factor for PSA recurrence, but not for cancer-specific survival after radical prostatectomy [19]. The restricted low range of preoperative PSA in the present study (25th and 75th percentile, 6.2 and 18.1) may have limited the predictive value of preoperative PSA levels on cancer-specific survival. Data reported by other groups were consistent with our results that only pT stage, and GS were significant prognostic factors about cancer specific survival after radical prostatectomy [19,20].

Expression of CYP2B6 has been recently investigated in a few carcinomas, including breast cancer and hepatocellular carcinoma (HCC). In breast cancer, CYP2B6 mRNA was down-regulated in the tumor tissue compared with normal adjacent tissue [10]. In addition, CYP2B6 mRNA was significantly lower in HCC with venous invasion than in HCC without venous invasion [21]. These findings suggest that CYP2B6 expression may be decreased in the development of cancers that are closely related to substrates of CYP2B6.

CYPs sensitize tumor xenograft to anticancer prodrugs such as cyclophosphamide (CPA). The anticancer activity of CPA in cultured tumor cells and in rodent and human xenograft models is substantially increased by introduction of cDNAs encoding CYP2B6, which are major catalysts of CPA activation in rat and human liver, respectively [22,23]. On the other hand Ikezoe et al. demonstrated that ritonavir blocked the docetaxel-induced expression of CYP3A4 at the mRNA level in prostate cancer DU145 cells and enhanced the antitumor effect of docetaxel in vitro and in BNX nude mice bearing DU145 tumors [24]. These findings showed that extrahepatic CYPs also biotransformed drugs like hepatic CYPs and that this biotransformation may be associated with activation or deactivation of anticancer drugs.

Testosterone is inactivated in the liver and the prostate. CYP2B6 are known to function in hepatic testosterone inactivation [7,25]. In the prostate, several enzymes are involved in the metabolism of testosterone. 5α-Reductase converts testosterone to DHT. DHT is converted by the enzymes 3α-and 3β-hydroxysteroid oxidoreductase into 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3βA-diol), respectively. The 3βA-diol is further hydroxylated to 6α-, 6β-, 7α-, and 7β-triols which are inactive as androgens and excreted from the prostate. In addition, rat CYPs catalyze the 6α-, 6β-, 7α-, and 7β-hydroxylation of the 3βA-diol in the ventral prostate [26–28] However little is known about the role of CYPs in testosterone metabolism in human prostate. We hypothesized that, consistent with the role of CYP2B6 in liver and the role of CYPs in rat prostate, CYP2B6 might play an important role in testosterone metabolism in the human prostate. If so, then decreased expression of CYP2B6 might contribute to elevated DHT levels in the prostate, thus promoting cancer progression. Consistent with this hypothesis, our present study showed that decreased expression of CYP2B6 significantly correlated with poor prognosis in human prostate cancer. Moreover, stable overexpression of CYP2B6 in LNCaP reduced their proliferation as well as testosterone-induced proliferation. Taken together, these results support the view that CYP2B6 interferes with proliferation of prostate cancer cells by either catabolize some pro-proliferative substances such as testosterones, or metabolize anti-proliferative substances.

CONCLUSIONS

In conclusion, our results indicate that decreased CYP2B6 expression was an independent prognostic factor for prostate cancer and that CYP2B6 overexpression interfered with proliferation of LNCaP cells. Immunoreactivity of CYP2B6 may therefore be useful in selecting patients with more aggressive tumor for adjuvant therapy. These results suggest that CYP2B6 has significant anti-tumor effects in prostate cancer. Therefore, compounds which highly induce CYP2B6 such as phenobarbital, rifampicin, clotrimazole, phenytoin and carbamazepine [29–31] might be of clinical benefit in the treatment of recurrent prostate cancer.

