

# Expression of ALDH1 in axillary lymph node metastases is a prognostic factor of poor clinical outcome in breast cancer patients with 1–3 lymph node metastases

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## Abstract

**Background** Recently, evidence in support of the cancer stem cell (CSC) hypothesis has been accumulating. On the other hand, it has been reported that the expression of aldehyde dehydrogenase 1 (ALDH1) in primary breast cancer is a powerful predictor of a poor clinical outcome, and that breast cancer stem cells express ALDH1. According to the CSC hypothesis, development of metastases requires the dissemination of CSC that may remain dormant and be reactivated to cause tumor recurrence. In this study, we investigated whether the detection of CSC in axillary lymph node metastases (ALNM) might be a significant prognostic factor in patients with breast cancer.

**Methods** From 1998 to 2006, 40 primary breast cancer patients with ALNM, the number of metastatic nodes varying in number from 1 to 3, underwent surgery at Okayama University; of these, 15 patients developed tumor recurrence. We retrospectively evaluated the common clinicopathological features and the expression of ER, HER2, ALDH1, and Ki67 in both the primary lesions and the ALNM, and analyzed the correlations between the

expression of these biological markers and the disease-free survival (DFS).

**Results** Expression of ALDH1 in the ALNM was significantly associated with the DFS ( $P = 0.037$ ).

**Conclusion** Evaluation of biomarker expression in ALNM could be useful for prognosis in breast cancer patients with 1–3 metastatic lymph nodes.

**Keywords** Cancer stem cell · ALDH1 · Axillary lymph node metastases · IHC

## Introduction

Although the cancer stem cell (CSC) hypothesis was first proposed almost 150 years ago, it is in recent years that the hypothesis has rapidly gained ground. Advances in stem cell biology and development of new animal models to measure self-renewal have contributed to the renewed recognition of this hypothesis [1]. Cancer stem cells were first documented in acute myeloid leukemia by taking advantage of the cell sorting technology using various surface markers [2]. Subsequently, the presence of CSC has been reported in solid tumors, including breast cancer, brain cancer, lung cancer, and colon cancer, as well [3–6]. Al-Hajj et al. [3] were the first to distinguish between tumorigenic cancer cells and non-tumorigenic cells in breast cancers by using the cell surface markers CD44 and CD24. They showed that following inoculation into mice, as few as 500 tumor cells with the CD44+/CD24– phenotype were able to form tumors in NOD/SCID mice, whereas even as many as  $10^5$ – $10^6$  tumor cells with other CD44/CD24 phenotypes were unable to form tumors. Subsequently, Ginestier et al. [7] reported that aldehyde dehydrogenase 1 (ALDH1) may be a better marker of

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breast cancer stem cells on the basis of the finding that fewer ALDH1-positive than CD44+/CD24- tumor cells were needed to form tumors in immunodeficient mice. According to the CSC hypothesis, metastases require the dissemination of cancer stem cells that may remain dormant and be reactivated to cause tumor recurrence. In contrast, dissemination of differentiated tumor cells produces only micrometastasis that do not progress [1]. In breast cancers, metastasis often first appears in the axillary lymph nodes. Hence, it may be crucial importance to detect the presence of CSC in the axillary lymph nodes [8]. Axillary lymph node metastases (ALNM) are considered the most important of prognostic factors in breast cancer patients, and the number of metastatic lymph nodes as the most powerful guide to selection of the most appropriate strategy for adjuvant therapy. When the number of ALNM was over 3, the risk of recurrence was considered to be high and adjuvant chemotherapy was considered to be necessary. The patients without ALNM were regarded as being at a low risk for recurrence and to therefore not need intensive adjuvant therapy. On the other hand, there has been much debate about the appropriate treatment for breast cancers with 1–3 lymph node metastases, because of the lack of definitive evidence [9].

