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

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(Received November 30, 2006/Revised December 14, 2006/Accepted December 24, 2006/Online publication March 16, 2007)

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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doi: 10.1111/j.1349-7006.2007.00444.x

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).

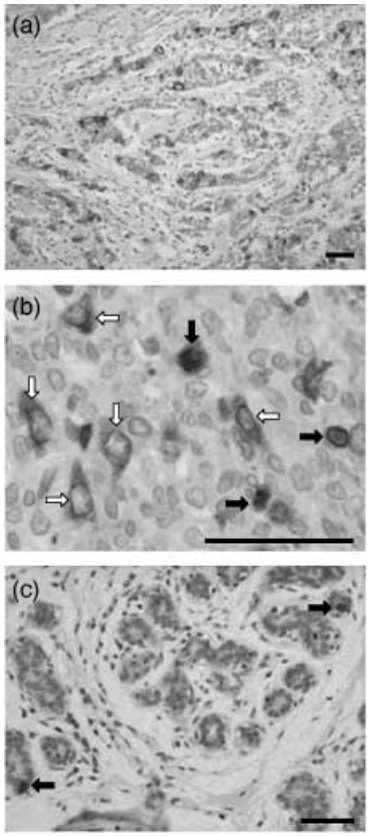

Fig. 1. Immunohistochemistry for cyclin B1 in the invasive ductal carcinoma. Cyclin B1 immunoreactivity was detected in the nucleus and/or cytoplasm of carcinoma cells: (a) lower magnification, (b) higher magnification. (b) Closed arrows represent nuclear cyclin B1 immunoreactivity, and open arrows show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

doi: 10.1111/j.1349-7006.2007.00444.x

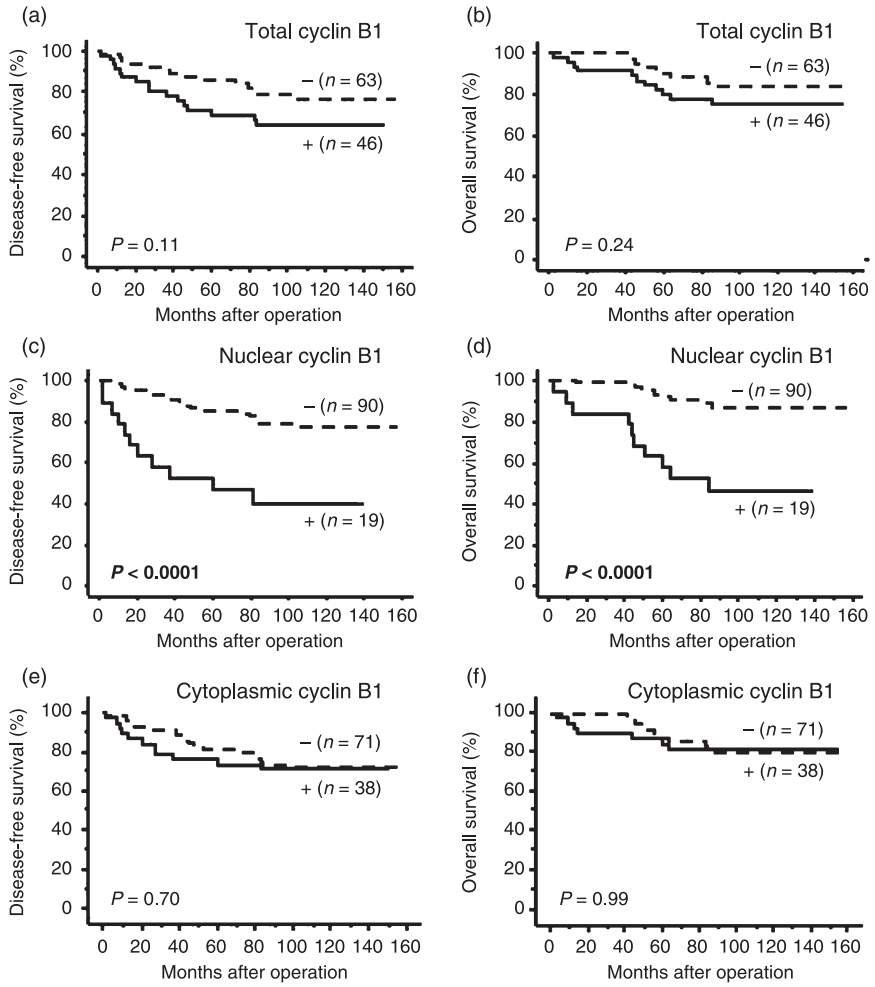

Fig. 2. Disease-free and overall survival of 109 patients with breast carcinoma according to the intracellular localization of cyclin B1 immunoreactivity (Kaplan–Meier method). Total cyclin B1 was not significantly associated with (a) disease-free or (b) overall survival. Nuclear cyclin B1 was significantly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

