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Characterization of CD44$^+$ALDH1$^+$Ki-67$^-$ Cells in Non-malignant and Neoplastic Lesions of the Breast

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Abstract. *Background: Cancer stem cells are tumor cells that present self-renewal, clonal tumor initiation capacity and clonal long-term repopulation potential. We have previously demonstrated that the co-expression of the breast cancer stem cell (BCSC) markers hyaluronan receptor (CD44) and aldehyde dehydrogenase-1 (ALDH1) in ductal carcinomas in situ could be determinant for disease progression. Combining these established BCSC markers with Ki-67 to evaluate quiescence we sought to identify, evaluate the distribution and estimate the mean percentages of CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells. Materials and Methods: Triple-immunohistochemistry for CD44, ALDH1 and Ki-67 was applied in a series of 16 normal, 54 non-malignant and 155 malignant breast tissues. Clinical relevance was inferred by associations with markers of breast cancer behavior, progression and survival. Results: The mean percentages of cells with this phenotype increased significantly from non-malignant lesions to high-grade ductal carcinomas in situ, decreasing in invasive ductal carcinomas, as also evidenced by an inverse correlation with histological grade and tumor size. The mean percentage of CD44$^+$ALDH1$^+$Ki-67$^-$ cells was also significantly higher in women who developed distant metastasis and died due to breast cancer, and a significant association with human epidermal growth factor type 2 (HER2) negativity was observed. Conclusion: Our novel findings indicate that CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells may favor distant metastasis and can predict overall survival in patients with ductal carcinomas of the breast. More importantly, quiescence may have a crucial role for tumor progression, treatment resistance and metastatic ability of BCSCs.*

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The cancer stem cell (CSC) model is an attractive concept to explain several poorly understood clinical phenomena due to its inherent theoretical properties. Such properties are based on the molecular features of normal stem cells. Thus, CSCs have the ability to renew themselves and last a lifetime but also to be resistant to electromagnetic and chemical insults. Such resistance ability allows them to survive for long periods of time and, consequently, colonize other parts of the body (1). The existence of tumor cells presenting stem cell features has been well established in the literature for specific cancer types; however, and due to functional heterogeneity existing in all tumors, no single CSC phenotype can be generalized. As a consequence, distinct cell populations within a unique tumor may exhibit CSC features, which has led to identification of other putative CSC subsets in a diversity of solid tumor types (2). Despite this, no single protocol or even combined protocols are guaranteed to obtain pure CSC subsets and most recent iterations to define CSCs have embraced a definition of the CSC phenotype as a dynamic cell state rather than a distinct cell type (3).

Apart from the clonogenic features of CSCs (self-renewal and differentiation tumorigenicity), several associations have been made between putative CSCs and their normal counterparts. In order to identify CSCs within the heterogeneous tumor bulk, numerous factors such as surface marker expression, cell-cycle state, migratory properties, immune

escape, or metabolic and transporter activities are being studied (4). In breast cancer, a population of hyaluronan receptor $(CD44)^{+}CD24^{-low}$ tumor cells has been demonstrated to have tumor-initiating properties. This tumorigenic phenotype has been associated with stem cell-like characteristics, enhanced invasive properties, radiation resistance and with distinct genetic profiles suggesting association with adverse prognosis (5-7). However, the notion that the $CD44^{+}CD24^{-low}$ surface markers are enriched for tumorigenic cells cannot be applied to all cases of breast cancer. Thus, the validity of the combination of these markers as a definition of breast cancer stem cells (BCSCs) has been called into question and additional markers such as aldehyde dehydrogenase-1 (ALDH1) have been reported (8, 9).

More recently, subpopulations of $ALDH1^{high}CD44^{+}$ cells were identified in several human breast cancer cell lines, which contributed to both chemotherapy and radiation resistance, suggesting a much broader role for ALDH1 in treatment response than previously reported (10). Indeed, an increase in the population of $ALDH1^{+}$ cells but not $CD44^{+}CD24^{-low}$ cells has been observed in breast cancer tumor biopsies after neoadjuvant treatment (11).

Nevertheless, a potentially more challenging problem is the recent observation that CSCs that display quiescent properties may exist. The isolation of adult stem cells revealed new insights on the epigenetic, transcriptional and post-transcriptional control of quiescence, proposing an actively preserved state of quiescence, which is regulated by signaling pathways that sustain a controlled state, allowing rapid activation (12). Although these quiescent cells are slowly dividing, they possess increased sphere-forming aptitude *in vitro*, suggesting that these cells are enriched in CSCs, being able to repair DNA damage induced by chemotherapeutic agents and radiotherapy (13). Such dormant cells were also identified in pancreatic adenocarcinoma and shown to be enriched for CSC markers such as CD133, CD44, CD24 and ALDH1 (12).

We recently reported a higher combined expression of CD44 and ALDH1 in ductal carcinomas *in situ* (DCIS) when compared with invasive ductal carcinomas (IDCs) (14). Regarding these results and combining these established BCSC markers with Ki-67 as a marker of quiescence, we aimed to identify, evaluate its distribution and estimate the mean percentage of $CD44^{+}ALDH1^{+}Ki\text{-}67^{-}$ breast cells in a series of normal, non-malignant and malignant breast tissues. This phenotype was further correlated with clinicopathological markers of breast cancer prognosis and overall survival.

