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).

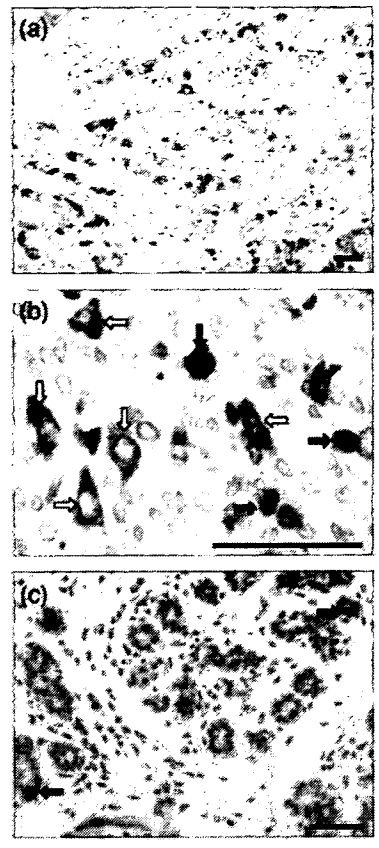

Fig. 1. Immunohistochemistry for cyclin B1 in the invasive ductal carcinoma. Cyclin B1 immunoreactivity was detected in the nucleus and/or cytoplasm of carcinoma cells: (a) lower magnification, (b) higher magnification. (b) Closed arrows represent nuclear cyclin B1 immunoreactivity, and open arrows show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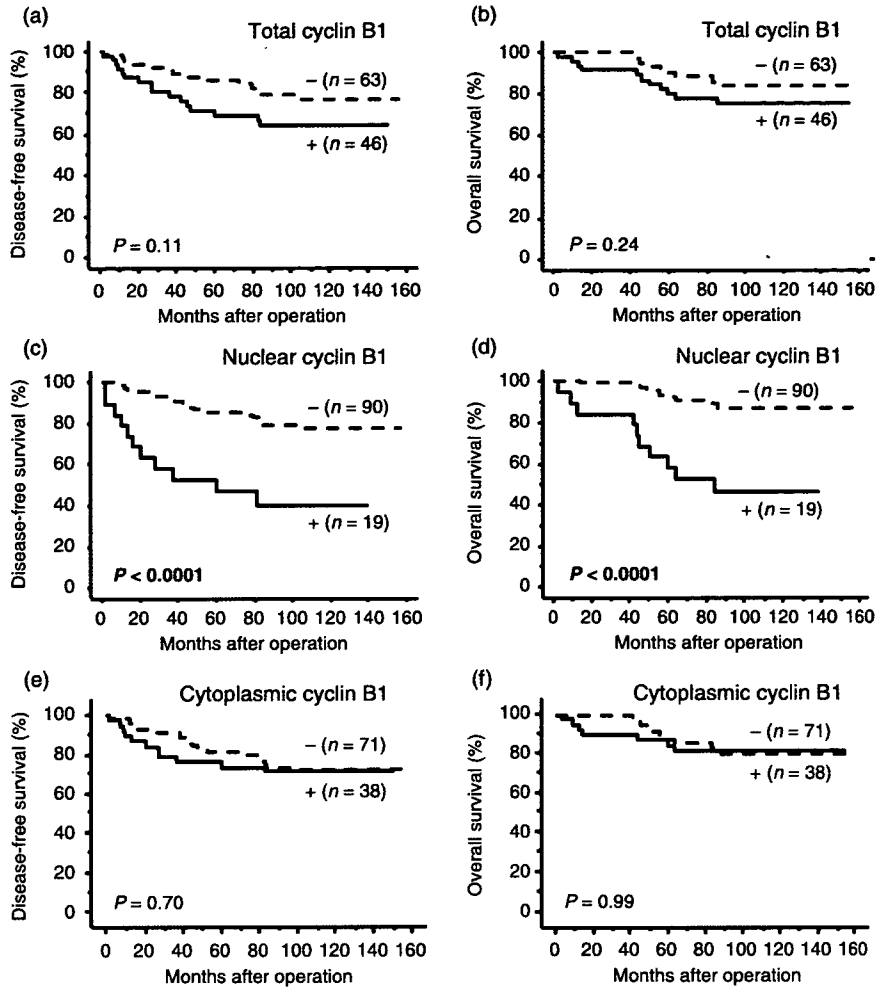

Fig. 2. Disease-free and overall survival of 109 patients with breast carcinoma according to the intracellular localization of cyclin B1 immunoreactivity (Kaplan–Meier method). Total cyclin B1 was not significantly associated with (a) disease-free or (b) overall survival. Nuclear cyclin B1 was significantly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

