

# Expression of CD133 and ALDH 1 proteins as cancer stem cells markers in a random sample of patients with breast cancer

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**Abstract.** Breast cancer is associated with a considerable amount of distress among women and with a risk of mortality worldwide; it is a major cause of cancer-related mortality among women. Cancer stem cells play crucial roles in cancer initiation, maintenance, behavior and response to therapy. The main aim of the present study was to assess the immunohistochemical expression of CD133 and aldehyde dehydrogenase 1 (ALDH1) as stem cells markers in breast invasive (ductal) carcinoma and to evaluate the significance of this expression in association with key clinicopathological parameters. For this purpose, a retrospective study was carried out on 52 cases of breast invasive ductal carcinoma of an archived random sample of patients. Immunostaining for CD133 and ALDH1 was performed on these samples, and the results were used to evaluate the association with the age, tumor grade and stage, as well as the hormonal receptor state [estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)/neu] of the patients. The results revealed that CD133 expression was elevated in relation to the less differentiated tumors and an advanced stage; in addition, CD133 expression was markedly elevated in relation to cases with a negative ER/PR and a positive HER2 hormonal status. While ALDH1 expression was associated with an advanced tumor stage, there was no significant association with tumor grade. ALDH-1 expression was significantly associated with the ER/PR positive status and was not associated with HER2/neu expression. On the whole, the present study demonstrates that the expression levels of CD133 and ALDH1 are associated with an advanced clinical stage. In addition, the significant expression of CD133 in an aggressive subtype of breast cancer that lacks ER and PR hormone receptors but overexpresses HER2 protein may explain the resistance to treatment and the

recurrence of this subtype of tumor. These findings may aid in the development of effective targeted therapy.

## Introduction

Breast cancer is associated with a considerable amount of distress for women, as well as with a risk of mortality worldwide (1). It is the major cause of cancer-related mortality among Iraqi patients, accounting for approximately one-third of all cases of cancer registered in the country (2). Breast cancer is currently considered as a group of heterogeneous diseases that vary in their clinical behavior, response to therapy and outcome. Even with the progress being made in diagnosis and treatment that favors early management, metastasis is inevitable for certain patients, rendering breast cancer a major burden on public health (3,4).

Cancer stem cells (CSCs) are a special type of population of cancer cells that carry characteristics of stem cells; they may also initiate the development of breast cancer. CSCs give rise to daughter cells at any time during cell division that can occur following the self-renewal of these stem cells, maintaining the full capability to divide and differentiate from the ordinary tumor cells that represent the major element of the tumor (5).

The need to identify specific biomarkers for CSCs has been considered in previous studies. A number of proteins have been identified as promising markers, including nestin, CD133, CXCR-4, EpCAM, ABCB1, aldehyde dehydrogenase 1 (ALDH1), Oct-4, c-Met, ABCG2, CD24 and CD44 (5-7).

The present study evaluated the expression of the representative CSC markers, CD133 and ALDH1, using immunohistochemical staining and their distribution in invasive (ductal) carcinoma of the breast. The present study also assessed their association with clinical and pathological parameters of patients, such as the age, stage and tumor grade tumor, as well as hormonal receptor state [estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)/neu].

Prominin-1 (also known as CD133) is one of the key members of pentaspan trans-membrane glycoproteins. The CD133 encoding gene in human beings is located on chromosome 4 (8). Its expression is frequently found on multipotent primitive progenitor cells and immature hematopoietic stem cells. CD133 is an acknowledged marker for CSCs.

The tumorigenic role of CD133 expression can be identified when CD133-expressing cells exhibit the majority of

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features of stem cells, such as self-renewal ability, a high proliferation rate and resistance to cancer drugs. This marker may also participate in cell differentiation, proliferation and apoptosis (9,10).

