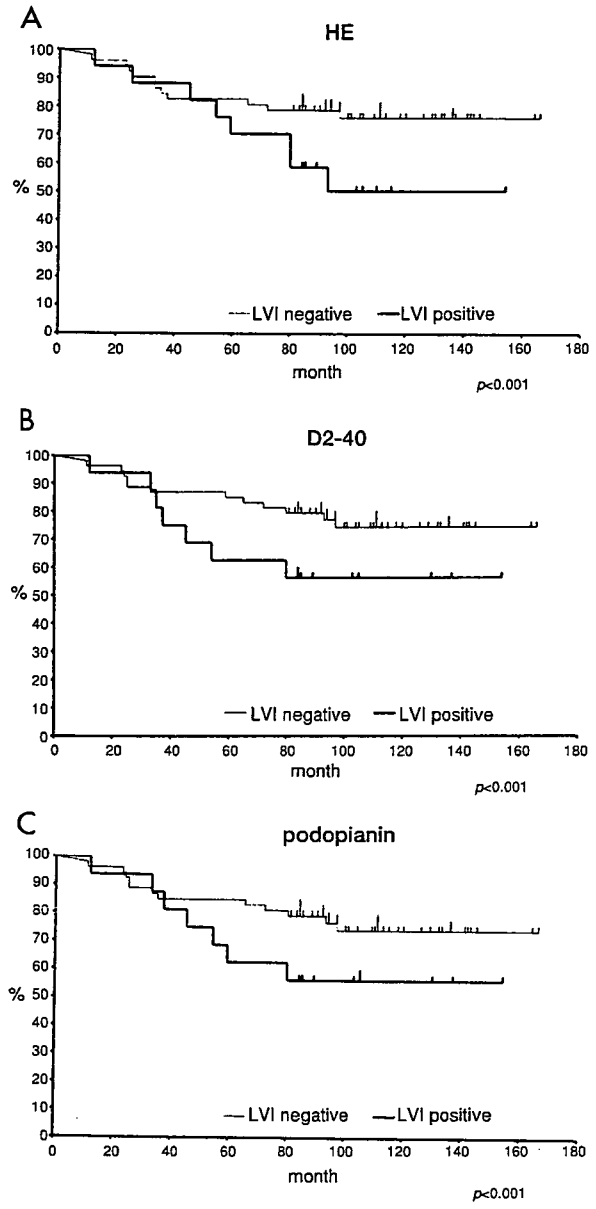

Fig 4. Disease free survival in 69 breast carcinoma cases with (LVI+) or without (LVI-) lymphatic vessel invasion. A: HE stain, B: D2-40, C; podoplanin.

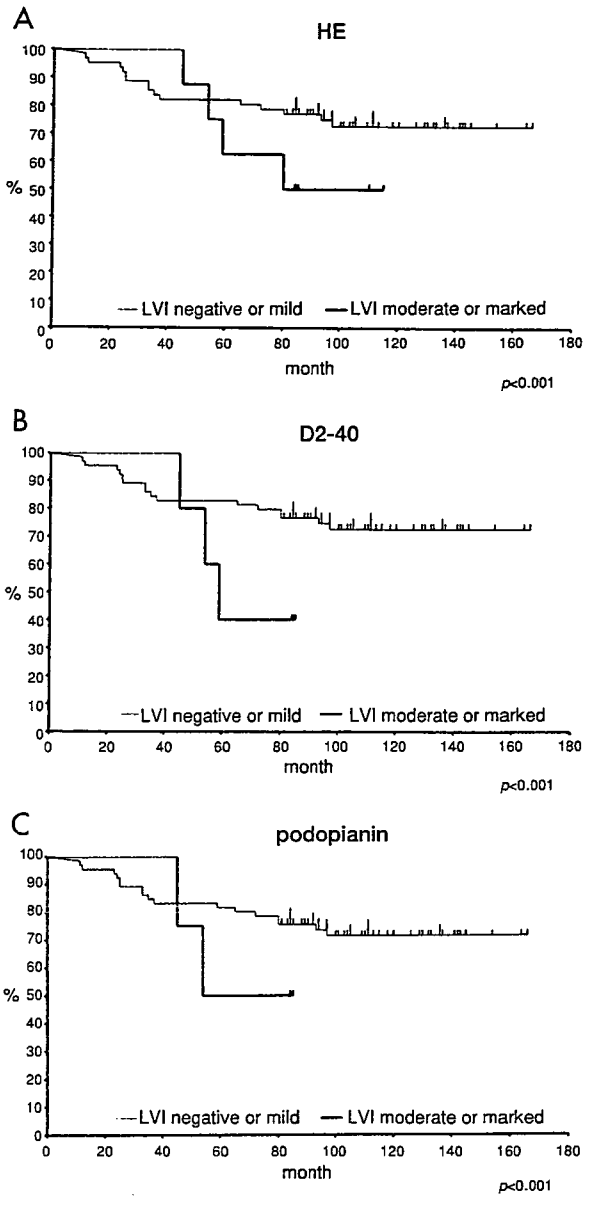

Fig 5. Disease free survival in 69 breast carcinoma cases with moderate to marked lymphatic vessel invasion, or with none or mild lymphatic invasion. A: HE stain, B: D2-40, C; podoplanin.

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.

Acknowledgements

The authors thank Tomomi Nakano MT, Akiko Yokozawa MT, and Hiromi Fuji MT, for their technical assistance.

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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).