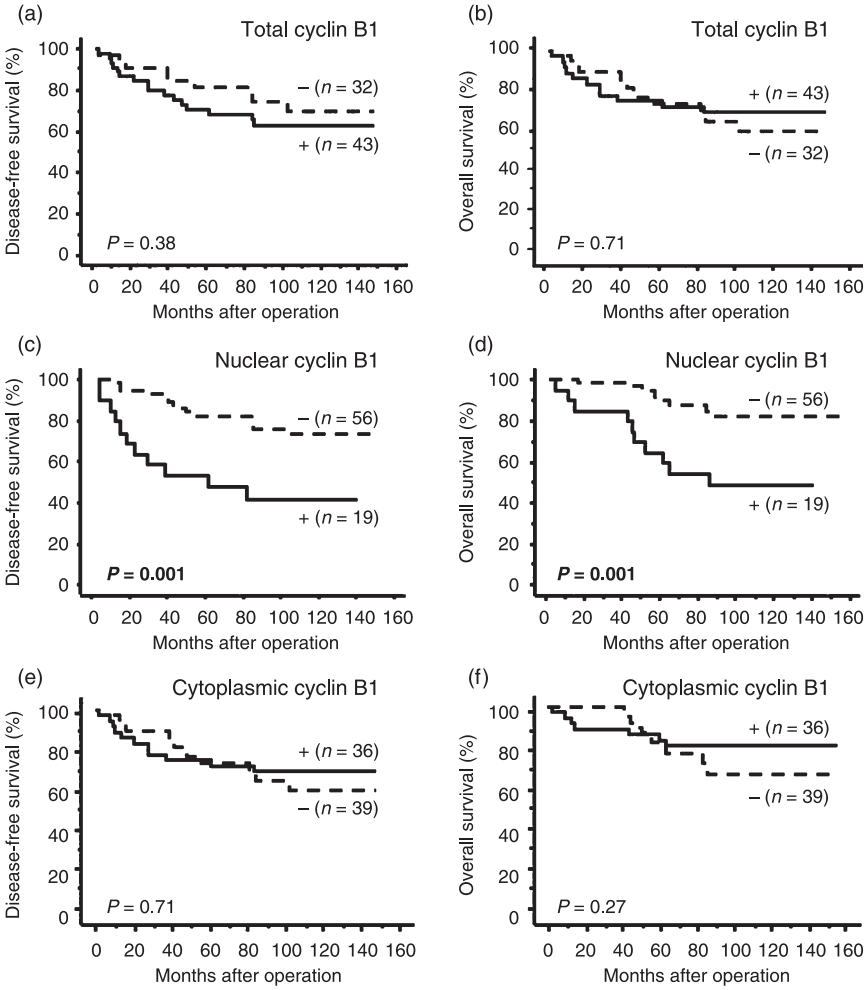

Fig. 3. Association between intracellular localization of cyclin B1 immunoreactivity and clinical outcome of the 75 patients showed high (>5 cells) mitotic count in the breast carcinoma (Kaplan–Meier method). There was no significant association between total cyclin B1 and (a) disease-free or (b) overall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

doi: 10.1111/j.1349-7006.2007.00444.x

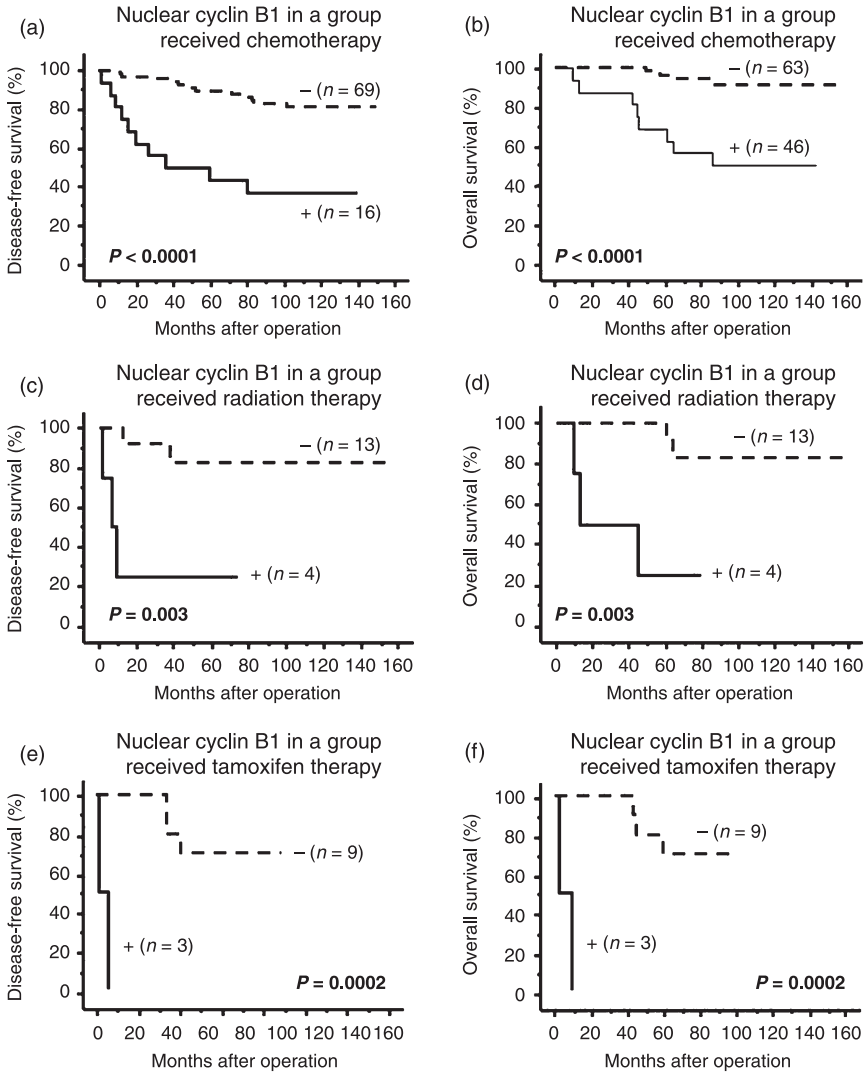

Fig. 4. Association between nuclear cyclin B1 immunoreactivity and clinical outcome of 109 breast carcinoma patients according to the adjuvant therapy (Kaplan–Meier method). Nuclear cyclin B1 immunoreactivity was significantly associated with adverse prognosis in the groups of patients receiving (a,b)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

Acknowledgment

We appreciate the skillful technical assistance of Mr Katsuhiko Ono (Department of Pathology, Tohoku University School of Medicine).

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