Evaluation of aldehyde dehydrogenase 1 and transcription factors in both primary breast cancer and axillary lymph node metastases as a prognostic factors

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Abstract

Background: Aldehyde dehydrogenase 1 (ALDH1) is a marker of breast cancer stem cells, and the expression of ALDH1 may be a prognostic factor of poor clinical outcome. The epithelial–mesenchymal transition (EMT) may produce cells with stem cell-like properties promoted by transcription factors. We investigated the expression of ALDH1 and transcription factors in both primary and metastatic lesions, and prognostic value of them in breast cancer patients with axillary lymph node metastasis.

Method: Forty-seven breast cancer patients with axillary lymph node metastasis who underwent surgery at Okayama University Hospital from 2002 to 2008 were enrolled. We retrospectively evaluated the levels of ALDH1 and transcription factors, such as Snail, Slug and Twist, in both primary and metastatic lesions by immunohistochemistry.

Results: In primary lesions, the positive rate of ALDH1, Snail, Slug and Twist was 19%, 49%, 40% and 26%, respectively. In lymph nodes, that of ALDH1, Snail, Slug and Twist was 21%, 32%, 13% and 23%, respectively. The expression of ALDH1 or transcription factors alone was not significantly associated with a poor prognosis. However, co-expression of ALDH1 and Slug in primary lesions was associated with a shorter DFS ($P = 0.009$).

Conclusions: The evaluation of the co-expression of ALDH1 and transcription factors in primary lesions may be useful in prognosis of node-positive breast cancers.

Key words: ALDH1, EMT, transcription factors, IHC, breast cancer
**Introduction**

Axillary lymph node status is one of the most important prognostic factors for breast cancer patients, and adjuvant therapy is decided in consideration of the number of axillary lymph node metastases (ALNM). Adjuvant chemotherapy is recommended to patients with four or more ALNM because they have a high recurrence risk [1].

Moreover, the breast cancer subtype which classified according to estrogen receptor (ER)/progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) positivity is regarded as a predictive factor of adjuvant therapy. We started to use this subtype and Ki67 expression for decisions of adjuvant chemotherapy; however, the validity and sufficiency of those parameters for this purpose is under debate. Further discussion is necessary to achieve a more precise treatment strategy for breast cancer.

According to the cancer stem cell theory, a small cell population with the characteristic of cancer stem cells has abilities of self-renewal, differentiation and heterogeneity[2]. The presence of cancer stem cells may be a poor prognostic factor because of their specific properties, and Ginestier et al. reported that aldehyde dehydrogenase 1 (ALDH1) is a marker of breast cancer stem cells and that its expression is strongly correlated with poor prognosis [3]. Considering that metastases often first appear in the axillary lymph nodes, and that several studies identified a discordance of biomarker expression between primary
and metastatic lesions, we demonstrated that the expression of ALDH1 in ALNM is significantly associated with a short disease-free survival (DFS) in breast cancer patients with 1–3 positive nodes [4].

The epithelial–mesenchymal transition (EMT) is a part of process in which a cell loses epithelial traits to acquire mesenchymal properties and is essential for organogenesis during embryonic development [5]. In the course of breast cancer progression, cell-cell adhesions are lost through the EMT process, thus leading to acquisition of migratory and invasive properties [5, 6]. Moreover, EMT has also been suggested to produce cell with stem-cell-like properties that are promoted by transcription factors, such as Snail, Slug, Twist, the so-called EMT markers [5, 7, 8]. Although some prior studies have shown that increased expression of such EMT markers is associated with aggressive clinicopathological features and poor outcome in breast cancer [9-11], other studies have demonstrated an absence of significant associations between the expression of EMT markers and disease prognosis; [12, 13] moreover, most of these reports have described the evaluation of biomarkers in the primary lesions. The expression rates of EMT markers and the relations between the expression of such EMT markers and clinical outcome may be controversial. Although EMT has been shown to participate in the production of cancer stem cells, there are no clinical data regarding the association between the expression of
ALDH1 and EMT markers

The present study was undertaken to determine whether the expression of ALDH1 and EMT markers such as Snail, Slug, and Twist, are correlated in both primary lesions and ALNM and whether the co-expression of ALDH1 and EMT markers has a significant effect on the clinical outcome in breast cancer with ALNM.
**Materials and Methods**

Patients and tissue samples

Forty-seven primary breast cancer patients with ALNM who underwent surgery at Okayama University Hospital between 2002 and 2008 were enrolled in this study. We selected these forty-seven cases consecutively, without setting the criteria. Patients were treated with surgery consisting of total or partial mastectomy with axillary dissection. Adjuvant chemotherapy and/or hormone therapy was given based on the nodal and hormone receptor status. Patients who underwent partial mastectomy were received radiation therapy for the residual breast tissue and patients who underwent mastectomy with four or more ALNM were received postmastectomy radiation therapy. Patients with distant metastases or preoperative chemotherapy were not eligible for this study.