Recently, evaluation of biomarkers to assess the responses to particular breast cancer therapeutic strategies has received much attention. Currently, the selection of therapeutic drugs for recurrent breast cancers is based only on the biomarker expression profile in the primary lesion evaluated at the time of the initial operation for the primary tumor. However, discordance of biomarker expression between primary and distant metastatic tumors has been increasingly reported.

In this study, we investigated whether the presence of cancer stem cells in ALNM, especially when the number of metastatic lymph nodes was under 4, might be a significant clinicopathological prognostic factor in patients with breast cancer, and the concordance of biological features between the breast tumors and the ALNM.

## Materials and methods

### Patients and sample studied

Tumor tissue samples from the primary lesions and ALNM were obtained from 40 primary breast cancer patients who were primarily treated by surgery between 1998 and 2006 at Okayama University Hospital (OUH). Curative surgery, namely, total or partial mastectomy with axillary dissection, was performed in all patients, and all patients had less than 3 metastatic lymph nodes in the axilla. After the surgery, the premenopausal patients with estrogen receptor

(ER)-positive tumors were administered a selective estrogen receptor modulator (SERM) and luteinizing hormone-releasing hormone (LH-RH) agonist, and the postmenopausal patients were administered an aromatase inhibitor (AI) for 5 years. ER-negative and/or histological grade 3 and/or >pT2 patients were administered adjuvant chemotherapy (AC or AC followed by paclitaxel). Patients who underwent partial mastectomy were also administered radiation therapy for the residual breast tissue. After the adjuvant therapy, all the patients were periodically followed up at our hospital. Recurrences were diagnosed by radiological and pathological examination.

Tumor tissues obtained at surgery were fixed in 10% buffered formalin and embedded in paraffin. The ALNM which had the largest metastases were examined. A routine histological examination was performed in sections stained with hematoxylin–eosin (H&E). We retrospectively evaluated the common clinicopathological features and the status of expression of ER, HER2, ALDH1, and Ki67 in both the primary lesion and the ALNM, and analyzed the discordance rate between the two for each marker. Furthermore, we evaluated the correlation between the expression status of these biological markers and the disease-free survival (DFS).

### Histological grade, ER, and HER2

The histological grade was determined using the Scarff–Bloom–Richardson grading system [10]. ER expression (Ventana Japan) was defined as positive when at least 10% of the tumor cells showed positive immunohistochemical staining. HER2 was detected by immunohistochemical staining using the HercepTest kit (Dako Japan). In this study, we considered the specimen to be HER2-positive when more than 30% of the cells showed positive immunohistochemical staining.

### Immunohistochemical staining for ALDH1 and Ki67

Immunohistochemistry was performed on formalin-fixed paraffin sections (4  $\mu$ m) of tumor tissues with the BOND<sup>TM</sup> automated immunostainer (Leica Microsystems). The protocol was in accordance with IHC-FP H1 (30). The antibodies and dilutions used were ALDH1 (BD Biosciences) at 1:200 dilution, and Ki67 (Dako Japan) at 1:250 dilution. Imaging analysis of the breast tumors for ALDH1 expression was performed in one selected area ( $\times$ 400 high power field) per case. That of the ALNM was performed in 3–7 randomly selected areas ( $\times$ 400 high power field) per case. We calculated the percentage of ALDH1-positive cells and divided the intensity of the immunohistochemical staining for ALDH1 into positive (more than 5% tumor cells showing positive staining). In the ALNM, Ki67 expression was

analyzed in 3–5 selected areas ( $\times 400$  high power field) per case. Ki67 expression was considered to be positive when at least 20% of the cancer cells showed positive staining [11].

### Statistical analyses

The SAS software JMP 7.0.2 was used for all the statistical analyses. Regression analysis was used for analyzing the correlations in the expression of the biomarkers between the primary tumors and the ALNM. Associations between the ALDH1 expression status and the clinicopathological parameters were evaluated by the  $\chi^2$  test. Agreement for ALDH1 expression between the primary tumors and the ALNM was assessed by Cohen's kappa coefficient. The log-rank test was used for comparison of the survival curves, and the Cox proportional hazards model was used for the univariate and multivariate analysis. Statistical significance was assumed at  $P$  less than 0.05.

