Co-expression of Stem Cell Markers ALDH1 and CD44 in Non-malignant and Neoplastic Lesions of the Breast

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Abstract. *Background/Aim: The Cancer Stem Cell (CSC) model proposes that cancer is driven by a cellular component which possesses stem cell (SC) properties, cancer stem cells (CSCs), a distinct cell-type which is tumorigenic and capable of invasion and metastasis. Enzymatic activity of aldehyde dehydrogenase-1 (ALDH1), a de-toxifying enzyme that oxidizes intracellular aldehydes, has been used as a marker of normal and malignant breast stem cells (BSCs). CD44-transmembrane protein has already been shown to possess the ability to identify breast epithelial cells with stem properties. Materials and Methods: In order to compare two of the currently most reliable BSCs markers, ALDH1 and CD44, and to correlate their expression within different breast lesions, 190 samples from breast cancer specimens were analyzed by tissue microarrays. Results: ALDH1 expression was observed in 85.43% and CD44 in 90.3% of all samples. No overexpression was observed for ALDH1 between invasive tumors, ductal carcinomas in situ and non-malignant lesions of breast, although ALDH1 had a significant negative correlation with estrogen-receptor (ER) and progesterone-receptor (PR) status ($p=0.002$ and $p=0.001$, respectively) and a positive correlation with CD44 ($p<0.001$). Moreover, combined overexpression of ALDH1 and CD44 was observed in ductal in situ tumors ($p<0.001$). Conclusion: The combined overexpression of these markers in ductal carcinomas in situ is in agreement with the CSC model in breast cancer.*

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Although the concept that cancer may arise from a small cell population with stem cell (SC) characteristics has been proposed since more than 150 years, new evidence has given an impetus to it through new advancements on SC research (1). According to the cancer stem cell (CSC) model, CSCs have the ability to maintain the growth and expansion of the tumor mass, and originate a differentiated cell population, with none or limited proliferation capacity (2), through de-regulation of the self-renewal process. There are two ways by which CSCs can be formed: mutations allowing for abnormal expansion of normal SCs or mutations in progenitor cells that re-acquire the ability to self-renew (3-6). Either way, the CSC model is capable of explaining the phenotypic heterogeneity observed in many tumors, since self-renewal conducts the tumorigenesis and differentiation (albeit aberrant) of the tumor (1, 3).

Developmental biology has advanced greatly through the discovery of markers that distinguish, phenotypically, SCs from their differentiated progeny. These discoveries have allowed SCs to be isolated and studied separately, using techniques such as flow cytometry or immunohistochemistry, while providing critical insight into the regulatory mechanisms of SC function (1). However, there is relatively little overlap between the different CSC markers reported in different tumor types and so, the choice of marker can vary greatly, depending on tissues or species (2-5). ALDH1 has been shown to be a reliable SC marker based on its ability to isolate a sub-population of cells displaying SC properties from normal human breast tissue and breast carcinomas (6). Furthermore, CD44 is also overexpressed in basal-like breast cancers having the ability to identify breast epithelial cells with stem properties (7).

ALDH1 is a de-toxifying enzyme that oxidizes intracellular aldehydes and confers resistance to alkylating agents (8-11). In fact, the de-toxification capacity of ALDH1, protecting SCs against oxidative insult, might underlie the well-recognized longevity of these cells. ALDH1 also converts retinol to retinoic acid, a modulator of cell proliferation, which may also control stem cell proliferation (12).

CD44 is a transmembrane receptor protein that participates in many cell–cell and cell–matrix interactions (13, 14), known to be expressed in most cell types (6, 7) and reported to enhance tumor invasion and metastasis (15).

In the present study, we aimed to investigate whether a higher expression of the SC markers ALDH1 and CD44 is associated with particular types of breast lesions, more specifically: non-malignant, ductal carcinomas *in situ* (DCIS) and invasive ductal carcinomas (IDC).

