane staining was scored as 1. Weak to moderate staining observed in >10% of the tumor cells was defined as 2. Strong staining of the complete membrane in >10% of the tumor cells was scored as 3. Staining was considered positive only if samples scored 3^+ according to the American Society of Clinical Oncology of American Pathologists recommendation. For basal cytokeratins (CK 5/6) and claudin-7, samples were considered positive if ≥10% of tumor cells were reactive.

Specimens that exhibited a positive staining in ≥20% of cells were considered as Ki-67 positive. Negative controls were performed by omission of the respective primary antibody. Normal breast tissue usually presents 2^+ pattern of claudin-7 expression and 1^+ or 0 of CD24 and CD44. Therefore, 3^+ usually represents overexpression.

CD44 and claudin-7 were considered positive when membranes were stained in a distinct and delicate pattern without reactive cytoplasm or nuclei. We used a HERcept test model for reporting results and the scoring was: 0, totally negative; 1^+, 1-10% positive neoplastic cells; 2^+, moderate staining in 10-30% of neoplastic cells; and 3^+, >30% of strongly reactive neoplastic cells. CD24 was detected mainly in the cytoplasm and scoring was conducted as for CD44. Normal breast tissue usually presents a 2^+ pattern of claudin-7 expression and 1^+ or 0 for CD24 and CD44. Therefore, 3^+ usually represents overexpression.

For the prognostic investigation and survival analysis, each individual immunophenotype pattern was evaluated for single $CD44^+$ (CD44 positive cells), single $CD24^+$ (CD24 positive cells) and four combinations, $CD44^+/CD24^+$, $CD44^-/CD24^-$, $CD44^+/CD24^-$, $CD44^-/CD24^+$.

Statistical methods. Correlation between antigen expression and other clinicopathological parameters was examined by the χ^2 test. Survival probabilities were estimated by the univariate Kaplan-Meier method, survival curves were compared by the log-rank (Mantel-Haenszel method). SSPS version 10.0 for Windows was used. Statistical significance was set at $p<0.05$.

Results

A total of 95 patients with IDC were evaluated. The demographic and clinical data are summarized in Table I.

The frequency of the biological markers analyzed is shown in Table II. Nuclear immunoreactivity for ER and PR and Ki-67 was expressed in 47.4% and HER2 in 34.7% of the cases. Claudin-7 was detected in 38.9%, basal cytokeratin 5 in 17.9% and CK18 in 61.1% of the cases. Tumors were mostly positive for either CD44 (57.9%) or CD24 (74.7%).

The pattern of CD44 expression was mainly membranous whereas CD24 was expressed predominantly in the cytoplasm. Claudin-7 was also expressed at the membrane and Ki-67 immunostaining was exclusively nuclear. Positive cytokeratins 5 and 18 were found in 17.9 and 61.1% of the cases, respectively. CK6 was expressed in only 2.1% of the cases. Examples of immunoreactivity of $CD44^+$, $CD24^+$, Ki-67 and claudin-7 are displayed in Fig. 1.

To determine if the expression of CD44 and CD24 is associated with tumor characteristics the clinicopathological

Table II. Frequency of immunostaining among arrayed breast carcinoma cases.

Parameters	Patients (%)
Estrogen receptor	
Positive	45 (47.4)
Negative	35 (36.8)
ND	15 (15.8)
Progesterone receptor	
Positive	45 (47.4)
Negative	35 (36.8)
ND	15 (15.8)
HER2	
Positive	33 (34.7)
Negative	51 (53.7)
ND	11 (11.6)
Ki-67	
Positive	45 (47.4)
Negative	40 (42.1)
ND	10 (10.5)
CD44	
Positive	55 (57.9)
Negative	23 (24.2)
ND	17 (17.9)
Phenotypes	
$CD44^+/CD24^+$	43 (45.3)
$CD44^+/CD24^-$	8 (8.4)
$CD44^-/CD24^+$	16 (16.8)
$CD44^-/CD24^-$	6 (6.3)
ND	22 (23.2)
CD24	
Positive	71 (74.7)
Negative	19 (20.0)
ND	5 (5.3)
Claudin-7	
Positive	37 (38.9)
Negative	49 (51.6)
ND	9 (9.5)
CK5	
Positive	17 (17.9)
Negative	68 (71.6)
ND	10 (10.5)
CK6	
Positive	2 (2.1)
Negative	80 (84.2)
ND	13 (13.7)
CK18	
Positive	58 (61.1)
Negative	27 (28.4)
ND	10 (10.5)
Subgroups	
Luminal A	24 (25.3)
Luminal B	23 (24.2)
HER2	9 (9.5)
Triple negative	20 (21.5)
ND	19 (20.0)

