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The Prognostic Significance of ALDH-1 and SOX9 Expression in Early Breast Cancer

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Abstract

Background: Aldehyde dehydrogenase 1 (ALDH1) is an enzyme accountable for the detoxification of aldehydes. Sex-determining region Y-box 9 (SOX-9) plays a role in many biological and pathological processes. In this study, we aimed to evaluate the prognostic significance of ALDH1 and SOX9 expression in early breast cancer.

Method: The expression of ALDH1 and SOX-9 was evaluated through immunohistochemistry derived from 50 eligible patients with early breast cancer included in the current prospective cohort study.

Results: Positive expression of ALDH1 and SOX-9 were detected in 29 (58%) and 34 (68%) patients, respectively. The positive expressions of both markers were statistically significant associated with increasing the stage, lymph nodes metastasis, high Ki67 labeling index, and molecular subtypes ($P < 0.001$), along with with the biological markers; estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 over-expressions, and large tumor size ($P = 0.039$, $P = 0.022$, $P = 0.024$ and $P = 0.003$ for ALDH1 expression and $P = 0.012$, $P = 0.007$, $P = 0.004$, and $P = 0.002$ for SOX-9 expression, respectively). There is a significant positive association between the expression of ALDH1 and SOX-9, r (correlation coefficient) = +0.806 ($P < 0.001$). Local recurrence was associated with the positive expression of ALDH1 only ($P = 0.045$) and the disease progression was statistically significant and associated with the positive expression of both ALDH1 and SOX-9 ($P = 0.038$, $P = 0.023$, respectively). There was significant association of positive expression of SOX-9 with reduced 3-y disease-free survival ($P = 0.039$).

Conclusion: Positive expression of ALDH-1 and SOX9 were associated with aggressive histopathological features and poor outcome in early breast cancer and can be considered potential prognostic markers in this group of patients.

Keywords: Early breast cancer, Neoplastic stem cells, Aldehyde dehydrogenase 1, SOX9, Prognosis

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Introduction

Breast cancer (BC) is the most prevalent malignancy and the leading cause of cancer-related deaths in females worldwide.[1]

The strategy of adjuvant combined therapy has been found to be effective in decreasing BC recurrence risk within five years after diagnosis. However, the recurrence could occur in some patients regardless of taking adjuvant therapy. Therefore, accurate and reliable estimates of the risk of recurrence are essential to make the most accurate decisions.[2]

Cancer stem cells (CSCs) were found to have the ability for self-renewal and differentiation. CSCs were first detected in acute myeloid leukemia using specific cell surface markers.[3] Later, they were identified in various solid tumors. Aldehyde dehydrogenase 1 (ALDH1) was described as a marker of CSCs in BC, which have tumorigenic and metastatic potential to distant sites.[4] ALDH1 is an enzyme catalysing aldehydes to carboxylic acids.[5] ALDH1A1 isozyme oxidises retinaldehyde to retinoic acid, responsible for the regulation of the expression of the genes involved in tumour-initiating stem-like cells, tumour growth, and resistance to drugs.[6-7] ALDH1-expressing cells were found to be responsible for resistance to adjuvant chemotherapy and the aggressive behavior of malignant tumors.[2] On the other hand, in several other studies, ALDH1 expression was found to be associated with favorable outcomes. Thus, the value of ALDH1