Materials and Methods

Patient samples. Formalin-fixed, paraffin-embedded (FFPE) breast specimens of 229 patients who underwent breast surgery prior to systemic treatment from 2004 to 2012 were obtained from the archives of the Pathology Department of Santo António Hospital (Porto Hospital Centre, Porto, Portugal). At the time of diagnosis, 59 patients were considered to have benign breast lesions (mean age of 43.63 years), including adenosis (ADs), fibroadenomas (FADs) and ductal hyperplasias (DHPs) without signs of malignancy. Regarding the malignant cases, 170 were retrieved including 28 pure DCIS (without signs of invasion), 34 DCIS within IDC and 108 IDCs. As controls, normal breast tissue was obtained from 20 women who underwent reduction mammoplasty with no previous history of breast cancer (mean age of 32 years). Clinicopathological information was obtained by reviewing pathology reports, hematoxylin and eosin (H&E)-stained sections and also H&E-stained tissue microarray sections for a more accurate information of each tissue core. The following histopathological variables for the pure DCIS and DCIS within IDC samples were available in the interim records: nuclear grade, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor type 2 (HER2) status. For IDC cases, we retrieved the Elston–Ellis histological grade, tumor size, lymph-node status, local recurrences, distant metastasis, mortality, ER, PR and HER2 status. All patients were female, with a mean age of 55.33 years. Regarding the presence of distant metastasis, none of the patients had distant metastasis at the time of diagnosis; hence, M1 was analyzed in patients who developed distant metastasis during follow-up, of which the mean time was 68.18 (SD=23) months. All cases were reviewed by two pathologists (JG and CL). FFPE tissue samples were arrayed using a tissue-arraying instrument (Thermo Scientific, USA) with a punch extractor of 2 mm. Each sample was arrayed in duplicate or in triplicate to minimize tissue loss and compensate for tumor heterogeneity. Representative areas with malignant lesions from DCIS and IDC were classified as pure DCIS or IDC alone, respectively. DCIS cores retrieved from IDC samples, without the invasive compartment, were classified as DCIS within IDC. The current study was approved by the following ethical boards: Porto Hospital Centre Research Ethics Health Committee (reference 203-CES) and by Porto Hospital Centre Department of Education, Development and Research (reference 135-DEFI).

Triple immunohistochemistry. Considering our previous results obtained by single-staining immunohistochemistry of CD44 and ALDH1, our cohort for this study was composed of some autologous samples of DCIS and IDCs from those previously used (14), which served as a control for triple immunohistochemistry validation.

For CD44, ALDH1 and Ki-67 triple immunohistochemistry, 3 μm-thick tissue microarray sections were cut, deparaffinized, rehydrated in a series of graded ethanol and washed in water. Target retrieval was achieved with citrate buffer (pH 6.0) in a microwave until boiling. Slides were then incubated for 10 min with 3% peroxide hydrogen in methanol, and further with blocking solution (Ultra Vision LP Detection System; Thermo Fisher Scientific, Cheshire, UK), for 5 min. CD44 (mouse monoclonal anti-human, 0.01 mg/ml concentration, MRQ-13, dilution: 1100, Cell Marque, Rocklin, CA, USA) and Ki-67 (rabbit monoclonal anti-human, 0.01 mgml concentrate, SP6, dilution: 1100, Cell Marque) were incubated together in a humid chamber for 2 hours at room temperature. Following this, immunohistochemistry was performed according to HiDef Detection™ Horseradish Peroxidase Polymer System procedures (Cell Marque). Enzyme reactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, Saint