Tumor tissues had been fixed in 10% buffered formalin and embedded in paraffin. The largest ALNMs were examined. A routine histological examination was performed using hematoxylin and eosin (H&E) staining. We retrospectively evaluated the common clinicopathological features and the expression of ER, PgR, and HER2 in primary lesions and ALDH1, Ki67, and transcription factors, such as Snail, Slug, and Twist, in both primary lesions and the ALNM. Moreover, we analyzed the correlation between the expressions of these biological markers and prognosis (overall survival [OS] and disease-
free survival (DFS).

**ER, PgR and HER2 status**

Pathology reports were reviewed to extract pathologic data including ER, PgR and HER2 status. ER and PgR were defined as being positive when at least 1% of the tumor cells showed positive immunohistochemistry (IHC) for these molecules. The HER2 status was defined as positive when scored to 2+ and 3+ with IHC.

**IHC for ALDH1, Snail, Slug, Twist and Ki67**

Immunostaining was performed as follows. Tissue sections (4-μm-thick) were cut from paraffin-embedded blocks. After deparaffinisatioin and rehydration, antigen retrieval and blocking of endogenous peroxidase were performed. The antibodies and dilutions used were: anti-ALDH1 (611195; BD Biosciences, Franklin Lakes, NJ) at 1:1000 dilution, anti-Snail (ab85936; Abcam, Cambridge, UK) at 1:500 dilution, anti-Slug (ab38551; Abcam) at 1:500 dilution, and anti-Twist (ab50887; Abcam) at 1:50 dilution and anti-Ki67 (Dako Japan, Tokyo, Japan) at 1:300 dilution. The sections were incubated at room temperature for 60 minutes with anti-ALDH1 antibody, for 15 minutes with anti-Snail antibody and overnight at 4 °C with anti-Slug, anti-Twist, and anti-MIB1 antibodies. For ALDH1, tissue from liver was used as positive control. Positive controls for Snail, Slug and Twist were used as suggested in the supplier’s sheet. The primary antibody was
omitted in negative-control experiments.

IHC analysis was performed by two independent observers who were blind to the clinical data and patients outcomes. For ALDH1, we evaluated staining of cytoplasm, whereas for Snail, Slug, Twist, and Ki67, only nuclear staining was assessed. We scored four biomarkers (i.e. ALDH1, Snail, Slug and Twist) according to the percentage of stained tumor cells, as follows: 0%–5% = 0, 5%–25% = 1, 25%–50% = 2, 50%–75% = 3, 75%–100% = 4. We judged only a cell stained clearly to be positive, and did not consider the intensity of staining. For all analysis, score 1–4 were lumped and classified as positive. Ki-67 expression was considered as positive when at least 14% of the cancer cells exhibited positive staining, according to the suggestion of St Gallen international expert consensus 2013 [1].

Statistical analysis

A software package (JMP 9.0.2; SAS Institute Inc., Tokyo, Japan) was used for all statistical analysis. The association between the expression of ALDH1, Snail, Slug and Twist and between the levels of such biomarkers and clinicopathological parameters were evaluated using the \( \chi^2 \) test. The log-rank test was used to compare the survival curves, and the Cox proportional hazards model was used for the univariate and multivariate analysis. Statistical significance was assumed at \( P < 0.05 \).
Results

Patient characteristics

The median age of patients was 54 years (range, 26–84 years). 32 (68%) patients were over 50 years of age, and 15 (32%) were under 49 years of age. All patients had a diagnosis of invasive carcinoma. The average number of metastatic lymph nodes was eight (range, 1–39). The median follow-up period was 61 months (range, 10–152 months). Fifteen patients among 20 recurrent patients died of breast cancer (Table 1).

ALDH1, Snail, Slug and Twist expression status in primary lesions and ALNM.

