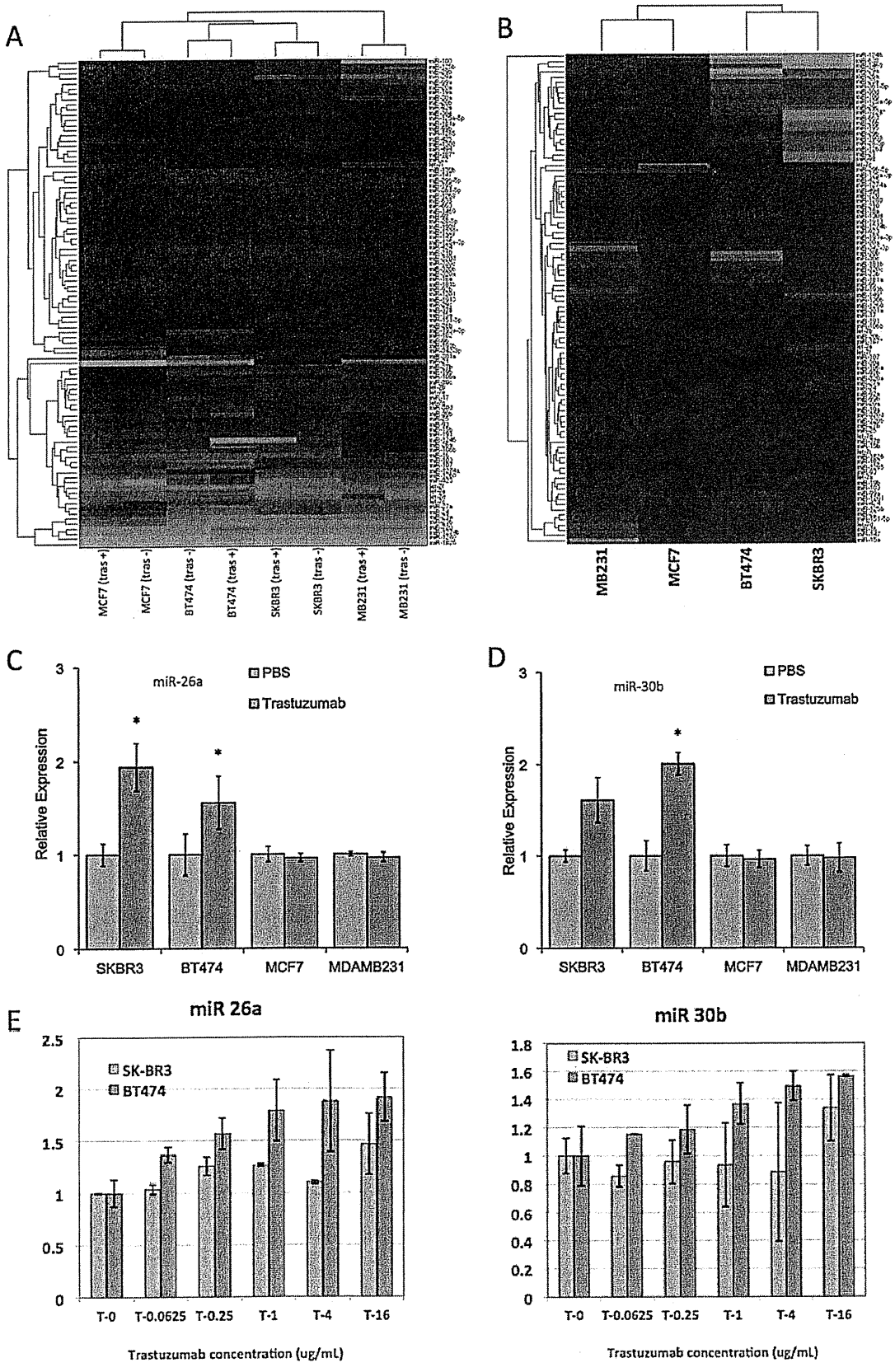
A
MCF7 (tras +)
MCF7 (tras -)
BT474 (tras -)
BT474 (tras +)
SKBR3 (tras +)
SKBR3 (tras -)
MB231 (tras +)
MB231 (tras -)
B
MB231
MCF7
BT474
SKBR3
C
miR-26a
PBS
Trastuzumab
Relative Expression
SKBR3
BT474
MCF7
MDAMB231
D
miR-30b
PBS
Trastuzumab
Relative Expression
SKBR3
BT474
MCF7
MDAMB231
E
miR 26a
SK-BR3
BT474
T-0
T-0.0625
T-0.25
T-1
T-4
T-16
Trastuzumab concentration (ug/mL)
miR 30b
SK-BR3
BT474
T-0
T-0.0625
T-0.25
T-1
T-4
T-16
Trastuzumab concentration (ug/mL)

Figure 2. Identification of trastuzumab-responsive microRNAs. 2A: A heat map and clustergram of the expression profile of 71 pre-filtered microRNAs. The red and green represent higher and lower expression levels, respectively. (tras +): with trastuzumab treatment, (tras −): without trastuzumab treatment. 2B: A heatmap and clustergram of the fold-change of microRNA expression by trastuzumab treatment. The red and green represent up- and down-regulation. 2C and 2D: The expression levels of miR-26a (2C) and miR-30b (2D) were validated by qRT-PCR (n = 3). The data are shown as microRNA expression levels relative to a control treatment (PBS). 2E: The expression level of miR-26a and miR-30b in different trastuzumab concentrations was measured (n = 2). The microRNA expression levels were normalized against miR-16. All bars and error bars represent means ± SEM. *: $p<0.05$.
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both two miR-30b binding sites was used, 11–35% reporter actively was recovered, which represented the total suppressive effect of endogenous miR-30 family through *CCNE2* 3′UTR.

Figure S3 showed that exogenous miR-30b mimic-oligos and inhibitors did not change mRNA levels of *CCNE2*. One of possible reasons is that miR-30b may regulate *CCNE2* only by translational inhibition. Another reason would be the change of cell cycle proportion of treated cells. The *CCNE2* is upregulated in G1 phase of cell cycle in a normal condition. Because introduction of miR26a/30b oligos increase G1 phase, *CCNE2* expression will be affected both by change of cell cycle phase proportion and post-transcriptional suppression due to these microRNAs. Because the two luciferase genes in reporter vector and internal control vector (pGL4.73) were driven by the same promoter (SV40), this system can assess the post-transcriptional regulation without any cell cycle-related bias.

Discussion

Recent evidence has shown that altered patterns of miRNA expression are correlated with carcinogenesis, malignant potential, prognosis [14], and the treatment response of various human cancers. In breast cancers, a high expression level of miR-10b [15] and miR-21 [16] are associated with metastasis and a poor outcome. Regarding the treatment response of breast cancer, the in vitro experiments showed that miR-34a [17] and miR-221/222 [18,19] are involved in the actions of docetaxel and tamoxifen, and that multidrug resistance–associated protein (MRP) was targeted by miR-7, mir-326, and miR-345 [20,21]. However, little has been reported in terms of microRNAs associated with the molecular mechanisms of trastuzumab treatment. This was the aim of this study.

At the beginning of this study, we confirmed the genome amplification and mRNA expression status of HER2 among the 11 breast cancer cell lines. SKBR3 andBT474 cells have high levels of genomic amplification and mRNA expression, and also exhibited trastuzumab sensitivity. This finding was also consistent with previous studies [22,23].

To screen the microRNAs related to the mechanisms of trastuzumab treatment, we initially set two selection criteria. The first one was microRNAs that were differentially expressed between trastuzumab sensitive and resistant *HER2*-positive breast cancer cells, and the second was microRNAs that were induced or reduced by trastuzumab treatment only in *HER2*-positive cells. For the former criterion, all of the *HER2*-positive breast cancer cells were trastuzumab sensitive. Furthermore, to establish trastuzumab-resistant *HER2*-positive cells, we administered trastuzumab to SKBR3 andBT474 cells at a concentration of 32 μg/mL for more than three months. However, these long-treated cells gained only 10–20% resistance as compared to the original cells, which were still moderately sensitive, similar to the MDA-MB-453 cells (data not shown). This was the reason why we chose the latter criteria in this study.

Using microarray-based microRNA profiling analysis and these screening criteria, we obtained a list of trastuzumab responsive microRNAs, as shown in Table 1. The validation of the RT-PCR demonstrated that most of the seven microRNAs had expression results consistent with the microarray data. Among the seven

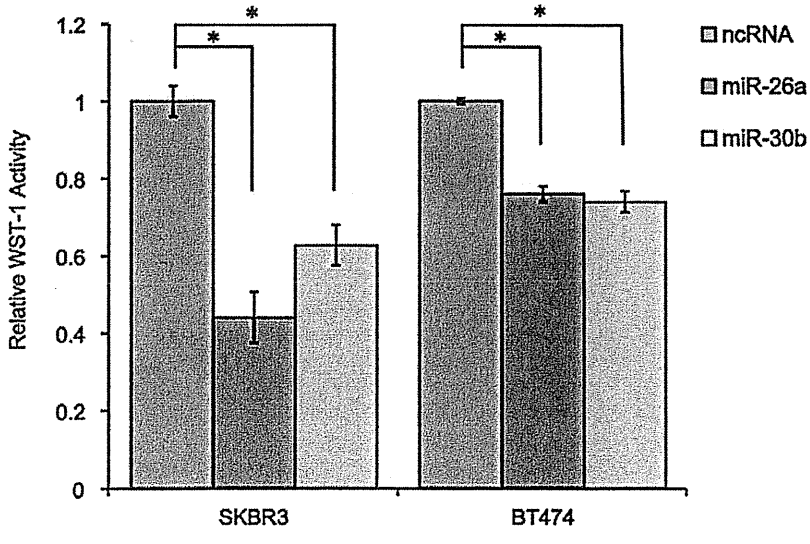

Figure 3. Effects of miR-26a and miR-30b on cell proliferation. The cells were transfected with negative control RNA (ncRNA), miR-26a, or miR-30b. At 72 hours after the transfection, the amount of viable cells was assessed by the WST-1 assay. The WST-1 activity values were normalized against that of the ncRNA-treatment. All bars and error bars represent means ± SEM (n = 4). *: $p<0.05$.
doi:10.1371/journal.pone.0031422.g003