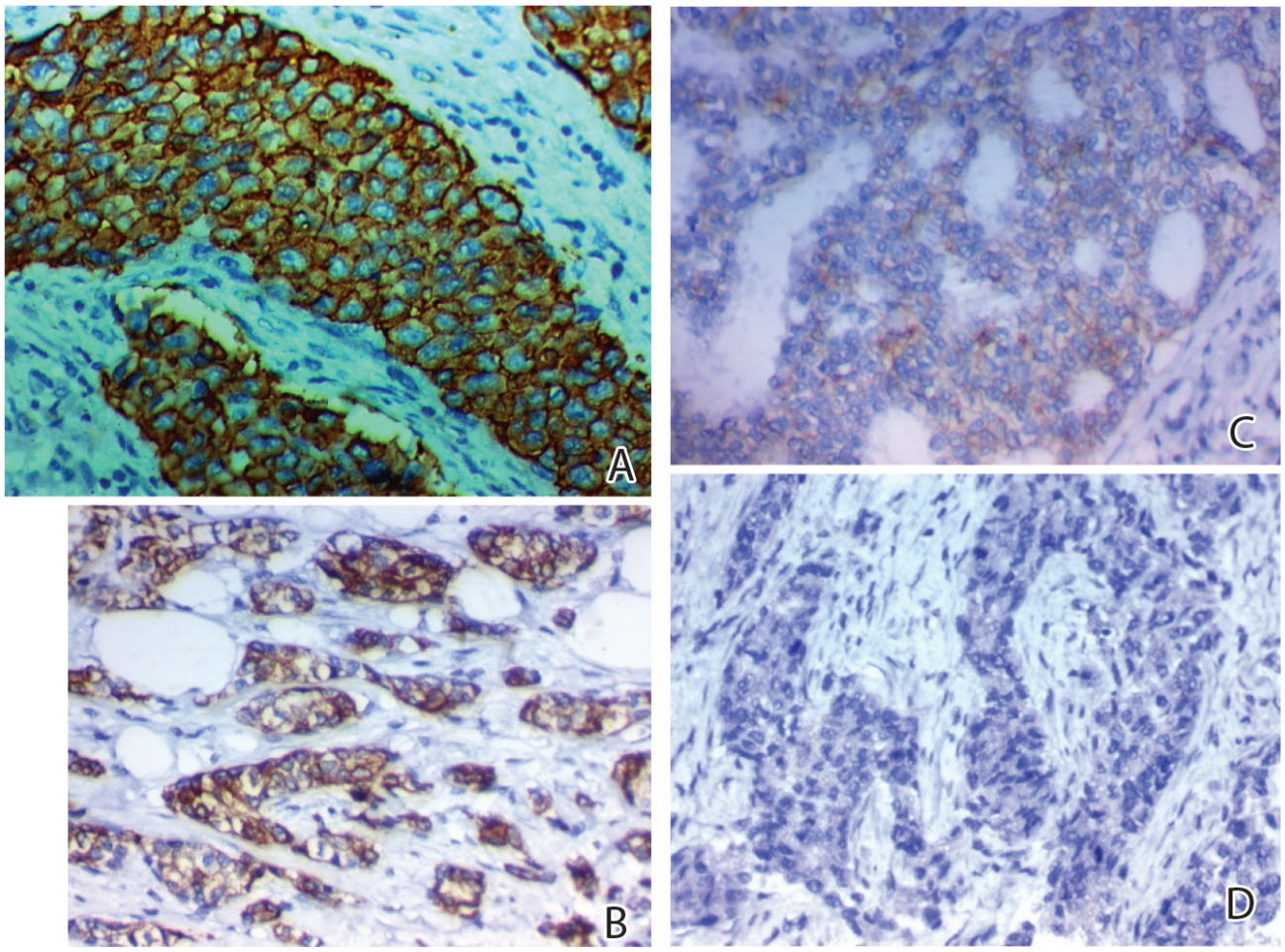

Figure 1. Immunohistochemical staining of ALDH1 in carcinoma of the breast: (A) high positive expression in the cytoplasm of high-grade infiltrating duct carcinoma of the breast stage IV ×400; (B) High positive expression in the cytoplasm of high-grade infiltrating duct carcinoma of the breast stage III ×400; (C) low expression in the cytoplasm of low-grade infiltrating duct carcinoma of the breast stage II ×400; and (D) negative expression in the cytoplasm of low-grade infiltrating duct carcinoma of the breast stage II ×400.
ALDH-1: Aldehyde dehydrogenase 1

expression as a predictor of BC recurrence has yet to be elucidated.

Sex-determining region Y-box9 (SOX9) belongs to a family of master regulators of sex-determining function in the gonads.[8] It is considered as a transcription factor that plays a

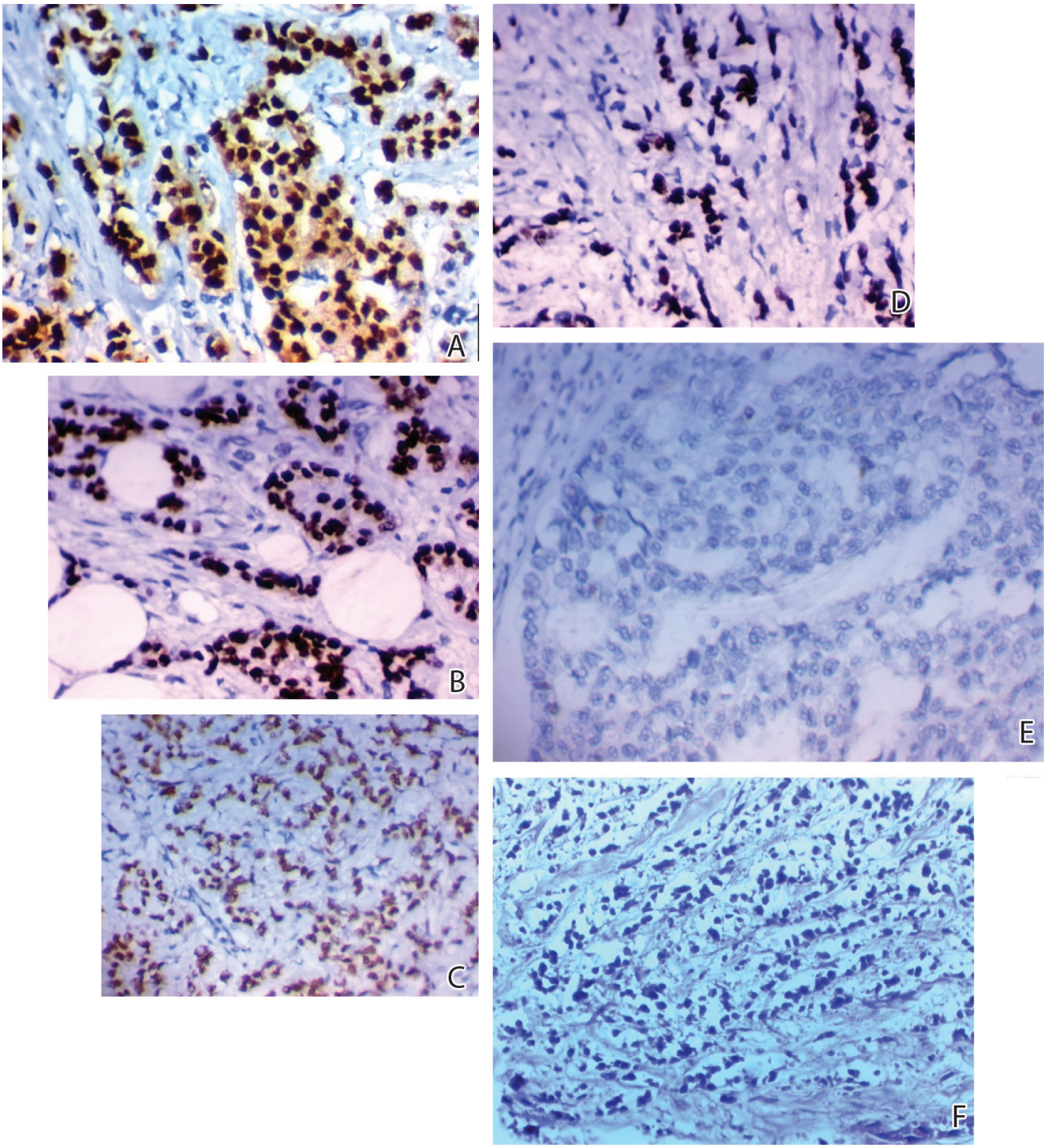

Figure 2. Immunohistochemical expression of SOX9 in carcinoma of the breast: (A) high expression in the nucleus of high-grade infiltrating lobular carcinoma of the breast stage IV ×400; (B) high expression in the nucleus of high-grade infiltrating duct carcinoma of the breast stage III ×400; (C) high expression in the nucleus of high-grade infiltrating lobular carcinoma of the breast stage IV ×400; (D) high expression in the nucleus of high-grade infiltrating lobular carcinoma of the breast stage III ×400; (E) negative expression in the nucleus of low-grade infiltrating duct carcinoma of the breast stage II ×400; and (F) negative expression in the nucleus of infiltrating lobular carcinoma of the breast stage II ×400.
SOX9: Sex-determining region Y-box 9

central role in the development and differentiation of multiple cell lineages.[9] Mutation or abnormal expression of the SOX gene leads to the occurrence of many cancers. There are many mechanisms, either by reactivating the WNT/β-catenin signaling as in prostate cancer or by inactivation of GKN1 as in gastric cancer. In addition, inducing the proliferation and tumorigenicity by increasing the expression of phosphorylated Akt and its downstream targets, such as phosphorylated forkhead box O (FOXO) 1 and phosphorylated FOXO3, two members of FOXO family of transcription factors as in oesophageal cancer.[9]

Recently, there have been reports of a correlation between SOX9 expression and clinical outcome in some cancer types, including early BC.[10] However, no studies have assessed the expression of both ALDH1 and SOX9 markers together in early BC. Hence, the current work aimed to evaluate the immunohistochemical (IHC) expression of both ALDH1 and SOX9 protein in early BC and to correlate their expression with clinicopathological characteristics and clinical outcome.

