



# Stem Cell Marker Aldehyde Dehydrogenase 1A1 Expression in Triple-negative Breast Carcinoma

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## Abstract

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**BACKGROUND:** Aldehyde dehydrogenase 1A1 (ALDH1A1) has recently been identified as a marker for cancer stem cells in some human malignancies including breast cancer. Triple negative breast cancer (TNBCs) is group of primary breast tumors with aggressive clinical behavior that have no targeted therapy at present.

**AIM:** The assessment of immunohistochemical expression of ALDH1A1 in TNBC and its correlation with the clinicopathological features of TNBC.

**MATERIALS AND METHODS:** A retrospective study that was carried out on formalin fixed; paraffin embedded tissue blocks of sixty cases of modified radical mastectomy that previously diagnosed as TNBC. Immunohistochemical cytoplasmic staining was carried out using ALDH1A1 monoclonal antibody. A total score of ALDH1A1 expression was calculated by multiplying the score of staining intensity and percentage of stained cells to obtain score ranging from 0 up to 300. Due to the relatively large number of positive cases, a statistical analysis was performed with a negative (score  $\leq 10$ ) and positive (score  $> 10$ ) cutoff.

**RESULTS:** Evaluation of the results of immunostaining for ALDH1A1 showed that 88.3% of total cases (53 cases) revealed a positive cytoplasmic reactivity. Statistical analysis for a possible correlation between ALDH1A1 expression and prognostic clinicopathological parameters; age, size, tumor grade, histologic subtypes, lymphovascular invasion, intraductal components, tumor infiltrating lymphocytes, and TNM stage grouping revealed a non-significant correlation.

**CONCLUSION:** ALDH1A1 couldn't be used solely as a diagnostic or prognostic marker in TNBCs. Further research combining with other biomarkers and with a greater number of patients is necessary to confirm the role of ALDH1A1 in TNBC.

## Introduction

Breast cancer remains a life-threatening disease despite the advanced achievement in breast cancer therapy [2].

Triple-negative breast cancer (TNBC) represents 15% of all breast cancers in united states and is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) receptor expression. TNBC is the most aggressive phenotypic subtype of breast cancer [3]. Gene expression profiling analysis often classifies TNBC as a subtype of basal-like breast cancer (BLBC). Approximately, 56% of TNBC and BLBC gene expression profiles overlap. The overlap ratio can be as high as 60–90% between TNBC and BLBC, compared to only 11.5% between non-TNBC and BLBC [4].

Cancer stem cells (CSCs) are a subpopulation of cells within the primary tumor mass, which possess self-renewal, differentiation and potential tumorigenic properties [5].

Aldehyde dehydrogenase 1A1 (ALDH1A1) isozyme oxidizes retinaldehyde to retinoic acid, which

regulates the expression of the genes involved in tumor-initiating stem-like cells, thereby initiating tumor growth and resistance to drugs [6].

ALDH1 is the most common BCSC marker and has been studied in both *in-vitro* and *in-vivo* BC models [7].

High expression of ALDH1A1 has been reported as a poor prognostic marker in several tumor types and is associated with poor patient outcomes [8].

The aim of the present study is to evaluate immunohistochemical expression of ALDH1A1 in 60 cases of TNBC, then to correlate between expression of ALDH1A1 and other clinicopathological features (Age, tumor size, lymph node status, etc.).

## Materials and Methods

### Retrieval of cases

This is a retrospective study that was carried out on formalin fixed, paraffin embedded tissue blocks of sixty cases of modified radical mastectomy that previously diagnosed as TNBC, retrieved from the

archives of the Pathology Department, Kasr Al Ainy Hospital, Cairo University during the time period from January 2015 to June 2016.

Data were collected from pathology reports regarding personal data (age and sex), histopathological data (tumor size, grade, nodal metastasis, etc.). Slides stained by ER and PR and HER2/neu state were also collected.

Serial sections of 5  $\mu$  thick were prepared from each paraffin block, one of them stained by Hematoxylin and Eosin (H and E) for histological evaluation; another one was mounted on a charged slide for immunohistochemical staining by monoclonal antibody against ALDH1A1 antigen. All slides were examined under light microscope.

### Histopathologic assessment of cases

Histopathological examination was done for revision and confirmation of diagnosis.

Tumors were typed according to the criteria described by the World Health Organization (WHO) [9].

