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Primary breast cancer patients with high risk clinicopathologic features have high percentages of bone marrow epithelial cells with ALDH activity and CD44⁺CD24^{lo} cancer stem cell phenotype

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Abstract

Background—Cancer stem cells (CSCs) are purported to be epithelial tumor cells expressing CD44⁺CD24^{lo} that exhibit aldehyde dehydrogenase activity (Aldefluor⁺). We hypothesized that if CSCs are responsible for tumor dissemination, disseminated cells in the bone marrow (BM) would be positive for putative breast CSC markers. Therefore, we assessed the presence of Aldefluor⁺ epithelial (CD326⁺CD45^{dim}) cells for the presence of the CD44⁺CD24^{lo} phenotype in BM of patients with primary breast cancer (PBC).

Methods—BM aspirates were collected at the time of surgery from 66 patients with PBC. Thirty patients received neoadjuvant chemotherapy (NACT) prior to aspiration. BM was analyzed for Aldefluor⁺ epithelial cells with or without CD44⁺CD24^{lo} expression by flow cytometry. BM aspirates from 3 healthy donors (HD) were subjected to identical processing and analyses and served as controls.

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Results—Patients with triple-receptor-negative (TN) tumors had a significantly higher median percentage of CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cell population than patients with other immunohistochemical subtypes (P=0.018). Patients with TN tumors or with pN2 or higher pathologic nodal status were more likely to have a proportion of CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cell population above the highest level of HD. Furthermore, patients who received NACT were more likely to have percentages of Aldefluor⁺ epithelial cells greater than the highest level of HD (P=0.004).

Conclusion—The percentage of CD44⁺CD24^{lo} CSC in the BM is higher in PBC patients with high risk tumor features. The selection or enrichment of Aldefluor⁺ epithelial cells by NACT may represent an opportunity to target these cells with novel therapies.

Introduction

Approximately 5% of patients with breast cancer have clinically detectable metastases at the time of initial diagnosis and 30–40% of patients who appear clinically free of metastases harbor occult metastases (1–4). It is presumed that tumor cells that shed from the primary lesions are released into the peripheral circulation as circulating tumor cells (CTC) that express epithelial-lineage markers, such as CD326 (i.e. EpCAM, epithelial cell adhesion molecules). CTC migrate to the bone marrow microenvironment where there is a selection to maintain a non-proliferative stem-cell like phenotype or to be induced to become cancer-initiating stem cells (CSC) that initiate metastases (5,6).

Evidence for the existence of CSC, a limited population of tumor cells responsible for giving rise to heterogeneous tumor, was first demonstrated in patients with acute myeloid leukemia (7,8). Later, Weissman et al. provided proof of principle that inhibiting the tumor stem cell can prevent the recurrence of leukemia (9). Al-Hajj and colleagues used cell-surface markers to isolate a subpopulation of highly tumorigenic breast cancer cells from the bulk of human breast tumor (10). They observed that CD44⁺CD24^{lo} human breast tumor cells have an increased ability to form tumors when injected into the cleared mammary fat pad of NOD/SCID mice than cells without this phenotype. While as few as 10² CD44⁺CD24^{lo} human breast tumor cells recapitulated the human tumors from which they were derived, injection of 10⁴ cells of other phenotypes failed to form tumors (10).

It has been demonstrated that the majority of cytokeratin positive (CK⁺) tumor cells in the bone marrow, also known as disseminated tumor cells (DTC), are CD44⁺CD24^{lo} even though these cells were observed in only a small proportion of bone marrow aspirates (11). Furthermore, the tumor outgrowth potential of CD44⁺CD24^{lo} cells resides within a subpopulation of epithelial cells with ALDH activity measured by the Aldefluor® method (STEMCELL Technologies, Vancouver, BC) (8). As bone marrow serves as a reservoir for occult disease and CD44⁺CD24^{lo} CSC within the Aldefluor⁺ epithelial (CD326⁺CD45^{dim}) cells may serve as prognostic factors for breast cancer, we measured these cells in bone marrow (BM) of patients with primary breast cancer (PBC) and correlated the findings with their clinicopathological characteristics.

