doi:10.4149/neo_2014_045

Association of aldehyde dehydrogenase 1 expression and biologically aggressive features in breast cancer

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Received July 9, 2013 / Accepted November 7, 2013

Aldehyde dehydrogenase 1 (ALDH1) has been regarded as a breast cancer stem cell marker. Several studies have reported that ALDH1 expression is associated with poor prognosis in breast cancer. We aimed, therefore, to determine the prognostic value of ALDH1 expression and its association with several biomarkers in breast cancer tissue using immunohistochemistry. Furthermore, we investigated the characteristics of and differences between cellular and stromal expression of ALDH1. We performed tissue microarray (TMA) analysis of 425 breast cancer tissue samples collected during surgery. Immunohistochemical staining was then performed to measure the expression of ALDH1 and other breast cancer biomarkers. Statistical analysis of the relationship between ALDH1 expression and clinicopathologic characteristics was performed for 390 TMA samples. We found that ALDH1 was expressed in 71 cases (18.2%) in the tumor cells and/or stroma. Of these cases, 38 (9.7%) showed ALDH1 expression in tumor cells and 38 (9.7%) showed ALDH1 expression in the stroma. ALDH1 expression was significantly associated with markers of a poor prognosis, such as young age, estrogen receptor negativity, progesterone receptor negativity, a high histological grade, and a high Ki-67 index. However, ALDH1 expression was not associated with p53, transforming growth factor-beta, Gli-1, YKL-40, or sonic hedgehog expression status. With regard to the expression site, the clinical characteristics did not differ between cases of cellular expression and those of stromal expression. However, ALDH1 expression in tumor cells was correlated with hormone receptor status, histological grade, molecular subtype, epidermal growth factor receptor expression status, and cytokeratin 5/6 expression status while stromal expression of ALDH1 was only correlated with hormone receptor status. Overall, these findings suggest that ALDH1 expression in tumor tissue is associated with a biologically aggressive phenotype.

Key words: ALDH1, biologically aggressive, breast cancer

Cancer comprises a heterogeneous assembly of cells that possess different characteristics. According to the cancer stem cell concept, a very small portion of cancer cells have the ability to self-renew and undergo differentiation into diverse cell types within tumors [1]. As highlighted in an acute myeloid leukemia study [2], cancer stem cells are present in a variety of solid tumors [3, 4]. For example, Al-Hajj et al. first reported the presence of cancer stem cells in breast cancer, describing them as a subset of cells presenting a $CD44^+/CD24^-$ phenotype [5]. More recently, Ginestier et al. reported that aldehyde dehydrogenase 1 (ALDH1) activity might be a potential marker for cancer stem cells, on the basis of their *in vivo* and *in vitro* results [6].

ALDH1 is a detoxifying enzyme that oxidizes intracellular aldehydes, thereby protecting stem cells against oxidative damage [7]. In addition, ALDH1 converts retinol to retinoic acid, consequently acting at an early stage in stem cell differentiation and proliferation [8]. Generally, cancer stem cells are known to play a role in the early stages of cancer, metastasis, and chemoresistance development [9, 10]. Several studies have demonstrated a similar role for ALDH1 in breast cancer. Some studies have reported the association of ALDH1 expression with poor prognosis and factors associated with aggressive disease such as estrogen receptor (ER) negativity and the expression of basal-like markers [11, 12]. ALDH1 positivity has also been shown to be correlated with poor prognosis, especially in lymph node-positive cancers, triple-negative cancers, and inflammatory breast cancers [13-15]. Furthermore, ALDH1 expression has been shown to be associated with resistance to chemotherapy [16, 17]. Although widely

studied, the exact prognostic value of ALDH1 expression has not yet been confirmed.

The aim of this study was to investigate ALDH1 expression status and the characteristics of stage I to III sporadic early-stage breast cancer patients in Korea. We regarded ALDH1 expression as a characteristic of cancer stem cells. Several reports have shown that biomarkers such as Gli-1, TGF, and YKL-40 are associated with a poor prognosis in breast cancer [18-20]. Accordingly, we analyzed the association of ALDH1 expression with various breast cancer markers such as transforming growth factor (TGF)-beta, Gli-l, YKL-40, and sonic hedgehog (shh), the exact prognostic value of which has not yet been proven. Further, we analyzed the correlation of ALDH1 expression with well-known prognostic markers such as hormone receptor (HR) status and Her2/*neu* expression.

Previous studies have shown that ALDH1 is expressed in both cancer cells and the surrounding stroma of tumors. Tumor stromal cells are a part of the local microenvironment, which includes myoepithelial cells, fibroblasts, myofibroblasts, endothelial cells, and inflammatory cells. The tumor stroma is known to play a role in breast cancer metastasis [21]. However, few studies have evaluated the prognostic value and related characteristics of both tumor cell and stromal expression. Therefore, in this study, we sought to investigate any differences between these two distinct expressions.

Materials and methods

We retrospectively acquired breast cancer tissue samples from patients who had undergone surgical resection for breast cancer at the Korea University Medical Center Guro Hospital between 1992 and 2006. A total of 425 tissue samples were available. A single pathologist at the Department of Pathology performed tissue microarray (TMA) analysis with tissue blocking and immunohistochemical staining. We reviewed the patients' medical records for clinical information such as age, gender, operation type, survival data, and tumor stage, which accounted for the status of lymph node metastasis and distant organ metastasis. Of the 425 samples, damaged tissue samples found difficult to analyze via immunohistochemical staining, samples of cases with inadequate medical records, and samples from stage IV cases were excluded. In total, we were able to obtain clinical and pathological information for 390 of the 425 samples. This study was approved by the Institutional Review Board of our institution.