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

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

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

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

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

Materials and Methods

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

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

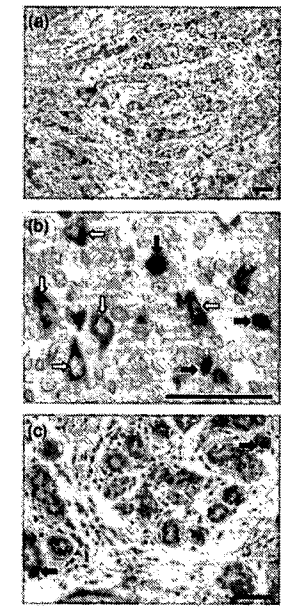

Fig. 1. Immunohistochemistry for cyclin B1 in the invasive ductal carcinoma. Cyclin B1 immunoreactivity was detected in the nucleus and/or cytoplasm of carcinoma cells: (a) lower magnification, (b) higher magnification. (b) Closed arrows represent nuclear cyclin B1 immunoreactivity, and open arrows show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

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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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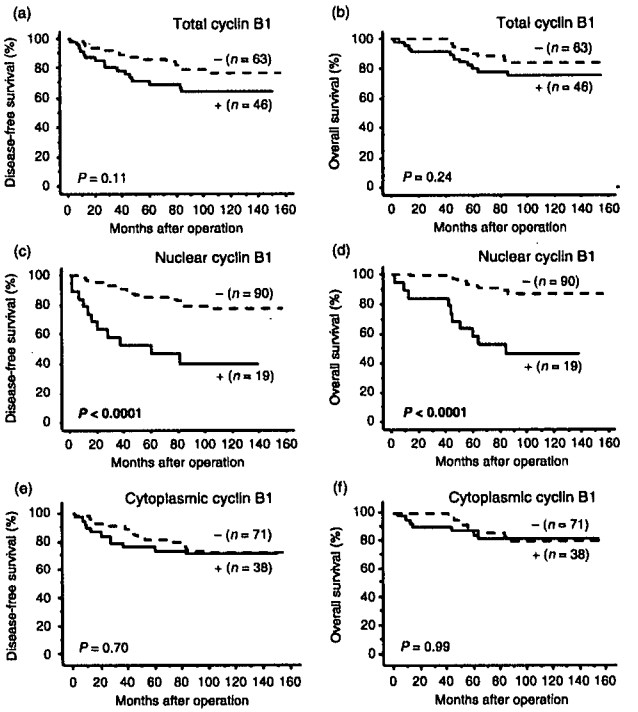

Fig. 2. Disease-free and overall survival of 109 patients with breast carcinoma according to the intracellular localization of cyclin B1 immunoreactivity (Kaplan–Meier method). Total cyclin B1 was not significantly associated with (a) disease-free or (b) overall survival. Nuclear cyclin B1 was significantly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

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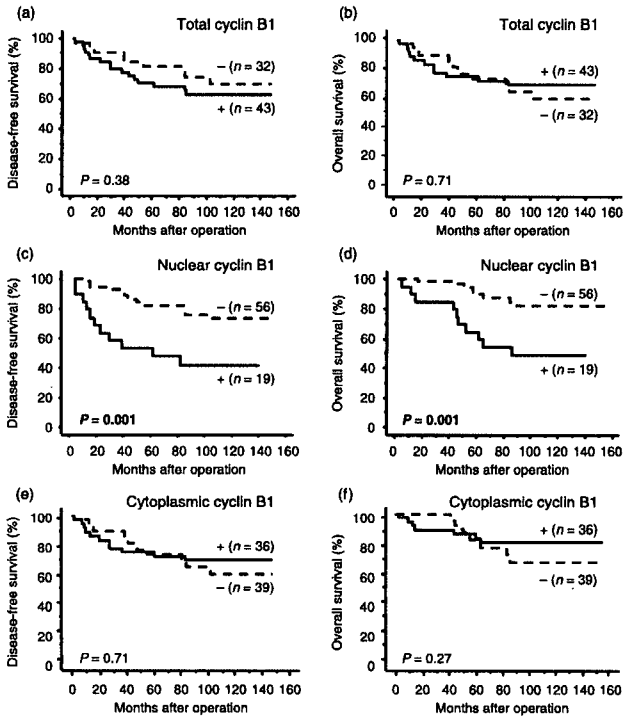