Materials & Methods

Study population

We conducted a prospective laboratory-based study (Lab04-0657, Principal Investigator: Anthony Lucci, M.D.) that was approved by the institutional review board of The University of Texas MD Anderson Cancer Center. Enrollment eligibility criteria included patients with a diagnosis of PBC, stages I–III without metastatic disease, and elected to undergo definitive surgery for primary tumor and lymph nodes dissection. All patients provided informed

consent according to institutional guidelines. From September 2006 to October 2008, 66 PBC patients were enrolled and provided a BM specimen either at the time of placement of a central venous catheter for delivering neoadjuvant chemotherapy (NACT) (N=6) or at the time of surgery (N=60). Thirty patients received NACT prior to bone marrow aspiration. We noted that NACT group had a significantly higher proportion of patients with N2 or higher nodal status ($P=0.000$) or with HER-2+ primary tumor ($P=0.001$). As a control, BM aspirates from three healthy donors (HD) were purchased from STEMCELL Technologies (Vancouver, BC) and analyzed in the same manner as patient BM samples.

Primary breast tumor characterization

The primary breast tumors were graded histologically on a scale from 1 to 3 based on a modified, combined Nottingham histologic grading system. Immunostaining for ER, PR, and HER2 was performed using primary antibodies against ER (clone 6FII, dilution 1:35; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), PR (clone PgR 1294, dilution 1:200; Dako, Carpinteria, CA) and HER2/neu (Lab Vision, clone neu Ab8, dilution 1:300; Lab Vision, Fremont, CA). 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 1+ when <10% of the tumor cells had complete membranous staining; as 2+ when weak-to-moderate, complete membranous staining was present in >10% of tumor cells; and as 3+ when strong, complete membranous staining was present in >30% of tumor cells. All 2+ and 3+ cases were evaluated by fluorescence in situ hybridization (FISH) for HER2 gene amplification using the HER2 DNA probe kit (Abbott Laboratories, Abbott Park, Ill). A HER2/CEP17 ratio >2.2 was regarded as positive for HER2 gene amplification.

Detection of epithelial cells in BM

To assess for the presence of epithelial cells in BM, 5–8 mL of BM were aspirated from bilateral iliac crests of 66 PBC patients, the specimen dispensed into EDTA-coated tubes, and transported to the laboratory immediately for processing within 4 hours. To maximize epithelial cell recovery, the bilateral BM samples were pooled prior to the isolation of mononuclear cells (MNC) by ficoll-hypaque density gradient centrifugation at $400 \times g$ for 30 minutes. The MNC were harvested and washed twice with ice-cold phosphate-buffered saline (PBS).

MNC samples were interrogated for ALDH activity using the Aldefluor[®] assay and manufacturer's protocol (STEMCELL Technologies). Briefly, 4×10^6 MNC from patients and HD were suspended in Aldefluor buffer which contains a proprietary ATP-binding cassette transport inhibitor and reacted with 5 μ L of the Aldefluor reagent. One third of the cells were reacted with 5 μ L of the ALDH inhibitor, diethylamino-benzaldehyde (DEAB), as a negative control. Both the test reaction and the negative control were incubated for 35 minutes at 37 °C in a 5% CO₂ atmosphere. Purified anti-CD44 monoclonal antibody (BD Pharmingen, San Diego, CA) was conjugated with Alexa700 using the Zenon[®] antibody labeling kit (Invitrogen, Carlsbad, CA) prior to reaction with the Aldefluor-labeled cells. Additionally, pre-conjugated antibodies to CD24 (PE) and CD45 (PE-Cy7) both from BD Pharmingen (San Diego, CA), and CD326 (APC, Miltenyi Biotec, Auburn, CA) were used to label cells at room temperature protected from light for 30 minutes. An additional tube of Aldefluor-labeled cells was stained with the appropriate isotype-matched controls. The stained cells were washed with Aldefluor buffer and suspended in 300 μ L of buffer prior to analysis on a LSR-II flow cytometer capable of discriminating 6-color fluorescence (BD Biosciences, San Jose, CA). Cellular debris was excluded from the analysis based on low forward light scatter. For analysis and throughout the manuscript, epithelial cells in BM were defined as cells exhibiting the phenotype CD326⁺ and CD45^{dim}. Within the Aldefluor⁺

epithelial cell population, a subset of cancer-initiating stem cells (CSC) was defined as cells with a CD44⁺CD24^{lo} phenotype.