Tumor grading was done according to Nottingham grading system [10].

According to the American Joint Committee on Cancer (AJCC) TNM staging system of breast cancer, 8<sup>th</sup> edition [11].

Presence of ductal carcinoma *in situ* and specification of its type and its percentage from the tumor bulk.

- Tumor infiltrating lymphocytes (TILs).
- Lymphovascular invasion
- Perineural invasion

Regional lymph node status as regards the absence or presence of metastatic tumor deposits.

Retrieval of immunostained slides of ER and PR was done. Positive nuclear staining in  $\geq 1\%$  of tumor cells according to ASCO/CAP guidelines recommendations were considered positive [12].

Assessment of Her2/neu state was done according to the criteria in (Table 1) [13].

**Table 1: HER2/neu score used to evaluate Hercept Test [13]**

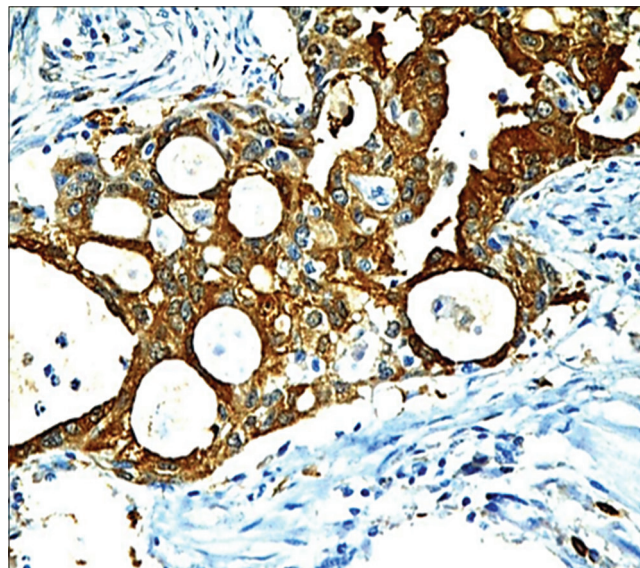
Score	Criteria
0 (Negative)	No immunoreactivity or immunoreactivity in $\leq 10\%$ of tumor cells.
1+ (Negative)	Faint weak immunoreactivity in $> 10\%$ of tumor cells but only a portion of the membrane is positive (incomplete).
2+ (Weak positive)	Incomplete and/or weak to moderate complete membrane immunoreactivity in $> 10\%$ of tumor cells or intense complete immunoreactivity in $\leq 10\%$ of tumor cells.
3+ (Positive)	Moderate to strong complete immunoreactivity in $> 10\%$ of tumor cells.

### Immunohistochemical assessment of cases

Immunostaining for ALDH1A1 was performed on all tissue of the biopsies.

Procedure: Sections from paraffin blocks were treated with antigen retrieval solution and then treated

with monoclonal antibody for ALDH1A1 [EP1933Y clone, Rabbit IgG, 0.1 ml concentrated, diluted at 1:200–400, manufactured by BIOCARE MEDICAL] using Automated Dako FLEX Ready-to-Use system. DAB was used as a chromogen and hematoxylin as a counter stain. A section of breast cancer (Figure 1) known to be positive for ALDH1A1 was used as positive control.



**Figure 1: Strong positive expression of ALDH1A1 of invasive duct carcinoma, breast (positive control) (400 $\times$  original magnification)**

### ALDH1A1 immunostaining interpretation

One ALDH1A1 immunostained slide was examined per case. Semi-quantitative analysis of ALDH1A1 immunostaining in tumor cells was performed using Olympus light microscope and the percenting of positively stained cells was recorded in consecutive fields at  $\times 400$  magnification.

The cytoplasmic staining of tumors cells was considered positive. The immunoreactivity was evaluated according to;

The staining intensity was scored according to the following scale: 0 score for no signal, 1 score for weak, 2 score for moderate, and 3 score for strong staining.

The percentage of cells with positive staining for the ALDH1A1 antibody was quantized (from undetectable level or 0%, to homogeneous staining or 100%).

Results were scored by multiplying the percentage of positive cells by intensity score by so called Quick score (Q) ( $Q = P \times I$ ; Maximum = 300). Due to the relatively large number of positive cases, a statistical analysis was performed with a negative (score  $\leq 10$ ) and positive (score  $> 10$ ) cutoff [1].