Among the 47 breast primary tumors, ALDH1 expression was observed in 9 (19%), Snail in 23 (49%), Slug in 19 (40%) and Twist in 12 (26%) cases. Among ALNM, ALDH1 expression was observed in 10 (21%), Snail in 15 (32%), Slug in 6 (13%) and Twist in 11 (23%) cases. Table 2 shows positive and negative number about ALDH1 and each transcription factors in both primary lesions and ALNM. The results of IHC for ALDH1, Snail, Slug and Twist in breast tumors are shown in Fig. 1.

In the primary lesions, Twist expression alone was significantly associated with that of ALDH1 ($P = 0.035$), and there was a significant positive association between several EMT markers: Snail and Slug ($P = 0.005$), Snail and Twist ($p < 0.001$) and Slug and Twist
In ALNM, the expression of three EMT markers was not correlated with ALDH1, and no correlation was observed between the three EMT markers.

Association between ALDH1, Snail, Slug and Twist expression in primary lesions and clinicopathological parameters

ALDH1 positivity in tumor cells was significantly associated with ER negativity ($P = 0.013$). The expression of ALDH1 had significant association with triple negative subtype ($P = 0.012$). No significant association was observed between ALDH1 positivity in primary lesions and age of the patient, tumor size, lymph node status, PgR, HER2 and Ki67. Primary lesions that had four or more ALNM were significantly more likely to express Snail ($P = 0.002$), Slug ($P = 0.039$) and Twist ($P = 0.049$). Twist positivity alone was significantly associated with the age of the patient ($P = 0.034$). No significant association was observed between any of the three EMT markers and tumor size, ER, PgR, HER2, Ki67 and subtype (Table 3).

Concordance rate of ALDH1, Snail, Slug and Twist expression between primary lesions and ALNM

The concordance rate of marker expressions between primary lesions and ALNM was 77% for ALDH1, 57% for Snail, 60% for Slug and 64% for Twist (Table 4).

Association between ALDH1, Snail, Slug Twist and patient prognosis
The expression of the markers studied here (ALDH1, Snail, Slug and Twist) in both primary lesions and ALNM had no significant prognostic value in terms of both OS and DFS. Univariate analysis showed a significance association between the DFS and the number of ALNM (four or more) \( (P = 0.041) \), Ki67 positivity \( (P = 0.029) \) and PgR negativity \( (P = 0.004) \). Multivariate analysis showed a significance association between the DFS and Ki67 positivity \( (P = 0.023) \) and Slug positivity in primary lesions \( (P = 0.034) \) (Table 5). In the survival curves, the group of Slug positivity in primary plus PgR negativity has no impact on DFS but the group of Slug positivity plus Ki67 positivity showed a significance association with short DFS \( (P = 0.017, \text{data not shown}) \). The co-expression of ALDH1 and Slug in primary lesions was associated with a shorter DFS \( (P = 0.009, \text{Fig. 2}) \), whereas the combinations of ALDH1 and Snail, ALDH1 and Twist, Snail and Slug, Snail and Twist and Slug and Twist in breast tumors as well as the combinations of ALDH1 and the three EMT markers in ALNM had no prognostic impact.
Discussion

The expression of ALDH1 in primary lesions was associated with ER negativity \((P = 0.013)\), consistent with previous studies reported [15-17]. Although none of other clinicopathological indicator significantly correlated with ALDH1-positivity, patients with four or more ALNM demonstrated a trend toward the expression of ALDH1 \((P = 0.072)\). In both primary lesions and ALNM, no association was found between ALDH1 expression and DFS \((primary: P = 0.198, \text{ALNM: } P = 0.616)\) and OS \((primary: P = 0.591, \text{ALNM: } P = 0.063)\). Regarding the correlation between ALDH1 positivity and prognosis, various studies reported different views. Dong et al. reported that ALDH1 expression in both primary lesions and ALNM had an impact on both DFS and OS [15], whereas Yoshioka et al. reported that ALDH1 expression in primary lesions had an impact on prognosis only in patients aged 65 years or younger [16].