TMAs were constructed using a 2-mm core size for the 425 cases. For immunohistochemical analysis, serial 4-μm sections were mounted on electrostatic slides, heat-dried at 56°C for 30 min, deparaffinized in xylene, and rehydrated in a graded ethanol series. Slides were incubated with 3% hydrogen peroxidase in methanol for 15 min to block endogenous peroxidase activity and with 0.3% bovine serum albumin/1× Tris-buffered saline (TBS) for 20 min thereafter to minimize non-specific staining. The following primary antibodies were applied for 30 min at room temperature: ALDH1 (1:100 dilution; BD biosciences, San Jose, CA, USA), p53 (1: 500 dilution; Novocastra, Newcastle Upon Tyne, UK), TGF-beta (1:100 dilution; AbDserotec, Oxford, UK), Gli-1 (1:50 dilution; Abcam, Cambridge, MA, USA), YKL-40 (1:100 dilution; Quidel, San Diego, CA), and shh (1:200 dilution; Abcam, Cambridge, MA, USA). After a series of TBS rinses, bound antibody was detected using a polymer secondary antibody from the Dako EnVision+ system (Dako, Carpinteria, CA, USA). Slides were rinsed with TBS and visualized following a 10-min incubation in liquid 3,3'-diaminobenzidine in buffered substrate (Dako). Slides were counterstained with hematoxylin.

The immunohistochemical staining of TMA sections was semiquantitatively assessed. Specifically, cancer cells with nuclear ER and progesterone receptor (PR) staining were considered immunoreactive and were scored according to the Allred scoring method. Her2/*neu* membranous staining was evaluated according to the guidelines of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP). Cases with a score of 3+ were considered Her2/*neu*-positive. Those with a score of 2+ were further evaluated for Her2/*neu* gene amplification by silver-enhanced in situ hybridization (SISH), performed on an automated Ventana Benchmark instrument (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer instructions for the INFORM Her2/*neu* DNA probe and chromosome 17 probes. SISH results were interpreted according to ASCO/CAP guidelines [22].

Immunohistochemical staining for cytokeratin (CK) 5/6 and epidermal growth factor receptor (EGFR) was performed to determine basal-like marker expression. Cases with weak or strong cytoplasmic and/or membranous expression of CK5/6 were considered positive. Membranous staining of EGFR was evaluated according to the HercepTest scoring method (DAkocytomation, Carpinteria, CA, USA). Membrane staining intensity was scored as follows: 0, negative staining; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. Cases with positive immunohistochemical results for CK5/6 and EGFR were considered positive for basal-like marker expression. The entire staining process for ER, PR, Her2/*neu*, CK5/6, EGFR, and Ki-67 was automated, using Ventana Benchmark Autostainers (Ventana Medical Systems, Tucson, AZ, USA).

Due to lack of universal scoring guidelines for ALDH1, Gli-1, TGF-beta, YKL-40, and shh expression, we describe herein our method for assigning scores. We evaluated cytoplasmic and/or nuclear ALDH1 staining in tumor cells and stroma. ALDH1-positive cells showed cellular and/or stromal staining (Fig. 1). Because staining intensity was similar in the tumor and stromal cells, we were not able to score staining intensity, and instead regarded cells with positive staining as ALDH1-positive cells. We further categorized samples according to their major ALDH1 expression site: samples that showed positive expression in stromal cells and negative expression in tumor cells (Fig. 1A), samples that showed positive expression in tumor cells and negative expression in stromal cells (Fig. 1B), samples that showed positive expression in both tumor cells and stromal cells (Fig. 1C). YKL-40 immunoreactivity was recognized as brown staining

