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Original Article

Significance of Pathological Evaluation for Lymphatic Vessel Invasion in Invasive Breast Cancer

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Background: Lymphatic vessel invasion (LVI) has been conventionally assessed on hematoxylin-eosin (HE) stained sections, but this assessment tends to be subjective. The aim of this study is to investigate the significance of LVI in invasive breast cancers, primarily using immunohistochemical lymphatic endothelial markers.

Methods: We studied 69 invasive breast carcinoma cases. Using D2-40 and podoplanin, we investigated the distribution of lymphatic vessels around the tumor and LVI, and they were compared with the HE sections. The correlation between LVI, lymph node metastasis and disease free survival (DFS) was also investigated.

Results: Lymphatic vessels were most frequently seen outside the tumor (86%), whereas lymphatic vessels were not seen in the central zone of the tumor. LVI was found in 22 cases, of which nineteen was seen in the peripheral zone (87%). For both HE and lymphatic markers, the rates of mild LVI tended to be high. The concordance rate between D2-40 and podoplanin was 94.2% (65/69). LVI assessed on HE sections was corresponded to 54/69 cases (78.2%) using either D2-40 or podoplanin. There were 25 axillary lymph node positive cases. Lymph node metastasis significantly correlated with LVI assessed by HE section, but did not correlate with LVI assessed by the lymphatic markers. The tumor recurred in 19 cases during the mean follow-up period of 47.5 months. Disease free survival was significantly better for LVI negative cases on HE analysis, and LVI negative or mildly positive by any staining procedure.

Conclusion: The lymphatic endothelium markers, D2-40 and podoplanin, are very useful for detecting LVI, but careful examination by routine HE sections may be enough for routine practice. Moderate or marked degree of LVI may be of value to predict survival.

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Key words: Breast, Breast cancer, Lymph vessel invasion, Pathology, Immunohistochemistry

Introduction

Lymph node metastasis is one of the most important prognostic factors for breast carcinomas[1]. In general, lymph node metastasis initially occurs by migration of carcinoma cells into the lymphatic vessels at the primary site. Then carcinoma cells are carried to regional (axillary in most cases) lymph nodes through the lymphatic system as tumor emboli. Thus, the recognition of peritumoral lymphatic vessel invasion (LVI) on histological sections is very important. Indeed, LVI has been included as new risk factors for patients who have undergone surgery for breast cancer at the St. Gallen consensus meeting in January 2005[2], and the presence of peritumoral LVI may be a predictor of postoperative prognosis[3-7].

Histopathologically, LVI has routinely analyzed using hematoxylin and eosin (HE) slides. LVI is recognized as tumor cell nests floating within empty spaces, which are surrounded by thin, spindle-shaped endothelial cells. In addition, immunohistochemical markers specific for lymph vessel

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Abbreviations:
HE; hematoxylin-eosin, LVI; lymphatic vessel invasion, VEGF; vascular endothelial growth factor, LVD; lymph vessel density

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endothelium have been established recently. D2-40, podoplanin, LYVE-1, Prox-1, and vascular endothelial growth factor (VEGF)-3 have been examined, to detect LVI more objectively[6, 8-17]. The lymph vessel density (LVD) has been correlated with the patients' prognosis in various carcinomas, including breast, and LVI has been considered as prognostic indicator in some studies[6, 11-15]. However, utilization of an immunohistochemical approach to detect LVI in invasive breast carcinoma has not been elucidated well.

Thus, the aim of this study is to detect the lymph vessels as well as LVI in invasive breast carcinoma appropriately, in routine pathological practice. The distributions of lymph vessels are characterized and the clinicopathological significance of LVI by the different staining procedures (HE and immunohistochemistry) is compared.

Material and Method

We studied 69 cases of invasive breast carcinoma, resected at Tohoku University Hospital between 1992 and 1999. All cases were women, and the age distribution was between 27 and 80 years old (mean: 52.1 years). Sixty-eight cases underwent quadrantectomy, and subcutaneous mastectomy was performed for one case. After formalin fixation, serial sections at 5 mm intervals were made, and all were subject to routine pathological review. The histological findings of the main tumors are listed in Table 1. There were 60 cases of invasive ductal carcinoma, not otherwise specified, and 9 cases with a special histological type (4 of invasive lobular, 2 of mucinous, one each medullary, tubular, and invasive micropapillary). The tumor grade was determined according to the criteria of Elston and Ellis[18]. Analysis for hormone receptors was performed by enzyme immunoassay.

Axillary lymph node dissection was performed in 68 cases, and the number of lymph node per case ranged from 5 to 35 (median 16). Histological examination for lymph node metastasis was evaluated by examining a single section at the maximum diameter for each dissected lymph node. Lymph node metastasis was detected in 25 cases, with the number of positive nodes ranging from 1 to 21 (mean: 2). After surgery 43 patients received radiotherapy. Patients with positive axillary lymph node metastasis, or with surgical carcinoma exposure received radiotherapy from 1992 to 1996 (10 of 24 cases). After 1997 (-1999), patients with extensive intraductal components (>2 cm), moderately to marked degree of lymphatic vessel invasion on HE specimens, and/or bilateral cancers, in addition to those with the previous criteria, received radiotherapy (33 of 45 cases).

Table 1. Histopathological Characters of Examined Cases

	Number of cases
Histological type	
IDC-NOS	60
Papillotubular	10
Solid-tubular	3
Scirrhous	47
Special types	9
Tumor size	
<2cm	62
≥2cm	7
Histological grade	
G1	13
G2	28
G3	18
Hormone receptor	
Positive	32
Negative	13
Unknown	24
Lymph vessel invasion (LVI) by HE	
LVI+	17
LVI-	52
Lymph node metastasis	
Positive (n1-n2)	25
Negative (n0)	44

We evaluated the distribution of lymphatic vessels and the presence of lymphatic tumor invasion using a section of the primary tumor at maximal invasive diameter. After selecting the appropriate tissue block, it was stained with hematoxylin-eosin (HE) and the immunohistochemical stains anti-D2-40 as well as anti-podoplanin. For immunostains, formalin-fixed and paraffin-embedded sections were cut at 4 μm intervals, deparafinized in xylene, and hydrated with graded alcohols and distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 minutes. Antigen retrieval was performed for D2-40 using microwaves in a citrate buffer for 15 minutes and subsequent washing in phosphate-buffered saline. Sections were incubated with anti-D2-40 monoclonal antibody (Signet Laboratories,

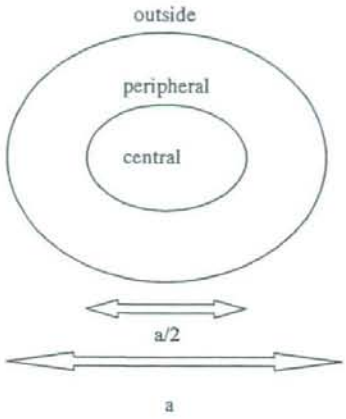

Fig 1. Distribution of lymph vessels on breast carcinoma: The maximum diameter of invasive carcinoma was divided into inner (central) and outer half (peripheral). The number of the lymph vessels was calculated individually using anti-D2-40 immunohistochemistry. The breast parenchyma surrounding and just close to the tumor (outside of the tumor) was also evaluated.

Dedham, MA, USA, dilution 1:200) or anti-podoplanin monoclonal antibody (Angiobio, Del Mar, CA, USA, dilution 1:100), for 16 hours at 4℃. The antigen-antibody reaction was visualized by Envision (DAKO, Carpentaria, CA, USA) for D2-40 and a histo-fine kit (Nichirei, Tokyo, Japan) and diaminobenzidine was used as the chromogen. After that, the sections were counterstained with hematoxylin.

The numbers of lymphatic vessels within and outside the tumor, and the distribution of LVI were calculated by anti-D2-40 immunohistochemistry. The distribution within the tumor was subdivided into an inner half (central zone) and outer half (peripheral zone) (Fig 1). The outside of the tumor implied that the breast parenchyma surrounded and was close to the tumor. Under middle power magnification, the number of the vessels was calculated using the micrometer. At least four sets of 1 mm squares were calculated, and the average was employed as the score.

LVI was assessed on both HE and immunohistochemical stained sections (D2-40 and podoplanin) individually. The degree of LVI was semiquantitatively scored as mild (one positive lymph vessel within the specimen), moderate (two or three positive lymph vessels) and marked (four or more positive lymph vessels) at the section of maximum tumor diameter. The number of the cases with positive/negative LVI and the degree of positive LVI was compared according to the different staining procedures. In addition, the presence as well as the degree of LVI positivity was compared with lymph node metastasis and disease free survival.

The statistical analysis was performed using the chi-square test. Univariate analysis of disease-free survival was performed by the log-rank test, and $p<0.05$ was considered significant.

Results

The lymph vessels were not easily detected on the HE specimen, but sometimes they were noticed on the HE specimen if they were dilated. The lining of thin endothelial cells was essential. However, immunohistochemiscal procedures more easily revealed the lymph vessels (Fig 2). Additionally, LVI was recognized if the carcinoma cells were circumscribed by thin endothelial cells, that were positive for either D2-40 or podoplanin (Fig 3) .

Lymphatic vessels detected by D2-40 were most frequently seen outside the tumor (87.0%), less frequently in the peripheral zone (13.0%), and not seen at all in the central zone. LVI was found in 13 cases, and was most frequently seen in the peripheral zone (81.3%), and less frequently outside (18.7%) (Table 2) . In addition, LVI was statistically seen more frequently in the peripheral zone, rather than the outside tumor area which contains most of the lymph vessels ($p<0.001$) .

LVI as assessed by HE and immunohistochemical procedures (D2-40 and podoplanin) is shown in Table 3. The frequency of LVI positive cases was not significantly different among the various staining procedures (17/69 by HE ; 16/69 by D2-40 and podoplanin). Mild LVI was more frequent than marked LVI by any of the staining procedures examined. There were 25 axillary lymph node positive cases (36.2%). The proportion of node metastatic cases by LVI status and staining procedure is summarized in Table 3. LVI positive cases were more frequently associated with lymph node metastasis than LVI negative cases by any staining procedure, but only statistically significant ($p<0.05$) on HE staining.