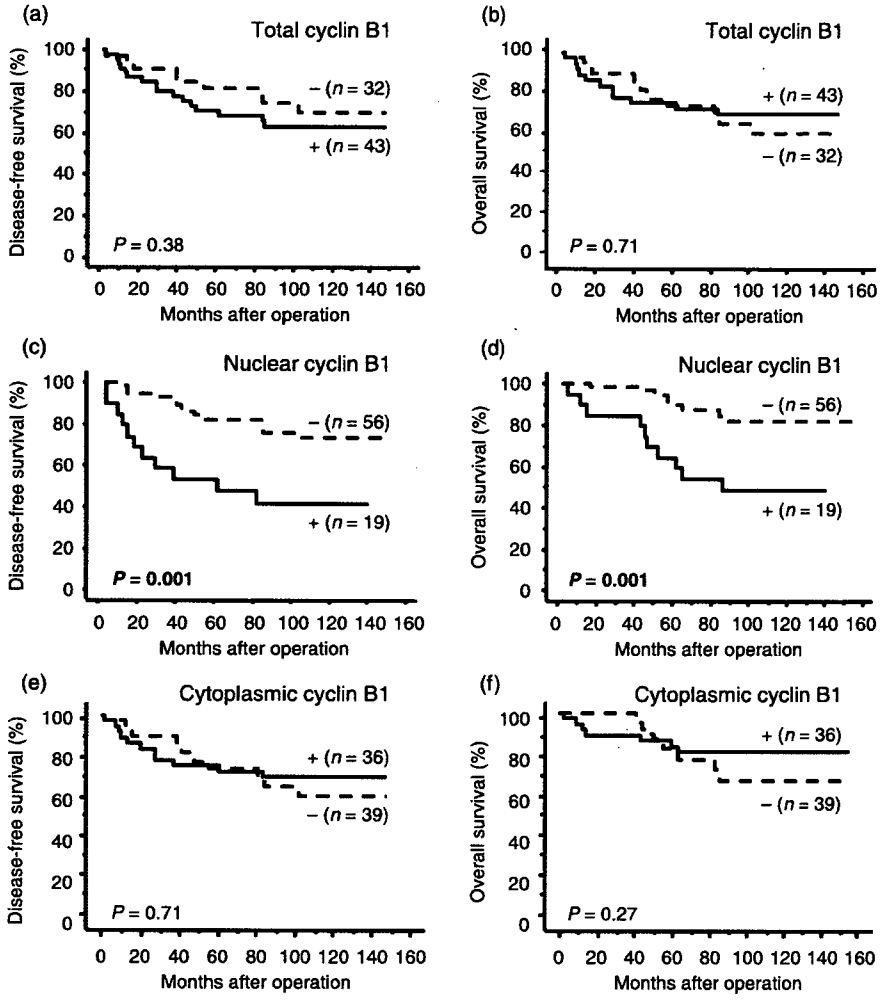

Fig. 3. Association between intracellular localization of cyclin B1 immunoreactivity and clinical outcome of the 75 patients showed high (>5 cells) mitotic count in the breast carcinoma (Kaplan–Meier method). There was no significant association between total cyclin B1 and (a) disease-free or (b) overall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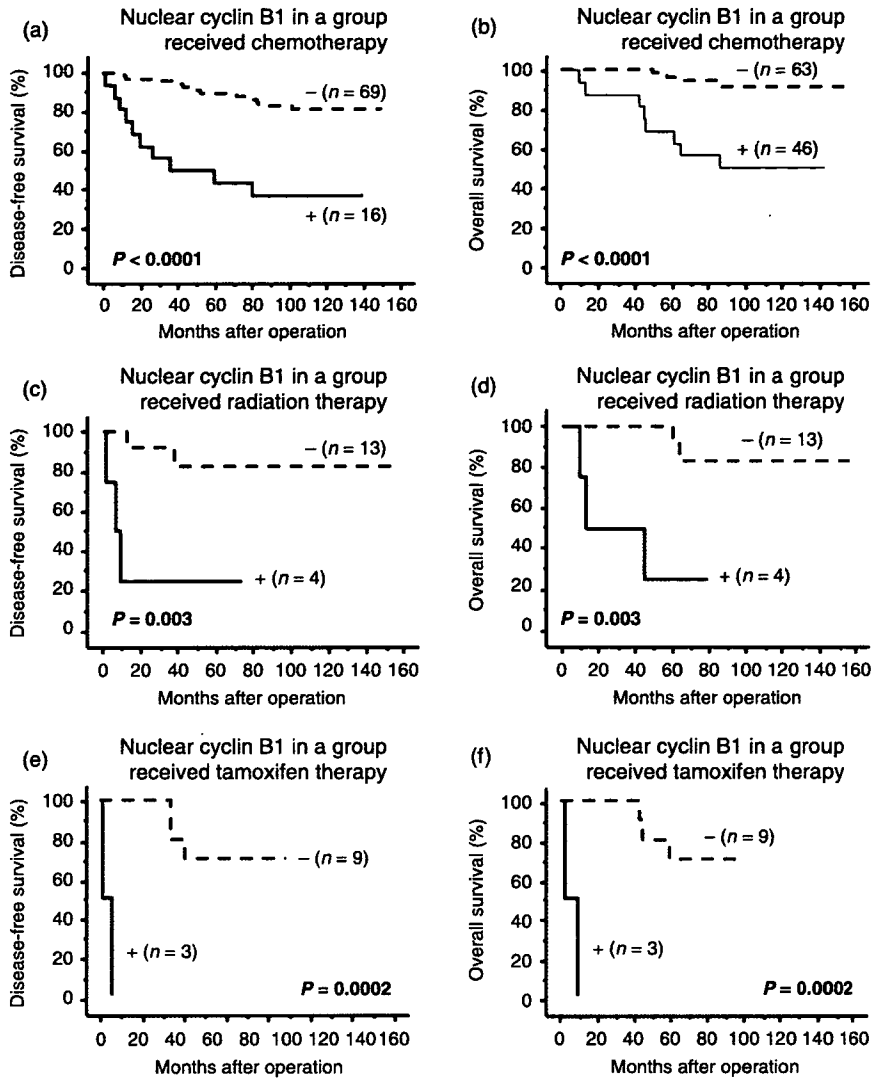

Fig. 4. Association between nuclear cyclin B1 immunoreactivity and clinical outcome of 109 breast carcinoma patients according to the adjuvant therapy (Kaplan–Meier method). Nuclear cyclin B1 immunoreactivity was significantly associated with adverse prognosis in the groups of patients receiving (a,b)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

Association of the *HTRA1* gene variant with age-related macular degeneration in the Japanese population

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Abstract The purpose of this investigation was to determine whether the high-temperature requirement A-1 (*HTRA1*) gene polymorphism is associated with age-related macular degeneration (AMD) in native, unrelated Japanese patients. A total of 123 patients with AMD and 133 control subjects without AMD were recruited for this study. The single-nucleotide polymorphism (SNP) rs11200638 in the *HTRA1* gene was assessed using a TaqMan assay. The risk A allele frequencies in the AMD cases and control patients were 0.577 and 0.380, respectively, and were associated with a significant risk of developing AMD ($p=7.75\times10^{-6}$). The results were more significant in subtype analyses with wet AMD ($p=5.96\times10^{-7}$). We conclude that the rs11200638 variant in the *HTRA1* gene is strongly associated with AMD in the Japanese population. This result supports the hypothesis that the *HTRA1* gene may increase susceptibility to AMD development and can participate in a potential new molecular pathway for AMD pathogenesis by extending this association across diverse ethnicities.

