Other randomized trials showed that ALND provided no clinical benefit in some breast cancer patients with positive sentinel lymph nodes. The American College of Surgeons Oncology Group (ACOSOG) Z0011 demonstrated that in patients with micrometastases or 1–2 macrometastases in sentinel lymph nodes who received SNB, breast-conserving surgery, breast irradiation and adjuvant therapy, the 5-year recurrence rate of axillary lymph nodes was 0.9% and not different from patients treated with ALND (3). Based on the results of the International Breast Cancer Study Group (IBCSG) 23-01, patients with micrometastases should be treated with SNB alone, since the regional recurrence rate at 5 years was 1% (4). The AMAROS trial suggested that regional node irradiation was effective for loco-regional control in sentinel node-positive breast cancer patients treated with SNB alone instead of SNB + ALND (5). In some institutes, patients who are eligible for ACOSOG Z0011 undergo no ALND in clinical practice (6). However, each one of these trials had some limitations. Patients registered in ACOSOG Z0011 and AMAROS tended to have small breast tumors and a small tumor burden in sentinel lymph nodes. Such patients might have no additional metastases in non-sentinel lymph nodes. How to incorporate these results into clinical practice is still unclear (7). In IBCSG 23-01, the histological diagnosis of micrometastases was strictly performed using lymph node specimens cut at 50–200 μm. This method is far from actual clinical practice. Indeed, a SNB registry trial in the Netherlands found that the 5-year regional recurrence rate after SNB alone in patients with micrometastases in sentinel lymph nodes was 5.6% (8).

On the other hand, personalized medicine based on the intrinsic subtype of breast cancer can eliminate breast tumor and nodal metastases. In ~30% of cases of triple-negative breast cancer and HER2-enriched breast cancer, neoadjuvant chemotherapy could achieve a complete pathological response of breast tumor and nodal metastases (9). These results again raised the question of whether ALND is always needed for node-positive breast cancer patients after neoadjuvant chemotherapy. The Japanese Society for Sentinel Node Navigation Surgery was founded in 1996 and a prospective study on SNB in breast cancer was reported (10). To evaluate the clinical benefit of SNB without ALND in sentinel node-positive breast cancer, we planned a cohort study to register patients with positive sentinel nodes prospectively. This study was approved by the institutional review board at Kyorin University in September 2013 and registered at the UMIN Clinical Trials Registry as UMIN000011782 (http://www.umin.ac.jp/ctr/index.htm).

PROTOCOL DIGEST OF THE STUDY

OBJECTIVES

The purpose of this study is to evaluate the prognosis of sentinel node-positive breast cancer patients treated with SNB alone. The secondary purpose is to compare the prognosis of

the patients treated with SNB alone to those treated with SNB followed by ALND. To reduce the bias associated with the lack of randomization, we use the propensity score matching method to adjust unbalanced clinicopathological factors in the both groups.

STUDY SETTING

A multi-institutional prospective cohort study.

ENDPOINTS

The primary endpoint is the recurrence rate of regional lymph nodes for patients treated with SNB after primary treatment of breast cancer. The secondary endpoint is the 5-year overall survival. Primary treatment is defined as breast surgery including SNB with or without ALND, neoadjuvant therapy or SNB to diagnose lymph node metastases prior to neoadjuvant therapy. The time to regional lymph node recurrence is counted from the date of primary treatment. It is censored at the earliest day of either local recurrence, contralateral breast cancer, distant metastases, other malignant disease or death from any cause. Overall survival is defined as the duration from primary treatment to death from any cause. It is censored at the last day when the patient is alive.

ELIGIBILITY CRITERIA

INCLUSION CRITERIA

(i) Female patients aged 20–70 years.
(ii) T1-3N0-1M0 in the eighth edition of the UICC TNM classification.
(iii) Histological confirmation of invasive disease in the breast.
(iv) SNB was performed or scheduled after 1 January 2012.
(v) SNB or SNB followed by ALND should be performed. SNB and the sampling of Level I lymph nodes is acceptable and considered SNB.
(vi) One to three positive sentinel lymph nodes with micrometastases and/or macrometastases confirmed by histological or molecular diagnosis.

EXCLUSION CRITERIA

(i) Ductal carcinoma *in situ* or lobular carcinoma *in situ* in the breast.
(ii) Synchronous or metachronous bilateral breast cancer.
(iii) Four or more sentinel lymph nodes with micrometastases and/or macrometastases except for isolated tumor cells
(iv) Past history of invasive disease within 5 years before registration.
(v) Physician's discretion due to the patient's condition (e.g. severe co-morbidity, psychiatric disorder, pregnancy, refusal to undergo appropriate surgery for breast cancer).

(vi) Failure of SNB, or histologically false-negative sentinel lymph nodes.

TREATMENT METHODS

Breast cancer treatment consists of breast surgery, adjuvant therapy and radiation therapy. Breast surgery includes SNB, ALND or both, and partial or total mastectomy with or without breast reconstruction. Adjuvant therapy includes chemotherapy, endocrine therapy and anti-HER2 therapy before and after breast surgery. Radiation therapy covers fields that include the breast, chest wall or regional lymph nodes. In this study, physicians will follow clinical practice for breast cancer patients according to imaging diagnosis and the intrinsic subtype of breast cancer as confirmed by core-needle biopsy or resected specimens. There is no surgical protocol with regard to SNB, ALND, type of mastectomy or breast reconstruction. Adjuvant therapy and radiation therapy also depend on the physician's discretion.

A histological diagnosis of sentinel lymph nodes is performed following the institution protocol, but it is recommended that physicians use lymph node specimens sliced at 2 mm intervals and stained with hematoxylin—eosin. Molecular diagnosis by the one-step nuclear amplification (OSNA) method is used worldwide instead of the histological examination of sentinel lymph nodes, and is allowed in this study (11).

OBSERVATION

The participants will be followed-up every 6 months until 5 years after primary treatment. Routine examination is recommended following the American Society of Clinical Oncology Clinical Practice Guidelines. If recurrence is suspected, an appropriate imaging diagnosis and histological confirmation should be performed.

STUDY DESIGN

Our objectives are to estimate regional lymph node recurrence of the patients treated with SNB and to compare them to patients treated with SNB followed by ALND. Although an observational study cannot provide the same definitive evidence as a randomized trial, some statistical methods should be able to reduce the bias associated with the lack of randomization. In this study, we use the propensity score matching method to compare SNB to SNB followed by ALND in sentinel node-positive patients.

UTILITY OF THE PROPENSITY SCORE MATCHING METHOD

In an observational study, treatment selection could be influenced by the patient's characteristics. Therefore, the distributions of risk factors such as age, stage, and severity differ between the treatment groups. To compare the outcome between groups without the effect of bias due to treatment selection, we use a propensity score matching method in this study. The propensity score is defined as a patient's probability of receiving a specific treatment conditional on the observed risk factors (12). In this study, an individual probability of being treated with SNB alone is the propensity score. It is estimated for each patient using the logistic model based on the observed risk factors. Risk factors included in the logistic model are selected from the observed baseline data after the close of enrollment. We plan to implement 1:1 or 1:2 greedy matching (13) with the propensity score.