The results of LVI assessed by HE corresponded to 54/69 cases (78.2%) assessed by either D2-

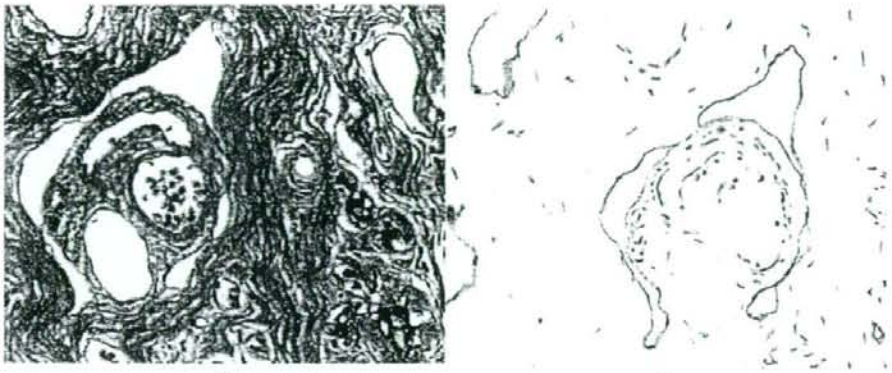

Fig 2. Histological detection of lymph vessels. Empty spaces close to the blood vessels, with thin, endothelial cell lining on HE (A). However, it is easier to recognize by immunohistochemistry (anti-D2-40) (B).

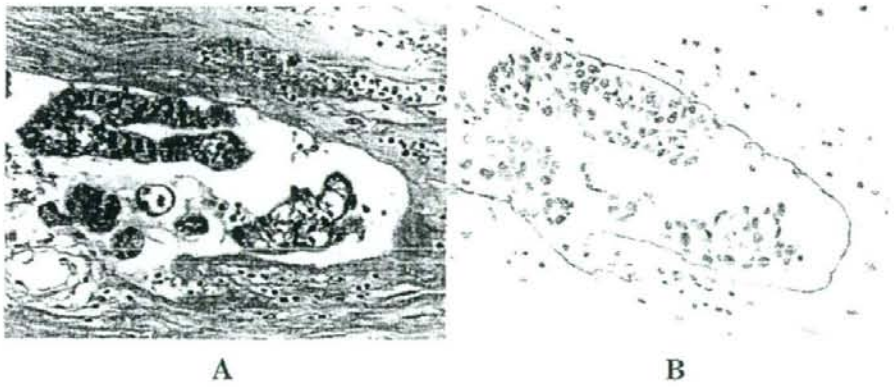

Fig 3. Lymph vessel invasion (LVI). The recognition of lymphatic vessel invasion, by HE (A) and immunohistocemistry (anti-D2-40) (B).

Table 2. Distribution of Lymphatic Vessel and Lymphatic Vessel Invasion

Number of case (%)	Central zone	Peripheral zone	Outside the tumor
Lymphatic vessels	0	9 (13.0%)	60 (87.0%)
Lymphatic vessel invasion	0	13 (81.3%)	3 (18.7%)

Table 3. Presence and Degree of LVI, and the Proportion of Positive Node Metastasis Cases among Various Lymphatic Vessel Invasion Status, by Different Staining Procedures

	LVI(-)	LVI(+)	mild	moderate	marked
HE	15/52* (28.8%)	10/17* (58.8%)	5/9 (55.6%)	4/6 (66.7%)	1/2 (50.0%)
D2-40	16/53 (30.2%)	9/16 (56.2%)	5/11 (45.4%)	3/3 (100%)	1/2 (50.0%)
podoplanin	16/53 (30.2%)	9/16 (56.2%)	5/12 (41.7%)	2/2 (100%)	2/2 (100%)

LVI: Lymphatic vessel invasion *: $p<0.05$

Table 4. Comparison of the Results of LVI by Different Staining Procedures

A: HE vs, D2-40			B: HE vs. podoplanin			C: D2-40 vs. podoplanin		
	D2-40 LVI(+)	D2-40 LVI(-)		Podoplanin LVI(+)	Podoplanin LVI(-)		Podoplanin LVI(+)	Podoplanin LVI(-)
HE LVI(+)	9	8	HE LVI(+)	9	8	D2-40 LVI(+)	14	2
HE LVI(-)	7	45	HE LVI(-)	7	45	D2-40 LVI(-)	2	51
$P<0.001$			$P<0.001$			$P<0.001$		

LVI: lymphatic vessel invasion

40 or podoplanin staining ($p<0.001$). The concordance rate between D2-40 and podoplanin was 94.2% (65/69) (Table 4) ($p<0.001$). The reasons for discrepancy between HE and immunohistochemistry were retraction artifacts on HE, overestimation of invasive micropapillay pattern on HE, or the different level of the tissue examined.

The carcinomas recurred in 19 cases (27.5%), by the mean follow-up period of 47.5 months. These included 3 cases of regional lymph node recurrences, 4 cases of ipsilateral breast recurrence, and 12 cases of distant metastasis (7 of bone, 4 of lung and one of liver). LVI negative cases, by any staining procedure, showed significantly better DFS than LVI positive cases ($p<0.001$) (Fig 4). In addition, moderate or marked LVI cases were much worse DFS than either LVI negative or mild, in any staining procedures ($p<0.001$) (Fig 5). The incidence of radiotherapy was not statistically different between LVI positive (13/17; 76.5%) and LVI negative cases (31/52; 59.6%).

Discussion

It is well known that lymph node metastasis is one of the most significant prognostic markers for patients with breast carcinoma[1]. Although the clinical significance of minute metastasis in sentinel nodes had not been elucidated well, the presence of obvious metastasis (more than 2 mm in diameter) as well as the numbers of positive nodes may be reliable and independent prognostic indicators[2, 19].

LVI and LVD may be additional parameters associated with lymph node status. Both have been considered to be of prognostic significance in breast carcinoma cases[6, 8-10, 14]. However, lymph vessels were not evident within the tumor (intratumor)[8], as in this study. Even if they exist ed in an entrapped manner, their proliferative index was minimal. In addition, the intratumoral LVD was less than in non-malignant breast lesions[8]. Thus, it have been speculated that lymph angiogenesis does not occur in the invasive areas of breast carcinoma. Indeed, a "hot spot" , the area of the the greatest number of distinct highlighted microvessels, have been used to analyze the clinicopathological significance of LVD[6].

Most of the lymph vessels visualized in invasive breast carcinoma were located outside the tumor, followed by the peripheral portion of the invasive component. However, most of the LVI was seen at the peripheral portion, especially at the peritumoral area. Almost all of the cases in this study underwent breast conserving surgery. If the cases with more advanced stage (carcinomas with extensive LVI such as inflammatory carcinoma) were to be added, the proportion of the LVI-positive zones would change.

Immunohitochemical markers specific for the endothelial cells of the lymph vessels are currently used in pathological practice. Among the various antibodies, anti-D2-40 and anti-podoplanin are relatively widely used, and they are specific for lymphatic endothelium but do not stain vascular endothelial cells[6, 8, 11-13, 15-17, 20]. So, immunohistochemical procedures may avoid underestimation of LVI by tumor emboli which completely fill lymph vessels, or overestimation of the retraction artifacts caused by fixation, or overestimation of invasive micropapillary patterns. However, LVI assessed by HE corresponded relatively well to immunohistochemical procedures (54/69 cases; 78.3%; either to D2-40 or podoplanin). The concordance rate between D2-40 and podoplanin was also high (65/69; 94.2%). Thus, careful evaluation of the peri-tumoral area on HE slides may be a convenient and reliable method to detect LVI.

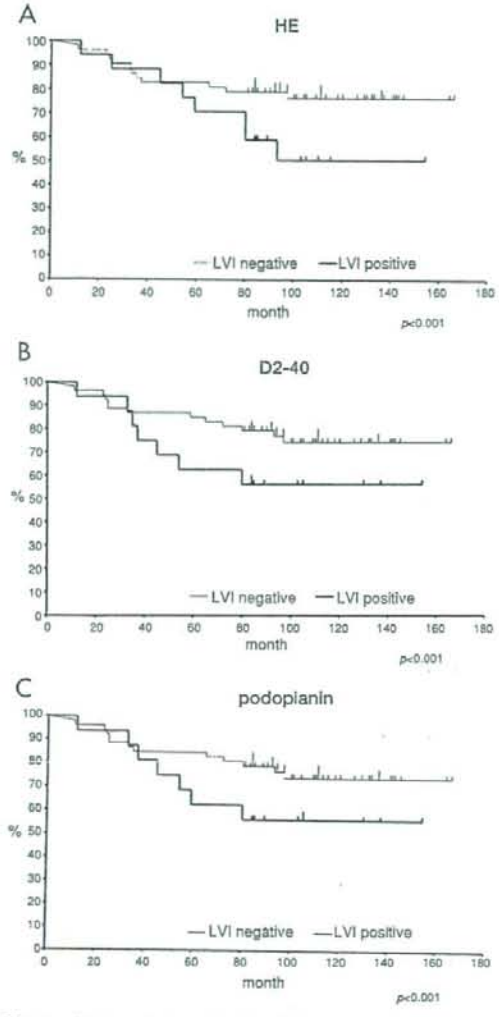

Fig 4. Disease free survival in 69 breast carcinoma cases with (LVI+) or without (LVI-) lymphatic vessel invasion. A: HE stain, B: D2-40, C; podoplanin.

