Cancer stem cell markers ALDH1 and CD44^{+}/CD24^{-} phenotype and their prognosis impact in invasive ductal carcinoma

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Abstract

Breast cancer is a very heterogeneous disease. The intrinsic molecular subtypes can explain the intertumoral heterogeneity and the cancer stem cell (CSC) hypothesis can explain the intratumoral heterogeneity of this kind of tumor. CD44^{+}/CD24^{-} phenotype and ALDH1 expression are the major CSC markers described in invasive breast cancer. In the present study, 144 samples of invasive breast carcinoma, no special type were distributed in 15 tissue microarrays (TMA) and then evaluated for expression of the CD44^{+}/CD24^{-} phenotype and ALDH1 to understand the importance of these CSC markers and the clinical aspects of breast cancer. The samples were classified into four molecular subtypes according to clinicopathological criteria: Luminal A, Luminal B, HER2, and Basal-like. A statistical association was found between the molecular subtypes and the CSC markers, with HER2 the most frequent subtype for both markers. ALDH1 was also associated with other poor prognostic variables, such as a high histological grade and larger tumors, but it was not associated with the patients' prognosis in this sample nor was the CD44^{+}/CD24^{-} phenotype in a multivariate analysis. There are still many controversies about the role of these markers in breast cancer molecular subtypes. The identification of these populations of cells, through immunohistochemical markers, can help to better understand the CSC theory in clinical practice and, in the near future, contribute to developing new target therapies.

Introduction

The "cancer stem cell" (CSC) hypothesis considers cancer to originate in a small subset of cells which have the abilities of self-renewal and differentiation into other cancer cells, stimulating tumor formation, growth and spread.[1,2] These groups of cells are also thought to be involved in the resistance mechanism to different cancer treatments, such as chemotherapy and radiotherapy.[3] The complete eradication of these cells is essential in cancer treatment and, to achieve this, development of new-targeted therapies to CSC is necessary.[2] One stem cell feature is that they are quiescent while chemotherapeutic agents, on the other hand, act on proliferating cells, explaining the chemoresistance of these cells.[4,5] Furthermore, the CSC has high levels of ATP binding cassette (ABC) transporter proteins, such as ABCG2, which actively expels the drugs from the cells, protecting the cell during the chemotherapy action.[6]

The first description of the CSC existence was in acute myeloid leukemia 3 (AML) by Lapidot *et al*.[7] In breast cancer they were described by Al-Hajj in 2003, with the cell membrane markers CD44 and CD24.[8] The authors demonstrated that cells expressing CD44 (CD44^{+}), but with low or absent expression of CD24 (CD24$^{-/low}$) were able to form tumors in immunodeficient mice with as few as 100 cells.[8] In 2007, Ginestier *et al*., using *in vitro* and *in vivo* experimental systems found that cells with increased aldehyde dehydrogenase activity (ALDH) have stem cell properties, both in normal and in cancer mammary epithelial cells.[9] ALDH1 is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes.[10] CD44^{+}/CD24^{-} phenotype and ALDH1 are the principal markers of CSC in breast cancer until now. Very few studies have evaluated both markers at the same time, and although both are important biomarkers in representing CSC, the overlap between CD44^{+}/CD24$^{-/low}$ phenotype and ALDH1 expression in primary tumors is very low at approximately 1%.[9] However, cells with both phenotypes seem to be more tumorigenic than other cells, being able to generate tumors from as few as 20 cells.[9]

The clinical impact and prognostic value of these markers is still uncertain. Indeed, it is probable that each CSC population will have distinct clinical values in different subgroups of breast cancer.[11,12] In this study, we aimed to investigate the association between the breast CSC markers, CD44^{+}/CD24^{-} and ALDH1, and the breast cancer molecular subtypes, disease-free survival and overall survival.