## Results

### Patient characteristics

The median age of the patients was 53 years (range 28–78 years). The median time on study with follow-up was 46 months (range 6–143 months). Of the total, 15 (24%) patients were over 50 years old, and 25 (76%) were under 51 years old. The diagnosis in all patients was invasive carcinoma with ALNM, classified as N1 on the basis of the seventh edition of the TNM classification. Out of the 40 patients, 32 (80%) were ER-positive and 8 (20%) were ER-negative, 9 (22.5%) patients were HER2-positive and 31 (77.5%) were HER2-negative, 11 (27.5%) patients were histological grade 1, 16 (40%) patients were histological grade 2, and 13 (32.5%) patients were histological grade 3, 16 (40%) patients had some recurrences [bone 7 (18%), liver 4 (10%), brain 1 (3%), breast 2 (5%), lung 2 (5%), skin 1 (3%), lymph nodes 6 (15%)], 6 (15%) patients died of cancer [breast cancer 5 (12.5%), other cancer 1 (2.5%)], 13 (32.5%) patients received adjuvant chemotherapy [anthracycline 10 (25%), taxane 7 (18%), anthracycline plus taxane 7 (18%), and cyclophosphamide plus methotrexate plus 5-fluorouracil (CMF) 3 (8%)], 22 patients received endocrine therapies [SERM 9 (28%) and AI 15 (38%)], and 7 (17.5%) patients received no adjuvant treatment (Table 1).

ER, HER2, Ki67, and ALDH1 expression status in the breast tumors and ALNM

Of the 40 breast tumors, 32 (80%) breast tumors were ER-positive and 8 (20%) were ER-negative; 28 (70%)

**Table 1** Patients characteristics

Parameters	<i>n</i> (%)
Median	53 (28–78)
Operation	
Total	13 (32.5)
Partial	27 (67.5)
Nodal status	
<i>n</i> = 1	23 (57.5)
<i>n</i> = 2	7 (17.5)
<i>n</i> = 3	10 (25)
Histology	
IDC	37 (92.5)
ILC	2 (5)
Other	1 (2.5)
Adjuvant therapy	
Chemotherapy	13 (32.5)
Anthracycline	10 (25)
Taxane	7 (17.5)
Anthracycline + taxane	7 (17.5)
CMF	3 (7.5)
Hormonal therapy	22 (55)
SERM (tamoxifen)	9 (22.5)
AI	15 (37.5)
None	7 (17.5)
Recurrence	16 (40)
Bone	7 (17.5)
Liver	4 (10)
Brain	1 (2.5)
Lung	2 (5)
Breast	2 (5)
Lymph node	6 (15)
Death	6 (15)
Breast cancer	5 (12.5)
Other	1 (2.5)

IDC invasive ductal carcinoma, ILC invasive lobular carcinoma

ALNM were ER-positive and 12 (30%) were ER-negative; 9 (22.5%) breast tumors were HER2-positive and 31 (77.5%) were HER2-negative; 10 (25%) ALNM were HER2-positive and 30 (75%) were HER2-negative; 30 (75%) breast tumors were Ki67-positive and 10 (25%) were Ki67-negative; 31 (77.5%) ALNM were Ki67-positive and 9 (22.5%) were Ki67-negative; 7 patients (17.5%) were ALDH1-positive and 33 patients (82.5%) were ALDH1-negative; 10 patients (25%) were ALDH1-positive and 30 patients (75%) were ALDH1-negative (Table 2). The results of immunohistochemical staining for ALDH1 in the breast tumor and in the ALNM are shown in Figs. 1 and 2.