STATISTICAL ANALYSIS

Based on an estimated regional lymph node recurrence rate of 5% at 5 years among patients treated with SNB, 240 patients are needed to give 80% power to reject the null hypothesis that the recurrence rate is 10% with a one-sided type I error rate of 2.5%. If we consider that some patients will be lost to follow-up or become ineligible, a total of 250 patients treated with SNB only will be needed to comprise the sample. At the same time, as many eligible patients as possible who are treated with SNB followed by ALND are also enrolled to constitute a control pool for comparison of regional lymph node recurrence.

Regional lymph node recurrence is estimated by considering all eligible patients with SNB only by the Kaplan—Meier method and the 95% confidence interval (CI) is computed using Greenwood's formula (14). The hazard ratio and its 95% CI of regional lymph node recurrence is estimated by the Cox regression model and a robust sandwich estimate of variance with matched samples. Basically, multivariate adjustment with other prognostic variables is not planned for comparison the patients treated with SNB only and SNB followed by ALND.

PARTICIPATING INSTITUTIONS

Asahikawa Medical University, Keio University, Kyorin University, Kyoto Prefectural University of Medicine, National Cancer Center Hospital East, Osaka National Hospital, Tonan Hospital, University of Kurume Faculty of Medicine.

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Conflict of interest statement

Toh has received consulting fees from Chugai Pharma and AstraZeneca plc. Other authors have no conflict of interest to declare.

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APPENDIX

List the other authors: Takashi Aikou, Seiunkai Hospital, Masaki Kitajima, International University of Health and Welfare.

CLINICAL TRIAL

Different prognostic significance of Ki-67 change between pre- and post-neoadjuvant chemotherapy in various subtypes of breast cancer

Nobuaki Matsubara · Hirofumi Mukai ·
Satoshi Fujii · Noriaki Wada

Abstract In a neoadjuvant setting, three parameters for Ki-67 could be obtained: pre-treatment Ki-67, post-treatment Ki-67 and Ki-67 change between pre- and post-treatments. It is uncertain which of the three parameters has the greatest prognostic significance, and whether this parameter has significance in each subtype of breast cancer. A total of 385 patients who received neoadjuvant anthracycline followed by taxane chemotherapy and subsequent surgery for breast cancer were analyzed retrospectively. By immunohistochemistry (IHC), patients were divided into four subtypes (Luminal A, Luminal B, Triple negative, and HER2). Ki-67 was examined by IHC in pre-treatment core needle samples and post-treatment surgical excision specimens. The relapse-free survival (RFS) rate was compared among each subtype. The median follow-up period was 56 months. The rate of pathological complete response was higher for HER2 (34.8 %) and Triple negative (24.3 %) subtypes than for Luminal B (8.3 %) and Luminal A (3.8 %) subtypes ($p < 0.0001$). A reduction in Ki-67 was observed in 58.5, 83.4, 70.2, and 74.2 % of patients in the Luminal A, Luminal B, Triple negative, and HER2 subtypes, respectively. Ki-67 change between pre- and post-treatments was an independent prognostic factor, but pre-treatment Ki-67 and post-treatment Ki-67 were not independent prognostic factors in a multivariate analysis. The RFS was significantly different between patients whose Ki-67 was reduced and those not reduced for Luminal B (81.4 vs. 50.0 %, $p = 0.006$), Triple negative (74.8 vs. 43.5 %, $p = 0.006$) and HER2 (82.7 vs. 59.0 %, $p = 0.009$). However, for Luminal A, the difference in RFS was not associated with changes of Ki-67 (78.8 vs. 75.3 %, $p = 0.193$). Ki-67 change between pre- and post-neoadjuvant chemotherapy is an independent prognostic factor in patients of Luminal B, Triple negative, and HER2 subtypes. Pre-treatment Ki-67 and post-treatment Ki-67 were not independent prognostic factors in a multivariate analysis.

Keywords Breast cancer · Neoadjuvant chemotherapy · Ki-67 · Prognostic factors · Intrinsic subtype

Introduction

Neoadjuvant chemotherapy is now well established as a standard treatment option in patients with locally advanced and operable breast cancer [1]. The purpose of neoadjuvant chemotherapy in patients with breast cancer is to not only achieve tumor shrinkage to facilitate the subsequent surgical procedure but also improve clinical outcome by eradicating micrometastases [2–4]. Also, biological and pathological analyses of surgical specimens after neoadjuvant chemotherapy can provide information on predictive and prognostic markers [5–7]. Further progressive research might help to introduce more accurate individualized treatment.

Ki-67 is a nuclear protein with nuclear function that is expressed in all phases of the cell cycle except G0, and it is one of the major markers of tumor proliferation, as assessed by IHC and the Ki-67 antibody, MIB-1 [8, 9].

N. Matsubara (✉) · H. Mukai
Department of Oncology and Hematology, National Cancer
Center Hospital East, 6-5-1 Kashiwanoha,
Kashiwa city, Chiba 277-8577, Japan
e-mail: nmatsuba@east.ncc.go.jp

S. Fujii
Pathology Division, National Cancer Center for Innovative
Oncology at Kashiwa, Chiba, Japan

N. Wada
Division of Breast Surgery, National Cancer Center
Hospital East, Chiba, Japan

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In addition, Ki-67 has been reported to be an independent predictive and prognostic marker in patients with operable breast cancer [10, 11]. Recently, a meta-analysis concluded that Ki-67 overexpression was associated with worse survival rates both in patients with both lymph node metastasis-positive and in those with metastasis-negative breast cancer [12]. Assessment of Ki-67 is now introduced into daily practice, and used to divide the cancer into subtypes or as a decision-making tool for adjuvant treatment [13].

However, in a neoadjuvant setting, especially neoadjuvant chemotherapy, the role of Ki-67 seems to be somewhat confusing. Physicians can obtain three parameters of Ki-67: pre-treatment Ki-67, post-treatment Ki-67, and Ki-67 change between pre- and post-treatments. It has been reported that a higher pre-treatment value of Ki-67 is associated with a better response to neoadjuvant treatment, and a significant reduction in Ki-67 was observed [14, 15]. On the other hand, previous studies have also reported that Ki-67 assessment performed on samples already exposed to treatment more accurately predicts clinical outcome than does the assessment in pre-treatment biopsy samples [16, 17]. However, this rationale is well established in neoadjuvant hormonal therapy, but not in neoadjuvant chemotherapy. In addition, it is still uncertain which of those three parameters of Ki-67 have the greatest prognostic significance in neoadjuvant chemotherapy settings. To the best of our knowledge, this question has not been investigated until now.