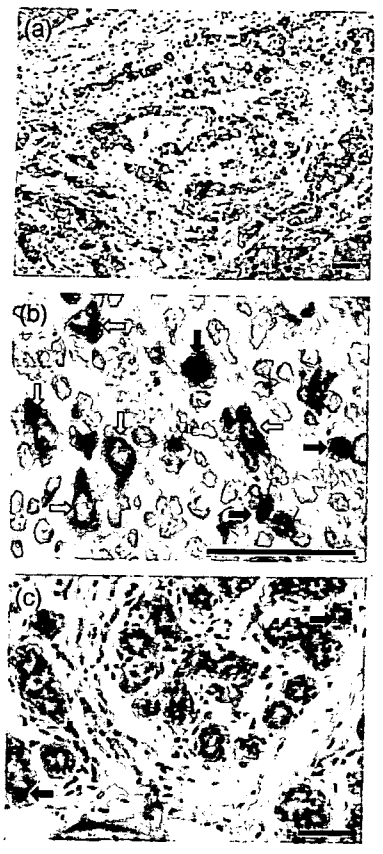

Fig. 1. Immunohistochemistry for cyclin B1 in the invasive ductal carcinoma. Cyclin B1 immunoreactivity was detected in the nucleus and/or cytoplasm of carcinoma cells: (a) lower magnification, (b) higher magnification. (b) Closed arrows represent nuclear cyclin B1 immunoreactivity, and open arrows show 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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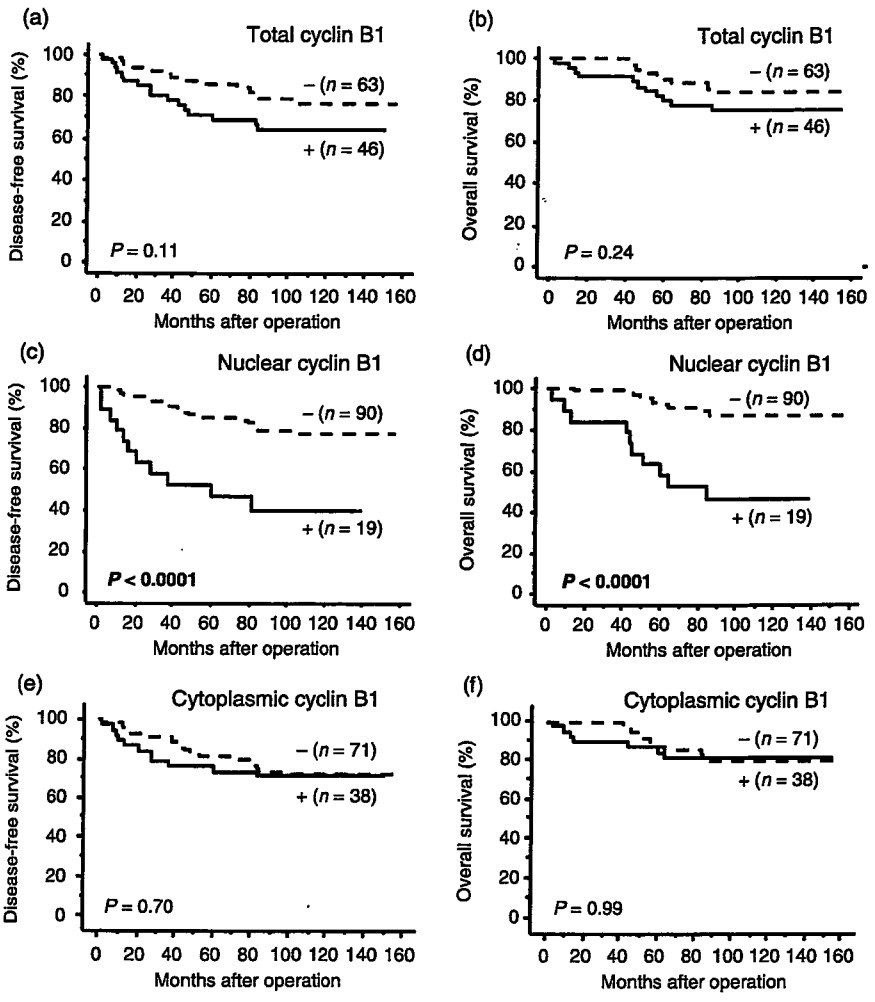

Fig. 2. Disease-free and overall survival of 109 patients with breast carcinoma according to the intracellular localization of cyclin B1 immunoreactivity (Kaplan–Meier method). Total cyclin B1 was not significantly associated with (a) disease-free or (b) overall survival. Nuclear cyclin B1 was significantly 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

