Breast Cancer Stem Cell Identified By Aldehyde Dehydrogense-1 And Their Association With Pathological Response In Breast Cancer Patients After Treatment With Neoadjuvant Chemotherapy

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Abstract

Background: Aldehyde dehydrogenase 1 (ALDH1) is a breast cancer stem cell (BCSC) marker related to clinical outcomes in breast cancer and have been proposed to chemo-resistance. The aim of this study was to analyze the breast cancer stem cells identified by ALDH1 and their association with clinico-pathological characteristics and outcomes after NAC.

Patients and Methods:

This is a prospective study conducted at medical oncology out-patient clinics of the National Cancer Institute, Cairo University (The **NCI's,** Tagamoa Al Awal branch) between January 2018 and June 2018. Women with locally advanced breast cancer were assigned to receive four cycles of anthracycline-based chemotherapy, followed by four cycles of taxane therapy with or without tarstuzumab. Tumor specimens were collected at baseline, and then at surgical resection. Cancer Stem Cells were determined by positive expression of ALDH1 by immunophentyping and correlated with pathological response after NAC, rate of breast conserving surgery, and disease free survival.

Results:

Eighty-one female patients were enrolled between January 2018 and June 2018. Mean age of the patients at time of diagnosis was 45.8 ± 9.1 years. Main pathologic subtype was duct carcinoma. Median follow up was 39.2 months.

There was a positive association between BCSCs identified by ALDH1 expression and pathological response (p value = **.026),** and BCS (p value = .037**).** Also there was association between ALDH1 expression and ER negativity (p value = .016). No significant association between ALDH1 and DFS (p value = 0.839). HTIL were associated with ER negativity (p value = .006), high tumor proliferation marker ki67 (p value = .009), and pCR (p value = .001).

Conclusions:

The results of this study may indicate a role for BCSCs identified by ALDH1 expression in chemo-responsiveness, rather than chemo-resistance, after NAC in breast cancer patients. Further research remains necessary to confirm this result

Keywords: Breast Cancer Stem Cells, BCSCs; Aldehyde dehydrogenase 1, ALDH1; Neo-adjuvant chemotherapy, NAC

Introduction

In women all around the world, breast cancer is the most frequent kind of cancer. Vitally important advancements in breast cancer prevention, early diagnosis, and treatment have led to dramatically lower mortality rates. Unfortunately, breast cancer patients still face an unacceptablely high mortality rate due to high rates of incidence and drug resistance, which leads to cancer relapse and metastasis.**(1)**.

Multimodal treatment that accounts for molecular subtype and regional tumour load is preferred. Since most patients present at an early stage of disease, surgical resection is frequently the first line of defence. However, not every patient will benefit most from having surgery as their first line of defence, even if their disease is caught early. To make previously inoperable or locally advanced breast cancers treatable, neoadjuvant chemotherapy (NAC) was developed. The use of NAC was extended to encompass individuals with operable illness when the efficacy of adjuvant chemotherapy in node-positive (and later node-negative) breast cancers was proven.**(2).**

In patients who have reacted well to NAC, the illness in the breast and axilla can be downstaged, increasing the rate of breast conservation and decreasing the need for complete axillary dissections.**(3)**.

Breast cancer relapses and metastasis are thought to be caused by a small population of highly tumorigenic cancer cells within the tumour bulk, generally referred to as breast cancer stem cells (BCSCs). The metastatic progression must be stopped, and it may be possible to do so if BCSC-targeting medicines can be improved. However, there is a paucity of particular biomarkers for BCSCs, and most clinical techniques are developed for generally changed BCSCs signalling pathways, making the design of effective and specific BCSC-targeting medicines difficult.**(1)**.

We aimed at this work to analyze the breast cancer stem cells identified by ALDH1 and their association with clinico-pathological characteristics and outcomes after NAC.

Subjects and Methods:

In the present study, we conducted a prospective trial at medical oncology out-patient clinics of the National Cancer Institute, Cairo University (the NCI's Tagamoa Al Awal branch) between January 2018 and June 2018 to evaluate the impact of ALDH1 positive cancer stem cells as a prognostic factor for pathological response in breast cancer after neoadjuvant chemotherapy. This was done through a correlation between breast cancer stem cells and pathological response.

Inclusion Criteria:

Written informed consent, Female patients aged 18 years or over, Histologically confirmed invasive carcinoma of the breast, Women with locally advanced breast cancer (any T, any N, M0), Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, Adequate hematological, renal, hepatic and cardiac function, Non metastatic breast cancer patients and No history of secondary malignancies.

The exclusion criteria include:

Patients who meet any of the following criteria were excluded from study entry: Male patients, Metastatic disease, Patients unfit for surgery, Medical comorbidities or organ dysfunction precluding chemotherapy administration, and Concurrent participation in any other investigational/experimental drug trial.

Before starting a treatment, patients underwent an evaluation by:

Careful history taking:

1. Age, menopausal status, family history.
2. Symptoms of metastases.
3. History of other diseases and relevant medical conditions.

Clinical examination:

1. For assessment size of tumor, axillary and supraclavicular involvement
2. For assessment performance status and co-morbidities

Standard studies:

3. Including bilateral breast ultrasound and mammography, chest, abdomen images, bone scan, routine lab and echocardiography.
4. Others: including MRI and PET/CT according to the case clinical situation

Patients were offered an anthracycline based chemotherapy regimen for 4 cycles with clinical assessment following each cycle then they were offered another 4 cycles of taxane-based regimen (either Taxan 175 mg/m² every 3 weeks or Taxan 80 mg/m² every week) as neoadjuvant chemotherapy. Patients with Her2/neu score 3 (IHC) or positive (FISH) were offered trastuzumab in neoadjuvant chemotherapy with taxan. Surgery had been done after completion of the chemotherapy course.