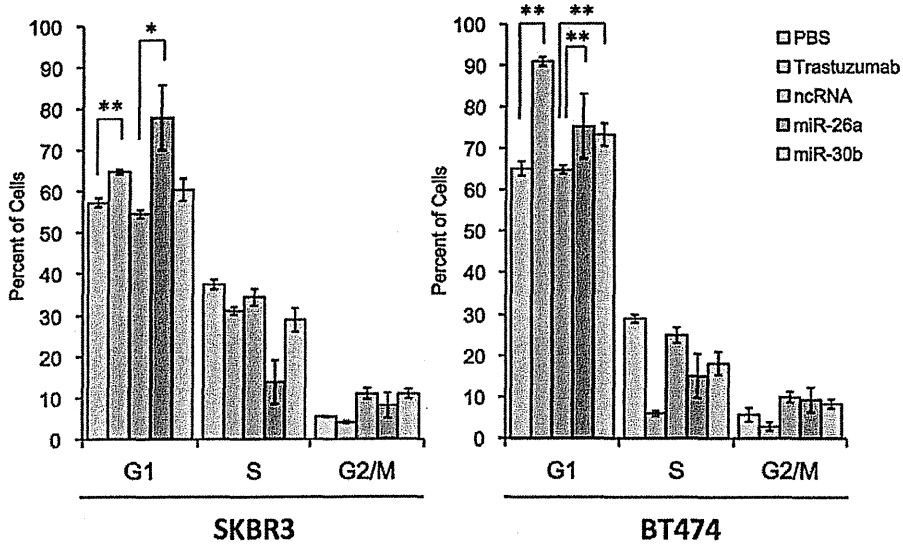

Figure 4. Effects of miR-26a and miR-30b on the cell cycle. The PI-stained DNA content of the cells was evaluated using a FACS Calibur (BD Biosciences) at 72 hours after transfection. All bars and error bars represent means ± SEM (n = 6). *: $p<0.05$, **: $p<0.005$.
doi:10.1371/journal.pone.0031422.g004

microRNAs, we focused on miR-26a as a microRNA up-regulated in bothSKBR3 andBT474 cells, and on miR-30b, because three out of five miR-30 family members were up-regulated inBT474 cells.

A down-regulation of miR-26a has been observed in various human malignancies, such as thyroid [24], liver cancer [25] and rhabdomyosarcoma [26], indicating that miR-26a is a tumor-suppressor microRNA. This study showed that the up-regulation of miR-26a by trastuzumab induced G1 arrest and apoptosis,

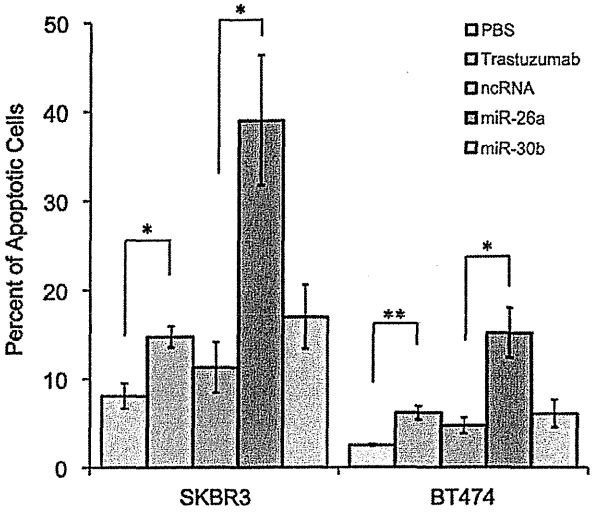

Figure 5. Effects of miR-26a and miR-30b on apoptosis. The apoptotic cells were detected using FITC-Annexin V at 72 hours after microRNA transfection. The percentage of Annexin V-FITC positive cells to the total cells was shown in the bar graphs. All bars and error bars represent means ± SEM (n = 4). *: $p<0.05$, **: $p<0.005$.
doi:10.1371/journal.pone.0031422.g005

which was consistent with previous observations. Some papers have reported the genes that were targeted by miR-26a, and are related to cell cycle and apoptosis. miR-26a regulated the cell cycle by targeting *cyclin D2* and *CCNE2* [27], and induced apoptosis by silencing the *enhancer of zeste, drosophila, homolog 2 (EZH2)*, and *metadherin* (*MTDH*) [28].

The expression of miR-30b was suppressed in invasive bladder cancer [29] and lung squamous cell carcinoma [30], as compared with superficial bladder cancer and the adjacent normal lung tissues, respectively. This suggests that miR-30b is also a tumor-suppressor microRNA. Transfecting with miR-30b had a cell growth suppressive effect and induced G1 cell cycle arrest, which was in agreement with the previous reports. Although information regarding the target genes of miR-26a was available, little has been known in terms of miR-30 target. Therefore, we screened the target genes of miR-30b that contributed to the miR-30b-induced G1 arrest. In this study, we demonstrated that miR-30b interacts directly with two binding sites in the 3′-UTR of *CCNE2*, and suppresses the expression of CCNE2. Cyclin E as well as Cyclins A and D are required for mammalian cells to transverse G1 and enter the S phase. Cyclin E1 and E2 activate cyclin-dependent kinase 2 (CDK2) by forming a CCNE-CDK2 complex [31], and initiate DNA synthesis. Therefore, it was a reasonable finding that the downregulation of CCNE2 by miR-30b induced G1 arrest. In Table 1, miR-30c and miR-30d were up-regulated by trastuzumab in BT474 cells. These miR-30 family members share the same sequence, 5′-GUAAACA-3′, in their seed regions. Thus, CCNE2 would be reduced in trastuzumab-treated BT474 cells not only by an up-regulation of miR-30b and miR-26a, but also by that of miR-30c/d. Recently, Scaltriti et al. demonstrated that gene amplification and overexpression of CCNE1 were associated with resistance of trastuzumab treatment for breast cancer [32], suggesting that cell cycle check-point system by CCNE is a key function for HER2-positive breast cancer. Thus, our finding that trastuzumab-inducible miR-26a/30b are regulating *CCNE2* was consistent with the their finding.

As shown in table 1, miR-125a-5p level was up-regulated both in SKBR3 and BT474 cells by trastuzumab exposure. Nishida et

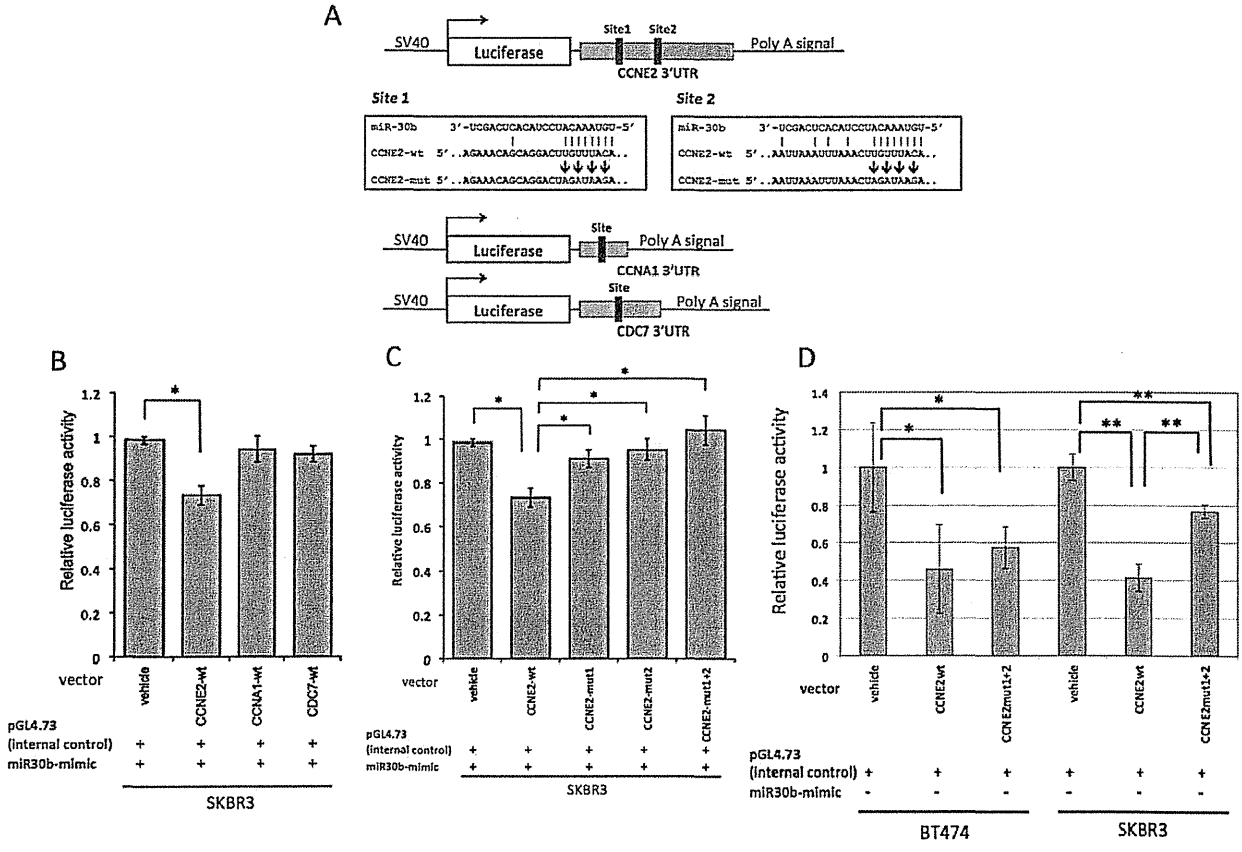

Figure 6. *CCNE2* is a direct target of miR-30b in breast cancer cells. 6A: A diagram of the 3'UTR-containing reporter constructs for *CCNE2*, *CCNA1*, and *CDC7* and their derivatives. The 3'UTRs of the three genes were inserted just downstream of the firefly luciferase gene in the pGL4.13 vector (wt). Next, the mutated derivatives (mut1, mut2, and mut1+2) of CCNE2-wt were generated by inserting mutations into two putative binding sites corresponding to the seed-sequence of miR-30b. 6B and 6C: SKBR3 and BT474 cells were co-transfected with reporter constructs, internal control vector (pGL4.73), and synthetic miR-30b oligomer. 6D: assessment of endogenous microRNA's inhibitory effects to *CCNE2*. Only reporter constructs and pGL4.73 were transfected into SKBR3 and BT474 cells. Twenty-four hours after the transfection, the reporter luciferase activity was measured. The data were shown as the luciferase activity relative to that of vehicle (pGL4.13+pGL4.73) transfection. All bars and error bars represent means ± SEM (n = 3). *: $p<0.05$, **: $p<0.005$.
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al. recently showed that miR-125a-5p targets HER2, and that it acts synergistically with trastuzumab in gastric cancer [33]. Our result suggested that the same mechanism would underlie trastuzumab therapy for breast cancer.