Statistical analysis

In this prospective study descriptive statistics were computed for Aldefluor⁺ epithelial cells and CD44⁺CD24^{lo} CSC in BM specimens of PBC patients and HD. Frequency and percentages of epithelial cells and CSC subsets were summarized for categorical variables and means, medians, standard deviations and standard errors of mean were used to summarize the continuous variables. The Wilcoxon rank sum tests (12) and Mann-Whitney U tests were used to compare the difference of continuous variables among and between clinicopathological groups, respectively (13). The Fisher's exact test was used to compare the categorical variables between groups. Statistically significant differences were set at $P < 0.05$. All analyses were conducted in SPSS 16 (SPSS Inc, Chicago, IL).

Results

Patient population

A total of 66 PBC patients, stages I–III, and undergoing surgery with (N=30) or without (N=36) prior NACT were studied (Table 1). The median age of the patient population was 52 years (range: 27–77 years). There were 45 patients with Hormonal Receptor (HR)+/HER2–, 4 patients with HR+/HER2-amplified (HER2+), 7 patients with HR–/HER2+, and 10 patients with triple-receptor-negative (TN) tumor subtypes according to the immunohistochemical (IHC) staining of the primary tumor using ER/PR and HER2-neu status. Tumor grade consisted of 8 grade 1, 28 grade 2, and 30 grade 3. Tumor sizes consisted of 32 T1, 20 T2, 6 T3, and 8 T4 (Table 1). Lymph node status of patients consisted of 36 N0, 15 N1, 3 N2, and 12 N3. Staging was based on the revision of the American Joint Committee on Cancer staging system for breast cancer (14).

Assessment of Epithelial cells and CSC for ALDH activity

Patient BM samples were analyzed for the presence of ALDH activity using the Aldefluor[®] assay kit (Figure 1). Cells were initially analyzed for their ALDH activity (Aldefluor⁺) and then assessed for the percentage of epithelial (CD326⁺CD45^{dim}) cells within the Aldefluor⁺ population. The mean percentage ($\pm$ SEM) of Aldefluor⁺ cells in MNC, irrespective of their epithelial or hematopoietic origin, was 1.40% ($\pm$ 0.11%) (Table 2). The mean percentage of Aldefluor⁺ epithelial cells was 48.6% ($\pm$ 2.21%; alternatively expressed as 0.68% of total MNC counted) and the mean percentage of CD44⁺CD24^{lo} CSC within the Aldefluor⁺ epithelial cell population was 43.0% $\pm$ 3.59% (Table 2; alternatively expressed as 0.30% of total MNC counted).

Relationship between immunohistological subtypes and percentage of CSC within the Aldefluor⁺ epithelial cells

Comparing the entire patient cohort to the HD samples, there was a statistically significantly higher median percentage of Aldefluor⁺ epithelial cells (independent of CD44⁺CD24^{lo} expression) in BM of PBC patients when compared with that of HD (50.6% vs. 37.7% $P=0.045$). Overall, the proportion of the CD44⁺CD24^{lo} CSC subset within Aldefluor⁺ epithelial cells was not statistically different between patients and HD (Figure 2b). However, the subgroup of patients with TN tumors had a significantly higher percentage of CD44⁺CD24^{lo} CSC when compared with patients with other tumor IHC subtypes (66.8% vs. 35.6%, $P=0.02$) (Figure 2d). In addition, these patients were more likely to have the proportion of CSC greater than the highest level of the HD (35.1%) ($P=0.019$, Table 4).

Relationship between the tumor size and percentage of CSC in bone marrow

There were 34 patients with T2 or greater tumors. There were no differences in the percentage of Aldefluor⁺ epithelial cells or proportion of CD44⁺CD24^{lo} CSC within the Aldefluor⁺ epithelial cell population between patients with T1 tumors and patients with T2 or greater tumors. However, compared with patients with grade 1–2 tumors (24/36 or 66.7%), patients with grade 3 breast cancer (27/30 or 90%) were more likely to have a proportion of Aldefluor⁺ epithelial cells above 39.3%, the highest level of HD bone marrow aspirates (P=0.023, Table 3). Nevertheless, patients with grade 1–2 and grade 3 tumors had similar proportion of CD44⁺CD24^{lo} CSC within the Aldefluor⁺ epithelial cell population.

Relationship between lymph node status and CD44⁺CD24^{lo} CSC in bone marrow

Compared with patients with pN1 or negative nodes, the percentage of Aldefluor⁺ epithelial cells (46.6% vs. 57.3%; P=0.018) and the proportion of CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cell cluster (33.1% vs. 66.2%; P=0.008) were significantly higher for patients with pN2 or higher nodal status (Figure 2f). Furthermore, patients with pN2 or higher nodal status were more likely to have the proportion of CD44⁺CD24^{lo} CSC higher than 35.1%, the highest level of HD bone marrow aspirates (P=0.031, Table 4).