HER2, human epidermal growth factor receptor 2; CK, cytokeratin; ND, not known.

features and biological markers were compared in the CD44 and CD24 positive and negative cases (Table III).

Presence of CD44 was not associated with ER, PR, Ki-67, claudin-7 nor with clinical characteristics, but a marginal association with high grade (P=0.08) was observed (Table III). Positive expression of CD24 was associated with ER (P=0.05), PR (P=0.02) and Ki-67 positivity (P=0.01). There was a trend although not statistically significant, for the presence of CD24 to correlate with claudin-7 and CK18.

Cases were categorized into four phenotypes according to CD44 and CD24 positive/negative expression. Of the 95 patients, 8 (8.4%) were categorized into the $CD44^+/CD24^-$ phenotype; the proportion of double positive ($CD44^+/CD24^+$) and double negative ($CD44^-/CD24^-$)tumors was 43 (45.3%) and 6 (6.3%) respectively, while the $CD44^-/CD24^+$ phenotype was detected in 16.8% of the tumors (Table II).

We used an immunohistochemical surrogate for the gene array expression to define the subgroups of breast carcinoma. Among 95 IDC, a total of 47 tumors were categorized within the luminal subgroups: luminal A (25.3%) and luminal B (24.2%); 20 (21.5%) of 95 had a triple negative profile and the HER2 subgroup contributed to 9.5% of the tumors (Table II).

Next, we explored the expression of CD44/CD24 phenotypes across the breast cancer subgroups (Table IV). No association with a particular subtype of breast cancer was observed. However, $CD44^+/CD24^-$ expression was more common in the triple negative subgroup, absent in the $HER2^+$ group and presented a low frequency in the luminal B subgroup (10%). Double positive tumors were more frequent in the luminal subgroups, and we noted that these subgroups contained a high proportion of $CD44^-/CD24^+$ cells. Our evaluation did not show any association of the frequency of different phenotypes with the clinicopathological parameters, pTNM stage, lymph node status, CK7, CK5, CK18, claudin-7 expression. However, we observed a marginal association (P=0.08) of a high proportion of double positive cases with high nuclear grade and of Ki-67 positive frequency with $CD44^-/CD24^+$ tumors (P=0.06). Concerning the association with the frequency of metastasis, the higher frequency was related to the $CD44^+/CD24^+$ phenotype (82.4%) followed in descending order by $CD44^-/CD24^+$ (11.8%), $CD44^-/CD24^-$ (5.9%) and $CD^+/CD24^-$ (0%).

We also evaluated whether the expression of claudin-7, Ki-67, CK5 and CK18 differed in the tumor subgroups (Table V). Results indicated a similar frequency of Ki-67

Table III. Association of CD44 and CD24 expression in breast carcinomas with clinicopathological characteristics and biological markers.

