

# Combined expression of aldehyde dehydrogenase 1A1 and $\beta$ -catenin is associated with lymph node metastasis and poor survival in breast cancer patients following cyclophosphamide treatment

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**Abstract.** This study investigated the expression of ALDH1A1 and  $\beta$ -catenin in breast cancer patients, and analyzed the correlation of their combined expression with clinicopathological features, chemotherapeutic responses, and prognosis of breast cancer patients. In total 276 human breast cancer tissues and 80 benign hyperplasia tissues were included. The expression of ALDH1A1 and  $\beta$ -catenin was examined using tissue microarray-based immunohistochemistry. ROC curve analysis was performed to determine an optimal cut-off score for the expression of ALDH1A1 and  $\beta$ -catenin, based on the survival status of breast cancer patients. Survival probabilities were estimated by the Kaplan-Meier method. ALDH1A1 expression was higher, but  $\beta$ -catenin showed no significant difference in breast cancer samples compared to controls. Compared with the membrane expression of  $\beta$ -catenin [ $\beta$ -catenin(m)], the cytoplasmic expression of  $\beta$ -catenin [ $\beta$ -catenin(c)] occurred significantly more frequently in breast cancer with the high expression of ALDH1A1 [ALDH1A1(high)] than in breast cancer with the low expression of ALDH1A1 [ALDH1A1(low)] ( $P=0.014$ ). The expression level of ALDH1A1 was significantly higher in  $\beta$ -catenin(c) breast cancer than in  $\beta$ -catenin(m) breast cancer ( $P=0.020$ ). ALDH1A1(high) expression or  $\beta$ -catenin(c) expression alone was associated with lymph node metastasis, and worse clinical outcome in breast cancer patients, especially in patients receiving cyclophosphamide treatment.

Combined expression of ALDH1A1(high) and  $\beta$ -catenin(c) was associated with lymph node metastasis, poor outcome, and resistance to cyclophosphamide treatment.  $\beta$ -catenin may regulate ALDH1A1 expression in a subtype of breast cancer with ALDH1A1(high) and  $\beta$ -catenin(c) expression. ALDH1A1(high) and  $\beta$ -catenin(c) expression may be used as a biomarker for predicting poor prognosis in breast cancer following cyclophosphamide treatment.

## Introduction

Breast cancer is one of the leading causes of cancer-related deaths in women. Breast cancer is a group of heterogeneous diseases with various responses to chemotherapy (1). Cancer stem cells (CSCs) are the tumor-initiating cells that are believed to be responsible for breast cancer recurrence, metastasis, and drug resistance (2,3). CSCs express specific biomarkers such as CD44, aldehyde dehydrogenase 1A1 (ALDH1A1), and  $\beta$ -catenin (4-6). Expression of these CSC markers has prognostic values for the recurrence, metastasis, and therapeutic resistance in various tumors such as lung, hepatocellular, and breast cancer (7-9).

ALDH1, a member of a family of NAD(P)<sup>+</sup>-dependent enzymes that detoxify a wide variety of aldehydes, have been found to be associated with poor prognosis in breast cancer (10). ALDH1A1 has been extensively studied, and is found to be associated with tumor progression and poor prognosis of breast cancer patients (11,12). ALDH1A1 plays an important role in the proliferation of breast CSCs and resistance to chemotherapy, particularly cyclophosphamide (13,14). However, the signaling pathways that regulate the expression of ALDH1A1 in breast cancer remain unclear.

Recently, Condello *et al* have reported that  $\beta$ -catenin upregulates the expression of ALDH1A1, and  $\beta$ -catenin-regulated ALDH1A1 contributes to the maintenance of ovarian cancer spheroids (15).  $\beta$ -catenin is located in the intracellular side of the cytoplasmic membrane, and plays an important role in cell-to-cell adhesion by linking the cytoskeleton and

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cadherin (16). It is well known that  $\beta$ -catenin is involved in the Wnt signaling pathway that plays an active role in CSCs and carcinogenesis of various tumors such as breast cancer (17,18) and ovarian cancer (19). Activation of Wnt signaling leads to translocation of  $\beta$ -catenin from the membrane to the cytoplasm and nucleus, where it promotes transcription of many genes that are associated with cancer growth, invasion, progression, and invasion (20). However, it remains unclear whether  $\beta$ -catenin regulates the expression of ALDH1A1 in breast cancer patients.

In the present study, we investigated the expression of ALDH1A1 and  $\beta$ -catenin in 276 breast cancer patients. We studied the association between the expression of ALDH1A1 and  $\beta$ -catenin in breast cancer patients, and analyzed the correlation of their combined expression with clinicopathological features, chemotherapeutic responses, and prognosis of breast cancer patients.

## Materials and methods

**Patients and tissue samples.** The Medical Ethics Committee of China Medical University approved this study. Due to the retrospective nature of the study, the Medical Ethics Committee waived the need of written informed consents by the patients.

This study included human breast tissues from 276 female patients with breast cancer, who underwent surgery at the First Affiliated Hospital of China Medical University between 2004 and 2008. Breast cancer was diagnosed based on pathological staining. The patient age, menopausal status, tumor type, tumor size, and lymph node metastasis were retrospectively obtained from medical records. Of the 276 breast cancer patients, 220 had invasive ductal carcinoma, 30 had invasive lobular carcinoma, and 26 had other types of tumors including mucinous, medullary, and cribriform carcinoma. The histological grade of the cancer was determined according to the World Health Organization grading system. The stage of the cancer was evaluated according to the TNM staging system.

No patients underwent radiation therapy and chemotherapy before surgery. After surgery, 165 patients received adjuvant chemotherapy, and the remaining 11 patients did not receive chemotherapy. All chemotherapy regimens contained cyclophosphamide, including CMF (cyclophosphamide + methotrexate + fluorouracil, n=65), AC (adriamycin + cyclophosphamide, n=32), CEF (cyclophosphamide + epirubicin + fluorouracil, n=32), CE (cyclophosphamide + epirubicin, n=20), and CAF (cyclophosphamide + adriamycin + fluorouracil, n=18).

**Tissue microarray (TMA).** Paraffinized donor blocks were stained with hematoxylin and eosin, and representative breast cancer samples were selected by reviewing the hematoxylin and eosin-stained blocks. Tissue cores with a diameter of 1.5 mm were extracted from each donor block, and precisely arrayed into a new paraffin recipient block with a maximum of 200 cores, using the Organization Microarrayer (Pathology Devices, USA).

