ISSN NO. 2320-5407

*Journal homepage: http://www.journalijar.com*

*INTERNATIONAL JOURNAL OF ADVANCED RESEARCH*

**RESEARCH ARTICLE**

# Immunohistochemical Expression of Aldehyde Dehydrogenase-1 and Hypoxia- Inducible Factor-1α in Breast Cancer

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## *Manuscript Info*

***Manuscript History:***

Received: 15 May 2014
Final Accepted: 26 June 2014
Published Online: July 2014

***Key words:***
Cancer stem cells, Hypoxia, ALDH-1, HIF-1α, Breast cancer, Immunohistochemistry

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## *Abstract*

**Aim of the work:** to evaluate the expression of ALDH-1 and HIF-1α in primary breast cancer and their relation to clinicopathological parameters using immunohistochemical methods.

**Material and Methods**: Fifty paraffin blocks of primary breast cancer immunostained by ALDH-1 and HIF-1α immunohistochemical markers.

**Results:** Increasing expression of HIF-1α was positively associated with nodal metastasis and histological grade, *P* value =0.001 and 0.036 respectively. There was a significant negative association between ALDH-1 expression and ER and PR expression, *P* value = 0.002 and 0.004 respectively. Also, there was a significant negative association between HIF-1α expression and ER and PR expression, *P* value =0.029 and 0.018 respectively.

**Conclusions:** An association was found between ALDH-1 expression and HIF-1α expression in breast carcinomas. Their negative association to hormonal (estrogen and progesterone) receptors suggests that they might play an important role in drug resistance, and may be used as prognostic markers.



## INTRODUCTION

Breast cancer occurs at a high frequency in women worldwide, accounting for 22.9% of all female cancers. In Egypt, it is estimated to be the most common cancer among females accounting for 37.7% of total female cancer, with 12,621 new cases in 2008 [1]. Early detection and anti-cancer treatment increase patient survival. However, cancer relapse and metastasis are the most common cause of mortality in breast cancer patients [2]. Breast cancer is not a single disease. It has multiple histological subtypes or entities (ductal, lobular, etc.). Based on the gene expression profiles, breast cancer has been divided into luminal, basal-like, HER2+ and normal breast-like subtypes. Even within each group, there is considerable variation in clinical outcome and response to therapy [3]. One of the suggested reasons for this variation and therapy resistance is cancer stem cells. There is increasing evidence that human breast cancers are driven by a tumor-initiating cancer stem cell component that may contribute to tumor metastasis and therapeutic resistance [4].

A stem-like cancer cell is defined as a cell with the capacity for self-renewal and the ability to generate different cell types that form a tumor. It has been proposed that aldehyde-dehydrogenase-expressing (ALDH+) breast cells include malignant stem-like cell populations that maintain and cause progression of cancer [5], and it has been suggested that this enzyme is also involved in stem cell preservation and initiation of differentiation [6].

ALDH-1 is a member of the ALDH superfamily responsible for catalyzing aldehyde oxidation (intracellular aldehyde metabolism); it is a cytosolic enzyme expressed in the liver and required for the conversation of retinol (vitamin A) to retinoic acids. It has been shown to be expressed in numerous normal tissues, including breast epithelium, where it appears to be localized to the myoepithelial cells [7]. Among many biomarkers, ALDH-1 appears to be a more effective predictive marker for identifying cancer stem cells (CSCs). Many existing studies have confirmed ALDH-1 expression in primary lesions of breast cancer and its significant association with poor clinical prognosis [8].

Regions of hypoxia are common in breast carcinoma as the rate of nutrient and oxygen delivery is often insufficient to meet the high metabolic demands of neoplastic cells due to the unrestricted proliferation of cancer cells, the formation of immature blood vessels and the impairment of microcirculation induced by inflammatory change in tumor tissue. The neoplastic cells can adapt to this hostile microenvironment using the activation of hypoxia-induced genes for angiogenesis, glycolysis and other processes involved in cell proliferation and survival [9].