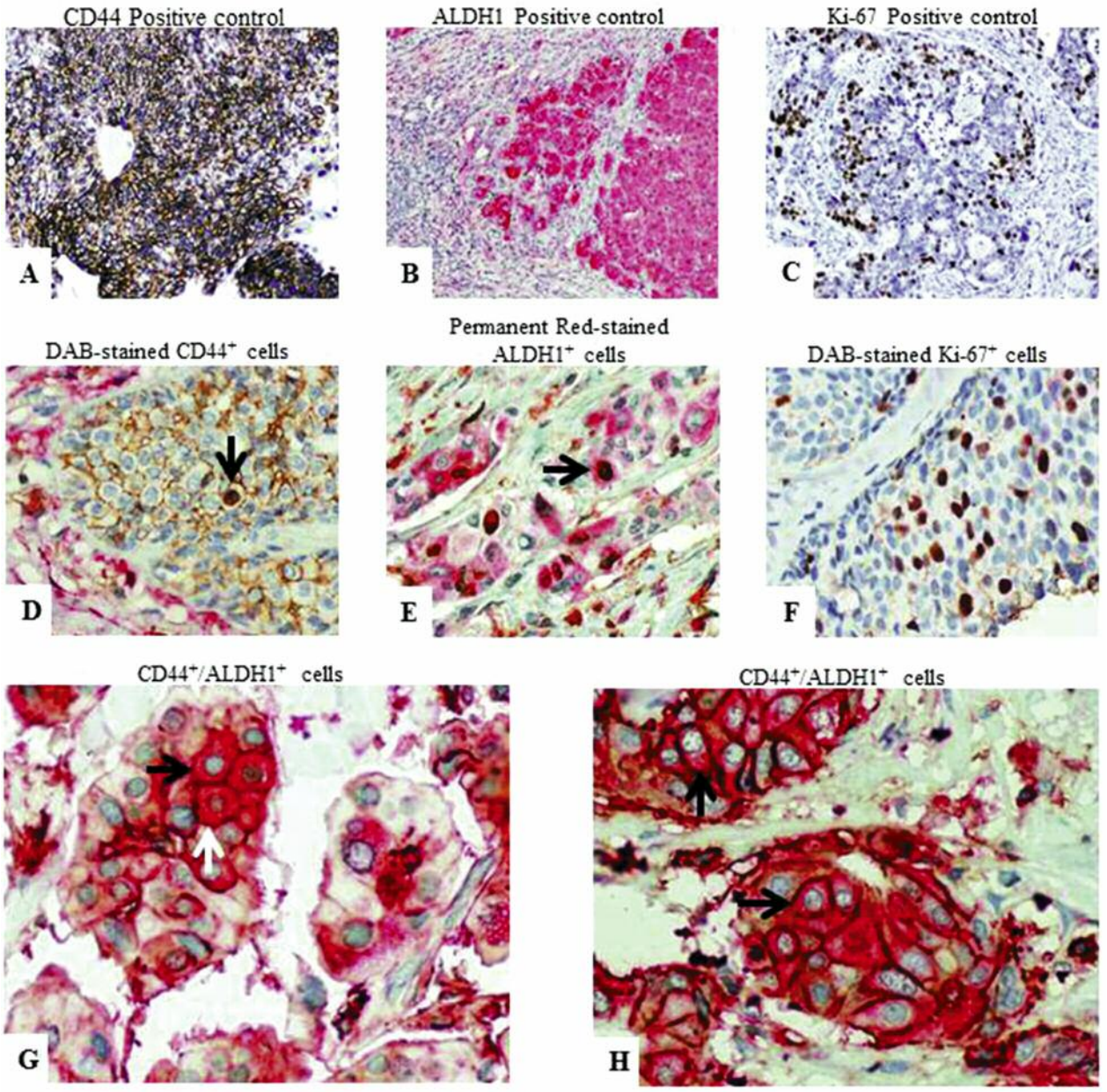

Figure 1. *Identification of hyaluronan receptor (CD44)$^+$ aldehyde dehydrogenase-1 (ALDH1)$^+$ Ki-67$^-$ breast cells. Positive controls for CD44 (A), ALDH1 (B) and Ki-67 (C) (×20). D: Illustrates a breast tissue sample without ALDH1 expression; black arrow points to a CD44$^+$ALDH1$^-$Ki-67$^+$ breast cell (×40). E: A breast tissue sample without CD44 expression; black arrow points a CD44$^-$ALDH1$^+$Ki-67$^+$ breast cell (×40). F: A breast tissue sample without CD44 and ALDH1 expression (×40). G, H: Breast tissue samples containing CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells (black arrows); white arrow in (G) points to a CD44$^+$ALDH1$^+$Ki-67$^+$ breast cell (×40).*

Louis, MO, USA). Slides were washed in running water and incubated with glycine buffer (pH 2.2) for 45 min at 37˚C. ALDH1 (rabbit monoclonal anti-human, 0.13 mgml concentrate, dilution: 1100; Abcam, Cambridge, UK) was then added and slides incubated in a humid chamber for 2 h at room temperature. HiDef DetectionTm Alkaline Phosphatase Polymer System procedures (Cell Marque) were followed and enzyme reactivity was visualized using Permanent Red chromogen (Permanent Red Chromogen Kit; Cell Marque). Finally, slides were counterstained with Mayer's hemalum solution (Merck Millipore, Darmstadt, Germany) and mounted with Aquatex Mounting Medium (Sigma-Aldrich, Darmstadt, Germany). For CD44, ALDH1 and Ki-67, positive controls were also used including chorion, liver and breast tissues, respectively (Figure 1A-C).