**Table 2** Relationship of ALDH1 positivity in the breast tumors with the clinicopathological parameters

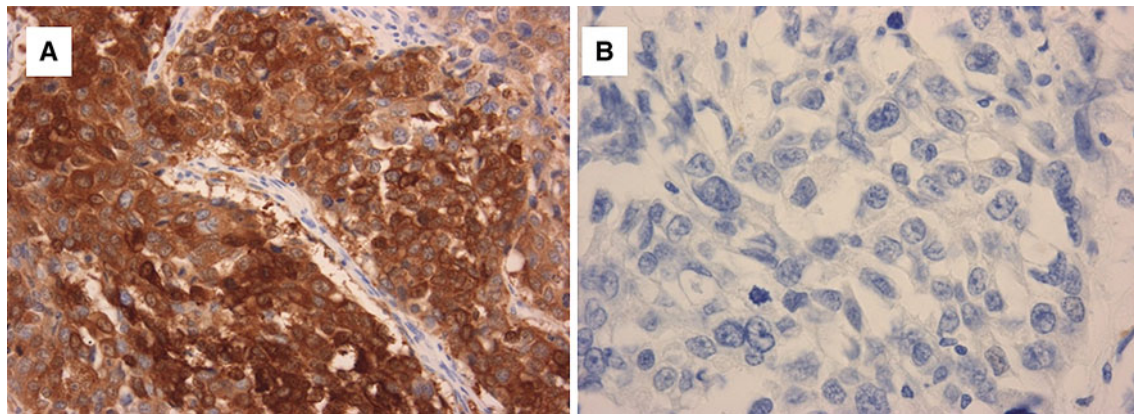
	<i>n</i>	ALDH1 (breast tumor <5%)		<i>P</i>	ALDH1 (ALNM <5%)		<i>P</i>
		Positive, <i>n</i> (%)	Negative, <i>n</i> (%)		Positive, <i>n</i> (%)	Negative, <i>n</i> (%)	
All breast tumor	40	7 (17.5)	33 (82.5)		–	–	
Lymph node	40	–	–		10 (25)	30 (75)	
Age (years)				NS			NS
≥50	15	2 (13)	13 (87)		6 (40)	9 (60)	
<50	25	5 (20)	20 (80)		4 (16)	21 (84)	
Histological grade				NS			0.02
1	11	0 (0)	11 (100)		0 (0)	11 (100)	
2	16	3 (19)	13 (81)		5 (31)	11 (69)	
3	13	4 (31)	9 (69)		5 (38)	8 (62)	
Tumor size (cm)				NS			NS
>2	11	0 (0)	11 (100)		3 (27)	8 (73)	
≤2	29	7 (24)	22 (76)		7 (24)	22 (76)	
Nodal status				NS			NS
<i>n</i> = 1	23	6 (26)	17 (74)		4 (17)	19 (83)	
<i>n</i> = 2	7	0 (0)	7 (100)		3 (43)	4 (57)	
<i>n</i> = 3	10	1 (10)	9 (90)		3 (30)	7 (70)	
ER							
Breast tumor				NS			NS
+	32	5 (16)	27 (84)		6 (19)	26 (81)	
–	8	2 (25)	6 (75)		4 (50)	4 (50)	
ALNM				0.012			0.002
+	28	2 (7)	26 (93)		3 (11)	25 (89)	
–	12	5 (42)	7 (58)		7 (58)	5 (42)	
HER2							
Breast tumor				NS			NS
+	9	2 (22)	7 (78)		3 (33)	6 (67)	
–	31	5 (16)	26 (84)		7 (23)	24 (77)	
ALNM				NS			NS
+	10	2 (20)	8 (80)		3 (30)	7 (70)	
–	30	5 (17)	25 (83)		7 (23)	23 (77)	
Ki67							
Breast tumor				0.031			NS
>20	30	3 (10)	27 (90)		7 (23)	23 (77)	
≤20	10	4 (40)	6 (60)		3 (30)	7 (70)	
ALNM				NS			0.002
>20	31	5 (16)	26 (84)		6 (19)	25 (81)	
≤20	9	2 (22)	7 (78)		4 (44)	5 (56)	

