Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression

Koji Morimoto,[1,2] Seung Jin Kim,[1] Tomonori Tanei,[1] Kenzo Shimazu,[1] Yoshio Tanji,[1] Tetsuya Taguchi,[1] Yasuhiro Tamaki,[1] Nobuyuki Terada[2] and Shinzaburo Noguchi[1,3]

[1]Department of Breast and Endocrine Surgery, Osaka University Graduate School of Medicine, Suita City, Osaka; [2]Department of Pathology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

(Received January 19, 2009/Revised February 24, 2009/Accepted March 2, 2009/Online publication March 30, 2009)

Recently, aldehyde dehydrogenase (ALDH) 1 has been identified as a reliable marker for breast cancer stem cells. The aim of our study was to investigate the clinicopathological characteristics of breast cancers with ALDH1$^+$ cancer stem cells. In addition, the distribution of ALDH1$^+$ tumor cells was compared on a cell-by-cell basis with that of estrogen receptor (ER)$^+$, Ki67$^+$, or human epidermal growth factor receptor type 2 (HER2)$^+$ tumor cells by means of double immunohistochemical staining. Immunohistochemical staining of ALDH1 was applied to 203 primary breast cancers, and the results were compared with various clinicopathological characteristics of breast cancers including tumor size, histological grade, lymph node metastases, lymphovascular invasion, ER, progesterone receptor, HER2, Ki67, and topoisomerase 2A as well as prognosis. Immunohistochemical double staining of ALDH1 and ER, Ki67, or HER2 was also carried out to investigate their distribution. Of the 203 breast cancers, 21 (10%) were found to be ALDH1$^+$, and these cancers were significantly more likely to be ER$^-$ ($P = 0.004$), progesterone receptor$^-$ ($P = 0.025$), HER2$^+$ ($P = 0.001$), Ki67$^+$ ($P < 0.001$), and topoisomerase 2A$^+$ tumors ($P = 0.012$). Immunohistochemical double staining studies showed that ALDH1$^+$ tumor cells were more likely to be ER$^-$, Ki67$^-$, and HER2$^+$ tumor cells. Patients with ALDH1 (score 3+) tumors showed a tendency ($P = 0.056$) toward a worse prognosis than did those with ALDH1$^-$ tumors. Breast cancers with ALDH1$^+$ cancer stem cells posses biologically aggressive phenotypes that tend to have a poor prognosis, and ALDH1$^+$ cancer stem cells are characterized by ER$^-$, Ki67$^-$, and HER2$^+$. (*Cancer Sci* 2009; 100: 1062–1068)

Evidence has recently been accumulating to support the cancer stem cell hypothesis for solid tumors, including breast cancer, which holds that cancers are driven by a small subpopulation of stem cells that are capable of self-renewal and give rise to multipotent progenitor cells that ultimately differentiate into all cell types within the tumor.[1] Al-Hajj *et al*. were the first to distinguish tumorigenic from non-tumorigenic cancer cells by using the cell surface markers CD44 and CD24.[2] They have shown that cancer stem cells in a population of tumor cells are enriched with the CD44$^+$ and CD24$^-$ phenotype because as few as 100 tumor cells with this phenotype were able to produce tumors in immunodeficient mice, whereas tumor cells with other CD44 and CD24 phenotypes were unable or rarely able to produce tumors even when as many as 10^5–10^6 tumor cells were inoculated into such mice. It was subsequently found by Ginestier *et al*. that aldehyde dehydrogenase (ALDH) 1 is a better marker of breast cancer stem cells as fewer ALDH1$^+$ tumor cells than CD44$^+$ and CD24$^-$ tumor cells are required to produce tumors in immunodeficient mice.[3]

It thus seems important to clarify the clinicopathological characteristics of breast cancers with ALDH1$^+$ cancer stem cells for a better understanding of the biological significance of cancer stem cells. However, this important issue has been addressed by only one reported study to date. Ginestier *et al*. demonstrated that breast cancers with ALDH1$^+$ cancer stem cells are associated with biologically aggressive phenotypes such as estrogen receptor (ER) negativity, high histological grade, human epidermal growth factor receptor type 2 (HER2) positivity, as well as poor prognosis.[3] If their observation is confirmed, determination of ALDH1$^+$ cancer stem cells may well be clinically useful for patient prognosis. In the study reported here, we therefore investigated the clinicopathological characteristics of breast cancers with ALDH1$^+$ cancer stem cells and also compared ALDH1 expression in primary tumors and axillary metastases. In addition, the ER, Ki67, and HER2 status of ALDH1$^+$ tumor cells was investigated on a cell-by-cell basis by means of double immunohistochemical staining for further characterization of the phenotype of breast cancer stem cells.

Materials and Methods

Patients and breast tumor tissues. Tumor tissue samples were obtained from 203 primary breast cancer patients (mean age, 52.6 years; range, 32–86 years) who underwent mastectomy or breast-conserving surgery between January 1993 and December 1997 at Osaka University Hospital, Osaka, Japan. Tumor tissues were fixed in 10% buffered formalin and embedded in paraffin. This study protocol was approved by the Ethics Committee of Osaka University.