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Materials and Methods

Patients and tumors

One hundred and sixty-four tumor samples were included from patients submitted to surgery at Nossa Senhora das Graças Hospital (HNSG) Breast Unit, Curitiba, Brazil, from January 1998 to July 2011. All patients had undergone surgery (mastectomy or breast conserving surgery with sentinel node or axillary dissection) and had an invasive breast carcinoma, no special type diagnosis. Cases submitted to neoadjuvant chemotherapy were excluded, as were the cases that had no sufficient paraffin-embedded material for a new analysis. The patients' ages were analyzed as a continuous variable by arithmetic mean and standard deviation. The histological grade was classified according to the Nottingham-Bloom-Richardson grading system.[13] Tumor size was reported according to TNM (8th edition, 2017) classification of the

American Joint Committee on Cancer.[14] Lymph node status was considered positive or negative, according to the presence or absence of tumor cells in at least one lymph node. Disease-free survival was considered as the period (months) between the surgery and the diagnosis of a disease recurrence at any site. Overall survival was considered as the time (months) between the first operation and the date of death.

This study was approved by the Ethics Committee of the Pontifical Catholic University of Paraná (PUCPR, Registration number: 5365).

Tissue microarray

Tumor samples were distributed in 15 TMAs using 4 mm tissue cores at the Experimental Pathology Laboratory of Pontifical Catholic University of Paraná (PUCPR). From each donor block was extracted one or two cylinders 4 mm in diameter and deposited in the receiver blocks, previously prepared. In each TMA, a sample of normal breast tissue was included as an internal control. Thereafter, 4 µm tissue sections from the TMA blocks were transferred to electrically charged Star Frost® (Braunschweig, Germany) slides and incubated with primary antibodies [anti-CD44 (anti-human mouse monoclonal, clone DF1485, dilution 1/40, Novocastra, Newcastle, UK), anti-CD44v6 (anti-human mouse monoclonal, clone VFF-7, dilution 1/100, Novocastra, Newcastle, UK), anti-CD24 (anti-human rabbit polyclonal, dilution 1/200, Abbiotec, San Diego, CA, USA), and anti-ALDH1 (anti-human rabbit monoclonal, clone E-P1932y, dilution 1/100, Epitomics, Cambridge, Massachusetts, USA)] for 12 h in a humidified chamber at 2−8°C. An Advance Dako (Caripenteria, CA, USA) secondary antibody was incubated with the slides for 30 min at 2−8°C. The reactions were developed using a DAB chromogen-substrate solution (Dako). Harris hematoxylin was used for counterstaining. Positive and negative (incubated without primary antibody) controls were run in parallel with all reactions.

Immunohistochemistry

The estrogen receptor(ER) and progesterone receptor (PgR) were evaluated at the time of diagnosis using the Streptavidin-Biotin-Peroxidase method. In this study the ER and PgR expressions were considered positive when nuclear expression was detected in at least 1% of tumor cells.[15] HER2 was analyzed at the time of diagnosis according to the American Society of Clinical Oncology (ASCO) / College of American Pathologists (CAP) protocols, when the HER2 scored 2+, the result was determined by fluorescence *in situ* hybridization (FISH).[16] The basal markers: cytokeratin 5/6 (CK5/6), cytokeratin 14 (CK14), cytokeratin 17 (CK17), c-kit, and epidermal growth factor receptor (EGFR) were carried out in an earlier study of our group.[17] They were considered positive when detected in at least 1% of tumor cells.

Immunohistochemistry was considered positive in CD44, CD44v6, and CD24 if it was expressed in more than 5% of tumor cells, as previously reported.[12] CD44 and CD44v6 showed mainly membranous staining, and CD24 was mostly cytoplasmic. The $CD44^{+}/CD24^{-}$ phenotype was established when a positive expression of CD44 or CD44v6 and a negative expression of CD24 was found. ALDH1 was considered positive when it showed cytoplasmic expression in more than 1% of tumor cells.[9,11] The slides were examined by two investigators (IR and APMS) simultaneously in a multi-observer microscope, without knowledge of the corresponding clinicopathological data.