CD133 is one of the markers that can be used in the, as well as in the prognosis of various neoplasms. In breast cancer, the increased expression of this marker is relatively related to a poor prognosis. CD133 is a key promising marker for the identification of CSCs in breast cancer subtypes, including the aggressive HER2-positive and triple-negative phenotypes (11).

ALDH1 belongs to a NADP-dependent enzyme family as a detoxifying enzyme that plays essential roles in the oxidation of extracellular and intracellular aldehydes to their corresponding carboxylic acids. It is essential in protecting cellular homeostasis by scavenging the reactive aldehydes that result from lipid peroxidation (12). A high expression level of ALDH1 is associated with resistance to cancer drugs by interfering with the cytotoxic effects of drugs used in cancer treatment (13).

Beyond their utility as diagnostic indicators, CD133 and ALDH1 function as active drivers of the aggressive phenotypes observed in invasive ductal carcinoma. CD133 (prominin-1) is not merely a surface protein; it functions as a critical scaffold that modulates intracellular signaling, specifically by activating the PI3K/Akt and Wnt/ $\beta$ -catenin pathways to promote self-renewal and epithelial-mesenchymal transition (9,12).

Simultaneously, ALDH1 plays a vital metabolic role by catalyzing the oxidation of retinol to retinoic acid, a potent regulator of gene expression that maintains cells in an undifferentiated, stem-like state. Furthermore, ALDH1 provides a 'metabolic shield' by detoxifying reactive oxygen species and metabolic byproducts of chemotherapy, such as aldehydes, thereby directly facilitating resistance to treatment and a rapid cellular turnover characteristic of high-stage, hormone-insensitive tumors (11,13).

## Patients and methods

*Study design and sample criteria.* In the present retrospective study, paraffin-embedded blocks of breast tissue of an archived random sample group of Iraqi female patients diagnosed with invasive (ductal) carcinoma of the breast were randomly selected from the archives of the Pathology Department at Al-Kadhimain Teaching Hospital/Al-Yarmouk Teaching Hospital (the primary teaching affiliate of the College of Medicine, Al-Mustansiriyah University) between the periods of April, 2023 and June, 2024 in Baghdad, Iraq.

These paraffin-embedded blocks represented 52 cases of invasive (ductal) carcinoma of the breast; 32 of the cases were true cut biopsies, while the remaining 20 specimens were mastectomies, excluding those who received pre-operative neoadjuvant chemotherapy, recurrent cases, poorly preserved tissue samples, and those with insufficient clinical data.

The authors reviewed and analyzed all clinical and pathological data, including age (according to the menopausal status in the patient history), hormonal receptors status (ER, PR and HER2/neu), tumor grading was performed according to the Nottingham modification of the Bloom-Richardson system as defined by the WHO Classification of Tumours (14) and tumor stage was determined using the AJCC TNM staging 8th edition (15).

The Institutional Review Board (IRB) of the Al-Yarmouk Teaching Hospital and College of Medicine/Al-Mustansiriyah University in Baghdad, Iraq, approved the application (Reference no: 5889 in 17th October 2022) no. 156 in 3/12/2024). The Al-Kadhimain Teaching Hospital Al-Yarmouk Teaching Hospital was used as the site for recruitment and for required investigations, due to the availability of immunohistochemistry facilities there, which are not available at the authors' institution or its affiliated hospital (Al-Yarmouk Teaching Hospital). The Institutional Review Board (IRB) of the Al-Kadhimain Teaching Hospital in Baghdad, Iraq, approved the application (Reference no. 855 in 9/11/2022).

A written informed consent to participate in the study, as specified in the Declaration of Helsinki, was obtained from each patient.

*Immunohistochemical staining.* Representative paraffin blocks containing breast tissue samples were sectioned at a thickness of 4  $\mu$ m. For each block, three sections were obtained: The first section was dedicated to hematoxylin and eosin staining for morphological evaluation. The subsequent two sections were transferred onto positively charged slides to be processed for immunohistochemistry using antibodies against CD133 and ALDH1.