HIF-1 is a heterodimer, consisting of a HIF-1α subunit and a HIF-1β subunit. While HIF-1β is constitutively expressed, HIF-1α levels are tightly regulated with rapid upregulation and degradation [10]. Overexpression of HIF-1α is detected in many types of human cancers [11]. Moreover, significant correlations between overexpression of HIF-1α and patient mortality has been shown in certain human cancers, including brain, breast, cervical, oropharyngeal, ovarian and endometrial cancers. In addition, other studies have indicated that HIF-1 mediates resistance to chemotherapy and radiation. These findings suggest that HIF-1α has a crucial role in cancer progression [12,13].

The conversion to a stem-like state can be driven by the epithelial-to-mesenchymal transition (EMT). Microenvironmental changes and signaling, such as tissue hypoxia and hypoxia-inducible factor-mediated signaling, can trigger EMT, thereby promoting metastasis and stemness [14]. It was recently demonstrated that differentiated cells could convert into stem-like cells in normal and neoplastic tissues [15]. Hypoxia-inducible factors (HIFs) mediate the processes involved in oxygen homeostasis. HIFs transcriptionally regulate the expression of target genes and represent a link between oxygen sensors and effectors in the cellular adaptation to hypoxia. Such observations support the idea that tumor hypoxia and hypoxia signaling are important for cancer heterogeneity, aggressiveness, and dissemination [16].

The aim of this work was to evaluate the expression of aldehyde dehydrogenase-1 (ALDH-1) and hypoxia inducible factor (HIF-1α) in primary breast cancer and their relation to clinicopathological parameters using immunohistochemical methods.

## MATERIAL AND METHODS

### *Tissue specimens*

Fifty formalin-fixed and paraffin-embedded female breast cancer tissues with their related data were randomly collected from the archive of Pathology Department, Faculty of Medicine, Zagazig University in the period 2011 - 2013. We obtained the clinical, pathological, and immunohistochemical (estrogen receptor [ER], progesterone receptor [PR]) information from the medical records of the patients. None of the patients had received chemotherapy or radiation therapy before surgery. Histological typing and grading were performed according to the World Health Organization classification and modified Bloom-Richardson grade [17]. Ethical approval and patient consent were obtained.

All paraffin blocks were cut in 4 microns thickness and stained with ordinary H&E stain to confirm the diagnosis.

### *Immunohistochemical procedures*

Immunostaining was performed using the avidin-biotin peroxidase technique. Paraffin sections mounted on coated slides were de-paraffinized with xylene, then the sections were rehydrated through 100 %, 90 %, 70 % and 50 % ethanol. The sections then treated with 0.01 M citrate buffer (pH 6.0) for 30 minutes to unmask antigens before further treatment. After a quick rinse in phosphate buffered saline (PBS), the sections were incubated in 0.3% $H_2O_2$ for 30 minutes to abolish endogenous peroxidase activity (Dako ko411 kit) before blocking with 5% horse serum for 2 hours at room temperature to inhibit the nonspecific immunoreactions.

Primary monoclonal antibodies were incubated overnight in a humidity chamber using the following dilutions: (ADLH-1) anti-aldehyde dehydrogenase-1 (1: 100, clone: 44 from BD Biosciences, CA, USA) and mouse anti-human HIF-1α (1: 300, H1alpha67, Abcame, Cambridge, MA). After washing in PBS they were incubated with biotinylated secondary antibodies for 30 minutes, and then followed by avidin-biotin peroxidase complex for

another 30 minutes, according to the instructions of the manufacturer (Universal Detection Kit, Dako, Denmark). Finally the immune reaction was visualized as a brown color with 3,3 – diaminobenzidine tetra hydrochloride (DAB, Dako K0114 Kit) for 5 minutes, then washed in distilled water. Then the slides were counterstained with Mayer's hematoxylin for one minute before mounting.

The entire procedures were performed at room temperature. Additionally, a negative control for both markers in which the primary antibody was omitted and replaced by phosphate buffered saline was used. In addition, normal liver tissue sections were used as positive control for ALDH-1 and tonsil for HIF-1α were added to be processed with the breast tissue sections in the same run for precision and standardization of the elaborated IHC results of both markers.

