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Effect of ALDH1 on prognosis and chemoresistance by breast cancer subtype

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Abstract Aldehyde dehydrogenase 1 (ALDH1) has been identified as a breast cancer stem cell marker, but its value as a predictor of prognosis and chemoresistance is controversial. This study investigated the effect of ALDH1 on prognosis and chemoresponse by breast cancer subtype. We immunohistochemically analyzed 653 invasive breast cancer specimens and evaluated correlations among clinicopathological factors, survival status, response to neoadjuvant chemotherapy, and ALDH1 expression. Of 653 specimens, 139 (21.3 %) expressed ALDH1 in tumor cells. ALDH1 expression was correlated significantly with larger tumor size, node metastasis, higher nuclear grade, and with HER2$^+$ and progesterone/estrogen receptor (HR)$^-$ subtypes. ALDH1 expression was significantly observed in HER2 type and triple-negative breast cancer (TNBC). Patients with ALDH1$^+$ cancers had significantly shorter disease-free survival ($P < 0001$) and overall survival ($P = 0.044$). ALDH1 expression significantly affected prognosis of luminal types, but not TNBC and HER2-enriched types. For the 234 patients treated with neoadjuvant chemotherapy, pathological complete response (pCR) rate was significantly lower in ALDH1$^+$ cases (13.5 vs. 30.3 %, $P = 0.003$). pCR and ALDH1 expression were significantly correlated in TNBC patients ($P = 0.003$). ALDH1$^+$ breast cancers tended to be aggressive, with poor prognoses. Although ALDH1$^+$ TNBC showed higher chemoresistance, ALDH1 had significant impact on prognosis in the luminal type but not in TNBC.

Keywords Breast cancer · Stem cell · ALDH1 · Prognosis · Chemoresistance · Subtype

Introduction

Aldehyde dehydrogenase 1 (ALDH1) has been identified as a marker of breast cancer stem cells (CSCs) [1]. CSCs are rare tumor cells that are capable of self-renewal and are multipotent [2–5], and are thought to initiate cancer and propagate metastasis [6–8]. The CD44$^+$/CD24$^-$ phenotype was initially used to identify CSCs in breast cancer. Although some cells from this population showed high tumorigenicity by initiating cancers in immunodeficient mice [9], in the original study, the CD44$^+$/CD24$^-$ phenotype did not successfully identify CSCs in patients, which implied that other CSC markers existed. Subsequently, ALDH1 was suggested as a possible marker for breast CSCs [1].

Despite convincing data in cell lines and animals, the clinical significance of CSCs remains controversial [10–13]. ALDH1 expression is reportedly associated with poor clinical prognosis by some researchers [1, 14], but not others [15].

A meta-analysis of 12 studies with 898 ALDH1$^+$ cases and 1853 controls found that ALDH1$^+$ breast tumors were significantly associated with higher histological grade, estrogen receptor (ER)$^-$ and progesterone receptor (PR)$^-$

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tumors, and HER2$^+$ tumors, but not with tumor size or nodal status; ALDH1$^+$ and CD44$^+$/CD24$^-$ tumor cells were significantly associated with shorter overall survival (OS)[16]. In a later (2014) review and meta-analysis about ALDH1A1 (the isoform considered responsible for ALDH1-mediated stemness in breast cancer) that included 15 studies with 921 ALDH1A1$^+$ cases and 2353 controls, higher ALDH1A1 expression was associated with larger tumor size, higher histological grade, higher rates of lymph node metastasis, greater expression of HER2, lower expression of ER/PR, and poorer prognosis [13].

Although both of these meta-analyses concluded that ALDH1 was a biomarker that could be used to predict tumor progression and poor OS in breast cancer patients, the influence of intrinsic subtype on its effect was not analyzed. In fact, in line with these two meta-analyses, most previous studies reported that HER2-enriched and triple-negative types, which are generally known to have poor prognoses, were more prevalent in ALDH$^+$ cancers [1, 17].