Effect of neoadjuvant chemotherapy on CSC in bone marrow

Patients who received NACT had a significantly higher median percentage of Aldefluor⁺ epithelial cells (55.5% vs. 43.2%; P=0.002), but not CD44⁺CD24^{lo} CSC, when compared with those of untreated patients. In addition, compared with patients who did not receive NACT (25/36 or 69.4%), NACT-treated patients (27/30 or 90%) were more likely to have a proportion of Aldefluor⁺ epithelial cells greater than 39.3%, the highest level of HD bone marrow aspirates (P=0.039, Table 3). Among the 30 patients who received NACT, 10 patients achieved pathological complete responses (pCR); however, the responses were not associated with lower percentage of Aldefluor⁺ epithelial cells (Table 3) or lower proportion of CD44⁺CD24^{lo} CSC (Table 4).

Discussion

As part of an on-going prospective trial to study the prognostic value of disseminated epithelial cells in patients with operable breast cancer, we report for the first time the distribution of putative breast cancer stem cell markers measured by flow cytometry in the bone marrow of patients with PBC. This cohort represents an unselected group with stage I–III breast cancer and the expected distribution of clinical tumor markers. In this study, we demonstrated the presence of epithelial cells (CD326⁺CD45^{dim}) with ALDH activity (Aldefluor⁺) in the bone marrow of PBC patients is significantly greater than that seen in HD. In particular, the percentage of epithelial cells expressing Aldefluor and putative CSC markers was highest in patients with TN tumor and advanced nodal disease. This is consistent with the hypothesis that CSCs are associated with early micrometastatic disease in patients who eventually relapse. These are preliminary but important translational data confirming the presence of these phenotypes in clinical samples and supporting the need for further studies to examine the mechanisms of chemotherapy resistance of these cells.

In our study, we used multi-parameter flow cytometry to detect CD326⁺CD45^{dim} epithelial cells and to enumerate the CD44⁺CD24^{lo} cells within the Aldefluor⁺ epithelial cells population in BM of PBC patients. We detected epithelial cells in the BM of all patients examined. While others used immunocytochemistry (ICC) and PCR-based technology for the detection of CSCs in the BM (3), we employed multi-parameter FACS analysis and found it to be sensitive and reliable for detecting of putative CSC in BM of PBC patients. A major disadvantage of employing the current putative markers (CD326, CD44, CD24,

Aldefluor) to identify CSC is that these characteristics are not lineage specific. Indeed, CD326 can be expressed by CD45⁺ hematopoietic cells and by CD34⁺ early progenitor cells in BM of HD (15) and we find them at lower levels in HD samples. Nevertheless, these markers represent the current state of the art for immunophenotypic characterization of CSC and have been reported by others as well (15).

CD44, a cell surface receptor for hyaluronic acid, is involved in cell adhesion, migration, and metastasis of cancer cells (16), and the lineage negative CD44⁺CD24^{lo} phenotype prospectively and selectively identified cells which re-generated breast tumors when transplanted into immunocompromised mice (10). In further studies of primary tumors, the CD44⁺CD24^{lo} phenotype was more common in basal-like tumors (17) and associated with BRCA1 hereditary breast cancer (17). Others have also reported that patients with the CD44⁺CD24^{lo} phenotype had TN tumors (18). Thus, it is consistent that the proportion of CD44⁺CD24^{lo} CSC within the Aldefluor⁺ epithelial cells in the bone marrow was indeed highest in patients with TN tumors. In addition, patients with pN2 or higher nodal status, regardless of immunohistological subtypes, tend to have a higher percentage of CD44⁺CD24^{lo} CSC within the Aldefluor⁺ epithelial cell population when compared to patients with pN0 or pN1 nodal status (Table 4). This finding is unprecedented and provocative given the strong correlation between nodal status and outcome.

