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Original Article

Expression of ALDH1 in atypical ductal hyperplasia, carcinoma, usual ductal hyperplasia and normal breast duct

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Abstract: Background: Aldehyde dehydrogenase 1 (ALDH1), which is an enzyme functioning in many metabolism process, has been suggested as a breast cancer (BC) stem cell marker. However, its potentiality as a prognostic predictor for BC is yet to be characterized. We compared ALDH1 expression in normal ducts, usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and invasive ductal carcinoma (IDC) to assess its prognostic value in breast cancer. Methods: We determined the expression of ALDH1 in 22 cases with normal duct, 20 UDH, 50 ADH and 160 IDC by immunohistochemical staining and assessed its correlation with patient outcome. Result: Of all specimens, the positive rates for cytoplasmic staining of ALDH1 in UDH, ADH, IDC were 80%, 64% and 35% respectively, whereas for stroma staining, the positive rates were 70%, 52% and 40% respectively of UDH, ADH and IDC. We also found that expression of ALDH1 in IDC luminal epithelial cytoplasm correlated with relapse-free and overall survival in BC patients ($P < 0.05$), while ALDH1 expression in stroma showed no correlation with patient outcome. Chi-square test indicated that cytoplasmic and stromal ALDH1 expression differed among IDC, ADH and UDH ($P < 0.05$). Conclusion: Expression of ALDH1 might participate in evolution of ADH to breast cancer, and ALDH1 expression might predict breast cancer outcome.

Keywords: Stem cells, aldehyde dehydrogenase 1, invasive ductal carcinoma, atypical ductal hyperplasia, usual ductal hyperplasia

Introduction

Aldehyde dehydrogenase 1 (ALDH1) plays very important roles in the metabolism of many cells and has been identified as a stem cell marker of the breast cancer (BC) [1]. Breast cancer stem cells (CSCs) as well as tumor initiated cells (TICs) are believed to be the origins of breast tumors [2]. Breast CSCs are capable of proliferating infinitely and multi-lineage differentiation, and can be insensitive to chemotherapy or hormone therapy, causing tumor recurrence and metastasis after primary treatment [3]. Genetic mutations drive the malignant transformation of the normal stem cells or progenitors to CSCs [4-6]. Tomasetti and Vogelstein [7] reported the association of stem cell division with the cancer risk of the same tissue, suggesting that the inhibition of proliferation for stem cells might reduce the possibility of the carcinogenesis [8].

Atypical ductal hyperplasia (ADH) is a frequent precancerous lesion in the breast tissues of women aged above 40 [9-12]. Over 30% of women aged 45-54 were found with moderate or severe hyperplasia in autopsy [13-15] and these women have about five-fold more risk than normal population in developing breast cancer [11, 16-18]. In addition, gene expression profiling indicates highly similar transcriptional profiles among ADH, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) [19]. However, the stem cell profile in these precancerous lesions has not been widely studied. Thus, measuring the stem cell number in nor-

Table 1. Basic information of the patients

Information	IDC N (%)	ADH N (%)	UDH N (%)	Normal duct, N (%)
Total	160	50	20	22
Age ≤ 50	94 (58.8)	38 (76)	18 (90)	19 (86.4)
Age > 50	66 (41.2)	12 (24)	2 (10)	3 (13.6)
Pre-menopause	99 (61.9)	37 (74.0)	16 (80.0)	17 (77.3)
Post-menopause	61 (38.1)	13 (26.0)	4 (20.0)	5 (22.7)
Age at last pregnancy (yrs)	26.1	25.8	26.3	26.2
No. of full-term pregnancies	2.3	2.1	2.4	2.2
Tumor stage				
T1	55 (34.4)			
T2	95 (59.4)			
T3	2 (1.2)			
T4	8 (5.0)			
N1	79 (49.4)			
N2	57 (35.6)			
N3	24 (15)			
Distant metastases	42 (26.3)			
Bone	4 (2.5)			
Brain	2 (1.3)			
Live	7 (4.4)			
Lung	12 (7.5)			
More than two sites	17 (10.6)			
Deaths	37 (23.0)	0	0	0

mal ducts, ADH and usual ductal hyperplasia (UDH), may aid the development of clinical strategies for the BC treatment.