Specimen collection:

Tumor specimens were obtained from each patient at two different time points. The first ultrasound-guided core biopsy was taken at baseline, before any chemotherapy. The second sample was obtained from the final surgical excision sample.

Histopathologic assessment of cases:

Hematoxylin and Eosin stained slides were prepared from the biopsy and resection specimen tumor blocks of each studied case. For each slide, the pathologist revised the adequacy of the tissue material and tumor classification according to the WHO classification of breast tumors **(4).**

All pathological materials were reviewed blindly for the tumor grade, the tumor-infiltrating lymphocytes (TILs), and the pathological response to neoadjuvant therapy in resection tumor slides. For TILs evaluation, all mononuclear cells (including lymphocytes and plasma cells) in the stromal compartment within the borders of the invasive tumor were evaluated and reported as a percentage value using 30% as the cutoff for high (HTILs) or low (LTILs). TILs outside of the tumor border, around DCIS and normal breast tissue, as well as in areas of necrosis, if any, were not included in the scoring **(5).** Complete pathological response to neoadjuvant chemotherapy (cPR) was defined as a grade 5 Miller-Payne scoring system: no malignant cells identifiable in sections from the site of the tumor; only vascular fibroelastotic stroma remains often containing macrophages. However, ductal carcinoma in situ (DCIS) may be present. Grades 1–4 were categorized as a partial pathological response (pPR) **(6).**

Immunohistochemical assessment of cases:

Each tumor specimen was stained with standard antibodies for the expression of ER, PR, HER2, and Ki67 in accordance with local clinical practice. HER2 gene amplification was determined by an in situ hybridization technique using the INFORM HER2 Dual ISH DNA Probe (VentanaW Medical System, Tucson, AZ, USA). Scoring for HER2 was performed as per American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines **(7)**.

We divided ER+ tumors into either luminal A (Her2− / Ki67low) or luminal B (HER2+ or Ki67 high) subtypes, as previously described. All ER− and HER2+ were classified as HER2 subtypes, while ER− /PR− /HER2− was classified as a triple-negative subtype **(8).**

All immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded tissues. From each case, 2 unstained sections at 4 microns thickness were prepared on positively charged slides for the immunohistochemical assessment of ALDH1 on biopsy and tumor resection tissues. Positive control slides were included within each batch of slides. Immunostaining was done for all cases using BenchMark Ultra (Ventana) autostainer and the following steps occurred automatically:

- Deparaffinization by using the EZ-prep solution.
- Cell conditioning (standard cell conditioning CC1) for 80 minutes.
- Antigen retrieval using reaction buffer (PH 7.4-7.8).
- Application of 100µ of the diluted (1:500) mouse monoclonal antibody (IgG_{2B} Clone # 703410 R&D systems USA) incubated for 40 minutes at room temperature.
- Application of Diaminobenzidine (DAB) as a chromogen (NexES iView DAB Detection Kit).
- Counterstaining with Hematoxylin II for 8 minutes.
- Post counter staining with bluing reagent for 4 minutes.

Slides were extracted and arranged in racks. Slides were washed in tap water and soap for 5 minutes and then dehydrated in the ascending grades of alcohol for 5 minutes in each container. Slides were cleared in Xylene, and then cover slips were applied.

Semiquantitative assessment of immunostaining was performed using Olympus light microscope (CX 31). First, the average positivity was estimated by reviewing the whole slide and by assessing at least 10 high power fields. In a second step, the slides were investigated for highly proliferative clones. The percentage of positive cells in these clones was scored. Tumor sections were counted as positive if cytoplasmic staining was clearly observed in more than 10% of tumor cells **(9).**

Statistical Analysis:

Statistical analysis was done using IBM SPSS® Statistics version 26 (IBM® Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Pearson's Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test).

Mc-Nemar test was used to compare the epithelial and stromal ALDH in the biopsy and after neoadjuvant chemotherapy.

Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. All tests were two-tailed. A p-value < 0.05 was considered significant.

Results

During the study period, 90 patients were recruited into the study diagnosed with locally advanced BC. Tumor core biopsies were successfully obtained in all patients at baseline. Baseline core biopsies from nine patients contained inadequate tissue for ALDH1 staining and were excluded, leaving a total of 81 informative subjects.

All our patients were female with the mean age of our included patients was 45.8 ± 9.1 years. Among the 81 patients, 36 (44.4%) patients aged ≤ 45 years, and most of our patients, 73 cases (90.1%) were invasive ductal carcinoma and 7 cases (7.4%) were invasive lobular carcinoma. Estrogen Receptor (ER) positive, PR-positive, and HER2neu positive tumors were observed in 53 (65.4%), 55 (67.9%), and 25 patients (30.9%), respectively. Twenty one (25.9%) underwent breast-conserving surgery, while 60 patients (74.1%) were subjected to MRM.

Aldehyde dehydrogenase 1 positive expression (ALDH1) in tumor was observed in 46 patients (56.8%), while ALDH1 positivity in stroma was observed in 51 patients (63%). Tumor-infiltrating lymphocytes were observed in fifty one patients (63%). Complete pathological response was observed in 27 patients (33.3%), while 54 (66.7%) had residual tumors. There were 11 relapses (13.6%) and no death occurred among the patients. Detailed patient demographics and tumor characteristics of all 81 eligible patients are shown in Table 1.