The CSC hypothesis also implies a role of CSCs in chemoresistance [18]. Accumulating evidence indicates that CSCs are naturally resistant to chemotherapy because of their quiescence, more efficient DNA repair, resistance to apoptosis, and expression of drug-resistance proteins [19]. If so, a small population of chemoresistant CSCs may resist being killed by conventional chemotherapy and thereafter regrow into a more chemoresistant tumor. However, CSC-associated chemoresistance in human breast cancer has not been widely studied [20, 21], although several in vitro studies have supported this idea [22–25].

A possible reason for the clinical uncertainty about the value of ALDH1 in predicting prognosis and chemoresistance is the heterogeneity of breast cancer and the corresponding potential effect of intrinsic subtype. Whereas chemoresponse and prognosis in breast cancer largely depends on subtype, the effect of ALDH1 in each subtype has not been addressed, to our knowledge. This study investigated the impact of ALDH1 on chemoresistance and prognosis according to intrinsic subtype in invasive breast cancer.

Materials and methods

Patients and tumor specimens

A total of 653 patients with primary breast cancer who had been treated between November 2004 and June 2013 at the Yokohama City University Medical Center were enrolled in this study. All patients were diagnosed from core needle biopsies with invasive breast carcinoma prior to any treatment. Patient characteristics are shown in Table 1. Their ages ranged from 24 to 94 years of age. Disease-free survival (DFS) interval was the time from diagnosis to the date of breast cancer-derived relapse or metastasis; overall survival (OS) was the number of days from diagnosis to breast cancer-related death. The median follow-up period was 2491 days.

Of the 653 enrolled patients, 234 received neoadjuvant chemotherapy (NAC). Of those 234 patients, 171 also received anthracycline followed by taxane-based therapy. All HER2$^+$ patients also received trastuzumab. Patients underwent breast-conserving surgery or mastectomy and axillary lymph node dissection about a month after final chemotherapy. Clinical data, tumor characteristics, lymph node status, and prognostic information were retrieved from the clinical database. The Ethics Committee of Yokohama City University approved the study. Informed consent was obtained from each patient.

Histopathological and immunohistological staining

Core needle biopsy specimens from patients were fixed in phosphate-buffered formalin and embedded in paraffin. Serial Sects. (3–4 µm) were mounted on silane-coated slides (Muto-Glass, Tokyo, Japan) and dried at 37 °C overnight. Hematoxylin and eosin (H&E)-stained sections from each block were prepared to determine histological diagnosis, histological type, and nuclear grade. Immuno-histochemical (IHC) staining was performed using an automatic staining machine (Dako-Autostainer; Dako, Kyoto, Japan). Slides were incubated at 95 °C for 40 min and deparaffinized four times for 5 min in xylene, followed by 5 min each in 95, 90, and 70 % ethanol, and twice for 1 min in distilled water. Antigen retrieval was achieved by microwaving slides in citrate buffer (pH 6.0) for 40 min at 95 °C. Endogenous peroxidase was blocked with a perox-idase-blocking reagent (Dako). Tissue sections were incubated with primary antibodies for 30 min, and with secondary reagents for 30 min. 3,3′diaminobenzidine (DAB; Dako) was used as a chromogenic substrate; slides were lightly counterstained with Mayer's hematoxylin.

Pathological evaluation

Three board-certified pathologists assessed the pathological diagnosis and IHC evaluation blindly. ER and PR status was evaluated using the Allred score [26]. HER2 status was examined using the Dako HercepTest^{TM} (K5204; Dako) or PathVysion^{TM}, (Abbott). HER2 positivity (overexpression or amplification) was scored according to the American Society of Clinical Oncology/College of American Pathologists