Molecular subtypes

The molecular subtypes were classified based on clinicopathological criteria as described in Table 1.[18]

Statistical analysis

Statistical analysis was performed using Stata/SE v.14.1. Age was described by mean, standard deviation and range. For categorical variables frequencies and percentages were presented. Associations between two categorical variables were evaluated by Fisher's exact test, a Chi-square test or a logistic regression model followed by a Wald test. Analysis of age was performed using Student's *t*-test. The Fine and Gray model (based on the hazard of the sub distribution) was used to estimate cumulative incidence functions for death caused by disease (other causes of death as competing risks) and for recurrence of cancer (all causes of death as competing risks). A P value <0.05 was considered significant.

Results

A total of 144 cases were included in this cohort. The mean age of the patients was 56.4±13.5 years (27-88 years). Most patients were post-menopausal (97 patients: 67.4%). Seventy-three (50.7%) cases were grade II, 47 (32.6%) were grade III, and 24 (16.7%) were grade I. Tumor size was classified as T1 in 50% (72 cases), and T2 in 44,4% (64). Only eight cases (5.6%) exhibited a locally advanced disease (T3-T4). The lymph node status was negative in eighty patients (55.6%) and positive in sixty-four patients (44.4%). In terms of molecular subtypes, the most common subtype of this cohort was Luminal B, with sixty-two patients (43.1%), 27 (18.8%) cases were Luminal A, 24 (16.7%) were HER2, and 31 (21.5%) were Basal-like.

The expression of CD44, CD44V6, CD24, and ALDH1 was analyzed and the immunostaining of these markers is represented in Figure 1. The $CD44^{+}/CD24^{-}$ phenotype was expressed in eighteen patients (12.9%). Twenty patients (14.5%) expressed the ALDH1. Only five cases (3.7%) expressed both stem cell markers simultaneously. The correlation between the CSC markers and the clinicopathological parameters are described in Table 2. There was a significant correlation between the

Table 1. Molecular subtype definition according to clinicopathological criteria.

Molecular subtype	Clinicopathological definition
Luminal A	• ER and/or PR positive • HER2 negative • Ki-67 low (<14%)*
Luminal B	Luminal B (HER2 negative): • ER and/or PR positive • HER2 negative • Ki-67 high (≥14%)* Luminal B (HER2 positive): • ER and/or PR positive • Any Ki-67 • HER2 over-expressed or amplified
HER2 overexpression	• ER and PR negative • HER2 overexpressed or amplified
"Basal-like"	• ER and PR negative • HER2 negative • At least one basal marker positive: CK 5/6, CK 14, CK 17, c-kit, EGFR

*The Ki-67 cut-off labeling index was established based on the Cheang *et al.* study,[42] through comparison to gene array data (PAM50).

ALDH1 expression and the variables histological grade, and tumor size. The grade three tumors and the locally advanced tumors (T3 and T4) more frequently expressed the ALDH1. Regarding CD44 expression, the variant isoform CD44v6 was much more sensitive than the standard isoform CD44, with 119 positive cases (85.6%) for CD44v6 versus 78 (46.1%) for CD44. CD24 was positive in one hundred and thirteen cases (80.7%). The correlation between the expression of each of CD24, CD44 and CD44v6 and the clinicopathological parameters (age, menopausal status, histological grade, tumor size, and lymph node status) was not significant.