However, generally, each microRNA can target potentially hundreds of genes. Therefore, the cell cycle/apoptosis may not be the only processes affected/regulated by miR-26a/miR-30b. In addition, this study is not suggesting that the suppressive effect in endogenous level of these microRNA is a main mechanism of trastuzumab therapeutic effect. Direct blocking effect of HER2 signal pathway is still the major mechanism of trastuzumab therapy, and alteration of microRNA expression could play a supporting role in the downstream of HER2 signal.

On the other hand, it is largely unknown how miR-26a and miR-30b are up-regulated by trastuzumab treatment. One possible explanation of this phenomenon is regulation via c-myc (MYC) [34]. MYC is located downstream of the HER2 signal pathway [35]. Thus, trastuzumab treatment can reduce the levels of phospho-MYC [36]. According to the MYC ChIP-seq data registered in the UCSC genome browser [37], there are c-myc binding peaks around the transcriptional start sites of the miR-26a primary genes (*CTDSPL* in chromosome 3p22.2 and *CTDSP2* in 12q14.1). Actually, a report showed that miR-26a was repressed by MYC [38]. Furthermore, there is a MYC-binding site in a CpG island located upstream of the intergenic and polycistronic miR-30b and miR-30d. Thus, we hypothesized that inactivation of *MYC* may upregulate miR-30b/d expression. However, knock down of *MYC* by siRNA down regulated miR-30b expression (Figure S4 and S5). Therefore, unknown mechanisms rather than *MYC* upregulate miR-30b expression in trastuzumab treatment.

The present study demonstrated that a subset of microRNAs played a biological role in the mechanisms responsible for trastuzumab's antitumor effects. This finding suggests that trastuzumab-resistant *HER2*-positive breast cancer cells could be sensitized to trastuzumab therapy by modulating the expression of these microRNAs [39]. Alternatively, some microRNAs would be biomarkers to predict the treatment response of trastuzumab.

In summary, trastuzumab treatment for breast cancer cells modulated the expression of a subset of microRNAs, including miR-26a and miR-30b. The up-regulation of miR-30b by trastuzumab may play a biological role in trastuzumab-induced cell growth inhibition by targeting *CCNE2*.

Supporting Information

Figure S1 Taqman RT-PCR to validate the microarray results. The fold change in the log2 values are shown in the Y-axis.
(TIFF)

Figure S2 Effect of microRNA inhibitors on the CCNE2-3′UTR reporter assay. SKBR3 cells were transfected with CCNE2-wt construct and microRNA inhibitors to assess the suppressive effect of endogenous microRNAs. Twenty-four hours after the transfection, the reporter luciferase activity was measured. NTC: non-specific control oligos. The data were shown as the luciferase activity relative to that of NC. All bars and error bars represent means ± SEM (n = 3). *: $p<0.05$, **: $p<0.005$.
(TIFF)

Figure S3 Effect of knockdown and overexpression of miR-26a and 30b on CCNE2 mRNA expression. SKBR3 and BT474 cells were transfected with microRNA mimic oligos and inhibitors. Twenty-four hours after the transfection, mRNA level of *CCNE2* was measured by quantitative RT-PCR. *GAPDH* mRNA level was used for normalization of data. The data using inhibitor and mimic oligo were shown as relative expression to each non-specific control (NC) oligo. All bars and error bars represent means ± SEM (n = 4).
(TIFF)

Figure S4 Knocking down efficiency of *MYC* by siRNA. *MYC* mRNA level was measured by quantitative RT-PCR after 72 hours later than control siRNAs (siCont) or 4 different siRNAs (Qiagen) against *MYC* gene that were purchased from Qiagen, designated as siMYC1, siMYC5, siMYC7, and siMYC8. The siMYC5 and siMYC7 were selected for further study. Y-axis: *MYC* expression level relative to siCont transfection. All bars and error bars represent means ± SEM (n = 3).
(TIFF)

Figure S5 Effect of *MYC* knockdown on miR26a and miR30b expression. The microRNA (miR26a and 30b) expression level was measured by Taqman RT-PCR system after 72 hours later than the transfection of siCont or siMYCs. Y-axis: microRNA expression level relative to that of siCont transfection. All bars and error bars represent means ± SEM (n = 3).
(TIFF)

Table S1 Primer sequences for quantitative PCR.
(DOCX)

Table S2 Primer sequences for generating luciferase reporter constructs.
(DOCX)

Table S3 Trastuzumab responsive microRNAs in HER2-negative cells. MicroRNAs with more than 1.5-fold change in HER2-negative cells but not in HER2-positive cells. *: RFC, relative fold change = (Fold change of miR) − (average fold change of the miR in SKBR3 and BT474)
(DOCX)

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Author Contributions

Performed the experiments: TI. Analyzed the data: TI FS. Contributed reagents/materials/analysis tools: MT GT KS. Wrote the paper: FS. Conceived the study: FS. Designed the experiments: TI FS. Assisted the experiments technically: KT ST.

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ORIGINAL ARTICLE – BREAST ONCOLOGY

Identifying Gaps in the Locoregional Management of Early Breast Cancer: Highlights from the Kyoto Consensus Conference

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ABSTRACT A consensus conference was held to investigate issues related to the local management of early breast cancer. Here, we highlight the major topics discussed at the conference and propose ideas for future studies. Regarding axillary management, we examined three major issues. First, we discussed whether the use of axillary reverse mapping could clarify the lymphatic system of breast and whether the ipsilateral arm might help avoid lymphedema. Second, the use of an indocyanine green fluorescent navigation system was discussed for intraoperative lymphatic mapping. These new issues should be examined further in practice. Finally, some agreement was reached on the importance of "four-node diagnosis" to aid in the diagnostic accuracy of sentinel nodes. Regarding breast treatment, there was general agreement that the clinical value of surgical margins in predicting local failure was dependent on the tumor's intrinsic biology and subtypes. For patients treated with preoperative chemotherapy, less extensive excision may be feasible in those who respond to systemic therapy in an acceptable manner. Most trials of preoperative chemotherapy lack outcome data on local recurrence. Therefore, there is a need for such data for overview analysis. We also agreed that radiation after mastectomy may be beneficial in node-positive cases where more than four nodes are involved. Throughout the discussions for both invasive and noninvasive disease, the investigation of nomograms was justified for major issues in the decision-making process, such as the presence or absence of microinvasion and the involvement of nonsentinel nodes in sentinel node-positive patients.

This study is conducted on behalf of the 2009 Kyoto Breast Cancer Consensus Conference panelists.

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When the paradigm for breast cancer treatment shifted from the localized Halstedian view to Fisher's systemic vision, the role of surgery in the local management of breast cancer changed simultaneously. Appropriate local management is critical for the effective treatment of early breast cancer, because local recurrence might be a marker for the development of distant disease. In addition, reducing the failure of local treatment might result in the reduction of systemic treatment failure. Understanding the biological and pathological phenotype of breast cancer helps in constructing systemic therapeutic plans as well as in achieving successful individualized local management strategies.

Among the aspects of breast cancer treatment that have recently drawn attention, we have focused on the local

management of primary noninvasive and invasive breast cancer, including: breast conservation in conjunction with preoperative systemic treatments; axillary management; radiation therapy for the breast, chest wall, and regional lymph nodes; and the pathological assessment of excised tissues. At the Kyoto Breast Cancer Consensus Conference, held in 2009, we clarified these issues for purposes of discussion and sought to reach a consensus.

PATHOLOGICAL ANALYSIS

Tumor extension to the surgical margins of the resected specimen should be examined meticulously using appropriate inking protocols. Ductal carcinoma in situ (DCIS) found at an inked margin should be considered as a positive margin. The best method of manipulating the specimen to reveal the status of the margin (e.g., the use of a perpendicular cut versus Carter's orange peel technique) is controversial. Furthermore, no consensus was attained on the definition of negative margin. The definitions of a negative margin ranged from no tumor at the inked margin to an invasive tumor at a minimum of 5 mm from the edge. In addition, even greater margins have been proposed for DCIS when postoperative radiation therapy was not performed.

There was a lack of agreement about the number of levels of a frozen section required to adequately examine the sentinel lymph nodes. Other points of discussion included the appropriate use of cytokeratins and the type of methodology used (e.g., molecular or immunohistochemical analysis) (Table 1). Despite the differences in the definition of isolated tumor cells (ITC) and micrometastasis (MIC), there was general agreement that the presence of ITC should be considered node negative, whereas the presence of MIC (0.2–2 mm) should be considered node positive for staging purposes.[1]

In addition to histological grading according to the Nottingham criteria, the analysis of the status of cell proliferation using biomarkers such as the MIB1/Ki67 index provides important prognostic information.[2] To collect the data necessary to reach a consensus regarding controversial issues such as the definition of positive margins, it is recommended that each institution maintain precise records.

AXILLARY SURGERY

Sentinel lymph node biopsy (SLNB) partially reduces the complications related to axillary staging by avoiding level I axillary lymph node dissection (ALND), level II ALND, and full ALND in the case of sentinel node-negative patients based on the reports of the ALMANAC experience.[3]

TABLE 1 Pathological factors to be recorded while analyzing breast cancer specimens

Tumor size
Measured microscopically in orthogonal directions including the largest size of invasion
Margin
Method used to assess (orange peel or perpendicular cut)
Definition of positive margin
Distance of margin from cut edge (mm)
Additional treatment in positive cases (re-excision or boost RT)
Biological markers
ER (%)
PR (%)
HER-2 (IHC or FISH)
MIB1/Ki67 index (%)
Other conventional factors
Nuclear grade
Vessel invasion
Fixation
Time to fixation
Time for fixation
Sentinel lymph nodes (SLNs)
Techniques to identify SLNs (RI, dye, fluorescent or others)
Method of diagnosis (HE, IHC, molecular analysis or others)
Definition of metastasis
Number of excised SLNs
Number of positive SLNs
Number of frozen sections
Was ALND performed?