For adjuvant therapy, 85 patients were treated with hormonal therapy (tamoxifen, $n = 74$; toremifene, $n = 7$; gosereline, $n = 3$; or gosereline + tamoxifen, $n = 1$), 22 with chemotherapy (fluoropyrimidine, $n = 8$; cyclophosphamide + methotrexate + 5-fluorouracil, $n = 7$; cyclophosphamide + adriamycin + 5-fluorouracil, $n = 4$; or high-dose chemotherapy, $n = 3$), and 84 with chemo-hormonal therapy (fluoropyrimidine, $n = 37$; cyclophosphamide + methotrexate + 5-fluorouracil, $n = 25$; cyclophosphamide + adriamycin + 5-fluorouracil, $n = 20$; or high-dose chemotherapy, $n = 2$; plus tamoxifen, $n = 78$; toremifene, $n = 1$; gosereline, $n = 3$; or gosereline + tamoxifen, $n = 2$). Twelve patients received no adjuvant therapy.

[3]To whom correspondence should be addressed.
E-mail: noguchi@onsurg.med.osaka-u.ac.jp

doi: 10.1111/j.1349-7006.2009.01151.x

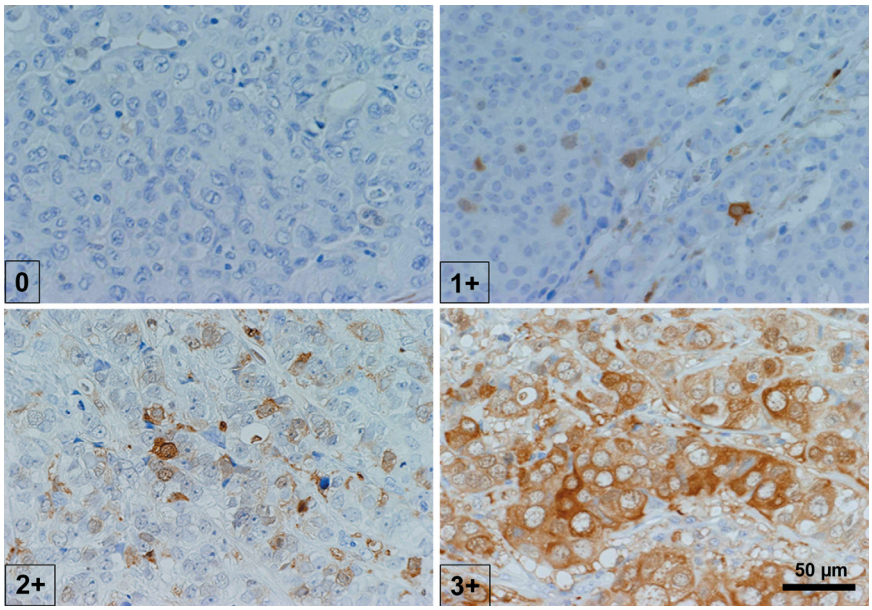

Fig. 1. Immunohistochemical identification of aldehyde dehydrogenase (ALDH) 1+ tumor cells. Representative results of immunostaining of ALDH1: 0 (negative), 1+ (weakly positive), 2+ (intermediately positive), and 3+ (strongly positive).

Antibodies. The antibodies used were: ALDH1 (monoclonal, IgG isotype, 1:100; BD Biosciences, San Jose, CA, USA), ER (polyclonal, IgG isotype, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), progesterone receptor (PR) (clone 636, monoclonal, IgG isotype, 1:800; Dako, Kyoto, Japan), c-erbB2 (HER2) (polyclonal, IgG isotype, 1:100; Nichirei, Kyoto, Japan), Ki67 (clone MIB-1, monoclonal, IgG isotype, 1:100; Dako), topoisomerase (TOP) 2A (clone Ki-S1, monoclonal, IgG isotype, 1:50; Dako), and TSA Biotin System (Tyramide Signal Amplification Biotin System, 1:50; Perkin Elmer, Wellesley, MA, USA).

Immunohistochemical staining. Formalin-fixed paraffin sections (3 μm) of the tumor tissues were subjected to immunohistochemical staining with the avidin–biotin–peroxidase method. In brief, antigen retrieval for ER, PR, Ki67, and ALDH1 was carried out by heating the samples in a target retrieval solution (Dako) at 98°C for 40 min or antigen retrieval for TOP2A was achieved with the same procedure for 60 min. Sections to be stained for HER2 were not pretreated. After quenching endogenous peroxidase with 3% H_2O_2 in methanol for 20 min, non-specific binding was blocked by incubating the slides with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, followed by incubation of the sections with the primary antibody at 4°C overnight. Next, the sections were treated with a biotin-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for ER, PR, Ki67, and HER2, or with a peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO; Nichirei) for ALDH1 and TOP2A, followed by incubation at room temperature for 1 h. Finally, after treatment with the avidin–biotin–peroxidase complex system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA), the sections were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany). The sections were then counterstained with hematoxylin. For ER, PR, Ki67, and TOP2A identification, nuclear-stained cancer cells in three non-overlapping fields (×200) were counted with the aid of WinROOF imaging software (Mitani, Tokyo, Japan).

Immunohistochemical staining of ALDH1 was classified as 3+ (≥50% positive tumor cells), 2+ (<50% but ≥10%), 1+ (<10%), or negative (0%).[3] The cut-off value was 10% for ER and PR. The cut-off value for Ki67 and TOP2A was also 10% because the strongest associations were demonstrated between ALDH1 and Ki67 status and between ALDH1 and TOP2A status using this cut-off value. Staining of HER2 was scored into four grades (0, 1, 2, and 3) according to the method described previously,[4] and tumors with scores of 2+ and 3+ were considered to be $HER2^+$.