We report here the results of the sequential assessment of Ki-67 in patients who received neoadjuvant anthracycline followed by taxane chemotherapy. Ki-67 was measured both in a pre-treatment biopsy sample and in surgical excision specimens, and then the relationship between three parameters of Ki-67 and clinical outcome was assessed. The primary objective of this investigation is to determine which of the three parameters of Ki-67 have the greatest prognostic significance in a neoadjuvant chemotherapy setting. The second objective of this investigation was to determine whether the most significant parameter of Ki-67 has a prognostic impact in each of the four subtypes characterized by estrogen receptor status (ER), progesterone receptor (PgR), and HER2 status. We hypothesize that the prognostic significance of Ki-67 might not be equivalent in all types of breast cancer but might be depend on subtypes.

Patients and methods

Patients

We retrospectively reviewed the clinical and pathological records of patients who received neoadjuvant anthracycline followed by taxane chemotherapy and subsequent curative

surgery at National Cancer Center Hospital East (Kashiwa, Japan) between January 2000 and December 2010. For inclusion in this study, the patients had to be in clinical stage IIA to stage IIIC with histological confirmation, according to the American Joint Committee on Cancer staging (7th edition). All chemotherapy regimens were allowed for this analysis, if the chemotherapy was administered as sequential anthracycline and taxane regimens. Basically, neoadjuvant chemotherapy was administered as four cycles each of anthracycline and taxane-based regimens.

Information on the following parameters had to be available for all patients from the pre-treatment assessment: age, clinical tumor size, clinical axilla nodes status, ER status, PgR status, HER2 status and pre-treatment Ki-67, and also parameters from the post-treatment assessment: chemotherapy agent, axilla nodes status, and post-treatment Ki-67. Patients whose clinical or pathological parameters were not available were excluded from this analysis. Patients who received neoadjuvant hormonal therapy or a combination of chemo-hormonal therapy and who had not undergone surgery were also excluded from this study. Finally, a total of 385 patients were eligible and analyzed for this study. However, only 23 patients (5.9 %) were enrolled from 2000 to 2002, and almost all the patients included in this study were treated between 2003 and 2010. Since most of the promising phase 3 trials with taxane were published after 2000, several standard neoadjuvant chemotherapy regimens existed between 2000 and 2002.

The indication for and type of post-surgical treatment (adjuvant treatment) was based on the St. Gallen Consensus Recommendation at that time. In brief, none of the patients received additional chemotherapy (adjuvant chemotherapy). Patients whose breast cancer was found to be ER and/or PgR positive by immunohistochemistry underwent adjuvant hormonal therapy for at least 5 years. After 2005, if HER2 positivity was confirmed by IHC or fluorescence in situ hybridization, trastuzumab was administered as neoadjuvant or adjuvant treatment for a total of 1 year. In contrast, from 2000 to 2005, none of the patients diagnosed with HER2-positive disease received trastuzumab as neoadjuvant or adjuvant treatment, because trastuzumab was not approved in Japan as adjuvant treatment until 2008 and as neoadjuvant treatment until 2011. All patients who underwent breast-conserving surgery had routinely received adjuvant radiotherapy. In cases of mastectomy, adjuvant radiotherapy was administered only at the discretion of the oncologist.

Immunohistochemistry

Immunohistochemistry (IHC) was routinely performed in our institution using formalin-fixed, paraffin-embedded tissue blocks with both pre-treatment core needle biopsy

samples and post-treatment surgical excision specimens. Immunohistochemical staining of tumors for ER (confirm anti-ER (SP1), rabbit monoclonal antibody, Ventana Medical Systems), PgR (confirm anti-PGR (1E2), rabbit monoclonal antibody, Ventana Medical Systems), and HER2 (pathway anti-HER2 (4B5), rabbit monoclonal antibody, Ventana Medical Systems) were performed using the automated Benchmark XT platform (Ventana Medical Systems) and according to the manufacturer's recommendations. For Ki-67 (Clone MIB1, Dako, Glostrup, Denmark; dilution 1:50), tumors were stained in accordance with the manufacturer's recommendation. All tumor samples and specimens were evaluated by two experienced pathologists belonging to our institution. A cutoff value of ≥ 1 % of positively stained nuclei was used as the definition of ER and PgR-positive disease. HER2 protein positivity was defined as a score of 3 by IHC or as positive by FISH. The methods and procedures of IHC were unchanged through the study period.

Ki-67 expression was quantified using a visual grading system. Cells stained for Ki-67 were counted and expressed as a percentage. If the staining was homogenous, the percentage of Ki-67 positive cells among the total number of carcinoma cells counted was determined at a magnification of 400× using an eye-piece graticule and counting 10 randomly selected fields. When hot spots, defined as areas in which Ki-67 staining was particularly prevalent, were present, pathologists assessed the whole section and recorded the overall average score. Each Immunohistochemical stainings included an external control to validate the Ki-67 protein expression status of each case. Therefore, the same section was used for the external control.

The cutoff level of Ki-67 was defined as ≥ 10 %, because the 10 % Ki-67 cutoff level provided a significant prognostic forecast in our institution in a previous investigation [18]. The categories of Ki-67 change were defined as follows; a reduction group was defined if post-treatment Ki-67 decreased by 1 % or less compared to pre-treatment Ki-67 and a no-reduction group was defined if post-treatment Ki-67 increased by 1 % or more compared to pre-treatment Ki-67 or if the Ki-67 index was unchanged between pre- and post-treatments. The representative figure samples of Ki-67 change with Ki-67 reduction and no-reduction are shown in Figs. 1 and 2.