Table 1 Patient characteristics

	ALDH1		P*
	Positive, n	Negative, n	
All Breast carcinomas (n = 653)	139	514	
Age			
≦40 years	18	52	0.32
>40 years	121	462	
Histologic type			0.45
Invasive ductal carcinoma	135	492	
Other invasive carcinoma	4	22	
Tumor size			≤0.001
T1	42	234	
T2	73	232	
T3	14	25	
T4	10	23	
Lymph node metastasis			0.004
N(−)	81	366	
N(+)	58	148	
Clinical stage			≤0.001
I	33	209	
II	76	246	
III	30	59	
ER(−)	75	159	≤0.001
ER(+)	64	355	
PgR(−)	93	196	<0.001
PgR(+)	46	318	
HER2(−)	95	441	<0.001
HER2(+)	44	73	
Intrinsic subtype			<0.001
Luminal (HER2−)	45	323	
Luminal-HER2	19	33	
HER2 enriched	25	41	
Triple negative	50	117	
Nuclear Grade			0.005
1	31	198	
2	36	117	
3	72	199	
ND			
Ki67			
≦13	27	73	0.748
13<	112	441	
ND			
Ki67			
<20	49	179	0.77
20≦	90	335	
ND			
Topo2A			
<20%	69	211	0.069
≧20 %	70	303	

Table 1 continued

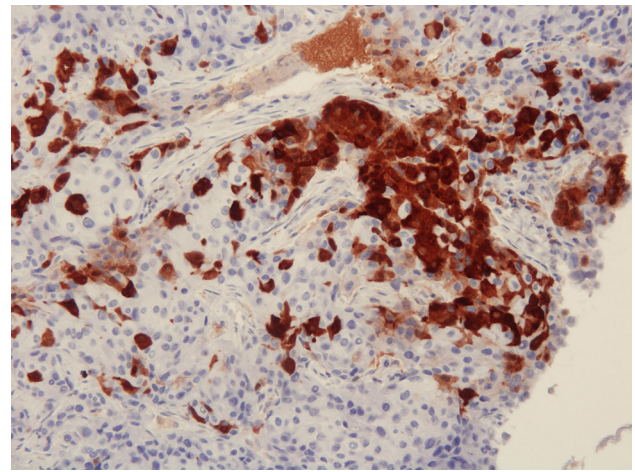|	ALDH1		P*
	Positive, n	Negative, n	
Basal phenotype			0.45
(CK5/6 or EGFR positive)			
Present	27	42	
Absent	20	41	
Chemotherapy treatment			*<0.001*
Present	113	236	
Absent	26	278	

Italic; statistically significant

guidelines [27]. Tumors were also classified into four subtypes using IHC results as surrogates, i.e., luminal type: ER $\geq$ 3 (Allred score) and HER2$^-$; luminal-HER2 type: ER $\geq$ 3 and HER2$^+$; HER2-enriched type: ER $\leq$ 2 and HER2$^+$; Triple-negative breast cancer (TNBC): ER $\leq$ 2 and HER2$^-$.

Immunostaining for MIB-1 (Dako), topoisomerase IIα (Topo2) (Ki-S1; Dako), and P53 (DO-7; Dako) was evaluated on the basis of the percentage of tumor cells with positive nuclear staining. For MIB-1, Topo2, and P53, only unequivocal nuclear staining was considered to be a positive reaction. After scanning the slides at low magnification to determine the most evenly labeled tissue areas, a minimum of 1000 tumor cells were counted at high power ($\times$ 350), and the number of labeled cells was calculated as a percentage of the total cell count. Ki-67, Topo2, and p53 were each dichotomized to high- and low-expression groups using their respective mean values as cut-off points.

Immunostaining of cytokeratin 5/6 (CK5/6) (D5/16 B4; Dako) and epidermal growth factor receptor (EGFR) (3C6; Roche Diagnostics, Tokyo, Japan) was semiquantitatively graded according to the proportion of tumor cells with positive nuclear staining (0, 0 %; 1, 0–10 %; and 2, >10 %). A score of 0 was defined as negative, and 1 or 2 was positive. Specimens that were CK5/6$^+$ and/or EGFR$^+$ were considered to be basal-type cancers.