	CD44			CD24		
Parameters	Negative (%)	Positive (%)	P-value[a]	Negative (%)	Positive (%)	P-value[a]
pTNM						
I/II	14 (35.0)	26 (65.0)	0.31	13 (28.3)	33 (71.7)	0.12
III	8 (22.2)	28 (77.8)		6 (14.0)	37 (86.0)	
Nuclear grading						
I	0 (0)	2 (100.0)		1 (50.0)	1 (50.0)	
II	14 (41.2)	20 (58.8)	0.08	8 (21.6)	29 (78.4)	0.52
III	8 (19.5)	33 (80.5)		9 (18.0)	41 (82.0)	
Nodal status						
Positive	16 (34.0)	31 (66.0)	0.30	13 (22.8)	44 (77.2)	0.79
Negative	6 (20.7)	23 (79.3)		6 (18.8)	26 (81.3)	
Estrogen receptor						
Positive	11 (26.2)	31 (73.8)	0.79	5 (11.4)	39 (88.6)	0.05
Negative	9 (31.0)	20 (69.0)		10 (30.3)	23 (69.7)	
Progesterone receptor						
Positive	7 (21.9)	25 (78.1)	0.42	2 (6.1)	31 (93.9)	**0.02**
Negative	12 (32.4)	25 (67.6)		12 (27.3)	32 (72.7)	
HER2						
Positive	6 (19.4)	25 (80.6)	0.12	5 (16.7)	25 (83.3)	1.00
Negative	16 (39.0)	25 (61.0)		10 (19.6)	41 (80.4)	
Ki-67						
Positive	13 (31.0)	29 (69.0)	1.00	4 (9.3)	39 (90.7)	**0.01**
Negative	9 (28.1)	23 (71.9)		13 (33.3)	26 (66.7)	
Claudin-7						
Positive	6 (18.8)	26 (81.3)	0.12	4 (11.8)	30 (88.2)	0.17
Negative	15 (35.7)	27 (64.3		13 (26.5)	36 (73.5)	
CK5						
Positive	4 (28.6)	10 (71.4)	1.00	2 (12.5)	14 (87.5)	0.51
Negative	17 (28.8)	42 (71.2)		14 (21.5)	51 (78.5)	
CK6						
Positive	0 (0)	2 (100.0)	1.00	0 (0)	1 (100.0)	1.00
Negative	19 (27.9)	49 (72.1)		14 (18.2)	63 (81.8)	
CK18						
Positive	12 (24.5)	37 (75.5)	0.28	9 (15.8)	48 (84.2)	0.14
Negative	9 (37.5)	15 (62.5)		8 (32.0)	17 (68.0)	

HER2, human epidermal growth factor receptor 2; CK, cytokeratin; [a]statistical significance was determined by the χ^2 test. P-values (two-sided) <0.05 were considered statistically significant and are indicated in bold.

(P=0.47) in all 4 subgroups; cytokeratin 18 (CK18) expression was clearly associated to the luminal cases (P=0.003) and CK5 positivity was more common in HER2 and triple negative tumors (P=0.1). However, only 30% (n=4) of the triple negative tumors displayed CK5 immunoreactivity, emphasizing the small number of basal tumors in our tumor material. Positive claudin-7 was less frequent in the triple negative subgroup.

Claudin-7 was not associated with known prognostic parameters such as pTNM, nuclear grade or nodal status, but on the other hand, showed a trend toward the presence of metastasis (P=0.07). Claudin-7 was very likely to be positive in HER2 positive tumors (P=0.07), and was inversely associated to progesterone receptor (P=0.02) and CK5 presence (P=0.03) (Table VI).

Table IV. Distribution of phenotypes according to immunohistochemically-defined subgroups of invasive ductal carcinomas, clinicopathological features and biological markers.