High ALDH1 activity in BC has been found to correlate with recurrence and metastasis, which leads to poor prognosis [20]. Patients with primary tumors and axillary lymph nodes metastases (ALNM) expressing ALDH1 have dramatically shorter overall (OS) and recurrence-free survivals (RFS) than those with ALDH1-negative tumors [21]. However, ALDH1 activity in stroma cells within the primary tumors correlates with longer disease-free (DFS) and metastasis-free survivals (MFS), as well as OS, suggesting that ALDH1 level might be a useful prognostic factor for DFS [22]. In addition, Epithelial-to-mesenchymal transition (EMT) has recently been related to the acquisition of stem cell properties of breast epithelial cells, and subsequent tumor metastasis or therapeutic resistance [23]. Furthermore, high ALDH1 expression is associated with tumor size, histological grade, lymph node metastasis, and worse prognosis [24]. Although ALDH1 has been regarded as a biomarker predicting tumor aggressiveness and poor prognosis, the significance of ALDH1 expression and localization in ADH has not been analyzed.

In this study, we measured expression of ALDH1 in the stroma and cytoplasm of luminal epithelial cells in UDH, ADH and IDC. Our results indicated that in IDC, the expression of ALDH1 in the cytoplasm of the epithelial cells correlated with OS and RFS whereas that in the stroma did not. Higher expression of ALDH1 was found in the luminal epithelial cytoplasm and stroma of ADH, than in UDH and normal duct tissues, suggesting that the ALDH1 in ADH may play a role in the ADH-BC transformation.

Materials and methods

Patients and tumor specimens

One hundred and sixty BC patients (age 31-70, median: 49) who underwent modified radical mastectomy at the First Hospital of Jilin University (Changchun, Jilin Province, China) between October 2003 and December 2008 were enrolled (**Table 1**, Supplementary Data). The patients were followed-up for 9-89 (mean 59) months after the surgery and those died of other diseases were excluded. The TNM staging was performed following the NCCN Clinical Practice Guidelines in Oncology (version 2015). Meanwhile, 50 ADH, 20 UDH, and 22 normal duct tissues were obtained from patients who underwent surgical resections at the First Hospital of Jilin University (**Table 1**, Supplementary Data). This study was approved by the ethics committee of the First Hospital of Jilin University and written informed consent was obtained from enrolled patients.

Tissue selection and preparation

Tissues obtained through surgical resection were immediately immersed in formalin and