PR progesterone receptor, *IHC* immunohistochemistry, *FISH* fluorescent in situ hybridization, *HE* hematoxylin and eosin

Lymphatic Mapping

SLNB causes arm lymphedema in approximately 5–8% of patients, even when they are assessed at 6 months postoperatively. The axillary reverse mapping (ARM) procedure, which can clarify the anatomical relationship between the lymphatic system of the breast and the ipsilateral arm, may provide a method to avoid this complication.[4] In nearly 98% of primary breast cancer cases, the lymphatics from the arm, which were identified with a subcutaneous injection of blue dye in the volar surface of the upper arm, did not drain into the sentinel lymph node of the breast. This method should be standardized for common practice.

Another novel and highly sensitive method for visualizing the lymphatic system and the sentinel lymph nodes involved indocyanine green fluorescent (ICGf) navigation.[5] A photodynamic eye that recognizes fluorescence emission from protein-binding ICG enables real-time mapping of the lymphatic network. It was generally agreed that further

studies, such as clinical trials and long-term outcome studies, are needed to elucidate the issue of lymphatic mapping and determine the ultimate impact of these modalities on the incidence of lymphedema. It is necessary to determine more precisely the value of combination of ICGf with radioisotope (RI) in prospective studies.

Number of Nodes Required for Diagnosis

Non-SLN metastases have been reported in 4–7% of SLN-negative cases.[6] It is crucial to consider the number of nodes that should be excised for diagnosis and staging and from the perspective of the therapeutic benefit of local control.[7–11] We agreed that examination of four SLN-containing nodes was sufficient to determine the status of metastases in the axilla. There were indications that four-node diagnosis would help to avoid unnecessary ALND and may enable less extensive axillary surgery (Fig. 1). Another important issue discussed was lymph node dissection for SLN-positive patients. Several studies have indicated that it may be possible to avoid subsequent axillary dissection in certain subgroups of node-positive patients.[12–14] Table 2 summarizes these options.

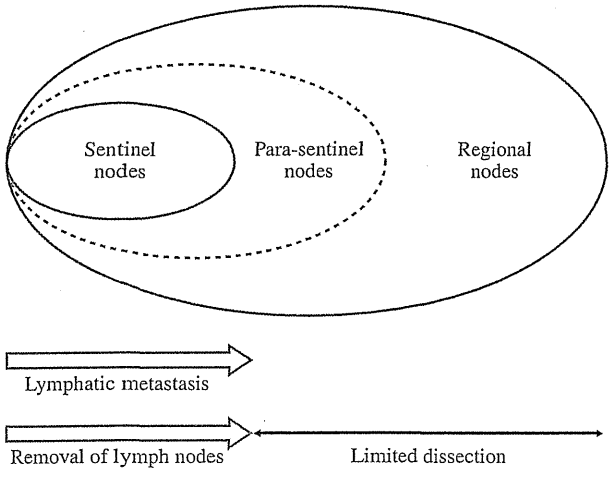

FIG. 1 Limited axillary lymph node dissection

TABLE 2 Impact of four-node diagnosis for sentinel nodes on subsequent ALND

No. of involved nodes	Requirement for completion of ALND
0 (ITC included)	Avoidable
1–3	Avoidable (individually)
More than 3	Inevitable

ITC isolated tumor cells

SLNB Prior to Systemic Therapy

Although SLNB before preoperative systemic therapy (PST) under local anesthesia is difficult, we concluded at the meeting that it is useful for the purpose of confirming the nodal status, especially in clinically node-negative cases. In clinically node-positive cases, SLNB before PST is controversial. The nodal information is important for designing and individualizing therapeutic plans for local and systemic treatment, because the nodal status can be altered by the treatment.

SLNB after PST is also controversial.[15] The major concerns are the relatively high false-negative rate and the uncertainty in the conversion of the positive nodes to negative. Future studies are warranted to clarify the accuracy of lymphatic mapping after PST, including anti-human epidermal growth factor receptor 2 (HER2) therapies, and to develop nomograms to facilitate the decision-making process (Table 3).

SLNB in DCIS

SLN metastases were identified by RI lymphatic mapping in approximately 1.4% of 854 patients with pure DCIS.[16] Most of these patients underwent complete ALND, and only one of these patients exhibited additional positive axillary lymph nodes. Several studies investigating the long-term outcomes of local control in proven DCIS cases determined that local failures were rare.[17] During the conference, there was general agreement that SLNB can be recommended for patients with DCIS who undergo mastectomy and for those diagnosed with invasive carcinoma upon final pathology. In addition, there was agreement that SLNB should be avoided in patients with needle biopsy-proven DCIS and without high risk factors for invasive cancer who undergo breast-conserving surgery (BCS). Therefore, the development of an algorithm to predict potential invasion and thus avoid SLNB for needle biopsy-proven DCIS (Table 3) is warranted.[18]

BREAST SURGERY

Ipsilateral Breast Tumor Recurrence (IBTR)

It is difficult to decide upon one margin width that is appropriate for all patients.[19] Opinions about the minimal acceptable margin in local breast cancer resection varied from less than 5 mm to more than 20 mm. The recommendations were divided into three major categories based on tumor location: within 5 mm, tumor within 2 mm, and tumor at the margin. With respect to the re-excision criteria in the case of BCS, the consensus was that a 2 mm radial margin was satisfactory and should not prompt

TABLE 3 Nomogram for breast cancer

Decision factors	Decision goal
DCIS	To determine whether SLNB is required by examining possible microinvasion[46]
PST	To determine the type of surgery required by examining possible pCR[47]
IBTR	To determine whether RT or re-excision is necessary[48]
SLNB	To determine whether ALND is required by predicting non-SLN metastasis[18,49–52]

re-excision.[20] For a close margin (i.e., 2–5 mm), boost irradiation can be considered.

The 20-year follow-up data from the National Surgical Breast and Bowel Project (NSABP) B-06 trial showed that 39.2% of the patients who received wide local excision without radiotherapy developed ipsilateral breast tumor recurrence (IBTR), as compared with 14.3% of those who received postoperative radiotherapy. Some believe that IBTR does not influence overall survival and that it can be considered a marker of distant metastases rather than a cause; its presence therefore cannot change the intrinsic risk of distant disease.[21] However, according to a meta-analysis performed by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), the impact of local radiation therapy (RT) to prevent local recurrence, either to the breast following BCT or to the chest wall after mastectomy, exhibited overall survival benefit in patients with greater than 10% risk of local recurrence, but it did not show any benefit in patients with less than 10% risk of local recurrence.[22] An analysis of hazard ratios for distant metastases in patients who had undergone breast-conservation surgery with or without postoperative radiotherapy indicated that local recurrence might be a cause of distant metastases.[23] These results suggested that the group with a high risk for locoregional recurrence gained a survival benefit from local radiotherapy. In addition, local relapse could be a crucial psychological stressor for a patient even if her long-term survival was unaffected.

PST

In the case of sequential chemotherapeutic regimens such as doxorubicin and cyclophosphamide (AC) followed by a taxane, the pathological complete remission (pCR) rates are higher in patients who responded to the preceding regimen than in nonresponders. Furthermore, combining chemotherapy with an anti-HER2 treatment such as trastuzumab resulted in even higher pCR rates in HER2-positive cases.[24] A multidisciplinary team, which included an attending surgeon, a radiologist, a medical oncologist, and a pathologist, was indispensable in making appropriate decisions regarding BCS after PST. The findings also led to the recommendation that long-term outcome data, particularly data related to local recurrence rates, and methodologies for assessing the response and success of treatment should be collected, analyzed, and clarified at each institution.[25]

The large majority of the attendees agreed that neoadjuvant endocrine treatment (NAET) is an acceptable approach for certain patients, including those with low-grade, estrogen receptor (ER)-positive breast cancers and postmenopausal patients. Recent studies have suggested that NAET provides higher breast-conservation rates. Nevertheless, because of a lack of randomized clinical trial data, especially on local recurrence, this issue remains to be studied with respect to the tailoring of treatment using biomarkers.[26] Future studies are required to investigate the factors that are predictive of a shrinkage pattern in tumors that have responded to NAET and to determine their postoperative prognosis.

Hereditary Breast Cancer

There was some consensus that patients at higher risk for local recurrence or development of breast cancer in the contralateral breast due to genetic mutations (e.g., *BRCA1* or *BRCA2*) require a more aggressive surgery than BCS. Although this is a controversial topic, the risks of IBTR and of developing contralateral breast cancer may be higher in patients with *BRCA* abnormalities. Therefore, performing a bilateral mastectomy may be preferable to BCS. In addition, performing a bilateral mastectomy would avoid use of RT in a majority of patients. Fifteen years of follow-up data from postoperative radiotherapy in *BRCA* patients suggested that there is a higher risk of radiation toxicity in these patients. Taking these data together, bilateral mastectomy for this specific subgroup could result in reducing cancer recurrence in the affected breast, decreasing new breast cancer development in the unaffected breast, and avoiding the late toxicity of radiotherapy.[27–29]

BCS for DCIS

The Van Nuys Prognostic Index (VNPI), originally proposed and validated by Silverstein et al., is a scoring system for predicting the risk of IBTR in DCIS patients undergoing BCS. Three major factors—margin status, high histological grade, and young age—were recognized as significant risk factors for IBTR after resection of DCIS. The distribution of the opinions as to the proper margin needed for DCIS was similar to that for invasive ductal carcinoma (IDC).

Several retrospective studies have suggested that RT after BCS is useful in avoiding IBTR, especially in patients with high-risk DCIS.[30,31] Tamoxifen in combination with RT has

also been reported to decrease IBTR in DCIS.[32] Prospective trials of neoadjuvant therapies for DCIS using trastuzumab or lapatinib have recently been initiated. These trials may elucidate the effect of anti-HER2 treatments on the local management of HER-2-neu-overexpressing DCIS.

Dunne et al. performed a meta-analysis of 4,660 cases identified from Medline with regard to the margins required for DCIS and RT. They found that a negative margin significantly reduced the risk of IBTR compared with a close margin, and a 2-mm margin was superior to a margin less than 2 mm. However, they observed no significant differences in the IBTR rates with margins over 2 mm.[33] Fisher et al. demonstrated the benefit of tamoxifen in the treatment of DCIS in NSABP B-24, a randomized controlled trial.[34] Because these data suggest that BCS alone is insufficient to prevent IBTR after surgery for DCIS, there was consensus at the meeting that RT and/or endocrine therapy is necessary after BCS.