The subtypes were defined by IHC of core needle biopsy samples as follows according to St. Gallen Consensus 2011 [13]. Luminal A was defined as negative HER2 status, ER positive, and/or PgR positive with Ki-67 ≤ 14 %. Luminal B was defined as negative HER2 status, ER positive, and/or PgR positive with Ki-67 >14 %. The triple negative
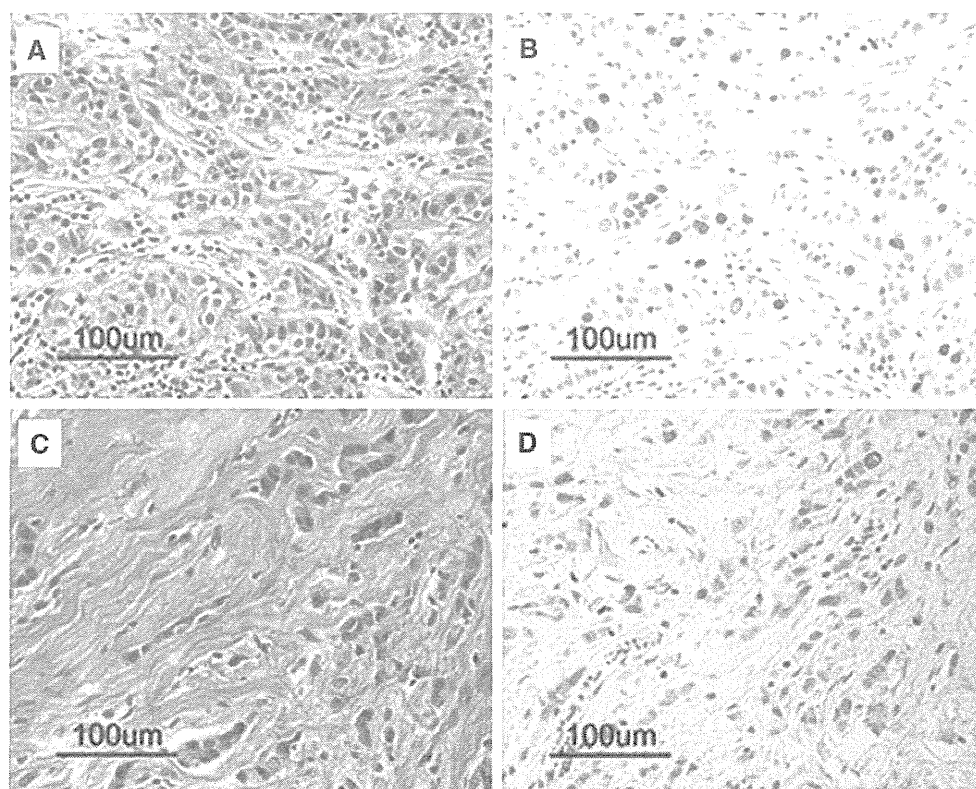

Fig. 1 Representative figure of Ki-67 staining with reduction group. a HE of pre-treatment core needle biopsy sample. b The Ki-67 score of pre-treatment core needle biopsy would be ∼30 %. c HE of post-treatment surgical excision specimens. d The Ki-67 score of post-treatment surgical excision specimens would be ∼4 %
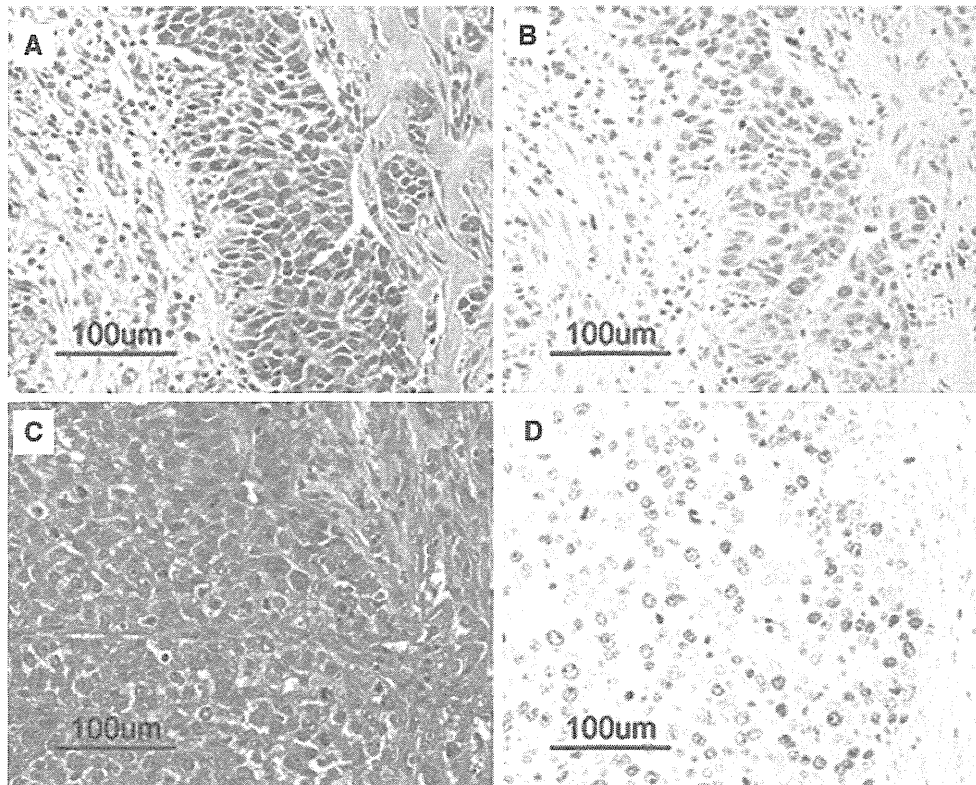
Fig. 2 Representative figure of Ki-67 staning with no-reduction group. **a** HE of pre-treatment core needle biopsy sample. **b** The Ki-67 score of pre-treatment core needle biopsy would be ∼20 %. **c** HE of post-treatment surgical excision specimens. **d** The Ki-67 score of post-treatment surgical excision specimens would be ∼60 %

subtype was defined as negative HER2 status, ER negative, and PgR negative. The HER2 subtype was defined as positive HER2 status regardless of ER and PgR status.

A pathological complete response (pCR) was defined by the absence of invasive carcinoma in the primary breast tumor irrespective of pathological axillary node status. And then, only the presence of residual ductal carcinoma in situ was included in the pCR category. Post-treatment Ki-67 with pCR was counted as 0 %.

Statistical methods

The definition of relapse excluded local breast relapse, axillary lymph node relapse and newly diagnosed contralateral breast cancer. The relapse-free survival (RFS) period was defined as the interval from the date of surgery to that of the first diagnosis of relapse or the last follow-up date without relapse.

Associations between prognostic factors and RFS were analyzed using Chi-squared test or Fisher's exact test, where appropriate. The Cox proportional hazards model was used for estimation of multivariate analysis. Only prognostic factors that were identified as showing statistical significance in a univariate analysis were included in the Cox proportional hazards regression model.

Survival distributions were estimated using the Kaplan–Meier method for RFS, and the Log-rank test was used to compare survival in different strata. All statistical tests were two-sided and had a 95 % confidence interval (CI), with the level of significance established at $p < 0.05$. Statistical analyses were performed using PASW (Predictive Analysis Software) 18.0 for Windows (SPSS, IBM, Chicago, Ill., USA).

Results

Patients and clinical outcomes

Table 1 shows the baseline characteristics of all 385 patients. The median and mean numbers of chemotherapy cycles were 8.0 and 7.06 (range 2–8), respectively. The patients were subdivided into four subtypes by IHC pattern. A total of 106 patients (27.5 %) were classified as Luminal A, 60 (15.5 %) were Luminal B, 107 (27.8 %) were Triple negative, and 112 (29.2 %) were HER2. The details of the distributions of baseline clinical and pathological characteristics among the subtypes are also listed in Table 1. There were significant differences in histological grade (HG) and pre-treatment Ki-67 among the subtype cohorts.