ALDH1 was evaluated by IHC with ALDH1A1 antibody (EP1933Y; Abcam). Specimens were considered ALDH1$^+$ when ALDH1 staining was >1 % in cancer cells. Expression in only stromal cells was considered negative. Representative results are shown in Fig. 1.

In pathological evaluation of NAC response, pCR was defined as any combination of (a) necrosis; (b) disappearance of all tumor cells; and (c) replacement of cancer cells by granulation and/or fibrosis. Pathological effect was defined as lack of histologic sign of invasive cancer cells in the primary tumor, using the NSABP B-18 criteria [28].

Fig. 1 Representative immunohistochemical results of ALDH1$^+$ tumor cells

Correlation of ALDH1 with clinicopathological factors

We analyzed associations among ALDH1 expression and other biomarkers with clinicopathological factors, including age, histologic type, tumor size, nodal status, ER/PR/HER2 status, nuclear grade (NG), Ki-67, Topo2, p53, CK5/6, and EGFR.

Statistical analysis

Findings were analyzed using the SPSS Statistics v.23 software (IBM SPSS Statistics for Windows, Version 23.0. IBM Corp, Armonk, NY, USA). We used χ^2 analysis (Spearman's coefficient) to test for associations between ALDH1 and clinicopathological factors, the Mann–Whitney U test for associations between ALDH1 and patient age and tumor size, and Fisher's exact test for ALDH1 associations with other biomarkers. Survival data were

evaluated using the Kaplan–Meier method and the log-rank test. Multivariate analyses of prognosis were evaluated using the Cox proportional hazards model. $P < 0.05$ was considered significant.

Results

Correlation between ALDH1 and clinicopathological factors

We performed IHC staining to examine ALDH1 expression in 653 specimens of invasive breast carcinoma from core needle biopsies taken prior to treatment, and found ALDH1$^+$ tumor cells in 139 cases (21.3 %). ALDH1 expression correlated significantly with larger tumor size, clinical node metastasis, higher clinical staging, higher nuclear grade, ER/PR positivity, and HER2 absence, but did not correlate with age, histologic type, expression of Ki67 or Topo2, or basal phenotype (Table 1). The percentages of subtypes with ALDH1$^+$ cells were luminal: 12.2 %; luminal-HER2: 36.5 %; HER2-enriched: 37.9 %; and TNBC: 30.0 %. ALDH1 expression was significantly more common in HER2 type and TNBC.

Prognostic study

Patients with ALDH1$^+$ specimens had significantly shorter DFS ($P < 0001$) and OS ($P = 0.044$) than the group with ALDH1$^-$ specimens (Fig. 2a). Cox multivariate analyses of DFS and OS, against tumor size, lymph node metastasis, clinical staging, nuclear grade, and ER, HER2, and ALDH1 expressions found that ALDH1 expression was an independent predictor for DFS ($P = 0.033$), but not for OS ($P = 0.124$; Table 2). Although ALDH1 expression significantly affected prognosis of patients with luminal-type cancers, it did not significantly affect those with TNBC and HER2$^+$ types (Fig. 2b–d).

Neoadjuvant study

We analyzed specimens from 234 breast cancer patients treated with NAC, including 63 with luminal type, 20 with luminal-HER2 type, 45 with HER2-enriched type, and 106 with TNBC type. ALDH1 was expressed in 88 specimens (37.6 %). For all subtypes, 56 patients (23.9 %) achieved pCR. The pCR rate was significantly lower in patients with ALDH1$^+$ tumors (13.5 vs. 30.3 %, $P = 0.003$). In univariate analysis, pCR was significantly correlated with ALDH1 expression ($P = 0.003$), ER negativity ($P = 0.001$), PR ($P = 0.004$) negativity, and higher expression of Ki67 ($P = 0.04$) and p53 ($P = 0.017$). In multivariate analysis, ALDH1 and ER correlated significantly with the pCR rate (Table 3).