The sections underwent deparaffinization and rehydration using a graded alcohol series. Following a 10-min wash in PBS (pH 7.2; MilliporeSigma), endogenous peroxidase activity was blocked with H<sub>2</sub>O<sub>2</sub> in methanol (3%) for 10 min at room temperature. Heat-induced antigen retrieval was subsequently performed by heating the slides to 95°C for 30 min, followed by a final PBS rinse.

Following several PBS washes, the sections were blocked with goat serum for 20 min at room temperature. The sections were then incubated overnight at 4°C in a humidified chamber with primary antibodies against CD133 (mouse monoclonal; cat. no. sc-sc-36553723908; Santa Cruz Biotechnology, Inc.) and ALDH1 (rabbit monoclonal; cat. no. sc-166362; Santa Cruz Biotechnology, Inc.), both used at a 1:200 dilution. Following incubation with a polymer enhancer (Reagent A, 20 min, at room temperature (25°C), the slides were washed in PBS and treated with a goat anti-mouse secondary antibody (Reagent B, 30 min).

Following a final PBS wash, immunoreactivity was visualized with freshly prepared diaminobenzidine (DAB; Dako; Agilent Technologies, Inc.) for 8 min. The slides were then stained with hematoxylin (MilliporeSigma) for 2 min at room temperature (25°C) hematoxylin as a counterstain, dehydrated and then mounted. Eosin staining was omitted to provide optimal contrast for the DAB chromogen.

Normal human kidneys tissues (n=2) were used as positive controls for CD133 and ALDH1. These samples were obtained from the archived paraffin-embedded blocks of the Pathology Department at Al-Kadhimain Teaching Hospital. The tissues were derived from the histologically normal cortex of nephrectomy specimens (Patients: 1 male, aged 52 years; 1 female, aged 48 years). Ethical approval and informed consent for the use of these archived tissues were obtained under the same protocol as the study samples were used as positive controls, while omitting the primary antibody (PBS used instead), used as the negative control; these controls were included in each run.

**Evaluation of immunohistochemistry.** Immunohistochemical expression was assessed independently by two pathologists using Olympus BX51 light microscope (Olympus Corporation). A systematic evaluation was performed for each sample, analyzing a minimum of five representative fields within the tumor core and the invasive front. Initial scanning was conducted using a 10X objective to identify hotspots, followed by a 40X objective for a detailed qualitative assessment of staining intensity and subcellular localization.

Staining intensity (scored as 0-3: nil, weak, moderate or strong) and the positive cell percentage (scored 1-4: <10, 11-50, 51-75 and >75%) were evaluated for membranous and/or cytoplasmic patterns; both patterns were considered positive. The final score, ranging from 0 to 12, was derived by multiplying the percentage and the intensity scores, according to the cited criteria (13).

A score of  $\geq 3$  was defined as positive for CD133 expression. The proportion of positive cells among tumor cells was determined by immunohistochemistry labeling for ALDH1A1. A semi-quantitative method was performed for the assessment of immunohistochemical expression, incorporating both the staining intensity (I) and the percentage of positive cells (P).

Intensity was scored as follows: 0, negative; 1, weak; 2, moderate; or 3, strong. The positive cell percentage was scored from 0 to 5, corresponding to increasing ranges from 0 to 100%. A final Quick score ( $Q=P + I$ ) was calculated, yielding a range of 0 to 8 (14).

For statistical purposes, a Quick score of 0 to 2 was considered as negative, and a score of  $\geq 3$  as positive (15).

**Statistical analysis.** The Chi-squared test with Phi and Cramer's test were used to assess the associations among the immunohistochemical expression of CD133 and ALDH1 and clinicopathological indices. SPSS 24.0 software for Windows (Dotmatics) (10) was used for this purpose. However, for variables where the expected cell count was <5 (such as HER2/neu status), Fisher's exact test was utilized to ensure statistical accuracy. A P-value <0.05 was considered to indicate a statistically significant difference.

