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Immunohistochemical Study of Stem Cell Marker ALDH1 and BRCA1 in Breast Cancer

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Abstract: BRCA1 gene is responsible for differentiation and maturation of mammary stem cells. Knockdown of this gene leads to evolution of a cell population with stem cell like characters (cancer stem cells) that leads to the emergence of breast cancer, mostly with poor prognosis. This cell population can be highlighted by the stem cell marker ALDH1. This study focused on detecting a possible relation between BRCA1 mutation and emergence of a considerable population of ALDH1 positive cancer stem cells and the correlation of this cell population with cancer prognostic factors. Thirty cases of female breast cancer were evaluated immunohistochemically for the expression of both ALDH1 and BRCA1 in the malignant epithelial cells and correlation of ALDH1 expression with clinic-pathological parameters was evaluated (age, positive family history for breast cancer, history of OCP intake, menopausal status, lactational history, tumor size, tumor type, tumor grade and lymph node status). The results indicated that nine cases out of 30 (30%) showed positive cytoplasmic expression for ALDH1 in malignant epithelium. BRCA1 expression was positive in 18 cases (60%). Concomitant positive expression of both ALDH1 and BRCA1 was detected in 2 cases (11.1%). The relationship between positive ALDH1 expression and clinic-pathological parameters were all non-significant. However the relationship between positive ALDH1 expression, positive Her2 and negative ER, PR expression, was highly significant. We also found that the protein expression profile; (ALDH1-, BRCA+, ER+, PR+, HER2-) was correlated with good prognosis and outcome of tumor while (ALDH1+,BRCA-, ER-, PR-, HER2+) was correlated with worse prognosis as well as tumor outcome. The current study revealed a significant inverse correlation between expression of ALDH1 and BRCA1 and established phenotypes combining expression of ALDH1, BRCA1, ER, PR and HER2, which correlate with prognosis and outcome. The importance of this and related studies emphasizes the possible utility of ALDH1 as a bio-marker to screen family members at risk for BRCA1 mutation testing, utility as a prognostic marker owing to the fact that its expression correlates with basal type phenotype. In addition, ALDH1 might be a possible therapeutic target in breast cancer as well. All these are feasible provided we standardize method of evaluation of immunohistochemical expression of ALDH1.

Key words: ALDH1 · BRCA1 · Breast cancer · Stem cells

INTRODUCTION

Stem cells play a role in repopulating the breast at several points in the human female lifespan. These primitive cells facilitate rapid expansion and regression in puberty and pregnancy and during the menstrual cycle. Mammary stem cells have been isolated, by evaluation of specific characteristics like multipotency, the ability to undergo both symmetrical and asymmetrical divisions as well as being long-lived, slow cycling cells [1]. Al-Hajj *et al.* [2] showed that only a small subpopulation of all cells in a tumor could be serially passaged, indicative of their tumor initiating capacity. These cells share many characteristics with stem cells and are therefore denoted cancer stem cells (CSC). Evidence has recently been accumulating to support the cancer stem cell hypothesis for solid tumors, including breast cancer, which holds that cancers are driven by a small subpopulation of stem cells that are capable of self-renewal and give rise to multipotent progenitor cells that

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ultimately differentiate into all cell types within the tumor [3]. The stem cell-like cells, designated as cancer stem cells, represent a minor subset of cells in the tumor and are distinct from the more differentiated tumor cells which may play an important role in cancer establishment, progression and resistance to current treatments. Traditional cancer therapies are effective at debulking some tumors but often fail to produce long-term clinical remissions, possibly due to their inability to eradicate the cancer stem cell population. Therefore, novel treatments aimed at targeting the cancer stem cell population could find use in treating both primary and metastatic tumors [4].