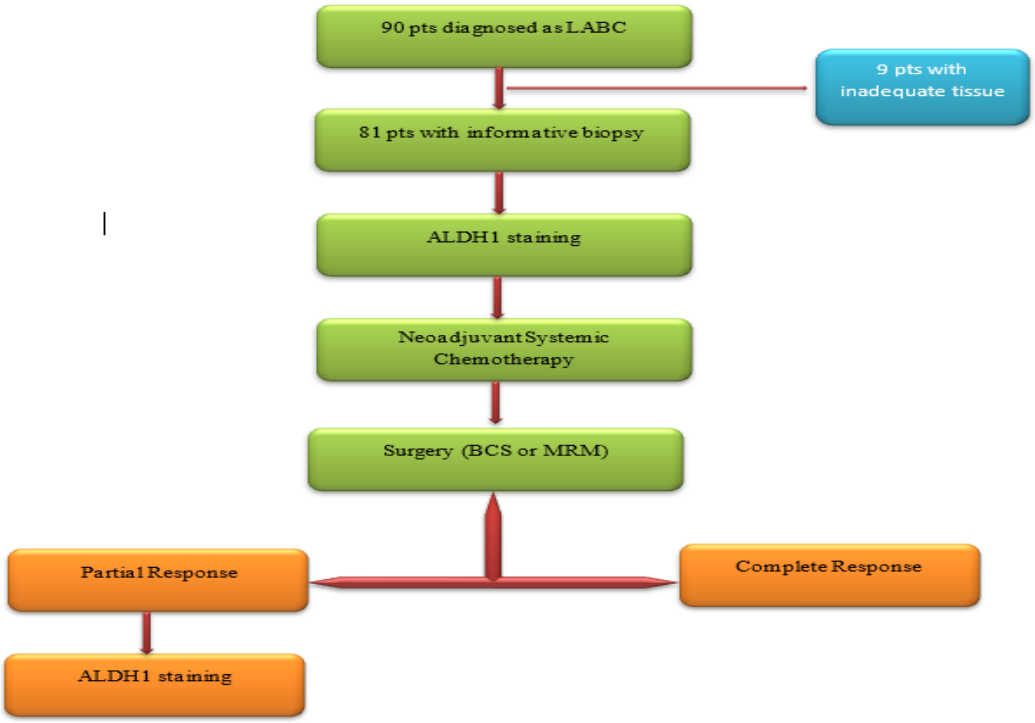

Figure 1: flowchart illustrated the process of the current study

Table 1: The clinico-pathological characteristics of the patients:

Variables	n = 81
Age	45.8 ± 9.1 years
Family history - Positive - Negative	 15(18.5%) 66(81.5%)
Menopausal status - Premenopausal - Postmenopausal	 56(69.1%) 25(30.9%)
Histological type - IDC - ILC - Others	 73(90.1%) 7(7.4%) 2(2.5%)
Grade - Low(I and II) - High (III)	 62(76.5%) 19(23.5%)
ER - Positive - Negative	 53(65.4%) 28(39.6)
PR - Positive - Negative	 55(67.9%) 26(32.1%)
HER2 - Positive - Negative	 25(30.9%) 56(69.1%)
Ki67(n=60) - >14% - ≤14%	 37(61.7%) 23(38.3%)

Biological type	
- Luminal A	20(24.7%)
- Luminal B	39(48.1%)
- TNBC	9(11.1%)
- HER2+	13(16.1%)
Tumor size	
- ≤5 cm	38(46.9%)
- >5 cm	43(53.1%)
Nodal status	
- Positive	72(88.9%)
- Negative	9(11.1%)
Site	
- Right	35(43.2%)
- Left	46(56.8%)
Surgery	
- MRM	60(74.1%)
- BBCS	21(25.9%)
Pathological response	
- Complete response	27(33.3%)
- Partial response	54(66.7%)
Relapse	
- Occurred	11(13.6%)
- Not occurred	70(86.4%)
ALDH expression	
- Positive	46(56.8%)
- Negative	35(43.2%)
TIL	
- LTIL	51(63%)
- HTIL	30(37%)

A)ALDH: aldehyde dehydrogenase, B)TIL: Tumor infiltrating lymphocytes, C)LTIL: low tumor infiltrating lymphocytes, and D)HTIL: high tumor infiltrating lymphocytes.

Tumor ALDH1positive expression and clinicopathological characteristics:

Aldehyde dehydrogenase 1 positive expression (ALDH1) in tumors was observed in 46 patients (56.8%), while 35 patients (43.2%) were negative for ALDH1 expression.

Pathological complete response was achieved in 27 patients (33.3%), while 54 (66.7%) had residual tumors. Among 27 patients twenty patients (74.1%) observed positive expression to ALDH1, while 7 patients (25.9%) showed negative expression to ALDH1.

A significant association was observed between ALDH1 positive expression with ER status (p value =.016), type of surgery (p-value =.037), and pathological response (p-value= .026).

There were no significant differences in age, family history, menopausal status, grade of tumor, size of tumor, and biological type.