Among the subtypes, pCR and ALDH1 expressions were very significantly associated in TNBC ($P = 0.003$). In the HER2-enriched and luminal-HER2 types, ALDH1 expression tended to correlate with low pCR rates, but not significantly so (Table 4). In the luminal (HER2$^-$) group, two patients achieved pCR; ALDH1 was not detected in either specimen. A univariate analysis of pCR predictors in TNBC correlated ALDH1 and Ki67 with pCR, but only ALDH1 survived in multivariate analysis ($P = 0.012$; Table 5).

Discussion

Cancer stem cells (CSCs) are considered to cause the initiation, progression, and recurrence of cancers [6, 29, 30]. ALDH1 expression has been correlated with increased tumor size, lymph node metastasis, nuclear grade, and clinical staging, all of which are associated with progression and poor prognosis. Our results conform to those of a meta-analysis published in 2014 [13]. However, in another meta-analysis published in 2010, tumor size and nodal status did not correlate with ALDH1 expression, which differs from our results and the 2014 meta-analysis, in which both showed a positive correlation.

We found the clinical significance of ALDH1 to differ by subtype. ALDH1 positivity significantly varied with poor prognosis in luminal breast cancers, but not for the other subtypes. In ER$^+$/PR$^+$ breast cancers, CSCs rarely exist and their clinical significance is generally unknown. Although several reports concerning the prognostic importance of CSCs in luminal breast cancer have been published, they were limited by small sample sizes and yielded questionable results. Kim et al. reported that non-TNBC patients with ALDH1$^+$ tumors had significantly shorter OS than those with ALDH1$^-$ tumors, whereas TNBC patients showed no statistical differences in DFS and OS according to ALDH1 expression [31]. Although the main limitation of this study is its small sample size ($n = 70$), we showed a similar tendency by subtype.

Hashimoto et al. reported that neither ALDH1 positivity nor CD44$^+$/CD24$^-$ status of tumor cells was related to progression-free survival in luminal metastatic breast cancers [32]. In our study, ALDH1 positivity was found in 13.9 % of luminal-type tumors and CSCs were shown to be an especially pessimistic prognostic indicator in patients with this subtype. To our knowledge, this is the largest study to report on the prognostic impact of CSCs on luminal breast cancers. Luminal breast cancers that are refractory to chemotherapy and endocrine therapy—the so-called "unmet needs"—have become a focus of much