In this study, we evaluated the expression of Snail, Slug and Twist by IHC only in nuclear staining because these molecules are transcription factors that specifically bind to DNA. As stated in a previous study [18], staining of normal cells were occasionally observed; however, we excluded these cells while comparing them with the H&E-stained slides. In several ALNM, we found that the sub-capsular area showed stronger staining than did the central area, which may reflect the fact that cancer cells form a cancer nodule
after the arrival of a cancer cell at the sub-capsular area of lymph nodes. The expression of the three EMT markers was correlated with each other in primary lesions, but not in ALNM, suggesting that each transcription factor was correlated with each other at the stage at which metastasis caused. Some studies indicated that after their migration to metastatic sites, cancer cells may be stabilized to establish metastatic foci through the process of mesenchymal-epithelial transition (MET) [19, 20]. This mechanism may explain why the expression of the three EMT markers was correlated with each other in primary lesions but not in ALNM. All three markers showed significantly elevated expression in primary lesions with four or more lymph node metastases (Snail: $P = 0.002$, Slug: $P = 0.039$, Twist: $P = 0.050$); hence, our results support the previous contention that these factors contribute to lymph node metastases [6, 9, 14, 21]. There was no significance correlation between the expression of single EMT markers in both primary lesions and ALNM and patients’ outcome. The association between the expression of ALDH1 and EMT markers and prognosis in breast cancer remains controversial; nevertheless, several factors may explain our results. Here we set the cut-off value used to classify positivity and negativity at 5%, although the cut-off value for both ALDH1 and EMT markers that is appropriate for an impact on prognosis of breast cancer has never been reported.

Furthermore, the expression of EMT markers has been examined using varying cut-off
values, and the staining area evaluated has also varied among reports (including the nucleus and the cytoplasm).

We found no associations between the expression of ALDH1 and EMT markers, with the exception of that observed between ALDH1 and Twist expression in primary lesions ($P = 0.035$). Regarding patient prognosis, co-expression of ALDH1 and slug ($P = 0.009$) in primary lesions yielded a significantly short DFS. Our data indicate that the expression of ALDH1 or EMT markers alone does not have a strong impact on prognosis; however, the evaluation of the co-expression of these two factors may be more valuable. In other words, the existence of cancer stem cells alone or the function of transcription factors may not be sufficient as prognostic factors, but coexistence of them may induce a recurrence more easily. It has been suggested that the EMT process contributes not only to the acquisition of stemness properties but also to their maintenance [22-24]; therefore, stemness maintenance, which is regulated by the activity of transcription factors, may confer more aggressive properties to cancer stem cells. Even if cancer stem cells can be selectively targeted by effective drugs, these cells may be produced anew and acquire more aggressive behavior through the EMT process. Thus, it is important that new strategies consider the interference of the signal pathway that induces EMT, leading to the production of cancer stem cells, which represent a therapeutic target. The EMT
process has also been reported to contribute to the mechanism of drug resistance through
induction of ABC transporter overexpression [6, 23, 25]. The evaluation of the correlation
between drugs used, the expression of biomarkers and patient outcomes may lead to the
identification of a more specific therapeutic target and establishment of a new treatment
strategy.

The concordance rates of the expression of the three EMT markers between primary
lesions and ALNM were lower than that of ALDH1. This data indicate that the process of
EMT plays a more important role in the acquisition of heterogeneity in breast cancer; in
particular, the positive-to-negative conversion of EMT markers may also support the
hypothesis of MET, which is the process of the loss of mesenchymal properties and re-
acquisition of epithelial characteristics at the metastatic sites.

This study had some limitations. The limited number of samples may have hampered
the detection of slight but crucial significant differences. In addition, pure
immunohistochemical studies imply a poor definition of causality because the expression
of proteins may not reflect their exact function at the molecular level. Nevertheless, we
are convinced that this study helped identify a new prognostic factor because this is the
first study that evaluated ALDH1 and transcription factors together. In this study, these
little number of cases provided the result such as the statement above, therefore, we will
be able to make more confidence examination when we increase the number of samples more. We would like to be able to connect to the next study.

In conclusion, our results indicate that evaluation of the breast cancer stem cell marker ALDH1 and of transcription factors that promote EMT in primary lesions may have an impact on prognosis in breast cancer patients with ALNM. A more thorough investigation of a larger cohort with respect to breast cancer stem cells, the function of transcription factors and their prognostic impact is required.
Conflict of Interest

The authors declare that they have no conflict of interest.
References


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Figure Legends

Figure 1. Immunohistochemical detection of aldehyde dehydrogenase 1 (ALDH1) (A), Snail (B), Slug (C) and Twist (D) in formalin-fixed, paraffin-embedded breast cancer tissues. A, Cytoplasmic staining of ALDH1 at invasion section. B, Nuclear staining of Snail at invasion section. C, Nuclear staining of Slug at invasion section. D, Nuclear staining of Twist at intraductal component.

Figure 2. Kaplan-Meier curve for disease-free survival according to the expression of aldehyde dehydrogenase 1 (ALDH1) and Slug in primary lesions. A: ALDH1 and Slug positivity. B: Other than a.