Patients and Methods

Study design and patients' eligibility

This is a prospective cohort study conducted between May 2016 and May 2019, at the Clinical Oncology and Nuclear Medicine, Medical Oncology and Pathology Departments, Faculty of Medicine, Zagazig University. The patients with early invasive BC and based on the American Joint Committee on Cancer (AJCC), 7th edition (2010) staging system (n = 50) underwent modified radical mastectomy or breast conservative surgery (BCS).[11] Early-stage BC is defined as clinical stage I or II or IIIA.[12]

The patients with adequate Eastern Cooperative Oncology Group (ECOG) performance status (= 0-2) were involved. Those with a prior neoadjuvant chemotherapy or having inadequate organs or bone marrow reservoirs were excluded. The Ethical Committee of Faculty of Medicine, Zagazig University (code: 4978) approved the study protocol.

Pathological diagnosis

All the samples either diagnostic true cut biopsy or after BCS or MRM were sent to Pathology Department, Faculty of Medicine, Zagazig

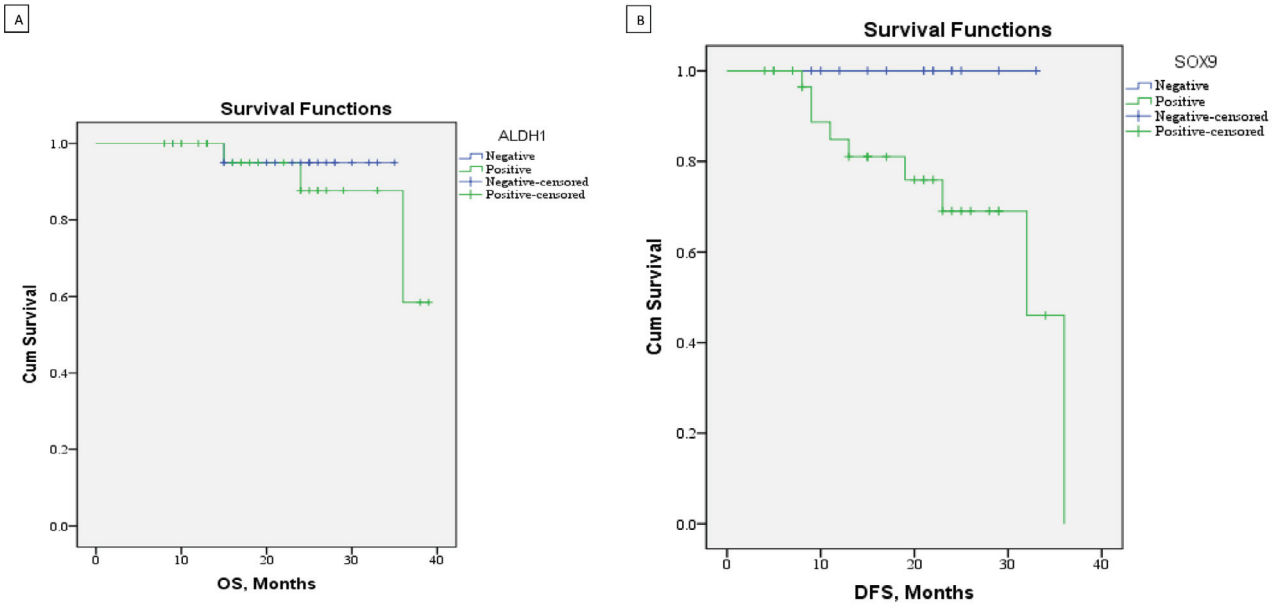

Figure 3. A) Kaplan- Meier survival curves illustrating the 3-y OS rate differences in the patients regarding the ALDH-1 expression and B): Kaplan- Meier survival curves illustrating 3-y OS rate differences in the patients regarding SOX9 expression.
ALDH-1: Aldehyde dehydrogenase 1; SOX-9: Sex-determining region Y-box 9; OS: Overall survival; Cum: Camulative; DFS: Disease-free survival

Table 1. The demographic characteristics and their association with ALDH-1and SOX9 expression in the included patients (N=50)