Table 1 Baseline characteristics of 385 patients

Characteristics	Total (%)	Luminal A (%)	Luminal B (%)	Triple negative (%)	HER2 (%)	p
No. of patients (%)	385 (100)	106 (27.5)	60 (15.5)	107 (27.8)	112 (29.2)	
Median age (range)	53 (25–71)	51 (25–68)	52 (33–70)	52 (28–68)	53 (32–71)	0.69
Menstrual status						0.74
Premenopausal	171 (44.4)	49 (46.2)	28 (46.6)	51 (47.7)	43 (38.4)	
Postmenopausal	214 (55.6)	57 (53.8)	32 (53.4)	56 (52.3)	69 (61.6)	
Tumor status						0.16
cT1	13 (3.4)	2 (1.9)	5 (8.3)	4 (3.7)	2 (1.8)	
cT2	220 (57.1)	67 (63.2)	30 (50.0)	63 (58.9)	60 (53.6)	
cT3	79 (20.5)	14 (13.2)	15 (25.0)	20 (18.7)	30 (26.8)	
cT4	73 (19.0)	23 (21.7)	19 (16.7)	20 (18.7)	20 (17.9)	
Nodal status						0.084
cN positive	260 (67.5)	61 (57.5)	44 (73.3)	76 (71.0)	79 (70.5)	
cN negative	124 (32.2)	45 (42.5)	16 (26.7)	31 (29.0)	33 (29.5)	
Histological grade						<0.001
1	13 (3.4)	12 (11.3)	0 (0)	1 (0.9)	0 (0)	
2	166 (43.1)	63 (59.4)	38 (63.3)	23 (21.5)	42 (37.5)	
3	130 (33.8)	12 (11.3)	12 (20.0)	57 (53.3)	49 (43.8)	
Missing	76 (19.7)	19 (17.9)	10 (16.7)	26 (24.3)	21 (18.8)	
Median Ki-67 pre-chemo (range)	20.0 (1–80)	9.0 (1–14)	25.0 (15–50)	40.0 (2–80)	20.0 (4–70)	<0.001
Neoadjuvant chemotherapy regimen						0.18
Anthra → PTX	282 (73.2)	77 (72.7)	42 (70.0)	79 (73.8)	84 (75.0)	
Anthra → DTX	103 (26.8)	29 (27.3)	18 (30.0)	28 (26.2)	28 (25.0)	

Follow-up ranged from 9 to 135 months, with a median follow-up of 56 months. During follow-up periods, disease relapse was observed in 105 patients (27.3 %). The RFS at median follow-up of Luminal A, Luminal B, Triple negative, and HER2 subtypes were 77.3, 76.2, 65.4, and 76.5 %, respectively, and the difference in RFS between subtypes was statistically significant (figure not shown; $p = 0.023$).

Response to neoadjuvant chemotherapy in subtypes

The pCR was observed in 74 patients (19.2 %). The rate of pCR was higher for HER2 (34.8 %) and Triple negative (24.3 %) subtypes than for Luminal B (8.3 %) and Luminal A (3.8 %) subtypes ($p < 0.0001$). The reduction in Ki-67 in post-treatment surgical excision specimens compared with pre-treatment biopsy samples was observed in 58.5, 83.4, 70.2, and 74.2 % of patients in the Luminal A, Luminal B, Triple negative, and HER2 subtypes, respectively. The proportion of patients with Ki-67 reduction was significantly different among the four subtypes ($p = 0.003$). The median reduction absolute values of Ki-67 were 5.0, 21.0, 30.0, and 15.5 % in the Luminal A, Luminal B, Triple negative, and

HER2 subtypes, respectively. More details on outcome after neoadjuvant chemotherapy are shown in Table 2.

Univariate and multivariate analyses

Various prognostic factors including Ki-67 change, that were commonly validated, were tested for RFS association by univariate analysis. Age, initial tumor size, initial nodal status, ER status, pre-treatment Ki-67, pathological nodal status, post-treatment Ki-67 and Ki-67 change were significant prognostic factors. However, HER2 status and type of taxane were not associated with RFS. More detailed results of the univariate analysis are shown in Table 3.

Prognostic factors that were significantly related to RFS by univariate analysis were then analyzed by multivariate analysis as shown in Table 3. Initial tumor size (Hazard Ratio (HR) 2.37; 95 % CI 1.57–3.45), ER status (HR 2.36; 95 % CI 1.48–3.73), pathological node status (HR 5.84; 95 %CI 3.37–10.12) and Ki-67 change (HR 1.96; 95 % CI 1.17–3.20) were independent unfavorable prognostic factors for RFS. On the other hands, pre-treatment Ki-67 and post-treatment Ki-67 were not independent prognostic factors.

Table 2 Treatment outcomes of neoadjuvant chemotherapy

Characteristics	Total (%)	Luminal A (%)	Luminal B (%)	Triple negative (%)	HER2 (%)	p
pCR	74 (19.2)	4 (3.8)	5 (8.3)	26 (24.3)	39 (34.8)	
Median Ki-67, post-chemo (range)	4.0 (0–80)	4.0 (0–50)	4.0 (0–60)	10.0 (0–80)	4.5 (0–70)	
Ki-67 change						
Decreasing	269 (69.8)	61 (57.5)	50 (83.4)	75 (70.2)	83 (74.1)	
Increasing	70 (18.2)	19 (18.0)	7 (11.7)	22 (20.5)	22 (19.6)	
No change	46 (12.0)	26 (24.5)	3 (5)	10 (9.3)	7 (6.3)	

Table 3 Univariate and multivariate analyses for factors related with RFS

Variables	Univariate			Multivariate		
	HR	95 %CI	P value	HR	95 %CI	P value
Age						
≥35 years	1			1		
<35 years	2.82	1.09–7.32	0.027	1.42	0.70–2.88	0.329
cT status						
≤T2	1			1		
>T2	3.04	1.92–4.83	<0.001	2.37	1.57–3.45	<0.001
cN status						
Negative	1			1		
Positive	2.98	1.70–5.24	<0.001	1.28	0.74–2.27	0.378
Pre-therapy ER						
Positive	1			1		
Negative	1.79	1.14–2.83	0.011	2.36	1.48–3.73	<0.001
Pre-therapy HER2						
Positive	1					
Negative	1.04	0.63–1.70	0.89	NS		
Ki-67 pre-chemo						
<10 %	1			1		
≥10 %	2.11	1.19–3.72	0.009	1.92	0.99–3.36	0.052
Taxane						
Paclitaxel	1					
Docetaxel	0.91	0.53–1.58	0.784	NS		
pN status						
Negative	1			1		
Positive	6.67	3.87–11.48	<0.001	5.84	3.37–10.12	<0.001
Ki-67 post-chemo						
<10 %	1			1		
≥10 %	3.94	2.46–6.31	<0.001	1.26	0.70–2.17	0.470
Ki-67 change						
Reduction	1			1		
No-reduction	2.67	1.68–4.23	<0.001	1.96	1.17–3.20	0.010

The prognostic association of Ki-67 change in each subtype

Figure 3 shows the RFS curves according to Ki-67 change (Ki-67 reduction or Ki-67 no-reduction) in all patients. The RFS rate is 91.2 % with Ki-67 reduction, and 59.4 % with Ki-67 no-reduction. The difference in RFS between two groups was statistically significant (Log-rank, $p < 0.0001$).