## Results

**Patient characteristics.** The present study evaluated the immunohistochemical expression of CD133 and ALDH1A1 in a sample of Iraqi female patients with breast cancer. The sample included 52 specimens of invasive (ductal) carcinoma; 33 patients were  $\geq 50$  years, and 19 patients were <50 years of age. The mean age of the patients was  $(56 \pm 6.3)$  years.

Among the specimens analyzed, 22 (42.3%) of the tumors were of grade I/II, and 30 (57.7%) tumors were of grade III. TNM stages I/II were identified in 24 (46.2%) cases, whereas 28 (53.8%) cases had advanced stage III disease.

As regards hormone receptor status, 24 (46.2%) cases were ER-positive, while 28 (53.8%) were ER-negative. In relation to the progesterone hormone receptor status, 25 (48.1%) cases were PR-positive and 27 (51.9%) were PR-negative. A total of 29 (55.8%) cases were HER2/neo-positive, and 23 (44.2%) were negative. The clinicopathological features of the patients and their corresponding tumors are presented in Tables I and II.

**Immunohistochemical staining.** Immunohistochemical analysis revealed CD133 expression in the cytoplasmic and membranous compartments of tumor cells, as illustrated in Fig. 1. It was expressed in 65.4% (34 of 52) of cases, while the remaining 34.6% (18 of 52) were negative (Table I).

A significant positive association was observed between CD133 expression and an advanced disease grade; CD133 positivity was found in 80% (24/30) of grade III tumors compared to 45.5% (10/22) of grade I/II tumors ( $P=0.01$ ). Similarly, CD133 expression was associated with an advanced disease stage, with positivity in 82.1% (23/28) of stage III tumors vs. 45.8% (11/24) of stage I/II tumors ( $P=0.006$ ). By contrast, no significant association was found with patient age ( $P=0.119$ ) (Table I).

Furthermore, the analysis demonstrated that a high expression of CD133 was significantly associated with a negative ER status ( $P=0.031$ ). However, no significant association was observed between CD133 expression and HER2 status ( $P=0.771$ ) (Table I).

Immunohistochemical staining for ALDH1 was primarily localized to the cytoplasm of tumor cells (Fig. 1). ALDH1 positivity was observed in 35 cases (67.3%), while the remaining 17 cases (32.7%) were negative (Table II).

A significant positive association was observed between ALDH1 expression and an advanced disease stage, with ALDH1 positivity in 82.1% (23/28) of stage III tumors compared to 50% (12/24) of stage I/II tumors ( $P=0.014$ ). By contrast, no significant associations were found with tumor grade ( $P=0.57$ ) or patient age ( $P=0.067$ ) (Table II).

As regards biomarker profiles, ALDH1 expression was significantly associated with both an ER-positive ( $P=0.004$ ) and PR-positive ( $P=0.014$ ), expression, but was independent of the HER2 status ( $P=0.134$ ) (Table II).

## Discussion

In the present study, the expression and distribution of the CSC markers, CD133 and ALDH1, in breast invasive ductal carcinoma were evaluated. Immunohistochemical analysis revealed that CD133 expression was localized to the membrane and cytoplasm of tumor cells. By contrast, this expression was absent in adjacent histologically normal breast tissue. The rate of expression progressively increased from normal to non-invasive to invasive carcinoma. These findings align with patterns previously observed in studies from Egypt and China (16,17).

In the present study, the analysis detected CD133 expression in 65.4% (34/52) of cases, consistent with the findings of a previous study in Egypt (68%) (16). Furthermore, the increased expression of CD133 exhibited a positive association with higher grade tumors ( $P=0.01$ ) and an advanced disease stage ( $P=0.006$ ). These associations align with previous research from Egypt ( $P=0.008$  and  $P=0.002$ , respectively) by Ahmed and Mohammed (16).