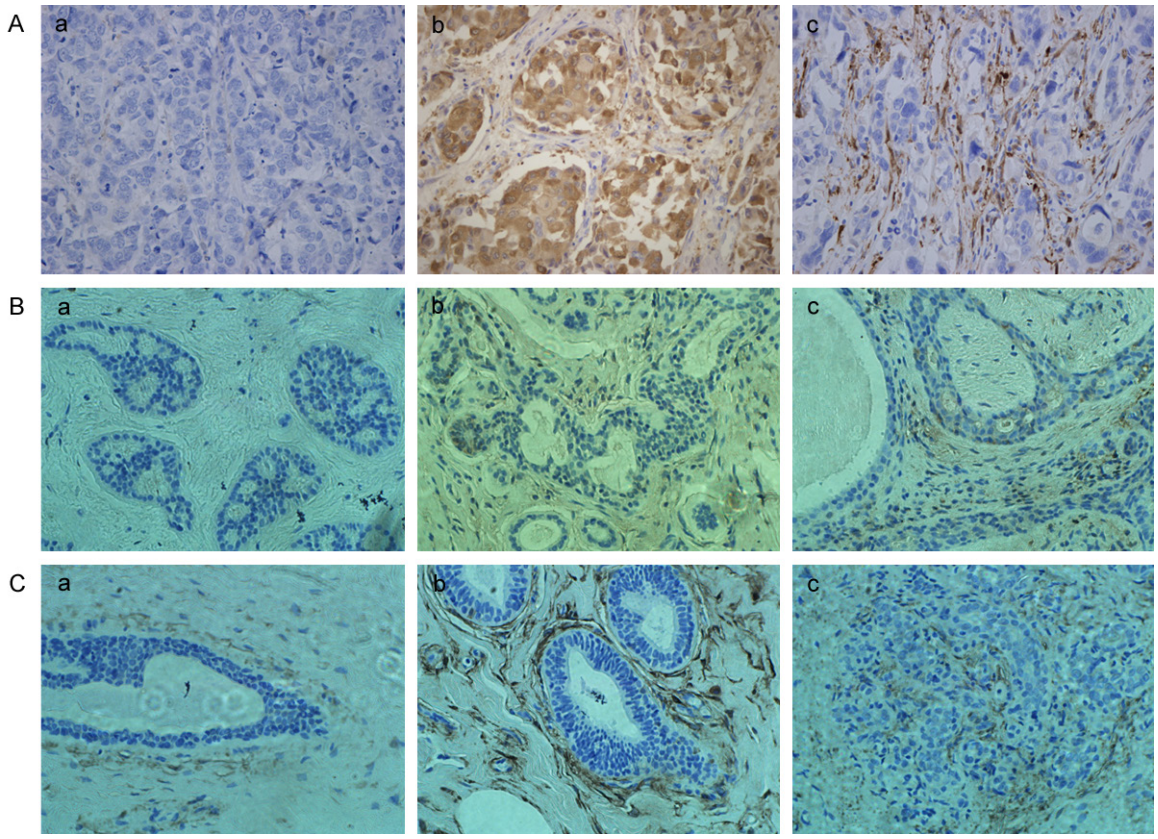

Figure 1. Characterization of ALDH1 expression in normal duct, IDC, ADH and UDH. Sections of IDC (A), ADH (B), UDH and normal duct (C) were stained with an anti-ALDH1A1 monoclonal antibody, visualized and imaged before being scored by two pathologists. Representative images of negative (a) and positive staining in cytoplasm (b) and stroma (c) are shown. The staining was scored based on intensity and percentage of positive cells as detailed in Materials and Methods.

Table 2. The expression of ALDH1 in the cytoplasm of IDC, ADH and UDH

	ALDH1 positive expression	ALDH1 negative expression	Expression rate	X^2	*P*
IDC	56	104	0.35	23.63	< 0.001
ADH	32	18	0.64		
UDH	16	4	0.80		

Table 3. The expression of ALDH1 in the stroma of IDC, ADH and UDH

	ALDH1 positive expression	ALDH1 negative expression	Expression rate	X^2	*P*
IDC	64	96	0.40	7.65	0.022
ADH	26	24	0.52		
UDH	14	6	0.70		

paraffin-embedded before ALDH1 expression was assessed by immunohistochemistry (IHC). H&E staining was also performed and sections observed and imaged using an Olympus IX51 microscope (X40; Olympus Company, Japan). Tissue specimens of UDH (n=20) and ADH (n=50) pathological lesions collected from noncancerous breasts were also analyzed in parallel using normal breast ducts as controls.