All data were collected and tabulated; ALDH1A1 expression and the other clinicopathological variables were correlated with each other and statistical analysis were performed to detect any significant correlation.

**Classification of breast cancer cases**

All cases were molecularly classified as TNBC. TNBC was defined as ER, PR and HER2/neu negative by immunohistochemistry.

**Statistical analysis**

All collected data were revised for completeness and consistency. Pre-coded data were entered on the computer using “Microsoft Office Excel Software” program (2013) for windows. Data were then transferred to the Statistical Package of Social Science Software program, version 24 (SPSS) to be statistically analyzed.

Data were summarized using mean, and standard deviation for quantitative variables and frequency and percentage for qualitative ones. Comparison between groups was performed using independent sample t-test or one-way ANOVA with post hoc Tukey’s test for quantitative variables and Chi-square or Fisher’s exact test for qualitative ones.

p < 0.05 were considered statistically significant and <0.01 were considered highly significant. Graphs were used to illustrate some information.

**Results**

This retrospective study was conducted on 60 cases of TNBC. Their ages ranged from 22 to 72 years with a mean age of 50.08 years. Thirty-five cases were 50 years or less (58.3%) and 25 cases were older than 50 years (41.7%).

The tumor size ranged from 1.5 to 9 cm with the mean size of 5.18 cm. 52 cases were found to be T2 (86.7%), 5 cases were T3 (8.3%), and 3 cases were T1 (5%).

About 53 cases (88.3%) are classified histologically as invasive ductal carcinoma (IDC), four cases (6.7%) were of lobular pattern (6.7%) and three cases (5%) displayed mixed duct and lobular pattern.

As regards to the degree of differentiation, 52 (86.7%) of cases were moderately differentiated (grade II) and 8 (13.3%) were poorly differentiated (grade III). The high-grade tumors included 5 cases of invasive duct carcinoma (no special type [NST]), 2 cases of lobular and one case of mixed pattern. 39 (65%) of cases showed no intraductal *in situ* components and 21 (35%) of cases showed intraductal *in situ* changes with 17 cases of minor components and 4 cases of major component. In the studied cases, 50 (83.3%) of cases showed TILs ≤50% and 10 (16.7%) showed TILs >50%. 57 (95%) of cases were negative for Lymphovascular invasion

and only 3 (5%) of cases showed Lymphovascular invasion carcinomas, all studied cases were negative for perineural invasion. Most of the studied cases revealed lymph nodes metastatic deposits; 16 (26.7%) of (N1), 25 (41.7%) of N2, and 4 (6.6%) of (N3) while 15 (25%) of cases were negative for lymph node metastasis. As regards AJCCTNM stage grouping, stage III was the dominant stage; 31 cases (51.67%) followed by Stage II; 26 cases (43.33%) then stage I; 3 cases (5%).

Immunohistochemical assessment of ALDH1A1 expression revealed Fifty-three cases (88.3%) showed positive ALDH1A1 expression while 7 cases (11.7%) were negative for ALDH1A1 expression (Figures 2-9).

Statistical analysis revealed no statistically significant correlations between ALDH1A1 expression and other clinicopathological parameters (Table 2).

**Table 2: Correlations between ALDH1A1 expression and clinicopathological data of the studied TNBC cases**

Clinicopathological Variable	ALDH1A1 Expression		Total	p-value
	Positive	Negative		
Age				
≤50	30 (85.7%)	5 (14.3%)	35 (58%)	0.455
>50	23 (92%)	2 (8%)	25 (42%)	
Tumor size (cm)				
T1 (≤2 cm)	3 (100%)	0 (0.0%)	3 (5%)	0.544
T2 (>2 cm–5 cm)	45 (86.5%)	7 (13.5%)	52 (86.7%)	
T3 (>5 cm)	5 (100%)	0 (0.0%)	5 (8.3%)	
Histological subtypes				
IDC	47 (88.7%)	6 (11.3%)	53 (88.3%)	0.387
Lobular carcinoma	4 (100%)	0 (0.0%)	4 (6.7%)	
Mixed	2 (66.7%)	1 (33.3%)	3 (5%)	
Tumor grade				
Grade II	46 (88.5%)	6 (11.5%)	52 (86.7%)	0.937
Grade III	7 (87.5%)	1 (12.5%)	8 (13.3%)	
DCIS				
No	34 (87.2%)	5 (12.8%)	39 (65%)	0.704
Yes	19 (90.5%)	2 (9.5%)	21 (35%)	
TILs				
≤50	45 (90.0%)	5 (10.0%)	50 (83.3%)	0.369
>50	8 (80.0%)	2 (20.0%)	10 (16.7%)	
LVI				
No	50 (87.7%)	7 (12.3%)	57 (95.0%)	0.518
Yes	3 (100.0%)	0 (0.0%)	3 (5%)	
Lymph node stage				
N0	14 (93.3%)	1 (6.7%)	15 (25%)	0.715
N1	14 (87.5%)	2 (12.5%)	16 (26.7%)	
N2	21 (84.0%)	4 (16.0%)	25 (41.7%)	
N3	4 (100.0%)	0 (0.0%)	4 (6.7%)	
TNM stage grouping				
Stage I	3 (100.0%)	0 (0.0%)	3 (5%)	0.801
Stage II	23 (88.5%)	3 (11.5%)	26 (43.33%)	
Stage III	27 (87.1%)	4 (12.9%)	31 (51.67%)	