**Immunohistochemistry.** Immunohistochemistry was performed as previously described (21). Briefly, sections (4- $\mu$ m thick)

were obtained from formalin-fixed and paraffin-embedded TMA recipient blocks, and mounted on poly-L-lysine-coated glass slides. Sections were deparaffinized with xylene, rehydrated in a graded alcohol series, and heated in a microwave oven to retrieve antigen. Sections were incubated with primary antibodies against ALDH1A1 (mouse anti-human monoclonal antibodies, 1:100 dilution; Abcam, UK) and  $\beta$ -catenin (mouse anti-human monoclonal antibodies, 1:200 dilution; Cell Signaling Technology, USA) overnight at 4°C, followed by incubation with biotinylated secondary antibodies for 30 min at 37°C. Sections were then incubated with streptavidin horseradish peroxidase for additional 30 min (LSAB kit; Dako, Glostrup, Denmark), and stained with 3,3-diaminobenzidine (DAB). Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative control sections were treated in the same conditions, except no primary antibodies.

**Evaluation of immunohistochemistry.** The immunostaining was examined under a light microscope, and was evaluated by two pathologists blinded to the experimental conditions. The immunoreactivity intensity was scored as follows: 0 for negative staining, 1 for weak positive staining, 2 for moderate positive staining, and 3 for strong positive staining. The percentage of stained cells was scored 0-100%. The final immunoreactive score was calculated by multiplying the intensity score with the score for the percentage of positively stained cells, ranging from 0 to 300%. The scores were assigned by using 5% increments (0, 5, 10, and 300%) as previously reported (22,23). The final immunoreactive scores were used to determine the cut-off value for discriminating tumors with the high expression of ALDH1A1 or  $\beta$ -catenin from those with low expression, using receiver operating characteristic (ROC) curves. To discriminate tumors with the cytoplasmic expression of  $\beta$ -catenin from tumor with the membrane expression of  $\beta$ -catenin, the percentage of  $\beta$ -catenin membrane expression for each sample was used to determine the cut-off value, using ROC curves. The percentage of  $\beta$ -catenin membrane expression was calculated as follows:  $\beta$ -catenin membrane expression % =  $\frac{\text{The number of cells with membrane expression}}{\text{the number of cells with membrane expression} + \text{the number of cells with cytoplasmic expression}} \times 100\%$ . The sensitivity and specificity for the survival status (alive or dead) of breast cancer patients was plotted to generate ROC curves.

**Statistical analysis.** Statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). For data with normal distribution, Student's t-test was used to compare the difference in the means. For data with unequal variance, Wilcoxon rank sum tests or Kruskal-Wallis tests were used to compare the difference. The association between the expression of ALDH1A1 and  $\beta$ -catenin and clinicopathological characteristics of breast cancer patients was evaluated using the Pearson's Chi-square test or Fisher's exact probability test. The Pearson's rank correlation analysis was applied to assess the association between the expression of ALDH1A1 and  $\beta$ -catenin. The Kaplan-Meier curves were plotted to demonstrate the survival difference, and the survival probabilities were assessed by a log-rank test. To assess the association of potential confounding variables with the prognosis, overall survival (OS), or disease-free survival (DFS), univariate and

Table I. Clinicopathological characteristics of breast cancer patients.

	Cases	
	n	%
Age (years)	276	
≤50	149	53.9
>50	127	46.1
Menopausal status	266	
Pre-menopause	150	56.4
Post-menopause	116	43.6
Tumor size (cm)	229	
≤2.0	142	62.0
>2.0	87	38.0
Tumor type	276	
Ductal	220	79.7
Lobular	30	10.9
Others	26	9.4
Histological grade	267	
I	136	50.9
II-III	131	49.1
TNM stage	198	
I	46	23.4
II	122	61.9
III	29	14.7
Lymph node metastasis	265	
No	175	65.8
Yes	91	34.2

multivariate Cox proportional hazards regression models were used. The OS was defined as the time between the

day of diagnosis and the disease-related death, or last known follow-up. The DFS was calculated as the time between the day of diagnosis and the occurrence of local recurrence or distant metastasis. Probability values  $\leq 0.05$  were considered statistically significant.

## Results

**Clinicopathological characteristics.** Table I summarizes clinicopathological characteristics of 276 breast cancer patients. The average age of breast cancer patients was  $50.1 \pm 7.9$  years (range, 31-82 years). Of the 276 patients, the age, menopausal status, tumor size, tumor type, histological grade, TNM stage, and lymph node metastasis were recorded in 276, 266, 229, 276, 267, 197, and 266 patients, respectively. The majority of these patients had a tumor with invasive ductal carcinoma (79.7%), and  $< 2$  cm in size (62.0%). Of the 267 patients with known histological grade, 136 (50.9%) patients had grade I breast cancer, and 131 (49.1%) patients had grade II-III breast cancer. The stage of the cancer was evaluated in 197 patients, including 46 patients with stage I breast cancer, 122 patients with stage II breast cancer, and 29 patients with stage III breast cancer. Lymph node metastasis occurred in 91 (34.2%) of 266 patients.

Follow-up information was available for 168 breast cancer patients. During the follow-up period of 9-118 months, relapses occurred in 62 cases and cancer-associated deaths were found in 48 cases. The 5-years survival rate was 84.5%. The mean OS and DFS were 98.5 and 91.5 months, respectively.

**Expression of ALDH1A1 and  $\beta$ -catenin.** We studied the expression of ALDH1A1 and  $\beta$ -catenin in 276 samples from breast cancer patients and 80 control samples from patients with benign hyperplasia, using immunohistochemistry (Fig. 1). ALDH1A1-immunopositive staining was mainly observed in the cytoplasm in both breast cancer and control samples. ALDH1A1-immunopositive staining was observed