The immunostaining was semiquanitatively evaluated by 2 pathologists (TI &SA). Positive expression of ALDH-1 was counted when tumor cells showed cytoplasmic staining. ALDH-1 staining was scored on a scale from 0 to 2 according to percentage of ALDH1-positive tumor cells: 0 – negative (0–5% of tumor cells showed ALDH-1 staining); 1 – weakly to moderately positive (5–50% of tumor cells showed ALDH-1 staining); and 2 – strongly positive (more than 50% of tumor cells showed ALDH-1 staining) [18].

HIF-1α was present in the cytoplasm and/or nuclei in a homogeneous pattern in tumor cells. The results were interpreted according to staining intensity and were scored as 0, 1+, 2+, or 3+. Any intensity of staining was considered positive [19].

***Statistical analysis***

Data were represented as numbers and percentages. The differences were compared for statistical significance by chi – square ($X^2$) test. Difference was considered significant at $P < 0.05$. The statistical analysis was performed using SPSS 16.0 for windows (SPSS Inc, Chicago, Illinois, USA).

## RESULTS

***Patients and their clinicopathological parameters***

Fifty female patients were submitted for this study, 60% of cases were equal or more than 50 years old while 40% were less than 50 years old. Invasive ductal carcinoma was diagnosed in 42 (84%) of patients and grade 2 was the most frequent (48%) histological grade. Lymph node metastasis was positive in 29 (58%) of our patients. The positive rates of ER and PR were 70% and 68% respectively (Table 1).

***ALDH-1 expression and localization***

Expression of ALDH-1 was defined as positive when tumor cells showed cytoplasmic staining. ALDH-1 expression was detected in 10 cases (20%), among these 9 cases showed weak to moderate expression and one case showed strong expression (Figure 1; Tables 1,2).

***Associations between ALDH-1 expression and clinicopathological parameters***

There was no association between ALDH-1 expression in primary breast tumors and patient's age, tumor size, lymph nodes, histological type and histological grade. However, there was a significant negative association between ALDH-I expression and ER and PR expression. *P* values= 0.002 and 0.004 respectively.

***HIF-1α expression and associations with clinicopathological parameters***

HIF-1α expressed in the cytoplasm and /or nuclei of malignant cells, it is detected in 32 cases (68%), 18 cases showed moderate to low expression and 14 cases showed high expression of HIF-1α (Figure 2).

HIF-1α expression was not associated with age, tumor size, clinical stage or histological type of primary breast cancer. Increasing expression of HIF-1α was positively associated with nodal metastasis and histological grade, *P* value = 0.001 and 0.036 respectively.

There was a significant negative association between HIF-1α expression and ER and PR expression, *P* value = 0.029 and 0.018, respectively (Table 1).

***Associations between ALDH-1 expression and HIF-1α in the studied cases***

There was a positive association between ALDH-1 and HIF-1α expression, where HIF-1α positivity was observed in 100% of ALDH-1 positive tumors and 55% (22 cases out of 40 cases) of ALDHI-negative tumors. Whereas, all HIF-1α negative tumors were ALDH-1 negative (*P* value =0. 008) (Table 2).