**Discussion**

TNBC is a breast cancer subtype that is characterized by poor prognosis and aggressive behavior. Meanwhile, CSCs are believed to contribute to tumorigenesis and metastasis.

Since the first report by Ginestier *et al.* [14] showing that ALDH1A1 expression was associated with poor clinical outcome in breast cancer, several studies have indicated that ALDH1A1+ breast cancer cells are related to TNBCs.



In the present study, we further analyzed the relationship between CSCs and cancer development in TNBC patients using ALDH1A1 as a CSC marker and correlated the immunohistochemical staining of ALDH1A1 stem cell marker with the clinicopathological characteristics of breast cancer in a random sample of Egyptian females.

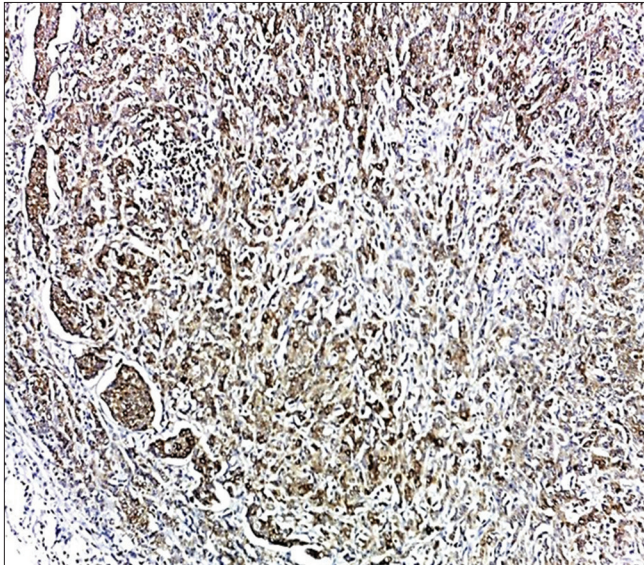


Figure 2: Strong Expression of ALDH1A1 in Invasive Duct Carcinoma Grade II (200× original magnification)

The present study included 60 cases of TNBC. The ages of the patients ranged from 22-72 with a mean age of 50.08 with 58.3% of patients were ≤50 years.

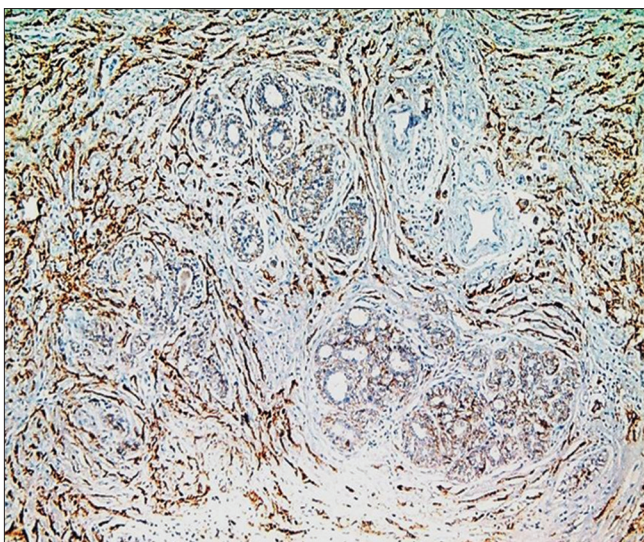


Figure 3: Strong Expression of ALDH1A1 in Invasive Lobular Carcinoma Grade II (200× original magnification)