For double immunohistochemical staining, the sections were incubated with anti-ALDH1 and anti-Ki67 antibody, anti-ALDH1 and anti-TOP2A antibody, anti-ALDH1 and anti-ER antibody, or anti-ALDH1 and anti-HER2 antibody. The sections were treated with alkaline phosphatase-conjugated secondary antibody (Histofine Simple Stain MAX PO) for ALDH1 staining, with peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX PO) using the TSA Biotin System for ER, Ki67, and TOP2A staining, and with biotin-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for HER2 staining. Finally, after treatment with the avidin–biotin–peroxidase complex system (Vector Laboratories), the sections were visualized with Fucshin (Dako) and subsequently with 3,3′-diaminobenzidine tetrahydrochloride (Merck) and counterstained with hematoxylin

Statistical analyzes. SPSS software version 12.1 (SPSS, Chicago, IL, USA) was used for all statistical analyses. Associations between ALDH1 status and clinicopathological parameters were assessed with the χ^2-test. Relapse-free survival (RFS) rates were calculated with the Kaplan–Meier method and differences in RFS rates were determined with the log-rank test. Univariate and multivariate analyses of RFS rates were carried out with the Cox proportional hazard model. All P-values of <0.05 resulting from two-sided tests were considered significant.

Results

Relationship of $ALDH1^+$ breast cancers with clinicopathological parameters. Representative results of immunohistochemical ALDH1 staining are shown in Figure 1. Of 203 tumors, ALDH1 was negative in 182 (90%), 1+ in 10 (5%), 2+ in three (1%), and 3+ in eight (4%). Breast cancers showing 1+ or higher staining of ALDH1 were considered to be $ALDH1^+$ in the following analysis. Of the 203 breast cancers, 21 (10%) were found to be $ALDH1^+$, and these breast cancers were significantly more likely to be ER^- ($P = 0.004$), PR^- ($P = 0.025$), $HER2^+$ ($P = 0.001$), $Ki67^+$ ($P < 0.001$), and $TOP2A^+$ tumors ($P = 0.012$) (Table 1). No significant association was observed between ALDH1 positivity and age, menopausal status, tumor size, lymph node status, or histological grade.

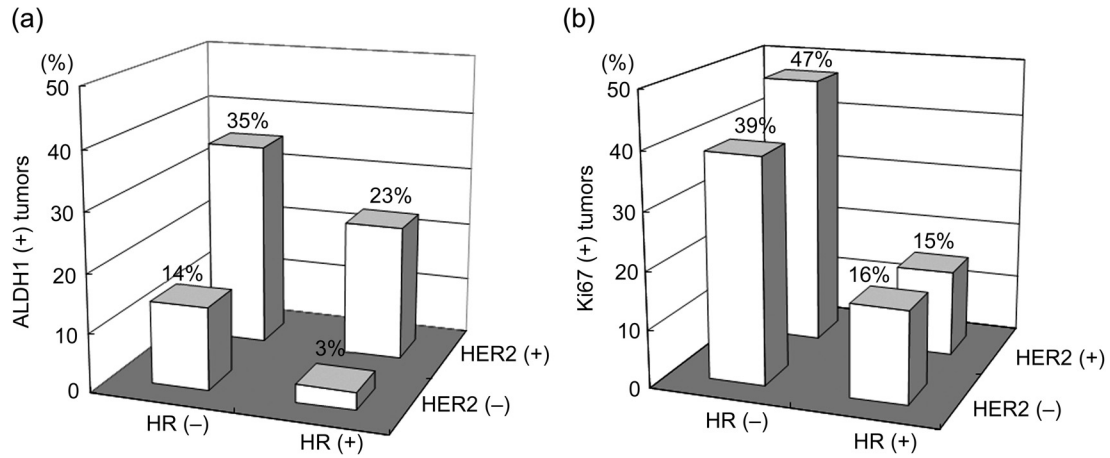

Fig. 2. Relationship between hormone receptor (HR)/HER2 status and aldehyde dehydrogenase (ALDH) 1 or Ki67. Breast cancers were classified into four categories according to their HR and HER2 status: HR^+ and $HER2^+$, HR^+ and $HER2^-$, HR^- and $HER2^+$, and HR^- and $HER2^-$. The frequencies of (a) $ALDH1^+$ and (b) $Ki67^+$ tumors were compared.

Table 1. Relationship between aldehyde dehydrogenase (ALDH) 1 expression and clinicopathological parameters of breast cancers

Characteristic	$ALDH1^-$	$ALDH1^+$	*P*-value
All cases	182	21	
Age (years)			
>50	89 (49)	10 (48)	1.000
≤50	93 (51)	11 (52)	
Menopausal status			
Pre	92 (51)	12 (57)	0.705
Post	86 (47)	9 (43)	
Unknown	4 (2)	0 (0)	
Tumor size			
≤2.0 cm	58 (32)	7 (33)	1.000
>2.0 cm	124 (68)	14 (67)	
Lymph node metastasis			
Negative	115 (63)	11 (52)	0.350
Positive	67 (37)	10 (48)	
Histological type			
DCIS	22 (12)	1 (5)	0.736
LCIS	1 (1)	0 (0)	
IDC	151 (83)	19 (90)	
ILC	1 (1)	0 (0)	
Others	7 (4)	1 (5)	
Histological grade			
1	41 (23)	3 (14)	0.577
2 + 3	141 (77)	18 (86)	
Estrogen receptor			
Negative (<10%)	68 (37)	15 (71)	0.004
Positive (≥10%)	114 (63)	6 (29)	
Progesterone receptor			
Negative (<10%)	121 (66)	19 (90)	0.025
Positive (≥10%)	61 (34)	2 (10)	
HER2			
Negative	161 (88)	12 (57)	0.001
Positive	21 (12)	9 (43)	
Ki67			
Negative	144 (79)	8 (38)	<0.001
Positive	38 (21)	13 (62)	
Topoisomerase 2A			
Negative	144 (79)	11 (52)	0.012
Positive	38 (21)	10 (48)	

Numbers in parentheses are percentages.