Variable	Total N=50	ALDH-1 Negative N=21	ALDH-1 Positive N=29	*P*	PSOX9 Negative N=16	PSOX9 Positive N=34	*P*
Age, years							
Mean ± SD	48.38 ± 9.5	50.1 ± 9.5	47.1 ± 9.4	0.282	50.3 ± 10	47.5 ± 9.3	0.345
Menopausal							
Pre-	23 (46.0%)	8 (38.1%)	15 (51.7%)	0.34	6 (37.5%)	17 (50.0%)	0.408
Post-	27 (54.0%)	13 (61.9%)	14 (48.3%)		10 (62.5%)	17 (50.0%)	
BMI							
Low	12 (24.0%)	4 (19.0%)	8 (27.6%)	0.4853	(18.8%)	9 (26.5%)	0.551
High	38 (76.0%)	17 (81.0%)	21 (72.4%)		13 (81.3%)	25 (73.5%)	
Family history							
No	42 (84.0%)	19 (90.5%)	23 (79.3%)	0.288	14 (87.5%)	28 (82.4%)	0.643
Yes	8 (16.0%)	2 (9.5%)	6 (20.7%)		2 (12.5%)	6 (17.6%)	
Diabetes Mellitus (DM)							
No	38 (76.0%)	15 (71.4%)	23 (79.3%)	0.52	11 (68.8%)	27 (79.4%)	0.41
Yes	12 (24.0%)	6 (28.6%)	6 (20.7%)		5 (31.3%)	7 (20.6%)	
Pathological subtype							
IDC	37 (74.0%)	15 (71.4%)	22 (75.9%)	0.683	10 (62.5%)	27 (79.4%)	0.311
IL	8 (16.0%)	3 (14.3%)	5 (17.2%)		3 (18.8%)	5 (14.7%)	
Others	5 (10.0%)	3 (14.3%)	2 (6.9%)		3 (18.8%)	2 (5.9%)	
Grade							
1	2 (4.0%)	2 (9.5%)	0 (0.0%)	0.083	2 (12.5%)	0 (0.0%)	0.06
2	32 (64.0%)	15 (71.4%)	17 (58.6%)		11 (68.8%)	21 (61.8%)	
3	16 (32.0%)	4 (19.0%)	12 (41.4%)		3 (18.8%)	13 (38.2%)	
Capsular invasion							
No	38 (76.0%)	18 (85.7%)	20 (69.0%)	0.171	13 (81.3%)	25 (73.5%)	0.551
Yes	12 (24.0%)	3 (14.3%)	9 (31.0%)		3 (18.8%)	9 (26.5%)	
Stage							
I	3 (6.0%)	3 (14.3%)	0 (0.0%)	<0.001	3 (18.8%)	0 (0.0%)	<0.001
II	14 (28.0%)	11 (52.4%)	3 (10.3%)		9 (56.3%)	5 (14.7%)	
III	33 (66%)	7 (33.3%)	26 (89.6%)		4 (25.0%)	29 (85.2%)	
T							
1	12 (24.0%)	7 (33.3%)	5 (17.2%)	0.003	6 (37.5%)	6 (17.6%)	0.002
2	17 (34.0%)	11 (52.3%)	6 (20.6%)		9 (56.2%)	8 (23.5%)	
3	21 (42.0%)	3 (14.2%)	18 (62.06%)		1 (6.3%)	20 (58.8%)	
N							
0	10 (20.0%)	8 (38.1%)	2 (6.9%)	<0.001	7 (43.8%)	3 (8.8%)	<0.001
1	12 (24.0%)	8 (38.1%)	4 (13.8%)		7 (43.8%)	5 (14.7%)	
2	2 (4.0%)	0 (0.0%)	2 (6.9%)		0 (0.0%)	2 (5.9%)	
3	26 (52.0%)	5 (23.8%)	21 (72.4%)		2 (12.5%)	24 (70.6%)	
ER							
Negative	15 (30.0%)	3 (14.3%)	12 (41.4%)	0.039	1 (6.3%)	14 (41.2%)	0.012
Positive	35 (70.0%)	18 (85.7%)	17 (58.6%)		15 (93.8%)	20 (58.8%)	
PR							
Negative	16 (32.0%)	3 (14.3%)	13 (44.8%)	0.022	1 (6.3%)	15 (44.1%)	0.007
Positive	34 (68.0%)	18 (85.7%)	16 (55.2%)		15 (93.8%)	19 (55.9%)	
HER2							
Negative	37 (74.0%)	19 (90.5%)	18 (62.1%)	0.024	16 (100.0%)	21 (61.8%)	0.004
Positive	13 (26.0%)	2 (9.5%)	11 (37.9%)		0 (0.0%)	13 (38.2%)	
KI 67							
Low	35 (70.0%)	20 (95.2%)	15 (51.7%)	0.001	16 (100.0%)	19 (55.9%)	0.001
High	15 (30.0%)	1 (4.8%)	14 (48.3%)		0 (0.0%)	15 (44.1%)	
Molecular subtypes							
Luminal A	27 (54.0%)	19 (90.5%)	8 (27.6%)	<0.001	16 (100.0%)	11 (32.4%)	<0.001
Luminal B	9 (18.0%)	0 (0.0%)	9 (31.0%)		0 (0.0%)	9 (26.5%)	
HER2 amplified	5 (10.0%)	0 (0.0%)	5 (17.2%)		0 (0.0%)	5 (14.7%)	
Triple -ve	9 (18.0%)	2 (9.5%)	7 (24.1%)		0 (0.0%)	9 (26.5%)	

SD: Standard deviation; BMI: Body mass index; ALDH-1: Aldehyde dehydrogenase 1; ER: Estrogen receptor; PR: Progesterone receptor, Her-2-neu: Human epidermal growth factor receptor 2; SOX-9: Sex-determining region Y-box 9; IDC: Invasive ductal carcinoma; IL: Invasive lobular

University where they were processed, diagnosed, graded, and staged.

Data collection

Clinical and pathological criteria as sex, age, tumor size, grade, and stage were identified. Estrogen receptors (ER), progesterone receptors (PR) hormonal receptors, human epidermal growth factor receptor 2 (Her-2 neu) expressions, and Ki 67 labeling index were evaluated for all the cases. The adjuvant treatment (chemotherapy,

Table 2. Markers co-expression in the studied population

Markers	SOX9	Total		*P*
		Negative	Positive	
ALDH1				
Negative	16 (100.0%)	5 (14.7%)	21 (42.0%)	< 0.001
Positive	0 (0.0%)	29 (85.3%)	29 (58.0%)	

ALDH-1: Aldehyde dehydrogenase 1; SOX-9: Sex-determining region Y-box 9

radiotherapy, and hormonal treatment with or without trastuzumab) was given according to their indication and staging. ER and PR were considered positive, if more than 1% of tumor cells have positive nuclear staining.[13]

HER-2/neu was considered positive, if immunohistochemically 3+ or equivocal 2+ cases which showed HER2 neu amplificationon on in situ hybridization (ISH). Ki67 labeling index was considered high, if > 14% and was considered low, if <14%.[14]

All the patients enrolled in this study gave written informed consent to participate.

Immunohistochemistry (IHC) staining

This study includes sections from formalin-fixed, paraffin-embedded tissue samples from early BC. IHC was performed.[15] We incubated sections with primary goat polyclonal anti-ALDH1A1 antibody (ab9883) and primary rabbit polyclonal Anti-SOX9 antibody (ab26414) 1: 200 dilutions (Abcam, campridge, UK).

Evaluation of both ALDH1 and SOX-9 IHC staining

Positive cytoplasmic ALDH1 expression of tumor cells and nuclear SOX-9 expression of tumor cells was considered to be positive expression.[16]

A semi-quantitative integral method was used to assess the results of both ALDH1 and SOX-9 staining. The staining intensity and the positive cell percentage points of SOX-9 were evaluated in a blinded manner. Grading standards were as follows: positive cell percentage points were scored as 0: no staining; 1: <10%; 2: 10%-50%; and 3: >50%. The staining intensity was scored as follows: 0: no staining; 1: mild staining; 2:

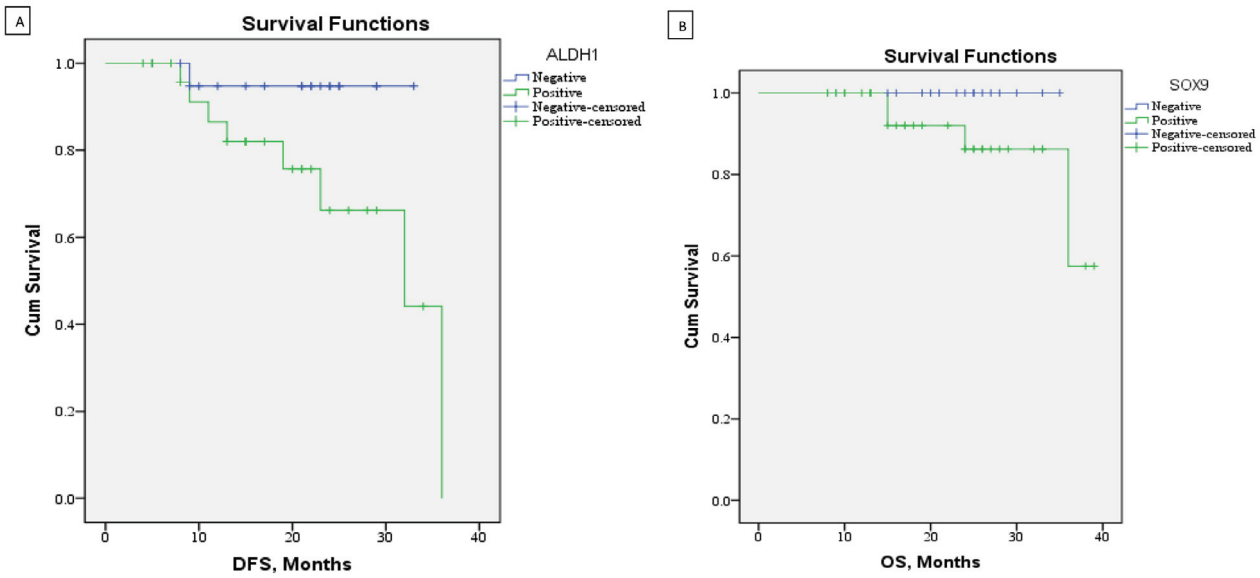

Figure 4. A) Kaplan- Meier survival curves portraying 3-y DFS rate differences in the patients regarding ALDH-1 expression and B) Kaplan- Meier survival curves portraying 3-y DFS rate differences in the patients regarding SOX9 expression.

ALDH-1: Aldehyde dehydrogenase 1; SOX-9: Sex-determining region Y-box 9; DFS: Disease-free survival; Cum: Cumulative

Table 3. Univariate Cox regression analyses of different prognostic factors for OS and DFS

	OS		DFS	
	Univariate Sig. Univariate	HR	Univariate Sig. Univariate	HR
Age	0.137	0.95	0.127	0.939
Menopausal Status	0.882	1.16	0.512	0.615
BMI	0.473	32.86	0.989	1.011
Family history	0.732	0.04	0.081	4.677
Diabetes Mellitus (DM)	0.533	2.17	0.107	3.48
Pathological subtype	0.826	0.81	0.409	0.474
Grade	0.475	2.07	0.031	5.782
Capsular invasion	0.288	2.91	0.408	1.835
Stage	0.012	18.55	<0.001	11.726
T	0.065	3.59	0.054	1.997
N	0.234	1.92	0.093	2.034
ER	0.342	0.01	0.02	0.082
PR	0.339	0.01	0.123	0.008
HER2	0.573	1.82	0.62	1.445
KI67	0.541	1.92	0.018	6.97
Molecular subtypes	0.091	2.43	0.018	2.111
ALDH1	0.586	1.95	0.104	5.732
SOX9	0.451	42.40	0.236	40.202

OS: Overall survival; DFS: Disease-free survival; BMI: Body mass index; ALDH-1: Aldehyde dehydrogenase 1; ER: Estrogen receptors; PR: Progesterone receptors; Her-2-neu: Human epidermal growth factor receptor 2; SOX-9: Sex-determining region Y-box 9; HR: Hazard ratio

moderate staining; and 3: strong staining. We calculated the results as the product of the staining intensity score and the positive cell percentage points. A product score of 3 was employed as the cut-off value. A score >3 was considered positive expression, while ≤3 was considered negative for both SOX-9 and ALDH1.[17]

Statistical analysis

The collected data were computerised and statistically analysed using SPSS program (Statistical Package for Social Sciences) version 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium).

Qualitative data were represented as frequencies and relative percentages.

Quantitative data were expressed as mean ± standard deviation (SD).

Quantitative data were compared using independent T test between both groups.

Chi square test (χ^2) and Fisher exact were applied to calculate the differences among qualitative variables as indicated.

Spearman's Rho Rank correlation test was used for correlating variables. The (+) sign was considered as the indication for direct correlation, namely increase frequency of independent lead to increase the frequency of dependent, and the (-) sign as the indication for inverse correlation, namely increase frequency of independent lead to decrease the frequency of dependent. We consider the values near to 1 as strong correlation and values near 0 as weak correlation

All the statistical comparisons were two tailed with a significance level of $P \leq 0.05$ indicating significant, $P < 0.001$ indicating highly significant difference, while $P > 0.05$ indicates non-significant differences.

Survival analysis

We employed Kaplan and Meier method to estimate the overall and disease-free survival and log rank test compared survival curves (P value was considered significant at ≤ 0.05 levels). Overall survival (OS) was calculated as the time from the date of diagnosis to the date of death or the last follow-up contact (censored). Disease-free survival (DFS) was calculated as the time from the end of primary treatment to the date of

Table 4. Outcome of patients in relation to markers expression

Outcome	Total	ALDH-1			SOX9		
		Negative	Positive	*P*	Negative	Positive	*P*
	N=50	N=21	N=29		N=16	N=34	
Local Recurrence							
No	45 (90.0%)	21 (100.0%)	24 (82.8%)	0.045	16 (100.0%)	29 (85.3%)	0.106
Yes	5 (10.0%)	0 (0.0%)	5 (17.2%)		0 (0.0%)	5 (14.7%)	
Progression							
No	41 (82.0%)	20 (95.2%)	21 (72.4%)	0.038	16 (100.0%)	25 (73.5%)	0.023
Yes	9 (18.0%)	1 (4.8%)	8 (27.6%)		0 (0.0%)	9 (26.5%)	
Death							
No	46 (92.0%)	20 (95.2%)	26 (89.7%)	0.473	16 (100.0%)	30 (88.2%)	0.153
Yes	4 (8.0%)	1 (4.8%)	3 (10.3%)		0 (0.0%)	4 (11.8%)	

ALDH-1: Aldehyde dehydrogenase 1; SOX-9 Sex-determining region Y-box 9

first failure of this treatment local or systemic. Stratification of OS and DFS was carried out according to IHC markers.