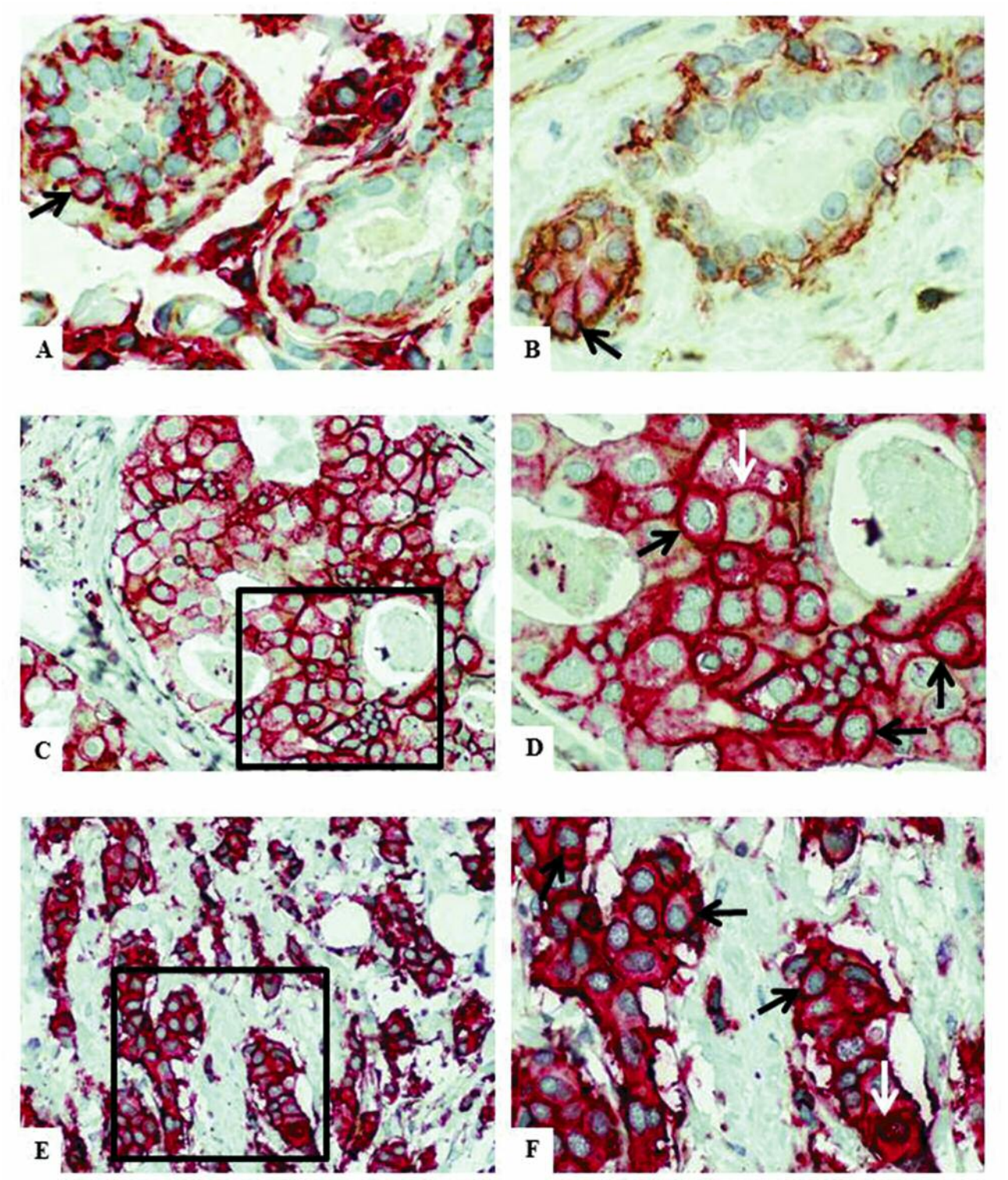

Figure 2. *Representative images of the triple immunohistochemistry for hyaluronan receptor (CD44), aldehyde dehydrogenase-1 (ALDH1) and Ki-67 in breast tissue sections. Normal (A) and non-malignant (Fibroadenoma) (B) breast tissue section; black arrows points to CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells (×40). Overview of ductal carcinoma in situ (C) and invasive ductal carcinoma (E) breast tissue (×20). Insets (D and F) show the same tissues at higher magnification (×40). D: Examples of CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells (black arrows); the white arrow demonstrates an example of a cell without ALDH1 expression. F: Examples of CD44$^+$ALDH1$^+$Ki-67$^-$ invasive tumor cells (black arrows); the white arrow demonstrates an example of a CD44$^+$ALDH1$^+$Ki-67$^+$ cell.*

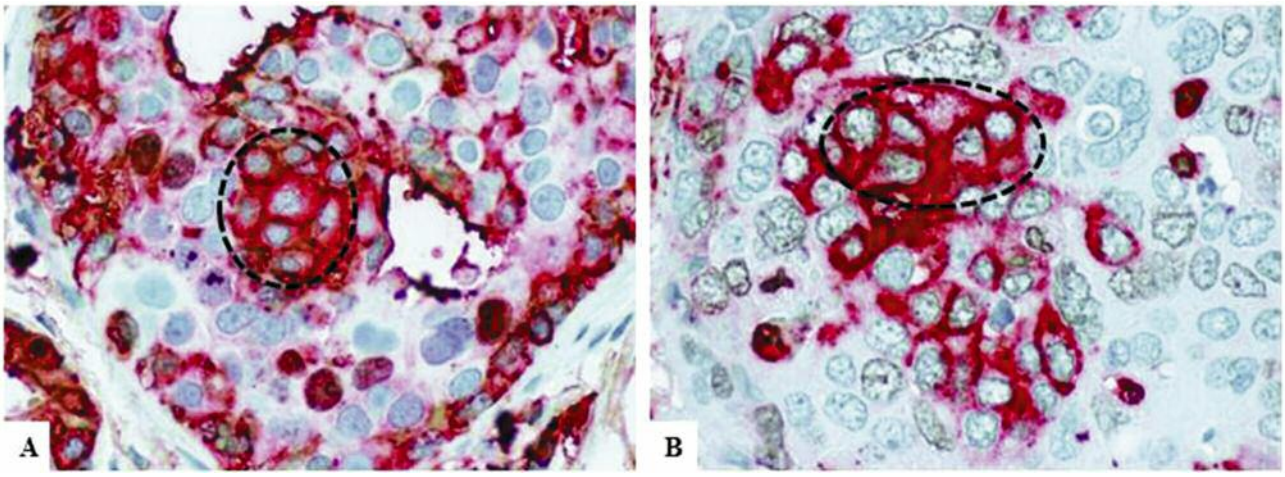

Figure 3. *Pools of hyaluronan receptor (CD44)$^+$ aldehyde dehydrogenase-1 (ALDH1)$^+$ Ki-67$^-$ breast cells (dotted circles) in ductal carcinoma in situ (A) and invasive ductal carcinoma (B) breast tissues respectively (×40).*

Definition of hormone receptor status. Primary breast tumors that expressed nuclear staining in 1% of tumor cells were regarded as positive for ER and PR. Immunostaining results for HER2 were scored as 0 when no staining was observed or when <10% of tumor cells had membranous staining; as 1 when a faint or barely membranous staining was present in >10% of tumor cells; as 2 when a weak to moderate membrane staining was observed in >10% of tumor cells and as 3 when a strong complete membrane staining was present in >10% of tumor cells. All cases classified previously as 2 were subjected to fluorescence *in situ* hybridization for determination of *HER2* gene amplification using the *HER2* DNA probe kit (Abbott Laboratories, Abbott Park, IL, USA). Scores of 0 and 1 were considered to be negative for HER2, while scores of 2 (if *HER2* gene amplification was observed) and 3 were considered to be positive.