**Table (1): Association between ALDH-1 and HIF-1α with clinicopathological parameters**

| Parameter | ALDH-1 | | *P* value | HIF-1α | | *P* value |
|---|---|---|---|---|---|---|
| | Negative (n=40) | Positive (n=10) | | Negative (n=18) | Positive (n=32) | |
| ***Age (years)*** | | | | | | |
| **<50 N=20** | 14 | 6 | | 9 | 11 | |
| **≥50 N=30** | 26 | 4 | 0.149 | 9 | 21 | 0.279 |
| ***Tumor size (cm)*** | | | | | | |
| **<2cm N=21** | 18 | 3 | 0.390 | 10 | 11 | 0.145 |
| **>2cm N=29** | 22 | 7 | | 8 | 21 | |
| ***Grade*** | | | | | | |
| **I N=16** | 14 | 2 | | 2 | 14 | |
| **II N=24** | 20 | 4 | 0.199 | 10 | 14 | 0.036 |
| **III N=10** | 6 | 4 | | 6 | 4 | |
| ***Histological type*** | | | | | | |
| ***Ductal N=42** | 35 | 7 | | 15 | 27 | |
| ***Lobular N=4** | 3 | 1 | 0.272 | 2 | 2 | 0.759 |
| ***Mixed N=4** | 2 | 2 | | 1 | 3 | |
| ***Lymph node metastasis*** | | | | | | |
| **Positive N=29** | 25 | 4 | 0.197 | 5 | 24 | 0.001 |
| **Negative N=21** | 15 | 6 | | 13 | 8 | |
| ***Estrogen receptor (ER)*** | | | | | | |
| **Negative N=15** | 8 | 7 | 0.002 | 2 | 13 | 0.029 |
| **Positive N=35** | 32 | 3 | | 16 | 19 | |
| ***Progesterone receptor (PR)*** | | | | | | |
| **Negative N=16** | 9 | 7 | 0.004 | 2 | 14 | 0.018 |
| **Positive N=34** | 31 | 3 | | 16 | 18 | |

**Table (2): Expression of ALDH-1 and HIF-1α in cancer breast patients**

| | HIF-1α negative (n=18) | HIF-1α positive (n=32) | $\chi^2$ | P value |
|---|---|---|---|---|
| ALDH-1 negative (n=40) | 18 | 22 | 7.03 | 0.008 |
| ALDH-1 positive (n=10) | 0 | 10 | | |