RADIATION THERAPY

RT as a Component of the Local Management of Breast Cancer

Postoperative RT reduces the risk of locoregional recurrence to approximately one-third of that without RT. Although the baseline risks have varied among existing reports, depending on the method of surgery and the pathological evaluation, the relative risk reduction related to RT was consistent.[22]

For each group of patients who received BCS, there have been continual efforts to find a subgroup of patients who do not require RT.[32,35–38] Unfortunately, such a subgroup had not yet been identified in a prospective trial. However, the eligibility criteria and systemic treatment used in early clinical trials were suboptimal in comparison with today's standards.[32] A clinical trial in a selected group of patients, which included individuals over 70 years old with hormone-responsive tumors treated with a suitable resection margin and appropriate hormonal therapy, demonstrated that the absolute reduction in the risk of local recurrence due to RT, although significant, was small enough that omission of RT could be considered.[32] It is suggested that the intrinsic subtype of breast cancer might be an independent predictive factor related to the benefit of postoperative RT.[39,40] At the meeting it was indicated that these findings should be verified in prospective trials.

Trends in Postoperative Irradiation for the Conserved Breast

Both hypofractionated whole-breast RT and accelerated partial-breast irradiation (APBI) were increasingly used after BCT. Hypofractionated whole-breast RT demonstrated equivalent tumor control and cosmetic results compared with conventional fractionation.[41,42] In the consensus conference, we discussed hypofractionation as an option for certain patients, such as those who are margin free. However, APBI is still considered an experimental treatment.

Indication for Boost to the Tumor Bed after BCT

Although a large randomized clinical trial demonstrated a significant reduction of IBTR in patients with a negative margin, we were unable to reach a consensus on the indications for an RT boost. The most important issue to be resolved was the definition of a "positive" margin after BCS. This definition varied by country and region.[43] Therefore, it should be further examined whether patients with positive margins benefit from routine administration of boost irradiation after whole-breast radiation therapy. At the consensus conference, approximately half of the participants responded that boost irradiation is not necessary if the margin is greater than 5 mm.

In addition to the dose dependency of the ipsilateral tumor control, the European Organisation for Research and Treatment of Cancer (EORTC) 22881-10882 trial clearly demonstrated that younger patients receive a greater benefit from boost irradiation secondary to their greater baseline risk of IBTR. However, in this consensus conference, approximately half of the participants answered that young age alone in not a sufficient criterion for providing a boost, if the margin is widely clear. To resolve this issue, we must standardize the definition of a positive margin, clarify the relationship between the distances required for a clear margin, and understand the magnitude of the effect of boost irradiation.

Survival Benefit of Postoperative RT for Breast Cancer

Meta-analyses performed by EBCTCG demonstrated that a reduction in the risk of locoregional recurrence at 5-year postoperative follow-up could eventually lead to a reduction in death from all causes at 15-year postoperative follow-up.[30] This survival benefit was attributed to prevention of secondary dissemination from local recurrence. However, the benefit was substantial only if the absolute risk reduction of the locoregional recurrence at 5 years exceeded 10%.

Currently, patients with four or more positive lymph nodes are regarded as being at high risk for local recurrence. Postoperative RT to the supraclavicular lymph nodes and the chest wall and breast are recommended in this group after both breast-conserving surgery and mastectomy. Furthermore, meta-analyses of existing trials have

suggested that patients with one to three positive lymph nodes might also receive a survival benefit from postoperative RT, although a randomized clinical trial investigating this hypothesis is ongoing. Patients with negative axillary lymph nodes generally exhibit a low risk of local recurrence. These patients do not benefit from such RT and may have increased risks of radiation side-effects if RT is given. Of note, the number of positive axillary lymph nodes in this context is only a surrogate for the risk of isolated locoregional recurrence. The indication for postoperative RT should ultimately be based on the absolute risk of local recurrence.

Postoperative Radiation Therapy in Patients Receiving PST

Recently, PST has been offered not only to patients with advanced disease but also to patients with early-stage breast cancer. The expansion of this practice has unveiled a new clinical question: What is the optimal RT dose for patients who respond favorably to PST? Randomized trials are needed to answer this question. However, the general consensus was that, for all patients who receive PST and BCS, postoperative RT is recommended. Retrospective studies of patients who received a mastectomy after PST showed that RT significantly improved local control even in patients with pCR after PST.[44] These investigators also found that RT improved survival in patients at higher risk of locoregional recurrence after PST and mastectomy.[45] These results provide insight that the decision to offer RT should be based on both the pretreatment assessment and the final pathologic findings. Postoperative RT is recommended for patients initially diagnosed as having a high risk of locoregional recurrence, regardless of their response to PST.

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Prognostic significance of tumor/stromal caveolin-1 expression in breast cancer patients

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Caveolin-1 (Cav-1) has been extensively characterized in cancer biological research. However, the role of Cav-1 in the interaction between tumor and stromal cells remains unclear. In the present study, we examined Cav-1 expression in tumor cells and stromal cells in breast cancer tissue by immunohistochemical analysis and evaluated its prognostic value in a training cohort. Immunohistochemical analysis of Cav-1 expression was scored as (++), (+) or (–) according to the proportion of positively stained tumor cells (T) and stromal cells (S). Correlation analysis between tumor/stromal Cav-1 expression and clinicopathological parameters revealed that only T(++) Cav-1 status was positively associated with tumor size and histological nodal status ($P = 0.019$ and 0.021, respectively). Univariate analysis revealed that combined T(++)/S(–) status was significantly correlated with unfavorable prognostic outcomes ($P < 0.001$). Multivariate analysis demonstrated that this combined status is an independent prognostic factor for primary breast cancer ($P = 0.002$). Clinical outcomes in different subgroups of breast cancer patients were also strictly dependent on this combined status ($P < 0.05$). The prognostic value of T(++)/S(–) Cav-1 status was also validated in the testing cohort. Collectively, our data indicate that high Cav-1 expression in tumor cells and lack of this expression in stromal cells could help identify a particular subgroup of breast cancer patients with potentially poor survival. Further studies are required to understand the regulatory mechanism of Cav-1 in the tumor microenvironment. (*Cancer Sci* 2011; 102: 1590–1596)

Breast cancer is the most common female cancer. Late-onset diagnosis, axillary lymph node metastases, tumor size, pathological type and resistance to antitumor therapy indicate a poor prognosis for breast cancer patients. Although treatment strategies for breast cancer have recently made great progress, recurrence and death rates remain unacceptably high.[1] Therefore, molecular biomarkers for recurrence and progression of breast cancer must be explored to help clinicians identify new diagnostic and therapeutic techniques to detect and treat breast cancer.[2]

Caveolins (Cav) are a family of scaffolding proteins that coat 50–100 nm plasma membrane invaginations. The Cav family is composed of three isoforms: Cav-1, Cav-2 and Cav-3. The Cav-1 gene is located on chromosome 7 (locus 7q31.1) and includes three exons (30, 165 and 342 bp) and two introns (1.5 and 32 kb).[3] Cav-1 expression depends on the type of tumor and its expression is downregulated in several human cancers such as sarcoma and lung cancer and might function as a tumor suppressor.[4,5] However, upregulation of Cav-1 expression has been reported in esophageal and pancreatic cancers and is also correlated with histopathological grade and poor prognosis.[6,7]

Cav-1 is mainly involved in vesicular transport, cholesterol homeostasis and signal transduction.[8] Furthermore, it might facilitate DNA repair and stabilize the insulin receptor against degradation. Cav-1 also plays a negative role in cell movement,[9] cellular senescence[10] and cell growth.[11] Endothelial cells from Cav-1–/– mice exhibit a diminished response to angiogenic growth factors.[12] Furthermore, Cav-1 overexpression is sufficient to induce premature cellular senescence in fibroblasts.[13,14] Cancer-associated fibroblasts (CAFs), which are derived from malignant or normal epithelial cells, promote tumor growth.[15] *In vitro* studies have shown that both stromal and epithelial Cav-1 play a protective role against mammary hyperplasia and tumorigenesis in breast cancer.[11,16,17] In addition, clinical studies have indicated that stromal loss of Cav-1 is a single independent predictor of early breast cancer recurrence and progression.[18,19] However, the value of combined tumor/stromal Cav-1 expression on the outcome of breast cancer patients is largely unknown.

In the present study, we investigated the clinical significance of Cav-1 expression (including tumor and stromal expression) in a training cohort and the correlation between tumor/stromal Cav-1 expression and clinicopathological characteristics. In addition, effects of combined tumor/stromal Cav-1 expression on outcomes in breast cancer patients were investigated. In addition, the prognostic value of combined tumor/stromal Cav-1 expression was also clarified in a testing cohort. Intriguingly, our results indicated that a counter balance of Cav-1 levels in the tumor microenvironment and epithelial compartment were the most strongly influenced clinical outcomes.