In this study, patients receiving NACT had a significantly higher median percentage of Aldefluor⁺ epithelial cells than untreated patients (55.5% vs. 43.2%, $P=0.008$), but no significant difference in CD44⁺CD24^{lo} CSCs. We highlight that these were not paired samples and as such comparisons are subject to biases between any two unrelated cohorts. In this case, the NACT group had a significantly higher proportion of patients with N2 or higher nodal status or with HER-2+ primary tumors. It is therefore possible that the higher % of Aldefluor⁺ epithelial cells seen in treated patients in these data reflects worse prognostic features or that while in some patients NACT is effective in reducing tumor burden, one may expect a concomitant enrichment of tumor cells with the stem cell phenotype in other patients (ALDH activity). Achieving pCR did not correlate with the percentages of Aldefluor⁺ epithelial cells nor with the proportion of CD44⁺CD24^{lo} CSC in patients who received NACT, however these are based on small numbers of patients. No prior studies have correlated putative CSC markers in the bone marrow to NACT, however several studies on primary tumor tissue suggest a relationship between CSCs and NACT. Tanei et al. (2009) observed similar results with an increase in ALDH1 expressing cells in the post chemotherapy primary samples, but no increase in CD44⁺CD24^{lo} cell population (19), while Li et al reported NACT increased the percentage of CD44⁺CD24^{lo} cells in PBC patients (20). In contrast to these studies, a recent comparative analysis of ALDH1 expression in tumor cells by Resetskova et al (2010) did not show a significant increase in the ALDH1 expressing population in the post chemotherapy samples (21). Nevertheless, in the bone marrow, the persistence of epithelial cells with ALDH activity following NACT implies resilience of these cells to chemotherapy which is concerning given the relevance of this phenotype to cancer-initiating properties. While the presence of Aldefluor⁺ epithelial cells in PBC patients who received NACT may select appropriate cohorts for future stem cell targeted therapy trials, the small sample size and lack of paired samples in the current study prohibit definitive conclusions regarding NACT.

It remains to be determined if putative CSCs in the bone marrow predict for distant metastases, a critical correlation, particularly among patients for whom standard clinical risk factors appear favorable. The small number of events (three patients have had relapse of disease) precludes this analysis here. While the correlation of putative CSCs in bone marrow with high risk features is important and consistent with the CSC hypothesis, patients with advanced disease and poor prognostic features can already be selected for additional

adjuvant therapies to eradicate occult disease. Finding a biomarker to select the small percentage of patients in the low risk cohort who will eventually relapse without further treatment is a major challenge. Rigorously testing a stem cell biomarker is particularly difficult as functional stem cell assays are needed to definitively prove the biomarker is a meaningful stem cell marker, and obtaining sufficient live cells from patients for functional studies is often not feasible. This is indeed a significant limitation of the work described here. Attempts to characterize functional endpoints from the material gained from the bone marrow were unsuccessful due to the limited material obtained. Herein, we report that the proportion of CD44⁺CD24^{lo} CSC in a subset of patients considered low risk is higher than that of HD controls. Consistent with these results, in the only previous study of cancer stem cell markers in the bone marrow of patients with early stage disease, Balic et al employed spectral imaging in conjunction with double marker immunohistochemistry to examine the simultaneous expression of CD44 and CD24 on cytokeratin-positive DTCs in the bone marrow of 50 patients with early stage breast cancer and detected CD44⁺CD24^{lo} cells in all cytokeratin positive BM samples with a median prevalence of 66% (11). It is tempting to speculate, but indeed remains unproven, that low risk patients that harbor putative CSCs in the bone marrow may be at higher risk for relapse.

A significant concern related to studies looking at DTCs or stem cell markers in the bone marrow is that, thus far, these studies appear to select a larger group of patients than are expected to relapse based on clinical data. In the Balic study (11), DTC selection with cytokeratin followed by stem cell marker assessment were selected in a small subset of patients and found the CSC in the majority of samples, consistent with our data. However, using a differentiation marker such as cytokeratin may increase the false negative rate by not detecting cancer stem cells lacking differentiation markers. Omitting cytokeratin as done herein leads to detection of putative CSCs in a larger population of patients even if a threshold for positivity is set well above that observed in HD controls. While this seems to refute the hypothesis or suggests this approach is not sufficiently specific, it is analogous to the accepted correlation between axillary metastases and clinical outcomes. Two randomized trials have clearly demonstrated a significant proportion of patients with pathologically demonstrable metastases in the axilla not subjected to local therapy will not experience an axillary recurrence (22,23). These data underscore how much is unknown about cancer cell biology and quiescence and demonstrate the danger of rejecting data that is inconsistent with our beliefs instead of challenging our beliefs in the face of new data.