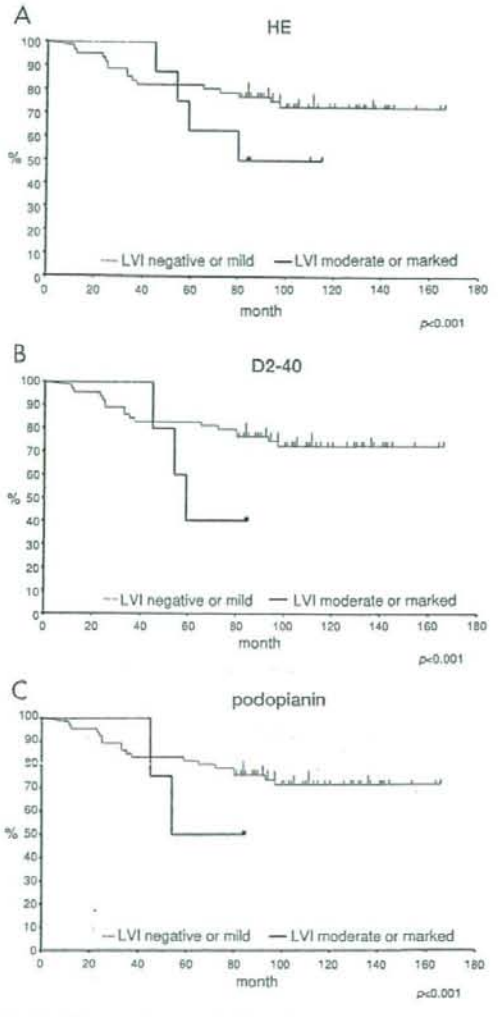

Fig 5. Disease free survival in 69 breast carcinoma cases with moderate to marked lymphatic vessel invasion, or with none or mild lymphatic invasion. A: HE stain, B: D2-40, C; podoplanin.

Another advantage of examining LVI in routine practice is to predict recurrence and/or metastasis after breast conserving surgeries. Especially, a moderate or marked degree of LVI correlated with a significantly worse prognosis (DFS) by any staining method examined. A significant difference between positive and negative LVI was evident only for the HE examination. Since mild LVI in this study was defined as only one positive lymph vessel on the glass slide, it may detect LVI by chance occurrence. Thus, for predicting patients' survival, the estimation for more than moderate LVI is a more reliable criterion.

In addition, moderate to marked LVI may be an indicator of postoperative irradiation after breast conserving surgery. Indeed, our initial criteria to avoid irradiation were pathological margin and node negativivity, but some locally recurred during the follow-up period. Thus we have changed the criteria, and now include moderate to marked

LVI as an indicator for irradiation. The recurrence rate was significantly reduced, although the follow-up period was limited[21].

In conclusion, the lymphatic endothelium markers D2-40 and podoplanin, are very useful and more objective methods for detecting LVI, but careful examinations by routine HE sections may be sufficient for routine practice, if one knows the distribution of lymph vessels and LVI in the tumor. In addition, moderate to marked LVI may predict the patients' survival.

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Nuclear cyclin B1 in human breast carcinoma as a potent prognostic factor

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Cyclin B1 is translocated to the nucleus from the cytoplasm, and plays an essential role in cell proliferation through promotion of mitosis. Although overexpression of cyclin B1 was previously reported in breast carcinomas, the biological significance of the intracellular localization of cyclin B1 remains unclear. Therefore, in this study, we examined cyclin B1 immunoreactivity in 109 breast carcinomas, according to the intracellular localization, that is, nucleus, cytoplasm or total (nucleus or cytoplasm). Total cyclin B1 was detected in carcinoma cells in 42% of breast carcinomas examined, whereas nuclear and cytoplasmic cyclin B1 were positive in 17 and 35% of the cases, respectively. Total or cytoplasmic cyclin B1 were positively associated with histological grade, mitosis, Ki-67, p53, c-myc or 14-3-3σ, and inversely correlated with estrogen or progesterone receptor. Nuclear cyclin B1 was significantly associated with tumor size, lymph node metastasis, histological grade, mitosis, Ki-67 or polo-like kinase 1. Only nuclear cyclin B1 was significantly associated with adverse clinical outcome of the patients, and multivariate analyses of disease-free and overall survival demonstrated nuclear cyclin B1 as the independent marker. A similar tendency was detected in the patients receiving adjuvant therapy after surgery. These results suggest that an onocogenic role of overexpressed cyclin B1 is mainly mediated in nuclei of breast carcinoma cells, and the nuclear translocation is regulated by polo-like kinase 1 and 14-3-3σ. Nuclear cyclin B1-positive breast carcinoma is resistant to adjuvant therapy, and nuclear cyclin B1 immunoreactivity is a potent prognostic factor in breast carcinoma patients. (*Cancer Sci* 2007; 98: 644–651)

Breast cancer is one of the most common malignancies in women worldwide. Invasive breast cancer has been generally regarded as a disease that metastasizes in an early phase, and clinical outcome of breast carcinoma patients is markedly influenced not only by metastasis of the tumor but also by proliferation activity of the tumor.[1] In fact, a multitude of prognostic factors identified for breast cancer have been demonstrated to be directly or indirectly related to proliferation of breast carcinoma cells.

It is well-known that proliferation of carcinoma cells is closely associated with altered regulation of the cell cycle.[2] Cell cycle progression is mediated by activation of a highly conserved family of cyclin-dependent kinases (Cdk),[3] and activation of a Cdk requires binding to a specific regulatory subunit, named a cyclin. Among the cyclins, cyclin B1 plays an essential role as a mitotic cyclin in the entry of mitosis from G_2 phase.[4] Overexpression of cyclin B1 has been reported in various human tumors, and some of these studies demonstrated the clinical significance of cyclin B1 as a poor prognostic factor for some cancers,[5–7] including lymph node-negative breast carcinoma.[8]

Cyclin B1 is initially localized in the cytoplasm, and is translocated to the nucleus at the beginning of mitosis.[9] Nuclear translocation of cyclin B1 is considered very important to facilitate access of the cyclin B–Cdc2 (also named Cdk1) complex to its nuclear substrate and promote mitosis.[4] Therefore, it becomes very important to examine the intracellular localization of cyclin B1 in tumor tissues, in order to obtain a better understanding of the biological roles of cyclin B1.[10] Previously, Winters *et al.* reported that nuclear cyclin B1 immunoreactivity was significantly associated with reduced disease-free survival of breast carcinoma patients in a log-rank analysis.[11] However, no other information is available regarding the intracellular localization of cyclin B1 in breast carcinoma tissue, and the biological significance of cyclin B1 remains unclear at this juncture. Therefore, in the present study, we examined the intracellular immunolocalization of cyclin B1, and correlated these findings with various clinicopathological parameters of the patients, including their clinical outcome.

Materials and Methods

Patients and tissues. One hundred and nine specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1984 to 1987 at the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from patients with a mean age of 53.1 years (range 23–82 years). The patients did not receive chemotherapy, irradiation or hormonal therapy prior to surgery. Review of the charts revealed that 85 patients received adjuvant chemotherapy (mitomycin C, methotrexate and fluorouracil, $n = 80$; cyclophosphamide, doxorubicin and fluorouracil, $n = 3$; and cyclophosphamide, mitomycin C and fluorouracil, $n = 2$). Seventeen patients received radiation therapy, and 12 patients received tamoxifen therapy after the surgery. The mean follow-up time was 106 months (range 4–157 months). The histological grade and tubule formation of each specimen was evaluated according to the method of Elston and Ellis.[12] All specimens were fixed with 10% formalin and embedded in paraffin wax. Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine.

Antibodies. A rabbit polyclonal antibody for cyclin B1 (H-433 [sc-752]) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). This antibody was raised against a recombinant peptide corresponding to amino acids 1–433 representing full-length human cyclin B1. Monoclonal antibodies for estrogen receptor α (ER; ER1D5), progesterone receptor (PR; MAB429), Ki-67 (MIB1), p53 (DO7) and c-myc (1-6E10) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), DAKO (Carpinteria, CA, USA), Novocastra Laboratories (Newcastle, UK) and Cambridge Research Biochemical (Cambridge, UK), respectively. Rabbit polyclonal antibodies for HER2 (A0485) and polo-like kinase 1.(PLK1; 06-813) were obtained

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from DAKO and Upstate Biotechnology (Lake Placid, NY, USA), respectively. Goat polyclonal antibody for 14-3-3σ (C-14 [sc-7683]) was purchased from Santa Cruz Biotechnology.

Immunohistochemistry. A Histofine Kit (Nichirei, Tokyo, Japan), which uses the streptavidin–biotin amplification method was used in this study. Antigen retrieval was carried out by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for cyclin B1, ER, PR, HER2, Ki-67 and p53 immunostaining, and antigen retrieval for PLK1 and 14-3-3σ immunostaining was done by heating the slides in a microwave oven for 15 min in the citric acid buffer. Dilutions of primary antibodies used in this study were as follows: cyclin B1, 1/500; ER, 1/50; PR, 1/30; HER2, 1/200; Ki-67, 1/50; p53, 1/200; c-myc, 1/600; PLK1, 1/1500; and 14-3-3σ, 1/1000. The antigen–antibody complex was visualized with 3.3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2), and counterstained with hematoxylin. As a negative control, normal mouse, rabbit or goat IgG was used instead of the primary antibodies, and no immunoreactivity was detected in these sections (data not shown).

Scoring of immunoreactivity and statistical analysis. Immunoreactivity of cyclin B1 was detected in the nucleus and cytoplasm, and was evaluated according to a report by Winters *et al.* with some modifications.[11] Briefly, cyclin B1 immunoreactivity was evaluated in the nucleus, cytoplasm or total (nucleus or cytoplasm) in more than 1000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity (i.e. the labeling index [LI]) was determined. ER, PR, Ki-67 and p53 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated as a LI. Cases with cyclin B1, ER, PR or p53 LI of more than 10% were considered positive in this study, according to a report for ER.[13] Immunoreactivity for c-myc, PLK1 and 14-3-3σ was detected in the cytoplasm, and cases that had more than 10% of positive carcinoma cells were considered positive. HER2 immunoreactivity was evaluated according to a grading system proposed in HercepTest (DAKO), and moderately or strongly circumscribed membrane staining of HER2 in more than 10% of carcinoma cells was considered positive.