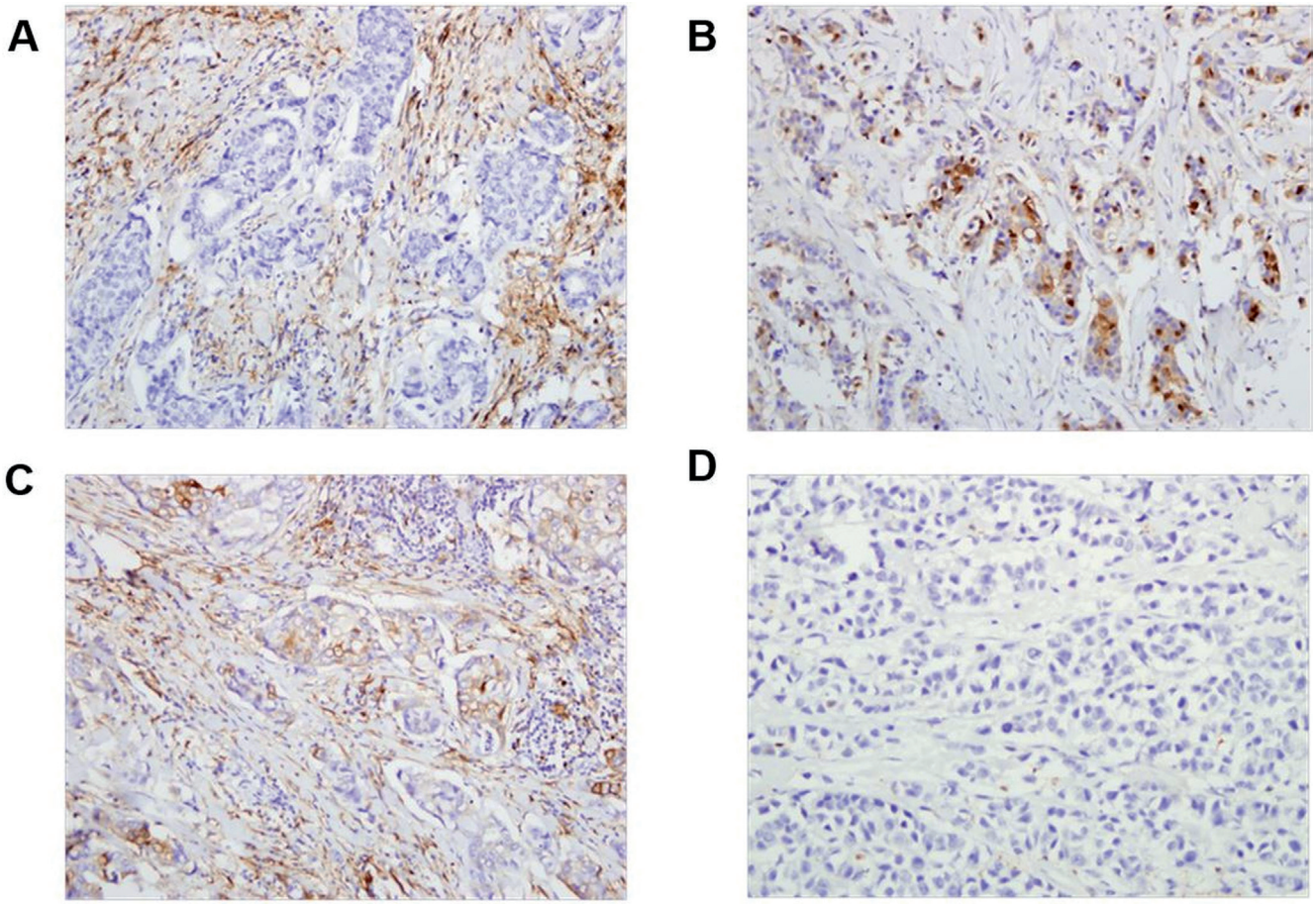

Figure 1. Representative results of immunohistochemical staining for ALDH1 in breast cancer
ALDH1 (negative tumor cell and positive stromal expression, 200×) (A), ALDH1 (positive tumor cell and positive stromal expression, 200×) (B), ALDH1 (positive tumor cell and negative stromal expression, 200×) (C), ALDH1 (negative tumor cell and negative stromal expression, 200×) (D).

localized mainly in the cytoplasm of tumor cells. Any granular, brown-colored staining of cancer cells was considered to indicate YKL-40-positive cells. The signal intensity of cytoplasmic staining was scored as follows: 0, negative staining; 1+, weak cytoplasmic staining of cancer cells; 2+, moderate cytoplasmic staining of cancer cells; and 3+, strong cytoplasmic and nuclear staining of cancer cells. Scoring of TGF-beta immunoreactivity was performed in the same manner. For Gli-1, cells with nuclear staining were considered positive. Cells positive for Ki-67 were counted and expressed as a percentage. Samples were scored on the basis of the percentage of Ki-67-positive nuclei as follows: 1+, 0–5%; 2+, 5–50%; and 3+, >50%.

We then categorized TMA samples as the luminal type, Her2/*neu*-enriched type, and basal-like type according to the status of hormone receptor expression, Her2/*neu* expression, CK5/6 expression, and EGFR expression on immunohistochemistry: ER and/or PR positivity and Her2/*neu* negativity, luminal type; Her2/*neu* positivity regardless of hormone receptor positivity, Her2/*neu*-enriched type; and triple negativity (for hormone receptors and Her2/*neu*) and CK5/6 and/or EGFR positivity, basal-like type. Tumors not meet the above criteria were classified in the "others" category.

The chi-squared test was used to analyze the association between ALDH1 expression and clinicopathologic variables. Associations between ALDH1 expression and other variables were analyzed using the chi-squared test and Fisher's exact test. Disease-free survival (DFS) was defined as time from surgery to the first locoregional or distant recurrence, and overall survival (OS) was defined as time from surgery to death from any cause. DFS and OS were calculated using the Kaplan-Meier method, and the difference between the curves was analyzed using the log-rank test. A P value of <0.05 was considered significant for all statistical tests. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Data on immunohistochemical staining were available for 404 of the 425 tumor samples (95.1%). Of these, clinicopatho-

logic information was available for 390 cases (91.8%). The median estimated follow-up duration was 74 months (range, 0–217 months). The median age of all patients was 45 years (range, 22–82 years). Breast-conserving surgery was performed in 67 patients (17.2%), while 300 patients (76.9%) underwent modified radical mastectomy. Twenty-one patients (0.5%) underwent excision only, and 32 patients (8.2%) were treated with neoadjuvant chemotherapy before surgery. Adjuvant chemotherapy was administered to 270 patients (69.2%), and adjuvant hormonal therapy was administered to 227 (58.2%). Twenty-one patients (5.4%) did not receive adjuvant treatment. The median tumor size was 2.5 cm (range, 0.5–11.3 cm). In total, 193 samples (49.5%) showed lymph node metastasis and 197 (50.5%) did not show lymph node metastasis at the time of surgery. Furthermore, the most common diagnosis was invasive ductal carcinoma (368 cases, 94.3%) followed by mucinous carcinoma (1.3%), medullary carcinoma (1.3%), and metaplastic carcinoma (0.8%). Ductal carcinoma in situ (DCIS) was diagnosed in 0.8% of cases, with 97 cases (24.9%) classified as stage I, 198 (50.8%) as stage II, and 95 (24.4%) as stage III.

Table 1. Correlation between ALDH1 expression and clinicopathologic variables (N = 390)