The tumor size in this study ranged from 1.5 to 9 cm with the mean size of 5.18 cm. T2 was the dominant size (52 cases representing 86.7%), while 5 cases were T3 (representing 8.3%) and 3 cases were T1 (representing 5%). Rhakha *et al.* [15] stated that the majority of their TNBC cases presented at T2 and attributed the large tumor size to the rapid growth of these tumors. However, in

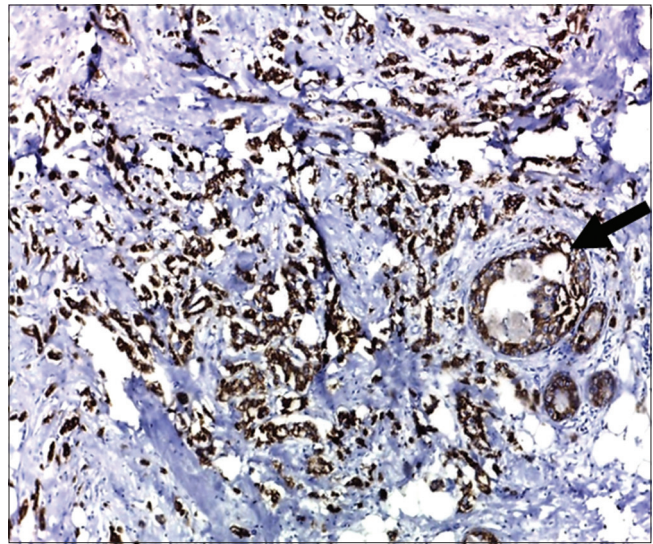


Figure 4: Strong Expression of ALDH1A1 in invasive duct carcinoma with intraductal component (arrow) (200× original magnification)

a study by Rebecca *et al.* [16], 66% of the TNBC cases were T3.

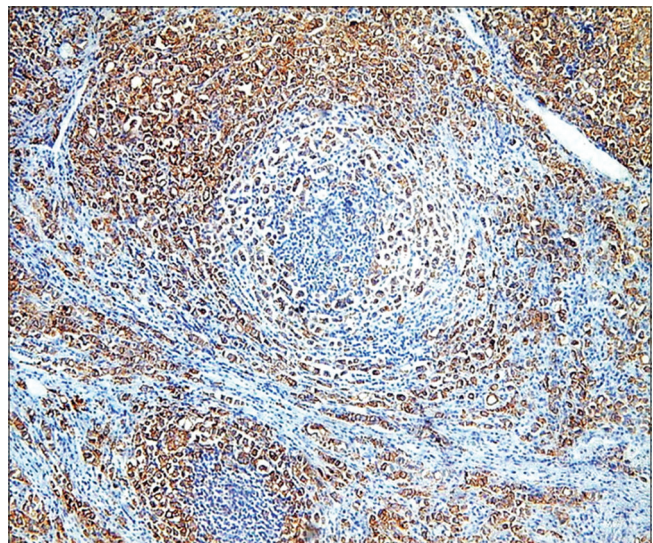


Figure 5: Strong Expression of ALDH1A1 in Metastatic lymph node by Invasive duct carcinoma (200× original magnification)

From a histologic standpoint, invasive duct carcinoma (NST) was the predominant subtype in this study and accounted for nearly 88% of all cases. As for the other variants in this present study, lobular carcinoma represented about 7% and mixed type represented 5%. This finding is in concordance with the published study of Bianchini *et al.* [3] which denoted that 95% of the TNBC cases are of invasive duct carcinoma (NST) histological type; another breast cancer comparative study by Thike *et al.* [17] stated that IDC (NOS) represented 77%, the medullary carcinoma cases represented 6%, and lobular carcinoma cases represented 2.4%. Interestingly, Thike *et al.* [17] postulated that prognosis in each of these appears to be distinct despite sharing the triple-negative phenotype.