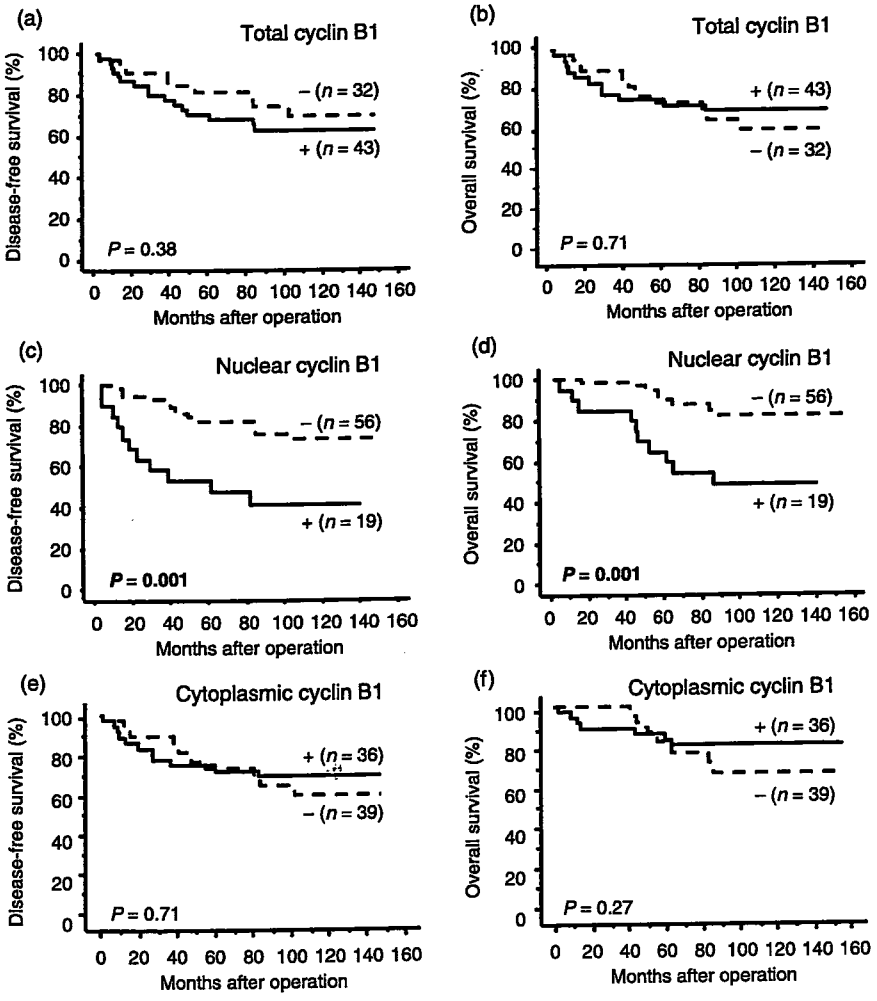

Fig. 3. Association between intracellular localization of cyclin B1 immunoreactivity and clinical outcome of the 75 patients showed high (>5 cells) mitotic count in the breast carcinoma (Kaplan–Meier method). There was no significant association between total cyclin B1 and (a) disease-free or (b) overall 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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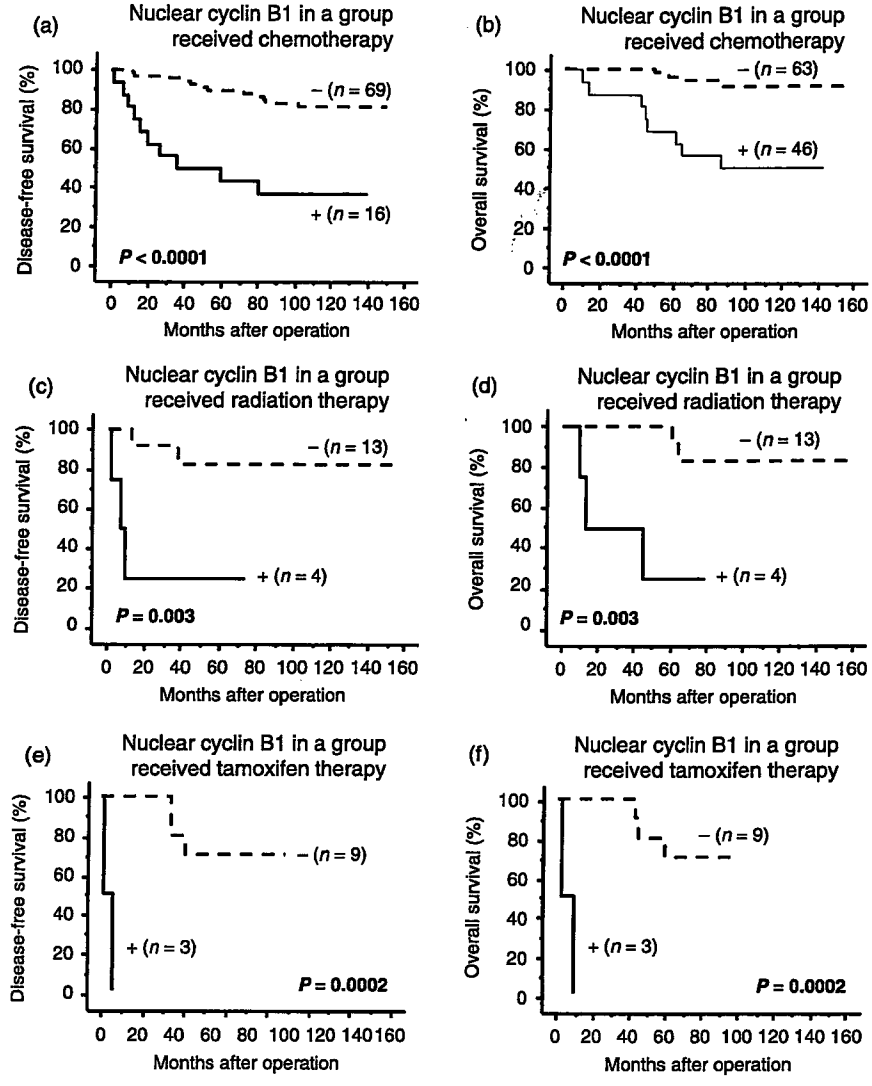

Fig. 4. Association between nuclear cyclin B1 immunoreactivity and clinical outcome of 109 breast carcinoma patients according to the adjuvant therapy (Kaplan–Meier method). Nuclear cyclin B1 immunoreactivity was significantly associated with adverse prognosis in the groups of patients receiving (a,b) 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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Endocrine-Related Cancer (2007) **14** 279–292