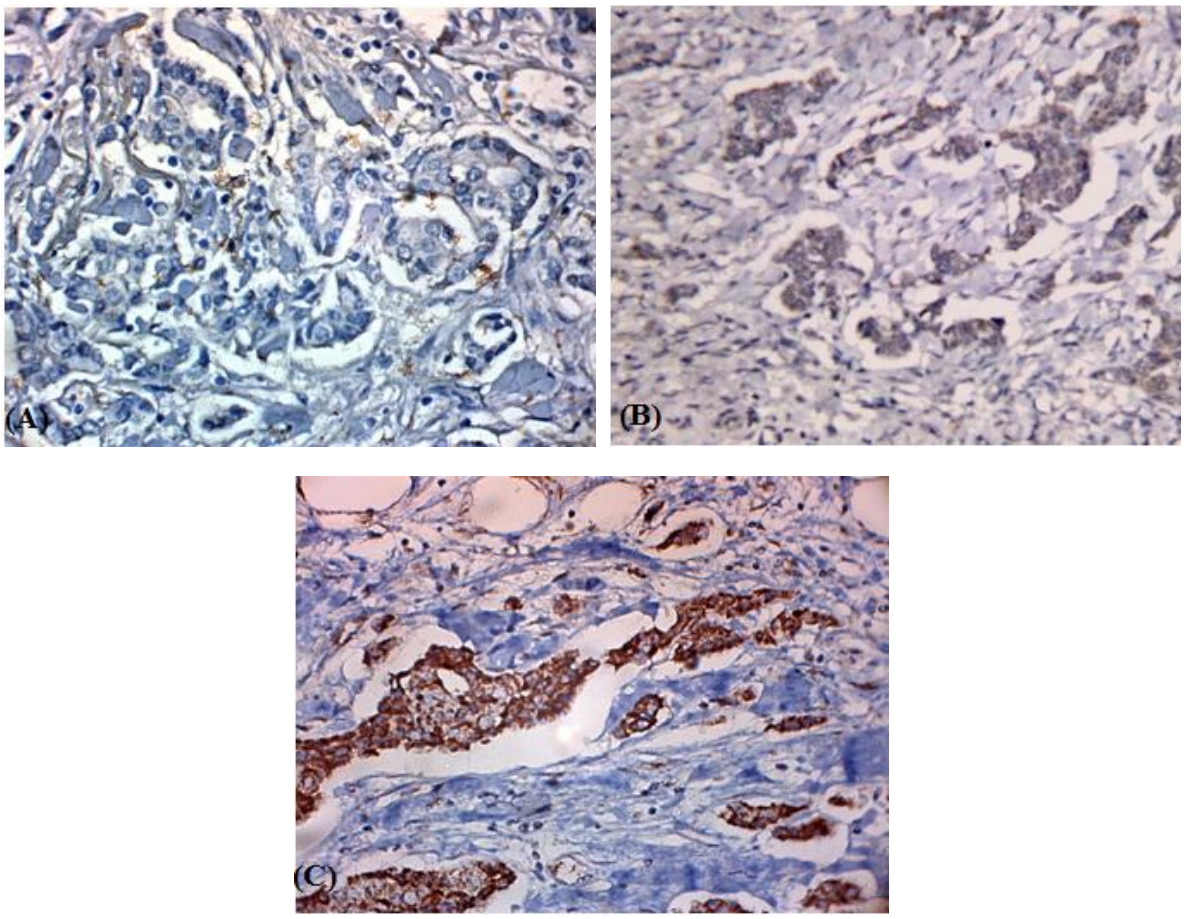


**Figure (1): ALDH-1 expression in breast carcinoma; A) Negative expression, score 0, B) Invasive carcinoma with moderate positive cytoplasmic expression, score 1, C) Invasive ductal carcinoma with more than 90% of cells showing positive cytoplasmic expression of ALDH-1, score 2.** ***Magnification of all samples (ABC, DAB chromogen X200)***