Pathological evaluation. After omitting 20 cases with uninterpretable immunohistochemistry results, a total of 209 cases were informative (10 ADs, 21 FADs, 23 DHPs, 25 pure DCIS, 30 DCIS within IDC and 100 IDCs). Using light microscopy (Olympus U-SPO3;Olympus Corporation, Tokyo, Japan), stained tissue sections were inspected by two pathologists (RS and CL) and a trained scientist (ACP) without knowledge of the diagnosis. To unambiguously identify CD44$^+$ALDH1$^+$Ki-67$^-$ cells, we needed to distinguish double-stained cells labeled with both DAB and Permanent Red, considered to be CD44$^+$ALDH1$^+$ cells from DAB-stained CD44$^+$ cells (Figure 1D) and Permanent Red-stained ALDH1$^+$ cells (Figure 1E). As for Ki-67, a clear distinction was seen between nuclear DAB-stained Ki-67$^+$ from Ki-67$^-$ cells (Figure 1F). Consequently, cells with brown membranes, red cytoplasm and hematoxylin stained-nucleus were considered to be CD44$^+$ALDH1$^+$Ki-67$^-$ (Figure 1G and 1H). Regarding the previous results obtained from single-immunohistochemistry of CD44 and ALDH1 and considering that we used the same antibodies, we discriminated our cells of interest only in breast epithelial cells, excluding artifacts staining tumor cell debris. All slides were scanned (Leica SCN400, Meyer Instruments, Houston, TX, USA) and digital images of each core were visualized through the SlidePath Gateway 2.0 System (Leica Biosystems, Wetzlar, Germany) program. These digitalized images allowed us to count more accurately the number of cells of interest. Percentages of such cells were estimated from the entire lesion areas. Cores from the same donor tissue diagnosed with the same histological type were grouped and their mean score calculated.

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics version 20.0 software (IBM Corp., Armonk, NY, USA). Sample distributions were compared using Kruskal–Wallis or Mann–Whitney tests. Pearson's chi-square test was used to evaluate the differences between categorical variables. Spearman's rank correlation coefficient was used to evaluate the relationship between variables, and the Kaplan–Meier method was used for univariate survival analysis. Statistical significance was accepted at $p<0.05$.

Results

CD44$^+$ALDH1$^+$Ki-67$^-$ cell populations in breast samples. ALDH1, CD44 and Ki-67 immunostaining was observed in epithelial cells and also in stromal inflammatory cells, both in normal, non-malignant and malignant samples. While CD44 exhibited mainly membranous staining, ALDH1 was almost exclusively detected in the cytoplasm and Ki-67 in the nuclei. We were able to identify CD44$^+$ALDH1$^+$Ki-67$^-$ cells in normal (Figure 2A), non-malignant (Figure 2B) and malignant breast tissues (Figure 2C-F). Interestingly, we were also able to identify small pools of these cells in some malignant breast tissues and although in rare cases, these pools of cells were observed completely isolated (Figure 3A and 3B).

Considering the low percentages of CD44$^+$ALDH1$^+$Ki-67$^-$ cells identified in normal and non-malignant breast tissues, raw percentages of these cells for mean calculation

Table I. *Prevalence of hyaluronan receptor (CD44)+ aldehyde dehydrogenase-1 (ALDH1)+ Ki-67− cells in breast tissue samples.*

Characteristic	CD44+ALDH1+Ki-67− breast cells			
	n (%)	<1%, n (%)	<10%, n (%)	≥10%, n (%)
Type of lesion				
Normal	16 (7.11)	14 (87.50)	2 (12.50)	0 (0)
Non-malignant	54 (24)	42 (77.78)	11 (20.37)	1 (1.85)
Malignant	155 (68.89)	52 (33.55)	67 (43.23)	36 (23.22)
Pure DCIS	25 (16.13)	7 (28)	14 (56)	4 (16)
DCIS within IDC	30 (19.35)	9 (30)	10 (33.33)	11 (36.67)
IDC alone	100 (64.52)	36 (36)	43 (43)	21 (21)

DCIS: Ductal carcinoma *in situ*; IDC: invasive ductal carcinoma.