Keywords High-temperature requirement A-1 (*HTRA1*) · Age-related macular degeneration · Single-nucleotide polymorphism · Japanese population · Smoking

Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries. There are approximately 8 million people in the United States with features of early or intermediate AMD, of whom approximately 1 million will develop advanced AMD within the next 5 years (Age-Related Eye Disease Study Research Group 2000, 2003, 2005). Currently, AMD is estimated to affect about 50 million people worldwide (Klein et al. 2004).

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AMD is a clinically heterogeneous and genetically complex disease with multiple genetic and environmental risk factors (Age-Related Eye Disease Study Research Group 2000; Zareparsi et al. 2005). Reported risk factors include ocular pigmentation, dietary factors, positive family history for AMD, high blood pressure, smoking, and several gene mutations, such as ATP binding cassette transporter retina, apolipoprotein E, angiotensin converting enzyme, and fibulin 5 (Age-Related Eye Disease Study Research Group 2000, 2005; Klein et al. 2004; Bok 2005; Allikmets et al. 1997; Allikmets 2000; Klaver et al. 1998; Souied et al. 1998; Hamdi et al. 2002; Stone et al. 2004). Moreover, family-based genome-wide and candidate region linkage studies have successfully identified several major chromosomal regions, including 1q31 and 10q26 (Klein et al. 1998; Weeks et al. 2000; Majewski et al. 2003; Seddon et al. 2003; Kenealy et al. 2004; Abecasis et al. 2004; Fisher et al. 2005).

Recently, the complement factor H (CFH) gene on chromosome 1q31 has been demonstrated as the first major AMD susceptibility gene, and may associate with 30–50% of AMD cases. In the CFH gene, the Y402H variant and other intron variants have been proposed as potentially causative factors in more than ten different Caucasian populations of European descendent (Zareparsi et al. 2005; Klein et al. 2005; Haines et al. 2005; Edwards et al. 2005; Hageman et al. 2005; Li et al. 2006; Maller et al. 2006). Several studies have reported a second major susceptibility genetic locus at chromosome 10q26 for AMD, contributing independently of CFH to disease (Jakobsdottir et al. 2005; Rivera et al. 2005; Schmidt et al. 2006). Very recently, studies of Chinese (DeWan et al. 2006) and Caucasian (Yang et al. 2006) populations have demonstrated the identification of a single-nucleotide polymorphism (SNP) rs11200638 in the promoter region of the high-temperature requirement A-1 (*HTRA1*) gene polymorphism at this locus.

The purpose of this study is to confirm the association between this novel SNP rs11200638 in the *HTRA1* gene and AMD in the Japanese population, as ethnic variation has been reported in AMD-associated Y402H variant and also in other diseases (Okamoto et al. 2006; Gotoh et al. 2006; Grassi et al. 2006; Lau et al. 2006; Uka et al. 2006; Fuse et al. 2006; Chen et al. 2006; Mori et al. 2005). In addition, an important question is whether the *HTRA1* variant and smoking are independent risk factors, and investigating this was the second objective of the present study.

Methods

Subjects

The case–control sample was composed of 123 consecutive cases with AMD ranging in age from 51 to 87 years [71.9±8.7; mean±standard deviation (SD)], 89 men and 34 women, and 133 controls without AMD ranging in age from 51 to 88 years (67.9±9.5; mean±SD), 68 men and 65 women, recruited from outpatient visits to the Department of Ophthalmology, Saitama Medical University Hospital in the Saitama prefecture, Japan. All case–control subjects were unrelated, native Japanese Asian. The study was approved by the Ethics Committee of Saitama Medical University, and all procedures were conducted in accordance with the principles of the Declaration of Helsinki. Each individual was fully informed of the purpose of, and the procedures involved in, the study. Informed written consent was obtained for each patient.

Ophthalmic examination, definition, and subtype classification of AMD

All patients with AMD and the control subjects underwent full ophthalmologic examination, including slit lamp biomicroscopy, funduscopy, and contact lens biomicroscopic examination of the retina. All AMD patients had fluorescein and/or indocyanine green fundus angiography. Complete information regarding diet, family history, systemic conditions, and lifestyle, including smoking, were documented on each subject in a predesigned questionnaire. The visual acuity of AMD patients ranged from hand motion to 20/32. AMD subtypes were diagnosed and classified using the AREDS criteria (Age-Related Eye Disease Study Research Group 2000). The inclusion criteria were as follows: (1) age of 50 years or older, (2) diagnosis of AMD in one or both eyes, (3) no association with other retinochoroidal diseases, such as angioid streaks, high myopia (greater than 6D of myopic refractive error), central serous chorioretinopathy, and presumed ocular histoplasmosis, and (4) positive family history within parents, children, or siblings. There were 104 patients with neovascular (wet form of) AMD and 19 patients with non-neovascular (dry form of) AMD. The control subjects were confirmed not to have clinical evidence of AMD by the same complete ophthalmologic examination that was used to identify the study cohort of AMD patients.

Genotyping and statistical analysis

Genomic DNA was extracted from the peripheral blood of each individual using a DNA extraction and purification kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA) according to the manufacturer's instructions. The samples were genotyped using a TaqMan genotyping assay with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The data were analyzed using the Allelic Discrimination Program (Applied Biosystems).

Table 1 Allele and genotype distribution for the single-nucleotide polymorphism (SNP) rs11200638 in the high-temperature requirement A-1 (*HTRA1*) gene

	Cases			Controls
	All AMD[a]	Wet AMD	Dry AMD	
n	123	104	19	133
Allele				
G	104 (42.3)	81 (38.9)	23 (60.5)	165 (62.0)
A	142 (57.7)	127 (61.1)	15 (39.5)	101 (38.0)
Genotype				
GG	26 (21.1)	18 (17.3)	8 (42.1)	54 (40.6)
GA	52 (42.3)	45 (43.3)	7 (36.8)	57 (42.9)
AA	45 (36.6)	41 (39.4)	4 (21.1)	22 (16.5)

The data are expressed as the number of subjects (% of the entire group)

[a] Age-related macular degeneration

Genotype and allele frequencies between AMD cases and controls were compared using the Chi-square test for quality of proportions. Hardy-Weinburg equilibrium tests were performed by Chi-square analysis. All analysis was performed using commercially available software (SNPAlyze ver. 6.0, Dynacom, Chiba, Japan).