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

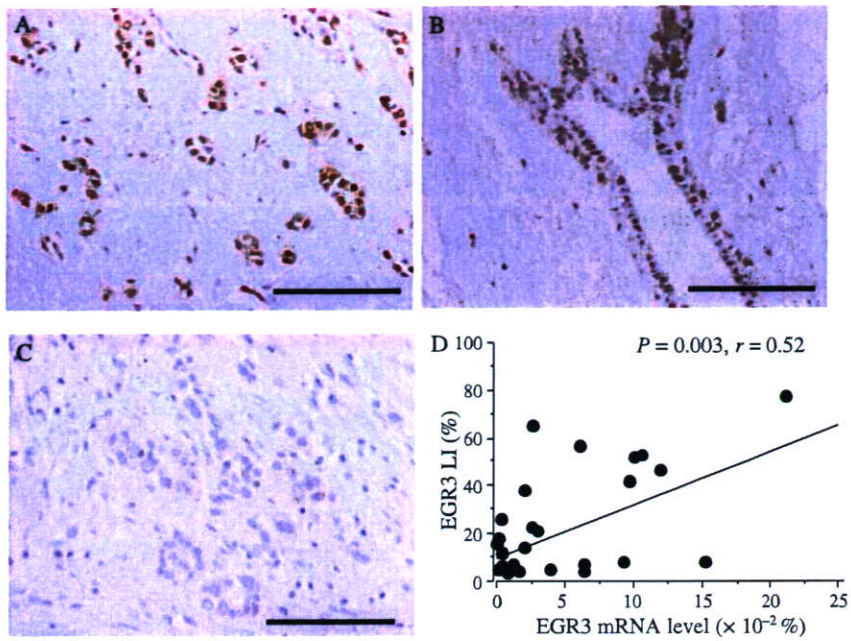

Figure 1 Immunohistochemistry for EGR3 in the invasive ductal carcinoma. (A) EGR3 immunoreactivity was detected in the nuclei of the carcinoma cells. (B) In morphologically normal mammary glands, immunoreactivity for EGR3 was focally and weakly detected in the nuclei of epithelial cells. (C) No significant 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,

Table 1 Association between Early growth responsive gene 3 (*EGR3*) immunoreactivity and clinicopathological parameters in 190 breast carcinomas

	EGR3 Immunoreactivity				
	+(n=99)	− (n=91)	*P* value	EGR3 LI	*P* value
Age (years)	52.3±1.2	54.4±1.2	0.23		0.59
Menopausal status					
Premenopausal	46 (24%)	38 (20%)		19.1±2.3	
Postmenopausal	53 (28%)	53 (28%)	0.61	19.2±2.2	0.96
Stage					
I	18 (10%)	26 (14%)		15.8±2.8	
II	56 (30%)	51 (27%)		18.8±2.1	
III	9 (5%)	9 (5%)		16.1±5.2	
IV	16 (8%)	5 (3%)	0.07	30.6±6.4	0.07
Tumor size (mm)	34.6±2.7	30.0±3.4	0.29		0.20
Lymph node status					
Positive	54 (28%)	29 (15%)		24.6±2.8	
Negative	45 (24%)	62 (33%)	**0.002**	14.9±1.7	**0.003**
Distant metastasis					
Positive	16 (8%)	5 (3%)		30.6±6.4	
Negative	83 (44%)	86 (45%)	**0.02**	17.7±1.6	**0.01**
Histological grade					
1 (well)	26 (14%)	24 (13%)		19.7±3.1	
2 (moderate)	40 (21%)	31 (16%)		21.5±2.7	
3 (poor)	33 (17%)	36 (19%)	0.60	16.3±2.6	0.48
Tubule formation					
1 (>75%)	12 (6%)	22 (12%)		13.2±2.9	
2 (10–75%)	23 (12%)	29 (15%)		14.9±2.5	
3 (<10%)	64 (34%)	40 (21%)	**0.01**	23.2±2.4	**0.20**
ERα status					
Positive	78 (41%)	57 (30%)		22.0±2.0	
Negative	21 (11%)	34 (18%)	**0.01**	12.2±2.3	**0.01**
ERα LI (%)	47.9±3.4	37.3±3.7	**0.02**	(r=0.23)	**0.001**
ERβ LI (%)	16.2±2.3	15.2±2.1	0.82		0.37
PR LI (%)	42.9±3.3	36.9±3.6	0.23		0.33
HER2 status					
Positive	29 (15%)	28 (15%)		15.4±2.5	
Negative	70 (37%)	63 (33%)	0.82	20.8±2.0	0.23
Ki-67 LI (%)	24.8±1.8	24.4±1.9	0.89		0.93
EGR3 immunoreactivity in recurrent lesions (n=13)				(r=0.65)	**0.02**
Positive	9 (69%)	1 (8%)			
Negative	1 (8%)	2 (15%)	**0.03**		

Data are presented as mean±95% confidence interval (95% CI) or the number of cases with percentage. *P* values <0.05 were considered significant, and described as boldface.

and these cases were ERα-positive breast cancers. The disease-free and overall survival curves in these patients were summarized in Fig. 2E and F respectively. EGR3 immunoreactivity was also associated with an increased risk of recurrence and worse prognosis in the group of breast cancer patients who received tamoxifen therapy (P=0.01 and 0.03 in the log-rank test respectively). An association between EGR3 immunoreactivity and clinical outcome of the patients was not significantly changed regardless of the status of adjuvant chemotherapy after surgery in this study (data not shown).