An association between cyclin B1 immunoreactivity and clinicopathological factors was evaluated using a correlation coefficient (r) and regression equation, Student's t-test, or a one-way ANOVA and Bonferroni test. Overall and disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using the log-rank test. Univariate and multivariate analyses were evaluated by a proportional hazard model (COX) using PROC PHREG in SAS software.

Results

Immunolocalization of cyclin B1 in breast carcinoma tissues. Immunoreactivity for cyclin B1 was detected in the nucleus or cytoplasm of breast carcinoma cells (Fig. 1a,b), and the mean values of cyclin B1 LI in the 109 breast carcinoma tissues examined were 12.8% (range 0–56%) in total, 5.4% (range 0–18%) in the nucleus, and 10.1% (range 0–52%) in the cytoplasm. The number of cyclin B1-positive breast carcinomas (i.e. cyclin B1 LI of more than 10%) was 46 cases (42%) in total, 19 cases (17%) in the nucleus, and 38 cases (35%) in the cytoplasm, respectively. Immunoreactivity of cyclin B1 was also detected in some epithelial cells of morphologically normal mammary glands (Fig. 1c), but its LI was less than 1% in all of the intracellular components examined in this study.

Significant associations ($P < 0.0001$) were detected among cyclin B1 LI of the intracellular components, and their correlation coefficients were as follows: $r = 0.95$ (total vs cytoplasm), $r = 0.64$ (total vs nucleus), and $r = 0.51$ (nucleus vs cytoplasm).

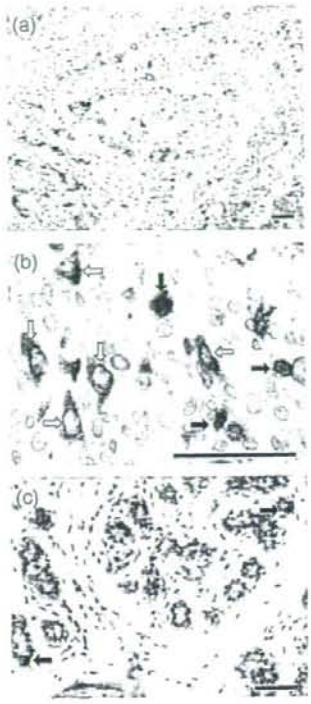

Fig. 1. Immunohistochemistry for cyclin B1 in the invasive ductal carcinoma. Cyclin B1 immunoreactivity was detected in the nucleus and/or cytoplasm of carcinoma cells: (a) lower magnification, (b) higher magnification. (b) Closed arrows represent nuclear cyclin B1 immunoreactivity, and open arrows show cytoplasmic cyclin B1 immunoreactivity. (c) In morphologically normal mammary glands, immunoreactivity for cyclin B1 was detected in some epithelial cells (arrows). Scale bar = 50 μm.

Association between cyclin B1 immunoreactivity and clinicopathological parameters in breast carcinoma. Associations between cyclin B1 immunoreactivity and clinicopathological parameters in 109 breast carcinomas are summarized in Table 1. Total cyclin B1 immunoreactivity was significantly associated with histological grade ($P = 0.001$), mitotic count ($P = 0.0001$) or Ki-67 LI ($P < 0.0001$), and inversely correlated with ER status ($P = 0.003$) or PR status ($P = 0.04$). There were no significant correlations between total cyclin B1 immunoreactivity and other clinicopathological parameters, such as patient age, menopausal status, clinical stage, tumor size, lymph node metastasis and HER2 status in this study.

However, immunoreactivity for nuclear cyclin B1 was positively associated with tumor size ($P = 0.01$), lymph node metastasis ($P = 0.003$), histological grade ($P = 0.003$), mitotic count ($P < 0.0001$) or Ki-67 LI ($P < 0.0001$), but no other significant association was detected. Cytoplasmic cyclin B1 immunoreactivity was positively associated with histological grade ($P = 0.001$), mitotic count ($P = 0.0001$) or Ki-67 LI ($P < 0.0001$), and an inverse association was detected between cytoplasmic cyclin B1 immunoreactivity and ER ($P = 0.003$) or PR status ($P = 0.01$), which was a similar tendency as that detected in the total cyclin B1 immunoreactivity.

Correlation between cyclin B1 immunoreactivity and its regulatory proteins in breast carcinoma. Previous studies have demonstrated that expression or intracellular localization of cyclin B1 is regulated by various proteins, including p53,[14,15] c-myc,[16]

Table 1. Association between cyclin B1 immunoreactivity and clinicopathological parameters in 109 breast carcinomas

Parameter	Cyclin B1 LI (%)					
	Total	*P*-value	Nucleus	*P*-value	Cytoplasm	*P*-value
Patient age*	$r = -0.14$	0.16	$r = -0.12$	0.20	$r = -0.11$	0.28
Menopausal status						
Premenopause ($n = 52$)	14.1 ± 1.9		6.0 ± 0.7		10.6 ± 1.6	
Postmenopause ($n = 57$)	11.7 ± 1.7	0.35	4.9 ± 0.6	0.23	9.6 ± 1.5	0.62
Clinical stage						
I ($n = 31$)	10.3 ± 2.4		3.3 ± 0.6		8.8 ± 2.1	
II ($n = 63$)	12.7 ± 1.9		5.9 ± 0.6		9.7 ± 1.7	
III ($n = 15$)	13.9 ± 4.3	0.68	5.3 ± 1.2	0.63	11.0 ± 3.8	0.86
Tumor size*	$r = 0.18$	0.08	$r = 0.24$	**0.01**	$r = 0.16$	0.10
Lymph node metastasis						
Positive ($n = 49$)	13.3 ± 1.8		6.9 ± 0.7		9.8 ± 1.5	
Negative ($n = 60$)	12.4 ± 1.1	0.70	4.3 ± 0.5	**0.003**	10.3 ± 1.5	0.83
Histological grade						
1 ($n = 29$)	5.5 ± 1.0		3.5 ± 0.7		5.0 ± 0.7	
2 ($n = 37$)	11.2 ± 1.8		5.0 ± 0.8		8.6 ± 1.4	
3 ($n = 43$)	18.1 ± 2.4	**0.001**	7.2 ± 0.7	**0.003**	14.4 ± 2.1	**0.001**
Mitotic count						
≤5 cells ($n = 34$)	3.6 ± 0.6		1.7 ± 0.4		3.1 ± 0.6	
5 < cells ≤ 10 ($n = 54$)	15.4 ± 1.8		6.7 ± 0.6		11.7 ± 1.6	
>10 cells ($n = 21$)	21.3 ± 3.1	**0.0001**	8.1 ± 0.7	**<0.0001**	17.1 ± 2.8	**0.0001**
ER status						
Positive ($n = 77$)	10.4 ± 1.2		4.9 ± 0.5		8.1 ± 1.0	
Negative ($n = 32$)	18.5 ± 3.0	**0.003**	6.7 ± 0.8	0.08	14.9 ± 2.6	**0.003**
PR status						
Positive ($n = 75$)	11.1 ± 1.4		5.1 ± 0.5		8.3 ± 1.1	
Negative ($n = 34$)	16.5 ± 2.6	**0.04**	6.1 ± 0.8	0.28	14.0 ± 2.3	**0.01**
HER2 status						
Positive ($n = 37$)	14.9 ± 2.2		6.1 ± 0.7		11.1 ± 1.9	
Negative ($n = 72$)	11.7 ± 1.5	0.24	5.1 ± 0.5	0.30	9.5 ± 1.3	0.49
Ki-67 LI*	$r = 0.51$	**<0.0001**	$r = 0.42$	**<0.0001**	$r = 0.56$	**<0.0001**

*The association was statistically evaluated utilizing a correlation coefficient (r) and regression equation. *P*-values less than 0.05 were considered significant, and are shown in bold. Mitotic count was evaluated in 10 high power fields. ER, estrogen receptor; LI, labeling index; PR, progesterone receptor.

PLK1[17,18] and 14-3-3σ.[19] Therefore, we next examined an association between the immunoreactivity of cyclin B1 and these proteins. As shown in Table 2, total cyclin B1 immunoreactivity was significantly associated with p53 ($P = 0.02$), c-myc ($P = 0.04$) and 14-3-3σ ($P = 0.001$), but not with PLK1. In contrast, nuclear cyclin B1 immunoreactivity was only correlated with PLK1 ($P = 0.02$). Cytoplasmic cyclin B1 was positively associated with p53 ($P = 0.01$), c-myc ($P = 0.01$) and 14-3-3σ ($P = 0.0002$), which was a similar tendency as in the total cyclin B1 immunoreactivity.

Association between cyclin B1 immunoreactivity and clinical outcome of breast carcinoma patients. No significant association was detected between total cyclin B1 immunoreactivity and risk of recurrence ($P = 0.11$) (Fig. 2a) or overall survival ($P = 0.24$) (Fig. 2b) in the 109 breast carcinoma patients examined. However, nuclear cyclin B1 immunoreactivity was significantly associated with an increased risk of recurrence ($P < 0.0001$) (Fig. 2c) and adverse clinical outcome of the patients ($P < 0.0001$) (Fig. 2d). Cytoplasmic cyclin B1 immunoreactivity was not significantly associated with clinical outcome of these patients ($P = 0.70$ in disease-free survival [Fig. 2e], and $P = 0.99$ in overall survival [Fig. 2f]) in our study. Nuclear cyclin B1 immunoreactivity was significantly associated with adverse clinical outcome of the patients showing high (more than 5 cells) mitotic count in breast carcinoma, but no significant association was detected between total or cytoplasmic cyclin B1 immunoreactivity and prognosis in these patients (Fig. 3).

Nuclear cyclin B1 immunoreactivity was also associated with an increased risk of recurrence and worse prognosis in the group of breast cancer patients who received adjuvant chemotherapy ($P < 0.0001$ in disease-free survival [Fig. 4a], and $P = < 0.0001$ in overall survival [Fig. 4b]), radiotherapy ($P = 0.003$ [Fig. 4c], and $P = 0.003$ [Fig. 4d]) or tamoxifen therapy ($P = 0.0002$ [Fig. 4e], and $P = 0.0002$ [Fig. 4f]) after surgery in this study.