Results

The distributions of rs11200638 genotype and allele frequencies are given in Table 1. The genotype frequencies in cases and controls were in Hardy-Weinburg equilibrium ($p>0.1$). The risk A allele frequencies in all AMD cases and control patients were 0.577 and 0.380, respectively, and were associated with a significant risk of developing AMD ($\chi^2=20.0$, $p=7.75\times10^{-6}$). The odds ratio (OR) was 2.23 (95% confidence interval (CI): 1.57–3.18). In comparison to the wild-type homozygous (GG genotype), the ORs for all AMD with the homozygous risk (AA) and heterozygous risk (GA) genotypes were 4.25 (95% CI: 2.13–8.49) and 1.89 (95% CI: 1.04–3.45), respectively. The results were more significant in subtype analyses with wet AMD. The allele frequency Chi-square test yielded a p value of $p=5.96\times10^{-7}$ in comparison between wet AMD cases and control patients ($\chi^2=24.9$). The OR was 2.56 (95% CI: 1.76–3.72). The ORs for wet AMD with AA and GA genotypes were 5.59 (95% CI: 2.66–11.76) and 2.37 (95% CI: 1.22–4.59), respectively, when compared to GG (Table 2).

HTRA1 SNP rs11200638 was also found to have a significant association for AMD in both smokers (subjects who had ever smoked) and nonsmokers (subjects who had never smoked). The association was more significant in nonsmokers than in smokers ($p=1.7\times10^{-4}$ and 1.9×10^{-2}, respectively) (Table 3).

Table 2 p values and odds ratio (OR) for the SNP rs11200638 in the *HTRA1* gene

	χ^2	p*	OR (95% CI)[a]
All AMD versus controls			
Allele frequency	20.00	7.75×10^{-6}	2.23 (1.57–3.18)
Genotype AA versus GG[b]	17.55	2.81×10^{-5}	4.25 (2.13–8.49)
Genotype GA versus GG[c]	4.40	3.59×10^{-2}	1.89 (1.04–3.45)
Wet AMD versus controls			
Allele frequency	24.92	5.96×10^{-7}	2.56 (1.76–3.72)
Genotype AA versus GG[b]	21.94	2.82×10^{-6}	5.59 (2.66–11.76)
Genotype GA versus GG[c]	6.68	9.76×10^{-3}	2.37 (1.22–4.59)

*Chi-square test

[a] Odds ratio (95% confidence interval)

[b] Homozygous risk (AA) versus wild-type homozygous (GG)

[c] Heterozygous risk (GA) versus wild-type homozygous (GG)

Table 3 Allele frequencies, p values and ORs for the SNP rs11200638 in the *HTRA1* gene in smokers and nonsmokers

	Smokers		Nonsmokers	
	Cases	Controls	Cases	Controls
Allele frequency				
G	0.441	0.588	0.385	0.640
A	0.559	0.412	0.615	0.360
p*	1.9×10^{-2}		1.7×10^{-4}	
OR (95% CI) [a]	1.81 (1.10–2.98)		2.88 (1.64–5.06)	

*Chi-square test

[a] Odds ratio (95% confidence interval)

Discussion

In this study, we have demonstrated that the rs11200638 variant in the *HTRA1* gene is strongly associated with AMD in the Japanese population. The results were more significant in subtype analyses with wet AMD. The OR for wet AMD associated with the AA and GA genotypes were 5.59 (95% CI: 2.66–11.76) and 2.37 (95% CI: 1.22–4.59), respectively, when compared to the GG genotype. These results are similar to the published data for Chinese (De-Wan et al. 2006) and Caucasian (Yang et al. 2006) populations. Replication in diverse ethnic groups worldwide may provide a better appreciation of the role of *HTRA1* in AMD pathogenesis. The results presented here support the hypothesis that the *HTRA1* gene associates with susceptibility to AMD development, and extends this association across diverse ethnicities. In addition, our data showed that *HTRA1* SNP rs11200638 was also found to have a significant association for AMD in smokers and nonsmokers, and the association was more significant in nonsmokers than in smokers. This suggests that *HTRA1* plays a role in

AMD pathogenesis in both smokers and nonsmokers, and probably more considerably in nonsmokers. Further studies are needed to determine this gene–environment interaction with a larger study population.

The spectrum of clinical presentation or phenotype of Japanese AMD bears some differences compared to that observed in Caucasian AMD. There are also apparent differences in some etiologic factors compared to Western World cultures. In our consecutive case series of patients presenting in an outpatient setting, we had 104 patients with wet AMD, but only 19 patients with dry AMD. These and other epidemiological features characteristic of Asian AMD have been previously reported and include; male predominance, unilateral presentation, a comparatively low incidence of soft drusen, and a greater prevalence of wet AMD (Uyama et al. 1999, 2002; Sho et al. 2003; Bird. 2003; Chang et al. 1999).