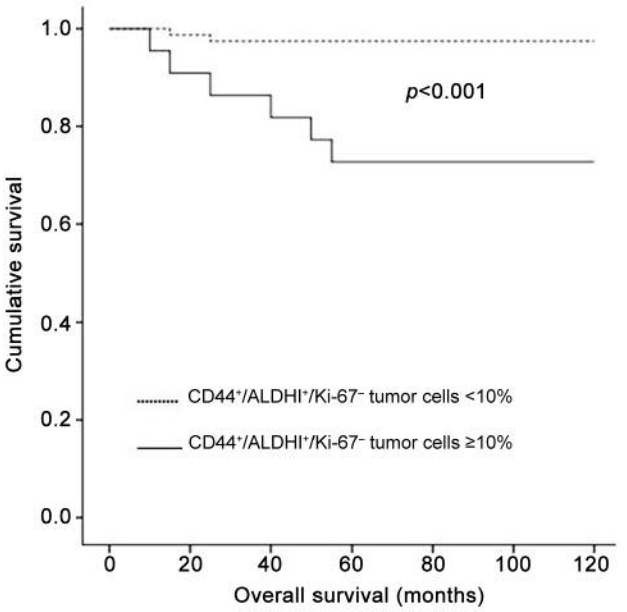

Figure 4. *Univariate analysis of overall survival according to hyaluronan receptor (CD44)+ aldehyde dehydrogenase-1 (ALDH1)+ Ki-67− tumor cell prevalence.*

were preserved instead of using cut-off values, except for prevalence description and overall survival representation. In control samples, obtained from reduction mammoplasties, percentages of CD44+ALDH1+Ki-67− cells ranged from 0% to 2%, with only two cases having more than 1%. Regarding the non-malignant tissues, these cells ranged from 0% to 11% with only one case having ≥10%. As for the malignant cases, CD44+ALDH1+Ki-67− tumor cells in pure DCIS (range: 0 to 50.7%), DCIS within IDC (range: 0 to 55.5%) and in IDC alone (range: 0 to 51%) were negative or presented scattered cells in 28%, 30% and in 36% of cases, respectively. In pure DCIS, 16% of cases had ≥10%; 36.67% of cases displayed ≥10% in DCIS within IDC and in IDC alone only 21% displayed ≥10% (Table I).

As described in Table II, although non-malignant tissues presented a higher mean percentage of CD44+ALDH1+Ki-67− cells when compared to normal breast tissues, this difference was not statistically significant. Conversely, the mean percentage of CD44+ALDH1+Ki-67− tumor cells in malignant specimens was significantly higher than that of normal (p=0.025) and non-malignant (p<0.001) breast tissues. Regarding only the malignant cases, DCIS within IDC cases presented a significantly higher percentage of CD44+ALDH1+Ki-67− tumor cells than IDC alone (p=0.016).

Clinical implications of CD44+ALDH1+Ki-67− tumor cells. Considering the percentage of CD44+ALDH1+Ki-67− tumor cells in DCIS lesions, a significant positive correlation was found between the mean percentage of these cells and nuclear grade (p=0.005, Table III).

From the 100 IDC cases analyzed, a significant negative correlation between the mean percentage of CD44+ALDH1+Ki-67− tumor cells and Elston-Ellis grading (p=0.027) and tumor size (p=0.007) were obtained. The follow-up analysis demonstrated that two patients developed local recurrences and 11 developed distant metastases in bone (n=3), liver (n=4) and lung (n=4); as for survival, eight patients died due to their disease. The mean percentage of CD44+ALDH1+Ki-67− tumor cells was strikingly higher in patients who developed distant metastases (p=0.001) and in those who died due to breast cancer (p=0.001, Table IV). Besides this, all patients presented CD44+ALDH1+Ki-67− tumor cells and those presenting ≥10% of these cells had a poorer overall survival (Figure 4).

We also explored the associations between the percentages of CD44+ALDH1+Ki-67− tumor cells and the hormone receptor status defined for each patient. The mean percentage of these cells was observed to be significantly higher in those with HER2− (p=0.002) breast tumors (Table V).

Discussion

One of the principles of the CSC model is that tumor growth, disease progression and the generation of heterogeneity in cancer is driven by a small population of tumorigenic cells within a tumor (8). Heterogeneity and plasticity in the phenotype of CSCs have been described in relation to their tissue of origin; as a consequence, few definitive markers have been isolated for CSCs from human solid tumors (15). In breast cancer, a plethora of studies involving the BCSC markers CD44, CD24 and ALDH1

Table II. *Mean percentage of hyaluronan receptor (CD44)$^+$ aldehyde dehydrogenase-1 (ALDH1)$^+$ Ki-67$^-$ breast cells in breast tissue samples.*

	CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells		
Characteristic	n (%)	Mean±SEM (%)	*p*-Value
Type of lesion			
Normal	16 (7.11)	0.43±0.12	Normal *vs*. non-malignant: 0.975
Non-Malignant	54 (24)	1.06±0.25	Normal vs. malignant: 0.025
Malignant	155 (68.89)	7.58±1.00	Malignant *vs*. non-malignant: <0.001
Pure DCIS	25 (16.13)	7.08±2.51	Pure DCIS *vs*. DCIS within IDC: 0.162
DCIS within IDC	30 (19.35)	13.13±2.88	Pure DCIS *vs*. IDC alone: 0.924
IDC alone	100 (64.52)	6.05±1.08	IDC alone *vs*. DCIS within IDC: 0.016

DCIS: Ductal carcinoma *in situ*; IDC: invasive ductal carcinoma; SEM standard error of the mean.