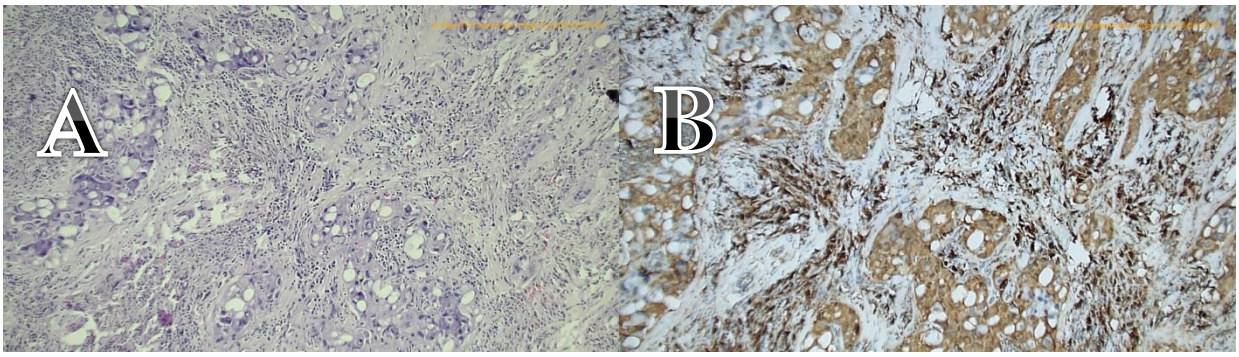

Figure 2: Representative immunohisto-chemical staining intensity of ALDH1 for patients with breast cancer: A) ALDH1 negative control. B) ALDH1 positive immunohistochemistry

Table 2: Association of tumor ALDH1 expression with clinicopathological characteristics:

Variables	ALDH1(+) (n=46)	ALDH1(-) (n=35)	p Value
Age	46.5±8.8	45.0±9.6(yrs)	.383
Family history			
- Positive	7(46.7%)	8(53.3%)	.381
- Negative	39(59.1%)	27(40.9%)	
Menopausal status			
- Premenopausal	31(55.4%)	25(44.6%)	.697
- Postmenopausal	15(60.0%)	10(40.0%)	
Grade			
- Low(I and II)	35(56.5%)	27(43.5%)	.912
- High (III)	11(57.9%)	8(42.1%)	
ER			
- Positive	25(47.2%)	28(52.8%)	**.016**
- Negative	21(75.0%)	7(25.0%)	
PR			
- Positive	29(52.7%)	26(74.3%)	.283
- Negative	17(65.4%)	9(34.6%)	
Her2			
- Positive	16(64.0%)	9(36.0%)	.381
- Negative	30(53.6%)	26(46.7%)	
Ki67(n=60)			
- >14%	21(56.8%)	16(43.2%)	.098
- ≤14%	8(34.8%)	15(65.2%)	
Biological type			
- Luminal A	8(40.0%)	12(60.0%)	.249
- Luminal B	23(59.0%)	16(41.0%)	
- HER2+	7(77.2%)	2(22.2%)	
- TNBC	8(61.5%)	5(38.5%)	
Tumor size			
- ≤5 cm	22(57.9%)	16(42.1%)	.850
- >5 cm	24(55.8%)	19(44.2%)	
Nodal status			
- Positive	39(58.2%)	28(41.8%)	.573
- Negative	7(50.0%)	7(50.0%)	
Site			
- Right	20(57.1%)	15(42.9%)	.955
- Left	26(56.5%)	20(43.5%)	
TIL			
- HTIL	17(56.7%)	13(43.3%)	.986
- LTIL	29(56.9%)	22(43.1%)	
Surgery			
- MRM	30(50.0%)	30(50.0%)	**.037**
- BCS	16(76.2%)	5(23.8%)	
Pathological response:			**.026**
- CR	20(74.0%)	7(26.0%)	
- PR	26(46.2%)	28(53.8%)	

Sequential changes in ALDH1 expression following NAC

At the time of surgery, twenty-seven (27) patients achieved a complete pathological response, and 54 had residual disease. From 54 patients with residual tumors, only 47 had informative ALDH1 staining as the other 7 patients with residual tumors had very low viable cells (<10%) and cannot be examined for ALDH1. Among 47 tumors, 27 tumors (57.4% %) were positive for ALDH1. From those 27 tumors, eight tumors were ALDH1 negative then they became positive after NAC. Twenty tumors (42.6%) were negative for ALDH1; six of those were positive and then became negative for ALDH1.

Aldehyde dehydrogenase 1 staining was observed in the tumor stroma. At biopsy, stromal ALDH1 expression was positive in 51 patients (63%), while 30 patients (37%) did not show stromal expression.

There was no significant association between stromal ALDH1 and any clinic-pathological characteristics.

Table 3: Association between stromal ALDH1 expression and clinico-pathological characteristics:

Variables	sALDH1+ (n=51)	sALDH1- (n=30)	p Value
Age	46.8±8.4(yrs)	44.3±10.2(yrs)	.147
Family history			
- Positive	9(60.0%)	6(40.0%)	.792
- Negative	42(63.6%)	24(36.4%)	
Menopausal status			
- Premenopausal	32(57.1%)	24(42.9%)	.104
- Postmenopausal	19(76.0%)	6(24.0%)	
Grade			
- Low(I and II)	41(66.1%)	21(70.0%)	.286
- High (III)	10(19.6%)	9(30.0%)	
ER			
- Positive	36(70.6%)	17(33.9%)	.203
- Negative	15(53.6%)	13(46.4%)	
PR			
- Positive	38(69.1%)	17(30.9%)	.097
- Negative	13(50.0%)	13(50.0%)	
Her2			
- Positive	16(64.0%)	9(36.0%)	.897
- Negative	35(55.4%)	21(44.6%)	
Ki67(n=60)			
- >14%	25(67.6%)	12(32.4%)	.597
- ≤14%	14(60.9%)	9(39.1%)	
Biological type			
- Luminal A	14(70.0%)	6(30.0%)	.094
- Luminal B	28(71.8%)	11(29.2%)	
- HER2+	4(44.4%)	5(55.6%)	
- TNBC	5(38.5%)	8(61.5%)	
Tumor size			
- ≤5 cm	23(59.0%)	16(41.0%)	.669
- >5 cm	28(65.1%)	15(34.9%)	
Nodal status			
- Positive	43(64.2%)	24(35.8%)	.620
- Negative	8(57.1%)	6(42.9%)	
Site			
- Right	23(65.7%)	12(34.3%)	.655
- Left	28(60.9%)	18(39.1%)	
TIL			
- HTIL	23(76.7%)	7(23.3%)	**.050**
- LTIL	28(54.9%)	23(45.1%)	
Surgery			
- MRM	39(65.0%)	21(35.0%)	.521
- BCS	12(57.1%)	9(42.9%)	
Pathological response			
- pCR	15(55.6%)	12(44.4%)	.329
- Non-pCR	36(66.7%)	18(33.3%)	

Tumor infiltrating lymphocytes and Clinicopathological Characteristics:

Tumor infiltrating lymphocytes evaluated during our study. High TILs were related to pathological complete response (p= .001). Similarly, HTILs was related to ER negativity (p= .006), and also related to high KI67 proliferation rate (p = .009). There were no significant differences in age, menopausal status, grade, and biological type. Table 4 summarizes association between TILs and clinicopathological features.

Table 4: Association between TILs with clinico-pathological features:

Variables	LTIL (n=51)	HTIL (n=30)	p Value
Age	45.2± 9.8(yrs)	46.4±7.9(yrs)	.282
Family history			
- Positive	7(46.7%)	8(53.3%)	.148

- Negative	44(66.7%)	22(33.3%)	
Menopausal status			
- Premenopausal	35(62.5%)	21(37.5%)	.897
- Postmenopausal	16(64.0%)	9(36.0%)	
Grade			
- Low(I and II)	39(62.9%)	23(37.1%)	.984
- High (III)	12(63.2%)	7(36.8%)	
ER			
- Positive	39(73.5%)	14(26.5%)	**.006**
- Negative	12(42.9%)	16(57.1%)	
PR			
- Positive	36(65.5%)	19(34.5%)	.499
- Negative	15(57.7%)	11(42.3%)	
Her2			
- Positive	14(56.0%)	11(44.0%)	.386
- Negative	37(66.1%)	19(33.9%)	
Ki67(n=60)			
- >14%	20(54.1%)	17(45.9%)	**.009**
- ≤14%	20(87.0%)	3(13.0%)	
Biological type			
- Luminal A	16(80.0%)	4(20.0%)	.230
- Luminal B	24(61.5%)	15(38.5%)	
- HER2+	5(55.6%)	4(44.4%)	
- TNBC	6(46.2%)	7(53.8%)	
Tumor size			
- ≤5 cm	24(63.2%)	14(36.8%)	.973
- >5 cm	27(62.8%)	16(37.2%)	
Nodal status			
- Positive	43(64.2%)	24(35.8%)	.620
- Negative	8(57.1%)	6(42.9%)	
Site			
- Right	22(62.9%)	13(37.1%)	.986
- Left	29(63.0%)	17(37.0%)	
ALDH1			
- Positive	29(63.0%)	17(37.0%)	.986
- Negative	22(62.9%)	13(37.1%)	
Surgery			
- MRM	37(61.7%)	23(38.3%)	.683
- BCS	14(66.7%)	7(33.3%)	
Pathological response			
- pCR	10(37.0%)	17(63.0%)	**.001**
- Non-pCR	41(75.9%)	13(24.1%)	

Disease free Survival analysis:

The median follow-up period was 39.2 months (ranging from 32.9 to 43.1 months). During the period of the study, no patients died. The median time to relapse was 32.2 months, (ranging from 5.4 to 36.1 months). The cumulative disease-free survival at 2 years was 92.6 %, and at the end of the study was 85.7 %.

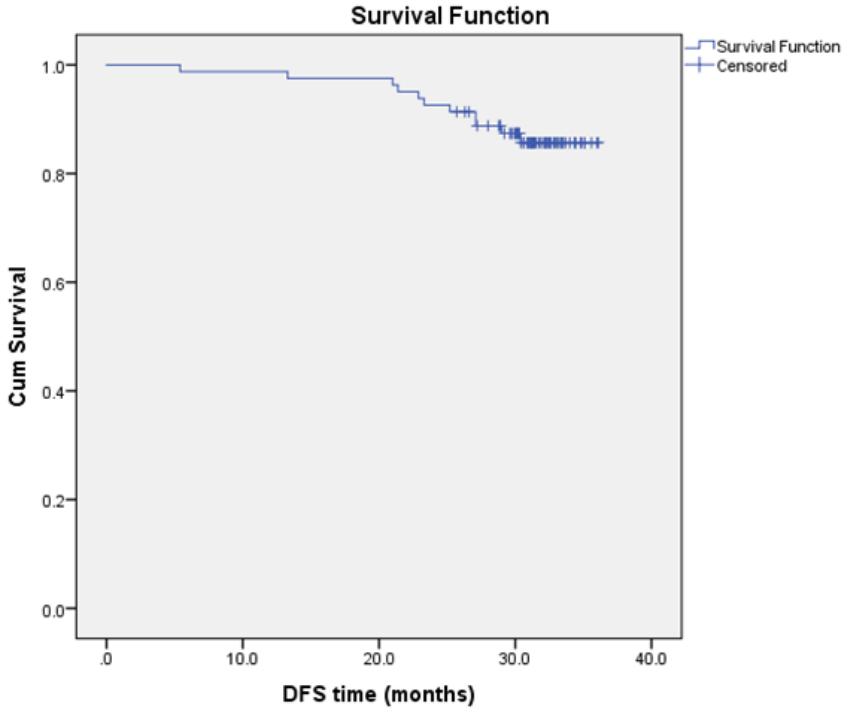

Figure 3: Disease free Survival analysis

Association of ALDH1 and Disease free survival:

The results showed that baseline tumor ALDH1 expression was not associated with DFS (p-value = 0.839). Also, there was no significant association between stromal ALDH1 expression and DFS (p-value = .179)

Table 5: Association of ALDH1 markers and Disease free survival:

	No.	No of events	Cumulative survival at 36 months (%)	p-value
Whole group	81	11	85.7 %	
Epithelial ALDH				
- Positive	46	6	86.9 %	
- Negative	35	5	83.0 %	.839
Stromal ALDH				
- Positive	51	5	89.6 %	
- Negative	30	6	78.9 %	.179

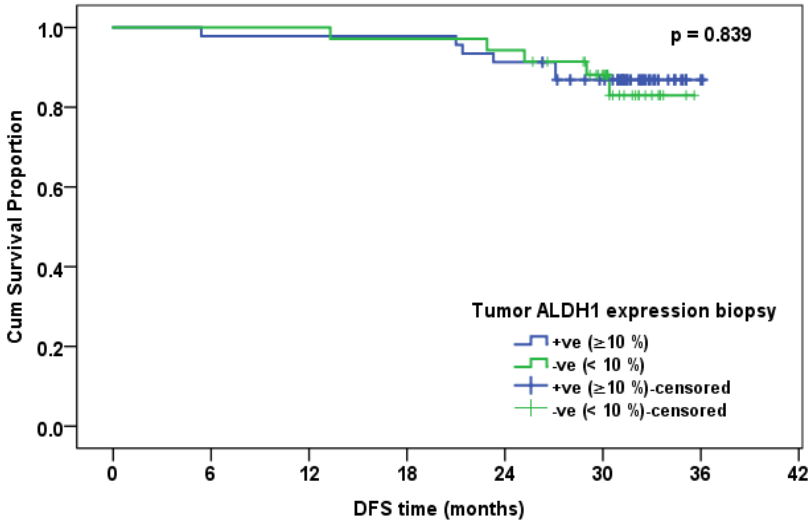

Figure 4: Association between DFS and tumor ALDH1

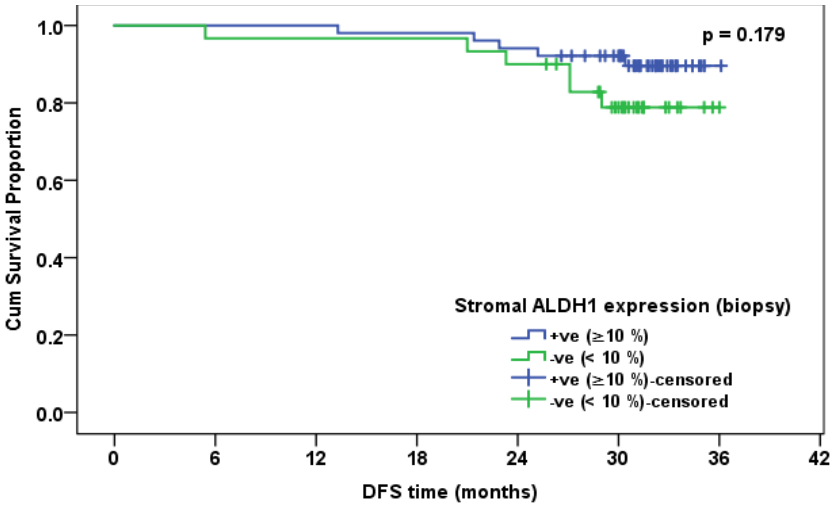

Figure 5: Association between DFS and Stromal ALDH1

Correlation of tumor infiltrating lymphocytes with DFS:

There was no significant correlation between tumor infiltrating lymphocytes and DFS. (p = .524)

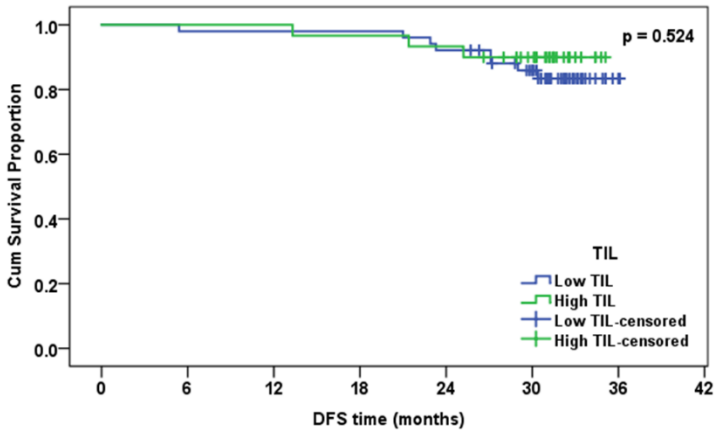

Figure 6: Association for DFS and TILs

Discussion

The metastatic progression must be stopped, and it may be possible to do so if BCSC-targeting medicines can be improved. However, there is a paucity of particular biomarkers for BCSCs, and most clinical techniques are developed for generally changed BCSCs signalling pathways, making the design of effective and specific BCSC-targeting medicines difficult.**(10).**