Factor	No. (%)	ALDH1 expression (cell and/or stroma)		
		Negative	Positive	*P*
Age (years)				0.001
>40	103 (26.4)	95 (24.4)	8 (2.1)	
≤40	287 (73.6)	224 (57.4)	63 (16.2)	
Tumor stage				0.985
1	94 (24.1)	77 (19.7)	17 (4.4)	
2	200 (51.3)	163 (41.8)	37 (9.5)	
3	96 (24.6)	79 (20.3)	17 (4.4)	
Tumor size (cm)				0.770
<2	145 (37.2)	116 (29.7)	29 (7.4)	
2–5	220 (56.4)	182 (46.7)	38 (9.7)	
>5	25 (6.4)	21 (5.4)	4 (1.0)	
Node status				0.966
0	198 (50.8)	163 (29.7)	35 (9.0)	
1–3	101 (25.9)	182 (46.7)	18 (4.6)	
4–9	53 (13.6)	43 (11)	10 (2.6)	
>9	38 (9.7)	30 (7.7)	8 (2.1)	
Histological grade				0.009
1	70 (20.3)	73 (18.7)	6 (1.5)	
2	180 (46.2)	147 (37.7)	33 (8.5)	
3	104 (6.9)	76 (19.5)	28 (7.2)	
Unknown	27 (6.9)	23 (5.9)	4 (1.0)	
ER status				0.000
Negative	141 (36.2)	98 (25.1)	43 (11)	
Positive	245 (62.8)	218 (55.9)	27 (6.9)	
Unknown	4 (1.0)	3 (0.8)	1 (0.3)	
PR status				0.002
Negative	146 (36.2)	103 (26.4)	38 (9.7)	
Positive	244 (62.8)	213 (54.6)	32 (8.2)	
Unknown	4 (1.0)	3 (0.8)	1 (0.3)	
Her2/*neu*				0.013
Negative	311 (79.17)	263 (67.4)	48 (12.3)	
Positive	77 (19.7)	54 (13.8)	23 (5.9)	
Unknown	2 (0.5)	2 (0.5)	0	
Molecular type				0.002
Luminal type	232 (59.5)	6 (1.5)	28 (7.2)	
Her2/*neu* enriched	77 (19.7)	7 (1.8)	23 (5.9)	
Basal-like type	37 (9.5)	3 (0.8)	9 (2.3)	
Others	43 (11)	2 (0.5)	11 (2.8)	
Unknown	1 (0.3)	1 (0.3)	0	
EGFR				0.005
0	314 (80.5)	267 (68.5)	47 (12.1)	
1+	40 (10.3)	27 (6.9)	13 (3.3)	
2+	24 (6.2)	17 (4.4)	7 (1.8)	
3+	11 (2.8)	8 (2.1)	3 (0.8)	
Unknown	1 (0.3)	0	1 (0.3)	
CK5/6				0.016
Negative	345 (88.5)	289 (74.1)	56 (14.4)	
Positive	43 (11)	29 (7.4)	14 (3.6)	
Unknown	2 (0.5)	1 (0.3)	1 (0.3)	
Ki-67				0.017
1+	217 (55.6)	188 (48.2)	29 (7.4)	
2+	123 (31.5)	96 (24.6)	27 (6.9)	
3+	45 (11.5)	31 (7.9)	14 (3.6)	
Unknown	5 (1.3)	4 (1.0)	1 (0.3)	
P53				0.253
0	285 (73.1)	237 (60.8)	48 (12.3)	
1+	34 (8.7)	29 (7.4)	5 (1.3)	
2+	32 (8.2)	26 (6.7)	6 (3.1)	
3+	38 (9.7)	26 (6.7)	12 (3.1)	
Unknown	1 (0.3)	1 (0.3)	0	
YKL-40				0.757
0	58 (14.9)	49 (12.6)	9 (2.3)	
1+	225 (57.7)	184 (47.2)	41 (10.5)	
2+	86 (22.1)	68 (17.4)	18 (4.6)	
3+	17 (4.4)	14 (3.6)	3 (0.8)	
Unknown	4 (1.0)	4 (1.0)	0	
TGF-beta				0.642
0	70 (17.9)	54 (13.8)	16 (4.1)	
1+	80 (20.5)	68 (17.4)	12 (3.1)	
2+	120 (30.8)	97 (24.9)	23 (5.9)	
3+	116 (29.7)	97 (24.9)	19 (4.9)	
Unknown	4 (1.0)	3 (0.8)	1 (0.3)	
Gli-1				0.811
0	15 (3.8)	11 (2.8)	4 (1.0)	
1+	89 (22.8)	75 (19.2)	14 (3.6)	
2+	212 (54.4)	173 (44.4)	39 (10.0)	
3+	72 (18.5)	58 (14.9)	14 (3.6)	
Unknown	2 (0.5)	2 (0.5)	0	
shh				0.616
0	33 (8.5)	30 (7.7)	3 (0.8)	
1+	125 (32.1)	100 (25.6)	25 (6.4)	
2+	181 (46.4)	148 (37.9)	33 (8.5)	
3+	48 (12.3)	38 (9.7)	10 (2.6)	
Unknown	3 (0.8)	3 (0.8)	0	
Total	390 (100)	319 (81.8)	71 (18.2)	

All specimens were categorized according to ALDH1 expression status as described in the Materials and methods section. In our study, 71 cases (18.2%) were positive for ALDH1. Of these, ALDH1 expression was observed in tumor cells in 38 samples (9.7%) and in stroma in 38 samples (9.7%). Only 5 samples showed both tumor cell and stromal cell expression. Of the ALDH1-positive tumors, 39.4% were of the luminal subtype, 32.4% were of the Her2/*neu* subtype, and 12.7% were of the basal-like subtype. Most ALDH1-positive tumors (88.7%) were from patients younger than 40 years. Of the 103 patients who were >40 years old, only 8 (7.8%) showed ALDH1 expression, whereas, 63 of 287 patients (22%) who were <40 years old showed ALDH1 expression. The clinical characteristics and associations between ALDH1 positivity and clinicopathologic variables are shown in Table 1. ALDH1 expression was not significantly associated with T stage, N stage, and overall tumor stage. However, age, hormone receptor status (i.e., ER and PR status), histological grade, Her2/*neu* status, molecular type, EGFR expression, CK5/6 expression, and Ki-67 status were associated with ALDH1 expression ($P < 0.05$). Further, ALDH1 expression was significantly associated with markers of poor prognosis such as ER negativity, PR negativity, a high histological grade, and a high Ki-67 index. However, this was not associated with p53, TGF-beta, Gli-1, YKL-40, or shh expression (Table 1).