We divided patients into four subtypes in accordance with IHC patterns (Luminal A, Luminal B, Triple negative, and HER2). In addition, they were re-classified according to Ki-67 change, and RFS was calculated. In the Luminal A
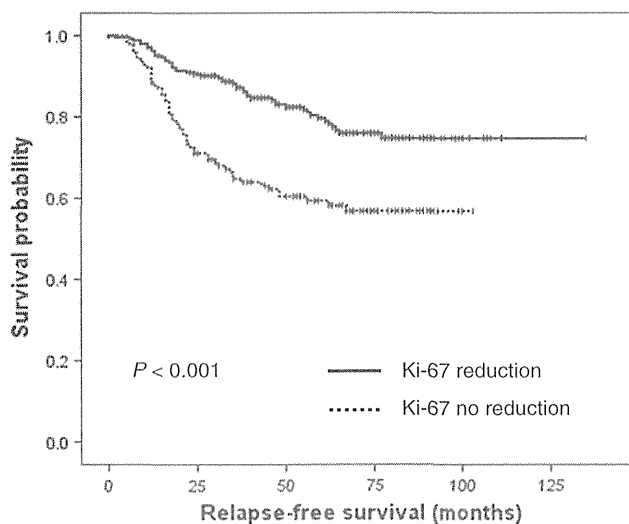
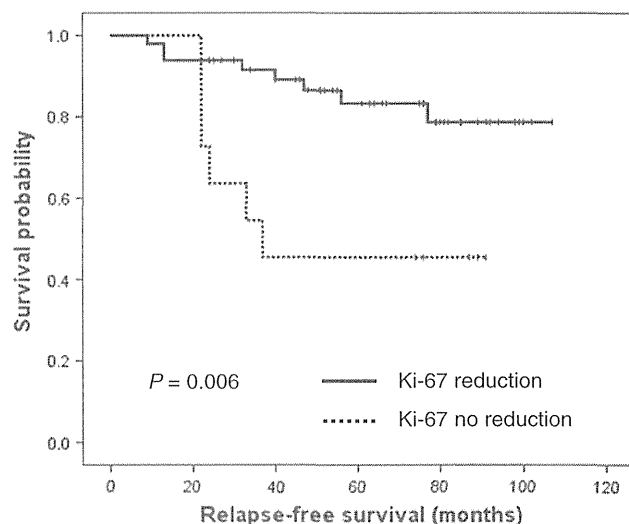Fig. 3 Kaplan-Meir curves of relapse-free survival by Ki-67 change in all patients

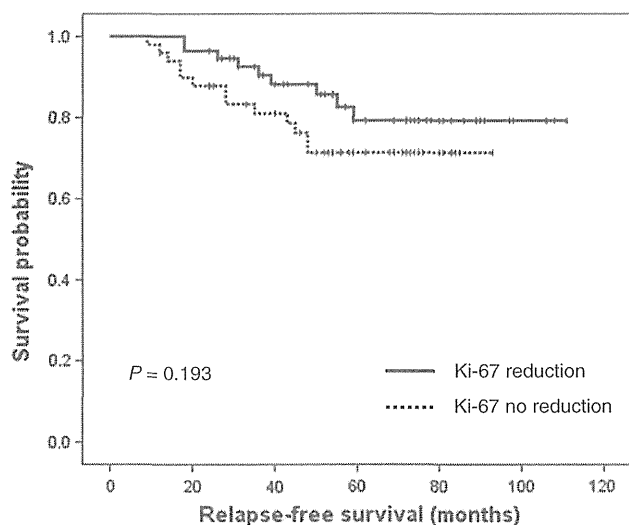Fig. 5 Kaplan-Meir curves of relapse-free survival divided from Ki-67 change in patients with Luminal B

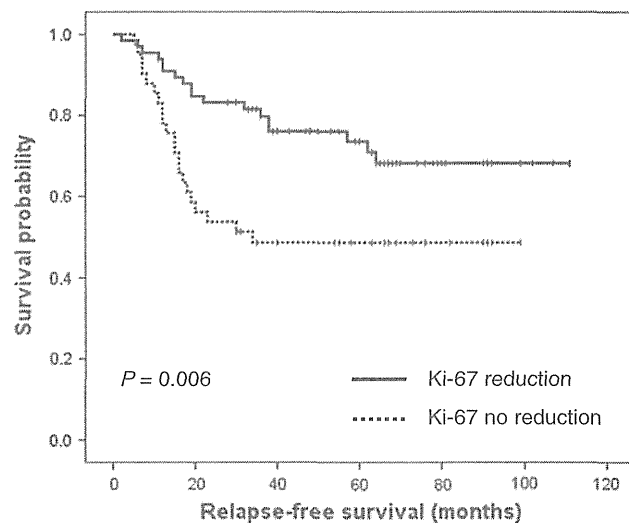Fig. 4 Kaplan-Meir curves of relapse-free survival divided from Ki-67 change in patients with Luminal A

Fig. 6 Kaplan-Meir curves of relapse-free survival divided from Ki-67 change in patients with Triple negative

subtype, no statistically significant RFS difference was observed between Ki-67 reduction and Ki-67 no-reduction in RFS (82.5 vs. 71.3 %, $p = 0.193$, Fig. 4). In contrast, the differences in RFS between Ki-67 reduction and Ki-67 no-reduction patients were statistically significant for Luminal B (83.3 % vs. 45.5 %, $p = 0.006$, Fig. 5), Triple negative (76.0 vs. 48.5 %, $p = 0.006$, Fig. 6), and HER2 subtype (83.0 vs. 60.9 %, $p = 0.009$, Fig. 7) subtypes.