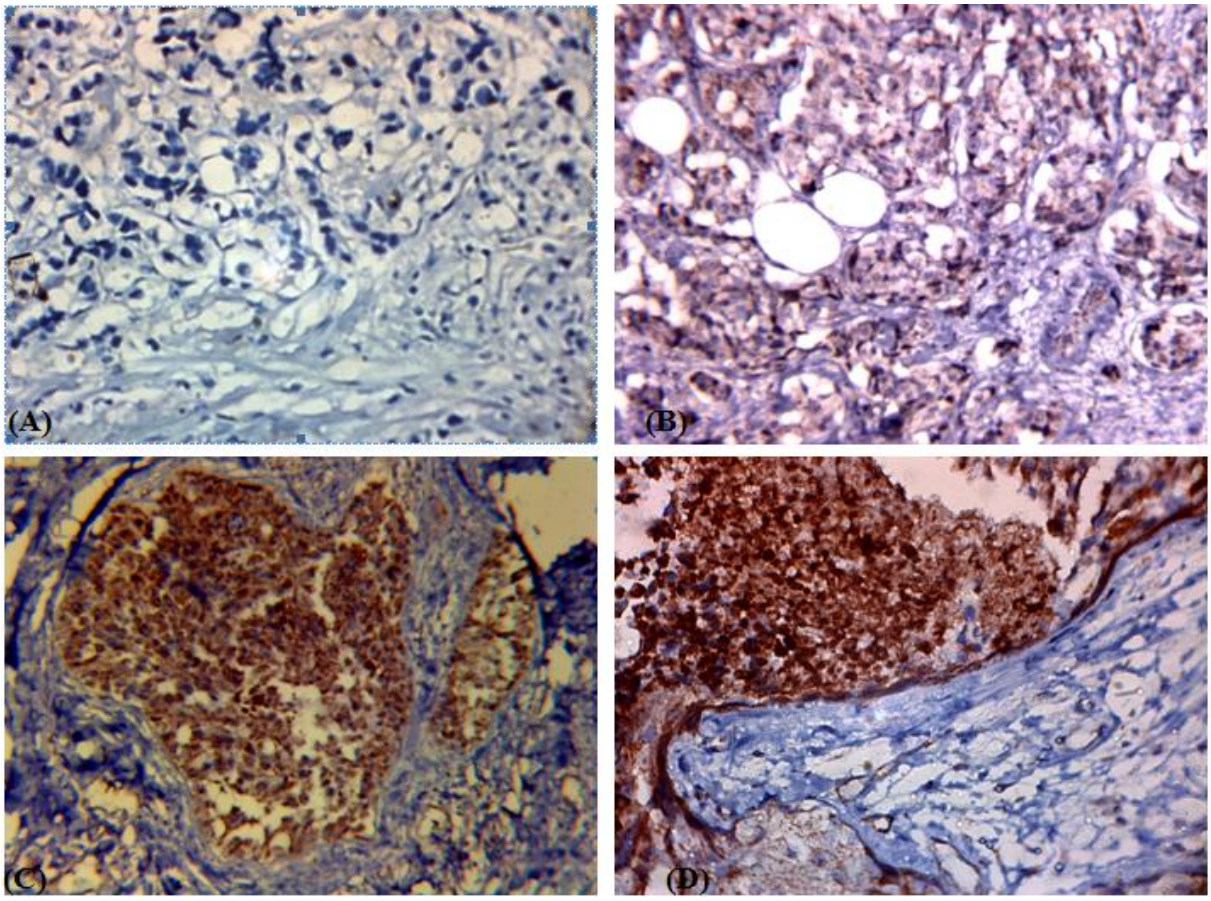


**Figure (2): HIF-1α (cytoplasmic and/or nuclear) expression in breast carcinoma, according to staining intensity. Tumors classified as 1+ to 3+ were considered positive HIF-1α expression. (A) Negative expression; (B) Weak positive (1+) expression; (C) Moderate positive (2+) expression; (D) Strong positive (3+) expression.** ***Magnification of all samples (ABC, DAB chromogen X200*****)**

## DISCUSSION

Breast cancer is a disease with an adverse prognosis. The presence of stem-like cells or tumor-initiating cells within tumors, defined as ALDH-positive or CD44+/CD24- cells, has been proposed as a relevant factor for treatment resistance as well as disease recurrence and dissemination [19,20]. The development of more effective cancer therapies may, thus, require targeting this important cancer stem cell population. Ginestier and colleagues showed that stem cell–like populations in breast tissue are characterized by the expression of ALDH-1, and breast cancer stem cells have been isolated on the basis of increased ALDH1 expression. This group further demonstrated that in the breast, ALDH-1 expression is considered to be a marker of both normal and malignant stem and progenitor cells [21].

The role of low oxygen tension in the resistance of cancer to therapy has been described for decades. Tumor hypoxia has long been considered to be a potential therapeutic problem because hypoxic cells are resistant to chemotherapy and radiation. Hypoxia is also associated with a more malignant tumor phenotype with increased potential for invasion and metastasis. Immunohistochemical assessment of endogenous hypoxia markers is a simpler method and assessable in archival tissue material [22,23].

Growing evidence highlights the role of hypoxia in stem cell dynamics in cancer progression. For example, cells in hypoxic tumor regions stabilized hypoxia- inducible factors (HIFs) and activated the expression of adaptive genes, such as MDR1, ABCG2, telomerase, Notch and c- Myc, that could promote further dedifferentiation and enhance stem cell-like properties, such as self-renewal [24,25]. In this work ALDH-1 expression was detected in 20% of our cases. Previous immunohistochemical analyses have shown that ALDH- positive cells varied from 12%

to 34% of cases [19,20,26]. Patients with different stages of the disease and different scoring methods may explain the differences among studies.

In the present study, there was no association between ALDH-1 expression in primary breast tumors and patient's age, tumor size, lymph nodes, histological type and histological grade. These results are consistent with a study by Dong et al. that reported that they did not find in their study any significant association between ALDH1-positivity in primary breast cancer and clinicopathological parameters [18]. However, there was a significant inverse relation between ALDH-1 expression and ER and PR expressions, *P* value =0.002 and 0.004 respectively

Approximately two-thirds of human breast cancers express the estrogen receptor, and the estrogen receptor status of a patient is highly predictive of response to antiestrogen therapy. In our study, we observed that ALDH-1 positive tumors were associated with absence of estrogen and progesterone receptor expression ($P < 0.05$). The data implicated that patients with a negative ALDH-1 status showed significant benefit from antiestrogen, whereas there was no significant benefit from antiestrogen in patients with positive ALDH-1 status. These results are in accordance with previous studies [20,21]. It was demonstrated that the acquired drug resistance was associated with the transcriptional activation of ALDH-1 expression in the cells. Thus, it was expected that ALDH-1 would be associated with poor prognosis in patients who received chemotherapy [21].