The frequency of $ALDH1^+$ tumors according to hormone receptor (HR) status and HER2 status is shown in Figure 2(a). The HR^+ (ER^+ and/or PR^+) and $HER2^-$ tumors showed the lowest frequency of $ALDH1^+$ tumors (3%, 4/116) and the HR^- and $HER2^+$ tumors showed the highest frequency of $ALDH1^+$ tumors (35%, 6/17). HR^+ and $HER2^+$ tumors and HR^- and $HER2^-$ tumors had intermediate frequencies of 23% (3/13) and 14% (8/57), respectively. The frequency of $Ki67^+$ tumors was higher in HR^- tumors than HR^+ tumors, irrespective of HER2 status (Fig. 2b).

Comparison of the distribution of $ALDH1^+$ tumor cells with that of $Ki67^+$, ER^+, or $HER2^+$ tumor cells. Double staining of ALDH1 and Ki67 was used to investigate the distribution of $ALDH1^+$ tumor cells and $Ki67^+$ tumor cells. Representative results are shown in Figure 3. In some tumors, $ALDH1^+$ tumor cells were seen in clusters (Fig. 3a), and in such tumors, $ALDH1^+$ tumor cells expressed Ki67 at a noticeably lower level (Fig. 3b) than did the surrounding $ADLH1^-$ tumor cells (Fig. 3c). In other tumors, where $ALDH1^+$ tumor cells were seen in a mosaic pattern (Fig. 3d), it was interesting that $ALDH1^+$ tumor cells (Fig. 3d, black arrows) rarely overlapped with $Ki67^+$ tumor cells (Fig. 3d, white arrows). Similar results were obtained for the distribution of $ALDH1^+$ tumor cells and $TOP2A^+$ tumor cells by double staining (Supporting Information Fig. S1).

Representative results of double staining of ALDH1 and ER are shown in Figure 4. Some tumors showed clusters of $ALDH1^+$ or $ALDH1^-$ tumor cells (Fig. 4a), and the latter often expressed ER (Fig. 4b), whereas the former rarely did (Fig. 4c). In other tumors where $ALDH1^+$ tumor cells were seen in a mosaic pattern (Fig. 4d), it was of interest that $ALDH1^+$ tumor cells (Fig. 4d, black arrows) rarely overlapped with ER^+ tumor cells (Fig. 4d, white arrows).

Representative results of ALDH1 and HER2 double staining are shown in Figure 5. The population of $ALDH1^+$ tumor cells was smaller than that of $HER2^+$ tumor cells, and $HER2^+$ tumor cells, unlike ER^+ or $Ki67^+$ tumor cells, overlapped with $ALDH1^+$ tumor cells (Fig. 5b, black arrows).

Comparison of ALDH1 expression between primary tumors and lymph node metastases. ALDH1 expression was compared between primary tumors and lymph node metastases in seven patients with $ALDH1^+$ tumors and in 13 patients with $ALDH1^-$ tumors. Of the seven patients with $ALDH1^+$ tumors, three (43%) showed $ALDH1^+$ lymph node metastases and four (57%) showed $ALDH1^-$ lymph node metastases (Fig. 6). All of the 13 patients with $ALDH1^-$ tumors showed $ALDH1^-$ lymph node metastases.