Materials and Methods

Collection of tissue samples. Tissue specimens of the training cohort were collected from the Department of Breast Surgery, Kyoto University Hospital (Kyoto, Japan) between July 2000 and February 2006. Informed consent was obtained from all patients prior to specimen collection and all study protocols were approved by the Ethics Committee for Clinical Research, Kyoto University Hospital. The clinical stage was assessed by The Japanese Breast Cancer Society classification.[20] For analysis of survival and follow up, the date of surgery was used to represent the beginning of the follow-up period. All patients who died from diseases other than breast cancer or from unexpected events were excluded from the case collection. Follow ups were terminated in June 2010. The median follow up was 74 months (range, 3–119 months). Clinicopathological parameters of the training cohort are listed in Table 1. In addition, we validated the results using an independent testing cohort of 193 consecutive patients (Table 1) who underwent surgical resection of breast cancer at Osaka Red Cross Hospital (Osaka, Japan). The protocols used in the testing group were approved by the Ethics Committee of the Osaka Red Cross Hospital. Follow ups

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Table 1. **Clinicopathological parameters of the training and testing cohort**

Parameter	Variable	*n* (100%)
Training cohort		
Age	>50	75 (72)
	≤50	29 (28)
Tumor size	≤2 cm	24 (23)
	2–5 cm	51 (49)
	>5 cm	10 (10)
	Unknown	17 (18)
Histological nodal status	Positive	46 (44)
	Negative	56 (54)
	Unknown	2 (2)
ER	Positive	80 (77)
	Negative	24 (23)
PgR	Positive	67 (64)
	Negative	37 (36)
HER2†	Positive	18 (18)
	Negative	81 (82)
ER/PgR/HER2†	Triple negative	12 (12)
	Others	92 (88)
Grade†	Grade 1	12 (12)
	Grade 2	41 (39)
	Grade 3	44 (42)
	Unknown	2 (7)
Recurrence	Yes	22 (21)
	No	72 (69)
	Unknown	10 (10)
Death	Yes	7 (7)
	No	97 (93)
Testing cohort		
Age	>50	147 (76)
	≤50	45 (23)
	Unknown	1 (1)
Tumor size	≤2 cm	85 (44)
	2–5 cm	97 (50)
	>5 cm	11 (6)
Histological nodal status	Positive	79 (41)
	Negative	109 (56)
	Unknown	5 (3)
ER	Positive	139 (72)
	Negative	50 (26)
	Unknown	4 (2)
PgR	Positive	95 (49)
	Negative	95 (49)
	Unknown	3 (2)
HER2	Positive	22 (11)
	Negative	170 (88)
	Unknown	1 (1)
ER/PgR/HER2	Triple negative	35 (18)
	Others	154 (80)
	Unknown	4 (2)
Grade	Grade 1	58 (30)
	Grade 2	50 (26)
	Grade 3	85 (44)
Recurrence	Yes	28 (14)
	No	165 (86)
Death	Yes	15 (8)
	No	178 (92)

†Patients with ductual carcinoma in situ (DCIS) were excluded. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor.

in the testing group were terminated in January 2011. The median follow up in the testing cohort was 42 months (range, 1–80 months).

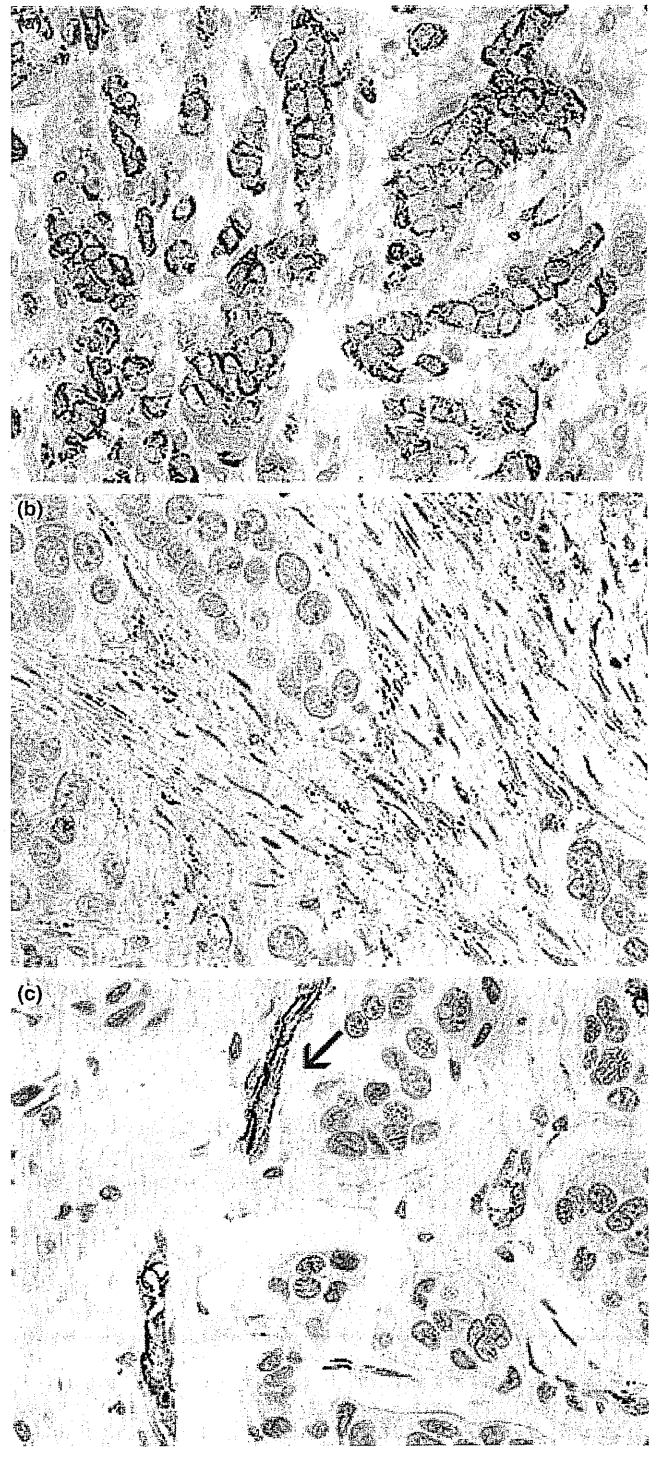

Fig. 1. Immunohistochemical analysis of caveolin-1 (Cav-1) expression (×400). (a) Tumor hot-spot expression. (b) Diffuse Cav-1 expression in stroma and (c) negative stromal Cav-1 expression. Endothelial cells indicate positive immunostaining for Cav-1 used as internal positive controls (arrows).

Immunohistochemical analysis. Immunohistochemical analysis was performed as described previously.[18,19] In brief, slides were incubated with an anti-Cav-1 monoclonal antibody (1:800; Cell Signaling Technology, Danvers, MA, USA). The

signals were detected by Envision kit (Dako, Glostrup, Denmark) and the sections were counterstained with haematoxylin. Negative control sections were incubated with phosphate-buffered saline plus 1% bovine serum albumin instead of primary antibody. Endothelial cells were used as internal positive controls because these cells commonly express Cav-1 in cancerous regions. Results of the analyses were evaluated by two pathologists, who were independent and blinded to the clinical features of the study. We determined three hot-spots at ×400 magnification, calculated the number of Cav-1-stained tumor cells (T) and stromal cells (S) and graded the cells as follows: negative expression (−), ≤5%; low expression (+), 5–50%; and high expression (++), >50%. For analyzing combined tumor/stromal Cav-1 expression, we determined tumors with a high tumor hot-spot grade that were stromal negative as T(++)/S(−).

Statistical analysis. The correlation between different types of Cav-1 expression was evaluated using Spearman's test. The correlation between Cav-1 and clinicopathological parameters was evaluated using the Kruskal–Wallis test. Disease-free survival was estimated using the Kaplan–Meier estimate and a comparison of stratified survival curves was performed using log-rank tests. Cox analysis was used to evaluate the correlation between Cav-1 and disease-free survival in the presence of various potential prognostic factors for disease-free survival. Differences were considered statistically significant at $P < 0.05$.[18,19]

Results

Immunohistochemical analysis of Cav-1 expression in the training cohort. All patients were Japanese women and their clinicopathological characteristics are listed in Table 1. Twenty-two patients developed recurrence and seven of them died as a result. Distributions of recurrence and survival parameters are also indicated in Table 1. Table S1 summarizes the number of patients in each subgroup stratified by Cav-1 grade. T(−) Cav-1 expression was observed in 16%, T(+) Cav-1 expression was observed in 67% and T(++) Cav-1 expression was observed in 17% of breast cancer patients. Strong positive staining showed a prevalent membrane pattern associated with cytoplasm positive. For total stromal Cav-1 expression, 57% were S(++/+) and 43% were S(−). In total, 72 patients were S(++/+) and 32 showed S(−) expression stratified by stromal hot-spot Cav-1 expression. For combined Cav-1 expression score grading, 5% of breast cancer patients were T(++)/S(−) and 16% were T(+)/S(−). Representative examples are illustrated in Figure 1.

Next, correlations between the different types of Cav-1 expression were analyzed. Stromal hot-spot Cav-1 expression was significantly correlated with total stromal Cav-1 expression ($R^2 = 0.517$, $P < 0.001$). However, stromal hot-spot Cav-1 expression was weakly correlated ($R^2 = 0.081$, $P = 0.003$) with tumor hot-spot expression. No significant difference was observed between total stromal and tumor hot-spot Cav-1 expression ($R^2 = 0.029$, $P = 0.083$). Because of the strong correlation between stromal hot-spot and total Cav-1 expression, we used the former as a representative for the following analysis.

Correlations between Cav-1 expression and clinicopathological parameters in the training cohort. Table 2 summarizes the correlation between Cav-1 expression and the clinicopathological parameters of breast cancer patients in the training cohort. T(++) Cav-1 expression was positively associated with tumor size and histological nodal status ($P = 0.019$ and 0.021, respectively). S(−) Cav-1 expression was independent of histological nodal and human epidermal growth factor receptor 2 (HER2) status ($P = 0.385$ and 0.055, respectively). No significant correlations were found between stromal hot-spot and tumor hot-spot Cav-1 expression and age, tumor stage, grade, estrogen receptor (ER) status or progesterone receptor (PgR)

Table 2. Associations between caveolin-1 (Cav-1) expression and clinicopathological parameters in the training and testing cohorts