In conclusion, we demonstrated that the CD326⁺CD45^{dim} epithelial cell population of PBC patients contained a subset of CD44⁺CD24^{lo} CSC. Clinicopathological characteristics such as TN tumor and higher nodal status (pN2 or higher) are factors that favored the presence of a higher percentage of CSC than found in healthy donors consistent with the hypothesis that these cells mediate recurrence. The markers examined are clearly present in the bone marrow of healthy donors at low levels and we were unable to determine if this is a true positive or false positive finding given the limitations of the current study. Further work is clearly needed to expand the study of healthy donors. Without feasible functional stem cells assays the correlation of biomarker studies to long-term clinical follow up is critical, and without them these findings are appropriately considered preliminary and hypothesis-generating. Nevertheless, they are consistent with the cancer stem cell hypothesis and lend credence to the hypothesis that current NACT regimens may not be effective in eliminating epithelial cells with stem cell phenotype. With further study we may well find that novel targeted therapy is required to eliminate CSC in some breast cancer patients.

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Figure 1.

Enumeration of CD44⁺ CD24^{lo} CSC within the Aldefluor⁺ epithelial (CD326⁺CD45^{dim}) cell population. BM mononuclear cells were analyzed for the ALDH activity using the Aldefluor® assay kit (Fig 1b). The percentage of epithelial (CD326⁺ CD45^{dim}) cells were calculated within the Aldefluor⁺ population (Fig. 1c) and the percentage of CD44⁺CD24^{lo} CSC were calculated within the Aldefluor⁺ epithelial cell population (Fig. 1d).

Figure 2.

Parentages of Aldefluor⁺ epithelial cells (a, c, e) and CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cells (b, d, f) in subsets of patients. While patients with different clinicopathological characteristics have similar percentages of Aldefluor⁺ epithelial cells (Fig. 2a), patients with TN tumors have a significantly higher median percentage of CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cells (Fig. 2d) than patients with other tumor subtypes. In addition, patients with pN2 or higher nodal status have a significantly higher median percentage of Aldefluor⁺ epithelial cells (Fig. 2e) and CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cells (Fig. 2f) than patients with pN1 or pN0 status.

Table 1

Characteristics of patients

Characteristics	Number	%
Median age (min, max)	52 (27, 77)	
Ethnicity		
Caucasian	52	78.8
Hispanic	7	10.6
Black	5	7.6
Asian	2	3.0
Tumor Markers		
ER	49	74.2
PR	36	54.5
HER2 (IHC 3+ or FISH)	11	16.7
Triple negative	10	15.2
Tumor grade		
Grade 1	8	12.1
Grade 2	28	42.4
Grade 3	30	45.5
Tumor size		
T1	32	48.5
T2	20	30.3
T3	6	9.1
T4	8	12.1
Lymph node status		
N0	36	54.5
N1	15	22.7
N2	3	4.5
N3	12	18.2
Neoadjuvant chemotherapy (NACT)	30	45.5
pCR after NACT	10	15.2

Table 2

The percentage of Aldefluor⁺ CD326⁺ epithelial cells and CSC of patients with different immunohistologic tumor subtypes

N=66	Median %	Mean %	S.E.M.
		Aldefluor ⁺	1.18	1.40	0.11
		CD326 ⁺ CD45 ^{dim}	47.0	48.6	2.21
		CD44 ⁺ CD24 ^{lo}	39.2	43.0	3.59
Tumor Histological Subtypes		% Aldefluor ⁺	% CD326 ⁺ CD45 ^{dim}	% CD44 ⁺ CD24 ^{lo}	
HR ⁺ HER2 ⁻	N	45	45	45	
	Median	1.25	46.90	36.10	
	Mean	1.37	46.61	40.39	
	Std. Error of Mean	0.13	2.71	4.36	
HR ⁺ HER2 ⁺	N	4	4	4	
	Median	1.07	61.15	21.49	
	Mean	1.48	61.65	33.64	
	Std. Error of Mean	0.47	7.51	18.74	
HR ⁻ HER2 ⁺	N	7	7	7	
	Median	.95	55.40	30.80	
	Mean	1.24	52.27	43.61	
	Std. Error of Mean	0.30	7.66	14.41	
Triple negative	N	10	10	10	
	Median	1.09	54.55	66.85	
	Mean	1.46	55.21	64.26	
	Std. Error of Mean	0.42	5.34	6.03	
HD	N	3	3	3	
	Median	2.39	37.30	19.60	
	Mean	1.98	30.47	22.73	
	Std. Error of Mean	0.58	7.85	6.43	

HR⁺ = hormone receptor⁺ (ER⁺ and/or PR⁺); HER2⁺ = HER2/neu amplified

Table 3

Relationship between clinicopathological characteristics and percentage of Aldefluor⁺ epithelial cell population.