Ethnic variation has been demonstrated in the AMD-associated Y402H variant of the CFH gene. Grassi et al. (2006) have reported the risk C allele frequencies in normal control populations among different ethnicities and they are as follows: Japanese 0.07±0.04, Hispanics 0.17±0.03, African Americans 0.35±0.04, Caucasians 0.34±0.03, and Somalis 0.34±0.03. This result is consistent with the international human haplotype map (HapMap) project database (The International HapMap Consortium 2003). Several Japanese case–control studies have not achieved significance in examining the association of the Y402H variant to AMD (Okamoto et al. 2006; Gotoh et al. 2006; Uka et al. 2006; Fuse et al. 2006). Although there remains a great deal to learn relating to CFH variants in the Chinese population, it appears that they more closely resemble CFH variants in a Japanese population than a Western Caucasian population (Lau et al. 2006; Chen et al. 2006). In contrast to CFH variants, our data demonstrate that the *HTRA1* variant in a Japanese population presents similar susceptibility to AMD development with the published findings for the Chinese and Caucasian populations. This finding is also consistent with those of another Japanese study published recently (Yoshida et al. 2007). Yang et al. (2006) have shown that this SNP in the *HTRA1* gene is the most likely causal variant for AMD at 10q26 in a Caucasian cohort. They have also found that drusen in the eyes of wet AMD patients were strongly immunolabeled with *HTRA1* antibody. DeWan et al. (2006) applied a whole-genome association mapping strategy to a Chinese population and have found a strong association of rs11200638 in the promoter region of the *HTRA1* gene and wet AMD. Importantly, this group has demonstrated that rs11200638 is functional in vitro by evaluating ARPE19 and HeLaS3 cells transfected with a relevant luciferase reporter plasmid. They hypothesized that CFH influences the drusen formation characteristic of dry AMD, whereas *HTRA1* influences choroidal neovascularization, the hallmark of wet AMD. Magnusson et al. (2006) have demonstrated that the CFH variant confers a similar risk of soft drusen and advanced forms of AMD, and has hypothesized that the CFH variant is a major risk factor for soft drusen formation, but that additional genetic and/or environmental factors may be required for progression to neovascular AMD. The results of our and other studies (Okamoto et al. 2006; Gotoh et al. 2006; Uka et al. 2006; Fuse et al. 2006) in the Japanese population may correlate with Japanese AMD characteristics of a comparatively low incidence of soft drusen and a greater prevalence of wet AMD, and support the hypothesis proposed by Magnusson et al. (2006), DeWan et al. (2006), and Yang et al. (2006).

In summary, this study indicates that the rs11200638 variant in the *HTRA1* gene is strongly associated with AMD in an ancestrally and geographically distinct population, as is represented by the Japanese population. This result supports the hypothesis that the *HTRA1* gene may increase susceptibility to AMD development and contribute in a potentially novel molecular pathway for AMD pathogenesis.

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ORIGINAL ARTICLES: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Association of a single nucleotide polymorphism in the steroid and xenobiotic receptor (*SXR*) gene (IVS1-579A/G) with bone mineral density

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Vitamin K2 plays an important role in the bone metabolism. The steroid and xenobiotic receptor (SXR) as a nuclear receptor activated by vitamin K2 as well as rifampicin could increase bone markers such as alkaline phosphatase in human osteoblastic cells. Thus, the SXR could mediate vitamin K2 signaling pathway in bone cells. Therefore, we analyzed expression of the SXR mRNA in human primary osteoblasts and chondrocytes. We also studied association of a single nucleotide polymorphism (SNP) in the *SXR* gene with bone mineral density (BMD). Expression levels of the SXR mRNA were analyzed during the culture course of human primary osteoblasts and chondrocytes. Association of a SNP in the *SXR* gene in intron 1 (IVS1-579A>G) with BMD was examined in 294 healthy postmenopausal Japanese women. The SXR mRNA increased at day 5 and then decreased at day 10 in human primary osteoblasts. Its mRNA gradually increased in human primary chondrocytes until day 10. As an association study of a SNP in the *SXR* gene (IVS1-579A/G), the subjects without the A allele (GG; $n = 47$) had significantly higher total BMD than the subjects bearing at least one A allele (AA + AG; $n = 247$) (*Z* score ± SD; 0.635 ± 1.031 *versus* 0.268 ± 1.061; $P = 0.0298$). The SXR mRNA was expressed and regulated in primary human osteoblasts and chondrocytes. A genetic variation at the *SXR* gene locus is associated with BMD, suggesting an involvement of the *SXR* gene in human bone metabolism.

Keywords: bone mineral density (BMD), osteoporosis, single nucleotide polymorphism (SNP), steroid and xenobiotic receptor (SXR), vitamin K2.

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture.[1] Twin and sibling studies have shown that bone mineral density (BMD) is under genetic control with estimates of heritability ranging 50–90%.[2–4] BMD is assumed to be determined by multiple genes with modest effects on bone mass and bone turnover as well as by environmental factors.[5,6] To date, various polymorphisms of candidate genes have been investigated in relation to BMD.[7,8] These include vitamin D receptor (*VDR*) gene,[9] estrogen receptor α (*ERα*) gene,[10] collagen type Iα1 (*COLIA1*) gene[11] and low-density lipoprotein receptor-related protein 5 (*LRP5*) gene.[12] Identification of candidate genes of which polymorphisms affect bone mass will be useful for early detection of

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individuals who are at risk for osteoporosis and early institution of preventive measures.

Vitamin K exerts an influence on the bone metabolism and is used as an anti-osteoporosis drug in Japan.[13] Moreover, vitamin K intake has been found to be associated with decrease of hip fracture risk.[14] In the bone homeostasis, a mechanism of vitamin K action is mediated through post-translation modification of proteins.[15] Vitamin K functions as an essential cofactor for carboxylation of glutamic acid residues to gammacarboxyglutamic acid residues. Recently, a novel mechanism was uncovered in the signaling that regulates the transcription of target genes by vitamin K through activation of a nuclear receptor, steroid and xenobiotic receptor (SXR, also known as PXR and NR1I2).[16] In the report, vitamin K2 was shown to bind to and activate the SXR that could induce bone markers such as alkaline phosphatase (ALP) and osteoprotegerin in the human osteoblastic cells.[16] Therefore, the SXR could be involved in the maintenance of bone homeostasis. In the present study, we examined the expression of the SXR in human primary osteoblasts and chondrocytes and the association between a polymorphism in the *SXR* gene and BMD in Japanese women to investigate possible contribution of the SXR in human bone metabolism.

Materials and methods

Cell culture

Primary human osteoblasts and chondrocytes were purchased from Cambrex (Charles City, IA, USA). Primary human osteoblasts were cultured 6-cm dishes in the osteoblast growth medium (OGM) medium (Cambrex) supplemented with SingleQuots for OGM, ascorbic acid and β-glycerophosphate for 2, 5, or 10 days according to the manufacturer's recommended protocol. Primary human chondrocytes were cultured in 6-cm dishes in the chondrocyte basal differentiation medium (CDBM) medium (Cambrex) supplemented with SingleQuots for CDBM (including insulin-like growth factor [IGF]-1, transforming growth factor [TGF]β1, insulin, transferrin and fetal bovine serum [FBS]) for 2, 5 or 10 days according to the manufacturer's recommended protocol.