Following univariate analysis by COX (Table 3a), lymph node metastasis ($P < 0.0001$), nuclear cyclin B1 immunoreactivity ($P = 0.0001$), tumor size ($P = 0.01$), 14-3-3σ ($P = 0.04$) and HER2 status ($P = 0.04$) were demonstrated to be significant prognostic parameters for disease-free survival in 109 breast carcinoma patients. A multivariate analysis (Table 3a) revealed that lymph node metastasis ($P = 0.0002$), nuclear cyclin B1 immunoreactivity ($P = 0.01$) and 14-3-3σ ($P = 0.01$) were independent prognostic factors with relative risks over 1.0.

For overall survival of the patients, lymph node status ($P = 0.0001$), nuclear cyclin B1 immunoreactivity ($P = 0.0001$), tumor size ($P = 0.01$), mitotic count ($P = 0.02$), c-myc ($P = 0.03$) and HER2 status ($P = 0.04$) turned out to be significant prognostic factors in a univariate analysis (Table 3b). However, multivariate analysis demonstrated that only lymph node status ($P = 0.004$) and nuclear cyclin B1 immunoreactivity ($P = 0.01$) were independent prognostic factors with a relative risk over 1.0, but other factors were not significant in this study (Table 3b).

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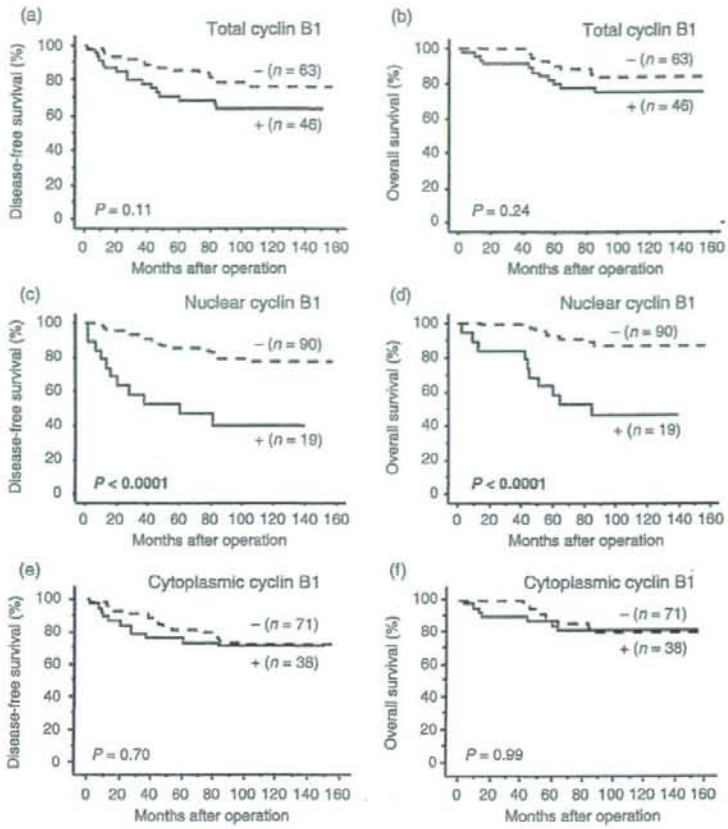

Fig. 2. Disease-free and overall survival of 109 patients with breast carcinoma according to the intracellular localization of cyclin B1 immunoreactivity (Kaplan–Meier method). Total cyclin B1 was not significantly associated with (a) disease-free or (b) overall survival. Nuclear cyclin B1 was significantly associated with (c) an increased risk of recurrence and (d) worse prognosis. Cytoplasmic cyclin B1 was not significantly associated with (e) disease-free survival or (f) overall survival. Statistical analysis was evaluated by a log-rank test. *P*-values less than 0.05 were considered significant, and are shown in bold.

Table 2. Association between cyclin B1 immunoreactivity and its regulatory proteins in 109 breast carcinomas

Immunoreactivity	Cyclin B1 LI (%)					
	Total	*P*-value	Nucleus	*P*-value	Cytoplasm	*P*-value
p53						
Positive ($n = 48$)	15.6 ± 2.3		6.4 ± 0.8		12.9 ± 2.0	
Negative ($n = 61$)	8.8 ± 1.6	**0.02**	4.9 ± 0.8	0.19	6.8 ± 1.2	**0.01**
c-myc						
Positive ($n = 50$)	16.5 ± 2.6		6.1 ± 0.8		14.0 ± 2.3	
Negative ($n = 59$)	11.1 ± 1.4	**0.04**	5.1 ± 0.5	0.28	8.3 ± 1.1	**0.01**
PLK1						
Positive ($n = 33$)	16.2 ± 3.1		6.9 ± 1.0		13.3 ± 2.7	
Negative ($n = 76$)	11.0 ± 1.5	0.11	4.5 ± 0.5	**0.02**	8.6 ± 1.3	0.09
14-3-3σ						
Positive ($n = 42$)	17.9 ± 2.3		5.6 ± 0.7		15.0 ± 2.0	
Negative ($n = 67$)	9.7 ± 1.3	**0.001**	5.3 ± 0.6	0.78	7.0 ± 1.1	**0.0002**

P-values less than 0.05 were considered significant, and are shown in bold. LI, labeling index.

In a univariate analysis, nuclear cyclin B1 immunoreactivity evaluated as a continuous variable was also a significant prognostic factor ($P < 0.0001$ in disease-free survival, and $P = 0.003$ in overall survival), and was an independent prognostic factor when it was included in a multivariate analysis instead of the dichotomized variable ($P = 0.03$ and $P = 0.001$, respectively).

Discussion

In the present study, cyclin B1 immunoreactivity was significantly associated with histological grade, mitotic count and Ki-67 LI in all intracellular components (i.e. total, nucleus and cytoplasm) of the breast carcinoma cases examined. Antibody Ki-67 recognizes

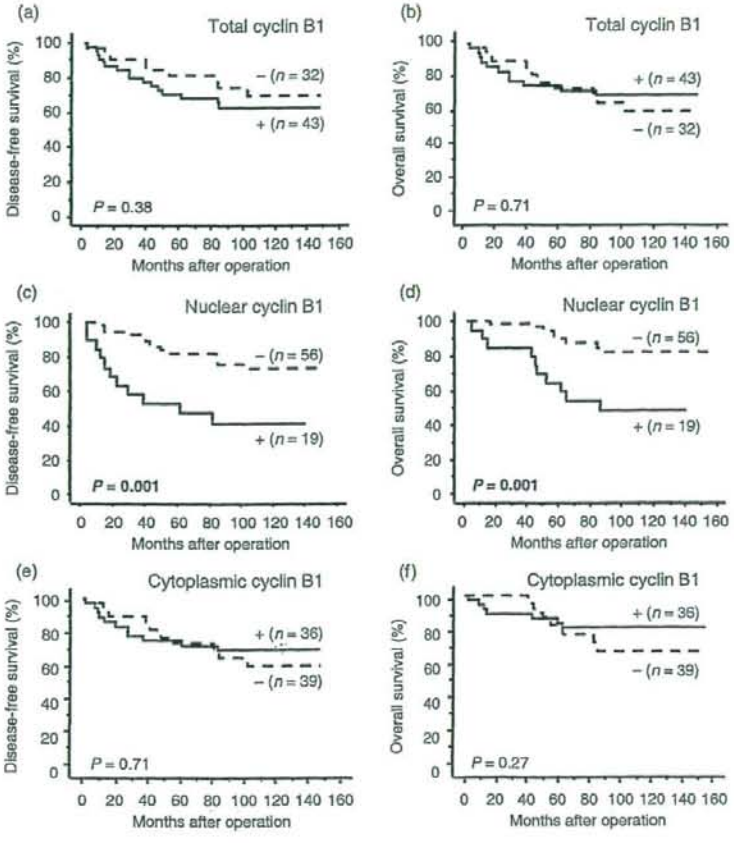

Fig. 3. Association between intracellular localization of cyclin B1 immunoreactivity and clinical outcome of the 75 patients showed high (>5 cells) mitotic count in the breast carcinoma (Kaplan–Meier method). There was no significant association between total cyclin B1 and (a) disease-free or (b) overall survival. In contrast, nuclear cyclin B1 was significantly associated with (c) an increased risk of recurrence and (d) worse prognosis in these patients. Cytoplasmic cyclin B1 was not significantly associated with (e) disease-free or (f) overall survival. Statistical analysis was evaluated by a log-rank test. *P*-values less than 0.05 were considered significant, and are shown in bold.

cells in all phases of the cell cycle except G_0 (resting) phase,[20] and Ki-67 LI is closely correlated with the S phase fraction and mitotic index.[1] Previously, Dutta *et al.* reported a positive correlation between cyclin B1 immunoreactivity and Ki-67 in breast carcinomas,[21] and Kuhling *et al.* showed that total cyclin B1 immunoreactivity is significantly associated with Ki-67 LI and histological grade in lymph node-negative breast carcinomas.[22] The results of our present study are in good agreement with these previous studies. Total cyclin B1 immunoreactivity is considered to reflect the physiological amount or aberrant expression of cyclin B1 protein,[22] and therefore, overexpression of cyclin B1 is postulated to play an important role in increased cell proliferation activity of human breast carcinoma.

The results of our study also demonstrated a significant association between total cyclin B1 and p53 or c-myc. Previous *in vitro* studies demonstrated that expression of cyclin B1 is suppressed by wild-type p53,[14,15,23] but is induced by mutant p53 or inactivation of p53.[24] The p53 antibody used in the present study (DO7) recognizes both the wild-type and mutated p53 proteins, but the accumulation of p53 protein is considered to be a good indicator of p53 mutation in breast carcinoma.[25] In addition, the *cyclin B1* gene is a direct transcriptional target of c-myc,[24] and overexpression of c-myc has been reported to induce cyclin B1 expression.[16] The results of our present study as well as the *in vitro* studies above all indicate that overexpression of cyclin B1 is, at least in part, regulated by mutant p53 and c-myc proteins in breast carcinoma.