Parameter	Variable	Tumor hot-spot Cav-1 expression (training cohort)			Stromal hot-spot Cav-1 expression (training cohort)			Tumor/stromal Cav-1 expression (training cohort)			Tumor/stromal Cav-1 expression (testing cohort)		
		T(+/−)	T(++)	P	S(−)	S(++/+)	P	T(++)/S(−)	Others	P	T(++)/S(−)	Others	P
Tumor size		27.0 ± 12.8	36.3 ± 17.4	0.019	30.5 ± 13.1	28.2 ± 14.6	0.498	36.6 ± 6.9	20.9 ± 3.3	<0.0001	40.9 ± 45.4	23.8 ± 12.4	<0.001
Age (%)	≤50	24 (28)	5 (28)	0.991	10 (31)	19 (26)	0.612	1 (20)	28 (28)	0.688	13 (59)	134 (79)	0.129
	>50	62 (72)	13 (72)		22 (69)	53 (74)		4 (80)	71 (72)		9 (41)	36 (21)	
T status (%)	T0/T1	24 (34)	2 (12)	0.095	5 (21)	21 (33)	0.258	0 (0)	26 (31)	0.253	5 (23)	80 (47)	0.033
	T2/T3/T4	47 (66)	14 (88)		19 (79)	42 (67)		3 (100)	58 (69)		17 (77)	91 (53)	
Histological nodal status (%)	Positive	34 (40)	12 (71)	0.021	16 (52)	30 (42)	0.385	5 (100)	41 (42)	0.012	5 (25)	104 (62)	0.002
	Negative	51 (60)	5 (29)		15 (48)	41 (58)		0 (0)	56 (58)		15 (75)	64 (38)	
Grade† (%)	Grade 1	12 (15)	0 (0)	0.140	2 (7)	10 (15)	0.264	0 (0)	12 (13)	0.208	4 (18)	54 (32)	0.113
	Grade 2	31 (39)	10 (59)		11 (37)	30 (45)		4 (80)	37 (40)		5 (23)	45 (26)	
	Grade 3	37 (46)	7 (41)		17 (56)	27 (40)		1 (20)	43 (47)		13 (59)	72 (42)	
ER (%)	Positive	69 (80)	11 (61)	0.081	21 (66)	59 (82)	0.359	3 (60)	77 (78)	0.360	13 (62)	126 (75)	0.201
	Negative	17 (20)	7 (39)		11 (34)	13 (18)		2 (40)	22 (22)		8 (38)	42 (25)	
PgR (%)	Positive	58 (67)	9 (50)	0.162	19 (59)	48 (67)	0.476	3 (60)	64 (65)	0.471	10 (48)	85 (50)	0.817
	Negative	28 (33)	9 (50)		13 (41)	24 (33)		2 (40)	35 (35)		11 (52)	84 (50)	
HER2† (%)	Positive	15 (19)	3 (17)	0.855	9 (30)	9 (13)	0.055	2 (40)	16 (17)	0.197	3 (29)	19 (33)	0.667
	Negative	66 (81)	15 (83)		21 (70)	60 (87)		3 (60)	78 (83)		18 (71)	152 (67)	

†Patients with ductual carcinoma in situ (DCIS) were excluded. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor; S, stromal hot-spot; T, tumor hot-spot.

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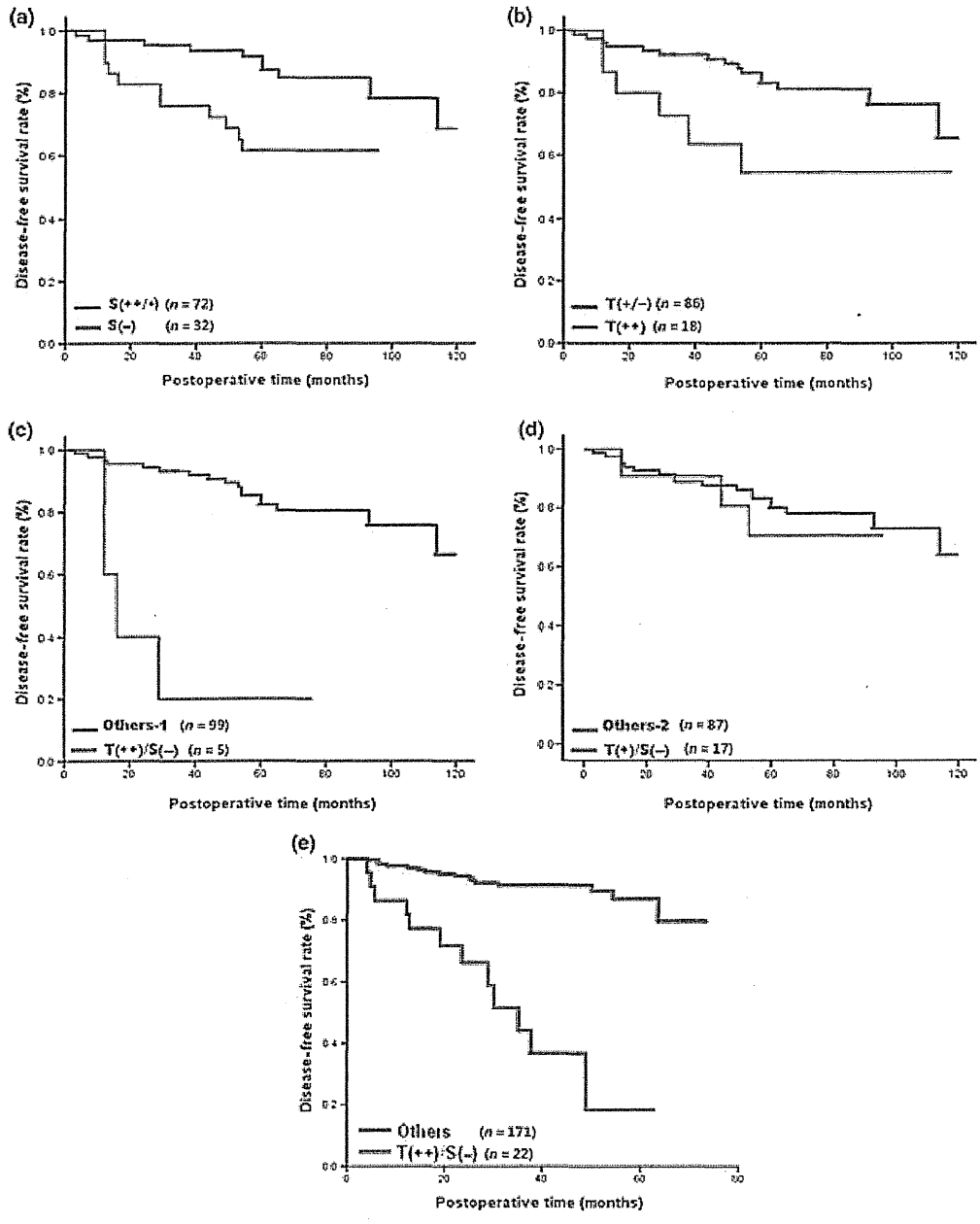

Fig. 2. Disease-free survival curves of the training cohort stratified by (a) stromal hot-spot caveolin-1 (Cav-1) expression status: S(−) *vs* S(++/+; $P = 0.009$, log-rank test); (b) tumor hot-spot Cav-1 expression status: T(++) *vs* T(+/−; $P = 0.019$, log-rank test); (c) combined Cav-1 expression: T(++)/S(−) *vs* Others 1 ($P < 0.001$, log-rank test); and (d) combined Cav-1 expression: T(+)/S(−) *vs* Others 2 ($P = 0.662$, log-rank test). Disease-free survival curves of the testing cohort stratified by (e) combined Cav-1 expression: T(++)/S(−) *vs* Others ($P < 0.001$, log-rank test).

status ($P > 0.05$). T(++)/S(−) Cav-1 expression was positively associated with tumor size and histological nodal status ($P < 0.001$ and 0.012, respectively). No significant correlations were found between T(++)/S(−) Cav-1 expression and age, tumor stage, grade, ER, PgR or HER2 status ($P > 0.05$).

Prognostic value of Cav-1 expression in the training cohort. Figure 2a–d illustrates the Kaplan–Meier curves of disease-free survival for the training cohort constructed on the basis of the Cav-1 expression level. T(++), S(−) and T(++)/S(−) status correlated closely with poor disease-free survival ($P = 0.009$, 0.019 and <0.001, respectively). No major differences were observed between S(+) and S(++) or between T(+) and T(−) Cav-1 expression on the predictive value of disease-free survival ($P > 0.05$; data not shown). T(+)/S(−) status also did not influence disease-free survival.

Kaplan–Meier analysis demonstrated a significant impact of certain clinicopathological prognostic factors such as HER2, tumor stage and ER on disease-free survival ($P = 0.015$, 0.010 and 0.010, respectively). No significant correlations were found between disease-free survival and other clinicopathological factors, including PgR, histological nodal status, grade and age ($P > 0.05$; Table 3). Cox analysis was performed to evaluate whether the correlation between Cav-1 expression and disease-free survival was related to the correlation of Cav-1

Table 3. Univariate analyses of factors associated with recurrence in the training and testing cohorts

Variable	Disease-free survival (*P*)
Training cohort	
Tumor/stromal Cav-1 expression T(++)/S(−) *vs* others	<0.001
Stromal hot-spot Cav-1 expression S(++/+) *vs* S(−)	0.009
Tumor stage (T0/T1 *vs* T2/T3/T4)	0.010
ER (positive *vs* negative)	0.010
HER2 (positive *vs* negative)	0.015
Tumor hot-spot Cav-1 expression T(++) *vs* T(+/−)	0.019
PgR (positive *vs* negative)	0.054
Histological nodal (positive *vs* negative)	0.069
Age (>50 *vs* ≤50)	0.411
Testing cohort	
Tumor/stromal Cav-1 expression T(++)/S(−) *vs* others	<0.001
Histological nodal status (positive *vs* negative)	<0.001
Grade (1 *vs* 2 *vs* 3)	<0.001
HER2 (positive *vs* negative)	<0.001
Tumor stage (T0/T1 *vs* T2/T3/T4)	0.008
ER (positive *vs* negative)	0.020
PgR (positive *vs* negative)	0.054
Age (>50 *vs* ≤50)	0.376

Cav-1, caveolin-1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor; S, stromal hot spot; T, tumor hot spot.