Stem cells markers and molecular subtypes

The CSC markers were related to the molecular subtypes, with statistical significance in both markers (Table 3). The HER2 subtype was associated with a greater likelihood of $CD44^{+}/CD24^{-}$ positivity compared to Luminal A (P=0.039), Luminal B (P=0.008), and Basal-like (P=0.039). The HER2 subtype was also associated with a greater probability of ALDH1 positivity compared to Luminal A (P=0.037), and

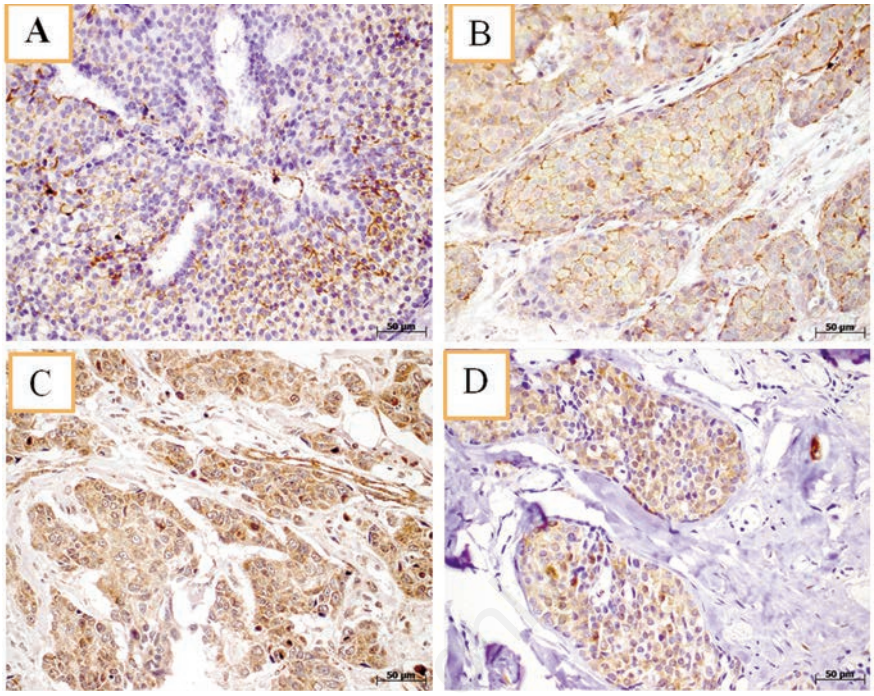

Figure 1. Immunohistochemistry analysis of CD44, CD44v6, CD24, and ALDH1 expression in tumor samples. Representative light photomicrographs showing positive staining of breast cancer tissue samples for CD44 (A); CD44v6 (B); CD24 (C) and, ALDH1 (D). Magnification: 40x; Scale bar: 50 µm.

Table 2. Correlation between stem cell markers and clinicopathological parameters.

Stem cell markers expression	Category	$CD44^{+}/CD24^{-}$				ALDH1			
		n	Negative	Positive	P*	n	Negative	Positive	P*
Age	Mean	122	56.4	55.2	0,726	118	56.3	58.5	0.503
	SD	18	13.3	15.2		20	13.7	14	
Menopausal status	Pre	45	38 (84.4)	7 (15.6)		44	38 (86.4)	6 (13.6)	
	Post	95	84 (88.4)	11 (11.6)	0.591	94	80 (85.1)	14 (14.9)	1
Histological grade	I	22	19 (86.4)	3 (13.6)		23	22 (95.7)	1 (4.3)	
	II	72	67 (93.1)	5 (6.9)		70	62 (88.6)	8 (8.4)	
	III	46	36 (78.3)	10 (21.7)	0.064	45	34 (75.6)	11 (24.4)	0.049
pT	T1	62	55 (88.7)	7 (11.3)		60	57 (95.0)	3 (5.0)	
	T2	70	62 (88.6)	8 (11.4)		70	56 (80.0)	14 (20.0)	
	T3-T4	8	5 (62.5)	3 (37.5)	0.100	8	5 (62.5)	3 (37.5)	0.009
Lymph node status	Negative	78	69 (88.5)	9 (11.5)		75	68 (90.7)	7 (9.3)	
	Positive	62	53 (85.5)	9 (14.5)	0.621	63	50 (79.4)	13 (20.6)	0.088

Results expressed as frequency (%); n, number of patients; *Fisher's exact test or Chi-square test, Student's *t*-test for independent samples, P<0.05.