The significant association between CD133 expression and an advanced histological grade ( $P=0.01$ ) identified in the present study suggests that CD133-positive cells contribute to the loss of cellular differentiation. This supports the hypothesis that grade III tumors harbor a larger pool of CSCs, which drive aggressive biological behavior and rapid cellular turnover (18,19).

Table I. Association between the immunohistochemical expression levels of CD133 and the pathological data of the patients.

Characteristic	CD 133 expression		Total (%) (n=52)	P-value
	Positive (n=34)	Negative (n=18)		
Age				
<50	15 (78.9%)	4 (21.1%)	19 (36.5%)	0.119
≥50	19 (57.6%)	14 (42.4%)	33 (63.5%)	
Tumor grade				
I + II	10 (45.5%)	12 (54.5%)	22 (42.3%)	0.01
III	24 (80%)	6 (20%)	30 (57.7%)	
Tumor stage				
I + II	11 (45.8%)	13 (54.2%)	24 (46.2%)	0.006
III	23 (82.1%)	5 (17.9%)	28 (53.8%)	
ER				
Positive	12 (50)	12 (50%)	24 (46.2%)	0.031
Negative	22 (78.6)	6 (21.4%)	28 (53.8%)	
PR				
Positive	12 (48)	13 (52%)	25 (48.1%)	0.011
Negative	22 (81.5)	5 (18.5%)	27 (51.9%)	
HER2/neo				
Positive	27 (93.1%)	13 (52%)	29 (55.8%)	0.771
Negative	7 (30.4%)	5 (18.5%)	23 (44.2%)	