NS not significant

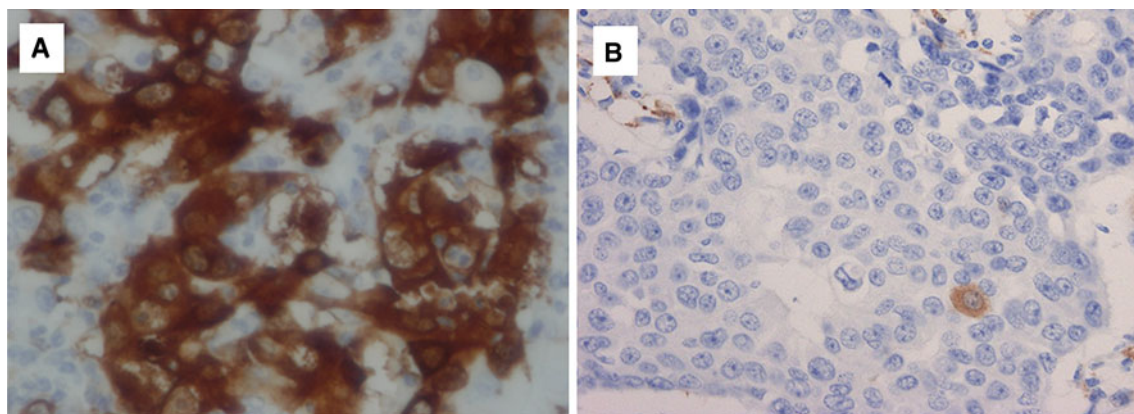
#### Relationship between ALDH1-positive expression in the breast tumors and the clinicopathological parameters

The ALDH1-positive breast tumors were significantly more likely to be ER-negative in the ALNM ( $P = 0.012$ ) and to be Ki67-positive in the primary tumor ( $P = 0.031$ ).

No significant association was observed between ALDH1 positivity in the primary tumor and the histological grade, age of the patient, size of the primary tumor, lymph node status, ER expression in the primary tumor, HER2 expression in the primary tumor, HER2 expression in the ALNM, or Ki67 expression in the ALNM. These ALDH1-positive ALNM were significantly more likely to depend



**Fig. 1** Immunohistochemical identification of ALDH1-positive tumor cells. The results of immunostaining of ALDH1 in breast cancer tissues: **a** positive, **b** negative



**Fig. 2** Immunohistochemical identification of ALDH1-positive tumor cells. The results of immunostaining of ALDH1 in ALNM: **a** positive, **b** negative

on high histological grade ( $P = 0.002$ ) or being ER-negative in the ALNM ( $P = 0.012$ ) and Ki67-positive in the ALNM (0.002). No significant association was observed between ALDH1 positivity in the ALNM and the tumor size, HER2 expression in the primary tumor, HER2 expression in the ALNM, Ki67 expression in the primary tumor, or Ki67 expression in the ALNM (Table 2).

Concordance rate of ER, HER2, Ki67, and ALDH1 expression between the breast tumors and the ALNM

The concordance rates of ER, HER2, Ki67, and ALDH1 expression between the breast tumors and the ALNM were 87.5, 82.5, 77.5, and 57.5%, respectively (Table 3). In order to show the associations for ALDH1-positive cancer cells between primary tumor and ALNM, we calculated Cohen's kappa coefficient. When the cutoff point between high and low ALDH1 expression level was set at 5%, they showed moderate agreement ( $\kappa = 0.481$ ).