Several markers have been identified for the selection of human (cancer) stem cells, of which Aldehyde dehydrogenase 1 (ALDH1) is among the most widely studied ones. ALDH1 is a cytosolic detoxifying enzyme responsible for the oxidation of (retin) aldehydes into retinoids [5]. In 2003, Al-Hajj *et al.* [2] distinguished tumorigenic from non-tumorigenic cancer cells using cell surface markers CD44 and CD24 in breast tumors. Consequently, Ginestier *et al.* [6] described that ALDH1 may be a better marker for characterization of breast cancer stem cells as fewer ALDH1+ tumor cells than CD44+ and CD24-tumor cells were required to produce tumors in immunodeficient mice. Expression of ALDH1 has been seen in stromal cells as well as epithelial cells of breast tumors, which might be associated with good outcome, concluding that tumor environment plays a crucial role in determining the prognostic impact of stem cells [7,8]. However, Ginestier *et al.*[6] stated that the exact function of ALDH1 in (mammary) stem cells remains largely unknown, but it is thought to play a role in cellular differentiation, mainly through the retinoid signaling pathway and in contrast to above statement he found that breast cancers with $ALDH1^+$ cancer stem cells are associated with biologically aggressive phenotypes such as estrogen receptor (ER) negativity, high histological grade, human epidermal growth factor receptor type 2 (HER2) positivity, as well as poor prognosis [9]. Germline mutation carriers of the BRCA1 gene locus harbor a high cumulative risk of developing breast and ovarian cancer of 57% and 40% by age 70, respectively [10]. BRCA1 related breast cancer shows a distinct histopathological and immunohistochemical phenotype. It has been shown to be more often of the ductal or medullary types, of high grade and to show a high mitotic activity index (MAI) and necrosis [11].These tumors usually do not express the estrogen (ER) and progesterone receptors (PR) and are almost always HER-2/neu negative ("triple negative") [12].

Increasing evidence indicates that BRCA1 is necessary for mammary stem cell differentiation, a function that could explain its tissue-specificity [13]. Knockdown of BRCA1 in primary breast epithelial cells leads to accumulation of cells expressing ALDH1 and a decrease in ER positive cells expressing luminal epithelial markers. Furthermore, in the normal tissue of BRCA1 mutation carriers, clusters of ALDH1 positive cells have been described that was ER negative and showed loss of heterozygosity (LOH) of BRCA1. BRCA1 might indeed serve as a stem cell regulator in the mammary epithelium and that the stem cell pool in the normal tissue of BRCA1 mutation carriers might be enlarged [14]. Cancer stem cells (CSCs) could be important therapy targets, due to their tumor initiating capacity and being therapy resistant and could be as well a monitor to prognosis and patient outcome. Therefore the aim of the study is to correlate immunohistochemical staining of ALDH1 stem cell marker and BRCA1, in malignant epithelium of breast cancer, with other clinic-pathological characteristics in a random sample of Egyptian females, to rule out a possible prognostic and therapeutic utility.

MATERIALS AND METHODS

Study Design: Retrospective cross-sectional study. Tumor tissue samples were obtained from paraffin blocks of 30 breast cancer patients (age range between 27 and 62 years), who underwent radical mastectomy or breast-conserving surgery between January and December 2012 at Kasr El-Ainy University Hospital, Cairo, Egypt. These tumor tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Clinical data of the patients were collected from the files (age, menopausal state, breast feeding, parity and family history of breast cancer), as well as histopathological data (tumor size and lymph node metastasis).

Histopathological Sudy: Formalin-fixed paraffin embedded sections (4 μm thick) were mounted on glass slides and subjected to H and E staining and then slides were examined pathologically to revise histopathological type and tumor grade. The already available ER, PR and Her2 immunohistochemical staining status for each case were retrieved from patient's files (initially done as a routine prognostic and theraputic step).