Materials and Methods

Tissue Microarrays. From the 190 patients selected for this study, 139 had invasive ductal carcinomas and 51 had *in situ* ductal carcinomas. The correspondent formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from 2004 to 2011, along with their corresponding hematoxylin and eosin (H&E)-stained slides, from the archives of Department of Pathology, Santo António Hospital (Porto Hospital Centre, Porto, Portugal). Hormone receptor status was obtained from routine performance on diagnostic specimens from the same Department of Pathology. Areas of DCIS and IDC of the breast were identified and marked from each block. Non-malignant lesions were selected from specimens that also contained invasive ductal carcinomas. DCIS sections were selected from patients with ductal carcinoma *in situ*. *In situ* and invasive lobular carcinomas were excluded from the study. A total of 414 FFPE breast tissue specimens were used for tissue microarray construction, out of which 350 cores were evaluable for ALDH1 and 365 cores for CD44. Tissue cores from human liver donor samples were included in each of these recipient blocks, for a correct slide orientation. 2-μm thick sections were routinely cut in a microtome. Pathological and clinical characteristics of the study population are provided in Tables I and II.

Table I. *Clinical characteristics of samples*.

Characteristics	Cohort population n (%)	CD44 expression samples n (%)	ALDH1 expression samples n (%)
Type of Lesion			
Non-Malignant	71 (17.2%)	67 (18.4%)	48 (13.7%)
DCIS	121 (29.2%)	115 (31.5%)	113 (32.3%)
IDC	222 (53.6%)	183 (50.1%)	189 (54.0%)
Total	414 (100%)	365 (100%)	350 (100%)

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

Table II. *Invasive ductal carcinoma patients' hormone receptor status*.

Characteristics	n (%)
ER status	
Positive	118 (84.9%)
Negative	21 (15.1%)
PR status	
Positive	106 (76.3%)
Negative	33 (23.7%)

ER, Estrogen receptor; PR, progesterone receptor.

Immunohistochemistry. ALDH1 and CD44 immunohistochemistry was performed on tissue microarray paraffin-embedded sections with the monoclonal antibodies ALDH1 (Rabbit monoclonal, IgG isotype, 0.13 mg/ml concentrate, Abcam, Cambridge, United Kingdom) and CD44 (Mouse monoclonal, IgG isotype, 0.01 mg/ml concentrate, MRQ-13, Cell Marque, California, United States). The sections were de-paraffinized, rehydrated in a series of graded ethanol and washed in water. Target retrieval was achieved with citrate buffer (pH 6.0), in a microwave at 850 W until boiling. After cooling, slides were washed three times in PBST (phosphate buffered saline, pH 7.4 – 0.05% Tween 20) each for 5 min. To avoid unspecific tissue peroxidase activity, slides were incubated, for 10 min, with 3% peroxide hydrogen in methanol and after incubation with a blocking solution (Ultra Vision LP Detection System, Thermo Fisher Scientific, Cheshire, UK), for 5 min. The ALDH1 (dilution: 1/100) and CD44 (dilution: 1/100) antibodies were incubated in a humid chamber, for 1 h at room temperature. The slides were then washed once in PBST for 5 min and incubated in a humid chamber with Primary Antibody Enhancer (Ultra Vision LP Detection System, Thermo Fisher Scientific, Cheshire, UK), for 10 min, and further washed three times, for 5 min each in PBST. Horseradish Peroxidase Polymer (Ultra Vision LP Detection System, Ultra Vision LP Detection System, Thermo Fisher Scientific, Cheshire, UK) was added, for 20 minutes in a humid chamber and washed three times, 5 minutes each in PBST. Enzyme reactivity was visualized using 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma, Saint Louis, United States). Slides were counter-stained with Mayers hemalumen solution (Merck), de-hydrated and mounted with Entellan (Sigma).

Staining. The reaction obtained in all samples was observed in a Nikon Eclipse E400 bright-field microscope. Each core was analyzed and classified according to its histological type. For the evaluation of staining patterns only breast epithelial cells were considered, excluding also artifacts staining tumor cell debris. A semi-quantitative evaluation method was applied: the percentage of positive cells (0 points: 0%; 1 point: 1-10%, 2 points: 11-20%, 3 points: 21-50% and 4 points: >50%) and the staining intensity (0 points: no staining, 1 point: weak staining, 2 points: moderate staining and 3 points: strong staining) were considered and multiplied. The presence or absence of immunoexpression for ALDH1 and CD44 was also considered. A combined score was also created by addition of ALDH1 and CD44 dichotomized scores in order to produce 3 categories (0 to 2). According to the American Society of Clinical Oncology (ASCO) for the hormone receptor status criteria, specimens having at least 1% positive tumor cells for ER or PR were considered positive.