Table 3. Association between stem cells markers and molecular subtypes.

Molecular subtypes	$CD44^{+}/CD24^{-}$				ALDH1			
	n	Negative	Positive	P*	n	Negative	Positive	P*
Luminal A	26	24 (92.3)	2 (7.7)		26	25 (96.2)	1 (3.8)	
Luminal B	59	54 (91.5)	5 (8.5)		59	53 (89.8)	6 (10.2)	
HER2	24	16 (66.7)	8 (33.3)		24	17 (70.8)	7 (29.2)	
Basal-like	31	28 (90.3)	3 (9.7)	0.032°	29	23(79.31)	6 (20.69)	0.039#

Results expressed as frequency (%); n, number of patients; *Logistic Regression Model and Wald test, P<0.05; °Luminal A *vs* HER2 (P=0.039); Luminal B x HER2 (P=0.008); Basal-like (P=0.039); other comparisons (P>0.05); #Luminal A *vs* HER2 (P=0.037); Luminal B x HER2 (P=0.038); other comparisons (P>0.05).

Luminal B (P=0.038), but not in comparison to Basal-like (P=0.477).

Stem cells markers and survival

A greater risk of relapse (P=0.011) and a worse outcome (P=0.019), were observed in the group that expressed the $CD44^+/CD24^-$ phenotype, but there was not a significant difference in the death risk (P=0.785). The ALDH1 expression did not show a statistically significant correlation with relapse, death, or outcome, as described in Table 4.

In this study, there were 42 cases of relapse (29.2%), 30 (20.8%) cancer death and 13 (9%) of death by other causes, in a mean time of 91 months (8-245). The death by other causes is a competitive risk to the event of relapse, or cancer death, because if the patients had lived long enough they could have presented our interest event. In order to establish the real prognostic value of our CSC markers, all the variables were analyzed using the Fine and Gray model for overall survival and disease-free survival. In the overall survival model, there was not a significant association between the CSC markers ($CD44^+/CD24^-$, ALDH1) and overall survival (Table 5, Figure 2). In the disease-free survival model, there was a significant association between the CD24 negativity [P=0.005, SHR 0.39 (0.20-0.76)], the $CD44^+/CD24^-$ phenotype [P=0.002, SHR 3.07 (1.51-6.22)] and a greater risk of relapse, in the univariate analyses, but in the multivariate analysis with the Fine and Gray model, only the histological grade and lymph node status remained as independent prognostic factors for recurrence (Table 6, Figure 2).

Discussion

One of the major limitations in the treatment of breast cancer is the heterogeneity of this disease. In the last fifteen years, much progress has been made in understanding the molecular subtypes of breast cancer, which has helped to explain the intertumoral heterogeneity. In addition, it is known that this heterogeneity not only happens between tumors but also happens inside the tumor; therefore in the same tumor two or more distinct populations of cells can be present, with very different molecular profiles, which further complicates the understanding of this disease.[19]

The CSC theory was postulated in an attempt to better understand this heterogeneity and explain the mechanism of recurrence and metastasis. The expression of CD44 and CD44v6 has been associated with the potential of progression and metastasis. CD44 is a glycoprotein transmembrane involved in many cellular processes including migration and adhesion, and hyaluronic acid is its principal ligand.[20] A single gene located at 11p13 chromosome

Table 4. Association of stem cells markers and prognosis.