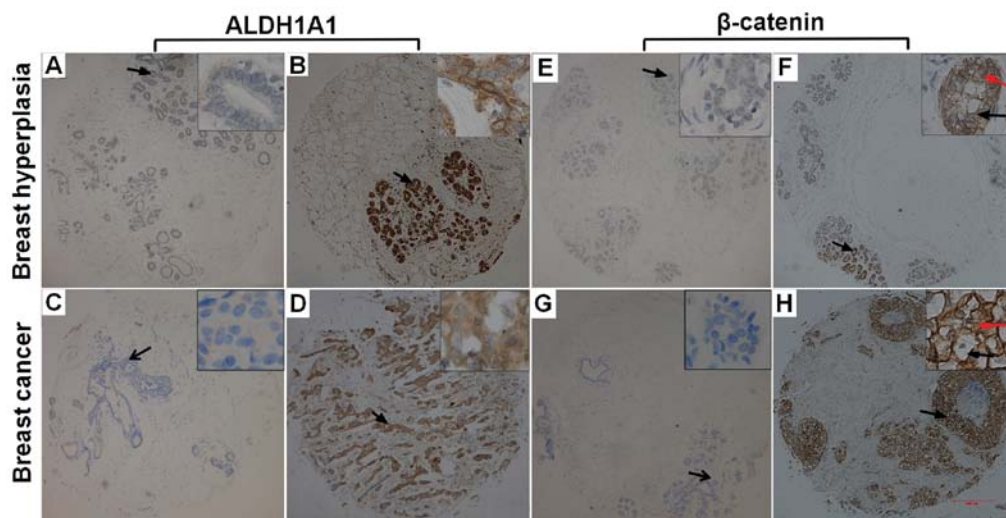


Figure 1. Representative micrographs showing negative (A, C, E and G) and positive (B, D, F and H) immunohistochemical staining of ALDH1A1 (A-D) and  $\beta$ -catenin (E-H) in breast hyperplasia and breast cancer. Magnification, x40. Arrows indicate the magnified regions in the insert (x400). Black arrow in the magnified regions indicate  $\beta$ -catenin membrane expression and red arrow indicate  $\beta$ -catenin cytoplasmic expression. Scale bar, 200  $\mu$ m. ALDH1A1, aldehyde dehydrogenase 1A1.

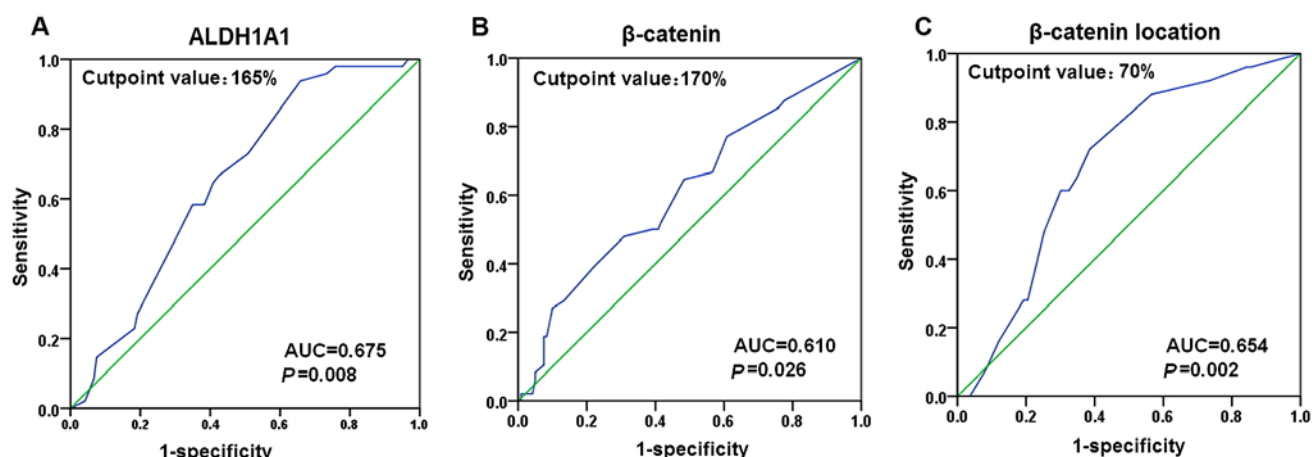


Figure 2. Receiver operating characteristic curves were used to determine the cut-off score for the expression of ALDH1A1 (A) and  $\beta$ -catenin (B), and subcellular expression of  $\beta$ -catenin (C) based on the survival status of breast cancer patients. The sensitivity and specificity for each outcome were plotted and the AUCs and p-value were indicated. ALDH1A1, aldehyde dehydrogenase 1A1; AUCs, areas under curve.

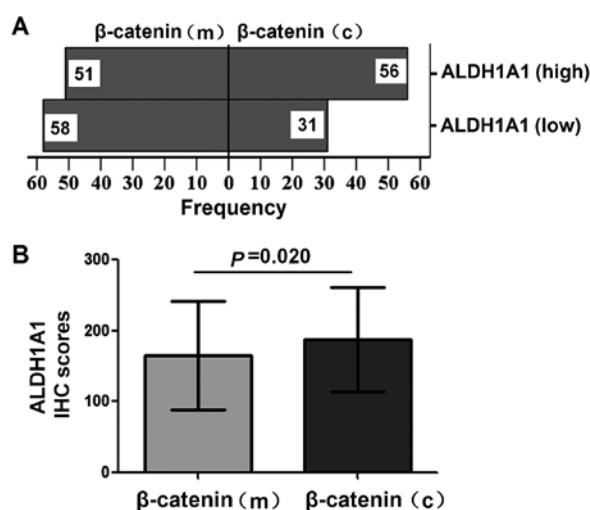


Figure 3. The association between the subcellular expression of  $\beta$ -catenin and ALDH1A1 expression in breast cancer. (A)  $\beta$ -catenin(m) and  $\beta$ -catenin(c) expression in breast cancer with ALDH1A1(high) and ALDH1A1(low) expression. (B) ALDH1A1 IHC scores in breast cancer with  $\beta$ -catenin(m) and  $\beta$ -catenin(c) expression. Data are presented as mean  $\pm$  SD.  $P=0.029$ . ALDH1A1, aldehyde dehydrogenase 1A1.

in 149 (54%) of 276 breast cancer samples and 25 (31.3%) of 80 control samples. ALDH1A1 immunoreactivity occurred significantly more frequently in breast cancer samples than in control samples ( $P<0.001$ ). In contrast,  $\beta$ -catenin immunoreactivity was observed in the membrane and cytoplasm in both breast cancer samples and control samples.  $\beta$ -catenin immunoreactivity was observed in 246 (89.1%) of 276 breast cancer samples and 72 (90.0%) of 80 control samples. There was no significant difference of  $\beta$ -catenin-immunopositive staining between breast cancer samples and control samples ( $P=0.824$ ).