We also investigated the association with clinical characteristics according to the expression site (cellular or stromal ALDH1 expression). However, we found no significant association between expression site and clinical characteristics (Table 2). Both cellular and stromal expression of ALDH1 were associated with hormone receptor negativity. Cellular ALDH1 expression was correlated with age, histological grade, molecu-

Table 2. Characteristics according to cellular and stromal ALDH1 expression

Factor	ALDH1 expression	
	Cell	Stroma
Age (years)		
>40	3	7
≤40	35	31
Tumor stage		
1	8	11
2	19	20
3	11	7
Tumor size (cm)		
<2	13	19
2–5	22	18
>5	3	1
Node status		
0	19	19
1–3	8	11
4–9	5	6
>9	6	2
Histological grade		
1	2	4
2	16	20
3	16	14
Unknown	4	0
ER status		
Negative	25	22
Positive	12	16
Unknown	1	0
PR status		
Negative	20	22
Positive	17	16
Unknown	1	0
Her2/*neu*		
Negative	26	25
Positive	12	13
Unknown	0	0
Molecular type		
Luminal type	13	16
Her2/*neu* enriched	12	13
Basal-like type	6	4
Others	7	5
Unknown	0	0
EGFR		
0	21	30
1+	10	4
2+	4	3
3+	2	1
Unknown	1	0
CK5/6		
Negative	28	32
Positive	9	6
Unknown	1	0
Ki-67		
1+	15	15
2+	16	14
3+	6	9
Unknown	1	0
P53		
Negative	27	24
Positive	11	14
Unknown	0	0
YKL-40		
Negative	5	4
Positive	33	34
Unknown	0	0
TGF-beta		
Negative	10	6
Positive	28	31
Unknown	0	1
Gli-1		
Negative	4	1
Positive	34	37
Unknown	0	
shh		
Negative	1	2
Positive	37	36
Unknown	0	0
Total	38	38

lar subtype, EGFR expression, and CK5/6 expression ($P < 0.05$). However, stromal ALDH1 expression was correlated only with hormone receptor status and p53 expression ($P < 0.05$) and not with histological grade, molecular subtype, EGFR expression, or CK5/6 expression ($P > 0.05$) (Table 3). Cellular ALDH1 expression showed similar correlation trends with overall ALDH1 expression, except with regard to Her2/*neu* expression and Ki-67 status. However, stromal ALDH1 expression trends differed from those of overall ALDH1 expression trends, except with regard to hormone receptor status.

As of May 2012, 80 of 390 patients (20.5%) died, and 116 of 390 of patients (29.7%) had relapsed. DFS and OS did not reach the median value (mean DFS, 150.52 months; confidence interval, 140.777–160.272; mean OS, 167.197 months; confidence interval, 157.697–176.697). In the DFS and OS Kaplan-Meier survival curves did not differ according to ALDH1 expression ($P > 0.05$) (Fig. 2). We then examined whether cellular and/or stromal expression of the tumor tissue correlated with survival. DFS and OS did not differ according to cellular or stromal expression ($P > 0.05$, Fig. 3).

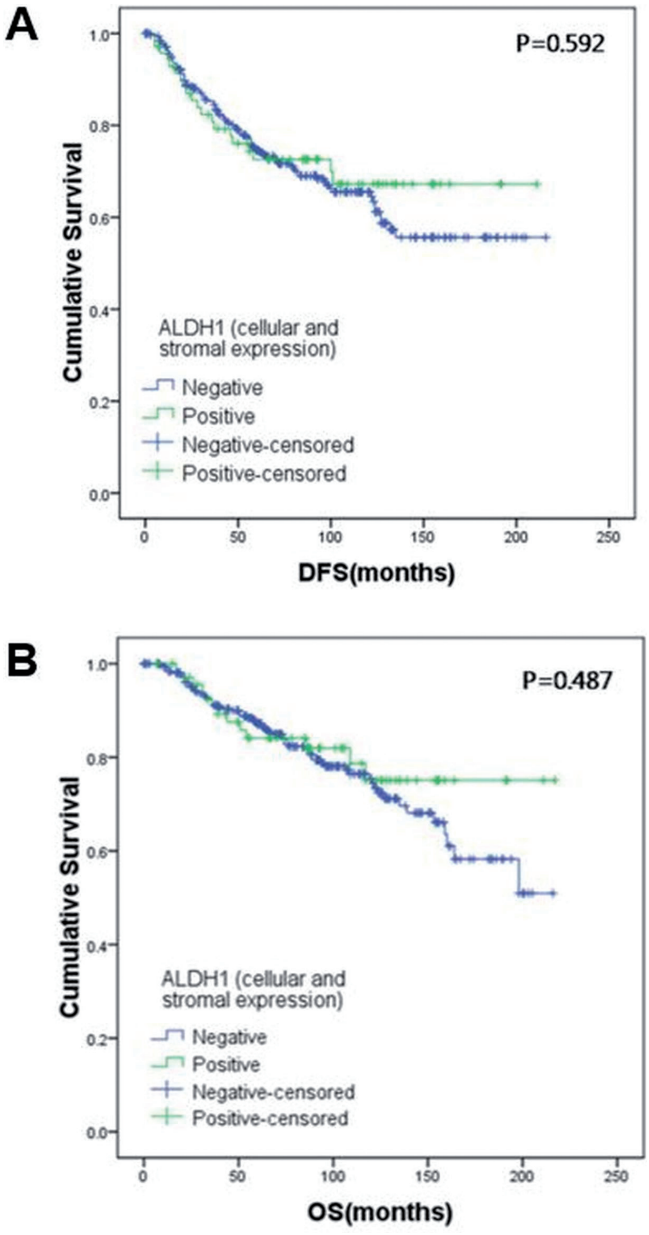

Figure 2. Kaplan-Meier curves for the association between ALDH1 expression (both cellular and stromal expression) and DFS (A) and OS (B)

Discussion

The incidence of breast cancer is rapidly increasing in Korea [23]. More accurate biomarkers to monitor disease status and predict prognosis are required to develop future therapies. In this study, we investigated the characteristics of biomarkers already associated with breast cancer, but focused on their relationship with ALDH1 expression. Although many studies have analyzed the role of TGF-beta, Gli-1, YKL-40, and shh in breast cancer, we currently do not fully understand the exact role of these markers or the relationships between them and the expression of other key biomarkers, such as ALDH1. Therefore, we evaluated these biomarkers in our study.

We examined a large sample size of tumor tissues using TMA, and measured the expression of various markers known to be related to breast cancer prognosis, whose exact role has not yet been established. In this study, we found that ALDH1 expression correlated with young age, hormone receptor negativity, Her2/*neu* positivity, a high histological grade, and a high Ki-67 index. ALDH1 expression was not related to TGF-beta, Gli-1, YKL-40, or shh expression. Furthermore, ALDH1 did not affect DFS or OS.