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

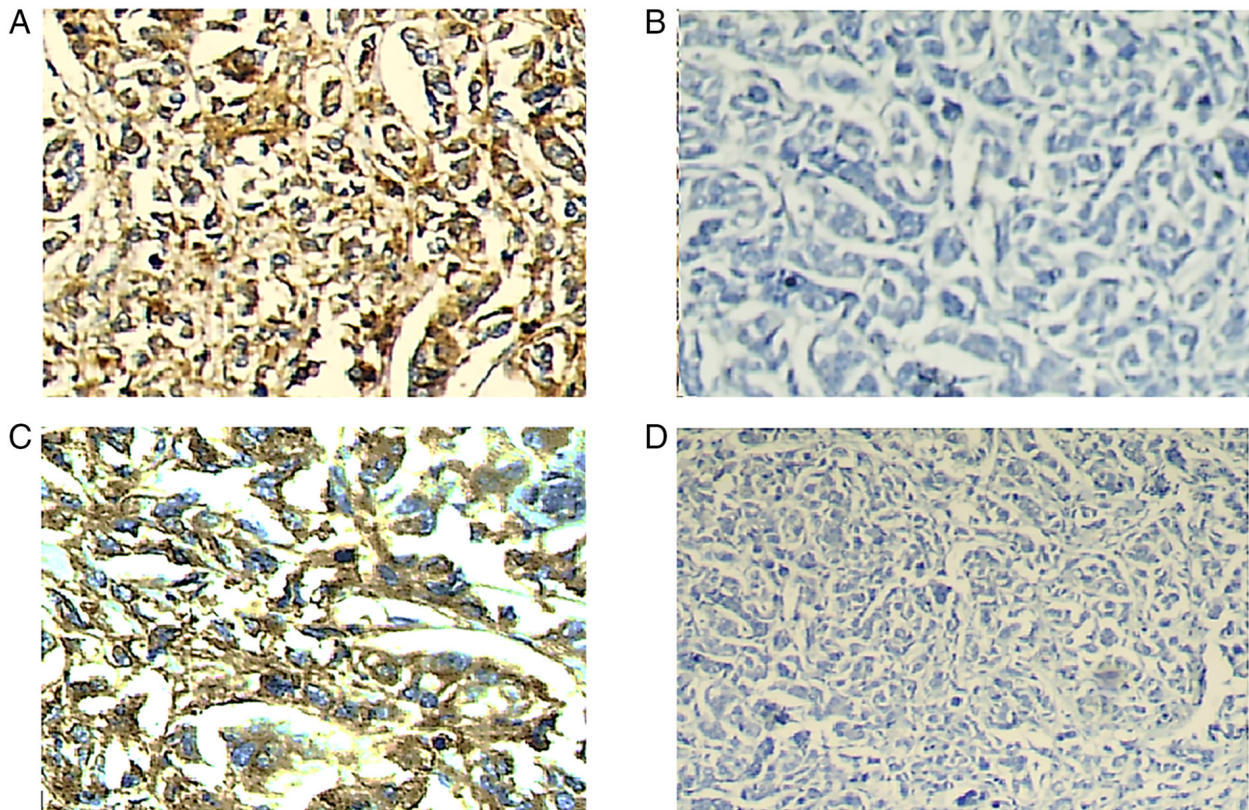


Figure 1. Microphotographs of invasive ductal carcinoma of breast cancer illustrating: (A) Positive immunohistochemical expression of CD133 with strong intensity (brownish discoloration of the cytoplasm with membranous expression (magnification, x10); (B) negative immunohistochemical expression of CD133 (magnification, x10); (C) positive immunohistochemical expression of ALDH1A1 with strong intensity (brownish discoloration of the cytoplasm with membranous expression (magnification, x10); and (D) negative immunohistochemical expression of ALDH1A1 (magnification, x10).

Table II. Association between the immunohistochemical expression levels of ALDH1 and the pathological data of the patients.

	ALDH1 expression		Total (%) (n=52)	P-value
	Positive (n=35)	Negative (n=17)		
Age				
<50	11 (57.9%)	8 (42.1%)	19 (36.5%)	0.067
≥50	24 (72.7%)	9 (27.3%)	33 (63.5%)	
Tumor grade				
I + II	13 (59.1%)	9 (40.9%)	22 (42.3%)	0.57
III	22 (73.3%)	8 (26.7%)	30 (57.7%)	
Tumor stage				
I + II	12 (50%)	12 (50%)	24 (46.2%)	0.014
III	23 (82.1%)	5 (17.9%)	28 (53.8%)	
ER				
Positive	21 (87.5%)	3 (12.5%)	24 (46.2%)	0.004
Negative	14 (50%)	14 (50%)	28 (53.8%)	
PR				
Positive	21 (84%)	4 (16%)	25 (48.1%)	0.014
Negative	14 (51.9%)	13 (48.1%)	27 (51.9%)	
HER2/neo				
Positive	17 (58.6%)	12 (41.4%)	29 (55.8%)	0.134
Negative	18 (78.3%)	5 (21.7%)	23 (44.2%)	

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

The enrichment of CD133 in advanced disease stages may be attributed to the survival advantages of CSCs. These cells exhibit resistance to cell-detachment-induced apoptosis, allowing them to survive lymphatic transport and establish secondary tumor deposits, which defines the clinical progression to stage III (19-21).

In the present study, significant associations were observed between CD133 expression and a negative ER status (P=0.031), a negative PR status (P=0.011). These findings align with previous reports from cohorts in Egypt and Korea (16,22). Conversely, no significant association was found between CD133 expression and patient age (P=0.119), a result consistent with prior studies in China (18).

A potential explanation for these findings is that ER/PR-negative tumors tend to be more undifferentiated, resembling normal breast tissue less than their hormone-receptor-positive counterparts, rendering them inherently more difficult to treat. This suggests that CD133-positive CSCs are more prevalent in triple-negative or hormone-insensitive subtypes, ultimately contributing to a poorer prognosis. Furthermore, evidence suggests that HER2 signaling actively drives the expansion of the CSC population. In these tumors, CD133 and HER2 likely function synergistically to fuel rapid cellular turnover and promote resistance to standard chemotherapy (22,23).