Parameters	$CD44^+/CD24^+$ n (%)	$CD44^+/CD24^-$ n (%)	$CD44^-/CD24^+$ n (%)	$CD44^-/CD24^-$ n (%)	P-value[a]
Luminal A					
$HER2^-/ER^+$ or PR^+	12 (57.1)	1 (4.8)	7 (33.3)	1 (4.8)	
Luminal B					
$HER2^+/ER^+$ or PR^+	14 (70.0)	2 (10.0)	3 (15.0)	1 (5.0)	0.30
$HER2^+/ER^-$ and PR^-	6 (85.7)	0 (0)	0 (0)	1 (14.3)	
Triple negative	7 (38.9)	4 (22.2)	5 (27.8)	2 (11.1)	
pTNM					
I + II	21 (55.3)	4 (10.5)	7 (18.4)	6 (15.8)	0.12
III	22 (64.7)	4 (11.8)	8 (23.5)	0 (0)	
Nuclear grading					
I	1 (50.0)	1 (50.0)	0 (0)	0 (0)	0.08
II	16 (51.6)	2 (6.5)	8 (25.8)	5 (16.1)	
III	26 (66.7)	5 (12.8)	8 (20.5)	0 (0)	
Nodal status					
Positive	25 (56.8)	4 (9.1)	10 (22.7)	5 (11.4)	0.57
Negative	18 (64.3)	4 (14.3)	5 (17.9)	1 (3.6)	
Metastasis					
M0	24 (53.3)	7 (15.6)	10 (22.2)	4 (8.9)	0.16
M1	14 (82.4)	0 (0)	2 (11.8)	1 (5.9)	
Ki-67					
Positive	24 (60.0)	3 (7.52)	12 (30.0)	1 (42.1)	0.06
Negative	17 (54.8)	5 (16.1)	4 (12.9)	5 (16.1)	
Claudin-7					
Positive	20 (69.0)	3 (10.3)	5 (17.2)	1 (3.4)	0.5
Negative	22 (52.4)	5 (11.9)	11 (26.2)	4 (9.5)	
CK5					
Positive	8 (61.5)	1 (7.7)	3 (23.1)	1 (7.7)	0.98
Negative	34 (60.7)	6 (10.7)	11 (19.6)	5 (8.9)	
CK18					
Positive	32 (66.7)	4 (8.3)	9 (18.8)	3 (6.3)	0.22
Negative	9 (40.9)	4 (18.2)	6 (27.3)	3 (13.6)	

HER2, human epidermal growth factor receptor; ER, estrogen receptor; PR, progesterone receptor; CK, cytokeratin; [a]statistical significance was determined by the χ^2 test. P-value (two-sided) <0.05 was considered statistically significant.

To determine whether CD24 and CD44 and their combined phenotypes may affect overall survival (OS) and disease-free survival (DFS), we constructed Kaplan-Meier survival curves which were analyzed statistically (log-rank test). The median follow-up for this study was 4.8 years (range, 0.36-10.9 years).

When the analysis was performed according to the frequency of combined phenotypes we found that they did not have an impact on patient DFS or OS. However, we observed that the $CD44^+/CD24^-$ phenotype was more favorable with respect to OS (87.5% of patients alive) or DFS (100% without metastasis) while the $CD44^-/CD24^+$ phenotype indicated a poorer survival (53.3% of alive patients) and reduced DFS (83% without metastasis).

No influence of $CD44^+$ immunophenotype on OS or DFS was observed. Considering the single $CD24^+$ phenotype we noted no association with OS, but there was a significant association to a reduction of DFS, although a border line one (P=0.07). CK5, CK18 and Ki-67 expression had no influence in OS or DFS. However, claudin-7 positivity although not statistically associated with OS, was associated with reduced DFS. 62.9% for positive claudin-7 vs. 82.5% for claudin-7 negative (P=0.05). Claudin-7 positivity was also related to a

Table V. Distribution of biological markers in accordance with the molecular subgroups defined by immunohistochemical features of breast carcinoma.

Parameters	Luminal A (HER2$^-$/ER$^+$ or PR$^+$) n (%)	Luminal B (HER2$^+$/ER$^+$ or PR$^+$) n (%)	HER2$^+$/ER$^-$ and PR$^-$ n (%)	Triple negative n (%)	P-value[a]
Claudin-7					
Positive	11 (45.8)	14 (60.9)	5 (55.6)	6 (30.0)	0.22
Negative	13 (54.2)	9 (44.4)	4 (44.4)	14 (70.0)	
Ki-67					
Positive	14 (60.9)	15 (65.2)	5 (55.6)	8 (42.1)	0.47
Negative	9 (39.1)	8 (34.8)	4 (44.4)	11 (57.9)	
CK5					
Positive	4 (17.4)	2 (8.7)	4 (44.4)	6 (31.6)	0.10
Negative	19 (82.6)	21 (91.3)	5 (55.6)	13 (68.4)	
CK18					
Positive	18 (78.3)	20 (87.0)	5 (55.6)	7 (36.8)	**0.003**
Negative	5 (21.7)	3 (13.0)	4 (44.4)	12 (63.2)	
Nodal status					
Positive	17 (73.9)	12 (54.5)	6 (66.7)	12 (60.0)	0.58
Negative	6 (26.1)	10 (45.5)	3 (33.3)	8 (40.0)	
pTNM					
I	2 (8.3)	3 (13.0)	1 (11.1)	1 (5.0)	0.96
II	9 (37.5)	10 (43.5)	4 (44.4)	9 (45.0)	
III	13 (54.2)	9 (39.1)	4 (44.4)	9 (45.0)	
IV	0	1 (4.3)	0 (0.0)	1 (5.0)	
Metastasis					
M0	14 (63.6)	16 (84.2)	1 (14.3)	15 (75)	0.05
M1	8 (33.3)	3 (13.0)	13 (13.0)	2 (10.0)	