**Selection of the cut-off value for the expression of ALDH1A1 and  $\beta$ -catenin.** ROC curve analysis was performed to determine an optimal cut-off score for the expression of ALDH1A1 and  $\beta$ -catenin in breast cancer samples. Based on the survival status, a cut-off score of 165 and 170% were selected for the expression of ALDH1A1 and  $\beta$ -catenin,

respectively (Fig. 2A and B). Tumors with immunohistological scores  $\geq 165$  and  $<165\%$  were defined as tumors with 'high' [ALDH1A1(high)] and 'low' [ALDH1A1(low)] expression of ALDH1A1, respectively. Tumors with immunohistological scores  $\geq 170$  and  $<170\%$  were defined as tumors with 'high' [ $\beta$ -catenin(high)] and 'low' [ $\beta$ -catenin(low)] expression of  $\beta$ -catenin, respectively. In total 149 tumors (54.0%) exhibited ALDH1A1(high) expression, and 127 (46.0%) tumors showed ALDH1A1(low) expression; 196 (71.0%) tumors exhibited  $\beta$ -catenin(high) expression, and 80 (29.0%) tumors showed  $\beta$ -catenin(low) expression.

We further performed ROC analysis to determine an optimal cut-off score for discriminating the membrane expression from the cytoplasmic expression of  $\beta$ -catenin in breast cancer with  $\beta$ -catenin(high) expression. Based on the survival status, a cut-off score of 70% was selected (Fig. 2C). Tumors with the membrane expression  $\geq 70$  and  $<70\%$  were defined as tumors with  $\beta$ -catenin(m), and  $\beta$ -catenin (c) expression, respectively. Of the 196  $\beta$ -catenin(high) breast cancers, 109 (55.6%) had  $\beta$ -catenin(m) expression, and 87 (44.4%) had  $\beta$ -catenin(c) expression.

**Association between the expression of ALDH1A1 and  $\beta$ -catenin.** Pearson's rank correlation analysis was used to analyze the association between the expression of ALDH1A1 and  $\beta$ -catenin in breast cancer. There was no significant correlation between the expression of ALDH1A1 and  $\beta$ -catenin in the breast cancer patients ( $n=276$ ). We then examined the subcellular expression levels of  $\beta$ -catenin in breast cancer with  $\beta$ -catenin(high) expression. In breast cancer with ALDH1A1(low) expression,  $\beta$ -catenin(m) expression was observed in 58 (65.2%) of 89 cases, and  $\beta$ -catenin(c) expression was found in 31 (34.8%) of 89 cases. In breast cancer with ALDH1A1(high) expression,  $\beta$ -catenin(m) expression was observed in 51 (47.7%) of 107 cases, and  $\beta$ -catenin(c) expression was found in 56 (52.3%) of 107 cases.  $\beta$ -catenin(c) expression occurred significantly more frequently in ALDH1A1(high) breast cancer than in ALDH1A1(low) breast cancer (Chi-square test,  $P=0.014$ ; Fig. 3A). Furthermore, the expression level of ALDH1A was significantly higher

Table II. Association of  $\beta$ -catenin localization and ALDH1A1 expression with clinicopathological features of breast cancer.

	ALDH1A1			$\beta$ -catenin localization		
	High (n/%)	Low (n/%)	P-value	$\beta$ -catenin (c) (n/%)	$\beta$ -catenin (m) (n/%)	P-value
Age at diagnosis (years)			0.690			0.364
≤50	80/55.2	65/44.8		42/36.2	61/63.8	
>50	67/52.8	60/47.2		43/47.3	48/52.7	
Menopausal state			0.736			0.908
Pre-menopause	82/54.7	68/45.3		47/43.5	61/56.5	
Post-menopause	61/52.6	55/47.4		35/42.7	47/57.3	
Tumor size (cm)			0.729			0.622
≤2.0	75/52.8	67/47.2		45/35.5	58/64.5	
>2.0	48/55.2	39/44.8		30/47.6	33/52.4	
Tumor type			0.065			0.102
Ductal	109/49.5	111/50.5		76/48.1	82/51.9	
Lobular	20/66.7	10/33.3		6/30.0	14/70.0	
Others	18/78.2	5/21.8		5/27.8	13/72.2	
Histological grade			0.876			<b>0.014</b>
I	75/55.1	61/44.9		35/36.1	62/63.9	
II-III	71/54.2	60/45.8		49/53.8	42/46.2	
TNM stage			0.267			0.139
I-II	87/51.5	82/48.5		48/40.0	72/60.0	
III	17/63.0	10/37.0		9/60.0	6/40.0	
Lymph node metastasis			<b>0.029</b>			<b>0.002</b>
No	85/48.6	90/51.4		48/36.5	84/63.5	
Yes	57/62.6	34/37.4		36/60.0	24/40.0	

P-value obtained from Pearson's Chi-square test or Fisher's exact test; ALDH1A1, aldehyde dehydrogenase 1A1.

Table III. Association of the combination of  $\beta$ -catenin location and ALDH1A1 with lymph node metastasis in breast cancer.

Features	Node metastasis		P-value <sup>b</sup>		
	No (%) <sup>a</sup>	Yes (%) <sup>a</sup>			
ALDH1A1(low) and $\beta$ -catenin(m)	47 (82.5)	10 (17.5)	0.003		
ALDH1A1(low) and $\beta$ -catenin(c)	37 (72.5)	14 (27.5)	0.216 <sup>c</sup>		
ALDH1A1(high) and $\beta$ -catenin(m)	21 (70.0)	9 (30.0)	0.181 <sup>c</sup>	0.806 <sup>d</sup>	
ALDH1A1(high) and $\beta$ -catenin(c)	27 (50.0)	27 (50.0)	<b>&lt;0.001<sup>c</sup></b>	<b>0.018<sup>d</sup></b>	0.076 <sup>e</sup>

<sup>a</sup>Numbers in parentheses are percentage; <sup>b</sup>p-value obtained from Pearson's Chi-square test or Fisher's exact test; <sup>c</sup>compared with group of ALDH1A1(low) and  $\beta$ -catenin(m); <sup>d</sup>compared with group of ALDH1A1(low) and  $\beta$ -catenin(c); <sup>e</sup>compared with group of ALDH1A1(high) and  $\beta$ -catenin(m); ALDH1A1, aldehyde dehydrogenase 1A1.

in  $\beta$ -catenin(c) breast cancer than in  $\beta$ -catenin(m) breast cancer (P=0.020, Fig. 3B).

*Association of the expression of ALDH1A1 and  $\beta$ -catenin with clinicopathological characteristics of breast cancer patients.* Table II summarizes the association between the expression of ALDH1A1 and  $\beta$ -catenin and the clinicopathological characteristics of breast cancer patients. The age, menopausal status,

tumor type, tumor size, histological grade, and TNM stage were not significantly associated with the expression of ALDH1A1. ALDH1A1(high) expression was associated with lymph node metastasis (P=0.029). The age, menopausal status, tumor size, tumor type, and TNM stage were not significantly associated with the subcellular expression of  $\beta$ -catenin.  $\beta$ -catenin(c) expression was associated with grade II-III tumors (P=0.014) and lymph node metastasis (P=0.002).