Relationship between various biological factors and the patient prognosis (DFS)

The associations between the DFS and various biological factors, such as the ALDH1 (in the primary tumor and ALNM), Ki67 (in the primary tumor and the ALNM), ER (in the primary tumor and the ALNM) expression status, age, histological grade, HER2 expression status (in the primary tumor and the ALNM), and the tumor size, were also studied. The ALDH1-positive ALNM group showed a poorer outcome in terms of the DFS ( $P = 0.148$ , primary tumor, Fig. 3a;  $P = 0.037$ , ALNM, Fig. 3b). Univariate analysis showed a significant association between the DFS and ER expression in the ALNM ( $P = 0.047$ ) and histological grade of differentiation of the tumor ( $P = 0.04$ ), and ALDH1 expression in the ALNM was likely to result in poor clinical outcome ( $P = 0.055$ ). Multivariate analysis showed no significant association between any of the variables and the DFS (Table 4). Further, we could not

**Table 3** Concordance rate of the biomarker expression between the primary tumors and the ALNM

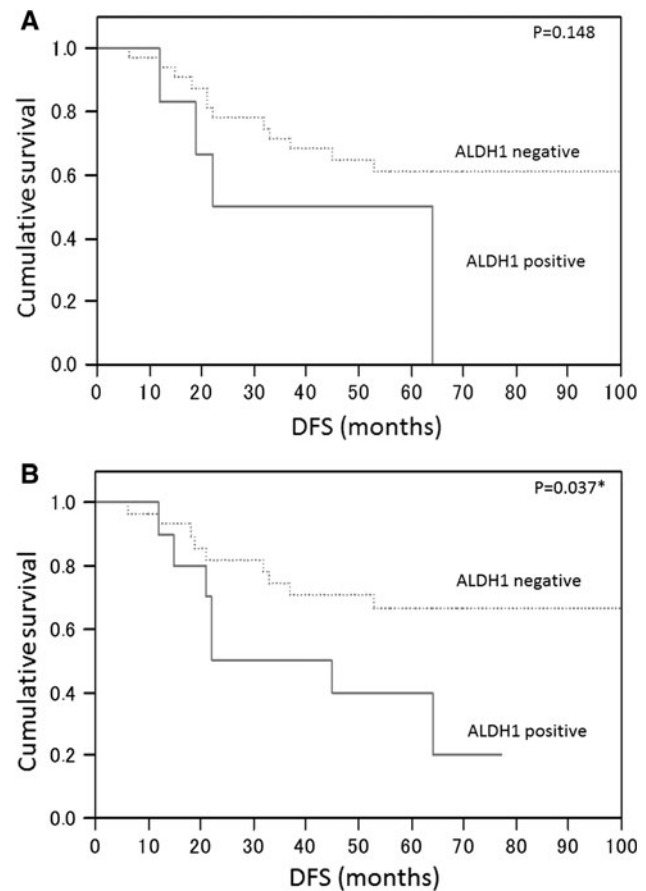
	Primary/metastatic tumor ( <i>n</i> = 40)			
	+/+	+/-	-/+	-/-
<b>ER</b>				
No. of patients	32	5	0	3
%	80	12.5	0	7.5
Concordance rate (%)	87.5			
<b>HER2</b>				
No. of patients	6	3	4	27
%	15	7.5	10	67.5
Concordance rate (%)	82.5			
<b>Ki67</b>				
No. of patients	5	5	4	26
%	12.5	12.5	10	65
Concordance rate (%)	77.5			
<b>ALDH1</b>				
No. of patients	7	0	17	16
%	17.5	0	42.5	40
Concordance rate (%)	57.5			

recognize any statistically significant association between these various biological factors and the overall survival (data not shown).