Total RNA isolation and cDNA synthesis

Total RNA were extracted from the cells using a ToTALLY RNA Kit (Ambion, Austin, TX, USA). cDNA was synthesized from 1 μg of total RNA of primary osteoblasts using first strand cDNA synthesis kit (Amersham, Chicago, IL, USA).

SYBR Green real time PCR

Primers were designed using PRIMER EXPRESS 1.0 software (Applied Biosystems, Foster City, CA, USA). Definitive primers were: human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-TGG ACCTCATGGCCCACA-3′, reverse 5′-TCAAGGGG TCTACATGGCAA-3′; human ALP, forward 5′-TCCC ACGTCTTCACATTTGGT-3′, reverse 5′-AAGGGC TTCTTGTCTGTGTCACT-3′; human collagen type II alpha 1 (COL2A1), forward 5′-TTGCCTATCTGG ACGAAGCA-3′, reverse 5′-CGTCATTGGAGCCCT GGAT-3′; and human SXR forward 5′-ACTGCC TTTACTTCAGTGGGAATC-3′, reverse 5′-ATTCTC TTGCTTTTCTCACTGTGAAC-3′. Quantitative polymerase chain reaction (PCR) was carried out using a 2 × master mix composed from the SYBR Green PCR Core Reagents (Applied Biosystems) and 50 nmol/L primers. PCR reactions were performed using an ABI Prism 7000 system (Applied Biosystems) with the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. ALP, COL2A1 or SXR signal was normalized to GAPDH signal.

Subjects

Genotypes were analyzed in DNA samples obtained from 294 healthy postmenopausal Japanese women (mean age ± SD; 65.5 ± 8.9). Exclusion criteria included endocrine disorders (e.g. hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease), use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin sodium), or unusual gynecological history. All were non-related volunteers and provided informed consent before this study. Ethical approval for the study was obtained from the ethics committee of University of Tokyo Hospital and the ethics committee of Research Institute and Practice for Involutional Diseases.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentration of Ca, ALP), intact-osteocalcin (I-OC, enzyme-linked immunosorbent assay [ELISA]; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT) and 1, 25(OH)2D3. We also measured urinary ratios of urinary deoxypyridinoline (DPD, high-performance liquid chromatography [HPLC] method) to creatinine. The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20 000 Japanese women.

Determination of a single nucleotide polymorphism in the SXR gene

We extracted a polymorphic variation in the *SXR* gene intron 1 region from the Assays-on-Demand single nucleotide polymorphism (SNP) Genotyping Products database (Applied Biosystems), and, according to its localization on the gene, denoted it IVS1-579A/G. We determined the IVS1-579A/G polymorphism of the *SXR* gene using the TaqMan (Applied Biosystems) PCR method.[17] To determine the *SXR* SNP we used Assays-on-Demand SNP Genotyping Products C_1834250–10 (Applied BioSystems), which contains sequence-specific forward and reverse primers and two TaqMan Minor Groove Binder (MGB) probes for detecting alleles. During the PCR cycle, two TaqMan probes competitively hybridize to a specific sequence of the target DNA and the reporter dye is separated from the quencher dye, resulting in an increase in fluorescence of the reporter dye. The fluorescence levels of the PCR products were measured with the ABI PRISM 7000, resulting in clear identification of three genotypes of the SNP.

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the A-allele and the group with only G-allele encoded at that locus were subjected to statistical analysis (Student's *t*-test; StatView-J 4.5). A *P*-value less than 0.05 was considered statistically significant.

Results

SXR mRNA expression is regulated during the course of primary osteoblasts and chondrocytes differentiation

At the inception of this study, we measured the SXR mRNA levels during the course of differentiation in human primary osteoblasts and chondrocytes. In the presence of ascorbic acid and β-glycerophosphate, primary osteoblasts proceed to differentiation normally with the deposition of a collagenous extracellular matrix that mineralizes.[18,19] The continual maturation of the osteoblasts was reflected by the increase of ALP mRNA (Fig. 1a). The SXR mRNA increased at day 5 and then decreased at day 10 in human primary osteoblasts (Fig. 1c). In the presence of insulin and transferrin, primary chondrocytes proceed to differentiation normally[20,21] and the continual maturation of the chondrocytes was reflected by the increase of COL2A mRNA (Fig. 1b). The SXR mRNA gradually increased in human primary chondrocytes until day 10 (Fig. 1c).