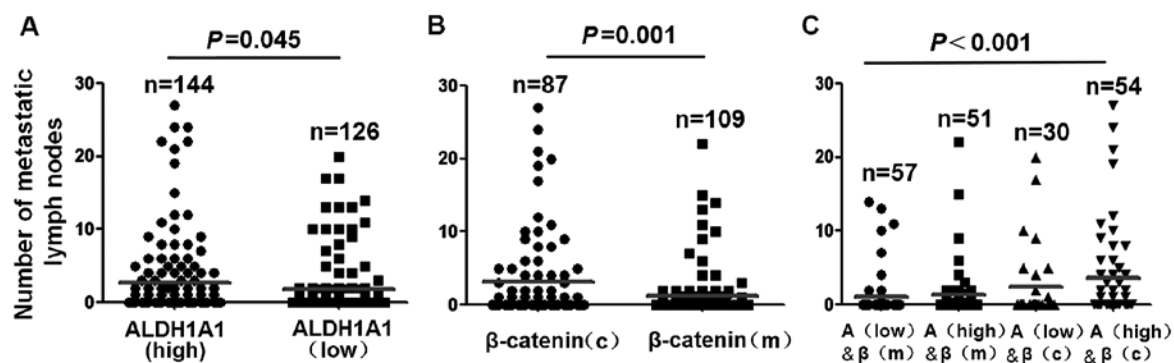


Figure 4. Breast cancer patients with ALDH1A1(high) and ALDH1A1(low) expression (A),  $\beta$ -catenin(c) and  $\beta$ -catenin(m) expression (B), and their combined expression (C). Red line denotes the median value. A and B, Wilcoxon rank sum test; C, Kruskal-Wallis H test.

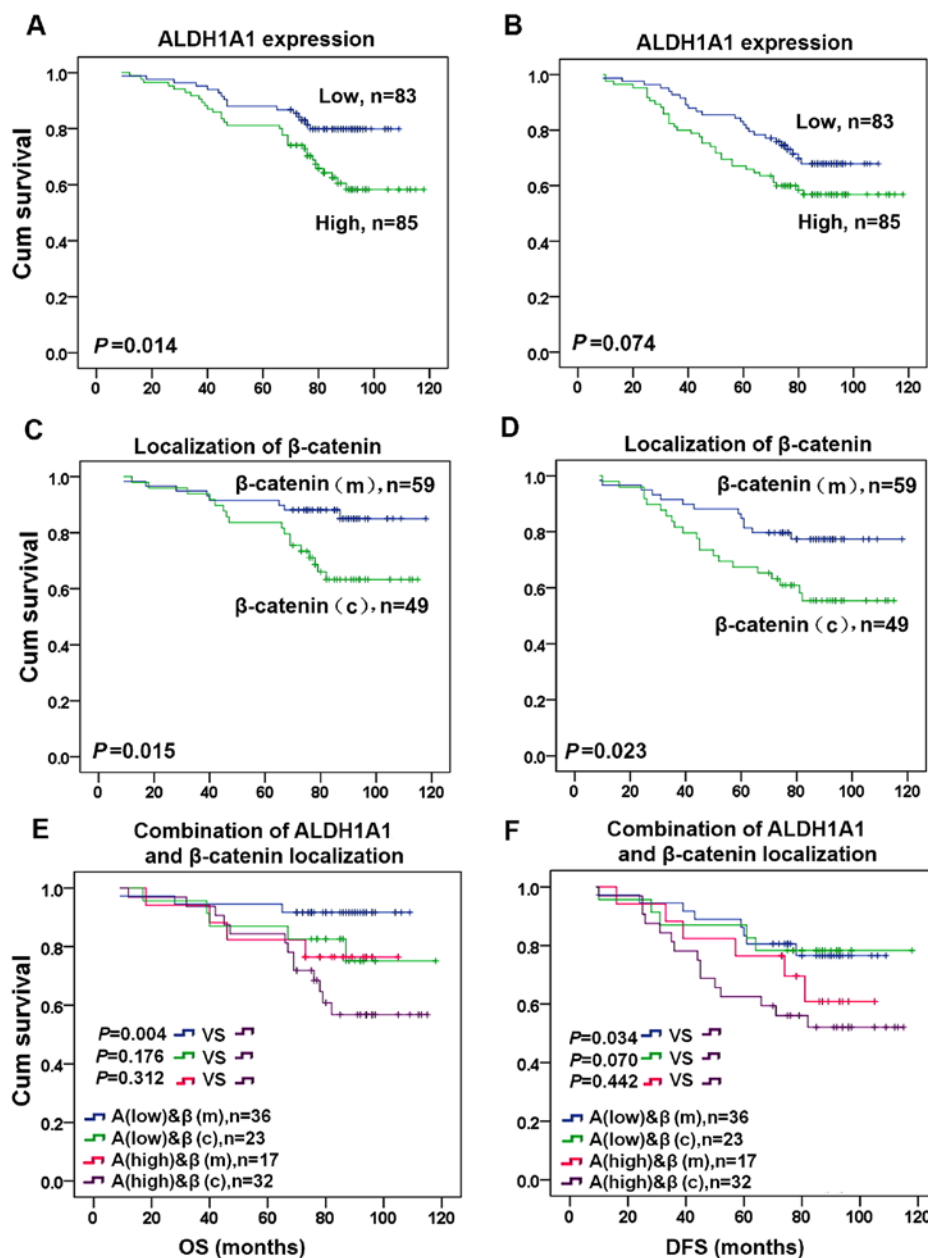


Figure 5. Kaplan-Meier survival analysis of ALDH1A1 and  $\beta$ -catenin expression in breast cancer patients. (A and B) Survival curves show the association between ALDH1A1 expression and OS (A) or DFS (B) in 168 breast cancer patients. (C and D) Survival curves show the association between the subcellular expression of  $\beta$ -catenin and OS (C) and DFS (D) in 108 breast cancer patients. (E and F) Survival curves show the association between combined expression of ALDH1A1 and  $\beta$ -catenin and OS (E) or DFS (F) in 108 breast cancer patients. ALDH1A1, aldehyde dehydrogenase 1A1; OS, overall survival; DFS, disease-free survival.