ALDH and treatment resistance in BCSC are closely connected. It can inhibit the metabolism of chemotherapy medications like cyclophosphamide, which is one of the causes. The high mitochondrial quality brought on by ALDH activity is another factor contributing to BCSCs' medication resistance.**(11).**

This study was performed for locally advanced breast cancer patients presented to Breast Hospital, first settlement, National Cancer Institute, Cairo University to determine the association between breast cancer stem cells identified by ALDH1 and pathological response in breast cancer patients after treatment with neoadjuvant chemotherapy.

We evaluated the association between breast cancer stem cells (BCSCs) identified by expression of ALDH1 and pathologic characteristics known as important for the clinical outcome, such as tumor size, nodal status, hormonal receptor status, HER2 status, Ki67 proliferation marker, biological type, histologic grade, type of surgery, and pathological response after neoadjuvant chemotherapy.

In our study population, among 81 breast tissue biopsies examined for BCSCs identified by ALDH1 expression in neoadjuvant sitting, 46 biopsies (56.8%) were positive for ALDH1. This was similar to the rate reported by a study performed by **Abdelaziz et al., (12).** which reported that the prevalence rate of BCSCs identified by ALDH1 expression in Egyptian patients was 58% **(12).**

López et al. had reported a slightly lower prevalence rate of BCSCs identified by ALDH1 expression (53%) **(13).**, while Zheng, et al. reported a higher rate (64%) (Zheng et al., 2014)**.**

The prevalence rate of BCSCs identified by ALDH1 was 21.3 % as reported by Kida et al., **(14).** while it was 47% as reported by Alamgeer et al. **(15).**

This variation in the rate of BCSCs identified by ALDH1 expression can be explained by the different clinical stages of the patients selected, the different antibodies used in studies, different cutoff values for ALDH1, and variations in methods of ALDH1 detection.

Regarding the association between tumor ALDH1 positive expression and clinicopathological features, we were able to demonstrate an association between tumor ALDH1 positive expression and ER negativity (p value= .016). This was in agreement with the results reported by Lee et al.,2018 which demonstrate that there was an association between ALDH1 (+) tumors and estrogen receptor negativity (p value= 0.026)**(16).** Also, Guan et al., 2021 reported a significant association between ALDH1 expression and ER negativity (P = 0.012) **(17).**

Two meta-analyses of 27 studies reported a significant association between tumor ALDH1 positive expression and ER negativity **(18,19**)**.**

In our study, there was no association between ALDH1 expression and age, menopausal status, histological grade of the tumor, progesterone receptor, HER2 status, Ki67 proliferation marker, biological type, tumor size, nodal status, site of the tumor, and tumor-infiltrating lymphocytes **(P > .05)**.

The association between the expression of ALDH1 and the clinicopathologic characteristics is controversial. A meta-analysis of 177 studies done by Liu et al, 2015 noticed a significant difference between ALDH1 expression with ER, PR status, and histological grade, but did not find a significant difference between ALDH1 expression and other clinicopathological features (**19)**. Another study performed by Neumeister et al. reported that ALDH1 expression was not associated with pathologic characteristics **(20).**

However, in other studies, the expression of ALDH1 was significantly associated with poor prognostic features such as high histologic grade, HER2 overexpression, and the absence of ER and PR expression **(12, 18).**

We investigated whether a correlation exists between ALDH1 expression and pathological response after neoadjuvant chemotherapy, our results showed a significant positive association between ALDH1-positive tumors and pathological response (**p value=.026**). Our results were in agreement with results reported by

López Flores et al., 2022 as they reported that positive ALDH1 expression achieved a higher pCR rate in comparison to negative ALDH1 carcinomas (60% vs. 37.1%; p = 0.048) **(13).** Also, Lee et al., reported the same positive correlation between ALDH1 positive and pathological response **(16).**

This result conflicted with most previous studies suggesting that ALDH1 expression was related to tumor aggressiveness and chemotherapy resistance. Pooled meta-analysis of 10 eligible studies including 1081 patients indicated an association between high ALDH1 expression and poor NAC responses (J. Li et al., 2018)**.** Also, Kida et al., showed that the pCR rate was significantly lower in patients with ALDH1-positive expression tumors **(14).**

The key to this difference might be in the BC subtypes included in our study. In our study, we included all BC subtypes presented in a non-metastatic locally advanced stage. Conventional neoadjuvant chemotherapy yielded a pCR in approximately 35–45% of patients with TNBC in 2020 **(21)**, also HER2 expression increases the positive ALDH breast cancer stem cells population which displays an increased expression of stem cell regulatory genes, increased invasion in vitro and tumorigenesis in animal models. Treatment with trastuzumab blocked this effect on sensitive cell lines but not on resistant ones. Furthermore, the clinical efficacy of trastuzumab may be related to its ability to target the cancer stem cell population in HER2-amplified tumors **(22).** In our series, all of the HER2-positive patients received trastuzumab as a part of their chemotherapy; this could be one of the reasons. Also, the low number of patients included in this analysis may be another reason for this difference.