In the present study, ALDH1 expression was infrequent in normal breast tissues matched with cancer tissues. Furthermore, it was found that ALDH1 expression was in 67.3% of cases, in agreement with this finding, (68.8%) cases were reported in another Iraqi study on breast cancer (24). A significant

association was also observed between ALDH1 expression and a high tumor stage (P=0.014); this finding aligns with previous studies in Korea (P=0.011) (22) and China (P=0.043) (23). By contrast, ALDH1 expression was not significantly associated with age (P=0.067) or histological tumor grade (P=0.57). These non-significant findings are supported by the findings of previous studies in China (P=1.000 and P=0.508) (25) and Africa (P=0.731 and P=0.0421) (26).

The findings of the present study indicate a biological decoupling between cancer cell stemness and cellular differentiation, suggesting they are regulated by independent genetic pathways. This divergence explains why a tumor can exhibit high invasiveness and an advanced clinical stage, driven by a robust stem cell population, while still maintaining a relatively organized histological structure or moderate grade (26).

The results presented herein demonstrate a significant association between ALDH1 expression and the ER (P=0.004) and PR (P=0.014) status. However, no significant association was found with HER2 expression (P=0.134). These findings align with the meta-analysis conducted by Liu *et al* (27), which similarly confirmed a robust association with ER (P<0.00001) and a non-significant association with HER2 (P=0.06).

The significant association between ALDH1 and the ER/PR status, which was in contrast to the lack of an association with HER2, may reflect the origin of these tumors from ALDH1-positive luminal progenitor cells. This suggests that ALDH1-driven stemness in the present study cohort was integrated into the hormone-signaling architecture of the tumor, rather than being driven by HER2-mediated growth factor pathways.

The present study had certain limitations, which should be mentioned. While the observed associations provide valuable insight, several limitations warrant consideration. First, the retrospective design and modest sample size may constrain the generalizability of these findings across more diverse patient populations. Nonetheless, these results establish an essential exploratory baseline. Second, the modest sample size (n=52) and the inclusion of only Iraqi patients may constrain the generalizability of these findings to broader, more diverse populations. Third, and most critically, the present study lacks patient outcome data, including survival analysis, recurrence rates and treatment response information.

However, the strong association between CSC markers and aggressive tumor characteristics suggests a potential link to treatment resistance, providing the necessary preliminary evidence to justify large-scale, prospective studies. The data presented herein highlight CD133 and ALDH1 as high-priority candidates for future research into predictive biomarkers and targeted therapeutic interventions.

In conclusion, the concomitant elevation of CD133 and ALDH1 expression is significantly associated with advanced clinical stages of breast cancer. As robust indicators of biological aggressiveness in invasive ductal carcinoma, these markers carry substantial prognostic value and may inform the selection of molecularly targeted therapies. Furthermore, the high prevalence of CD133 in aggressive phenotypes, particularly those characterized by hormone receptor (ER/PR) negativity and HER2 overexpression, underscores its role in driving therapeutic resistance and tumor recurrence. Consequently, characterizing these CSC populations may facilitate the development of more precise, evidence-based strategies to overcome chemoresistance.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

RHZ was involved in the conception and design of the study, in the literature search, clinical analysis, data analysis and statistical analysis, as well as in the preparation and reviewing of the manuscript. HAG was involved in the conception and design of the study, in the literature search, and in clinical analysis and data analysis. HSS was involved in the preparation and reviewing of the manuscript, in the conception and design of the study, and in data analysis. All authors have read and approved the final manuscript. RHZ and HAG confirm the authenticity of all the raw data.

### Ethics approval and consent to participate

The Institutional Review Board (IRB) of the Al-Kadhmain Teaching Hospital in Baghdad, Iraq, approved the application (Reference no. 855 in 9/11/2022). Al-Kadhmain Teaching Hospital was used as the site for recruitment and for required investigations, due to the availability of immunohistochemistry facilities there, which are not available at the authors' institution. A written informed consent to participate in the study as specified in the Declaration of Helsinki was sought from each patient.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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