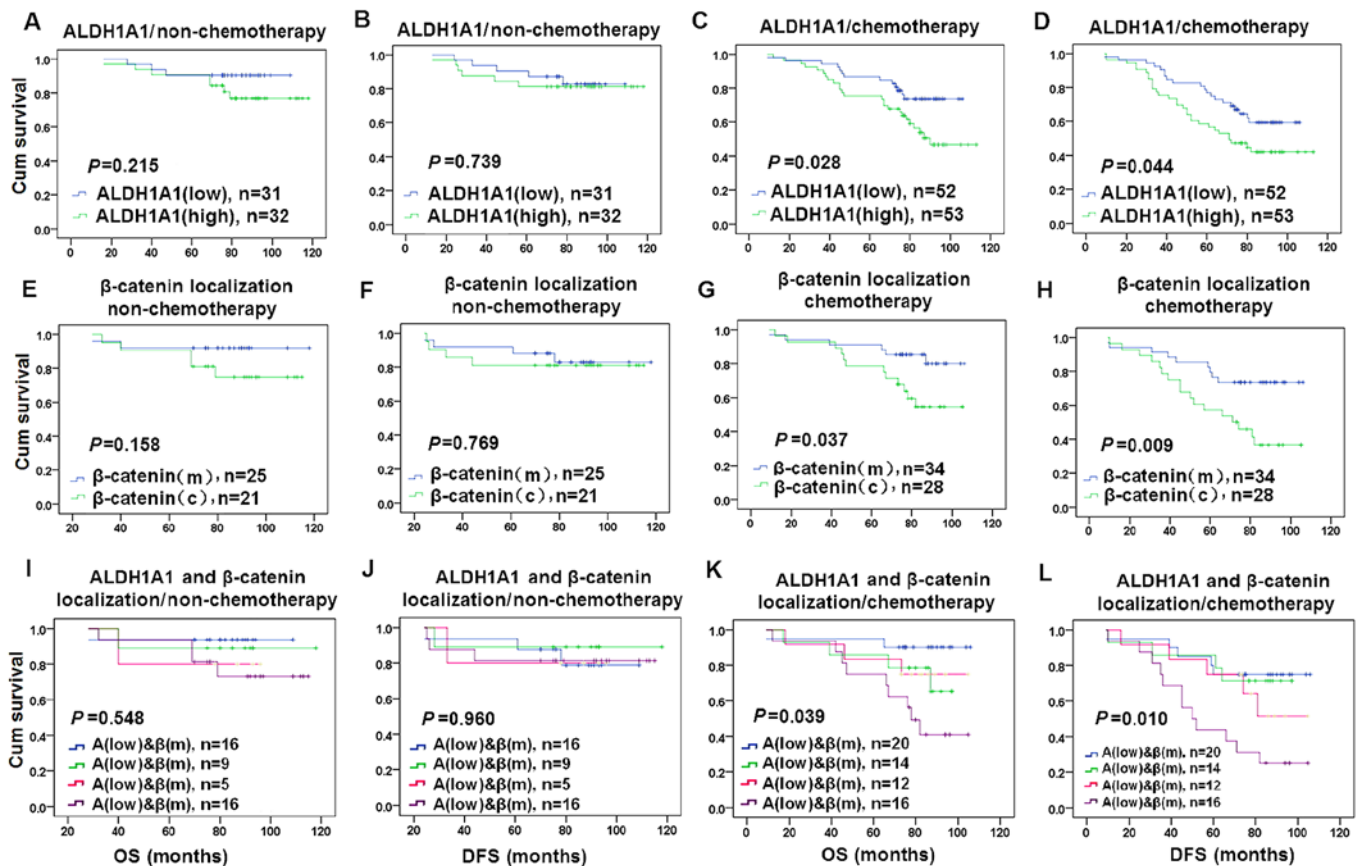


Figure 6. Kaplan-Meier survival analysis of ALDH1A1 and  $\beta$ -catenin expression in breast cancer patients with or without cyclophosphamide treatment. (A-D) Survival curves show the association between ALDH1A1 expression and OS (A and C) or RFS (B and D) in breast cancer patients receiving non-cyclophosphamide (A and B) or cyclophosphamide (C and D) therapy. (E-H) Survival curves show the association between the subcellular expression of  $\beta$ -catenin and OS (E and G) or RFS (F and H) in breast cancer patients receiving non-cyclophosphamide (E and F) or cyclophosphamide (G and H) therapy. (I-L) Survival curves show the association between the combined expression of ALDH1A1 and  $\beta$ -catenin and OS (I and L) or RFS (J and L) in breast cancer patients receiving non-cyclophosphamide (I and J) or cyclophosphamide (K and L) therapy. ALDH1A1, aldehyde dehydrogenase 1A1; OS, overall survival.

Table III summarizes the association of combined expression of ALDH1A1 and  $\beta$ -catenin with lymph node metastasis. Compared with tumors with ALDH1A1(low) and  $\beta$ -catenin(m) expression and ALDH1A1(low) and  $\beta$ -catenin(c) expression, tumors with ALDH1A1(high) and  $\beta$ -catenin(c) expression was associated with lymph node metastasis ( $P < 0.05$ , Table III). Compared with tumors with ALDH1A1(high) and  $\beta$ -catenin(m) expression, tumors with ALDH1A1(high) and  $\beta$ -catenin(c) expression exhibited a tendency toward more lymph node metastasis ( $P = 0.076$ , Table III).

We then examined the effect of ALDH1A1 and  $\beta$ -catenin expression on the number of metastatic lymph nodes in breast cancer. The number of metastatic lymph nodes was significantly higher in patients with ALDH1A1(high) expression compared with patients with ALDH1A1(low) expression ( $P = 0.045$ , Fig. 4A). The number of metastatic lymph nodes was significantly higher in patients with  $\beta$ -catenin(c) expression compared with patients with  $\beta$ -catenin(m) expression ( $P = 0.001$ , Fig. 4B). Patients with ALDH1A1(high) and  $\beta$ -catenin(c) expression had significantly more metastatic lymph nodes compared with tumors with other combined expression of ALDH1A1 and  $\beta$ -catenin ( $P < 0.001$ , Fig. 4C).