Immunohistochemical Staining: Formalin-fixed paraffin embedded sections (4 µm thick) of the tumor tissues were mounted on charged slides and subjected to immunohistochemical staining with the avidin-biotin-peroxidase method. Sections were then counterstained with hematoxylin. Semi-quantitative subjective assessment of independent observers was used in this study. The staining of the tissue sections was evaluated by two investigators. Cytoplasmic expression of ALDH1 in the malignant epithelial component was evaluated, whereas nuclear staining alone was considered nonspecific. Stromal immunoreactivity in the neoplastic growth and adjacent non-neoplastic breast tissue was observed as well, but not incorporated in scoring ALDH1 expression. The expected pattern of BRCA1 expression in neoplastic epithelial cells was either nuclear, cytoplasmic, or combined nuclear and cytoplasmic staining. For both ALDH1 and BRCA1, positivity in malignant epithelium will be considered only with moderate or strong staining in more than 10% of tumor cell population (which is equivalent to H-score ≥ 4 evaluated in some other series). The state of estrogen and progesterone immunoreactivity according to Quick score system was recorded from patient's files. Score for proportion of cells with positive staining (0= no staining, 1=< 1% nuclear staining, 2=1-10% nuclear staining, 3= 11-33% nuclear staining, 4=34-66% nuclear staining, 5=67-100% nuclear staining) and score for intensity (0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining). The scores are summed to give maximum of 8. Patients with scoring 2 or less are regarded as ER and PR negative and those with scoring above two are regarded as ER and PR positive [15]. Immunohistochemical staining for HER2 was scored according to both the intensity and the number of cells stained. No staining or membrane staining in < 10% was scored as 0; weak staining of the membrane in > 10% of cells was scored as 1+; moderate membrane staining in > 10% of cells was scored as 2+ and strong membrane staining in > 10% of cells as 3+. Cytoplasmic staining was excluded. A score of 2+ or 3+ was regarded as 'positive' and a score of 0 or 1+ as 'negative' [16].

Statistical Analysis: IBM SPSS software version 20 was used for all statistical analyses. Associations between ALDH1 status, BRCA1 status and clinic-pathological parameters were assessed with the Paired student test to compare means. Pearson Chi square test, Fischer Exact test and Kappa agreement were used to detect significance of correlations, 2-sided significance for each correlation was considered. All *P*-values of <0.05 resulting from two-sided tests were considered significant.

RESULTS

In this study the total cases were 30 female breast cancer patients, whose age range was between (27-62) years with mean age 41.4 years ± SD 9.1. Three patients were menopausal (3/30) (10% of all cases). Nine patients were nullipara (9/30) (30% of all cases). 17 patients (56.7%) had history of breast feeding. Patients having positive family history were 14 (46.7%). Females with history of oral contraceptive pills intake were 16 (56.7%). Invasive duct carcinoma constituted 24 cases (80%) of all the studied cases, while invasive lobular and mixed carcinoma (lobular and duct) constituted 3 cases each. Tumor grade II constituted the majority of all the studied cases, 22 cases (73.3%). Tumor sizes ranged from 1.5 to 13.5 cm with a mean 4 ± 2.5 cm. Twenty two cases had lymph node metastasis (73.3%). ER positive expression was detected in 19 (63.3%) of total cases, PR positive expression was detected in 19 (63.3%) of total cases and HER2 positive expression was detected in 11 cases (36.7%) of total cases. Nine cases out of 30 (30% of all cases) showed positive cytoplasmic expression for ALDH1 in malignant epithelium. Positivity of ALDH1 in neoplastic stroma was observed as well in 16 cases (53.3%), 14 cases (46.6%) showed stromal positivity in both neoplastic and adjacent non-neoplastic compartments, especially in lobular stroma. All 16 cases were negative for malignant epithelial ALDH1 expression except for one (3%); however this finding was not incorporated in scoring ALDH1 expression in each case (Fig. 1). BRCA1 immunohistochemical expression was positive in 18 cases (60%) (Fig. 2). Concomitant positive expression of both ALDH1 and BRCA1 was detected in 2 cases (11.1%) of total cases. The relationship between positive epithelial ALDH1 expression and clinicopathological parameters; age, positive family history for breast cancer, history of OCP intake, menopausal status, lactational history, tumor size, tumor type, tumor grade and lymph node status; were all non-significant (Table 1). The relationship between positive ALDH1 expression and negative ER, PR was highly significant and positive ALDH1expression with positive HER2 was highly significant e.g. predominant protein expression profile was (ALDH1-, ER+, PR+, HER2-) and vice versa (Table 2).