Cox proportional hazards regression models were summarised with hazard ratios using enter method.

Results

Patients' characteristics with ALDH1 and SOX-9 expressions

Among the eligible patients, positive expression of ALDH1 and SOX-9 were detected in 29 (58%) and 34 (68%) patients, respectively (Figures 1 and 2). Those positive expressions of both markers were statistically significant and associated with increasing the stage; lymph nodes (LNs) metastasis; high Ki67 labeling index; molecular subtypes ($P < 0.001$) (luminal B, Her2 amplified and triple negative); and the biological markers: ER, PR, Her-2 over-expressions, and large tumor size (T) ($P = 0.039$, $P = 0.022$, $P = 0.024$, and $P = 0.003$ for ALDH1 expression and $P = 0.012$, $P = 0.007$, $P = 0.004$, and $P = 0.002$ for SOX-9 expression, respectively).The demographic characteristics of the included patients and the association with ALDH-1and SOX9 expression in the included patients (N=50) are represented in table 1.

The association between the expression of SOX-9 and ALDH1

Table 2 demonstrates a significant positive association between the expression of ALDH1 and SOX-9. r (correlation coefficient) = +0.806 ($P < 0.001$).

Univariate analyses

The effects of all the factors on prognosis were evaluated through univariate survival analyses, revealing that stage, grade, ER, Ki-67 expression, and molecular subtype were significant prognostic indicators for DFS (Table 3).

The correlations between ALDH-1and SOX9 expression and outcome

While local recurrence was associated with positive expression of ALDH1 alone ($P = 0.045$), the disease progression was statistically significant and associated with the positive expression of both ALDH1 and SOX-9 ($P = 0.038$ and $P = 0.023$, respectively). Statistical analysis shed light on the significant association of positive expression of SOX-9 with reduced 3-year DFS ($P = 0.039$). In contrast, there were no (OS) benefits for ALDH-1 and SOX9 expressions ($P = 0.576$ and $P = 0.181$, respectively) (Tables 4 and 5, Figures 3 and 4).

Discussion

Herein, a positive expression of ALDH1 and SOX-9 were detected in 29 (58%) and 34(68%) patients, respectively. The positive expressions of both markers were statistically significant and associated with the following: increasing the stage; LNs metastasis; high Ki67 labeling index; molecular subtypes ($P < 0.001$); and with the biological markers ER and PR, Her-2 over-expressions, and large tumor size ($P = 0.039$, $P = 0.022$, $P = 0.024$, and $P = 0.003$ for ALDH1 expression and $P = 0.012$, $P = 0.007$, $P = 0.004$, and $P = 0.002$ for SOX-9 expression, respectively).There was a significant positive association between the expression of ALDH1 and SOX-9, r (correlation coefficient) = +0.806 $P < 0.001$). Local recurrence was associated with

Table 5. Overall and disease -free survival in relation to markers expression

Markers	Overall Survival				Disease-Free Survival			
	No. of Events	%	3-year Survival rate %	*P*	No. of Events	%	3-year Survival rate %	*P*
ALDH1								
Negative	1	4.8%	95%	0.576	1	4.8%	94.7%	0.065
Positive	3	10.3%	58.5%		8	27.6%	0.00%	
SOX9								
Negative	0	0.0%	100.0%	0.181	0	0.0%	100.0%	0.039
Positive	4	11.8%	57.5 %		9	26.5%	0.00%	
Overall	4	8.0%	60.80%	9		18.0%	0.00%	

ALDH-1: Aldehyde dehydrogenase 1; SOX-9: Sex-determining region Y-box 9

the positive expression of ALDH1alone (P = 0.045). The disease progression was statistically and significantly associated with positive expression of both ALDH1 and SOX-9 (P = 0.038 and P = 0.023, respectively). There was a significant association of positive expression of SOX-9 with reduced 3-y DFS (P = 0.039).

Recently, there are emerging data on the role of ALDH1 and SOX9 in various malignancies. ALDH1 may influence cellular proliferation, metastasis and even resistance to chemotherapy through its enzymatic action.[18-20]

In addition, SOX9 may inhibit apoptosis and consequently promote proliferation, invasion, and cancer spread through the interaction with WNT/betacatenin signaling pathway and controlling on cells adhesion and cytoskeleton remodeling.[21, 22]

In the current work, we reported that the expression of ALDH1 and SOX9 was associated with poor clinicopathological parameters in the form of large tumor size, high stage, LNs metastasis, high Ki-67 labeling index, molecular subtypes, along with with the biological markers: ER, PR, and Her-2 over-expression. These findings are consistent with those of numerous previous studies.[23, 24]

Lei B et al. in their study found that SOX9 expression was associated with LNs metastasis, ER, PR, Ki67, and p53 in BC.[17] Moreover, Chakravarty G et al. reported that SOX9 expression is significantly associated with higher tumor grade that provided an excellent rationale to use SOX9 as an identifying marker of aggressive behavior BC.[25] This is matched with our study revealing that positive expression of SOX9 was statistically and significantly associated with LNs metastasis, high Ki67 labeling index (P < 0.001), and with the biological markers, ER and PR (P = 0.012, P = 0.007).

On the contrary, Miyoshi Y et al. failed to detect a significant association between ALDH1 expression with conventional clinical features, such as tumor size, TNM stage, basal-like features, or the expression of ER, PR, or HER-2.[2]

Additionally, our study showed that DP was statistically and significantly associated with the positive expression of both ALDH1 and SOX9 (P = 0.038 and P = 0.023, respectively). Furthermore, statistical analysis demonstrated the significant association of positive expression of SOX9 with reduced 3-year DFS (P = 0.039). These findings are in concordance with that shown in many previous studies.[26-30]

Ginestier C et al. found that ALDH1-positive tumor cells are associated with poor clinical outcomes.[31] Furthermore, Zhong Y et al. stated that there was a significant association with ALDH1 expression with tumor recurrence and metastasis in patients with BC.[4] This may be due to the role of ALDH1- positive cells in developing resistance to adjuvant chemotherapeutic agents and tumor aggressiveness.[21, 32] Kadaja M et al., Lawson DA et al., and Dong et al. showed that ALDH1 expression is considered as an independent predictor of poor outcomes in BC.[33] This is consistent with our research which found that the positive expression of ALDH1 was statistically and significantly associated with local recurrence (P = 0.045) and the disease progression (P = 0.038).

A systematic review and meta-analysis using 15 publications showed that ALDH1 is a potential biomarker to predict poor survival in patients

with BC.[22] However, our work did not exhibit any significant associations between ALDH1 and survival.