Table III. *Mean percentage of hyaluronan receptor (CD44)$^+$ aldehyde dehydrogenase-1 (ALDH1)$^+$ Ki-67$^-$ breast cells according to ductal carcinoma in situ nuclear grade.*

	CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells			
Characteristic	n (%)	Mean±SEM (%)	Correlation coefficient	*p*-Value
Nuclear grade				
Low	10 (18.18)	0.36±0.30		
Intermediate	19 (34.55)	9.10±3.17	0.374	0.005
High	26 (47.27)	15.16±3.15		

SEM: Standard error of the mean.

have demonstrated, essentially through immunological and genetic approaches, the implications of this type of cell in tumorigenesis and their effect after therapy failure. In fact, attempts to identify and characterize CD44$^+$CD24^{-low} or ALDH1^{+high} cells revealed enhanced ability to form tumors *in vitro* (16) and enhanced metastatic capacity (5). From an immunological point of view, however, ALDH1 seems to be a more effective predictive marker than the CD44$^+$CD24^{-low} phenotype. In fact, few studies have characterized CD44$^+$ALDH1^{+high} breast cancer cells and even a lack of correlation among these three major BCSC markers was recently reported (14, 17).

In this study, beyond the characterization of CD44 and ALDH1 in a cohort comprising of a series of normal, non-malignant and malignant breast tissues, we also aimed to identify CD44$^+$ALDH1$^+$ breast cells in their quiescent state (Ki-67$^-$) to analyze not only their correlation with the clinicopathological information but also to localize them among the different breast lesions assessed. After our previous results, we were able to identify CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells through a triple-staining method along with the corresponding digital images which allowed us to better control color-staining differences and the precise proportion of these cells when comparing with the entire tumor tissue.

Normal breast tissues with no previous history of cancer presented very low percentages of CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells. In tissues with benign breast diseases, this percentage, although not significantly higher than in normal tissues, was greater than 1% and even in a few cases, these cells were present in more than 5% of the entire lesion. CD44 and ALDH1 have already been described in normal and non-malignant breast tissues and even a higher expression of ALDH1 in women who developed breast cancer from proliferative benign lesions was demonstrated (18). Considering the sequential progression of breast tumors, we sought to determine if there was any difference in the percentage of CD44$^+$ALDH1$^+$Ki-67$^-$ cells between pure DCIS and DCIS within IDC. As expected, the mean percentage of these cells in DCIS within IDC was higher although not statistically significantly so. Nonetheless, when grouping all the DCIS cases, a significant positive correlation between the mean percentage of CD44$^+$ALDH1$^+$Ki-67$^-$ cells and nuclear grade was observed. This enrichment of CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells supports the CSC model regarding their tumor-initiating capacity but more interestingly, highlights that these cells exist in a quiescent state, raising some important questions about the real role of dormancy. Recent discoveries

Table IV. *Mean percentage of hyaluronan receptor (CD44)$^+$ aldehyde dehydrogenase-1 (ALDH1)$^+$ Ki-67$^-$ breast cells according to invasive ductal carcinoma clinicopathological markers of clinical progression and outcome.*

	CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells			
Characteristic	n (%)	Mean±SEM (%)	Correlation coefficient	*p*-Value
Grade				
G1	15 (15)	9.40±3.50		
G2	53 (53)	7.12±1.64	–0.221	0.027[a]
G3	32 (32)	2.70±0.97		
Tumor size				
T1	51 (71)	7.47±1.57		
T2	41 (51)	5.04±1.72	–0.269	0.007[a]
T3-4	8 (8)	2.19±1.23		
Nodal status				
N0	55 (55)	5.36±1.16		
N1	30 (30)	7.13±2.43	–0.067	0.508[a]
N2-3	15 (15)	6.39±3.31		
Metastasis				
M0	89 (89)	4,64±0.91	-	0.001[b]
M1	11 (11)	17.48±5.34		
Status				
Alive	92 (92)	4,88±0.94	-	0.001[b]
Died	8 (8)	19.55±6.68		

SEM: Standard error of the mean. [a]Spearman's non-parametric correlation; [b]Mann–Whitney *U*-test.

Table V. *Mean percentage of hyaluronan receptor (CD44)$^+$ aldehyde dehydrogenase-1 (ALDH1)$^+$ Ki-67$^-$ breast cells according to the patient's hormone receptor and human epidermal growth factor receptor (HER)-2 status.*

	CD44$^+$ALDH1$^+$Ki-67$^-$ breast cells				
	Pure DCIS n (%)	DCIS within IDC n (%)	IDC alone n (%)	Mean±SEM (%)	*p*-Value
ER status					
ER$^+$	20 (80)	27 (90)	80 (80)	7.85±1.17	0.114
ER$^-$	5 (20)	3 (10)	20 (20)	6.39±1.56	
PR status					
PR$^+$	14 (56)	22 (73.33)	70 (70)	6.81±1.51	0.319
PR$^-$	11 (44)	8 (26.67)	30 (30)	7.94±1.28	
HER2 status					
HER2$^+$	8 (32)	8 (26.67)	24 (24)	4.03±1.15	0.002
HER2$^-$	17 (68)	22 (73.33)	76 (76)	8.82±1.27	

ER: Estrogen receptor; PR: progesterone receptor; SEM: standard error of the mean.

suggested that the quiescent state is not just a passive state but, instead, is actively regulated by different intrinsic mechanisms. As CSCs may adopt the quiescent state to resist metabolic stress and to preserve genomic integrity, quiescent CSCs may be prompted for activation by specific energetically favorable mechanisms that are compatible with the low metabolic state of quiescence. Such modulation of CSCs can consequently generate rapid and global responses needed for activation (19).