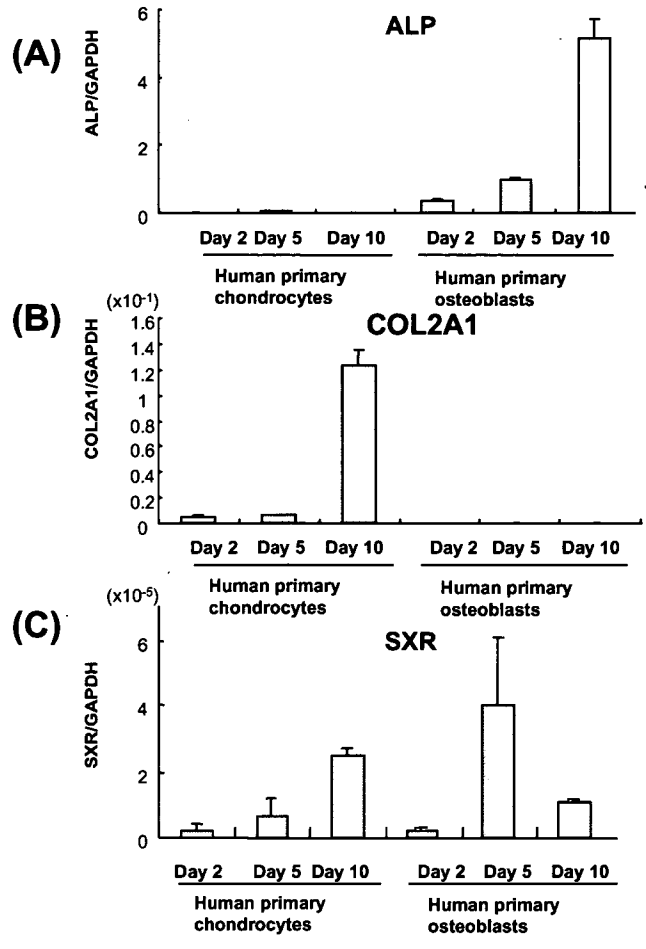

Figure 1 Expressions of the alkaline phosphatase (ALP), collagen type 2 α1 (COL2A1) and SXR mRNA during culture course of human primary osteoblasts and chondrocytes were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Human primary osteoblasts and chondrocytes were cultured with appropriate medium described in "Materials and methods" up to 10 days. At the indicated time, RNA was extracted and the expression levels of the ALP (a), COL2A1 (b) and steroid and xenobiotic receptor (SXR) (c) were analyzed by real-time PCR, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression ($n = 4$ for each group). Values are shown by means ± SD.

Association of the SXR gene polymorphism in intron 1 with BMD

During the search for SNP of human *SXR* gene by a SNP Genotyping database (http://www.appliedbiosystems.com), we noticed an SNP (IVS1-579A/G) in the *SXR* gene intron 1 region. We further studied this SNP for association analysis in Japanese women using the TaqMan methods, because it may affect transcriptional regulation of this gene. Among 294 postmenopausal volunteers, 112 were AA homozygotes, 135 were AG heterozygotes, and 47 were GG homozygotes.

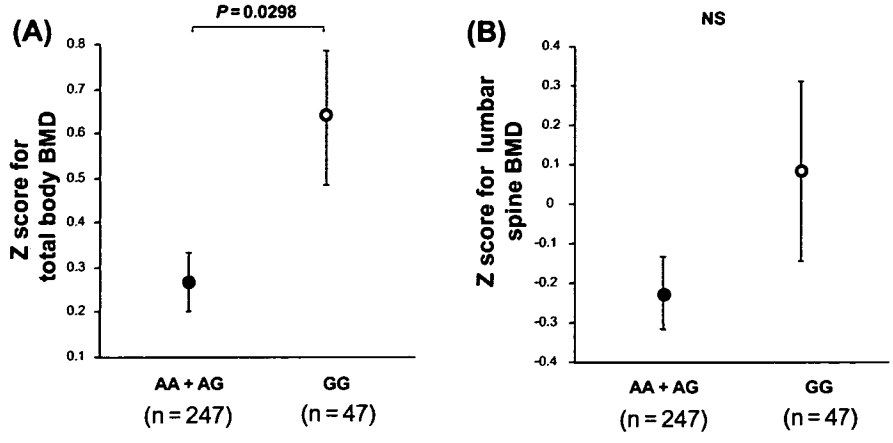

Figure 2 Z score values of total body and lumbar spine bone mineral density (BMD) in the groups of genotype AA + AG and genotype GG of the *SXR* polymorphism in intron 1 (IVS1-579). (a) Z score values for total BMD are shown for genotype AA + AG and for genotype GG. Values are expressed as mean ± SE. Number of subjects is shown in parentheses. (b) Z scores for lumbar BMD as shown in the same manner as (a).

Table 1 Comparison of background, bone mineral density and biochemical data between subjects bearing at least one A allele (AA + GA) and subjects with no A allele (GG) in the steroid and xenobiotic receptor (*SXR*) gene (IVS1-579A/G)

Items	Genotype (mean ± SD) AA + AG	GG	*P*-value
No. of subjects	247	47	
Age (years)	65.2 ± 9.0	66.7 ± 8.7	NS
Height (cm)	150.8 ± 6.5	151.6 ± 5.4	NS
Body weight (kg)	50.5 ± 8.1	51.5 ± 7.7	NS
Lumber spine BMD (Z score)	−0.224 ± 1.475	0.083 ± 1.547	NS
Total body BMD (Z score)	0.268 ± 1.061	0.635 ± 1.031	0.0298
ALP (IU/L)	190.9 ± 62.7	177.3 ± 57.8	NS
I-OC (ng/mL)	8.3 ± 4.2	7.5 ± 3.1	NS
DPD (pmol/μmol Cr)	7.8 ± 4.4	6.8 ± 2.5	NS
Intact PTH (pg/mL)	34.7 ± 16.8	33.7 ± 8.6	NS
Calcitonin (pg/mL)	22.4 ± 10.4	20.7 ± 14.7	NS
1,25 $(OH)_2D_3$ (pg/mL)	35.1 ± 11.3	34.5 ± 10.3	NS
% fat	31.6 ± 8.1	32.6 ± 6.2	NS
BMI	22.1 ± 3.2	22.4 ± 3.1	NS

ALP, alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; DPD, deoxypyridinoline; I-OC, intact-osteocalcin; NS, not significant; PTH, parathyroid hormone. Statistical analysis was performed according to the method described in the text.

We compared Z scores for BMD of total body and lumbar spine between the subjects bearing at least one A allele (AA + AG) and subjects without the A allele (GG). Comparison of the Z scores of the lumbar BMD between those with and without A allele showed a higher average value for GG homozygote group, but its difference was not statistically significant (Z score; 0.083 ± 1.547 vs −0.224 ± 1.475; $P = 0.195$) (Fig. 2b). On the other hand, Z score of the total body BMD in GG homozygote group was significantly higher than the other group (Z score; 0.635 ± 1.031 vs 0.268 ± 1.061; $P = 0.0298$) (Fig. 2a). The background and biochemical data were not statistically different between these two groups (Table 1).