Lei B et al. evaluated the role of SOX9 expression among 420 BC patients and reported the association with high-risk demographic feature and poor survival outcome either OS or DFS.[17] Those results matched with that reported previously by Chakravarty G et al., Lapierre M et al., Riemenschnitter C et al., and Willis S et al.[25, 34-36]

On the other hand, there is controversy about the association of ALDH with outcome in patients with BC. Some studies have revealed that positive ALDH-1 expression is associated with shorter survival and early recurrence.[37] Other reports failed to prove that association with poor outcome.[38, 39] Moreover, Miyoshi Y et al. reported that ALDH1 expression was associated with good outcomes.[2] Those differences among different study results might be related to the differences in sample sizes, follow-up period, and different cut-off values for ALDH1 staining. The importance of a longer term follow-up, cut-off value, and methods of ALDH1 expression evaluation has been highlighted in a long term follow-up study.[40] In addition, our study revealed that stage, grade, ER, Ki-67 expression, and molecular subtype were significant prognostic indicators for DFS via univariate survival analyses.

Our study is the first to evaluate the relation between the expression of ALDH1 and SOX9 and reveal a significant positive association between the expression of both markers. r (correlation coefficient) = +0.806 ($P < 0.001$).

Limitations

The major limitation in our study was the use of different adjuvant chemotherapy protocols that might have affected both tumor recurrences and outcomes. In addition, we evaluated ALDH1 and SOX9 through IHC not with microarray.

The number of patients was limited according to the rate in our hospital and we recommend to assess markers expression in larger cohort of patients for a better evaluation of the studied markers.

Additionally, we recommend to asses mRNA markers expression using Real-Time PCR for a better evaluation of the studied markers.

Conclusion

ALDH1 and SOX9 may represent new prognostic molecular and predictor markers of poor DFS in patients with early BC. Taken together, they may be employed as a therapeutic target for cancer treatment.

Conflict of Interest

None declared.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin.* 2017;67(1):7-30. doi: 10.3322/caac.21387.
2. Miyoshi Y, Shien T, Ogiya A, Ishida N, Yamazaki K, Horii R, et al. Differences in expression of the cancer stem cell marker aldehyde dehydrogenase 1 among estrogen receptor-positive/human epidermal growth factor receptor type 2-negative breast cancer cases with early, late, and no recurrence. *Breast Cancer Res.* 2016;18(1):73. doi: 10.1186/s13058-016-0731-3.
3. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature.* 1994;367(6464):645-8. doi: 10.1038/367645a0.
4. Zhong Y, Shen S, Zhou Y, Mao F, Guan J, Lin Y, et al. ALDH1 is a better clinical indicator for relapse of invasive ductal breast cancer than the CD44+/CD24- phenotype. *Med Oncol.* 2014;31(3):864. doi: 10.1007/s12032-014-0864-0
5. Althobiti M, El Ansari R, Aleskandarany M, Joseph C, Toss MS, Green AR, et al. The prognostic significance of ALDH1A1 expression in early invasive breast cancer. *Histopathology.* 2020;77(3):437-48. doi: 10.1111/his.14129.
6. Vassalli G. Aldehyde dehydrogenases: not just markers, but functional regulators of stem cells. *Stem Cells Int.* 2019; 3904645. doi.org/10.1155/2019/3904645.
7. Ciccone V, Terzuoli E, Donnini S, Giachetti A, Morbidelli L, Ziche M. Correction to: Stemness marker aldh1a1 promotes tumor angiogenesis via retinoic acid/hif-1alpha/vegf signalling in mcf-7 breast cancer cells. *J Exp Clin Cancer Res.* 2019;38;45. doi:10.1186/s13046-019-1045-y.
8. Hersmus R, Kalfa N, de Leeuw B, Stoop H, Oosterhuis JW, de Krijger R, et al. FOXL2 and SOX9 as parameters of female and male gonadal differentiation in patients with various forms of disorders of sex development (DSD). *J Pathol.* 2008; 215: 31-8. doi:

10.1002/path.2335.
9. Ruan H, Hu S, Zhang H, Du G, Li X, Li X, et al. Upregulated SOX9 expression indicates worse prognosis in solid tumors: a systematic review and meta-analysis. *Oncotarget.* 2017;8(68):113163-73. doi: 10.18632/oncotarget.22635.
10. Higo N, Okumura H, Uchikado Y, Omoto I, Sasaki K, Kita Y, et al. Expression of SOX9 is related to prognosis in patients with oesophageal squamous cell carcinoma. *In Vivo.* 2018;32:835-8. doi: 10.21873/in vivo. 112316.
11. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol.* 2010;17(6):1471-4. doi: 10.1245/s10434-010-0985-4.
12. Dimitrakopoulos FI, Kottorou A, Antonacopoulou AG, Makatsoris T, Kalofonos HP. Early-stage breast cancer in the elderly: Confronting an old clinical problem. *J Breast Cancer.* 2015;18(3):207-17. doi: 10.4048/jbc.2015.18.3.207.
13. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society Of Clinical Oncology/College of American Pathologists guideline recommendations for IHC testing of estrogen and progesterone receptors in breast cancer. *Arch Pathol Lab Med.* 2010;134:907-22. doi: 10.5858/134.7.e48.
14. Gutierrez C, Schiff R. HER2 biology, detection, and clinical implications. *Arch Pathol Lab Med.* 2011;135:55-62. doi: 10.5858/2010-0454-RAR.1.
15. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem.* 1981;29:577-80. doi: 10.1177/29.4.6166661.
16. Tingting Li, Huang H, Shi G, Zhao L, Li T, Zhang Z, et al. TGF-β1-SOX9 axis-inducible COL10A1 promotes invasion and metastasis in gastric cancer via epithelial-tomesenchymal transition. *Cell Death and Disease.* 2018;9:849. doi:10.1038/s41419-018-0877-2.
17. Lei B, Zhang Y, Liu T, Li Y, Pang D. Sox9 upregulation in breast cancer is correlated with poor prognosis and the CD44+/CD24-/low phenotype. *Int J Clin Exp Pathol.* 2016; 9(7):7345-51. doi:10.31557/APJCP.2020.21.9.2561.
18. Grantzdorffer I, Yumlu S, Gioeva Z, Von Wasielewski R, Ebert MP, Röcken C. Comparison of different tissue sampling methods for protein extraction from formalin-fixed and paraffin-embedded tissue specimens. *Exp Mol Pathol.* 2010;88(1):190-6. doi: 10.1016/j.yexmp.2009.09.009.
19. Wang Y, Zhe H, Gao P, Zhang N, Li G, Qin J. Cancer stem cell marker ALDH1 expression is associated with lymph node metastasis and poor survival in esophageal squamous cell carcinoma. a study from high incidence area of northern China. *Dis Esophagus.* 2012;25(6):560-5. doi: 10.1111/j.1442-2050.2011.01279.
20. Liu Y, Lv DL, Duan JJ, Xu SL, Zhang JF, Yang XJ, et al. ALDH1A1 expression correlates with clinicopathologic features and poor prognosis of breast cancer patients: a systematic review and meta-analysis. *BMC Cancer.* 2014;14:444. doi: 10.1186/1471-2407-14-444.
21. Kadaja M, Keyes BE, Lin M, Pasolli H, Genander M, Polak L, et al. SOX9 a stem cell transcriptional regulator of secreted niche signaling factors. *Genes Dev.* 2014;28:328-41. doi: 10.1101/gad.233247.113.
22. Larsimont JC, Youssef KK, Sánchez-Danés A, Sukumaran V, Defrance M, Delatte B, et al. SOX9 controls self-renewal of oncogene targeted cells and links tumor initiation and invasion. *Cell Stem Cell.* 2015;17:60-73. doi: 10.1016/j.stem.2015.05.008.
23. Morimoto K, Kim SJ, Tanei T, Shimazu K, Tanji Y, Taguchi T, et al. Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression. *Cancer Sci.* 2009;100(6):1062-8. doi: 10.1111/j.1349-7006.2009.01151.
24. Pan H, Wu N, Huang Y, Li Q, Liu C, Liang M, et al. Aldehyde dehydrogenase 1 expression correlates with the invasion of breast cancer. *Diagn Pathol.* 2015;10:66. doi: 10.1186/s13000-015-0301-5.
25. Chakravarty G, Moroz K, Makridakis N, Lloyd SA, Galvez SE, Canavello PR, et al. Prognostic significance of cytoplasmic SOX9 in invasive ductal carcinoma and metastatic breast cancer. *Exp Biol Med (Maywood).* 2011; 236:145. doi: 10.1258/ebm.2010.010086.
26. Strati A, Nikolaou M, Georgoulias V, Lianidou ES. Prognostic significance of TWIST1, CD24, CD44, and ALDH1 transcript quantification in EpCAM-positive circulating tumor cells from early stage breast cancer patients. *Cells.* 2019;8(7):652. doi: 10.3390/cells8070652.
27. Sarkar P, Basu K, Sarkar P, Chatterjee U, Mukhopadhyay M, Choudhuri M, et al. Correlations of aldehyde dehydrogenase 1(ALDH1) expression with traditional prognostic parameters and different molecular subtypes of breast carcinoma. *Clujul Med.* 2018;91(2):181-7. doi: 10.15386/cjmed-925.
28. Demir H, Dulgar O, Gulle BT, Turna H, Ilvan S. Prognostic value of aldehyde dehydrogenase 1 (ALDH1) in invasive breast carcinomas. *Bosn J Basic Med Sci.* 2019;7;18:313-9. doi: 10.17305/bjbms.2018.3094.
29. Domenici G, Aurrekoetxea-Rodríguez I, Simões BM, Rábano M, Lee SY, Millán JS, et al. A Sox2Sox9 signalling axis maintains human breast luminal progenitor and breast cancer stem cells. *Oncogene.*

2019;38(17):3151-69. doi: 10.15386/cjmed-925.
30. Grimm D, Bauer J, Wise P, Krüger M, Simonsen U, Wehland M, et al. The role of SOX family members in solid tumours and metastasis. *Semin Cancer Biol.* 2020;67(Pt 1):122-53. doi: 10.1016/j.semcancer.2019.03.004.
31. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell.* 2007;1(5):555-67. doi: 10.1016/j.stem.2007.08.014.
32. Lawson DA, Bhakta NR, Kessenbrock K, Prummel KD, Yu Y, Takai K, et al. Single-cell analysis reveals a stem-cell program in human metastatic breast cancer cells. *Nature.* 2015;526(7571):131-5. doi.org/10.1038/nature15260.
33. Dong Y, Bi LR, Xu N, Yang HM, Zhang HT, Ding Y, et al. The expression of aldehyde dehydrogenase 1 in invasive primary breast tumors and axillary lymph node metastases is associated with poor clinical prognosis. *Pathol Res Pract.* 2013;209(9):555-61. doi: 10.1016/j.prp.2013.05.007.
34. Lapierre M, Linares A, Dalvai M, Duraffourd C, Bonnet S, Boulahtouf A, et al. Histone deacetylase 9 regulates breast cancer cell proliferation and the response to histone deacetylase inhibitors. *Oncotarget*. 2016;7:19693-708. doi: 10.18632/oncotarget.7564.
35. Riemenschnitter C, Teleki I, Tischler V, Guo W, Varga Z. Stability and prognostic value of Slug, Sox9 and Sox10 expression in breast cancers treated with neoadjuvant chemotherapy. *Springerplus.* 2013;2:695. doi: 10.1186/2193-1801-2-695.
36. Willis S, De P, Dey N, Long B, Young B, Sparano JA, et al. Enriched transcription factor signatures in triple negative breast cancer indicates possible targeted therapies with existing drugs. *Meta Gene*. 2015;4:129-41. doi: 10.1016/j.mgene.2015.04.002.
37. Chatterjee D, Bal A, Das A, Singh G. Proliferation rate and breast cancer subtype, but not ALDH1 expression, predict pathological response to neoadjuvant chemotherapy in locally advanced breast cancer. *Virchows Arch.* 2015;467(3):303-10. doi: 10.1007/s00428-015-1794-8.
38. Ito M, Shien T, Omori M, Mizoo T, Iwamoto T, Nogami T, et al. Evaluation of aldehyde dehydrogenase 1 and transcription factors in both primary breast cancer and axillary lymph node metastases as a prognostic factor. *Breast Cancer.* 2016; 23(3):437-44. doi: 10.1007/s12282-015-0583-1.
39. Resetkova E, Reis-Filho JS, Jain RK, Mehta R, Thorat MA, Nakshatri H, et al. Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment. *Breast Cancer Res Treat.* 2010; 123(1):97-108. doi: 10.1007/s10549-009-0619-3.
40. Yoshioka T, Umekita Y, Ohi Y, Souda M, Sagara Y, Sagara Y, et al. Aldehyde dehydrogenase 1 expression is a predictor of poor prognosis in node-positive breast cancers: a long-term follow-up study. *Histopathology.* 2011;58(4):608-16. doi: 10.1186/1471-2407-14-444.