In this work HIF-1α expression detected in 32 cases (68%). Tiezzi et al. found HIF-1α expression in 38/75 (51%) [19]. Yamamoto et al. found in their study overexpression of HIF-1α in 63 (36.8%) out of 171 invasive breast cancer [27].

Correlations between HIF-1α expression and clinicopathological factors were also examined. We found that it was not associated with age, tumor size or histological type of primary breast cancer. Increasing expression of HIF-1α was positively associated with nodal metastasis and histological grade, *P* value =0.001 and 0.036 respectively. These results are consistent with that of Yamamoto et al. [27].

The correlation between HIF-1α expression and nodal status in breast cancer is controversial. Bos et al. did not show any evidence for a positive correlation between these two parameters [28]. In contrast, Giatromanolaki et al. reported a positive correlation between HIF-1α expression and nodal status [29]. In oropharyngeal cancer, it was shown that increased levels of HIF-1α correlated with lymph node metastasis [12]. The relationship between HIF-1α expression and tumor grade is also controversial. Bos et al. reported that increased levels of HIF-1α were positively related to tumor grade [28]. It is well accepted that hypoxia induces genetic alterations in tumor cells, allowing them to adapt to hypoxic conditions. Such genetic alteration also promotes morphological change of tumor cells and their nuclei. Consequently, these findings suggest that HIF-1α positive tumors are of a higher tumor grade than HIF-1α negative tumors. In this study, there was a significant negative association between HIF-1α expression and ER and PR expression, *P* values= 0.029 and 0.018 respectively; these results are consistent with Yamamoto et al. [27].

The status of two central steroid hormone receptors, namely ER and PR, is the most important thing in determining the relevance of hormonal therapy in patients with breast cancer. Kurebayashi et al. have reported that hypoxia reduced the expression levels of ER in a time dependent manner in human breast cancer cell lines, and also significantly reduced the growth-promoting effect of estoradiol and the growth-inhibitory effects of an antiestrogen in these cell lines [30]. Furthermore, they showed that ER expression was significantly lower in nuclear HIF-1α positive tumors than negative tumors. These findings suggest that these processes may lead to an acquired resistance to hormonal therapy in breast cancer. Also Generali et al. reported that tissue oxygen tension less than 20–30 mm Hg has been directly associated with increased resistance to radiation and chemotherapy [23].

In this study, there was a positive association between ALDH-1 and HIF-1α expression in primary breast cancer (*P* value =0.008). These results are in accordance with that of Tiezze et al., stating that a positive association was observed between ALDH-1 and HIF-1α expression ($P = 0.03$) [19].

The role of HIFs in controlling the stem-like cancer cell population has become evident in recent publications. Louie et al. demonstrated that the stem-like breast cancer cell subpopulation could be expanded through repetitive hypoxia/reoxygenation cycles without genetic manipulation [31]. These data support previous experimental findings that HIF signaling can control the ‘‘stemness’’ phenotype [32,33]. Therefore, the use of HIF-targeted therapy may be effective in reducing or eradicating stem-like cells, thereby improving overall survival.

## CONCLUSIONS

ALDH-1 immunostaining is a simple method for identifying breast cancer stem cell like populations. This marker may facilitate the application of cancer stem cell biology to the clinical practice. It may be useful to study the expression of HIF-1α using immunohistochemical analysis for better understanding of the tumor characteristics of breast cancer. An association was found between ALDH-1 expression in tumor cells and HIF-1α expression in breast carcinomas. There was a negative association between the expression of ALDH-1 and HIF-1α and hormonal

(estrogen and progesterone) receptors, so they might play an important role in drug resistance, and may be used as prognostic markers.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

TI, SA, AH: Provision of study material, design, tissue processing techniques, collection and assembly of data; TI, SA: Examination and histologic grading of the pathologic specimens. AH: Sharing in identification of the normal tissues and statistical analysis of results. TI wrote the manuscript; SA and AH critically revised the manuscript. All authors have read and approved the final manuscript.

**Acknowledgements**

The authors thank Prof. ***Yehia El-Alfy*** Head of pathology dept. and members of pathology and anatomy departments for their facilitation and invaluable assistance throughout the work.

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