Immunohistochemical staining

ALDH1 expression was detected by immunohistochemical staining. Specimens embedded in paraffin were cut into 3-mm sections, which were soaked and boiled in sodium citrate buffer (pH 6.0) for antigen retrieval. After 3% H_2O_2 treatment, sections were blocked and incubat-

Table 4. ALDH1 expression in IDC, ADH, UDH and normal duct

	Groups	Median (inter-quartile range)	X^2	*P*
ALDH1 in cytoplasm	IDC (n=160)	2 (4)	7.244	0.065
	ADH (n=50)	3 (2)		
	UDH (n=20)	2 (4)		
	Normal duct (n=22)	1 (4)		
ALDH1 in stroma	IDC (n=160)	3 (5)	3.134	0.371
	ADH (n=50)	3 (4)		
	UDH (n=20)	2 (4)		
	Normal duct (n=22)	2 (5)		

ed with anti-ALDH1A1 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ) at 4°C overnight, briefly washed, and then incubated with the secondary antibody. Fifteen min later the S-P IHC reagent (MaiXin Biotechnology, Fuzhou, China) was applied and excess washed. DAB staining was then performed for 2-4 min and sections counterstained with hematoxylin before being examined and scored by two experienced pathologists.

Assessment of ALDH1 expression

Previous studies [25-28] showed high expression of ALDH1 in the epithelial cell cytoplasm. In this study, we assessed ALDH1 expression in the cytoplasm of luminal epithelial and stroma cells. We also scored ALDH1 staining in normal ducts, UDH, ADH and IDC based on the percentage of ALDH1 positive cells and the intensity. The percentage of ALDH1 positive cells was scored as 0 (negative, 0-1% cells stained), 1 (positive, 1-10% cells positive), 2 (positive, 10-50% cells positive), or 3 (positive, > 50% cells positive), and the intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).

Statistical analysis

Statistical analyses were performed using a SPSS software package (version 19.0, SPSS Company, Chicago, IL). The association between ALDH1 expression and tumor development was analyzed by the Chi-square or Kruskal-Wallis rank sum test. OS and RFS rates were calculated using the Kaplan-Meier method, and compared by the log-rank test. Multivariate Cox regression analysis was performed to evaluate the risk of metastasis associated with cytoplasmic ALDH1 expression. $P < 0.05$ was considered to be statistical significance. Semiquantitative analysis of ALDH1 expression was assessed by the Kruskal-Wallis rank sum test. Each group of data is expressed as median (inter-quartile range).

Results

ALDH1 expression occurs more frequently in ADH and UDH than in IDC

To explore the significance of ALDH1 expression in breast tumors and ADH, we examined its expression in IDC, ADH and UDH by immunohistochemical analysis. The results indicated significant difference between IDC, ADH, and UDH in the expression of ALDH1 in cytoplasm and stroma (**Figure 1**). Fifty-six out of 160 IDC tumors demonstrated cytoplasmic ALDH1 compared to 32 of 50 ADH and 16 of 20 UDH samples. Chi-square tests demonstrated significant difference in cytoplasmic ALDH1 expression among IDC, ADH, and UDH ($P < 0.05$; **Table 2**, Supplementary Data).

Meanwhile, we also examined ALDH1 expression in stroma of IDC, ADH and UDH. As shown in **Figure 1**, immunohistochemical analysis suggested that ALDH1 was expressed in the stroma of 40% of IDC, 52% of ADH and 70% of UDH samples. Chi-square test indicated that stromal ALDH1 expression differed significantly among three categories ($P < 0.05$; **Table 3**, Supplementary Data). These results indicate significantly higher ALDH1 expression in the cytoplasm and stroma of ADH, and UDH, compared to IDC, suggesting a possible role of ALDH1 in ADH-BC transformation.

ALDH1 expression in normal duct, IDC, ADH and UDH tissues

The Kruskal-Wallis rank sum test was performed to compare ALDH1 expression in IDC, ADH, UDH, and normal duct (**Figure 1C**). The results indicated median ALDH1 expression in the luminal epithelial cytoplasm of 2, 3, 2 and 1 in IDC, ADH, UDH and normal duct, respectively, but with no statistically significant difference ($P=0.065$; **Table 4**, Supplementary Data). Meanwhile, median levels of ALDH1 of 3, 3, 2, and 2 were found in the stroma of IDC, ADH, UDH and normal ducts, respectively, again with no statistically significant difference ($P=0.371$;