*Association of the expression of ALDH1A1 and  $\beta$ -catenin with the survival of breast cancer patients.* The Kaplan-Meier anal-

ysis and log-rank test were used to evaluate the association of the expression level of ALDH1A1 and  $\beta$ -catenin with the OS or DFS in breast cancer patients. ALDH1A1(high) expression was associated with significantly shorter OS ( $P = 0.014$ , Fig. 5A). Although ALDH1A1(high) expression was associated with a tendency toward shorter DFS in breast cancer patients, no significant difference was found ( $P = 0.074$ , Fig. 5B). In addition,  $\beta$ -catenin(c) expression was associated with significantly shorter OS ( $P = 0.015$ , Fig. 5C) and DFS ( $P = 0.023$ , Fig. 5D). We further examined the association of the combined expression of ALDH1A1 and  $\beta$ -catenin with the OS or DFS in breast cancer patients. Compared with ALDH1A1(low) and  $\beta$ -catenin(m) expression, ALDH1A1(high) and  $\beta$ -catenin(c) expression was associated with shorter OS and DFS in breast cancer patients ( $P < 0.05$ , Fig. 5E and F).

We examined the association of the expression of ALDH1A1 and  $\beta$ -catenin with the therapeutic response in breast cancer patients who received cyclophosphamide treatment. ALDH1A1 and  $\beta$ -catenin alone and their combined expression were not significantly associated with the OS and DFS in breast cancer patients with non-cyclophosphamide therapy (Fig. 6A, B, E, F, I and J). ALDH1A1(high) expression was associated with shorter OS ( $P = 0.004$ ) and DFS ( $P = 0.002$ ) in patients with cyclophosphamide treatment (Fig. 6C and D).  $\beta$ -catenin(c) expression was associated with shorter OS

Table IV. Univariate Cox regression analysis of the association between clinicopathological data and OS and DFS in breast cancer patients.

	Total no.	OS			DFS		
		Events n (%)	RR (95% CI)	P-value	Events n (%)	RR (95% CI)	P-value
Age (years)				0.126			0.116
≤50	87	21 (24.1)	1 (reference)		27 (31.0)	1 (reference)	
>50	79	27 (34.2)	1.561 (0.883-2.763)		34 (43.0)	1.500 (0.905-2.487)	
Menopausal state				<b>0.029</b>			<b>0.034</b>
Pre-menopause	87	19 (21.8)	1 (reference)		25 (28.7)	1 (reference)	
Post-menopause	75	28 (37.3)	1.911 (1.067-3.424)		33 (44.0)	1.755(1.043-2.953)	
Tumor size (cm)				<b>&lt;0.001</b>			<b>&lt;0.001</b>
≤2.0	76	11 (14.5)	1 (reference)		19 (25.0)	1 (reference)	
>2.0	60	33 (55.0)	5.070 (2.557-10.054)		38 (63.3)	3.546(2.036-6.173)	
Tumor type				0.541			0.387
Ductal	134	37 (27.6)	1 (reference)		47 (35.1)	1 (reference)	
Lobular	17	6 (35.3)	1.309 (0.552-3.102)		7 (41.2)	1.178 (0.813-1.706)	
Histological grade				<b>&lt;0.001</b>			<b>&lt;0.001</b>
I	79	6 (7.6)	1 (reference)		13 (16.5)	1 (reference)	
II-III	84	42 (50.0)	8.699 (3.687-20.524)		48 (57.1)	4.673 (2.526-8.642)	
TNM stage				<b>&lt;0.001</b>			<b>&lt;0.001</b>
I-II	93	24 (25.8)	1 (reference)		31 (33.3)	1 (reference)	
III	20	15 (75.0)	5.039 (2.625-9.673)		16 (80.0)	4.727 (2.554-8.749)	
Lymph node metastasis				<b>&lt;0.001</b>			<b>&lt;0.001</b>
No	98	13 (13.3)	1 (reference)		18 (18.4)	1 (reference)	
Yes	62	32 (51.6)	4.997 (2.617-9.542)		40 (64.5)	4.900 (2.800-8.574)	
ALDH1A1 expression				<b>0.016</b>			0.077
Low	83	16 (19.3)	1 (reference)		25 (30.1)	1 (reference)	
High	85	32 (37.6)	2.085 (1.144-3.800)		36 (42.4)	1.585 (0.951-2.641)	
$\beta$ -catenin localization				<b>0.019</b>			<b>0.027</b>
$\beta$ -catenin(m)	59	8 (13.6)	1 (reference)		13 (22.0)	1 (reference)	
$\beta$ -catenin(c)	49	17 (34.7)	2.724 (1.175-6.316)		21 (42.9)	2.182 (1.092-4.360)	

RR, relative risk; 95% CI, 95% confidence interval; OS, overall survival; DFS, disease-free survival.

( $P=0.021$ ) and DFS ( $P=0.003$ ) in patients with cyclophosphamide treatment (Fig. 6G and H). Combined ALDH1A1(high) and  $\beta$ -catenin(c) expression was associated with shorter OS ( $P=0.020$ ) and DFS ( $P=0.012$ ) in patients with cyclophosphamide treatment compared with other combined expression of ALDH1A1 and  $\beta$ -catenin (Fig. 6K and L).

The univariate analysis identified that the menopausal status, tumor size, histological stage, TNM stage, and lymph node metastasis were significantly associated with the OS and DFS in breast cancer patients (Table IV). In addition, ALDH1A1(high) expression was significantly associated with shorter OS of breast cancer patients.  $\beta$ -catenin(c) expression was significantly associated with shorter OS and DFS of breast cancer patients (Table IV). Furthermore, multivariate

Cox regression analysis found that histological grade was an independent prognostic factor for OS and DFS in breast cancer patients ( $P<0.05$ , Table V). Lymph node metastasis and histological grade were independent prognostic factors for shorter DFS in breast cancer patients ( $P<0.05$ , Table V).

## Discussion

In this study, we performed tissue microarray-based immunohistochemistry to examine the expression of ALDH1A1 and  $\beta$ -catenin in 276 breast cancer patients. We found that ALDH1A1 overexpression was associated with lymph node metastasis and shorter OS in breast cancer patients. In addition, we found that the  $\beta$ -catenin(c) expression was associated

Table V. Multivariate Cox regression analysis of the association between clinicopathological data and OS and DFS in breast cancer patients.