doi: 10.1111/j.1349-7006.2009.01151.x

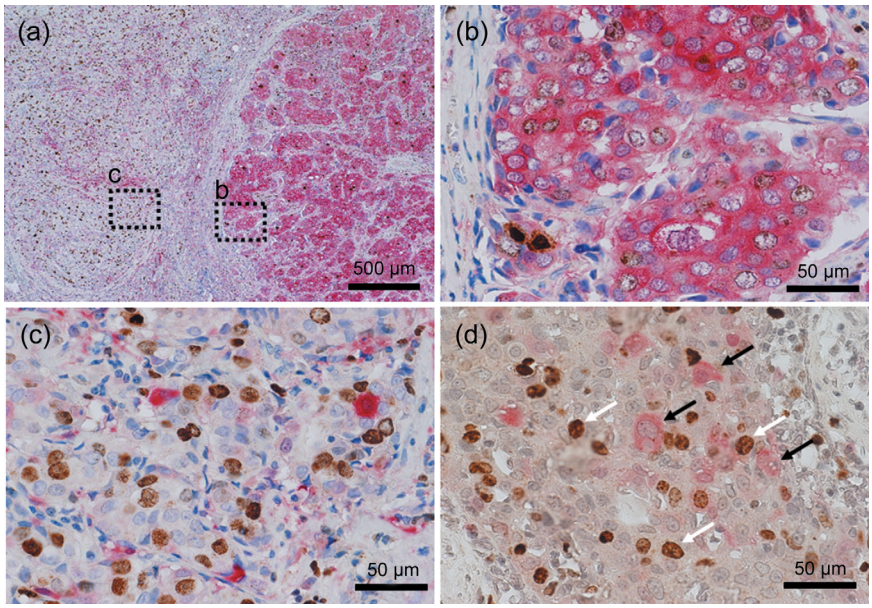

Fig. 3. Immunohistochemical double staining of aldehyde dehydrogenase (ALDH) 1 and Ki67. Representative results of immunohistochemical double staining of ALDH1 (red) and Ki67 (brown) in two breast cancers: (a–c) tumor #1, and (d) tumor #2. (a) ALDH1$^+$ and ALDH1$^-$ tumor cells were localized separately in tumor #1. Higher magnification of the ALDH1$^+$ and ALDH1$^-$ areas of this tumor revealed that (b) ALDH1$^+$ tumor cells were mostly Ki67$^-$ and (c) ALDH1$^-$ tumor cells were more likely to be Ki67$^+$. (d) In tumor #2, ALDH1$^+$ tumor cells are localized in a mosaic pattern, and ALDH1$^+$ tumor cells (black arrows) and Ki67$^+$ tumor cells (white arrows) rarely overlapped.

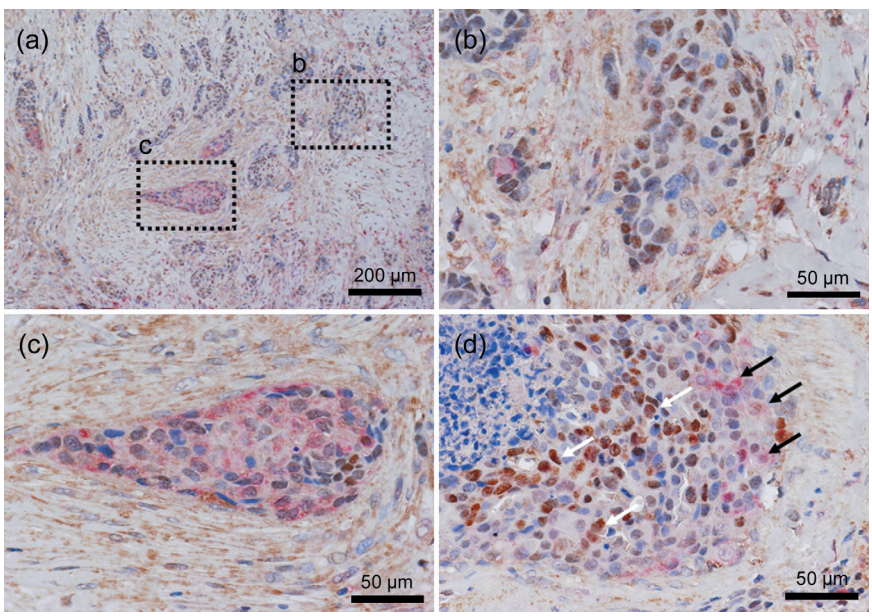

Fig. 4. Immunohistochemical double staining of aldehyde dehydrogenase (ALDH) 1 and estrogen receptor (ER). Representative results of immunohistochemical double staining of ALDH1 (red) and ER (brown) in two breast cancers: (a–c) tumor #3, and (d) tumor #4. (a) ALDH1$^+$ tumor cell clusters and ALDH1$^-$ tumor cell clusters were observed. (c) Higher magnification of an ALDH1$^+$ tumor cell cluster revealed that ALDH1$^+$ tumor cells were rarely ER$^+$, and (b) that of an ALDH1$^-$ tumor cell cluster revealed that ALDH1$^-$ tumor cells were mostly ER$^+$. (d) In tumor #4, ALDH1$^+$ tumor cells were localized in a mosaic pattern, and ALDH1$^+$ tumor cells (black arrows) and ER$^+$ tumor cells (white arrows) rarely overlapped.

ALDH1 expression and patient prognosis. RFS rates of patients with ALDH1$^+$ tumors tended to be lower than those with ALDH1$^-$ tumors although the difference was not statistically significant (Fig. 7a). Patients with ALDH1 (score 3+) tumors showed marginally significantly lower RFS rates than those with ALDH1$^-$ tumors ($P = 0.056$, Fig. 7c) whereas the RFS rates of patients with ALDH1 (scores 1+ and 2+) tumors were similar to those with ALDH1$^-$ tumors (Fig. 7b).

Univariate analysis of various prognostic factors showed that menopausal status, tumor size, histological grade, lymph node status, ER, PR, HER2, and Ki67 were significantly associated with prognosis (Table 2). The prognostic significance of ALDH1 (score 3+) was therefore assessed by multivariate analysis in order to adjust the other parameters significantly associated with prognosis. This analysis showed that ALDH1 (score 3+) was not significantly associated with prognosis ($P = 0.459$).

Discussion

The purpose of the present study was to clarify the clinicopathological characteristics of breast cancers that were ALDH1$^+$ and thus thought to contain cancer stem cells. We found that ALDH1$^+$ tumors are significantly associated with high ER negativity, PR negativity, HER2 positivity, high Ki67 expression, and high TOP2A expression. These observations are essentially consistent with those reported by Ginestier *et al.* indicating that ALDH1$^+$ breast tumors are characterized by a biologically aggressive phenotype.[3]

Recent studies based on gene expression profiling have clearly shown that breast cancers can be classified into intrinsic subtypes such as luminal A, luminal B, HER2, and basal,[5,6] and that these subtypes can be estimated by combining HR and HER2 status results.[7] We therefore studied the relationship