## Discussion

Abraham et al. [12] performed immunohistochemical studies of CD44+/CD24– tumor cells in human breast cancer and reported that breast tumors containing a high proportion of CD44+/CD24– cells were more frequently associated with the development of distant metastases, although no association with the event-free or overall survival was shown. Mylona et al. [13] reported that the prevalence of CD44+CD24– exerted no significant impact on the prognosis, although a tendency towards increase of the DFS was noted, because these cell populations might not originate from normal adult stem cells but from a transit cell. Moreover, the same authors reported that tumor cells with the CD44–CD24+ phenotype seemed to identify patients with worse disease-free and overall survivals among patients with tumors showing intermediate-grade differentiation. Their results were supported by Baumann et al. [14] who showed that with CD24 expression, breast cancer cells acquire enhanced ability for spreading, movement, and invasion, which facilitate the development of metastasis.

Ginestier et al. [7] documented that immunohistochemically identified tumor ALDH1 expression was associated with a poor prognosis in breast cancer patients.

**Fig. 3** Kaplan–Meier curve for disease-free survival (DFS) according to ALDH1 status in **a** breast tumors and **b** ALNM

ALDH1 in cancer stem cells may be closely involved in stem cell differentiation by regulating the conversion of retinoic acid to oxidizing retinol [15]. Consequently, we thought that immunohistochemically demonstrated CD44+CD24– cells may not have reliable prognostic significance. There have been no reports of the evaluation of ALDH1 expression in ALNM. Thus, we investigated the biological markers of breast tumors and ALNM. Our data showed that the expression of ALDH1 in ALNM was significantly associated with a shorter DFS. This result indicates that breast cancer patients with 1–3 lymph node metastases and expression of ALDH1 in ALNM would tend to have earlier relapse. In this study, we examined the findings in immunohistochemically stained slides of both the breast tumors and ALNM in comparison with those in the H&E-stained slides. It has been reported previously that ALDH1 is expressed in both normal and cancerous mammary epithelial cells [7]. In this study also, we observed ALDH1-positive cells in normal mammary tissues, and excluded these cells from the present evaluation morphologically. Furthermore, quite a few macrophages exist in lymph nodes, and it has been reported that macrophages also show ALDH1 expression [16]. Therefore, we paid

**Table 4** Univariate and multivariate analyses to identify predictors of the DFS

	Univariate analysis			Multivariate analysis		
	Odds ratio	95% CI	<i>P</i>	Odds ratio	95% CI	<i>P</i>
ALDH1 in the breast tumor (positive/negative)	2.26	0.63–6.54	0.19			
ALDH1 in the ALNM (positive/negative)	2.75	0.98–7.46	0.055			
Ki67 in the breast tumor (positive/negative)	1.89	0.64–5.08	0.24			
Ki67 in the ALNM (positive/negative)	2.07	0.65–5.71	0.2			
ER in the breast tumor (-/+)	2.85	0.13–1.13	0.076			
ER in the ALNM (-/+)	2.89	1.01–7.89	<b>0.047</b>	1.57	0.49–4.93	0.44
Age ( $\leq 50$ / $> 50$ )	1.24	0.46–3.64	0.68			
Histological grade (3/1, 2)	2.88	1.05–7.91	<b>0.04</b>	1.83	0.60–5.65	0.28
HER2 in the breast tumor (-/+)	1.7	0.54–4.70	0.34			
HER2 in the ALNM (-/+)	3.04	0.69–9.55	0.13			
Tumor size ( $> 2$ cm/ $< 2$ cm)	2.36	0.75–10.31	0.15			