Table III. *ALDH1 and CD44 positivity in breast tissue samples.*

	ALDH1 expression score			CD44 expression score		
Characteristics	Positive cases (%)	Negative cases (%)	*p*-Value	Positive cases (%)	Negative cases (%)	*p*-Value
Type of Lesion						
Non-Malignant	87.5	12.5	0.064[a]	81.1	11.9	0.003[a]
DCIS	91.2	8.8		94.8	5.4	
IDC	81.5	18.5		80.9	19.1	

DCIS, Ductal carcinoma *in situ*; IDC, invasive ductal carcinoma. [a]Pearson Chi-Square.

Statistical analysis. Results of the immunohistochemical analyses were statistically examined using IBM SPSS Statistics Version 20.0 (SPSS Inc., IBM, Chicago, IL, USA). Statistical significance was calculated using the Mann-Whitney or Kruskal-Wallis test, according to the number of groups considered, and Fisher's Exact Test or Pearson's Chi-Square to assess relationships between data sets. Significance was set at $p<0.05$.

Results

Details on the baseline characteristics of the samples selected for analysis are provided on Table I. Samples from 190 patients' FFPE blocks were included in the study. The cohort comprised of 71 non-malignant (17.2%), including normal, hyperplastic and other non-malignant samples, 121 DCIS (29.2%) and 222 IDC (53.6%) lesions. The ALDH1 immunoexpression evaluation cohort comprised of 48 non-malignant (13.7%), 113 DCIS (32.3%) and 189 IDC (54.0%) lesions. For CD44, 67 non-malignant (18.4%), 115 DCIS (31.5%) and 183 IDC (50.1%) lesions were analyzed (Table I).

ALDH1 and CD44 immunostaining was observed in epithelial cells of the breast and also in stromal inflammatory cells, both in non-malignant and neoplastic samples. Figure 1a shows ALDH1 immunoexpression in a papilloma where expression is observed in the cytoplasm of most cells. In a high-grade DCIS and in an invasive ductal carcinoma, ALDH1 is seen in cytoplasm of rare cells (Figure 1b) and in most of cells presented (Figure 1c). Figure 1d demonstrates CD44 immunoexpression also in a papilloma with a strong expression in the cells' membrane. A strong CD44 expression is also seen in the membrane of most cells in a high-grade DCIS (Figure 1e) as well as in an invasive ductal carcinoma (Figure 1f).

ALDH1 and CD44 expression and correlation with clinicopathological variables. Using positivity criteria, ALDH1 and CD44 expression was evaluated for each lesion type. CD44 expression was observed in 299 (85.4%) of the 350 samples and CD44 in 316 (90.3%) out of 365. A higher percentage of samples expressing ALDH1 (91.2%) and CD44 (94.8%) was seen in the DCIS group, followed by the non-malignant sample group (ALDH1 87.5%; CD44 81.1%) and then IDC (ALDH1 81.5%; CD44 80.9%). Differences between the groups were significant for CD44 expression only ($p=0.003$) (Table III).

Table IV. *ALDH1 and CD44 expression in breast tissue samples.*

	ALDH1 expression score		CD44 expression score	
Characteristics	Mean	*p*-Value	Mean	*p*-Value
Type of Lesion				
Non-Malignant	155.21		163.07	
DCIS	169.43	0.147[a]	190.43	0.209[a]
IDC	184.28		185.63	

DCIS, Ductal carcinoma *in situ*; IDC, invasive ductal carcinoma. [a]Kruskal-Wallis test.

ALDH1 and CD44 mean rank expression and correlation with clinicopathological variables. ALDH1 and CD44 mean expression was assessed for each type of lesion and for clinicopathological variables. For ALDH1, mean rank expression was higher in the IDC group (185.63), succeed by DCIS (169.43) and the non-malignant group (155.21). On the other hand, the DCIS group presented the higher CD44 mean rank expression (190.43), with the non-malignant group presenting the lowest mean rank expression (163.07). None of the SC markers mean rank expression was significantly different between the groups (Table IV). Considering the ER- and PR-status analysis, ALDH1 mean rank expression was significantly higher for ER-negative (86.65) and PR-negative (83.33) patients ($p=0.002$ and $p=0.001$, respectively). A significant negative correlation was obtained between ALDH1 expression and ER-negative ($p=0.002$) and PR-negative status ($p=0.001$). Even though a higher mean rank for CD44 expression was observed in ER- and PR-negative patients, the differences were not significant