Estrogen-mediated expression of EGR3 in MCF-7 breast carcinoma cells

As shown in Fig. 3A, EGR3 mRNA expression was induced by estradiol in a dose-dependent manner in MCF-7 cells. This induction became significant from 1 nM estradiol (P<0.001 versus control (non-treatment with estradiol)), and EGR3 mRNA level of MCF-7 cells treated with 10 nM estradiol (100.0±2.0%) was 14-fold increased when compared with the control level (7.2±2.0%). The estradiol-mediated mRNA expression of EGR3 was suppressed by addition of

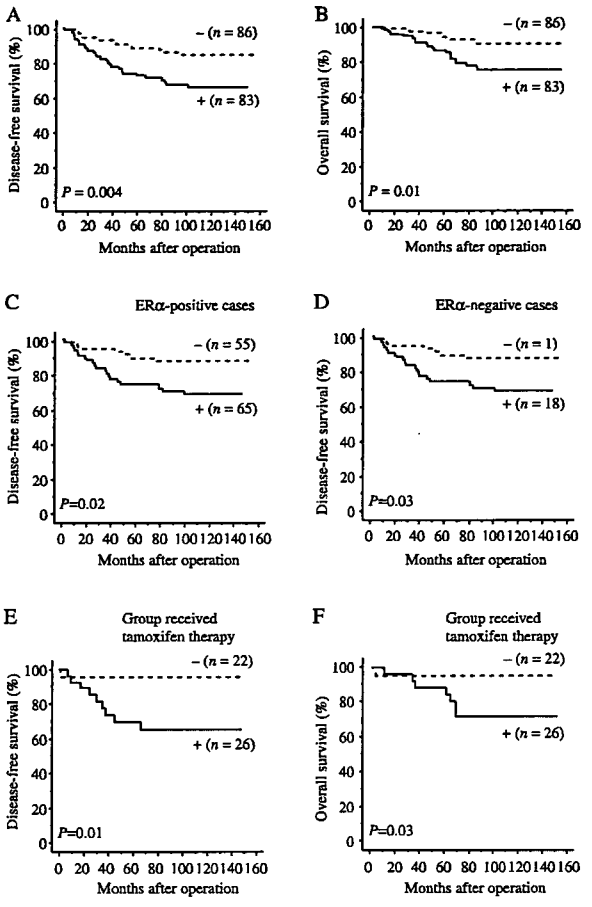

Figure 2 Disease-free and overall survival of 169 patients with breast carcinoma according to EGR3 immunoreactivity (Kaplan–Meier method). (A and B) EGR3 immunoreactivity was significantly associated with an increased risk of recurrence (P=0.0036, log-rank test) (A) and worse prognosis (P=0.0090, log-rank test) (B). (C and D) EGR3 immunoreactivity was significantly correlated with a risk of recurrence regardless of the ERα status (P=0.0154 in ERα-positive cases (C), and P=0.0348 in ERα-negative cases (D)). (E and F) EGR3 immunoreactivity was significantly associated with an increased risk of recurrence (P=0.0123) (E) and worse prognosis (P=0.0316) (F) in the 48 patients received tamoxifen therapy.

tamoxifen in a dose-dependent manner (Fig. 3B). EGR3 mRNA level in MCF-7 cells treated with 10 nM estradiol and 10 μM tamoxifen (21.8±6.2%) was decreased into 22% of that treated with 10 nM estradiol alone (100.0±2.0%), but its level remained significantly higher (P<0.001 and threefold) than the control level (neither estradiol nor tamoxifen; 7.2±2.0%). Tamoxifen (10 μM) alone did not significantly change the EGR3 mRNA level in MCF-7 cells.

Estradiol also induced EGR3 mRNA expression in LY-2 cells, a tamoxifen-resistant MCF-7 cell variant, in a dose-dependent manner (Fig. 3C), which was

Table 2 Uni- and multivariate analyses of disease-free survival in stages I–III breast cancer patients examined (n=169)

Variable	Univariate P	Multivariate P	Multivariate Relative risk (95% CI)
Lymph node status (positive/negative)	**<0.0001**	**0.0002**	3.8 (1.9–7.7)
EGR3 immunoreactivity (positive/negative)	**0.01**	**0.01**	2.5 (1.3–4.8)
HER2 status (positive/negative)	**0.01**	0.08	
Tumor size (≧20 mm/<20 mm)	**0.04**	0.48	
Ki-67 LI (≧10/<10)	0.15		
Adjuvant chemotherapy (no/yes)	0.34		
ERα status (negative/positive)	0.44		
Tamoxifen therapy (no/yes)	0.46		
Histological grade (3/1, 2)	0.78		

Data considered significant (P<0.05) in the univariate analyses were described as boldface, and were examined in the multivariate analyses.

significant from 10 pM estradiol (P<0.05 versus the control level). The level of EGR3 mRNA in LY-2 cells treated with 10 nM estradiol (1245±222%) was 31-fold higher than the control level (39.7±14.2%), and was 12-fold higher than that in MCF-7 cells treated

Table 3 Uni- and multivariate analyses of overall survival in stages I–III breast cancer patients examined (n=169)

Variable	Univariate P	Multivariate P	Multivariate Relative risk (95% CI)
Lymph node status (positive/negative)	**<0.0001**	**0.001**	6.0 (2.2–16.5)
Histological grade (3/1, 2)	**0.003**	**0.03**	2.5 (1.1–4.7)
HER2 status (positive/negative)	**0.004**	0.38	
EGR3 immunoreactivity (positive/negative)	**0.01**	**0.01**	3.0 (1.3–7.0)
Tumor size (≧20 mm/<20 mm)	**0.02**	0.28	
Ki-67 LI (≧10/<10)	0.11		
Adjuvant chemotherapy (no/yes)	0.21		
Tamoxifen therapy (no/yes)	0.37		
ERα status (negative/positive)	0.42		

Data considered significant (P<0.05) in the univariate analyses were described as boldface and were examined in the multivariate analyses.