When analyzing the tumor grade in this study, 52 cases were grade II, constituting (86.7%)



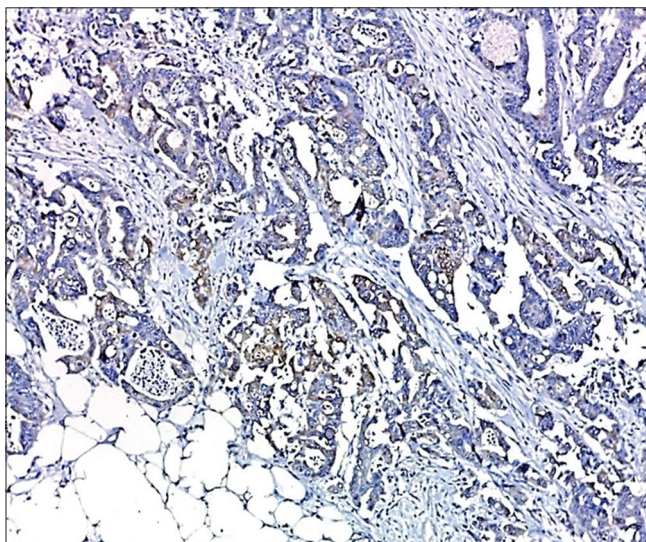


Figure 6: Moderate Expression of ALDH1A1 in Invasive Duct Carcinoma Grade II (200× original magnification)

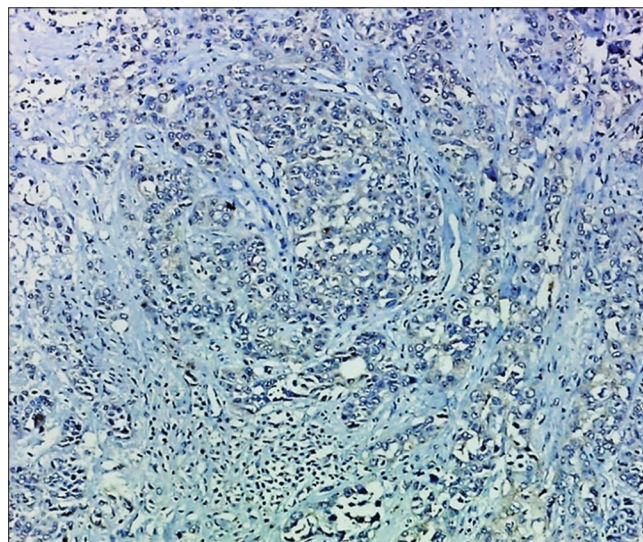


Figure 8: Weak Expression of ALDH1A1 in Invasive Duct Carcinoma Grade III (200× original magnification)

of the cases, and 8 cases were grade III (13.3%). Thus, all the cases included in this study were high grade (none of the cases were grade I). This agreed with Nassar *et al.* [18] who found that the majority (77%) of their triple negative cases were high grade. The higher grade involved in this study may be attributed to the later presentation of breast cancer among Egyptian patients together with genetic variability.

grade II and III may be due to subjective evaluation in grading system.

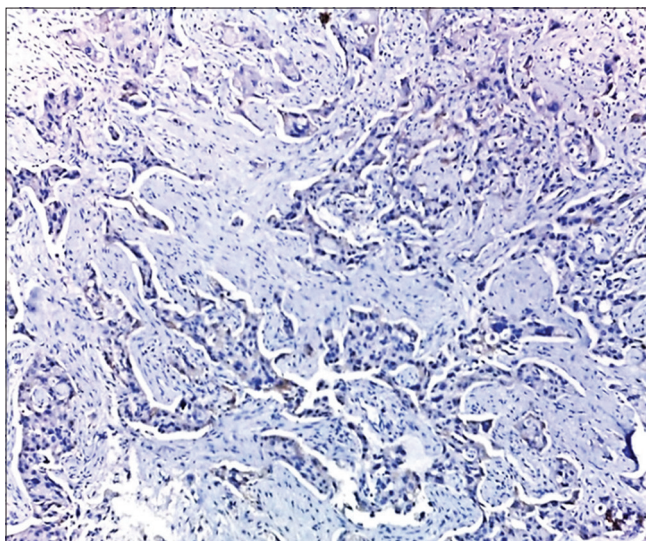


Figure 7: Weak Expression of ALDH1A1 in Invasive Duct Carcinoma Grade II (200× original magnification)