Since the first report by Ginestier et al. [6], many studies have reported the role of ALDH1 in breast cancer. Most studies have examined ALDH1 expression in tumor tissues using immunohistochemistry, but to date, ALDH1 expression has not been thoroughly assessed. ALDH1 is expressed in both the cytoplasm of tumor cells and in the stroma surrounding the tumor cells. The criteria for ALDH1 positivity differs between studies, so that there is an inconsistent reported frequency of ALDH1 expression in the literature.

ALDH1 expression has been reported in 18–20% of sporadic breast cancer patients [6, 15]. ALDH1 expression rates have been reported to be higher in inflammatory breast cancers, basal-like breast cancers, *BRCA1* mutation-associated cancers, and African patients, with a frequency of approximately 30–78%[12, 13, 24, 25]. In this study, ALDH1 positivity was observed in 18.2% of sporadic breast cancer patients, a value consistent with results of previous reports.

As with many other studies, we observed a correlation between ALDH1 expression and aggressive tumor features such as hormone receptor negativity, a high histological grade, and a high Ki-67 index. In addition, ALDH1 expression was more

Table 3. Associations between cellular/stromal ALDH1 expression and clinicopathological variables (N = 390)

Factor	No.(%)	ALDH1 expression (cellular expression)			ALDH1 expression (stromal expression)		
		Negative	Positive	*P*	Negative	Positive	*P*
Age (years)				0.006			0.240
>40	103 (26.4)	100 (25.6)	3 (0.8)		96 (24.6)	7 (1.8)	
≤40	287 (73.6)	252 (64.6)	35 (9.0)		256 (65.6)	31 (7.9)	
Tumor stage				0.780			0.582
1	94 (54.1)	86 (22.1)	8 (2.1)		83 (21.3)	11 (2.8)	
2	200 (51.3)	181 (46.4)	19 (4.9)		180 (46.2)	20 (5.1)	
3	96 (24.6)	85 (21.8)	11 (2.8)		89 (22.8)	7 (1.8)	
Tumor size (cm)				0.878			0.182
<2	145 (37.2)	132 (33.8)	13 (3.3)		126 (32.3)	19 (4.9)	
2–5	220 (56.4)	198 (50.8)	22 (5.6)		202 (51.8)	18 (4.6)	
>5	25 (6.4)	22 (5.6)	3 (0.8)		24 (6.2)	1 (0.3)	
Node status				0.578			0.759
0	198 (50.8)	179 (45.9)	19 (4.9)		179 (45.9)	19 (4.9)	
1–3	101 (25.9)	93 (23.8)	8 (2.1)		90 (23.1)	11 (2.8)	
4–9	53 (13.6)	48 (12.3)	5 (1.3)		47 (12.1)	6 (1.5)	
>9	38 (9.7)	32 (8.2)	6 (1.5)		36 (9.2)	2 (0.5)	
Histological grade				0.025			0.075
1	79 (20.3)	77 (19.7)	2 (0.5)		75 (19.2)	4 (1.0)	
2	180 (46.2)	164 (42.1)	16 (4.1)		160 (41.0)	20 (5.1)	
3	104 (26.7)	88 (22.6)	16 (4.1)		90 (23.1)	14 (3.6)	
Unknown	27 (6.9)	23 (5.9)	4 (1.0)		27 (6.9)	0	
ER status				0.000			0.016
Negative	141 (36.2)	116 (29.7)	25 (6.4)		119 (30.5)	22 (5.6)	
Positive	245 (62.8)	233 (59.7)	12 (3.1)		229 (58.7)	16 (4.1)	
Unknown	4 (1.0)	3 (0.8)	1 (0.3)		4 (1.0)	0	
PR status				0.026			0.016
Negative	141 (36.2)	121 (31.0)	20 (5.1)		119 (30.5)	22 (5.6)	
Positive	245 (62.8)	228 (58.5)	17 (4.4)		229 (58.7)	16 (4.1)	
Unknown	4 (1.0)	3 (0.8)	1 (0.3)		4 (1.0)	0	
Her2/*neu*				0.168			0.057
Negative	311 (79.7)	285 (73.1)	26 (6.7)		286 (73.3)	25 (6.4)	
Positive	77 (19.7)	65 (16.7)	12 (3.1)		64 (16.4)	13 (3.3)	
Unknown	2 (0.5)	2 (0.5)	0		2 (0.5)	0	
Molecular type				0.010			0.115
Luminal type	232 (59.5)	219 (56.2)	13 (3.3)		216 (55.4)	16 (4.1)	
Her2/*neu* enriched	77 (19.7)	65 (16.7)	12 (3.1)		64 (16.4)	13 (3.3)	
Basal-like type	37 (9.5)	31 (7.9)	6 (1.5)		33 (8.5)	4 (1.0)	
Others	43 (11.0)	36 (9.2)	7 (1.8)		38 (9.7)	5 (1.3)	
Unknown	1 (0.3)	1 (0.3)	0		1 (0.3)	0	
EGFR				0.000			0.940
0	314 (80.5)	293 (75.1)	21 (5.4)		284 (72.8)	30 (7.7)	
1+	40 (10.3)	30 (7.7)	10 (2.6)		36 (9.2)	4 (1.0)	
2+	24 (6.2)	20 (5.1)	4 (1.0)		21 (5.4)	3 (0.8)	
3+	11 (2.8)	9 (2.3)	2 (0.5)		10 (2.6)	1 (0.3)	
Unknown	1 (0.3)	0	1 (0.3)		1 (0.3)	0	
CK5/6				0.009			0.520
Negative	345 (88.5)	317 (81.3)	28 (7.2)		313 (80.3)	32 (8.2)	
Positive	43 (11)	34 (8.7)	9 (2.3)		37 (9.5)	6 (1.5)	
Unknown	2 (0.5)	1 (0.3)	1 (0.3)		2 (0.5)	0	
Ki-67				0.109			0.055
1+	217 (55.6)	202 (51.8)	15 (3.8)		202 (51.8)	15 (3.8)	
2+	123 (31.5)	107 (27.4)	16 (4.1)		109 (27.9)	14 (3.6)	
3+	45 (11.5)	39 (10.0)	6 (1.5)		36 (9.2)	9 (2.3)	
Unknown	5 (1.3)	4 (1.0)	1 (0.3)		5 (1.3)	0	