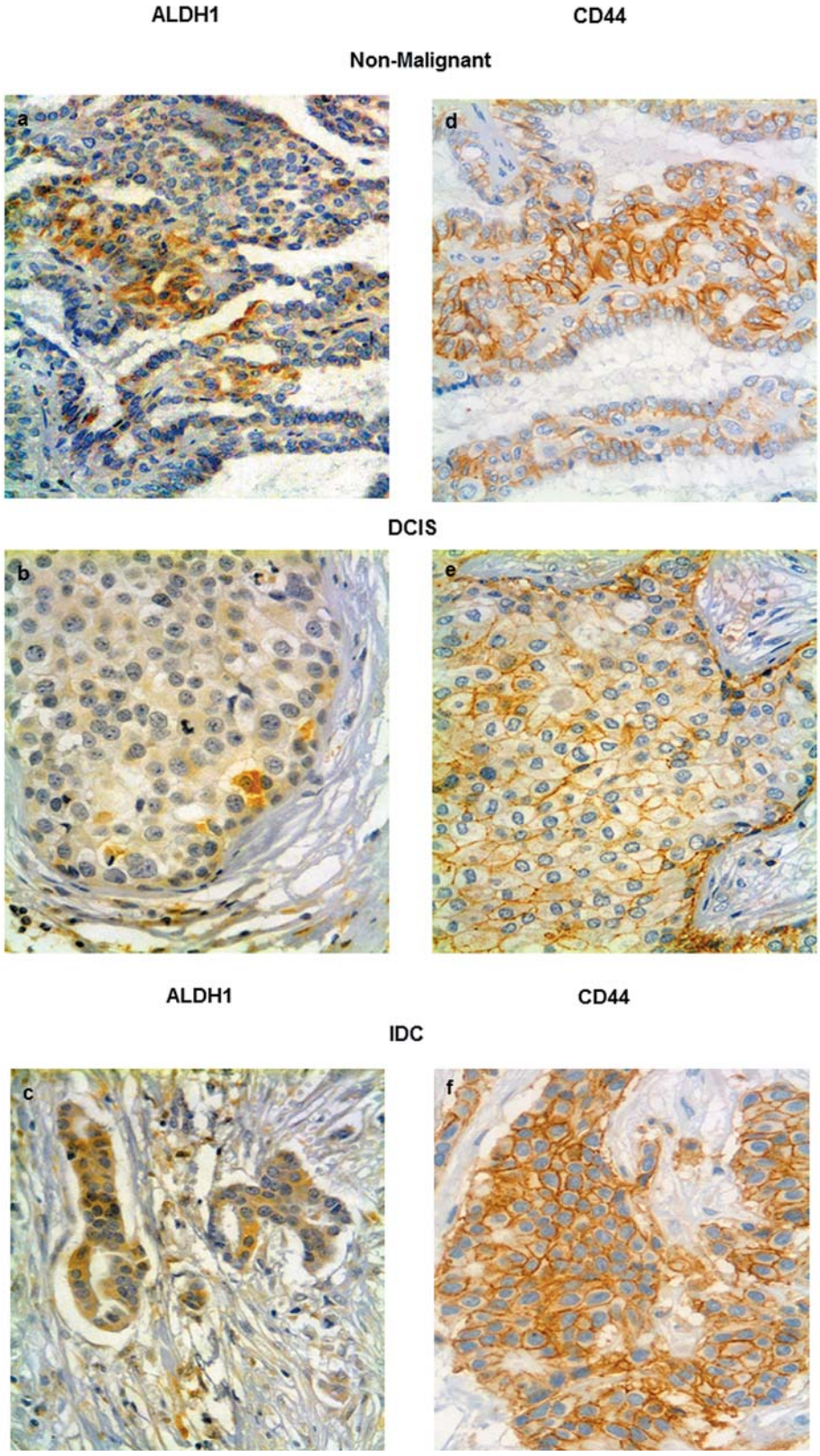

Figure 1. *Representatives images of ALDH1 staining in a) non-malignant (×20), b) DCIS (×40) and c) IDC samples (×20) and CD44 staining in d) non-malignant (×20), e) DCIS (×40) and f) IDC samples (×40).*

Table V. *ALDH1 and CD44 expression according to patients' hormone receptor status.*

	ALDH1 expression score				CD44 expression score			
Characteristics	Mean	*p*-Value	Correlation coefficient	*p*-Value	Mean	*p*-Value	Correlation coefficient	*p*-Value
ER status								
Positive	59.77	0.002[a]	–0.270	0.002[b]	61.11	0.511[a]	–0.059	0.514[b]
Negative	86.65				66.89			
PR status								
Positive	58.13	0.001[a]	–0.297	0.001[b]	62.19	0.915[a]	0.01	0.915[b]
Negative	83.33				61.40			

ER, Estrogen receptor; PR, progesterone receptor. [a]Mann-Whitney test; [b]Non-parametric correlation.