HER2, human epidermal growth factor receptor 2; [a]statistical significance was determined by the χ^2 test. A P-value (two-sided) <0.05 was considered statistically significant and is indicated in bold.

higher frequency of metastatic tumors (65%) as compared to claudin-7 negativity (40%, P=0.07). When we analyzed the distribution of cases relative to their subgroup profiles, we observed a larger proportion of patients who died or relapsed in the HER2 subgroup (P=0.03) within the follow-up period, whereas no differences in OS or DFS was verified among the other subgroups (Figs. 2 and 3).

Discussion

In this study we used a tissue microarray to examine the expression of hormone receptors and HER2 in 95 cases of invasive ductal carcinoma and categorized these cases into subgroups based on immunohistochemistry analysis. Next, we determined the expression of CD44 and CD24 in our tumor series and analyzed the association of these phenotypes with the different subgroups.

We found that luminal groups were the most common immunohistochemical subtypes within the range of other studies. There was also no significant association between subtypes and clinicopathological features, consistent with previous reports (18). In accordance to recent findings (19,20), the HER2-overexpressing subtype showed the worst OS and DFS, but the survival rate of patients with triple negative carcinomas was not different from that of other groups except for the HER2-overexpressing subtype. The luminal A group of patients showed an unexpectedly low OS and DFS. As in our study, this subgroup showed a high metastasis frequency (33.3%) as compared to the other subgroups and displayed a high proliferation score. These features may have contributed to the observed worse prognosis.

In contrast to results of Al-Hajj *et al* (9) reporting CD44$^+$/CD24$^-$ cells in all breast carcinomas studied, our evaluation of the conventional stem cell phenotype (CD44$^+$/CD24$^-$) indicated that only 8.4% of the analyzed tumors expressed this phenotype. The frequency of stem cells was expected to be low (21) and our frequency rate is lower than those reported by several authors (11,15,16,22). Analysis of several human breast cancer cell lines indicated that this particular phenotype was exhibited by only 25% (2/8) of the cell lines (23).