Discussion

Many previous studies have investigated a potential relationship between Ki-67 and survival outcome in early stage breast cancer [10, 11, 19]. However, the Ki-67 assessment seems to provide conflicting results in neoadjuvant chemotherapy settings, mainly because after surgery, three parameters of Ki-67 can be obtained, such as pre-treatment Ki-67 in core needle biopsy samples, post-treatment Ki-67 in surgical specimens and Ki-67 changes between pre- and post-treatment comparisons. It has been uncertain which of the Ki-67 parameters has the greatest prognostic significance. Recent progress might reveal biomarkers that depend on cancer cell biology, such as intrinsic subtypes in breast cancer. We theorized that the prognostic significance of Ki-67 parameters might not be equivalent but depend on subtypes, so therefore we investigated the Ki-67 prognostic
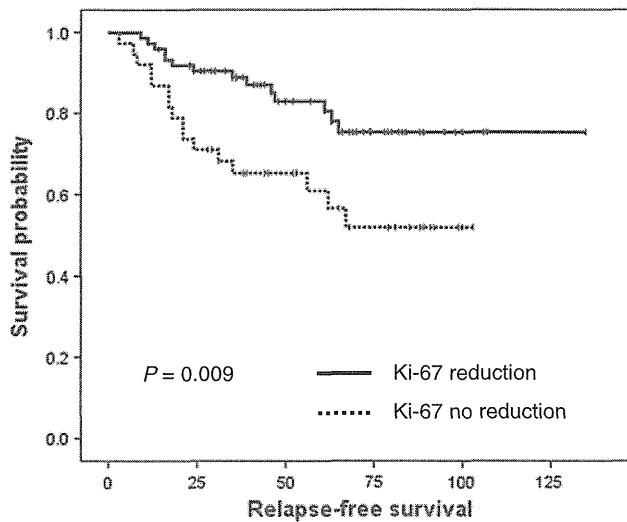
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Fig. 7 Kaplan-Meir curves of relapse-free survival divided from Ki-67 change in patients with HER2

association with the subtypes defined by ER, PgR, and HER2 status.

In the present investigation, the three Ki-67 parameters, such as pre-treatment Ki-67, post-treatment Ki-67 and Ki-67 change were significant prognostic factors in univariate analysis. However, in a multivariate analysis, Ki-67 change was the only independent prognostic factor. This result could be interpreted as showing that Ki-67 change is the most important predictor for clinical outcome rather than both absolute values of pre- and post-treatment Ki-67. However, some previous studies reported different results from ours. Lee et al. [20] reported that post-treatment Ki-67 was the only significant independent factor associated with overall survival. In contrast, other studies have not found an independent association between post-treatment Ki-67 and survival [21–23]. In addition, other studies have found the same results as ours that Ki-67 change was a significant independent predictor for disease-free survival (DFS) and RFS [24, 25]. There are some plausible reasons for the discordant results, such as patient population, chemotherapy regimens, and heterogeneous patient subtypes. Thus, the patient proportions of subtypes defined by ER, PgR, and HER2 status might have influenced the results. For example, 43.6 and 52.4 % of patients had ER positive status in the previous study and in our study, respectively, in which post-treatment Ki-67 was found to be an independent prognostic factor [20]. Further investigations assessing the prognostic significance of Ki-67 in each subtype might prove this hypothesis.

Another finding in the current investigation revealed that the reduction in Ki-67 as a favorable surrogate marker for RFS could be applied to Luminal B, Triple negative, and HER2 subtypes, but not to the Luminal A subtype.

To the best of our knowledge, this is the first analysis of the association between prognosis and Ki-67 change in each subtype. This finding also means that the significance of Ki-67 change differs among subtypes, and this difference depends on the breast cancer cell biology. Similar findings have been reported in a recently published pooled analysis that investigated the association between pCR and survival in each intrinsic subtype [26]. This pooled analysis, in which 6,377 patients with breast cancer received neoadjuvant anthracycline-taxane-based chemotherapy, demonstrated that pCR is a suitable surrogate marker for patients with Luminal B/HER2-negative, Triple negative, and HER2 subtypes but not for those with Luminal B/HER2-positive or Luminal A subtype. Both our results and this pooled analysis suggest that in low proliferating breast cancer, such as Luminal A, pathological outcomes after neoadjuvant chemotherapy, Ki-67 changes or pCR, are not surrogate markers for prognosis. In contrast, in high proliferating breast cancer, such as Luminal B, Triple negative, and HER2, those parameters could discriminate accurately between patients with good and poor prognosis.

In addition, in a neoadjuvant hormonal therapy setting, opposite results from ours were reported. A previous study on ER-positive breast cancer demonstrated that higher Ki-67 associated with worse RFS following just two weeks of neoadjuvant hormonal therapy compared to the baseline pre-treatment Ki-67 [17]. However, in our investigation, Ki-67 change was not independent prognostic factor for RFS in Luminal A subtype patients treated with neoadjuvant chemotherapy. From those results, the role of Ki-67 change might depend on not only subtype but also treatment procedure (chemotherapy or hormonal therapy).

Gene profiling assays, such as MammaPrint or Oncotype DX, also seem to be useful tools for predicting disease recurrence, and have already been introduced commercially in predictive tests. However these assays are unlikely to become widely used in daily practice at the present time due to issues of cost and insurance coverage. Moreover, to the best of our knowledge, few investigations have confirmed the utility of these gene profiling assays in neoadjuvant settings.

The strengths of our analysis are large sample size, long median follow-up period and treatment of all patients with anthracycline followed by taxane chemotherapy in a single institution. On the other hand, some study limitations are present, such as retrospective analysis, the lack of central pathological review of Ki-67 measurements, and not all HER2-positive patients received trastuzumab as neoadjuvant and/or adjuvant treatment. This treatment difference with or without trastuzumab might influence Ki-67 changes and PFS.

In conclusion, our investigation revealed that in Luminal B, Triple negative, and HER2 subtypes, a Ki-67 change

between pre- and post-treatments is an independent prognostic factor in patients receiving neoadjuvant anthracycline followed by taxane chemotherapy. Pre-treatment Ki-67 and post-treatment Ki-67 were not independent prognostic factors in this patient population.

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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

Tomohiro Nogami · Tadahiko Shien · Takehiro Tanaka · Keiko Nishiyama ·
Taeko Mizoo · Takayuki Iwamto · Hirokuni Ikeda · Naruto Taira ·
Hiroyoshi Doihara · Shinichiro Miyoshi

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

T. Nogami (✉) · T. Shien · K. Nishiyama · T. Mizoo ·
H. Ikeda · N. Taira · H. Doihara · S. Miyoshi
Department of General Thoracic Surgery and Breast
and Endocrine Surgery, Okayama University Graduate
School of Medicine, Dentistry and Pharmaceutical Sciences,
2-5-1 Kita-ku, Shikata-cho, Okayama 700-8558, Japan
e-mail: imagon12000@yahoo.co.jp