Fig. 2 Kaplan–Meier curves for disease-free survival (DFS) and overall survival (OS) among ALDH1⁻ and ALDH1⁺ patients with [A] all breast cancer subtypes [B] triple-negative type [C] luminal type, and [D] HER2 type (including HER2-enriched and luminal-HER2 types)
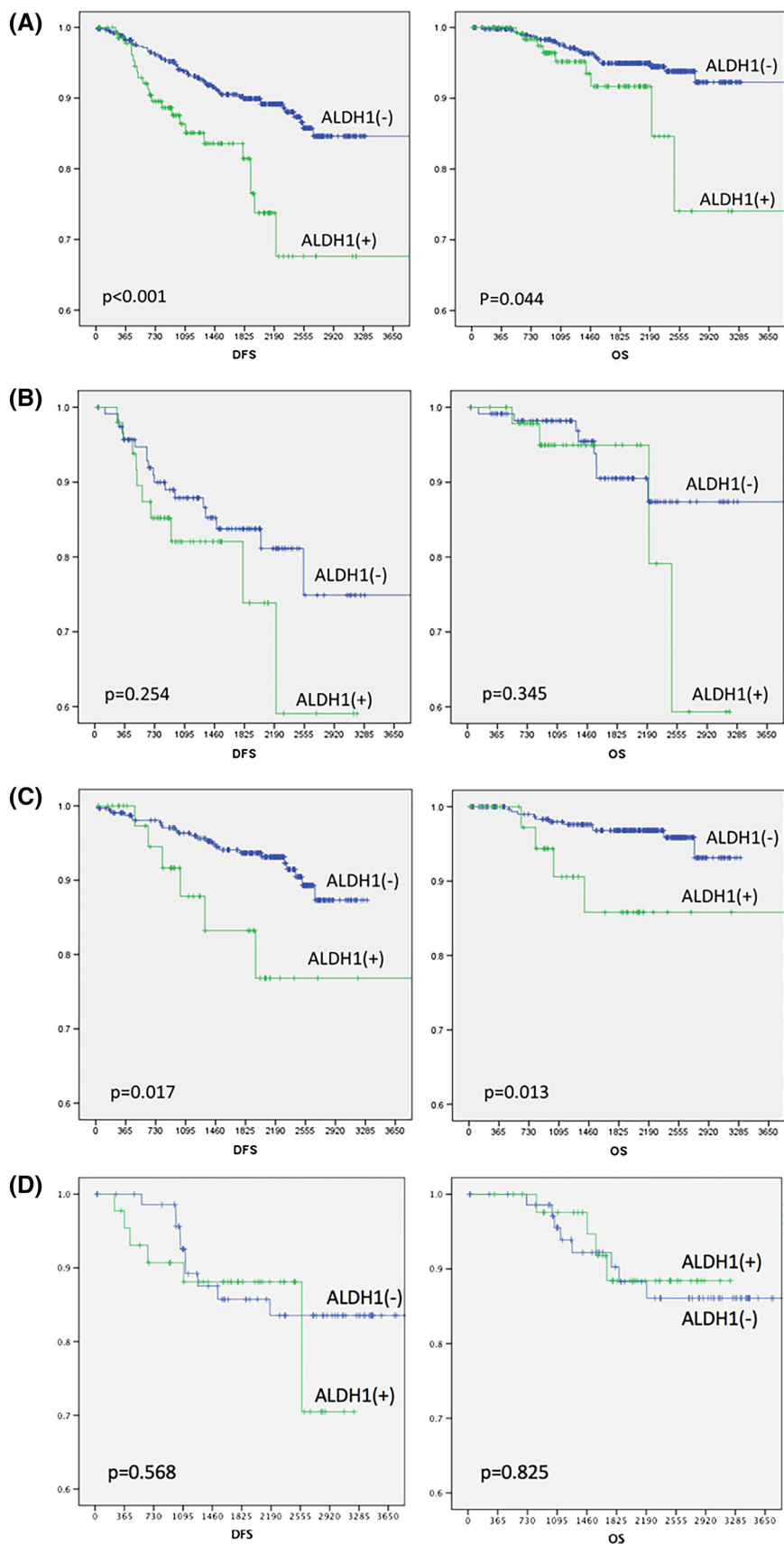
Table 2 Multivariate analysis of various prognostic indicators

	DFS P^*	OS P^*
ALDH1	*0.033*	0.124
Tumor size	0.247	0.314
Lymph node metastasis	0.196	0.047
Clinical staging	0.107	0.55
ER	0.7	0.976
HER2	0.604	0.856
Nuclear grade	0.855	0.251
Ki67	0.523	0.956

*Italic; statistically significant

Table 4 Correlation between chemoresponse and *ALDH1* expression by intrinsic breast cancer subtype

Subtypes	n	pCR rate (ALDH negative/positive)	P^*
Triple negative	106	43.5/10.8	*0.003*
HER2-enriched	45	36.0/20.0	0.239
Luminal	63	4.5/0	0.358
Luminal-HER2	20	42.9/23.0	0.357
All subtypes	234	30.3/13.5	*0.003*

*Italic; statistically significant

recent study. ALDH1 could be a biomarker for this particular subgroup.