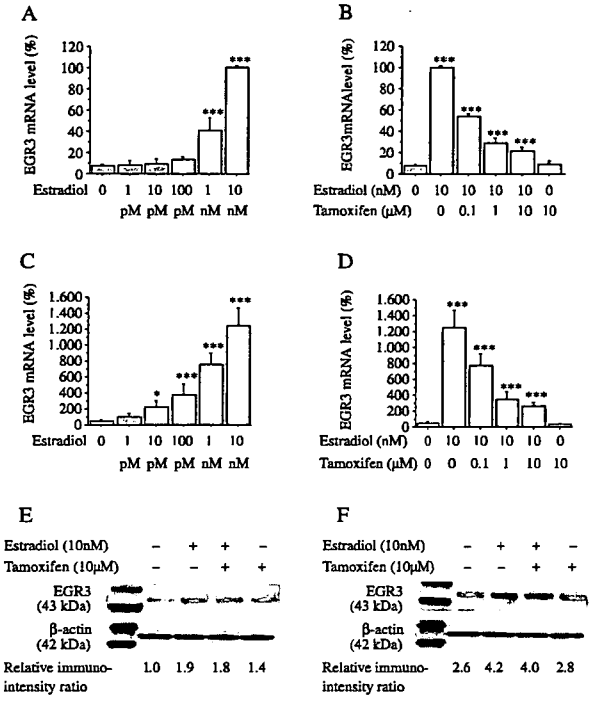

Figure 3 Induction of EGR3 expression by estradiol in MCF-7 cells. (A and C) MCF-7 (A) or LY-2 (C) cells were treated with indicated concentrations of estradiol for 3 days, and the EGR3 mRNA was evaluated as the ratio of RPL13A mRNA level (%). (B and D) MCF-7 (B) or LY-2 (D) cells were treated with estradiol (10 nM) with indicated concentrations of tamoxifen for 3 days, and the EGR3 mRNA was evaluated as the ratio of RPL13A mRNA level (%). Data are presented as mean ± S.D. ($n=4$). *$P<0.05$ and ***$P<0.001$ versus control (no treatment with estradiol or tamoxifen for 3 days; left column) respectively. The statistical analyses were performed using a one-way ANOVA and Bonferroni test. (E and F) Immunoblotting for EGR3 in MCF-7 (E) and LY-2 (F) cells. Cells were treated with estradiol (10 nM) and/or tamoxifen (10 μM) for 3 days. Amount of protein loaded in each lane was 25 μg (immunoblotting for EGR3) or 5 μg (immunoblotting for β-actin). Immunointensity of EGR3 in each sample was normalized to that of β-actin, and relative immunointensity ratio of EGR3 was summarized as a ratio compared with that of MCF-7 cells treated without estradiol or tamoxifen (left lane in E).

under the same condition. Tamoxifen dose-dependently suppressed the estradiol-mediated mRNA expression of EGR3 in LY-2 cells (Fig. 3D). EGR3 mRNA level in LY-2 cells treated with 10 nM estradiol and 10 μM tamoxifen (278 ± 35.0%) was decreased into 22% of that treated with 10 nM estradiol alone (1,245 ± 222%), but was still significantly higher ($P<0.001$ and sevenfold) than the control level (39.7 ± 14.2%). Tamoxifen (10 μM) did not significantly change the EGR3 mRNA level also in LY-2 cells. Similar tendency was detected at EGR3 protein levels both in MCF-7 and LY-2 cells by immunoblotting analyses (Fig. 3E and F).

Pure anti-estrogen, ICI 182 780, alone did not significantly change EGR3 mRNA level in MCF-7 cells (Fig. 4A). EGR3 mRNA level was slightly increased by addition of estradiol in MCF-7 cells under the treatment with 10 nM ICI 182 780, but no significant association was detected (2.3-fold and $P=0.06$, 10 nM estradiol versus control (Fig. 4B; non-treatment with estradiol).

On the other hand, as shown in Fig. 4C, ICI 182 780 alone significantly inhibited the EGR3 mRNA level of LY-2 cells in a dose-dependent manner, and the EGR3 mRNA level of LY-2 cells treated with 10 μM ICI 182 780 was decreased into 21% (7.8 ± 3.6%, $P<0.001$) of the basal level (non-treatment with ICI 182 780; 37.8 ± 11.8%). When LY-2 cells were treated with 10 nM ICI 182 780, the EGR3 mRNA level was significantly induced by estradiol in a dose-dependent manner (43-fold and $P<0.001$, 10 nM estradiol versus control (Fig. 4D; non-treatment with estradiol).

Estradiol (10 nM) did not significantly induce the EGR3 mRNA expression when MCF-7 or LY-2 cells were treated with 10 μM ICI 182 780 (0.9-fold and $P=0.83$ in MCF-7, and 1.2-fold and $P=0.77$ in LY-2).