Prognosis	Category	$CD44^+/CD24^-$ Negative (n=122)	$CD44^+/CD24^-$ Positive (n=18)	P*	ALDH1 Negative (n=118)	ALDH1 Positive (n=20)	P*
Relapse	No	92 (75.4)	8 (44.4)		83 (70.3)	14 (70)	
	Yes	30 (24.6)	10 (55.6)	0.011	35 (29.7)	6 (30)	1
Death	No	86 (70.5)	12 (66.7)		83 (70.4)	14 (70)	
	Yes	36 (29.5)	6 (33.3)	0.785	35 (29.6)	6 (30)	1
Outcome	Live	79 (64.8)	8 (44.5)		72 (61)	13 (65)	
	Cancer death	23 (18.9)	6 (33.3)		24 (20.4)	5 (25)	
	Death other causes	13 (10.6)	0 (0)		11 (9.3)	1 (5)	
	Live with relapse	7 (5.7)	4 (22.2)	0.019	11 (9.3)	1 (5)	0.812

Results expressed as frequency (%); n, number of patients; *Fisher's exact test or Chi-square test, P<0.05.

Table 5. Univariate and multivariate analyses of stem cells markers and other predictors of overall survival.

Variable	Variable	Univariate analyses P*	SHR	CI 95%	Multivariate analyses P*	SHR	CI 95%
Age		0.001	1.04	1.02-1.07	0.177	1.03	0.99-1.08
Molecular subtype	Luminal A						
	Luminal B	0.175	2.35	0.68-8.04	0.119	2.78	0.15-5.68
	Basal-like	0.884	0.89	0.18-4.46	0.923	0.91	0.15-5.68
	HER2	0.023	4.55	1.24-16.70	0.049	4.45	1.01-19.68
Menopause	Pre						
	Post	0.009	4.82	1.49-15.58	0.190	2.86	0.59-13.75
Lymph node	Negative						
	Positive	0.040	2.13	1.04-4.39	0.012	2.79	1.25-6.22
$CD44^+/CD24^-$	Negative						
	Positive	0.098	2.12	0.87-5.16	0.274	1.63	0.68-3.89
ALDH1	Negative						
	Positive	0.622	1.27	0.49-3.31	0.984	0.99	0.40-2.43

*Fine & Gray subdistribution hazards model and Wald test, P<0.05.

that has 20 exons encodes CD44. The first five and the last five exons are constant, while the 10 exons located between these regions are subject to alternative splicing, resulting in the formation of a variable region that produces a wide range of proteins.[21,22] The standard isoform (CD44s) is the most common, but approximately 20 variant isoforms have been described. The CD44v6 is one of the most common and best-studied CD44 variant isoforms and has been described as playing a role in cell migration and proliferation and, consequently, in cancer progression and metastasis.[23,24] In this study we included the isoform CD44v6 expression in the identification of the $CD44^+/CD24^-$ phenotype to try to achieve the two major isoforms that have prognostic implications and improve the identification of the CSC phenotype.

A major point in our study was the possibility to compare the two principal CSC markers in breast cancer, ALDH1 and $CD44^+/CD24^-$ phenotype, since they possibly identify different groups of stem cells, and both are important in the evaluation of CSC. Most of the previous studies evaluated just one stem cell marker. Recently another study also evaluated both cancer stem cells markers and identified that high CD44/CD24 ratio was mainly in charge of self-renewal, proliferation, and tumor growth, while ALDH1+ represented a stronger capability for invasion and metastasis. The authors also demonstrated that these two markers performed different functions during tumor progression and metastasis, corroborating that a single CSC marker alone was not enough to characterize the stem properties of breast cancer.[25]

Our study found that grade III tumors and the locally advanced tumors (T3-T4) were associated with a greater probability of expression of ALDH1 with a significant result, as previously described in other studies.[9,26] In this study, the $CD44^+/CD24$ and the ALDH1 were not associated with the lymph node status. One explanation for this fact is that these tumors exhibit a more aggressive behavior and tend to disseminate more frequently hematologically rather than lymphatically.