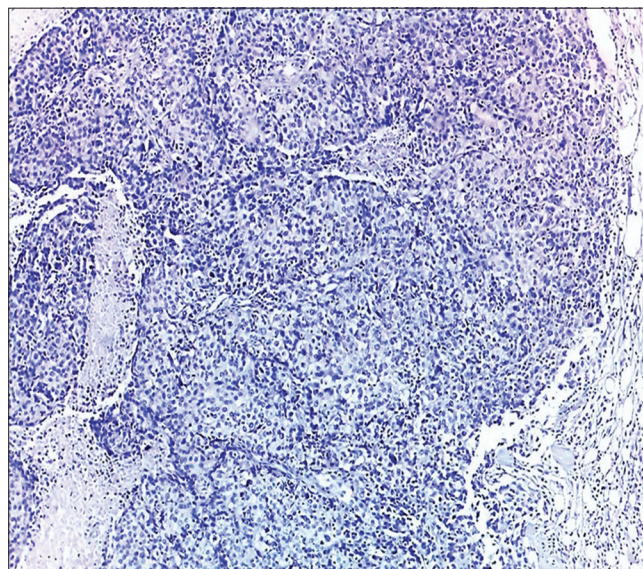


Figure 9: Negative Expression of ALDH1A1 in Invasive Duct Carcinoma Grade II (200× original magnification)

Wen *et al.* [19] showed in a retrospective case study that high histologic grade was more commonly observed in triple-negative breast tumors (68.1%) than in luminal breast tumors (23.5%). High mitotic count and high nuclear pleomorphism were also more prevalent in TNBC, as 44.7% and 78.7%, respectively. In the study done by Hegmane *et al.* [20], no grade I was detected as a finding similar to our study, in contrast, grade III constituted 71%, grade II comprised 25% and 4% were not graded. This discrepancy in

It is widely believed that breast cancer initiates as the pre-malignant stage of atypical ductal hyperplasia, progresses into the pre-invasive stage of DCIS, and culminates in IDC. In this study, *in situ* component was evident in 21 cases (35%). This percentage was much lower in a triple negative study performed in Japan where only 30% of a 97-case study showed intraductal component [21]

Tumors that have more than 50% lymphocytic infiltrate are called lymphocyte-predominant breast cancer (LPBC) and have the best prognosis [22]. In our study, the percentage of LPBC represented 16.7% of total cases. A retrospective study of 897 TNBC patients showed a median percentage of 20% TILs and 21.9% LPBC [23].

Regarding Lymphovascular invasion, only 3 cases (5%) out of sixty studied cases showed



Lymphovascular invasion. These results are quite different from those reported by Agarwal *et al.* [24]

Regarding metastatic tumor deposits to regional lymph nodes, 45 cases (75%) out of the studied sixty cases showed positive axillary lymph node metastatic deposits with the following distribution; N1: 16 cases (26.7%), N2: 25 cases (41.7%), and N3: 4 cases (6.6%).

Interestingly, the majority of TNBCs in the study by Na *et al.* [25] had metastatic deposits in 1-3 axillary lymph nodes (N1; 45.5%). Yet, the remaining cases in that study showed close results to this study; N2: 34.5% and N3: 10%. Other studies reported higher incidence of nodal metastasis as mentioned by Hegmane *et al.* [20] who reported nodal metastasis in 94%.

According to AJCC TNM stage grouping, stage III was the dominant stage Thirty-one cases, constituting (51.67%) of the cases, and only 3 cases were stage I (5%) these agreed with Atwa *et al.* [26] who found that 60% of the patients with stage III. These results are quite different from those reported by Edwin *et al.* [27] stated that the majority of their TNBC cases with stage II.

ALDH1A1 may be used as biomarker to identify breast CSC groups and is correlated with the malignant transformation of breast tissue and progression into a more aggressive triple-negative phenotype [28].

Evaluation of the results of immunostaining for ALDH1A1 in the present study showed that only 7 cases (11.7% of total cases) were negative for immunoreactivity. The remaining 53 cases (88.3% of total cases) showed positive cytoplasmic reactivity.

In the present study, positive ALDH1A1 expression was observed in 88.3% of patients. This is supported by the work of Madjd *et al.* [29] who found expression of ALDH 1A1 in 74% of cases. Atwa *et al.* [26] found ALDH1A1 expression in 66.6% of the patients and ALDH1A1 was expressed in both epithelial tumor cells and stromal cells. Interestingly, in study done by Fei *et al.* [30], a majority of the ALDH1A1+ cases showed low levels of ALDH1A1 expression that presented as focal positive expression, which may be compatible with the idea that CSCs represent only a minority of the tumor cell population. These differences may be attributed to different scoring methods used by the authors.