	Aldefluor ⁺ epithelial cells > 39.3% [*]		
	No	Yes	
Variable	N (%)	N (%)	P-values [#]
Lymph node status			
pN0	10 (27.8%)	26 (72.2%)	0.219
pN1-3	5 (16.7%)	25 (83.3%)	
Lymph node status			
pN0-1	13 (25.5.0%)	38 (74.5%)	0.270
pN2-3	2 (13.3%)	13 (86.7%)	
Tumor size			
pT1	9 (28.1%)	23 (71.9%)	0.236
pT2-4	6 (17.6%)	28 (82.4%)	
High tumor grade			
3	3 (10.0%)	27 (90.0%)	0.023
1-2	12 (33.3%)	24 (66.7%)	
Estrogen receptor			
Negative	3 (17.6%)	14 (82.4%)	0.416
Positive	12 (24.5%)	37 (75.5%)	
Progesterone receptor			
Normal	6 (20.0%)	24 (80.0%)	0.428
Amplified	9 (25.0%)	27 (75.0%)	
HER2 status			
Normal	13 (23.6%)	42 (76.4%)	0.262
Amplified	1 (9.1%)	10 (90.9%)	
Triple receptor negative			
No	13 (23.2%)	43 (76.8%)	0.319
Yes	1 (10.0%)	9 (90.0%)	
IHC subtypes			
HR+/HER2−	12 (26.7%)	33 (73.3%)	0.352
HR+/HER2+	0 (0.0%)	4 (100%)	
HR−/HER2+	1 (14.3%)	6 (85.7%)	
Triple receptor negative	1 (10.0%)	9 (90.0%)	
Neoadjuvant therapy			
No	11 (30.6%)	25 (69.4%)	0.039
Yes	3 (10.0%)	27 (90.0%)	
pCR			
No	2 (10.0%)	18 (90.0%)	0.719
Yes	1 (10.0%)	9 (90.0%)	

* The highest value of the HD

Fisher Exact test

Table 4

Relationship between clinicopathological characteristics and percentage of CD44⁺CD24^{lo} CSC within Aldefluor⁺ epithelial cell population.

	CD44 ⁺ CD24 ^{lo} CSC > 35.1% [*]		
Variable	No N (%)	Yes N (%)	P-values [#]
Lymph node status			
pN0	16 (44.4%)	20 (55.6%)	0.563
pN1-3	13 (43.3%)	17 (56.7%)	
Lymph node status			
pN0-1	26 (51.0%)	25 (49.0%)	0.031
pN2-3	3 (20.0%)	12 (80.0%)	
Tumor size			
pT1	13 (40.6%)	19 (59.4%)	0.391
pT2-4	16 (47.1%)	18 (52.9%)	
High tumor grade			
1–2	17 (47.2%)	19 (52.7%)	0.368
3	12 (40.0%)	18 (60.0%)	
Estrogen receptor			
Negative	5 (29.4%)	12 (70.6%)	0.132
Positive	24 (49.0%)	25 (51.0%)	
Progesterone receptor			
Negative	11 (36.7%)	19 (63.3%)	0.201
Positive	18 (50.0%)	18 (50.0%)	
HER-2 status			
Normal	22 (40.7%)	32 (59.3%)	0.215
Amplified	7 (58.3%)	5 (41.7%)	
Triple receptor negative			
No	28 (50.0%)	28 (50.0%)	0.019
Yes	1 (10.0%)	9 (90.0%)	
IHC subtypes			
HR+/HER2–	22 (48.9%)	23 (51.1%)	0.114
HR+/HER2+	3 (60.0%)	2 (40.0%)	
HR–/HER2+	4 (57.1%)	3 (42.9%)	
Triple receptor negative	1 (10.0%)	9 (90.0%)	
NACT			
No	16 (44.4%)	20 (55.6%)	0.563
Yes	13 (43.3%)	17 (56.7%)	
pCR			
No	9 (45.0%)	11 (55.0%)	0.554
Yes	4 (40.0%)	6 (60.0%)	

^{*}The highest value of the HD

Fisher Exact test