In this cohort we observed a significant association between CSC markers and the molecular subtypes, with the HER2 subtype more frequently expressed in both markers. Several studies correlated the $CD44^+/CD24^-$ phenotype to the more undifferentiated subtypes as Basal-like and Claudin-low.[11,26,27] The basal-like subtype is possibly related to the most primitive cell lines, so it was expected that the CSC phenotype would be enriched in this kind of tumor.[11,27-29] The $CD44^+/CD24^-$ phenotype has been described in association with the HER2

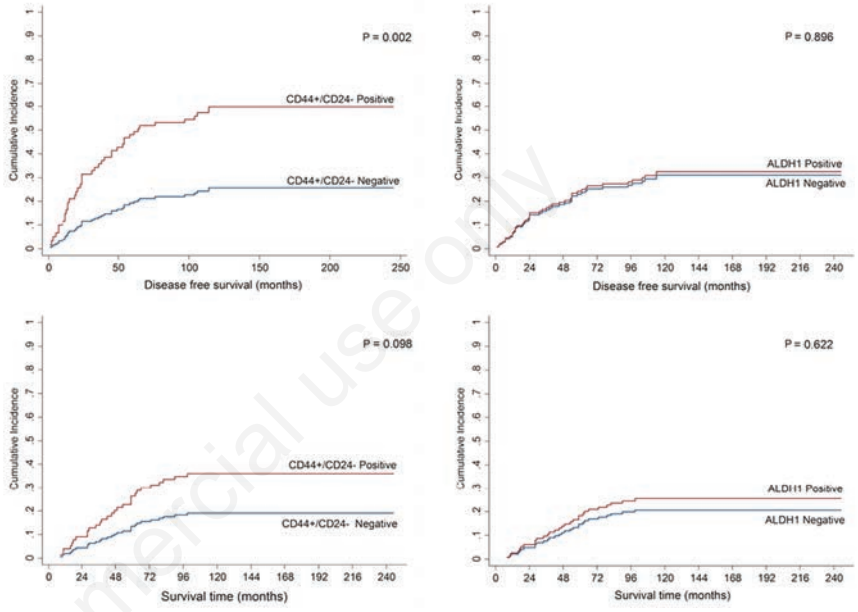

Figure 2. Cumulative incidence curves for recurrence and cancer death by $CD44^+/CD24^-$ and ALDH1 (positive and negative).

Table 6. Univariate and multivariate analyses of stem cells markers and other predictors of disease-free survival.

		Univariate analyses			Multivariate analyses		
Variable	Variable	P*	SHR	CI 95%	P*	SHR	CI 95%
Age		0.051	1.02	1.00-1.05	0.002	1.05	1.02-1.08
Molecular subtype	Luminal A						
	Luminal B	0.108	2.37	0.83-6.81	0.168	2.48	0.68-9.05
	Basal-like	0.293	1.87	0.58-6.03	0.458	1.63	0.45-5.99
	HER2	0.010	4.31	1.42-13.11	0.119	2.86	0.76-10.71
Histological grade	I						
	II	0.086	2.71	0.87-8.49	0.065	3.52	0.92-13.47
	III	0.013	4.31	1.35-13.70	0.023	5.97	1.27-27.95
Lymph node	Negative						
	Positive	0.011	2.21	1.20-4.08	0.032	2.21	1.07-4.57
CD24	Negative						
	Positive	0.005	0.39	0.20-0.76	0.833	0.85	0.19-3.80
$CD44^+/CD24^-$	Negative						
	Positive	0.002	3.07	1.51-6.22	0.197	2.85	0.58-14.06
ALDH1	Negative						
	Positive	0.896	1.06	0.44-2.57	0.250	0.59	0.24-1.44

*Fine & Gray subdistribution hazards model and Wald test, P<0.05.

molecular subtype, only in some *in vitro* studies.[30] In our study we classified the molecular subtypes according to the clinicopathological criteria, and considered the Ki67 expression to be part of the Luminal B group definition, as recommended in the St. Gallen consensus,[18] which resulted in more cases classified as Luminal B that would had been classified as Luminal A for other authors.[27,31] The Luminal A group is the best prognosis group and, in our sample, it was negatively associated with the CSC markers. Some studies included in their samples other histological types, which made them more heterogeneous.[19,27] In our study, only the invasive breast carcinoma, no special type was included.