The next argument concerns the impact of CSCs on TNBC. Ohi et al. stated that ALDH1$^+$ cancer cells were found in 59 % of TNBC cases and correlated significantly with high histological grade. We also found Cox multivariate analysis to show that ALDH1 expression was an independent prognostic indicator of relapse-free survival [33]. Although the relationship between ALDH1 and prognosis has been controversial in earlier studies [31, 34, 35], the present study found no significant association between prognosis and ALDH1 expression, although ALDH1$^+$ TNBC patients had a significantly lower pCR rate. This result may have been caused by the ambiguity of using the pCR rate as a surrogate prognostic marker in TNBC. TNBC also encompasses a heterogeneous group within the subtype, which should be considered when performing future analyses of this particular subtype.

The relationship between HER2 and ALDH1 in HER2$^+$ breast cancers has recently become a focus of study. Several studies have shown HER2 to be an important regulator of the CSC population in breast cancer [36–39]. These

studies indicate that the clinical efficacy of trastuzumab may be based on its ability to target the CSC population in *HER2*-amplified tumors. However, the impact of ALDH1 on HER2$^+$ breast cancer prognosis is not widely reported. In our current research, all HER2$^+$ patients received trastuzumab; they showed no correlation between ALDH1 positivity and prognosis, regardless of ER status. If trastuzumab can indeed target CSCs, our clinical finding could be considered reasonable. As our HER2$^+$ sample size was small, additional studies with larger cohorts are needed to verify our findings. Furthermore, the efficacy of trastuzumab has been suggested to rely on its ability to target CSCs in a process that does not depend on *HER2* gene amplification [39]. If so, trastuzumab may be effective against CSC$^+$/HER2$^-$ cancers. This hypothesis bears exploring and further research in this area is expected.

Our study had the advantages of having a moderate sample size and performing accurate analyses by breast cancer subtype. However, it was limited by its retrospective design; moreover, chemotherapy regimens were not the same for all patients, though most were anthracyclines followed by a taxane.

In conclusion, ALDH1 expression in tumor cells correlated significantly with tumor size, lymph node

Table 3 Univariate and multivariate analyses of pCR against various characteristics

	pCR rate (%)	Univariate analysis P^*	Multivariate analysis P^*
ALDH1 (−)/(+)	30.3/13.5	*0.003*	*0.005*
ER(−)/(+)	31.1/10.8	*0.001*	*0.002*
PR(−)/(+)	28.6/10.2	*0.004*	0.114
HER(−)/(+)	22.4/28.1	0.356	0.17
Ki67(<20 %/≧20 %)	13.5/27.7	*0.04*	0.167
Topo2(<20 %/≧20 %)	20.6/25.4	0.455	0.179
p53		*0.017*	0.081
Basal phenotype(−)/(+)	25.0/29.6	0.536	0.381
Nuclear grade		0.05	0.981
Tumor size		0.123	0.526
Node metastasis(−)/(+)	21.9/25.4	0.539	0.5
Clinical staging		0.298	0.393

*Italic; statistically significant

Table 5 Multivariate and univariate analyses for outcome predictors against pCR results in patients with triple-negative type breast cancer

	pCR rate (%)	Univariate analysis P*	Multivariate analysis P*
ALDH1 (-)/(+)	43.5/10.8	*0.003*	*0.012*
Ki67		0.068	0.897
Topo2(<20 %/≧20 %)	23.1/34.5	0.053	0.052
p53		0.144	0.424
Basal phenotype(−)/(+)	35.7/29.9	0.666	0.637
Nuclear grade		0.405	0.916
Tumor size		0.651	0.501
Node metastasis(−)/(+)	24.4/37.7	0.148	0.214

*Italic; statistically significant

metastasis, nuclear grade, poor prognosis, and response to NAC. Although the correlation between ALDH1 expression and pCR rate was much higher in TNBC than other subtypes, ALDH1 expression was also correlated with prognosis in the luminal type. Our results indicate that ALDH1 is a potential biomarker for poor survival, and its significance varies by breast cancer subtype.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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