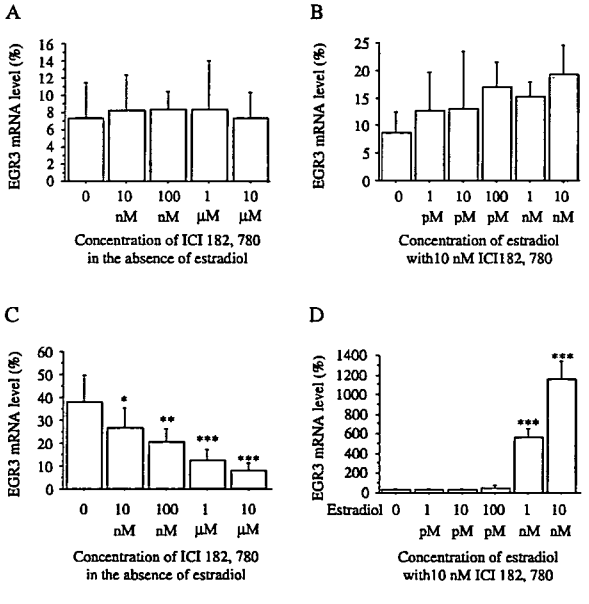

Figure 4 Effects of ICI 182 780 on EGR3 mRNA expression in MCF-7 cells. (A and C) MCF-7 (A) or LY-2 (C) cells were treated with indicated concentrations of ICI 182 780 for 3 days in the absence of exogenous estradiol. (B and D) MCF-7 (B) or LY-2 (D) cells were treated with indicated concentrations of estradiol and ICI 182 780 (10 nM) for 3 days. Data are presented as mean ± S.D. ($n=4$). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ versus control (non-treatment with ICI 182 780 (A and C) or 10 nM ICI 182 780 in the absence of estradiol (B and D) for 3 days (left column in each figure)). The statistical analyses were performed using a one-way ANOVA and Bonferroni test.

Increased invasion properties in *Egr3*-expressing MCF-7 Tet-Off cells

In order to further characterize the biological functions of EGR3 in breast carcinoma cells, we then employed Eg-10 and Eg-11 *Egr3*-expressing transformants derived from MCF-7 Tet-Off cells (Inoue *et al.* 2004). EGR3 mRNA levels of these transformants were 57 and 540% in Eg-10 and Eg-11 respectively. As a control, we used Ctl-7, which was stably transfected with empty vector in the MCF-7 Tet-Off cells (Inoue *et al.* 2004), and the EGR3 mRNA level was negligible (2.2×10^{-3}%). In the immunohistochemistry, EGR3 immunoreactivity was detected in the nuclei of Eg-10 and Eg-11 cells (Fig. 5A), but not in Ctl-7 cells (Fig. 5B). The EGR3 mRNA levels decreased into negligible levels in both Eg-10 (5.0×10^{-3}%) and Eg-11 (1.4×10^{-2}%) cells, when these cells were treated with doxycyclin (50 ng/ml) for 3 days.