It was recently demonstrated that CSC could possibly play an important role in the metastasis process, due to their self-renewal capability and potential to differentiate and adapt to different organ microenvironments[32] and an association between CSC and metastasis of breast cancer lines has been demonstrated *in vitro*.[32,33] In our study an association between the CSC markers and overall survival was not demonstrated. The $CD44^{+}/CD24^{-}$ phenotype was initially associated with a greater risk of relapse in the univariate analysis along with CD24 negativity, molecular subtype, histological grade, and lymph node status, but in the multivariate analysis, it was not demonstrated as an independent prognostic factor. Contradicting our study recently was showed significant correlate with 5-year relapse in ER-positive breast cancer patients during adjuvant tamoxifen treatment, suggesting that CD24 expression may reflect tamoxifen resistance.[34] The $CD44^{+}/CD24^{-}$ phenotype was previously correlated to metastasis-free survival by other authors,[35,36] but there are still many controversies about the true role of these markers, with very conflicting results in the studies.[37,38] According to Riaz *et al*., the androgen receptor (AR), and CD24 significantly correlated with favorable clinicopathological features and an improved survival whereas CSC markers such as $CD44^{+}$, $CD44^{+}/CD24^{-}$, and $ALDH1^{+}$ were not effective prognostic indicators for outcome prediction.[39] Zhong *et al.* considered the ALDH1 a better predictor of relapse than the $CD44^{+}/CD24^{-}$ phenotype, and demonstrated that the ALDH1 expression was associated with a high rate of metastasis or recurrence.[32] An issue that could influence these heterogeneous results is the lack of standardization in the analysis of these markers that define $CD44^{+}/CD24^{-}$ phenotype, and the use of multiple cut-off criteria. We used a 5% cut-off for CD44, CD44v6 and CD24, as previously described by other authors.[12] Ricardo *et al.* considered a score that evaluates the percentage of cells immunostaining positive: CD44 was considered positive when it has ≥10% of positivity, and CD24 was defined as negative or low when it scored up to 25% of positivity.[11] Adamczyk *et al.* considered a 10% cut-off for both CD44 and CD24 expressions.[40]

The CSC markers study, is also of extreme relevance in understanding the mechanism of multidrug resistance, that decrease the chemotherapeutic agents' efficacy in breast cancer treatment. Some studies have revealed some potential biomarkers of doxorubicin resistance in breast cancer stem cells, such as STAT3 that was recently described as a promising chemoresistance biomarker associated with the $CD44^{(+/high)}/CD24^{(-/low)}/ALDH(^{+})$ BCSCs-like subset of the triple-negative breast cancer (TNBC), classified as a claudin-low subtype.[41] The progress in this field is of major importance for the improvement of breast cancer treatment.

Our study presents some limitations that need to be highlighted. First, we did not have complete information of lymphovascular space invasion and we could not make the correlation with the several markers used in the present study. Second, a simple immunostaining was employed to establish the $CD44^{+}/CD24^{-}$ negative phenotype, as previously described by other authors,[26-28] instead of double immunolabeling. Even though, Ricardo *et al*., compared the simple and the double immunostaining to establish the $CD44^{+}/CD2^{4-}$ phenotype in the same sample and did not find any difference between those techniques.[11]

There are still many controversies about the prognostic value of CSC markers that has to be elucidated. Our study demonstrated that despite these markers being associated with some indicators of bad prognosis they cannot be considered as independent prognostic factors. Considering the CSC theory, and the accessibility of these CSC markers, it is important to better understand their role in breast cancer to be able to develop in the future new target therapies for this cell population.

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