As a result of a significantly good pathological response of our cases who showed ALDH1 positive expression to neoadjuvant chemotherapy, there was a positive significant positive correlation with breast-conserving surgery in our cases (**p value=.037**).

Regarding changes in ALDH1 expression after NAC, eight tumors were ALDH1 negative then they became positive after NAC and this increase the rate of ALDH1-positive expression after NAC tumors to 57.4% (27/47). Chatterjee et al. reported a significant increase in the rate of expression in ALDH1 post-NAC **(23).** Also, Klintman et al. had reported a significant increase in ALDH1 post-NAC **(24).**

The possible mechanism that can sustain and hence propagate the ALDH1 positive stem cell pool is believed to be the autocrine production of inflammatory cytokines, such as interleukin 6 (IL-6) and interleukin 8 (IL-8), secondary to chemotherapy-induced cellular apoptosis **(25).**

Cancer Stem Cells interact with their niche and in turn, are regulated by cells in the tumor microenvironment. These interactions involve inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8, which in turn activate Stat3/NF-κB pathways in both tumor and stromal cells. Activation of these pathways stimulates further cytokine production, generating positive feedback loops that in turn drive CSC self-renewal, and in turn increase the expression of ALDH1 **(22).** Interestingly, some initially ALDH1 positive cases also converted to ALDH1 negative after chemotherapy and, hence, achieved improvement in their long-term outcome. These results may support a rare phenomenon of 'phenotypic switching', which indicates that stem cell-like and non-stem cell-like populations in breast cancer may be plastic and interconvertible **(26)**.

Lee A. et al. reported that there was a more positive conversion of ALDH1 in the residual post-NAC group. Based on these results, they ascertained the positive conversion of ALDH1 could be a more important factor than the existence of ALDH1-positive tumors before NAC in chemotherapy resistance **(16).**

The aldehyde dehydrogenase 1 (ALDH1) expression in the breast is not restricted to the epithelial cells and has been noted in stromal fibroblasts. However, the significance of this finding has not been evaluated in many studies. In the present study, we correlate stromal ALDH with clinicopathological characteristics and there was no significant correlation between stromal ALDH1 and any clinicopathologaical characteristics. This was in agreement with results reported by many authors **(16; 23; and 27).**

The association between ALDH1 expression and tumor-infiltrating lymphocytes was reported in a lot of studies. Polónia et al. reported that was a significant association between tumor-infiltrating lymphocytes and ALDH1 (p= .014) **(28)**. In our study, we tried to improve this hypothesis but due to the small sample size and different cutoff values, this hypothesis could not improve in our study (p=.986). Our results were in agreement with the results reported by Ibrahim et al. **(29).**

Although the BC's inflammatory infiltrate has been studied for several decades with conflicting results, large cohorts have shown an association between the presence of tumor-infiltrating lymphocytes (TILs) with improved prognosis and better response to neoadjuvant chemotherapy, regardless of the absence of information on its specific immune cells **(30).**

Regarding our study, there was an association between tumor-infiltrating lymphocytes (TIL) and ER negativity (p=0.006), this is in agreement with a lot of studies **(31,32).** However, a non-significant association was detected between tumor-infiltrating lymphocytes and age, menopausal status, histological grade, HER2 status, biological type, tumor size, and nodal status (p-value ≥ .05). **López Flores et al,** reported a non-significant correlation between tumor-infiltrating lymphocytes and clinicopathological features **(13).**

Tumor-infiltrating lymphocytes and the pathological response were noticed in many studies. A meta-analysis of 29 published studies consisting of approximately 9145 participants reported that an increased proportion of TILs predicted a higher pCR rate for NAC in total breast cancer (pooled OR = 3.18, 95% CI, 2.55–3.97, *P* = 0.000) **(18)**.

The result of our study goes with the results of the previously mentioned meta-analysis. We noticed a significant association between complete pathological response and higher tumor infiltration lymphocytes (p= 0.001)**.**

In the survival analysis, Published data had shown increased tumor ALDH1 expression, associated with a higher frequency of relapse and poor overall survival **(15; 18).** However, other authors did not find any correlation with survival **(23).** We were not able to find an association between ALDH1 expression and disease-free survival, although previous studies showed that the expression of ALDH-1 was associated with poor prognosis **(14).**

Regarding tumor-infiltrating lymphocyte survival analysis, Shenasa et al. assessed the interaction of chemotherapy with different biomarkers such as TILs in BC. These authors reported the association of TILs with improved invasive DFS **(33).** We could not prove this hypothesis in our study and there was a non-significant association between TILs and DFS. This was similar to what was reported by López Flores et al. **(13).**

This discrepancy between the studies may be related to the difference in sample size, the difference in study populations, study types, variations in evaluation methods, and variations in tumor subtypes

Conclusion

Patients with Breast cancer stem cells identified by Aldehyde dehydrogenase-1 expression achieved a higher pCR rate and increased breast conserving surgery after neo-adjuvant chemotherapy, indicating the role of Breast cancer stem cells identified by Aldehyde dehydrogenase-1 expression in chemo-response than chemo-resistance. Further research that includes a larger number of research subjects remains necessary to confirm this result. Tumor response to NAC might not be determined by a single cell expression, but be determined by a harmony of cells that compose the tumor microenvironment.

Tumor-infiltrating lymphocytes may be considered a suitable marker for pathological response to neo-adjuvant chemotherapy. Breast cancer stem cells identified by Aldehyde dehydrogenase-1 implications in tumor response and survival in BC patients need to be investigated in further studies.

Conflicts of Interest: The authors declare no conflict of interest.

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