Discussion

The nuclear receptor SXR (also known as PXR and NR1I2) plays a central role in the transcriptional regulation of xenobiotic detoxifying enzymes and transporters such as CYP3A4 and MDR1.[22–24] The SXR is activated by a diverse array of pharmaceutical agents, including Taxol, rifampicin, SR12813, clotrizole, phenobarbital, hyperforin, the herbal antidepressant Saint John's wort, and peptide mimetic HIV protease inhibitors such as ritonavir.[25–27] Recently, it was shown that vitamin K2 was a novel ligand for the SXR and could induce bone marker genes through the SXR.[16] The SXR is a member of nuclear receptor NR1I subfamily. The

vitamin D receptor (VDR, NR1I1) is a close relative of the SXR in terms of amino acid sequence similarity and belongs to the same subfamily.[22] Many reports showed that the VDR is expressed and regulated in the bone cells and *VDR* gene allelic variants could predict bone mineral density.[28] These data prompted us that the SXR may have a role in the bone homeostasis, especially in osteoporosis, as like other NR1I subfamily members.

In the present study, during the course of primary osteoblast differentiation, the increase of ALP expression, that is a marker of osteoblast differentiation[29] was followed by the increase of the SXR expression. A recent report also demonstrated that the SXR expression was detected in human osteosarcoma cell lines HOS, MG-63 and SaOS2.[16] Interestingly, the vitamin K2, one of the ligands for the SXR, upregulated the steady state mRNA levels for a panel of osteoblastic bone markers including ALP in these cells. Thus, it is possible that the SXR is involved in the differentiation of osteoblasts and the regulation of ALP gene. We have shown that the SXR expression was detected in human primary chondrocytes as well as in primary osteoblasts and increased in parallel with the increased expression of COL2A, which is a marker of chondrocyte differentiation.[20] It is also possible that the SXR is involved in the cartilage metabolism. Future studies should be required on how vitamin K and the SXR signaling could be delivered to the regulation of skeletal differentiation.

To our knowledge, the present study is the first to investigate the influence of a polymorphism of the *SXR* gene on the BMD. We demonstrated that the Japanese postmenopausal women who had two alleles of an intronic change of A-G transition showed significantly higher total-body BMD. Lumbar BMD was also higher in the subjects bearing at two G alleles, although the difference was not statistically significant. Lower BMD in postmenopausal women can be considered as a result of abnormally rapid bone loss and/or lower peak bone mass. The SNP analyzed in this study would be useful as a genetic marker for low BMD and the susceptibility to osteoporosis. Although the biological meanings of this polymorphism should be revealed by functional studies, the *SXR* IVS1-579A/G polymorphism may modulate BMD by influencing transcription and/or expression levels of the SXR.

In conclusion, our findings suggest that the *SXR* gene may be a genetic determinant of BMD in postmenopausal women as is the case with its related nuclear receptor, VDR. Examining the variation in the SXR gene will hopefully enable us to elucidate one of mechanisms of involutional osteoporosis. Furthermore, the variation may be a potential genetic susceptibility factor that need to be further evaluated with regard to the condition of other metabolisms in which the *SXR* have been clearly implicated, including lipid and drug metabolisms.[22,24]

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MINI REVIEW

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Steroid and xenobiotic receptor mediates a novel vitamin K_2 signaling pathway in osteoblastic cells

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Abstract The nuclear receptor steroid and xenobiotic receptor (SXR) is a transcriptional regulator activated by various biological and xenobiotic substances. We have recently shown that SXR is expressed in bone and that this receptor is critical for bone metabolism, particularly in osteoblastic cells. Vitamin K_2, one of the critical nutrients in bone metabolism, has been demonstrated that it is a potent SXR agonist and modulates the expression of various bone-related genes in osteoblastic cells. Using microarray analysis, we identified novel SXR target genes that were activated by vitamin K_2 in osteoblastic cells. Among them, a small leucine-rich repeat proteoglycan, tsukushi, has been shown to contribute to collagen accumulation, and the protein may interact with another vitamin K_2-inducible SXR target, matrilin-2, a member of the matrilin family that functions as collagen adaptors. Besides functioning as a xenobiotic biosensor, our findings show that SXR is also a vitamin K_2 target and an important transcriptional factor that regulates bone homeostasis in bone cells.

Key words steroid and xenobiotic receptor · vitamin K_2 · osteoblastic cells · collagen accumulation

Introduction

Steroid and xenobiotic receptor (SXR) is a nuclear receptor that activates transcription in response to a diversity of natural and synthetic compounds. We have recently shown that SXR is expressed in bone and that this receptor is critical for bone metabolism. In particular, it has been demonstrated that vitamin K_2, one of the effective osteoporosis therapeutic agents, is a potent SXR agonist and modulates the expression of various bone-related genes in osteoblastic cells. This article reviews the current findings in regard to the SXR signaling pathways and vitamin K_2 actions in osteoblastic cells.

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Structure and ligand specificity of SXR

SXR [1] (also termed pregnane X receptor, PXR [2]) belongs to the nuclear receptor subfamily 1, group I (NR1I). Similar to other nuclear receptors, SXR has a structure of N-terminal DNA-binding domain (DBD) and C-terminal ligand-binding domain (LBD). Compared with the high homology of human SXR DBD with mouse PXR DBD (95% identity), human SXR LBD shares lower homology (73%) with mouse PXR LBD. This difference in LBD structure among species provides one explanation for ligand specificity [1] (Fig. 1). SXR forms a heterodimer with the 9-*cis*-retinoic acid receptor RXR and binds to specific SXR response elements (SXREs) in the genome, regulating the transcription of its target genes in the vicinity of the SXR-binding sites [1] (Fig. 2). Direct or everted repeats of the 6-bp half-site sequence 5′-AG(G/T)TCA-3′ are known as SXRE, including DR-3, DR-4, ER-6, or ER-8 with the corresponding number of spacer bases between the half-sites [2].

SXR is predominantly expressed in liver and intestine and plays an important role in the metabolism of endogenous substances such as bile acids and steroid hormones, as well as in the detoxification of various chemical agents and xenobiotics. This SXR function is mediated by its downstream responsive genes including drug-metabolizing enzyme genes. Phase I drug-metabolizing enzymes such as cytochrome P450 3A (CYP3A) genes, phase II drug-conjugating enzymes such as UDP-glycosyltransferase