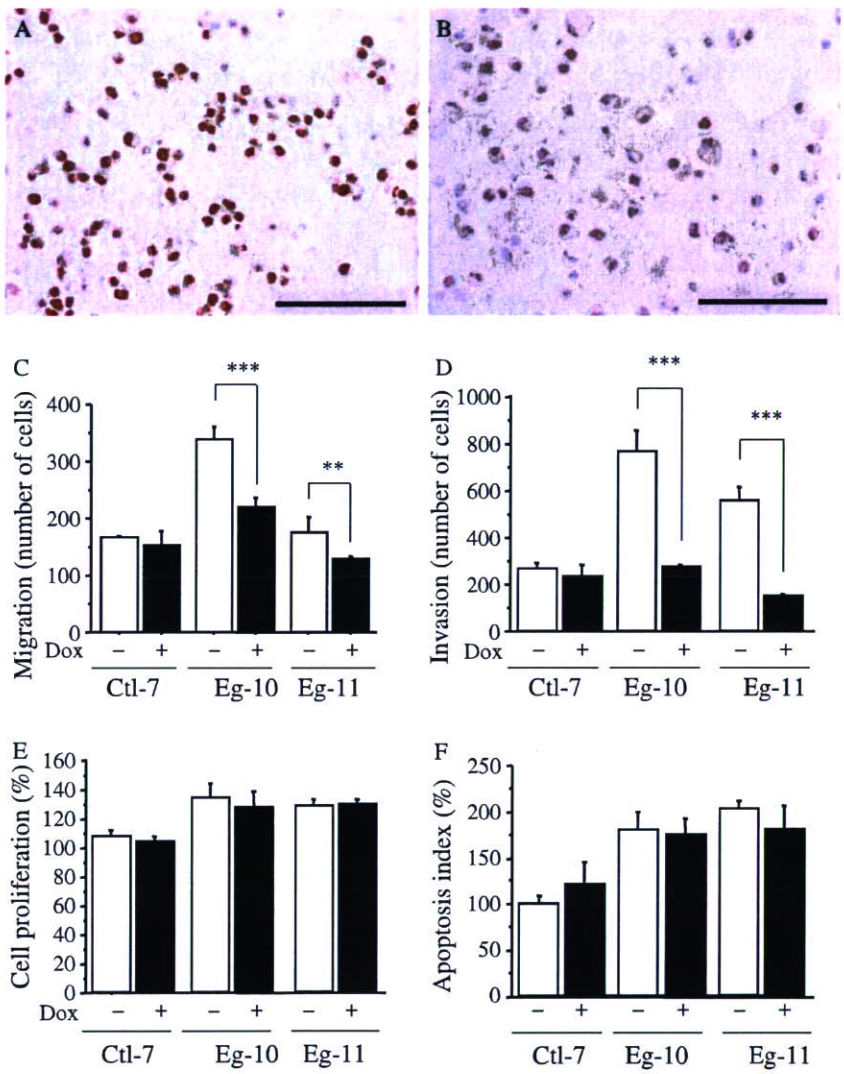

Figure 5 (A and B) Immunohistochemistry for EGR3 in *Egr3*-expressing MCF-7 Tet-Off cells. Immunoreactivity of EGR3 was detected in the nuclei of Eg-11 cells (A), but not in Ctl-7 cells (B). Immunohistochemistry was performed using cell blocks from formalin-fixed and paraffin-embedded specimens. Bar = 100 μm. (C–F) Migration assay (C), invasion assay (D), cell proliferation assay (E), and apoptosis analysis (F) in *Egr3*-expressing cells. Eg-10, Eg-11, and Ctl-7 cells were incubated with 6 h, 24 h, 3 days, and 3 days at 37 °C respectively, without or with the treatment of doxycyclin (50 ng/ml). A total number of cells on the lower surface of membrane of Chemotaxicell was counted in the migration and invasion assays (C and D). While, the cell number and apoptosis index were calculated and evaluated as a ratio (%) compared with that at 0 day after the treatment in the cell proliferation assay and apoptosis analysis (E and F). Data are presented as mean ± S.D. ($n=4$). An open bar represents the value of cells treated without doxycyclin, and a closed bar shows that under the treatment with doxycyclin. The statistical analyses were performed between the values in the absence and presence of doxycyclin using a one-way ANOVA and Bonferroni test, and $^{**}P<0.01$ and $^{***}P<0.001$ respectively.

Figure 5C shows the result of migration assay in *Egr3*-expressing MCF-7 cells. The number of migrated cells was significantly higher in Eg-10 ($P<0.001$) and Eg-11 ($P<0.01$) when compared with that in the treatment with doxycyclin. However, a number of migrated Ctl-7 cells were not significantly altered between the absence and presence of doxycyclin. Moreover, the number of invaded cells was significantly higher in Eg-10 and Eg-11 ($P<0.001$) than that under the treatment with doxycyclin, and it was 3.3-fold higher in Eg-10 and 3.7-fold in Eg-11 (Fig. 5D). Invasion property was not significantly altered in Ctl-7 cells according to the treatment with doxycyclin.

Cell proliferation (Fig. 5E) and apoptosis index (Fig. 5F) of these three cells were not significantly altered between the absence and presence of doxycyclin for 3 days.

Morphological features of Egr3-expressing MCF-7 cells in athymic mice xenograft model

In order to study the biological roles of EGR3 in breast carcinoma cells *in vivo*, we injected Eg-11 and Ctl-7 cells into female nude mice, and the tumor tissues were resected after 2 months. As shown in Fig. 6A and B, Ctl-7 showed a well-circumscribed tumor, and tubule formation was remarkable in the carcinoma tissue. Invasion into the surrounding tissue was not detected in any of the cases examined. On the other hand, Eg-11 cells arranged in clusters and trabeculae with focal glandular formation, and irregularly invaded into the adjacent peritumoral tissue such as adipose tissue in all of the cases examined (Fig. 6C and D). As shown in Table 4, Eg-11 carcinoma tissues significantly demonstrated invasion ($P=0.01$) and less tubule formation ($P=0.03$) when compared with the Ctl-7 carcinoma tissues. However, tumor volume, largest dimension histologically determined, and Ki-67 LI of the tumor were not significantly different between these two types of carcinoma. The tumor volumes of Eg-11 and Ctl-7 were monitored weekly, but no significant changes were detected when compared with the original volume (data not shown).

Discussion

In this study, EGR3 immunoreactivity was closely correlated with the mRNA level, and was significantly associated with the ERα status, but not with ERβ, in the breast carcinoma tissues. In the previous studies using cDNA microarray, EGR3 mRNA was significantly

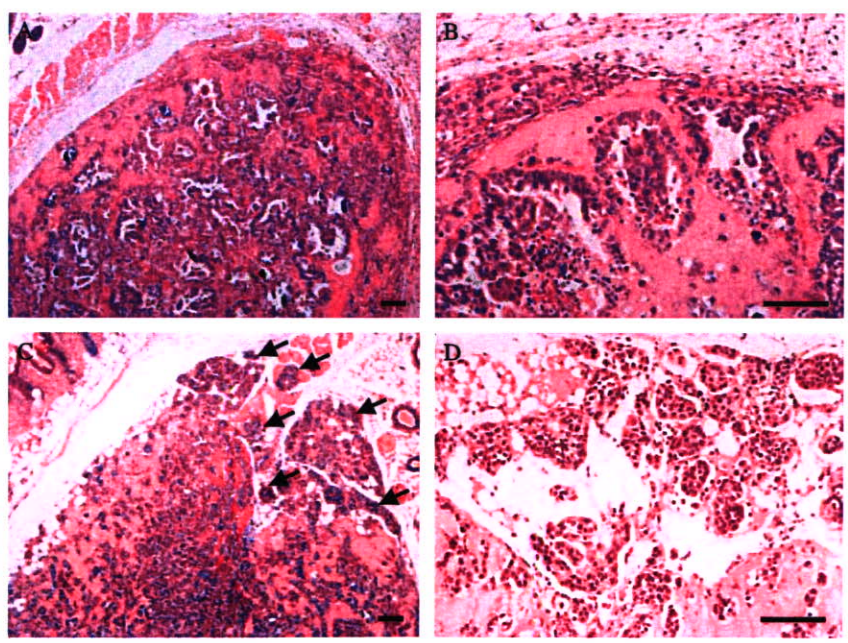

Figure 6 Histological features of *Egr3*-expressing MCF-7 cells in athymic mouse xenograft model. (A and B) Tubule formation was remarkable in the Ctl-7 carcinoma and no invasive components were detected. (C and D) Eg-11 carcinoma cells mainly arranged in clusters and trabeculae, and frequently invaded into a tissue which surrounded the primary carcinoma lesion (arrows). (D) shows invasive components of Eg-11 cells in the adipose tissue adjacent to the primary carcinoma site. Hematoxylin and eosin stain. Tumor tissues were resected at 2 months after the injection, and were fixed in 10% formalin and embedded in paraffin wax. Bar=100 μm.