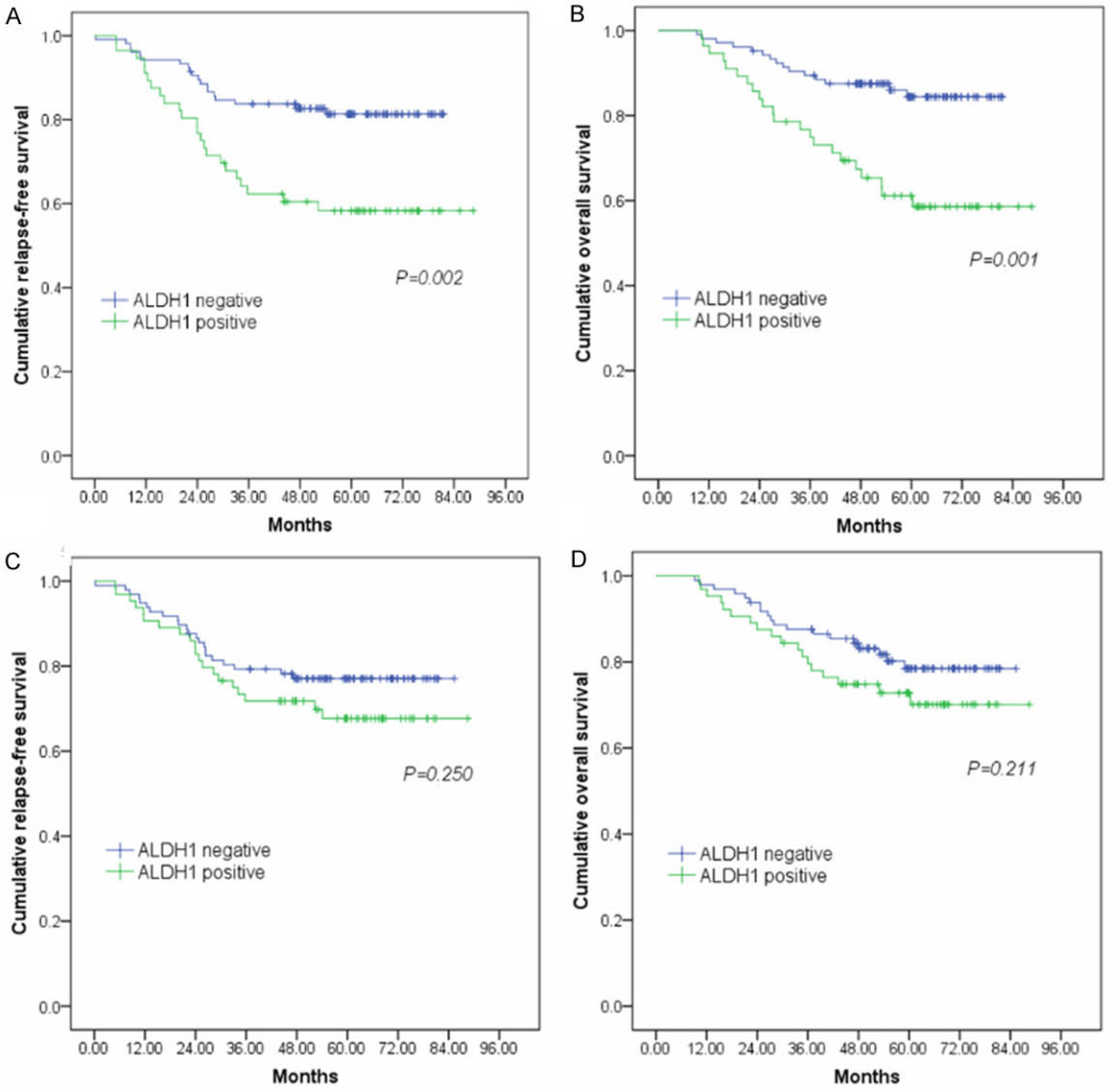

Figure 2. Kaplan-Meier analysis of relapse-free survival (RFS) and overall survival (OS) in patients with IDC. Relapse-free survival (A) or Overall survival (B) curves of patients with IDC negatively expressing ALDH1 or positively expressing ALDH1 in the cytoplasm ($P < 0.05$); Relapse-free survival (C) or Overall survival (D) curves of patients with IDC negatively expressing ALDH1 or positively expressing ALDH1 in the stroma ($P > 0.05$).