Table 4. Multivariate analysis of factors that might affect disease-free survival in the training and testing cohorts

	Disease-free survival		
	HR	95% CI	*P*
Training cohort			
Stromal hot-spot Cav-1 expression	0.322	0.098–1.065	0.063
ER	1.092	0.306–3.901	0.893
HER2	3.362	1.035–10.923	0.044
Tumor stage	7.772	0.985–61.288	0.052
Histological nodal status	1.536	0.900–2.619	0.115
Grade	3.807	1.018–7.472	0.021
Tumor hot spot-Cav-1 expression	0.370	0.108–1.269	0.114
ER	0.844	0.248–2.867	0.785
HER2	4.778	1.337–17.081	0.016
Tumor stage	6.437	0.806–51.392	0.079
Histological nodal status	1.406	0.814–2.430	0.222
Grade	0.322	1.220–11.881	0.046
Tumor/stromal Cav-1 expression	0.041	0.006–0.297	0.002
ER	0.774	0.232–2.580	0.677
HER2	3.665	1.037–12.954	0.044
Tumor stage	7.234	0.900–58.129	0.063
Histological nodal status	1.327	0.753–2.341	0.328
Grade	5.868	1.523–22.614	0.010
Testing cohort			
Tumor/stromal Cav-1 expression	0.249	0.107–0.582	0.001
ER	0.846	0.362–1.982	0.701
HER2	2.141	0.875–5.238	0.096
Tumor stage	1.551	0.965–2.494	0.070
Histological nodal status	4.124	1.517–11.208	0.005
Histological grade	3.150	1.416–7.007	0.005

Cav-1, caveolin-1; CI, confidence interval; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hazard ratio.

expression with other prognostic factors. The results revealed that S(−) Cav-1 expression was not an independent prognostic factor for disease-free survival ($P = 0.063$). No significant correlation was found between T(++) Cav-1 expression and disease-free survival ($P = 0.114$). Consistent with the univariate analysis results, the multivariate analysis revealed that combined T(++)/S(−) status was strongly associated with an unfavorable prognosis ($P = 0.002$; Table 4). We conducted further subgroup analysis stratified by histological nodal, ER and HER2 status, which were associated with poor clinical outcomes. As a result, S(−) Cav-1 showed clear trends for predicting disease-free survival in histological node+ patients ($P = 0.008$). However, T(+) Cav-1 expression did not influence disease-free survival in histological node+ patients ($P = 0.088$). Notably, histological node+ patients with T(++)/S(−) Cav-1 expression exhibited lower disease-free survival ($P = 0.001$). Furthermore, T(++)/S(−) Cav-1 expression also served as an important predictor of disease-free survival for ER+ and HER2+ patients ($P = 0.001$ and 0.045, respectively). Moreover, patients with T(++)/S(−) Cav-1 expression who were in the ER−, HER2−, PgR (+ and −), tumor size (>5 and ≤5 cm), age (>50 and ≤50 years) and grade (Grade 2 and 3) subgroups had poorer disease-free survival (Table S2).

Validation of the prediction power of T(++)/S(−) Cav-1 status in the testing cohort. In the testing cohort, all patients were also Japanese women and their clinicopathological characteristics are listed in Table 1. T(++)/S(−) Cav-1 expression was observed in 11% and other expressions were observed in 89% breast cancer patients. T(++)/S(−) Cav-1 expression was significantly related to tumor size ($P < 0.001$), tumor stage ($P = 0.033$) and histological nodal status ($P = 0.002$). We could not find a statistically significant association between T(++)/S(−) Cav-1 expression and age, grade, ER, PgR or HER2 ($P > 0.05$; Table 2).

We then examined the association of T(++)/S(−) Cav-1 expression with the clinical outcome. Kaplan–Meier survival analysis showed patients with T(++)/S(−) Cav-1 expression had shorter disease-free survival than those with other expressions ($P < 0.001$, Fig. 2e). The significant impact of clinicopathological prognostic factors such as tumor stage, ER and HER2 on disease-free survival ($P = 0.008$, 0.020 and <0.001, respectively) was also validated. These results were consistent with the above findings. In addition, a significant correlation between disease-free survival and grade or histological nodal status was observed in the testing cohort ($P < 0.001$ and <0.001, respectively; Table 3).

Table 4 provides the multivariate analyses of factors related to patient disease-free survival. Cox analysis indicated that T(++)/S(−) Cav-1 status was an independent predictor of disease-free survival ($P = 0.001$), as were histological nodal status ($P = 0.005$) and grade ($P = 0.005$). Moreover, the role of T(++)/S(−) Cav-1 expression in disease-free survival in the ER (+ and −), HER2 (+ and −), PgR (+ and −), tumor size (>5 and ≤5 cm), age (>50 and ≤50 years) and grade (Grade 1 + 2 and 3) subgroups is shown in Table S2.

Discussion

The Cav-1 gene is colocalized at the D7S522 locus on human chromosome 7q31.1 and is commonly deleted in breast, colon, kidney, prostate, ovary, head and neck cancers. Thus, it seems feasible to propose that the Cav-1 gene might serve as a candidate tumor suppressor gene.[21] In the present study, we focused on breast cancer patients to determine the correlation of tumor/stromal Cav-1 expression with clinicopathological parameters and survival. Cav-1 expression was evaluated semiquantitatively based on the proportion of positively stained tumor and stromal cells. We found that tumor Cav-1 demonstrated a prevalent membrane pattern associated with cytoplasm positive and that T(−) Cav-1 expression was noted in 16% of

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cases, which is consistent with previous reports.[22] The stromal Cav-1 expression rate and pattern in the present study are similar to a previous report.[23]

According to previous reports, total tumor Cav-1 expression has no prognostic value in primary breast cancer patients.[18,24] In the present study, we examined stromal hot-spot, total stromal and tumor hot-spot Cav-1 expression. First, we analyzed correlations between stromal hot-spot and total stromal and tumor Cav-1 expression and found a weak but significant correlation between stromal and tumor expression, indicating that Cav-1 expression could be regulated differently between tumor and stromal cells and that Cav-1 might influence different functions in those cells.[25,26] T(++) Cav-1 expression was positively associated with tumor size and histological nodal status. Previous reports revealed that tumor Cav-1expression was negatively associated with HER2 status.[27–29] Several studies have indicated that Cav-1 might function as a negative signal transduction regulator to HER2/neu and that it might play a negative regulatory role in mammary tumor development. In addition, activation of HER2/neu might downregulate Cav-1 expression *in vitro*.[30,31] However, this finding was not supported by our results. Stromal Cav-1 expression showed no significant correlation with any of the clinicopathological parameters, which was inconsistent with a previous report,[18] and therefore we focused on combined tumor/stromal Cav-1 expression. T(++)/S(−) was observed frequently in large-size tumors and histological node+ cases, indicating that tumor/stromal Cav-1 expression is involved in breast cancer progression. Interestingly, survival analyses revealed that patients with T(++)/S(−) Cav-1 expression had the shortest disease-free survival among various Cav-1 expression subgroups. Multivariate analysis confirmed an independent prognostic value of the combined status. Consistent with these results, T(++)/S(−) Cav-1 expression was significantly related to tumor size, histological nodal status and disease-free survival in the testing cohort. Besides, the positive correlation between T(++)/S(−) Cav-1 expression and tumor stage was also indicated in the testing cohort. A possible explanation for the discrepancy could be due to the difference in sample size. Furthermore, T(++)/S(−) Cav-1 expression also impacted the clinical outcomes stratified by ER status, PgR status, HER2 status, tumor size, age, histological nodal status and grade in both the training and testing cohorts. Therefore, these results indicate that combined Cav-1 status has a more potent prognostic value than either stromal or tumor hot-spot alone. We believe that these results are important when considering breast cancer biology. Given the limited number of cases, prospective studies with long-term follow-up data are warranted.[32]

Tumor Cav-1 expression with respect to tumorigenesis seems more complex than originally believed. Cav-1 loss-of-function induces ligand-independent hyperactivation of Ras-p42/44 MAPK and Smad signaling pathways as well as enhanced matrix metalloproteinase-2/9 secretion. Each of these pathways is likely to contribute to cell cycle progression, growth factor independence, cell invasiveness and epithelial–mesenchymal transition.[33] Despite extensive evidence supporting the role of Cav-1 as a tumor suppressor, several studies have suggested an alternative view of Cav-1 expression in tumors. In breast cancer, Cav-1 protects tumor cells from anoikis, promotes tumor cell survival and abrogates detachment-induced p53 activation.[34,35] Furthermore, Cav-1 expression is upregulated in multidrug-resistant MCF-7 cells.[36,37] A hypothesis has been proposed to explain the divergent roles of Cav-1; even if an initial loss of Cav-1 is observed in breast cancer, re-expression of Cav-1 at later stages might correlate with more malignant characteristics.[35,38]

Stromal Cav-1 plays a vital role in tumorigenesis. Loss of stromal Cav-1 is an independent predictor for therapeutic resistance and poor prognosis in primary breast cancers.[12,18,19,39] Woodman *et al.*[12] reported that endothelial cells from Cav-1−/− mice exhibit a disrupted response to angiogenic growth factors. Senescent human diploid fibroblasts exhibit increased levels of the Cav-1 protein.[10] In addition, loss of Cav-1 in stromal cells of various organs directly leads to disorganised stromal compartments and dysfunctional organ systems.[40]

Furthermore, recent studies have revealed a role played by Cav-1 in the interaction between tumor and stromal cells in breast cancer. During tumor formation, cancer cells and adjacent fibroblasts are metabolically coupled. A new model has been proposed in which glycolytic CAF promote tumor growth by secreting energy-rich metabolites that can be taken up by adjacent tumor cells.[41] Loss of Cav-1 *in vitro* induces metabolic coupling between CAF and tumor cells and leads to the formation of a host–parasite relationship. Martinez-Outschoorn *et al.*[42] showed that Cav-1 expression is downregulated in fibroblasts co-cultured with MCF-7 cells and that it mediates autophagic/lysosomal degradation. Furthermore, autophagy induced by loss of Cav-1 in fibroblasts provides cancer cells with essential chemical building blocks.[42,43] Loss of stromal Cav-1 fibroblasts protects adjacent cancer cells via decreased apoptosis, increased TP53-induced glycolysis and apoptosis regulator expression.[44] Furthermore, loss of Cav-1 induces oxidative stress in CAF, which is the root cause of mitochondrial dysfunction in CAF and promotes DNA damage. In the present study, the predictive value of T (++)/S(−) was demonstrated in luminal-type cancers and HER2+ cancers. Its value was stratified by an intrinsic subtype and warrants an examination with a greater number of cases.

The regulatory mechanism of Cav-1 expression in breast cancer remains to be elucidated. Pro-autophagic stimuli such as hypoxia, oxidative stress and nuclear factor κB activation might cause the loss of Cav-1.[45] Conversely, multiple factors are present during Cav-1 upregulation.

In conclusion, we provide evidence that T (++)/S(−) Cav-1 expression is closely associated with unfavorable prognostic outcomes in primary breast cancer patients. This particular subgroup seems to be engaged in rapid disease progression. Further studies involving analysis of molecular mechanisms of Cav-1 expression are required. The interaction between tumor and stromal cells and Cav-1 in the tumor microenvironment is also a key issue to investigate. Moreover, new therapies targeting Cav-1 expression might be a novel therapeutic approach, particularly for patients with T (++)/S(−) Cav-1 status.

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Disclosure Statement

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