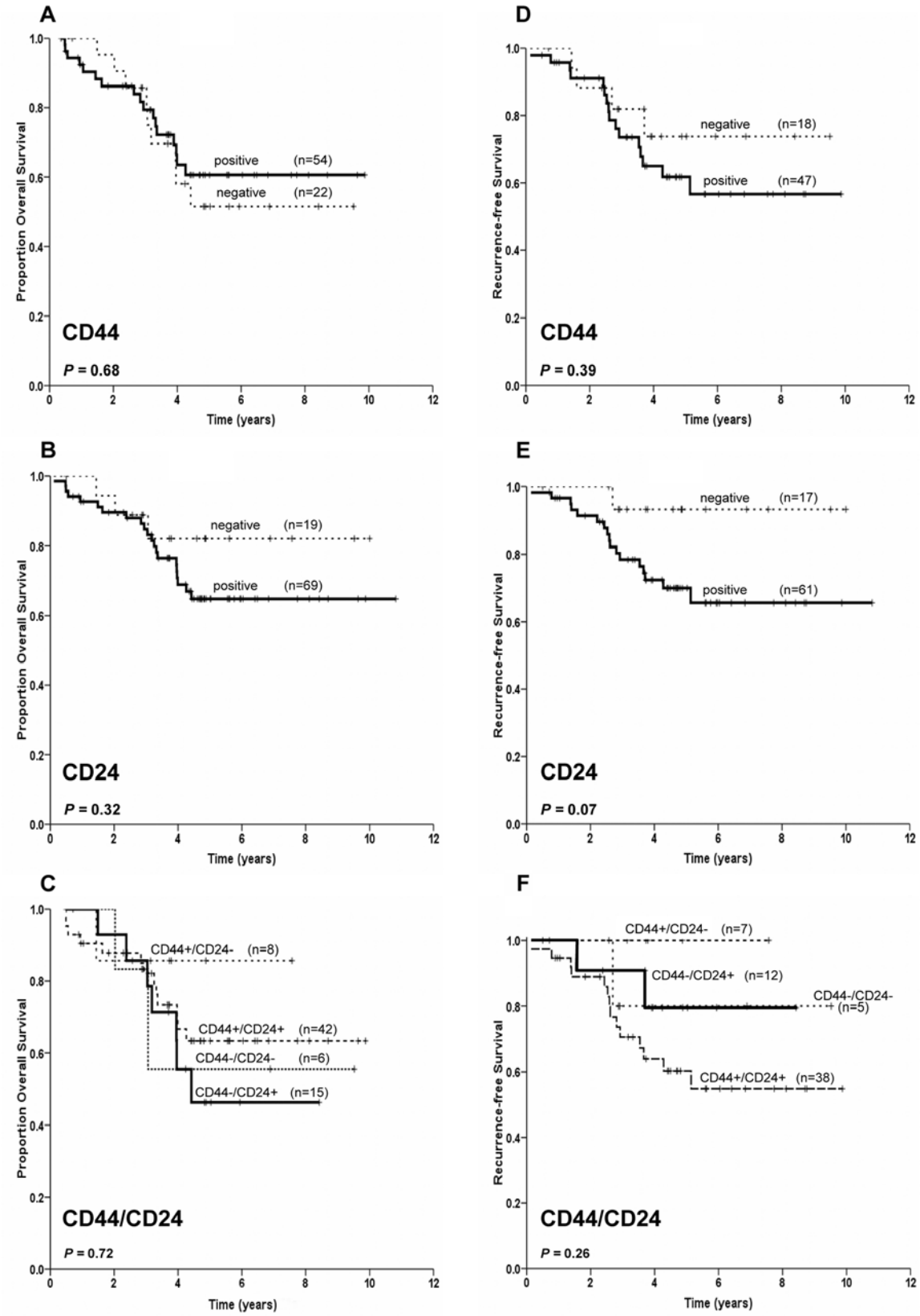

Figure 2. Overall survival and disease-free survival in all patients according to: CD44 (A and D); CD24 (B and E); and CD44/CD24 (C and F).

Several studies have delineated that the $CD44^{+}/CD24^{-}$ status is associated with the basal-like or triple negative subgroups of breast cancer (11,12,14,24). Although in our study this phenotype was more common in the triple negative subgroups, no statistical significance was seen in the comparison between phenotypes and subgroups. We also did not find an association with known prognostic parameters (pathological stage, nuclear grade and nodal status). Our results confirmed previous reports (11,15,16).