($p>0.05$) (Table V). No significant correlation was observed between CD44 expression and ER- and PR-status.

Correlation and simultaneous expression of the stem-cell markers ALDH1 and CD44. The presence of ALDH1 and CD44 immunoreactivity was positively correlated (Spearman's R=0.153; $p=0.008$), as well as CD44 mean rank expression was correlated with ALDH1 mean rank expression (Spearman's R=0.261; $p<0.001$) (data not shown). Based on these results, we created a score representing a composite measure for the expression of both markers. A higher mean rank expression for the combined SC score was observed in DCIS samples, followed by non-malignant lesions and then IDC. The mean rank expression difference between the groups is highly significant ($p<0.001$) (Table VI).

Table VI. *ALDH1 and CD44 combined score mean rank expression.*

Characteristics Type of Lesion	Mean	*p*-Value
Non-Malignant	161.82	<0.001[a]
DCIS	169.57	
IDC	134.58	

DCIS, Ductal carcinoma *in situ*; IDC, invasive ductal carcinoma. [a]Kruskal-Wallis test

Discussion

The aim of the present study was to explore a possible association between a high expression of SC markers and a determined type of lesion during breast cancer progression. For this purpose, ALDH1 and CD44 expression was immunohistochemically-evaluated in non-malignant breast lesions, ductal carcinoma *in situ* and invasive ductal carcinoma sample cores. However, our study has some limitations. Since putative CSCs were originally identified using flow cytometry we have assumed that this assay can be translated into an immunohistochemistry-based equivalent. Although we would expect these modalities to identify a population with a high degree of overlapping, it is probable that there will be some discordance. Besides that we have used TMAs to detect for a sub-population of cells of reputed scarcity and as a result it is probable there is some sampling error. In order to minimize these errors, we selected more than one area of breast cancer specimens to be representative of the entire tissue block, thus increasing our cohort population. We decided to exclude *in situ* and invasive lobular carcinomas due to the reduced number of blocks containing these types of lesions, not being representative for this expression study. In fact, invasive lobular carcinomas counts for 5 to 15% of different races worldwide, contrarily to 70 to 90% of women having invasive ductal carcinomas (16). Moreover, these two types of breast cancer are histologically- and genetically-different, which explains the restriction to the immunoexpression analysis performed in *in situ* and invasive ductal carcinomas.

Several studies have assessed ALDH1 expression by immunohistochemistry in breast tissue, either in non-malignant or malignant breast tumors in order to define ALDH1 role and impact in predicting cancer development. Kunju *et al*. reported that ALDH1 is expressed in epithelial and stromal cells in benign breast tissues, and discovered that ALDH1 positivity in breast epithelial cells is associated with increased risk of breast cancer (17). Madjd and his colleagues verified a high percentage of breast tumors with ALDH1 positive cells (86%) (18). In our study, ALDH1 expression was observed in 85.43% of all samples, with a homogenous distribution in the different groups of assessed lesions. Considering the fact that ALDH1 was already demonstrated to be a potential marker of normal and malignant human BSCs (6) a high percentage of non-malignant breast tissues samples with ALDH1-positive cells would be expected. However, samples with less than 5% of

ALDH1-positive cells were also counted as ALDH1-positive which increases the possibility of having a high number of positive samples. In fact, the vast majority of samples had a relatively small amount of ALDH1-positive cells, which can be consistent with the theory that SCs comprise of a minority of tumor tissues. Furthermore, all normal and hyperplasic breast tissue samples were selected and collected from cancer specimens. These may not be representative of truly benign tissue, due to genetic alterations not affecting cell architecture (19). Moreover, no significant differences were observed between the lesion groups regarding ALDH1 expression, but we obtained a negative significant correlation between ALDH1 expression and ER-negative and PR-negative status. These results are in accordance with the majority of others studies done so far regarding ALDH1 expression in breast carcinogenesis (23, 25, 26) and studies that have correlated ALDH1$^+$ tumors with negativity for estrogen and progesterone receptors (12, 25, 27-29).