Table 3. (continue)

Factor	No.(%)	ALDH1 expression (cellular expression)			ALDH1 expression (stromal expression)		
		Negative	Positive	*P*	Negative	Positive	*P*
P53				0.946			0.032
0	285 (73.1)	258 (66.2)	27 (6.9)		261 (66.9)	24 (6.2)	
1+	34 (8.7)	30 (7.7)	4 (1.0)		33 (8.5)	1 (0.3)	
2+	32 (8.2)	29 (7.4)	3 (0.8)		28 (7.2)	4 (1.0)	
3+	38 (9.7)	34 (8.7)	4 (1.0)		29 (7.4)	9 (2.3)	
Unknown	1 (0.3)	1 (0.3)	0		1 (0.3)	0	
YKL-40				0.514			0.586
0	58 (14.9)	53 (13.6)	5 (1.3)		54 (13.8)	4 (1.0)	
1+	225 (57.7)	206 (52.8)	19 (4.9)		199 (51)	26 (6.7)	
2+	86 (22.1)	75 (19.2)	11 (2.8)		78 (20)	8 (2.1)	
3+	17 (4.4)	14 (3.6)	3 (0.8)		17 (4.4)	0	
Unknown	4 (1.0)	4 (1.0)	0		4 (1.0)	0	
TGF-beta				0.525			0.725
0	70 (17.9)	60 (15.4)	10 (2.6)		64 (16.4)	6 (1.5)	
1+	80 (20.5)	75 (19.2)	5 (1.3)		71 (18.2)	9 (2.3)	
2+	120 (30.8)	108 (27.7)	12 (3.1)		107 (27.4)	13 (3.3)	
3+	116 (29.7)	105 (26.9)	11 (2.8)		107 (27.4)	9 (2.3)	
Unknown	4 (1.0)	4 (1.0)	0		3 (0.8)	1 (0.3)	
Gli-1				0.266			0.789
0	15 (3.8)	11 (2.8)	4 (1.0)		14 (3.6)	1 (0.3)	
1+	89 (22.8)	80 (20.5)	9 (2.3)		83 (21.3)	6 (1.5)	
2+	212 (54.4)	194 (49.7)	18 (4.6)		189 (48.5)	23 (5.9)	
3+	72 (18.5)	65 (16.7)	7 (1.8)		64 (16.4)	8 (2.1)	
Unknown	2 (0.5)	2 (0.5)	0		2 (0.5)	0	
Shh				0.229			0.421
0	33 (8.5)	32 (8.2)	1 (0.3)		31 (7.9)	2 (0.5)	
1+	125 (32.1)	107 (27.4)	18 (4.6)		117 (30.0)	8 (2.1)	
2+	181 (46.4)	167 (42.8)	14 (3.6)		159 (40.8)	22 (5.6)	
3+	48 (12.3)	43 (11.0)	5 (1.3)		42 (10.8)	6 (1.5)	
Unknown	3 (0.8)	3 (0.8)	0		3 (0.8)	0	
Total	390 (100)	352 (90.2)	38 (9.7)		352 (90.2)	38 (9.7)	

*ALDH1 expression in both tumor and stromal cells was observed in 5 samples

frequent (88.7%) in young patients (<40 years of age) in our study. Similar results have recently been reported by Tan et al. [26]. In their study, ALDH1 expression was observed in 25% of tumors from patients who were <35 years old and younger women were 14 times more likely to have ALDH1-positive tumors. It has been well documented that patients who are diagnosed with breast cancer at a young age have a relatively poor prognosis compared to patients diagnosed at an older age [27], which is further supported by our data.

According to a recent report by de Beca et al., enrichment of CD44+/CD24- cells and/or ALDH1 expression differs to some extent according to tumor histologic types. Further, CD44+/CD24- cells are valuable breast cancer stem cell markers. de Beca et al. showed that metaplastic and medullary carcinomas, which are classified as high-grade tumors, showed a significantly increased frequency in simultaneous CD44+/CD24- cell enrichment and ALDH1 expression, whereas most low-grade tubular carcinomas showed CD44+/CD24- cell enrichment alone [28]. These findings also support our data, in that cells expressing ALDH1 tend to be more aggressive than those expressing CD44+/CD24- alone.

In Korean patients, we found that ALDH1 expression was not associated with Her2/*neu* negativity or basal-like tumor characteristics. In contrast, we found that ALDH1 expression was associated with Her2/*neu* overexpression. With similar results currently emerging from groups in Japan, we propose that ALDH1 expression is associated with Her2/*neu*-overexpressing breast cancers in Asian patients [11, 14].

Several studies have reported that ALDH1 expression is associated with poor clinical outcomes in breast cancer [6, 13-15, 29, 30]. In previous studies, ALDH1 expression was detected in a higher proportion and appeared to be a poor prognostic factor for patients who had other indicators of poor prognosis such as lymph-node positive breast cancer, inflammatory breast cancer, or triple-negative cancer than in sporadic cancer. In this study, ALDH1 expression was not associated with DFS or OS. Our result is likely due to the fact that our tumor samples were all from sporadic breast cancer