Table 4, Supplementary Data). These results suggest no statistical variation in the expression of ALDH1 in luminal epithelial cytoplasm and stroma of IDC, ADH, UDH, and normal duct tissues although there was likely a trend of increase in ADH compared with UDH and normal ducts.

Cytoplasmic expression of ALDH1 in the IDC predicts metastasis and poor survival

To characterize whether ALDH1 expression in the luminal epithelium cytoplasm and stroma of IDC correlated with RFS and OS in BC patients, a Kaplan-Meier analysis was performed and a log-rank test used to compare the survivals of different groups. Our results indicated that cytoplasmic ALDH1 expression in the luminal epithelial cells in 56 of 160 patients predicted poorer prognosis (**Figure 2A** and **2B**). On the other hand, however, ALDH1 expression in stromal cells (64 of 160 patients) was not associated with patient survivals ($P > 0.05$; **Figure 2C** and **2D**). Multivariate Cox regression analysis showed significantly higher risk of metastasis associated with cytoplasmic ALDH1 expression (hazard ratio (HR): 3.985, 95% confidence interval (CI): 1.832-8.665, $P < 0.001$), as well

Table 5. Multivariate analyses of RFS

	B	SE	Wald	df	P	HR	95.0% CI for HR	
							Lower	Upper
ALDH1 in cytoplasm	1.382	.396	12.164	1	.000	3.985	1.832	8.665
ALDH1 in stroma	-.364	.380	.917	1	.338	.695	.330	1.464
Age	-.003	.030	.013	1	.908	.997	.940	1.056
Menopausal status	-.491	.469	1.097	1	.295	.612	.244	1.534
Tumor size	.332	.142	5.461	1	.019	1.394	1.055	1.841
pathological sub-stratification	.691	.128	29.265	1	.000	1.996	1.554	2.564
ER in primary breast tumors	.424	.514	.681	1	.409	1.528	.558	4.182
PgR in primary breast tumors	-1.023	.489	4.375	1	.036	.359	.138	.938
HER-2 in primary breast tumors	-.706	.425	2.767	1	.096	.493	.215	1.134
KI-67 in primary breast tumors	-.016	.333	.002	1	.962	.984	.513	1.890

Table 6. Multivariate analyses of OS

	B	SE	Wald	df	P	HR	95.0% CI for HR	
							Lower	Upper
ALDH1 in cytoplasm	1.489	.422	12.475	1	.000	4.435	1.940	10.135
ALDH1 in stroma	-.348	.401	.753	1	.386	.706	.322	1.549
Age	-.014	.032	.194	1	.660	.986	.926	1.050
Menopausal status	-.202	.487	.172	1	.678	.817	.315	2.121
Tumor size	.233	.150	2.419	1	.120	1.262	.941	1.693
pathological sub-stratification	.649	.136	22.699	1	.000	1.914	1.465	2.499
ER in primary breast tumors	.234	.539	.189	1	.664	1.264	.440	3.632
PgR in primary breast tumors	-1.030	.524	3.869	1	.049	.357	.128	.996
HER-2 in primary breast tumors	-.731	.460	2.521	1	.112	.482	.195	1.187
KI-67 in primary breast tumors	.005	.357	.000	1	.988	1.005	.500	2.023