T. Tanaka
Department of Pathology, Okayama University Hospital,
Okayama, Japan

T. Iwamto
Department of Breast and Endocrine Surgery, Okayama
University Hospital, Okayama, Japan

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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 ALMN, 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 ALMN 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 μm) of tumor tissues with the BOND^{TM} automated immunostainer (Leica Microsystems). The protocol was in accordance with IHCFP 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 (×400 high power field) per case. That of the ALNM was performed in 3–7 randomly selected areas (×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 (×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 χ^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	n (%)
Median	53 (28–78)
Operation	
Total	13 (32.5)
Partial	27 (67.5)
Nodal status	
$n = 1$	23 (57.5)
$n = 2$	7 (17.5)
$n = 3$	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

	n	ALDH1 (breast tumor <5%)		P	ALDH1 (ALNM <5%)		P
		Positive, n (%)	Negative, n (%)		Positive, n (%)	Negative, n (%)	
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
n = 1	23	6 (26)	17 (74)		4 (17)	19 (83)	
n = 2	7	0 (0)	7 (100)		3 (43)	4 (57)	
n = 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
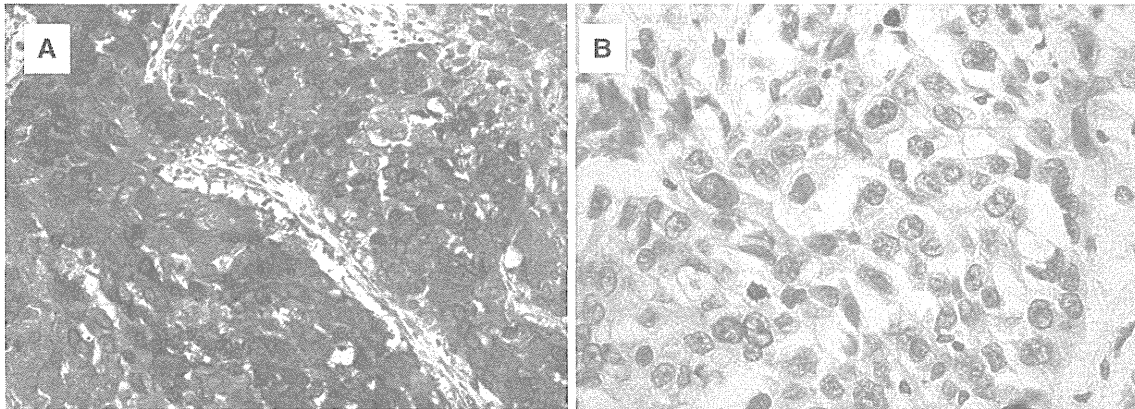
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Fig. 1 Immunohistochemical identification of ALDH1-positive tumor cells. The results of immunostaining of ALDH1 in breast cancer tissues: **a** positive, **b** negative
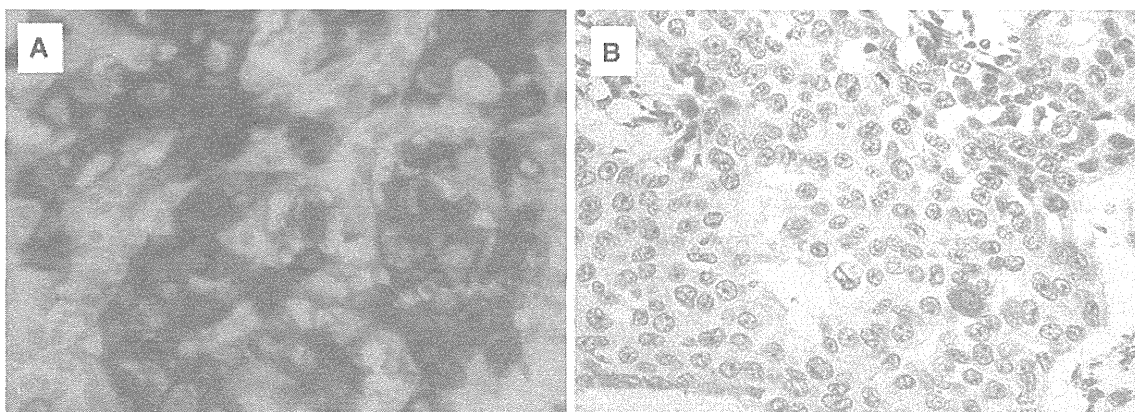

Fig. 2 Immunohistochemical identification of ALDH1-positive tumor cells. The results of immunostaining of ALDH1 in ALNM: **a** positive, **b** negative

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 ($n = 40$)			
	+/+	+/–	–/+	–/–
ER				
No. of patients	32	5	0	3
%	80	12.5	0	7.5
Concordance rate (%)	87.5			
HER2				
No. of patients	6	3	4	27
%	15	7.5	10	67.5
Concordance rate (%)	82.5			
Ki67				
No. of patients	5	5	4	26
%	12.5	12.5	10	65
Concordance rate (%)	77.5			
ALDH1				
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 immunohisto-chemically identified tumor ALDH1 expression was associated with a poor prognosis in breast cancer patients.
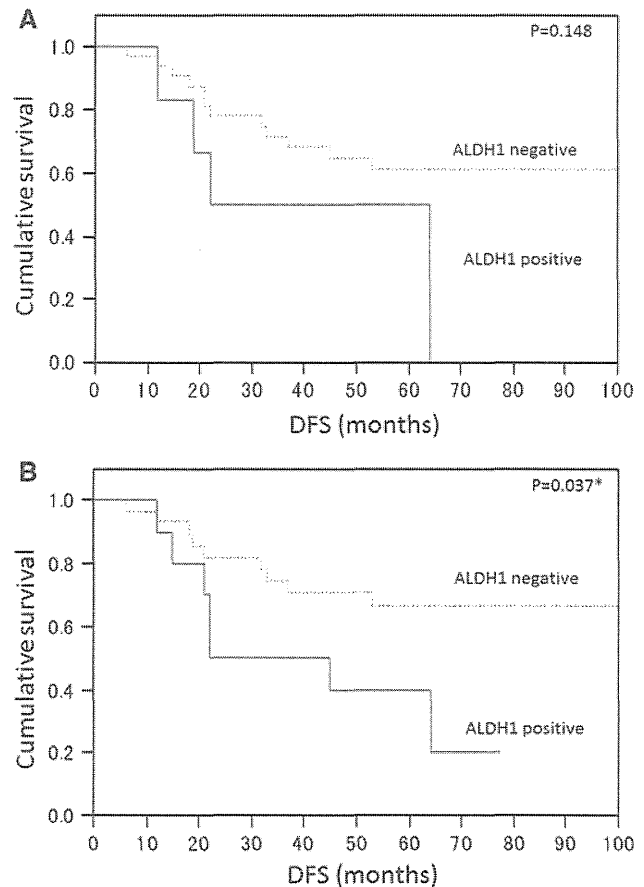

Fig. 3 Kaplan–Meier curve for disease-free survival (*DFS*) according to ALDH1 status in **a** breast tumors and **b** ALNM

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 be 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	P	Odds ratio	95% CI	P
ALDH1in 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	**0.047**	1.57	0.49–4.93	0.44
Age (≤50/>50)	1.24	0.46–3.64	0.68			
Histological grade (3/1, 2)	2.88	1.05–7.91	**0.04**	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