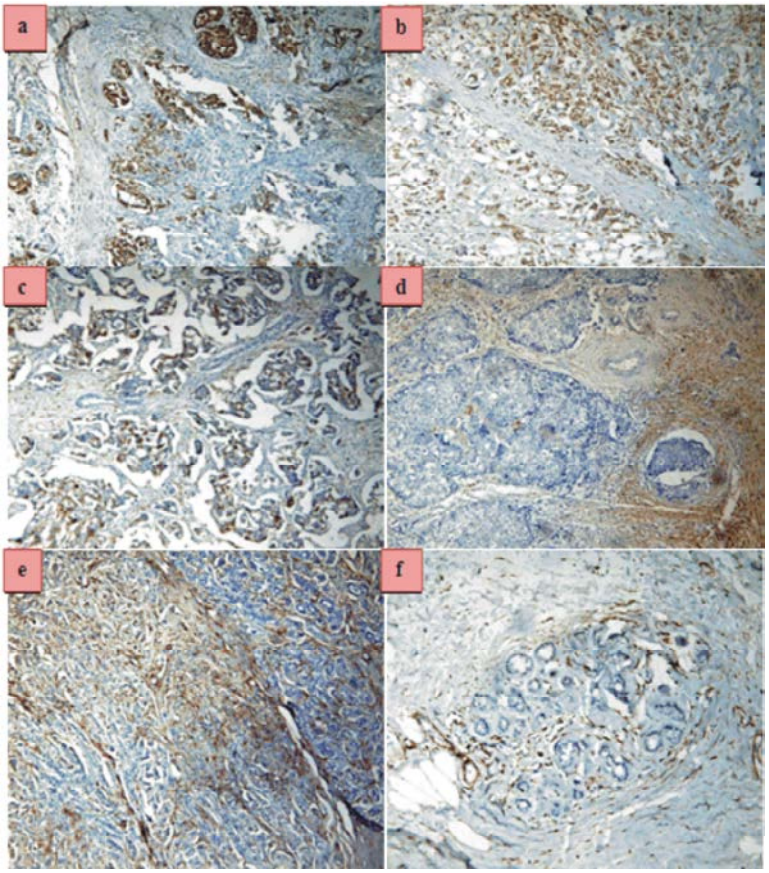

Fig. 1: Positive epithelial expression for ALDH1 in invasive breast carcinoma (a, b c). Negative epithelial expression with concomitant strong positive stromal expression for ALDH1 (d). Positive stromal expression in lobular stroma, whether with adenosis (e) or normal (f).

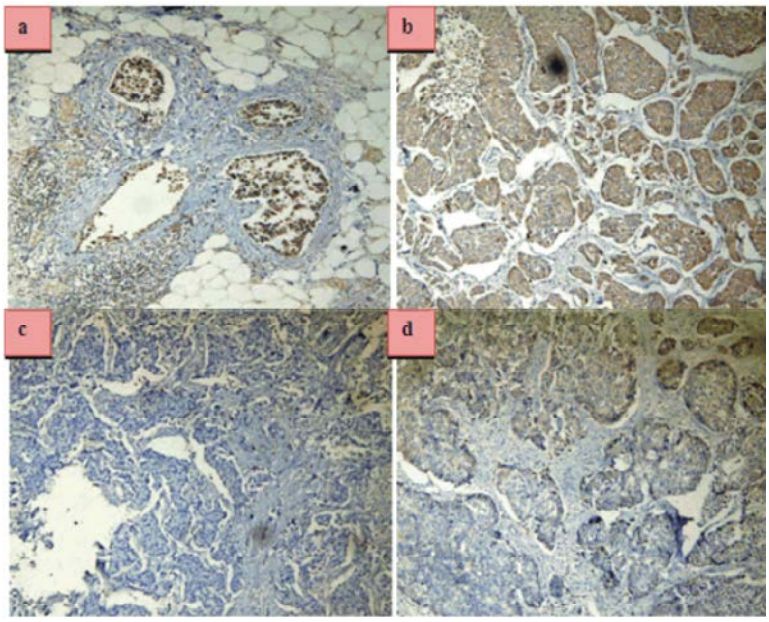

Fig. 2: Positive epithelial expression for BRCA1 in invasive breast carcinoma (a, b). Negative epithelial expression for BRCA1, absent staining (c) and faint focal staining (d).