In our present study, nuclear cyclin B1 was significantly associated with tumor size, lymph node metastasis and adverse prognosis, but total or cytoplasmic cyclin B1 was not associated with these clinicopathological factors. Regarding the relationship between intracellular localization of cyclin B1 and the clinical outcome of breast carcinoma, Winters *et al.* reported that both nuclear and cytoplasmic cyclin B1 were associated with reduced disease-free or overall survival in their univariate analyses, but a significant association was only detected between nuclear cyclin B1 and disease-free survival in log-rank analyses.[11] These findings were partly consistent with the results of our present study. Cytoplasmic cyclin B1 may induce mitosis, but it is much weaker than nuclear cyclin B1.[15] In addition, Nozoe *et al.*[10] reported that the prognosis in esophageal carcinomas with nuclear-dominant expression of cyclin B1 is significantly worse than that of tumors with cytoplasmic-dominant expression. Therefore, the malignant potential of cyclin B1 may be mainly mediated by nuclear cyclin B1 in breast carcinoma cells, and cyclin B1 immunoreactivity is required to be evaluated in the nucleus, rather than total or cytoplasm, in breast carcinoma.

The mean value of nuclear cyclin B1 LI was only approximately half that of total or cytoplasmic cyclin B1 LI in our study, which suggests that the biological functions of overexpressed cyclin B1 may be regulated by nuclear transportation from the cytoplasm. Previous *in vitro* studies demonstrated that nuclear entry of cyclin B1 was facilitated by PLK1 through the phosphorylation of cyclin B1,[17,18] and overexpression of PLK1 was also reported in breast carcinoma.[26,27] However, 14-3-3σ anchored cyclin B1 in the cytoplasm and prevented the nuclear transition of cyclin B1 or inhibited mitosis.[19,28] In our present study, a significant association was detected between nuclear

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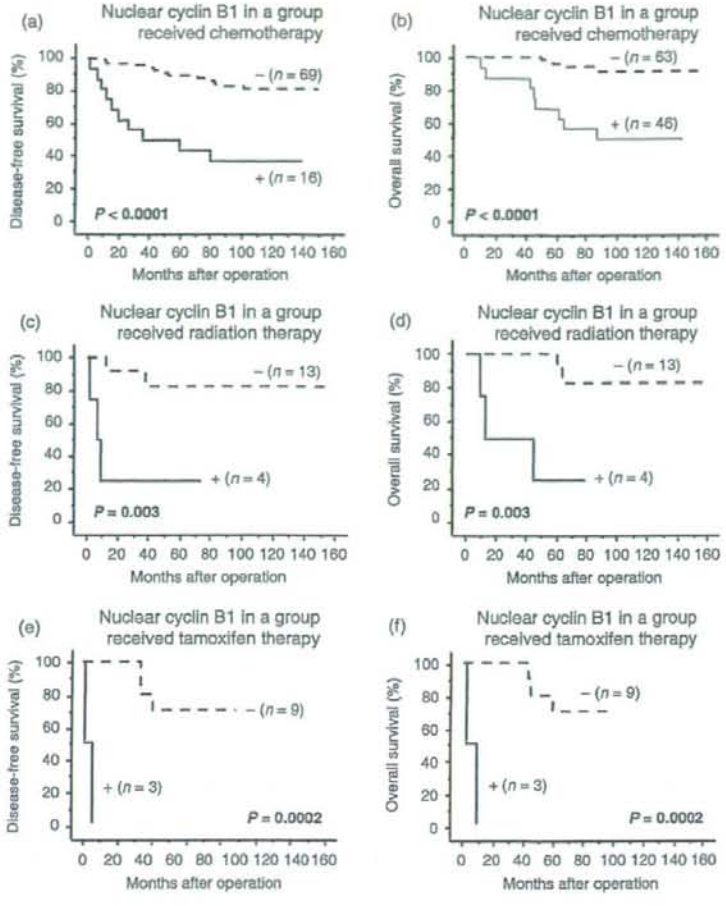

Fig. 4. Association between nuclear cyclin B1 immunoreactivity and clinical outcome of 109 breast carcinoma patients according to the adjuvant therapy (Kaplan–Meier method). Nuclear cyclin B1 immunoreactivity was significantly associated with adverse prognosis in the groups of patients receiving (a,b) adjuvant chemotherapy, (c,d) radiation therapy or (e,f) tamoxifen therapy after surgery. Statistical analysis was evaluated by a log-rank test. *P*-values less than 0.05 were considered significant, and are shown in bold.

cyclin B1 and PLK1, and between cytoplasmic cyclin B1 and 14-3-3σ immunoreactivity. These results are consistent with previous *in vitro* studies, and PLK1 and 14-3-3σ may play important roles in the regulation of intracellular localization of cyclin B1 in human breast carcinoma cells.

The results of our univariate analyses revealed that the prognostic value of nuclear cyclin B1 was more significant than that of other proliferation markers, such as mitotic count and Ki-67. Nuclear cyclin B1 was significantly associated with adverse clinical outcome of the patients showing high (more than 5 cells) mitotic count in breast carcinoma, and multivariate analyses demonstrated that nuclear cyclin B1 was an independent poor prognostic factor in both recurrence and overall survival of the patients as well as lymph node metastasis, a well-established diagnostic modality.[29] This may be partly due to the fact that nuclear cyclin B1 demonstrated worse prognosis even in a group of patients who received adjuvant therapy following surgery. Radiation or most anticancer drugs usually result in DNA strand breaks and induce cell cycle arrest or cell death. DNA damage of carcinoma cells by radiotherapy or chemotherapy resulted in the p53-mediated inhibition of cell cycle progression in either G_1 or G_2–M.[30,31] Irradiation of tumor cells was usually associated with a G_2 delay, a cellular response to DNA damage that allows time for repair and prevents mitosis of damaged cells. However, overexpression of cyclin B1 did not eliminate this G_2 delay in irradiated cells,[32] overrode G_2-M arrest, and made the cells enter into mitosis regardless of the status of p53 expression.[33] Cyclin B1 depletion has also been reported to inhibit proliferation and induce apoptosis of human breast carcinoma cells.[34] Hassan *et al.* reported that head and neck squamous cell carcinoma tumors overexpressing cyclin B1 were resistant to radiotherapy, which is similar to the results of our present study.[35] Therefore, residual carcinoma cells following surgical treatment in nuclear cyclin B1-positive breast carcinomas may grow rapidly regardless of the adjuvant therapy, thereby resulting in an increased recurrence and poor prognosis of these patients.

Escape from G_2–M arrest by overexpressed cyclin B1 may allow insufficient time for DNA repair and cause the accumulation of mutations. Previous *in vitro* studies demonstrated that elevated levels of cyclin B1 often precede the onset of tumor cell immortalization and aneuploidy,[24,36,37] and Kuhling *et al.*[22] reported that cyclin B1 immunoreactivity was significantly associated with DNA aneuploidy in lymph node-negative breast carcinomas. Therefore, nuclear cyclin B1 may induce chromosomal instability and enhance the aggressiveness of the carcinoma cells. Further examination is required to clarify the detailed functions of nuclear cyclin B1 in breast carcinoma, in addition to its effects on cell proliferation.

Table 3a. Univariate and multivariate analyses of disease-free survival in 109 breast cancer patients examined

Variable	Univariate		Multivariate
	P-value	*P*-value	Relative risk (95% CI)
Disease-free survival			
Lymph node metastasis (positive/negative)	**<0.0001***	**0.0002**	6.0 (2.4–15.4)
Nuclear cyclin B1 (positive/negative)	**0.0001***	**0.01**	2.9 (1.3–6.6)
Tumor size (>20 mm/≤ 20 mm)	**0.01***	0.18	
14-3-3σ (negative/positive)	**0.04***	**0.01**	4.2 (1.6–11.2)
HER2 status (positive / negative)	**0.04***	0.96	
Mitotic count (>5/≤ 5)	0.06*	0.20	
c-myc (positive/negative)	0.08*	0.11	
Total cyclin B1 (positive/negative)	0.11		
Ki-67 (≥10/<10)	0.13		
p53 (positive / negative)	0.50		
Histological grade (3/1, 2)	0.53		
Cytoplasmic cyclin B1 (positive/negative)	0.70		
PLK1 (positive/negative)	0.94		
Overall survival			
Lymph node metastasis (positive/negative)	**0.0001***	**0.004**	21.3 (2.6–87.6)
Nuclear cyclin B1 (positive/negative)	**0.0001***	**0.01**	4.7 (1.5–14.7)
Tumor size (>20 mm/≤20 mm)	**0.01***	0.38	
Mitotic count (>5/≤5)	**0.02***	0.45	
c-myc (positive/negative)	**0.03***	0.33	
HER2 status (positive/negative)	**0.04***	0.55	
PLK1 (positive/negative)	0.07*	0.46	
Histological grade (3/1, 2)	0.08*	0.40	
p53 (positive/negative)	0.10		
Total cyclin B1 (positive/negative)	0.25		
Ki-67 (≥10/<10)	0.36		
14-3-3σ (negative/positive)	0.57		
Cytoplasmic cyclin B1 (positive/negative)	0.99		

Data considered significant ($P < 0.05$) in the univariate analyses are shown in bold. *Significant ($P < 0.05$) and borderline-significant ($0.05 \leq P < 0.01$) values were examined in the multivariate analyses in this study.