Interest in CD44 has stemmed from reports showing that high levels of CD44 expression in combination with low-level expression of CD24 can be used to prospectively identify a population of breast cancer cells enriched in SC-like properties and tumor-initiating capacity (3). CD44 comprises a family of cell surface receptors that recognize hyaluronan, a component of the extracellular matrix, as their principal ligand (20). Multiple splice variants of CD44 exist, which have a more restricted expression (21). Moreover, CD44 has been associated with SC in normal breast tissue (22). In breast carcinomas, Auvinen and colleagues demonstrated that CD44 expression was common in carcinoma cells and only in 8% of cases, were all carcinoma cells CD44-negative (23). The same authors detected the expression of an isoform of CD44, CD44v6 in 20-30% of ductal epithelial cells in benign lesions of breast (24). Moreover, Bankfalvi *et al*. investigated whether the CD44 immunophenotype of breast lesions correlated with the clinical evolution and prognosis of breast cancer. They found that in normal breast tissue luminal epithelial cells lacked detectable CD44 in contrast to basal cells, which constitutionally expressed CD44s, v.3, v.5 v.6 and v.9 isoforms (25). Our results showed positivity for CD44 in 90.3% of all samples with homogenous distribution between the lesion groups. No significant differences were observed between the lesion groups relatively to CD44 expression and no significant correlation was seen between CD44 expression and ER and PR status.

Cell surface markers and enzymatic activity detected by fluorescence-activated cell sorting (FACS) have been widely used for the prospective isolation of putative CSCs. The idea of combining markers to increase the purity of sub-populations for CSCs was utilized by Ginestier *et al*. who showed that the combination of CD44$^+$/CD24$^{-/low}$ and ALDEFLUOR activity enabled for isolation of cells able to form tumors in NOD/SCID mice from as few as 20 cells, compared to 500 cells when sorted by ALDEFLUOR activity alone (12). Neumeister and colleagues developed an *in situ* method to define CSCs in FFPE breast cancer tissues, with the goal of assessing the prognostic value of the presence of breast cancer stem cells (BCSCs) using a multiplexed assay for CD44 and ALDH1. They found a strong co-expression between these two markers in breast tumors associated with poor prognosis (26). In our study, and considering the fact that both markers are positively-correlated we decided to combine ALDH1 and CD44 expression in order to determine if both markers had a significant co-expression in the different lesion groups. This co-expression was significantly higher in *in situ* carcinomas when compared against the other two groups.

According to the CSC model, cancers originate from the malignant transformation of an adult SC or a progenitor through the de-regulation of the normally tightly-regulated self-renewal program. This leads to clonal expansion of stem/progenitor cells that undergo further genetic or epigenetic alterations to become fully-transformed. As a consequence, tumors contain a cellular component of CSCs which retains the key stem cell properties that may initiate and drive carcinogenesis (1). Regarding the roles of ALDH1 and CD44, it is plausible to associate their expression in transformation of BSCs in to BCSCs. The progression to a malignant phenotype involves local metastasis and invasion, two processes in which the cell-cell and cell-extracellular matrix adhesion are altered. Along the tumoral progression, CD44 can be involved in the cellular matrix adhesion, cellular matrix degradation, cancer cells migration and angiogenesis (27). On the other hand, ALDH1 plays a role in early differentiation of stem cells by promoting the formation of retinoic acid where the retinoic signaling is been directly implicated in modulating BCSCs differentiation (28). If this cellular component is present in breast tumors with stem cell properties that initiate and drive carcinogenesis, after their differentiation through symmetrical division, the formed cancer cells will have a lower expression of ALDH1 than BCSCs. Besides that, cancer cells other than BCSCs can metastasize *in vivo* or in experimental models, indicating that cells with an invasive phenotype can be found outside the BCSC pool (29). Thus, this could explain the combined overexpression between ALDH1 and CD44 in *in situ* carcinomas obtained in this study, a type of lesion supposed to be a transition of non-invasive to an invasive phenotype in breast cancer (30).

In conclusion, no ALDH1 and CD44 overexpression was obtained between the different types of assessed breast lesions. Moreover, the co-expression between these markers was shown to be significantly higher in *in situ* carcinomas when compared to the other two lesions groups. Regarding the CSC model theory, it is plausible to associate these findings with the malignant transformation of BSCs in the

carcinogenesis process of the breast. Nevertheless more studies have to be performed, other than immunoexpression analysis, to consolidate this association.

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Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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