Table 1: The relationship between ALDH1 expression and clinic-pathological parameters

Item	ALDH1+	ALDH1-	Kappa value	P-value	Significance
LN metastasis +	6	16	000	0.157	NS
LN metastasis-	3	5			
Tumor grade 1	1	2	0.115	0.199	NS
Tumor grade 2	6	16			
Tumor grade 3	2	3			
Duct carcinoma	8	16	0	0.199	NS
Lobular carcinoma	1	2			
Mixed carcinoma	0	3			
Family history +	3	11	0	0.157	NS
Family history-	6	10			
OCP intake +	4	12	0	0.157	NS
OCP intake-	5	9			
Lactation history +	5	12	0	0.157	NS
Lactation history-	4	9			
Nullipara	3	6	-333	0.157	NS
Multipara	6	15			
Menopausal	0	3	0	0.157	NS
premenopausal	9	18			
Mean age	40.5556	39.1111	--	0.674	NS
Mean size	4.3556 cm	3.8444 cm	--	0.686	NS

Table 2: The relationship between ALDH1 expression and ER, PR and Her2 immunohistochemical expression

	ALDH1					
	+VE (n=9)		-VE (n=21)			
Item	N	%	N	%	Kappa value	P value
ER						
+VE	0	0.0	19	90.5	-0.687	<0.005
-VE	9	100.0	2	9.5	--	HS
PR						
+VE	0	0.0	19	90.5	-0.687	<0.005
-VE	9	100.0	2	9.5	--	HS
Her 2						
+VE	9	100.0	2	9.5	0.851	<0.005
-VE	0	0.0	19	90.5	--	HS

Table 3: Correlation between BRCA1, ALDH1 and hormone receptors expression

	BRCA1					
	+VE (n=18)		-VE (n=12)			
Item	N	%	N	%	Kappa value	P value
ER						
+VE	16	88.9	3	25.0	0.648	<0.005
-VE	2	11.1	9	75.0	--	HS
PR						
+VE	16	88.9	3	25.0	0.648	<0.005
-VE	2	11.1	9	75.0	--	HS
Her2						
+VE	2	11.1	9	75.0	-0.582	<0.005
-VE	16	88.9	3	25.0	--	HS
ALDH1						
+VE	2	11.1	7	58.3	-0.420	0.005
-VE	16	88.9	5	41.7	--	HS

We also noticed highly significant relationship between positive BRCA1 expression and positive ER, PR, as well as positive BRCA1 expression with negative Her2 and ALDH1 expression e.g. predominant protein expression profile was (ALDH1-,BRCA1+, ER+,PR+,HER2-) and vice versa (Table 3).