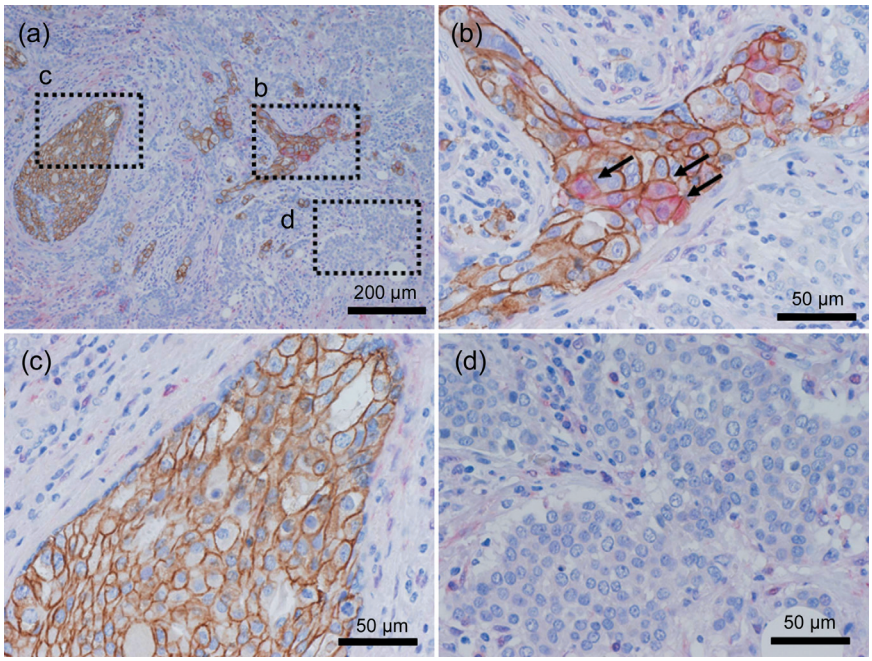

Fig. 5. Immunohistochemical double staining of aldehyde dehydrogenase (ALDH) 1 and HER2. Representative results of immunohistochemical double staining of ALDH1 and HER2 in breast cancer (tumor #5) are shown. (a) HER2$^+$ tumor cell clusters surrounded by HER2$^-$ tumor cells were observed. (b) Higher magnification of an HER2$^+$ tumor cell cluster revealed that ALDH1$^+$ tumor cells (black arrows) were included in this cluster. (c) There was also a cluster not containing ALDH1$^+$ tumor cells. (d) All HER2$^-$ tumor cells were ALDH1$^-$.

Table 2. Univariate and multivariate analysis of various prognostic factors

Characteristic	Univariate analysis			Multivariate analysis[†]		
	Hazard ratio	95% confidence interval	*P*-value	Hazard ratio	95% confidence interval	*P*-value
Menopausal status (pre vs post)	1.770	1.082–2.890	0.023	1.704	0.991–2.933	0.054
Tumor size (>20 vs ≤20 mm)	3.108	1.402–6.887	0.005	3.452	1.486–8.019	0.004
Histological grade (2, 3/1)	2.937	1.699–5.077	<0.001	2.182	1.120–4.253	0.022
Lymphatic vessel invasion (+/–)	1.650	0.934–2.914	0.084			
Blood vessel invasion (+/–)	1.955	0.882–4.335	0.099			
Lymph node metastasis (–/+)	2.805	1.609–4.892	<0.001	2.787	1.405–5.529	0.003
Estrogen receptor (–/+)	2.591	1.490–4.484	0.001	1.086	0.577–2.045	0.799
Progesterone receptor (–/+)	3.012	1.418–6.410	0.004	2.710	1.172–6.289	0.020
HER2 (+/–)	2.268	1.190–4.321	0.013	1.339	0.663–2.702	0.416
Ki67 (≥10 vs <10%)	1.891	1.090–3.281	0.023	1.214	0.631–2.339	0.561
Topoisomerase 2A (≥10 vs <10%)	1.638	0.280–2.893	0.089			
Aldehyde dehydrogenase 1 (3+/–)	2.706	0.975–7.509	0.056	1.516	0.504–4.559	0.459

[†]Adjusted for adjuvant therapies (none/hormotherapy/chemotherapy/chemo-hormonal therapy).

between these intrinsic subtypes and the frequency of ALDH1$^+$ tumors, and found that HR$^+$ and HER2$^-$ tumors (luminal A type) have the lowest frequency (3%) of ALDH1$^+$ tumors. Luminal A type tumors are the most differentiated form of breast tumors and their pathogenesis and progression are thought to be strongly influenced by estrogens. The fact that a very small proportion (only 3%) of luminal A type tumors are ALDH1$^+$ seems to be compatible with the hypothesis that luminal type A tumors originate from HR$^+$ mammary progenitor cells rather than from HR$^-$ mammary stem cells.[8]

On the other hand, the HR$^-$ and HER2$^-$ tumors (i.e. triple negative tumors) had a higher proportion (14%) of ALDH1$^+$ tumors than luminal A type (3%). Although triple negative tumors are heterogeneous, the majority have been shown to be basal type tumors.[9,10] These results seem to support the hypothesis that basal type tumors originate directly from HR$^-$ mammary

doi: 10.1111/j.1349-7006.2009.01151.x

stem cells. In order to characterize the ALDH1$^+$ tumor cells more accurately, we carried out immunohistochemical double staining of ALDH1 and Ki67 and were able to show that ALDH1$^+$ tumor cells were mostly Ki67$^-$, and, interestingly, ALDH1$^-$ tumor cells were more likely to be Ki67$^+$. These results indicate that breast cancer stem cells proliferate very slowly and the surrounding tumor cells (progenitor cells) proliferate more rapidly, which is consistent with the theory that cancer stem cells are relatively quiescent and give rise to a rapidly dividing population of progenitor cells.[1] Thus, our observation that ALDH1$^+$ tumors are more likely to be Ki67$^+$ tumors does not mean that this is true for ALDH1$^+$ tumor cells but that ALDH1$^+$ tumors have a higher proportion of ALDH1$^-$ and Ki67$^+$ tumor cells surrounding the ALDH1$^+$ tumor cells. Our present observation on the distribution of ALDH1$^+$ tumor cells and Ki67$^+$ tumor cells appears to be interesting but it needs to be confirmed by a future study.