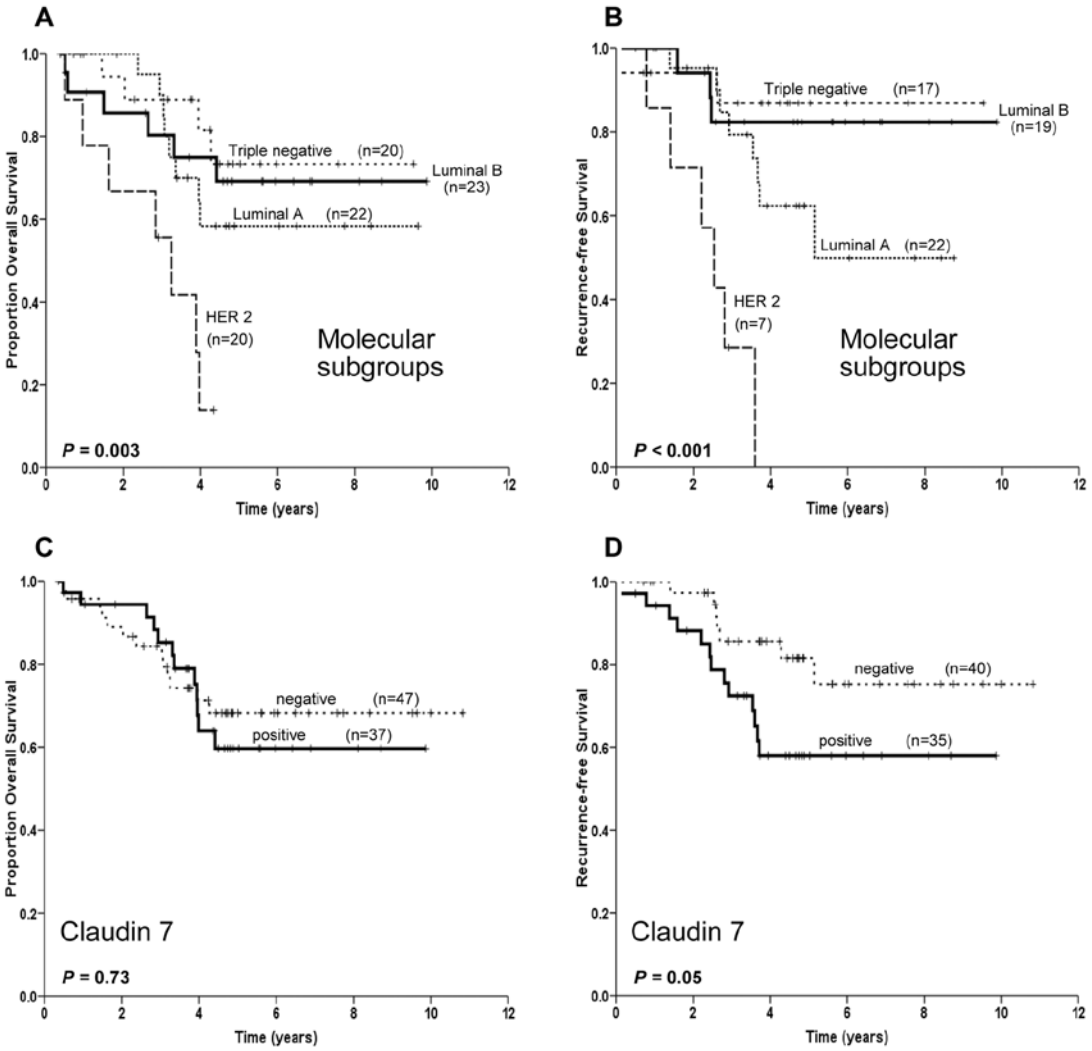

Figure 3. Overall survival and disease-free survival in all patients based on immunohistochemical subgroups (A and B) and claudin-7 (C and D).

Moreover, the HER2 subgroup which in our results presented the worst prognosis in terms of overall survival and disease-free recurrence was negative for the $CD44^+/CD24^-$ phenotype. Similar results have been previously reported suggesting that other markers indicative of a cancer stem cell for HER2 remain to be identified (11).

Other studies have indicated that $CD44^+/CD24^-$ breast cancer cell lines have highly invasive and metastatic properties (14,25,26) and thus should correlate with poor prognosis. We did not observe any association of $CD44^+/CD24^-$ in tumors expressing clinicopathological parameters of prognosis or with the potential to progress or recur or with high frequency of metastasis according to other reports (10,15,16,22,27).

In view of these results, we suggest that features of $CD44^+/CD24^-$ tumors in our study are not concordant with tumorigenic properties reported to characterize cancer stem cells (9) and therefore, are consistent with the ones suggesting that $CD44^+/CD24^-$ may not have the ability to identify cancer stem cells. These results highlight the need of other markers for the evaluation of cancer stem cells. Similar suggestions have been made by Mylona *et al* (15), Abraham *et al* (16) and Hwang-Verslues *et al* (23).