DISCUSSION

Among several markers which have been identified for the characterization of cancer stem cells, ALDH1 is one of the most widely reported ones [8,17-18]. It has been also suggested that knockdown of BRCA1 function in primary breast epithelial cells leads to an increase in cells expressing CSC marker ALDH1 [14]. The aim of the study was to correlate immunohistochemical staining of ALDH1 stem cell marker and BRCA1 related cases with clinicopathological characteristics of breast cancer in a random sample of Egyptian females. The current study revealed non-significant correlation between positive ALDH1 expression and other prognostic clinicopathological parameters; age, positive family history for breast cancer, history of OCP intake, menopausal status, lactational history, tumor size, tumor type, tumor grade and lymph node status. This is in agreement with several studies conducted by Resetkova *et al.* [8], Zhou *et al.* [18] and Madjd *et al.* [19] who were unable to verify significant correlations between intra-tumoral epithelial expression of ALDH1 and patient's age, tumor grade, lymph node metastasis or tumor size. Moreover, Madjd *et al.* [19] attributed this to limited sample size and therefore warranted further investigation with a larger number of samples and also they stressed that there is no agreement on the scoring method and cut-off used for ALDH1 positivity. This might be the same causes for the non-significant relations evaluated in the current study. On the other hand, Yoshioka *et al.* [20] in a study performed on 257 invasive ductal carcinomas (IDCs) showed that ALDH1 expression was correlated with larger tumor size in node-positive breast cancers, the large sample size in this study might have led to the significant correlations detected. In the current study we noticed positive expression of neoplastic stroma in 53.3% of cases, the majority (46.6%) showed concomitant expression in adjacent non neoplastic stroma especially lobular, this percent is slightly lower than that stated by Madjd *et al.* [19], who found moderate to strong stromal expression of ALDH1 in 74% of cases, which might be attributed to different positivity scoring methods adopted by different authors. The fact that all cases (except 1) were negative for epithelial ALDH expression and positivity was seen in both neoplastic and non-neoplastic, especially lobular stroma emphasizes that there is no association between epithelial and stromal ALDH1 expression. Heerma van voss *et al.* [21] stated that the biological role of ALDH1 apart from its potential role in stem cells and cellular differentiation might be another explanation for diverse epithelial and stromal ALDH1 expression. That is why we focused in this study on malignant epithelial expression only as a possible prognostic and therapeutic target. The current study revealed a significant inverse correlation between expression of ALDH1 and BRCA1. Consistent with prior published data by Madjd *et al.* [19], who found a significant inverse association between expression of ALDH1 and BRCA1 indicating that ALDH1 positive tumors are more likely to lose or express low level of BRCA1. This supports the idea that BRCA1 mutated breast cancers contain an enlarged CSC component. Liu *et al.* [14] suggested that loss of BRCA1 function in primary breast epithelial cells leads to accumulation of cells expressing ALDH1. Heerma van voss *et al.* [21] stated that intra-tumoral epithelial ALDH1 expression was clearly more present in BRCA1 mutation carriers, implying that this population indeed has an enlarged CSC component. He pointed out that ALDH1 tumor cell expression was an independent predictor of BRCA1 mutation status in a case-control study, in which they compared ALDH1 expression in malignant tissue of 41 BRCA1 related breast cancers with 41 age-matched sporadic breast cancers. Madjd *et al.* [19] concluded that loss of BRCA1 expression is a marker of tumor aggressiveness, potentially linked to BRCA1 status and a CSC phenotype in primary breast cancer. Breast CSCs are more likely to have low levels of BRCA1 expression than non-stem cells. Further to previous studies, we established phenotypes with combination of expression of ALDH1, BRCA1, ER,PR, Her-2 expression in malignant epithelium of breast cancer and found a trend for correlation between these populations; (ALDH1-, Berca1+, ER+, PR+, HER2-) which correlates with good prognosis and outcome and (ALDH1+, BRCA1-, ER-, PR-, HER2+) which correlates with worse prognosis and outcome (we evaluated prognosis in referral to hormone and HER2 receptor status). This is in agreement with those reported by Heerma van voss *et al.* [21], who found a correlation between intra-tumoral epithelial ALDH1 expression and basal-like subtype and ER-and PR-negative receptor status and Madjd *et al.* [19] found a trend for correlation between this population (ALDH1+

/BRCA1 low tumors) with high grade tumors, indicating that this phenotype tends more to occur in high grade tumors. In conclusion ALDH1 is a stem cell marker that might play role in cancer cell differentiation which may be utilized as prognostic marker, the study also emphasized the possible utility of ALDH1 as a biomarker for BRCA1 mutation carriers and thus might be able to screen family members at risk for BRCA1 mutation testing. In addition, ALDH1 could be a possible therapeutic target in breast cancer as well, retarding tumor growth and reducing incidence of metastasis.

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