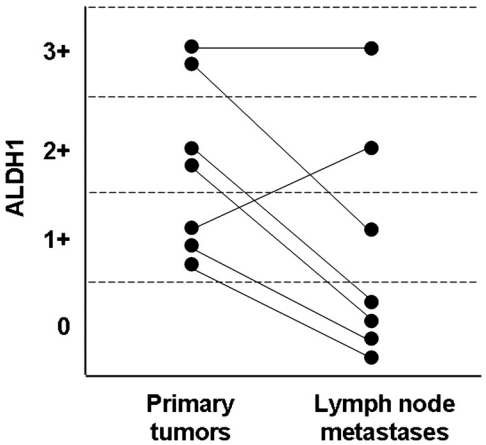

Fig. 6. Comparison of aldehyde dehydrogenase (ALDH) 1$^+$ tumor cells between primary tumors and lymph node metastases. In patients with ALDH1$^+$ primary tumors and lymph node metastases ($n = 7$), immunohistochemical study of ALDH1 in lymph node metastases was carried out for a comparison with primary breast tumors.

One of the most interesting findings of our study is that HER2$^+$ tumors are more likely to be ALDH1$^+$ tumors regardless of HR status. HER2$^+$ and HR$^-$ tumors are thought to originate from ER$^-$ mammary stem cells. The cancer stem cells transformed by *HER2* gene amplification seem to have a high capability for self-renewal and expansion. Immunohistochemical double staining in our study demonstrated that ALDH1$^+$ tumor cells commonly overlap with HER2$^+$ tumor cells, indicating that *HER2* gene amplification must have taken place in ER$^-$ mammary stem cells that transformed to cancer stem cells. The origin of HR$^+$ and HER2$^+$ tumors (luminal B type) is still controversial but it is speculated that luminal B type tumors with ALDH1$^+$ tumor cells originate from HR$^-$ mammary stem cells and show a certain tendency to differentiate into ER$^+$ tumor cells. Our observation that ALDH1$^+$ tumor cells are commonly ER$^-$ and that ALDH1$^-$ tumor cells are more likely to be ER$^+$ in a given tumor might imply that ALDH1$^+$ and ER$^-$ cancer stem cells could differentiate into ALDH1$^-$ and ER$^+$ tumor cells.[8,11]

Because it seemed to be of considerable interest to investigate whether ALDH1$^+$ cancer stem cells are more or less likely to metastasize, we compared ALDH1$^+$ cancer stem cells in primary tumors and lymph node metastases of seven patients with ALDH1$^+$ tumors and lymph node metastases. Lymph node metastases were ALDH1$^+$ in three of these patients (43%) and ALDH1$^-$ in four (57%), indicating that ALDH1$^+$ cancer stem cells do metastasize but seem not to have a stronger propensity to do so compared with ALDH1$^-$ tumor cells. The metastatic potentials of ALDH1$^+$ cancer stem cells will need to be clarified in more detail because it is speculated that the presence of cancer stem cells in metastases has important clinical implications for prognosis and response to chemotherapy.

It is speculated that breast cancers with ALDH1$^+$ cancer stem cells would show a poorer prognosis because of their association with biologically aggressive phenotypes and their inherent chemoresistant nature.[12,13] In fact, Ginestier *et al*. have reported that patients with ALDH1$^+$ tumors show a significantly poorer prognosis than those with ALDH1$^-$ tumors.[3] In the present study, we were able to show a marginally significant ($P = 0.056$) difference in RFS rates between ALDH1 (score 3+) tumors and ALDH1$^-$ tumors, suggesting that the presence of ALDH1$^+$ cancer stem cells has prognostic significance. This tendency,