A rising prevalence of ALDH1A1 expression with increasing age; most patients over 50 years representing 92% was observed in this study; however, no significant correlation was detected between ALDH1A1 and age ( $p = 0.455$ ); this is in agreement with several studies conducted by Resetkova *et al.* [31] and Hosni *et al.* [32].

Regarding tumor size, there was no significant positive correlation between ALDH1A1

expression and tumor size ( $p = 0.544$ ), which is similar to the findings of Madjd *et al.* [29]. However, these findings are in disagreement with results of Atwa *et al.* [26] who reported significant positive correlation between ALDH1A1 expression and tumor size. These outcome differences may be a result of varying sample sizes.

As for the correlation of the ALDH1A1 staining results to the histopathological variables, 88.7% of patients with IDC (NST) showed positive immunostaining. In the study done by Huihui *et al.* [33], ALDH1A1 was positive in 22 of 85 cases (25.9%) of IDC.

Fei *et al.* [30] found ALDH1A1 was positive in 87 of 152 cases (57%) of IDC and 4 of 31 cases (13%) of invasive lobular carcinomas and 1 of 6 cases (16.7%) of other histologic types.

Hosni *et al.* [32] found ALDH1A1 was positive in 8 of 24 cases (33.3%) of IDC and 1 of 3 cases (33.3%) of invasive lobular carcinomas.

Regarding the relation between tumor grade and ALDH1A1 expression, 88.5% of grade II tumors and 87.5% of grade III tumors showed positive immunostaining; however, no significant correlation was seen ( $p = 0.937$ ). Ohi *et al.* [34] showed that ALDH1A1 expression in carcinoma cells was correlated only with high histological grade ( $P < 0.006$ ) and therefore suggest that ALDH1A1 expression in carcinoma cells is an independent prognostic factor in TN breast cancer patients. This was different from other studies conducted by Hosni *et al.* [32]; Atwa *et al.* [26] and Fei *et al.* [30].

The TNBC cases in this study that had intraductal components accounted for 35% of the total cases and 90.5% of these tumors showed positive immunostaining for ALDH1A1. Yet, statistical analysis between ALDH1A1 expression and presence of intraductal components showed no significant correlation. With a similar standpoint, Kurbel [35] proved in a comparative study between TNBCs and non-TNBCs that intraductal components are more evident in luminal invasive cancers and that suggested that the quick tumor progression might be the main feature of the triple-negative breast tumors, leading to less intraductal evidence at the time of breast cancer diagnosis.

The present study revealed non-significant correlation between positive ALDH1A1 expression and other prognostic clinicopathological parameters; Lymphovascular invasion and TILs.

A non-significant correlation was detected between ALDH1A1 expression and nodal status in which ALDH1A1 expression was seen in 88.3% of the total cases ( $p = 0.715$ ). Thayer *et al.* [36] stated that tumors with positive lymph nodes expressed ALDH1A1 more frequently than did node-negative tumors ( $p = 0.006$ ).

Regarding the relation between AJCC TNM stage grouping and ALDH1A1 expression, 88.5% of Stage II tumors and 87.1% of stage III tumors showed positive immunostaining; however, no significant correlation was seen ( $p = 0.801$ ). This is in agreement with other studies conducted by Edvin *et al.* [27] and Atwa *et al.* [26].

Fei *et al.* [30] found that the expression of ALDH1A1 protein evaluated by immunohistochemistry staining in tumor cells significantly correlated with larger tumor size ( $p = 0.02$ ) and advanced stage ( $p = 0.04$ ).

Liu *et al.* [37] examined 15 publications and found that ALDH1A1 expression with large tumor size, higher histological grade, later stage, higher HER2 expression, and lower ER expression.

## Conclusion

In conclusion, the level of ALDH1A1 expression was relatively high in the majority of TNBCs with different staining percentages and intensities as most studies stated. There is no statistical significance between ALDH1A1 expression and other prognostic clinicopathological parameters; age, size, tumor grade, histologic subtypes, lymphovascular invasion, intraductal components, TILs, and TNM stage grouping. Documented statistics for TNBC in Egypt as well as its subsets are recommended to identify their incidence and risk factors.

## Ethical Committee

The project is approved by ethical committee of faculty of medicine, Cairo university.

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