In summary, nuclear cyclin B1 immunoreactivity was detected in carcinoma cells in 17% of human breast carcinomas, whereas total and cytoplasmic cyclin B1 immunoreactivities were detected in 42 and 35% of the cases, respectively. Cyclin B1 immunoreactivity in these three components (i.e. total, nucleus and cytoplasm) were all associated with histological grade, mitotic count or Ki-67 LI, and nuclear cyclin B1 was also correlated with tumor size and lymph node metastasis. Moreover, only nuclear cyclin B1 was significantly associated with adverse clinical outcome of the patients, and turned out to be an independent prognostic factor of both disease-free and overall survival by multivariate analyses. These results suggest that an onocogenic role of overexpressed cyclin B1 is mainly mediated in the nucleus of breast carcinoma cells, and nuclear cyclin B1 immunoreactivity is a potent prognostic factor in breast carcinoma patients.

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Early growth responsive gene 3 in human breast carcinoma: a regulator of estrogen-meditated invasion and a potent prognostic factor

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Abstract

Early growth responsive gene 3 (EGR3) is a zinc-finger transcription factor and plays important roles in cellular growth and differentiation. We recently demonstrated estrogen-mediated induction of EGR3 in breast carcinoma cells. However, EGR3 has not yet been examined in breast carcinoma tissues and its significance remains unknown. Therefore, in this study, we examined biological functions of EGR3 in the breast carcinoma by immunohistochemistry, *in vitro* study, and nude mouse xenograft model. EGR3 immunoreactivity was detected in carcinoma cells in 99 (52%) out of 190 breast carcinoma tissues and was associated with the mRNA level. EGR3 immunoreactivity was positively associated with lymph node status, distant metastasis into other organs, estrogen receptor α, or EGR3 immunoreactivity in asynchronous recurrent lesions in the same patients, and was negatively correlated with tubule formation. EGR3 immunoreactivity was significantly associated with an increased risk of recurrence and adverse clinical outcome by both uni- and multivariate analyses. *Egr3*-expressing transformant cell lines derived from MCF-7 Tet-Off cells (Eg-10 and Eg-11) significantly enhanced the migration and invasion properties according to the treatment of doxycyclin, but did not significantly change the cell proliferation. Moreover, Eg-11 cells injected into athymic mice irregularly invaded into the adjacent peritumoral tissues, although Clt-7, which was stably transfected with empty vector as a control, demonstrated a well-circumscribed tumor. Eg-11 cells were significantly associated with invasive components and less tubule formation in the xenograft model. These results suggest that EGR3 plays an important role in estrogen-meditated invasion and is an independent prognostic factor in breast carcinoma.

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Introduction

Breast carcinoma is one of the most common malignancies in women worldwide. Human breast tissue is a target for estrogens, and these sex steroids play an important role in development of hormone-dependent breast carcinomas (Thomas 1984, Vihko & Apter 1989). The biological effects of estrogens are mediated through an initial interaction with estrogen receptor (ER) α and/or β, members of a nuclear receptor superfamily (designated NR3A1 and NR3A2 respectively). ERs function as dimers, and activate transcription in a ligand-dependent manner by binding to estrogen responsive elements (EREs) located in the promoter region of various target genes (Tsai & O'Malley 1994). A variety of estrogenic functions

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are characterized by the expression of these genes (Hayashi *et al.* 2003), and therefore, it is very important to examine the expression and roles of estrogen responsive genes to obtain a better understanding of estrogenic actions in human breast cancer.

Early growth responsive gene 3 (EGR3) belongs to the EGR family of zinc-finger transcription factors, and shares a common sequence termed the EGR responsive element with other members involved in DNA binding and transactivation (Patwardhan *et al.* 1991, O'Donovan *et al.* 1999). Previous studies revealed that EGR3 was involved in the development of muscle spindle (Tourtellotte & Milbrandt 1998, Tourtellotte *et al.* 2001) and thymocyte proliferation (Xi & Kersh 2004), indicating that EGR3 plays important roles in cellular growth and differentiation. We have recently demonstrated that EGR3 was induced by estradiol in MCF-7 breast carcinoma cells from the cDNA microarray analysis (Inoue *et al.* 2004). These findings suggest a possible role for EGR3 in estrogen-dependent human breast carcinomas. However, EGR3 has not been examined in human breast carcinoma tissues, and its biological and clinical significance remains unknown. Therefore, in this study, we examined biological functions of EGR3 in the breast carcinoma using immunohistochemistry, *in vitro* study, and nude mouse xenograft model. From these results, here, we first report that EGR3 is a regulator of estrogen-mediated invasion, and is a potent prognostic factor in human breast carcinomas.

Materials and methods

Patients and tissues

About 190 specimens of invasive ductal carcinoma of the breast were obtained from female patients who underwent mastectomy from 1984 to 1992 in the Department of Surgery, Tohoku University Hospital, Sendai, Japan. Breast tissue specimens were obtained from patients with a mean age of 53.5 years (range 22–82). The patients did not receive chemotherapy or irradiation prior to surgery. About 62 patients received tamoxifen therapy after the surgery. The mean follow-up time was 102 months (range 3–157 months). The histological grade and tubule formation of each specimen was evaluated based on the method of Elston & Ellis (1991). Asynchronous recurrent lesions of the breast carcinoma were also available for examination in 13 cases (breast, 3 cases; lymph node, 3 cases; skin, 2 cases; liver, 2 case; lung, 1 case; bone, 1 case; and chest wall, 1 case). All specimens were fixed in 10% formalin and embedded in paraffin wax.

Thirty-one specimens of invasive ductal carcinoma were obtained from patients who underwent mastectomy in 2000 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Specimens for RNA isolation were snap-frozen and stored at −80 °C, and those for immunohistochemistry were fixed in 10% formalin and embedded in paraffin wax. Informed consent was obtained from all patients prior to their surgery and examination of specimens used in this study.

Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

Antibodies

A rabbit polyclonal antibody for EGR3 (C-24 (sc-191)) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). This antibody was raised against a peptide mapping at the carboxy terminus of human *EGR3*. The EGR3 antibody specially recognized human EGR3 by immunoblotting and immunohistochemistry, and was non-cross-reactive with EGR1, EGR2, or Wilms' tumor proteins (data from Santa Cruz Biotechnology). Monoclonal antibodies for ERα (ER1D5), progesterone receptor (PR; MAB429), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), and DAKO (Carpinteria, CA, USA) respectively. Rabbit polyclonal antibodies for ERβ (06-629) and HER2 (A0485) were obtained from Upstate Biotechnology (Lake Placid, NY, USA) and DAKO respectively.

Immunohistochemistry

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin–biotin amplification method was used in this study. Antigen retrieval was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate (pH 6.0)). Dilutions of primary antibodies used in this study were as follows: EGR3, 1/500; ERα, 1/50; ERβ, 1/50; PR, 1/30; HER2, 1/200; and Ki-67, 1/50. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H_2O_2) and counterstained with hematoxylin. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibodies. Immunohistochemical preabsorption test was also performed for EGR3 immunohistochemistry using the blocking peptide (sc-191 P; Santa Cruz Biotechnology).

Scoring of immunoreactivity and statistical analysis

EGR3, ERα, ERβ, PR, and Ki-67 immunoreactivity was detected in the nucleus, and the immunoreactivity was evaluated in more than 1000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, i.e. labeling index (LI), was determined. Cases with EGR3 or ERα LI of more than 10% were considered EGR3- or ERα-positive breast carcinomas in this study, according to a report on ERα (Goldhirsch *et al.* 2005).

An association between EGR3 immunoreactivity and clinicopathological factors was evaluated using a Student's *t*-test, cross-table using the χ^2-test, or correlation coefficient (r) and regression equation. Overall and disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using the log-rank test. Uni- and multivariate analyses were evaluated by a Cox's proportional hazard model using PROC PHREG in our SAS software.

Cells and chemicals

MCF-7 human breast cancer cell line and LY-2, which is a tamoxifen-resistant MCF-7 cell variant (Paik *et al.* 1994), were cultured in RPMI-1640 (Sigma–Aldrich) with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). We also used Eg-10 and Eg-11 cells which are *Egr3*-expressing transformants derived from MCF-7 Tet-Off cells (Inoue *et al.* 2004), and Ctl-7 cells which are MCF-7 Tet-Off cells stably transfected with empty vector (Inoue *et al.* 2004). Overexpression of *Egr3* in Eg-10 and Eg-11 cells was dramatically repressed by the treatment of doxycyclin (50 ng/ml; Inoue *et al.* 2004). These cells were also cultured in RPMI-1640 (Sigma–Aldrich) with 10% FBS. All the cells used in this study were cultured with phenol red-free RPMI-1640 medium containing 10% dextran-coated charcoal–FBS for 3 days before treatment of the experiment. Estradiol and tamoxifen were purchased from Sigma–Aldrich, while ICI 182 780 was obtained from Tocris Cookson Inc. (Ellisville, MO, USA).

Real-time PCR

Total RNA was extracted from breast carcinoma tissues or cultured cells using TRIzol reagent (Invitrogen Life Technologies), and a reverse transcription kit (Superscript II Preamplification system; Gibco-BRL) was used in the synthesis of cDNA.

The LightCycler System (Roche Diagnositics GmbH) was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin *et al.* 2000). Settings for the PCR thermal profile were as follows: initial denaturation at 95 °C for 1 min followed by 40 amplification cycles of 95 °C for 1 s, annealing at 68 °C (EGR3 and ribosomal protein L 13a (RPL13A)) for 15 s, and elongation at 72 °C for 15 s. Oligonucleotide primers for EGR3 (NM_004430) were designed in different exons to avoid the amplification of genomic DNA, and the primer sequences were FWD: 5′-CTGCCTGACAATCTGTACCC-3′ (cDNA position; 416–435) and REV: 5′-GTAGGT-CACGGTCTTGTTGC-3′ (cDNA position; 594–613). The primer sequences for RPL13A (NM_012423) were FWD: 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ (cDNA position; 487–509) and REV: 5′-TTGAG-GACCTCTGTGTATTTGTCAA-3′ (cDNA position; 588–612; Vandesompele *et al.* 2002). To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. EGR3 mRNA level was summarized as the ratio of RPL13A mRNA level (%).