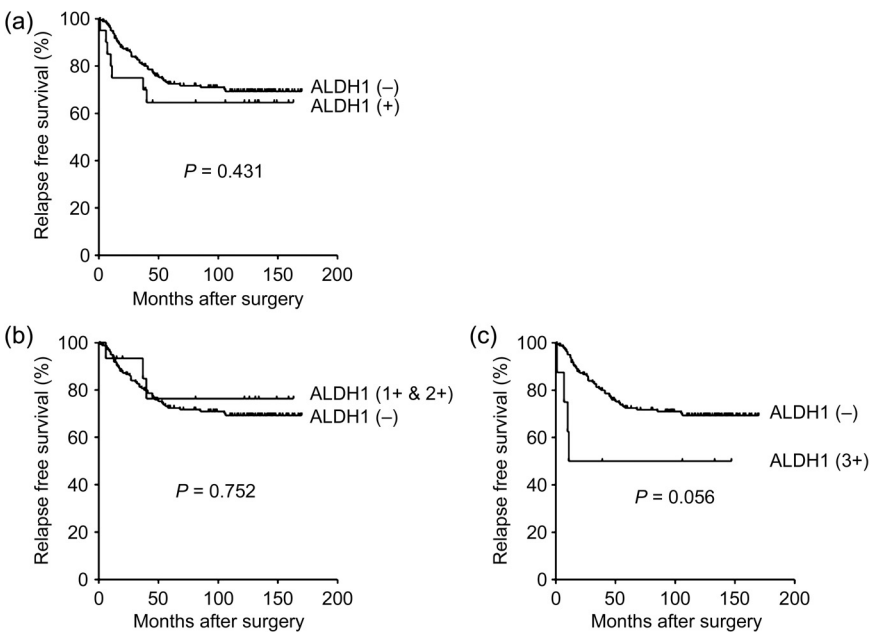

Fig. 7. Relapse-free survival (RFS) curves of breast cancer patients according to aldehyde dehydrogenase (ALDH) 1 expression. (a) RFS rates were compared for patients with ALDH1$^-$ tumors and those with ALDH1$^+$ tumors. Patients with ALDH1$^+$ tumors were categorized into those with ALDH1 (1+ and 2+) tumors and those with ALDH1 (3+) tumors, and their RFS rates were compared with those of ALDH1$^-$ tumors (b) and (c), respectively.

however, was no longer observed after adjustment for other prognostic factors, indicating that ALDH1 is not an independent prognostic factor. The limited number of patients enrolled in our study seems to largely account for our inability to demonstrate a statistically significant difference in prognosis between ALDH1$^+$ and ALDH1$^-$ tumors, unlike the study by Ginestier *et al.* who analyzed as many as 577 patients and were able to demonstrate a statistically significant difference.[3] Another limitation of the present study lines in the fact that it is a retrospective study conducted on patients who were treated with various types of adjuvant therapies that might affect their prognosis. Thus, in the present study, we have evaluated the prognostic significance of ALDH1 by multivariate analysis in order to adjust the various other parameters, including the types of adjuvant therapies. However, ideally the prognostic significance of ALDH1 should be evaluated in patients treated without adjuvant therapy, although it is actually very difficult to recruit enough of such patients because almost all patients are currently treated with some adjuvant therapy.

In conclusion, we have shown that breast cancers with ALDH1$^+$ cancer stem cells possess biologically aggressive phenotypes with a tendency leading to a poor prognosis, and that ALDH1$^+$ cancer stem cells are characterized by ER$^-$, Ki67$^-$, and HER2$^+$. Our results seem to indicate that immunohistochemical determination of ALDH1$^+$ cancer stem cells may be clinically useful for the identification of biologically aggressive breast cancers as well as a better understanding of the pathogenesis of breast cancers but our present preliminary observation needs to be confirmed by a future study including a larger number of patients.

Acknowledgements

We wish to thank Dr. M. Takeda (Department of Pathology, Yao City Hospital) for his helpful comments and encouragement.

References

1 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev* 2008; **8**: 755–68.
2 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983–8.
3 Ginestier C, Hur MH, Charafe-Jauffret E *et al*. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007; **1**: 555–67.
4 Miyoshi Y, Kurosumi M, Kurebayashi J *et al*. Topoisomerase IIα-positive and BRCA1-negative phenotype: association with favorable response to epirubicin-based regimens for human breast cancers. *Cancer Lett* 2008; **264**: 44–53.
5 Sorlie T, Tibshirani R, Parker J *et al*. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA* 2003; **100**: 8418–23.
6 Sotiriou C, Neo SY, McShane LM *et al*. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci USA* 2003; **100**: 10 393–8.
7 Carey LA, Perou CM, Livasy CA *et al*. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA* 2006; **295**: 2492–502.
8 Dontu G, El-Ashry D, Wicha MS. Breast cancer, stem/progenitor cells and the estrogen receptor. *Trends Endocrinol Metab* 2004; **15**: 193–7.
9 Anders C, Carey LA. Understanding and treating triple-negative breast cancer. *Oncology* 2008; **22**: 1233–9.
10 Cleator S, Heller W, Coombes RC. Triple-negative breast cancer: therapeutic options. *Lancet Oncol* 2007; **8**: 235–44.
11 Polyak K. Breast cancer: origins and evolution. *J Clin Invest* 2007; **117**: 3155–63.
12 Liu G, Yuan X, Zeng Z *et al*. Analysis of gene expression and chemoresistance of CD133$^+$ cancer stem cells in glioblastoma. *Mol Cancer* 2006; **5**: 67.
13 Ghods AJ, Irvin D, Liu G *et al*. Spheres isolated from 9L gliosarcoma rat cell line possess chemoresistant and aggressive cancer stem-like cells. *Stem Cells* 2007; **25**: 1645–53.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immunohistochemical double staining of aldehyde dehydrogenase (ALDH) 1 and topoisomerase (TOP) 2A. Representative results of immunohistochemical double staining of ALDH1 (red) and TOP2A (brown) in two breast cancers are shown: (a–c) tumor #6, and (d) tumor #7. (a) ALDH1$^+$ and ALDH1$^-$ tumor cells were localized separately in tumor #6. Higher magnification of the ALDH1$^+$ and ALDH1$^-$ areas of this tumor revealed that (b) ALDH1$^+$ tumor cells were mostly TOP2A$^-$, and (c) ALDH1$^-$ tumor cells were more likely to be TOP2A$^+$. (d) In tumor #7, ALDH1$^+$ tumor cells were localized in a mosaic pattern, and ALDH1$^+$ tumor cells (black arrows) and TOP2A$^+$ tumor cells (white arrows) rarely overlapped.

doi: 10.1111/j.1349-7006.2009.01151.x