Another interesting point is the significant decrease of CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells from DCIS within IDC to IDC alone and the negative correlation obtained with histological grade and tumor size. Such difference can be partly explained by other theoretical properties of CSCs: if these cells have the ability to self-renew and differentiate generating non-tumorigenic cancer cells that form a tumor mass, only a few cells would be required for invasion. Regarding the important roles of CD44 (self-renewal, niche preparation and resistance to apoptosis) and ALDH1 (self-renewal, stem cell proliferation control, protection against oxidative insults) in the stemness maintenance of CSCs, a decrease in the expression of these markers would then be expected. Again, only a few studies have analyzed ALDH1 and CD44 expression in DCIS. A higher expression of CD44 in DCIS when compared to IDCs was reported (20) and ALDH1 expression in DCIS associated with tumor-initiating properties was also demonstrated (21). Moreover, the increment of non-tumorigenic cancer cells obtained by symmetric divisions of CSCs can also explain the lower number of CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells seen in larger tumors.

Apart from the number of these cells, isolated pools of CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells were also detected in DCIS and IDCs. Even with the limitations of immunological techniques and considering the features described in literature for the so-called CSC niches, such isolated cells in malignant cases can be highly tumorigenic. If these cells happen to resist and prevail due to specific microenvironmental conditions, this advantage can allow them to gain malignant potential and thus to progress along the tumorigenic process.

Furthermore, the higher mean percentage of CD44$^+$ALDH1$^+$Ki-67$^-$ tumor cells among women who developed distant metastases and in those who died due to their disease is also noteworthy. A large body of evidence points to the fact that CSCs are particularly resistant to radiotherapy and chemotherapy, which can be partly explained by the clonal evolution model and tumor heterogeneity. Tumor progression has been related to 'Darwinian' evolution. The expansion of an established tumor can be explained by the generation of new clones as a result of mutations, genetic instability or epigenetic alterations. The prevalence of new CSCs and their clones will be determined by different selective pressures (nutritional or immune status, oxygenation and therapy) that modify the tumor microenvironment. If selected, these cells

can be responsible for tumor relapse or metastasis (22). Another factor that can compromise a good therapeutic response is the assumption of a fluid existence of 'stemness', with cells having the ability to both acquire and lose stemness. The acquisition of a stem cell phenotype has been demonstrated through the epithelial–mesenchymal transition induced either by paracrine signaling from cancer-associated fibroblasts or neighboring tumor cells (8).

In agreement with our results, the $CD44^+ALDH1^+Ki\text{-}67^-$ phenotype can potentially contribute to both chemotherapy and radiation resistance. These BCSCs markers were already described to be determinant for treatment resistance, recurrences and metastasis development through expression of high levels of therapy-resistance proteins (10, 23). ALDH1 activity has been shown to render cancer cells exquisitely resistant to some chemotherapy agents mainly due to its well-characterized role in differentiation through the retinoic acid pathway (24, 25). Moreover, $ALDH1^+$ tumor cells were shown to be more likely negative for Ki-67 (26), which is in part in accordance with our present study. In fact, quiescence may also have a determinant role in tumor progression and relapse. Several chemotherapeutic agents as well as radiotherapy work by inducing DNA damage. Thus, cells that have the ability to repair DNA damage are more prone to survive chemotherapy. Regarding the properties of quiescence, quiescent cells may have the potential and time to repair the damage inflected on them. Although quiescence is not an essential characteristic that defines stem cells, in BCSCs, there is increased expression of DNA-repair genes, indicating that high DNA-repair activity may aid in making CSCs resistant to tumor therapy (12).

Regarding the distribution of $CD44^+ALDH1^+Ki\text{-}67^-$ tumor cells according to hormone receptor status, a significant association with HER2 negativity was observed. Some controversial studies regarding the associations between ALDH1 and CD44 expression and hormone receptor status raise the question about the prognostic ability of these markers (27). Moreover, the studies carried out so far regarding the characterization of $ALDH1^{high}CD44^+$ tumor cells were *in vitro* studies; hence the correlation of these cells with poor prognosis remains to be clarified.

Despite our solid results concerning the *in situ* identification of $ALDH1^+CD44^+Ki\text{-}67^-$ tumor cells, some limitations of this work need to be addressed: the lack of validation precludes us from concluding that we have truly identified stem cells and BCSCs, in any case we referred to our cells of interest as BCSCs. In addition, we were not able to infer anything about breast tumor progression and $CD44^+ALDH1^+Ki\text{-}67^-$ cells in the same patient as this was a retrospective study. Finally, the limited number of patients enrolled in our study may have influenced the results, especially for patients who developed distant metastases (n=11) and for those who died (n=8).

In conclusion, $CD44^+ALDH1^+Ki\text{-}67^-$ tumor cells may have a greater tumorigenic effect in breast cancer than $CD44^+CD24^{-low}$ tumor cells. Due to its role, ALDH1 can determine the behavior of CSCs, their ability to resist chemotherapeutic agents and their dissemination to other parts of the body, which can be aided by the role of CD44. Additionally, quiescence seems to have a more preponderant role than previously expected, which may be crucial for tumor progression, resistance to chemotherapeutic agents and the metastatic spread of BCSCs. Despite improvements in knowledge of the adverse effects of ALDH1 and CD44 in breast cancer treatment, additional studies on the tumorigenic and metastatic ability of $CD44^+ALDH1^{+high}$ tumor cells combined with their quiescence status are still needed.

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Conflicts of Interest

The Authors state that no conflicts of interest exist in regard to this study.

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