as other factors such as tumor size (HR: 1.394, 95% CI: 1.055-1.841, *P*=0.019), pathological sub-stratification (HR: 1.996, 95% PI: 1.554-2.564, *P* < 0.001), and progesterone receptors (PR) expression (HR: 0.359, 95% PI: 0.138-0.938, *P*=0.036) (**Table 5**). As a result, patients with IDC that express ALDH1 in cancer cell cytoplasm more likely had shorter survival (HR: 4.435, 95% CI: 1.940-10.135, *P* < 0.001) (**Figure 2A** and **2B**), which also correlated with pathological sub-stratification (HR: 1.914, 95% CI: 1.465-2.499, *P* < 0.001) and PR expression (HR: 0.357, 95% CI: 0.128-0.996, *P*=0.049). ALDH1 expression in stroma cells was not associated with metastasis (*P*=0.338) and patient survival (*P*=0.386) (**Table 6**).

Discussion

Breast cancer is one of the most common malignances across the world [29], accounting for 25-30% of women malignancies in USA, and 8%-10% of all cancers in China [30]. Multiple lines of evidence indicated that ADH positively correlates with the breast cancer tumorigenesis [10, 18]. Therefore, it is urgent to identify the potential indicators for the diagnosis of ADH precancerous lesions of the mammary gland disease.

CSCs form a small population of cancer cells in tumor masses, but are responsible for tumorigenicity and metastasis in various tumors [31-38]. In 2006, Lapidot *et al*. firstly reported the human $CD34^{+}CD38^{-}$ CSCs [39]. CSCs have been isolated and characterized from more than 20 different types of cancer. Accumulating evidences indicated that these cells maintain certain level of stemness similar to epithelial stem cells, and they are naturally resistant to chemotherapy and very common to observe a recurrence [38, 40-43].

Several studies have indicated that ALDH1 can be a marker of breast CSCs. Balicki *et al*. used ALDH1 activity to isolate breast CSCs and rec-

ommended ALDH1 as a reliable marker for breast CSCs [1]. Another investigation demonstrated that transplantation of as few as 500 $ALDH1^+$ cells is enough to generate tumors in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice [20]. Representing only two percent of the cancer cells these small number of $ALDH1^+$ cells are featured with unlimited self-renewal, proliferation and differentiation [20].

In this study, the percentage of epithelial cells with cytoplasmic ALDH1 expression in IDC, ADH and UDH was determined to be 35%, 64% and 80% respectively, while the percentage in stroma was 40%, 52% and 70% respectively (**Tables 2** and **3**, Supplementary Data). Of clinical importance in IDC, ALDH1 in cytoplasm is associated with shorter OS and RFS ($P < 0.05$; **Figure 2A** and **2B**), whereas stromal ALDH1 expression did not correlate with the patient outcomes ($P > 0.05$; **Figure 2C** and **2D**), which is inconsistent with previous reports [22]. This discrepancy might be resulted from different specimens or different detection methods employed, which need to be further studied.

Interestingly, ALDH1 expression was detected in fewer IDC samples (35%) compared to benign and normal epithelial tissues ($P < 0.05$). ALDH1 may exist in both stem cells and CSCs in breast tissue, so ALDH1 positive cells in IDC might be CSCs, whereas the ALDH1 positive cells in ADH/UDH tissues might originate from normal stem cells. Moreover, there is likely an increase of ALDH1 expression in ADH, compared to IDC, UDH and normal ducts (**Table 4**, Supplementary Data), although future confirmation in larger cohorts of patients is needed. In breast tumors, ALDH1 has recently been reported to significantly correlate with histological grade, positive ERBB2 expression, and negative expression of estrogen and progesterone receptors in females [24, 45]. Therefore, our findings indicated that ALDH1 positive cells might have important roles in transformation of mammary gland hyperplasia and development of breast cancer. Thus, ALDH1 might represent a useful prognostic biomarker for breast cancer severity and represent a target for therapeutic interventions.

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Disclosure of conflict of interest

None.

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