Bold values are statistically significant

careful attention to excluding macrophages morphologically, especially in the ALNM. Ginestier et al. [7] reported that ALDH1 positivity (using a cutoff value for ALDH1 of 5%) in the primary tumors was significantly associated with a poor overall survival (OS). However, the appropriate cutoff value for ALDH1 in ALNM or the correlations between ALDH1 expression in ALNM and the clinical outcome has not yet been reported. In this study, we found no correlation between the expression of ALDH1 (positive defined as greater than 5%) in the primary tumors and the clinical outcome (DFS;  $P = 0.14$ ). In regard to the correlation between the expression of ALDH1 in ALNM and the DFS, a significant association was found ( $P = 0.037$ ). Moreover we analyzed using a lower cutoff value of 1%, because we thought it important whether CSC were present or not in ALNM. There was a significant difference in DFS (data not shown). It may be suggested that ALDH1-negative cells (not cancer stem cells) in ALNM do not survive or spread to other organs. Thus, the presence of ALDH1-negative cells in ALNM may indicate against a poor clinical outcome. On the contrary, a few cancer stem cells may survive for a long period and expand, resulting in worsening of the prognosis. The association between the presence of ALDH1-positive cells and a poor clinical outcome in breast cancer may be attributable to the cancer stem cells being more likely to be transferred to other organs. On the other hand, observation of cancer stem cells in ALNM provides practical evidence for the presence/absence of dissemination. In other words, evaluation of the expression of ALDH1 in ALNM provides direct evidence of dissemination, in view of the CSC hypothesis. The results of this study lend support to this hypothesis.

It was previously reported that ALDH1 expression was associated with features of aggressive tumors such as high histological grade and ER negativity [17, 18], and that

ALDEFLUOR-positive cells exhibited features of basal breast cancers [19]. Our results were consistent with those of previous reports; the ALDH1-positive breast tumors were significantly more likely to be ER-negative in the ALNM ( $P = 0.012$ ) and to be Ki67-positive in the primary tumor ( $P = 0.031$ ). These ALDH1-positive ALNM were significantly more likely to depend on high histological grade ( $P = 0.002$ ) or being ER-negative in the ALNM ( $P = 0.012$ ) and Ki67-positive in the ALNM ( $P = 0.002$ ).

In regard to the concordance rate between primary tumors and the ALNM, some reports have indicated that whereas the concordance rate between primary tumors and the ALNM for ER was 81–96.6%, that for HER2 was 82.5–100% [20–23]. In this study, the concordance rates for ER and HER2 were 87.5 and 82.5%, respectively; 4 patients were HER2-positive in the primary tumor and HER2-negative in the ALNM. The concordance rate for HER2 in this study seems to be slightly lower as compared with previous reports, perhaps because these patients may have received and shown good response to trastuzumab administered as postoperative adjuvant therapy. The genetic instability of breast cancer cells was likely to be a major cause for this diversity [24]. Moreover, we evaluated the expression of ER, ALDH1, and Ki67, a marker of cell proliferation, by immunohistochemistry. Our results revealed that the concordance rate between the primary tumor and the ALNM was 87.5% for ER, 57.5% for ALDH1, and 77.5% for Ki67. These results also support the notion of possible discrepancies between the primary tumor and the ALNM.

The low concordance rate of ALDH1 expression between the primary tumor and the ALNM suggests that ALDH1 expression plays an important role in the heterogeneity of breast cancers. When we assessed correlations of the expression between the breast tumors and the ALNM

by Cohen's kappa coefficient, it showed moderate agreement ( $\kappa = 0.481$ ), indicating that they might have relatively similar statistical power for predicting prognosis. However, a significant correlation between the expression of ALDH1 and the DFS was found only in the ALNM (odds ratio 3.79, 95% CI 1.37–12.1), which suggests that evaluation of ALDH1 in the ALNM is more likely to be useful for predicting prognosis in breast cancer patients with ALNM compared to primary tumors.

This study is relatively small and, therefore, some true but weaker prognostic variables may not have been detected as significant in this analysis. Also, protein levels from old samples might not represent the actual biological processes. Nevertheless we believe our findings are generalizable and are consistent with prognostic results observed in separate patients in previous publications [7, 25].

Thus, the results of this study indicate that evaluation of biomarker expression in the ALNM may have clinical significance in terms of prognosis for breast cancer patients with ALNM ( $n = 1-3$ ). We need to conduct a prospective study with a larger sample size to confirm the value and methods of evaluation of biomarker expression in ALNM.

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