On the other hand, although the $CD44^-/CD24^+$ phenotype has failed to identify a statistically significant association with overall survival, we noted a tendency of patients harboring $CD44^-/CD24^+$ tumors to present a worse prognosis. Moreover, despite the lack of relation to survival we found that $CD24^+$ alone was a prognostic indicator of decreased disease-free survival time, confirming results of a recent report (22).

The CD24 protein was expressed in several solid tumors. This protein has been associated with bad disease prognosis and metastatic behavior for several solid tumors including breast cancer (27-30). $CD24^+$ has also been associated with proliferation, adhesion and invasion in MCF-7 cells (31). We verified a statistical association between $CD24^+$ and Ki-67 (P=0.01). Therefore, its seems that $CD24^+$ is associated with an aggressive tumor behavior in spite of retaining a differentiated luminal epithelial cell phenotype and our data indicate a significant association between $CD24^+$ with ER^+, PR^+ and a marginal one with CK18.

Among the proteins analyzed in order to determine the association of its expression with the different phenotypes of breast cancer, claudin-7 expression was noteworthy, as a decreased expression of claudin-7 correlated with higher tumor

Table VI. Correlation between claudin-7 expression and biological markers and clinicopathological features.

Characteristics	Claudin-7 expression		P-value[a]
	Negative (%)	Positive (%)	
Stage			
I + II	23 (57.5)	17 (42.5)	1.00
III + IV	26 (56.5)	20 (43.5)	
Nodal status			
Positive	20 (62.5)	12 (37.5)	0.50
Negative	28 (53.8)	24 (46.2)	
Nuclear grade			
I	0 (0)	2 (100.0)	0.21
II	22 (62.9)	13 (37.1)	
III	27 (56.6)	21 (43.8)	
Metastasis			
M0	33 (60.0)	22 (40.0)	0.07
M1	7 (35.0)	13 (65.0)	
Estrogen receptor			
Positive	23 (52.3)	21 (47.7)	0.65
Negative	20 (58.8)	14 (41.2)	
Progesterone receptor			
Positive	29 (65.9)	15 (34.1)	**0.02**
Negative	14 (40.0)	21 (60.0)	
HER2			
Positive	14 (42.4)	19 (57.6)	0.07
Negative	31 (64.6)	17 (35.4)	
Ki-67			
Positive	25 (55.6)	20 (44.4)	0.82
Negative	22 (59.5)	15 (40.5)	
CK5			
Positive	14 (82.4)	3 (17.6)	**0.03**
Negative	32 (50.0)	32 (50.0)	
CK6			
Positive	1 (50.0)	1 (50.0)	1.00
Negative	43 (56.6)	33 (43.4)	
CK18			
Positive	31 (54.4)	26 (45.6)	0.64
Negative	16 (61.5)	10 (38.5)	

HER2, human epidermal growth factor receptor 2; [a]statistical significance was determined by the χ^2 test. P-values (two-sided) <0.05 were considered statistically significant and are indicated in bold.

grade and metastatic disease (32). Moreover, the claudin^{low} subtype, which has recently emerged in the molecular classification of breast cancer, is characterized by reduced expression of claudins including claudin-7 (6).

However, we did not find an association between claudin-7 with different phenotypes, or with overall survival. On the other hand, claudin positive carcinomas relapsed more frequently than claudin negative ones, (P=0.05). Although this observation seems to contradict the hypothesis that claudin^{low} cells are associated to poor outcome tumors, it is consistent with two recent studies that have highlighted the association of preserved claudin-4 and claudin-1 with poor prognosis in breast cancer (33,34).

In conclusion, our results suggest that the frequency of CD44$^+$/CD24$^-$ tumor cells in breast cancer may not be associated to outcome. Both CD24 and claudin-7 positivity were associated to reduced time of recurrence suggesting that these two investigated markers can be used in combinations with other clinicopathological information to improve the assessment of prognosis in breast carcinoma.

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