	OS		DFS	
	RR (95% CI)	P-value	RR (95% CI)	P-value
Age (years) (>50/≤50)	1.510 (0.172-13.260)	0.710	2.792 (0.506-15.416)	0.239
Menopausal state (post/pre)	0.658 (0.064-6.727)	0.724	0.249 (0.037-1.694)	0.155
Tumor size (cm) (>2.0/≤2.0)	2.382 (0.733-7.743)	0.149	1.647 (0.565-4.805)	0.361
Histological grade (II-III/I)	12.066 (2.488-58.518)	<b>0.002</b>	3.005 (1.123-8.045)	<b>0.029</b>
TNM stage (III/I-II)	2.735 (0.929-8.049)	0.068	2.049 (0.750-5.600)	0.162
Lymph node metastasis (yes/no)	3.851 (0.926-16.019)	0.064	5.734 (1.600-20.556)	<b>0.007</b>
ALDH1A1 expression (high/low)	1.259 (0.395-4.006)	0.697	0.654 (0.257-1.665)	0.373
β-catenin localization (c/m)	1.351 (0.446-4.089)	0.594	0.935 (0.337-2.591)	0.897

RR, relative risk; 95% CI, 95% confidence interval; OS, overall survival; DFS, disease-free survival.

with lymph node metastasis and shorter OS and DFS in breast cancer patients. Furthermore, combined expression of ALDH1A1(high) and β-catenin(c) was associated with shorter OS and DFS in breast cancer patients, especially in patients with cyclophosphamide treatment. Our findings suggest that translocation of β-catenin from the membrane to the cytoplasm may subsequently promote the expression of ALDH1A1 in breast cancer, and β-catenin-regulated ALDH1A1 may contribute to poor outcome of breast cancer patients.

In the present study, we examined the association of ALDH1A1 expression with clinical characteristics and outcomes of breast cancer patients. We found that ALDH1A1(high) expression was associated with poor clinical outcome of breast cancer patients in agreement with previous studies showing that ALDH1A1 is associated with poor prognosis of breast cancer patients (11,12). In addition, we found that ALDH1A1(high) expression was associated with lymph node metastasis, and patients with ALDH1A1(high) expression had more metastatic lymph nodes than those with ALDH1A1(low) expression. Consistent with our results, Khoury *et al* found that ALDH1A1 expression correlated with lymph node metastasis in breast cancer patients (24). Several studies have demonstrated that ALDH1 expression is associated with lymph node metastasis and poor survival in various tumors such as esophageal squamous cell carcinoma, gastric cancer, and rectal cancer (25-27). Our findings that ALDH1A1(high) was associated with lymph node metastasis and poor clinical outcome suggests that ALDH1A1 expression may define breast cancer with strong metastatic potential and be used for predicting disease outcome. Furthermore, ALDH1A1 is known to inactivate cyclophosphamide, and thus contribute to resistance to cyclophosphamide and poor clinical outcome of breast cancer patient (14,24). Moreover, we found that ALDH1A1(high) expression was associated with shorter OS and DFS in patients with cyclophosphamide treatment, but not in those with non-cyclophosphamide treatment. Therefore, ALDH1A1 may be used as a biomarker to predict poor clinical outcome after cyclophosphamide treatment.

The Wnt signaling pathway is known to be involved in the development of breast cancer (28). During Wnt signaling

activation, β-catenin is translocated from the membrane to the cytoplasm and the nucleus, where it promotes transcription of multiple protumorigenic genes via interaction with LEF/TCF transcription factors (20). The cytoplasmic accumulation of β-catenin has been found to be associated with poor clinical outcome of breast cancer patients (20,29-32). In the present study, we found that the cytoplasmic expression of β-catenin was associated with grade II-II tumors, lymph node metastasis, and shorter OS and DFS in breast cancer patients. Our study supports that the cytoplasmic expression of β-catenin can be used as a prognostic marker for poor outcome of breast cancer patients. Although the mechanisms by which β-catenin is translocated from the membrane to the cytoplasm in breast cancer remain largely unclear, Adenomatous polyposis coli (APC), a scaffolding protein that regulate the cellular level of β-catenin in the Wnt signaling pathway (33), may be involved. It has been reported that the low expression of APC is associated with overexpression of β-catenin in the cytoplasm of breast cancer cells (34). In breast cancer cell lines, APC truncation leads to an increase in the cytoplasmic expression of β-catenin (35).

Although ALDH1A1 is known to be a CSC marker that is associated with poor prognosis of many tumors including breast cancer (10-12), little is known about the regulation of ALDH1A1 expression. Recently, it has been reported that ALDH1A1 is a direct target of β-catenin in ovarian cancer spheroids (15). In the present study, we found that β-catenin(c) expression occurred more frequently in ALDH1A1(high) breast cancer, and the expression level of ALDH1A1 was higher in β-catenin(c) breast cancer, suggesting that β-catenin may regulate the expression of ALDH1A1 in breast cancer. This idea is supported by the finding that the ALDH activity was reduced by the inhibition of Wnt/β-catenin signaling by a TCF4-dominant negative construct in human breast cancer cells (36). Thus, β-catenin may regulate the expression of ALDH1A1 via interaction with the TCF4 transcription factor in breast cancer. In addition, we found that tumors with ALDH1A1(high) and β-catenin(c) expression were associated with lymph node metastasis and poor clinical outcome in

breast cancer patients. Consistent with our findings, Xu *et al* reported that in patients with colorectal carcinoma,  $\beta$ -catenin was preferentially expressed in tumors with high expression of ALDH1A1, and were associated with high potential of metastasis and poor outcome (37). Furthermore, we found that ALDH1A1(high) and  $\beta$ -catenin(c) expression was associated with shorter OS and DFS in patients with cyclophosphamide treatment, suggesting that tumors with ALDH1A1(high) and  $\beta$ -catenin(c) expression exhibited resistance to cyclophosphamide treatment. ALDH1A1(high) and  $\beta$ -catenin(c) expression may be used for predicting poor outcome of breast cancer patients.

In summary, we investigated the expression of ALDH1A1 and  $\beta$ -catenin in breast cancer patients, and analyzed the association of their expression alone and in combination with the clinicopathological characteristics and prognosis of breast cancer patients. We found that ALDH1A1(high) and  $\beta$ -catenin(c) expression was associated with lymph node metastasis and worse clinical outcome in breast cancer patients, especially those receiving cyclophosphamide treatment. Combined expression of ALDH1A1(high) and  $\beta$ -catenin(c) was associated with lymph node metastasis, poor outcome, and resistance to cyclophosphamide treatment. Our study suggests that  $\beta$ -catenin may regulate ALDH1A1 expression in a subtype of breast cancer with ALDH1A1(high) and  $\beta$ -catenin(c) expression. ALDH1A1(high) and  $\beta$ -catenin(c) expression may be used as a biomarker for predicting poor prognosis in breast cancer following cyclophosphamide treatment.

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