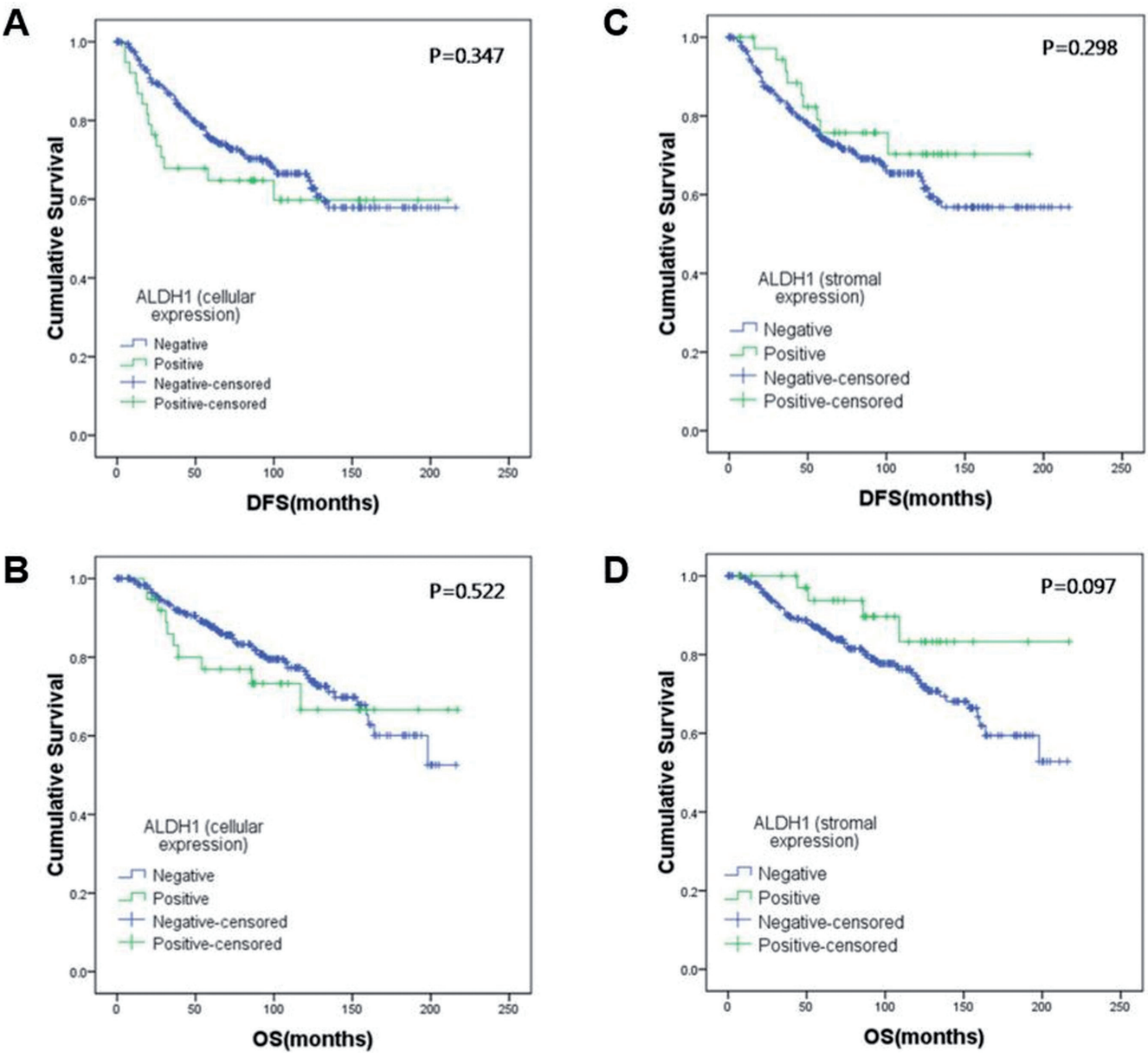

Figure 3. Kaplan-Meier curves for the association of cellular ALDH1 expression with DFS (A) and OS (B) and of stromal ALDH1 expression with DFS (C) and OS (D)

cases. Moreover, we examined tissue samples from operable cases, including stage I to III cases, but not stage IV cases. Therefore, our finding may not elucidate the prognostic value of ALDH1 expression for early-stage sporadic cancer.

ALDH1 expression has been detected in benign breast tissue as well as in tumor tissue [31, 32]. In tumor tissues, ALDH1 was expressed in both tumor and stromal cells. In previous studies, ALDH1 expression results were inconsistent because of the lack of an optimized scoring method for ALDH1 expression. Some studies reported cytoplasmic positivity for ALDH1 expression in tumor cells only [6, 11, 12], and some studies detected both cytoplasmic positivity of tumor cells and stromal positivity for ALDH1 expression [15, 24, 25]. Since tumor stromal cells are known to be involved in tumor growth and metastasis [21, 33], differences in the distribution and characteristics of cytoplasmic cellular or stromal expression of ALDH1 need to be elucidated to better understand the exact role of ALDH1 expression in breast cancer. A few previous reports have analyzed the implications of cellular and stromal expression. Resetkova et al. reported that stromal ALDH1 expression was correlated with improved outcomes in a triple-negative breast cancer cohort [25]. In addition, De

Brot et al. observed that ALDH1 expression was frequent in stromal cells and that this was associated with better overall outcome in triple-negative breast cancers [34]. Charafe-Jauffret et al. reported contrasting results that cellular expression of ALDH1 was an independent predictive factor for poor survival outcome in inflammatory breast cancers [13]. Similarly, cellular and stromal expression seems to affect different processes in carcinogenesis and cancer progression. However, the patient populations of these studies were different, which could account for the discrepancies. In our study, clinical characteristics did not differ between tumors with cellular and stromal expression. Both cellular expression and stromal ALDH1 expression were associated with hormone receptor negativity. In addition, neither cellular nor stromal expression affected survival outcomes. On the basis of our findings, we concluded that cellular and stromal ALDH1 expression did differ in terms of their associations with clinicopathological characteristics in cases of sporadic breast cancer. Additionally, we suggest that tumor stromal cells strongly expressing ALDH1 are similar to tumor cells themselves and are also associated with more aggressive features. These findings will be helpful in establishing standard criteria for ALDH1 positivity and in interpreting the effects of ALDH1 expression in future studies.

In conclusion, we have shown that breast cancers with ALDH1 expression display biologically aggressive phenotypes, including young age (<40 years), hormone receptor negativity, a high histological grade, and a high Ki-67 index. However, further studies with patients with all stages of breast cancer and standardized ALDH1 expression scoring are required to further validate our results.

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