Immunoblotting

The cell protein was extracted in triple detergent lysis buffer (LK-18) at 4 °C. About 25 μg (immunoblotting for EGR3) or 5 μg (immunoblotting for β-actin) of the protein (whole cell extracts) were subjected to SDS–PAGE (10% acrylamide gel). Following SDS–PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (Amersham Biosciences). The blots were blocked in 5% nonfat dry skim milk for 1 h at room temperature, and were then incubated with a primary antibody for EGR3 (C-24 (sc-191), Santa Cruz Biotechnology) or β-actin (AC-15 (Sigma #A-5411), Sigma–Aldrich) for 18 h at 4 °C. After incubation with anti-rabbit or anti-mouse IgG horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature, antibody/protein complexes on the blots were detected using ECL plus western blotting detection reagents (Amersham Biosciences). Immunointensity of specific bands was measured by LAS-1000 imaging system (Fuji Photo Film, Tokyo, Japan). Immunointensity of EGR3 in each sample was normalized to that of β-actin, and subsequently, relative immunointensity ratio of EGR3 was summarized as a ratio compared with that of MCF-7 cells in the absence of estradiol or tamoxifen.

Migration assay and invasion assay

Cell migration assay was performed using a 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ, USA) and Chemotaxicell (8 μm pore size; Kurabo, Osaka, Japan). The membrane of Chemotaxicell was coated with 0.3 mg/ml of collagen I (CELLGEN, Tokyo, Japan). After 3 days of the treatment with or without doxycyclin (50 ng/ml) in serum-free RPMI-1640 medium, 5×10^5 cells were plated at the upper chamber, while NIH/3T3 conditioned medium was in the lower chamber. After incubation for 6 h at 37 °C, cells on the upper surface of membrane were removed by wiping with a cotton swab, and those on the lower surface were subsequently fixed with 70% ethanol and stained with hematoxylin and eosin. The migration ability was evaluated as a total number of cells on the lower surface of membrane, which was counted under microscopy.

The cell invasion assay was performed by a modified migration assay. In this experiment, upper surface of the membrane of Chemotaxicell was coated with 80 μg/cm^2 of Matrigel basement membrane matrix (BD Biosciences, Two Oak Park, MA, USA; Albini *et al.* 1987, Taniguchi *et al.* 1989). About 5×10^5 cells at the upper chamber were incubated with 24 h at 37 °C, and the invasion ability was subsequently evaluated as the total number of cells on the lower surface of membrane.

Cell proliferation assay and apoptosis analysis

The status of cell proliferation of cells was measured using a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) method (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). The apoptotic status of cells was evaluated by an apoptosis screening kit (Wako, Osaka, Japan), which employed a modified TdT-mediated dUTP nick-end labeling (TUNEL) method. Optical densities (OD=450 nm for cell proliferation assay and OD=490 nm for apoptosis analysis) were obtained with a Model 680 microplate reader (Bio-Rad Laboratories). The cell number and apoptosis index were calculated according to the following equation: (cell OD value after test materials treated/vehicle control cell OD value), and subsequently evaluated as a ratio (%) compared with that at 0 day after the treatment.

Athymic mouse xenograft model

Eg-11 and Ctl-7 cells were resuspended in phenol-red free Matrigel (Becton Dickinson; 1×10^7 (0.1 ml)/site) and placed on superior side of BALB/c-nu/nu athymic female mice (5 weeks of age; Charles River Laboratories, Tokyo, Japan). Tumor tissues were resected after 2 months, and were subsequently fixed in 10% formalin and embedded in paraffin wax.

Results

Immunohistochemistry for EGR3 in breast carcinoma tissues

Immunoreactivity for EGR3 was detected in the nuclei of invasive ductal carcinoma cells (Fig. 1A). A mean value of EGR3 LI in the 190 breast carcinoma tissues examined was 19.1% (range 0–96%), and a number of EGR3-positive breast carcinomas (i.e. EGR3 LI of more than 10%) were 99 out of 190 cases (52%). EGR3 immunoreactivity was weakly and focally detected in epithelial cells of morphologically normal glands (Fig. 1B). Immunohistochemical preabsorption test for EGR3 demonstrated no specific immunoreactivity in a negative control (Fig. 1C). We also examined mRNA expression of EGR3 in 31 cases of invasive ductal carcinoma tissues using real-time PCR. EGR3 mRNA expression level was significantly ($P=0.003$, $r=0.52$) correlated with the EGR3 immunoreactivity in these cases examined (Fig. 1D).

Associations between EGR3 immunoreactivity and clinicopathological parameters in 190 breast carcinomas were summarized in Table 1. EGR3-positive breast carcinoma was significantly associated with synchronous lymph node status ($P=0.002$), distant metastasis into other organs ($P=0.02$), ERα status ($P=0.01$), ERα LI ($P=0.02$), or EGR3 immunoreactivity in asynchronous recurrent lesions in the same patients ($P=0.03$). On the other hand, a negative correlation was detected between EGR3 immunoreactivity and tubule formation ($P=0.01$). In this study, there were no significant correlations between EGR3 immunoreactivity and other clinicopathological parameters, including the patient age, menopausal status, clinical stage, tumor size, histological grade, ERβ LI, PR LI, HER2 status, and Ki-67 LI. Similar tendency was detected when EGR3 immunoreactivity was evaluated as a continuous variable (i.e. LI; Table 1).

Correlation between EGR3 immunoreactivity and clinical outcome of the breast carcinoma patients

In order to examine an association between EGR3 immunoreactivity and prognosis precisely, we excluded stage IV cases and used stages I to III breast carcinoma patients ($n=169$) in the following analyses. EGR3 immunoreactivity was significantly associated with an

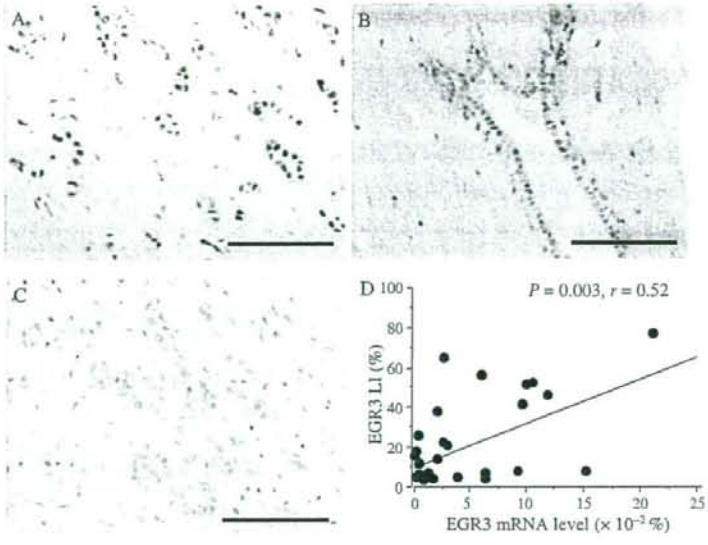

Figure 1 Immunohistochemistry for EGR3 in the invasive ductal carcinoma. (A) EGR3 immunoreactivity was detected in the nuclei of the carcinoma cells. (B) In morphologically normal mammary glands, immunoreactivity for EGR3 was focally and weakly detected in the nuclei of epithelial cells. (C) No significant immunoreactivity of EGR3 was detected in the sections of breast carcinomas in immunohistochemical preabsorption test as a negative control. Bar=100 μm. (D) Association between the mRNA level and relative immunoreactivity (LI) of EGR3 in 31 breast carcinoma tissues. Significant positive association was detected ($P=0.0029$, $r=0.517$). EGR3 mRNA level was summarized as the ratio of RPL13A mRNA level (%). Statistical analysis was performed utilizing a correlation coefficient (r) and regression equation.

increased risk of recurrence (Fig. 2A; $P=0.004$ in the log-rank test). Following univariate analysis by COX (Table 2), lymph node status ($P<0.0001$), EGR3 immunoreactivity ($P=0.01$), HER2 status ($P=0.01$), and tumor size ($P=0.04$) were demonstrated significant prognostic parameters for disease-free survival in 169 breast carcinoma patients. A multivariate analysis (Table 2) revealed that only lymph node status ($P=0.0002$) and EGR3 immunoreactivity ($P=0.01$) were independent prognostic factors with relative risks over 1.0.

Similar tendency was detected when EGR3 immunoreactivity was further categorized into three groups (0–9, 10–49, and 50–100% of positive cells; $P=0.01$ in both uni- and multivariate analyses), EGR3 immunoreactivity was evaluated as a continuous variable ($P=0.003$ in both uni- and multivariate analyses), or EGR3 immunoreactivity was evaluated in all the cases from stages I to IV ($n=190$; $P=0.0002$ in univariate analysis and $P=0.002$ in multivariate analysis).

Overall survival curve was demonstrated in Fig. 2B, and a significant correlation was detected between EGR3 immunoreactivity and adverse clinical outcome of the patients ($P=0.01$ in the log-rank test). Utilizing a univariate analysis (Table 3), lymph node status ($P<0.0001$), histological grade ($P=0.003$), HER2 status ($P=0.004$), EGR3 immunoreactivity ($P=0.01$), and tumor size ($P=0.02$) turned out to be significant prognostic factors for overall survival in this study. Multivariate analysis revealed that lymph node status ($P=0.001$), EGR3 immunoreactivity ($P=0.01$), and histological grade ($P=0.03$) were independent prognostic factors with a relative risk over 1.0, but other factors were not significant in this study (Table 3).

Similar tendency was detected when EGR3 immunoreactivity was categorized into the three groups ($P=0.002$ in both uni- and multivariate analyses), EGR3 immunoreactivity was evaluated as a continuous variable ($P=0.002$ in univariate analysis and $P=0.001$ in multivariate analysis), or EGR3 immunoreactivity was evaluated in stage I to IV cases ($n=190$; $P=0.0003$ in univariate analysis and $P=0.001$ in multivariate analysis).

An association between EGR3 immunoreactivity and clinical outcome of the patients was similar regardless of the ERα status in this study (Fig. 2C and D). About 48 out of 169 patients received tamoxifen therapy after surgery,