Fig. 3. Association between intracellular localization of cyclin B1 immunoreactivity and clinical outcome of the 75 patients showed high (>5 cells) mitotic count in the breast carcinoma (Kaplan–Meier method). There was no significant association between total cyclin B1 and (a) disease-free or (b) overall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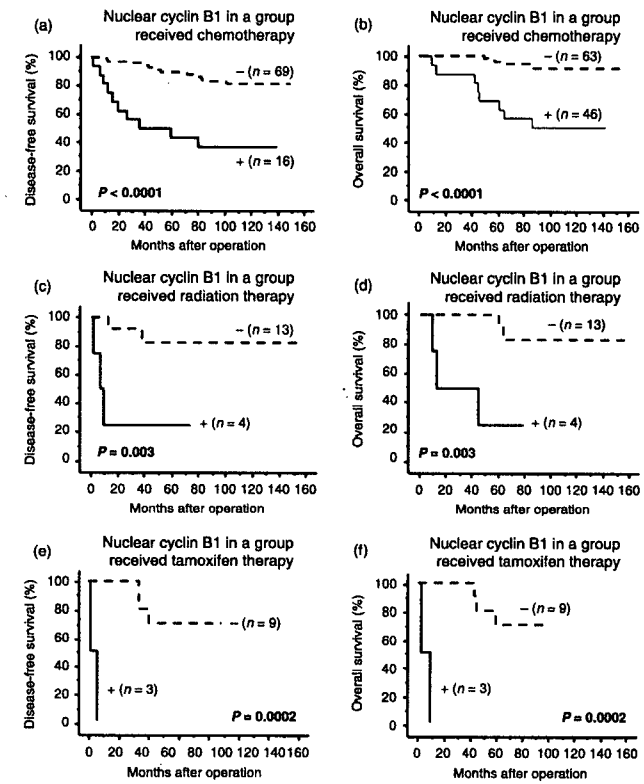

Fig. 4. Association between nuclear cyclin B1 immunoreactivity and clinical outcome of 109 breast carcinoma patients according to the adjuvant therapy (Kaplan–Meier method). Nuclear cyclin B1 immunoreactivity was significantly associated with adverse prognosis in the groups of patients receiving (a,b)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

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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=7.75×10^{-6}). The results were more significant in subtype analyses with wet AMD (p=5.96×10^{-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

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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).

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 (DeWan 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 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/cm²) 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]; Tcijin, 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.

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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).

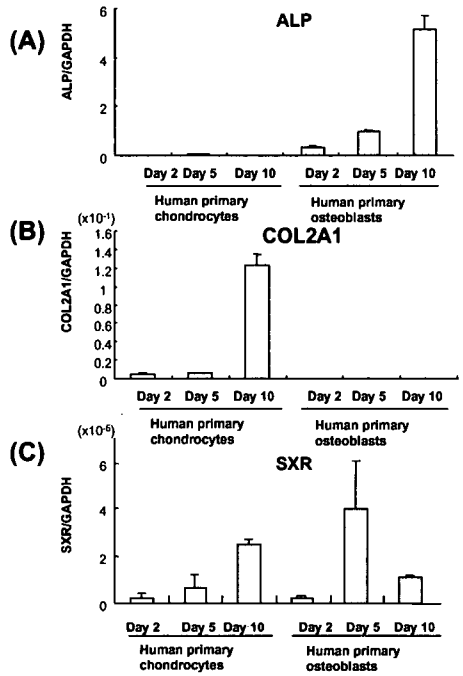

Figure 1 Expressions of the alkaline phosphatase (ALP), collagen type 2 α1 (COL2A1) and SXR mRNA during culture course of human primary osteoblasts and chondrocytes were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Human primary osteoblasts and chondrocytes were cultured 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.

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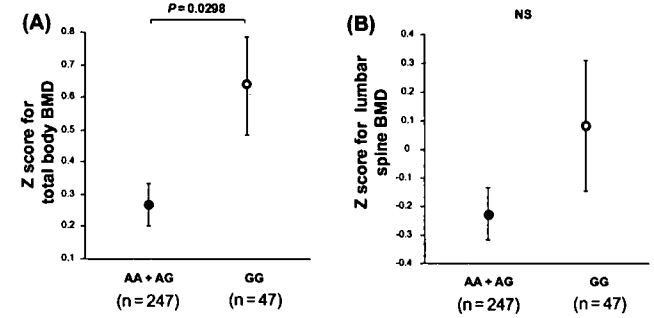

Figure 2 Z score values of total body and lumbar spine bone mineral density (BMD) in the groups of genotype AA + AG and genotype GG of the *SXR* polymorphism in intron 1 (IVS1-579). (a) Z score values for total BMD are shown for genotype AA + AG and for genotype GG. Values are expressed as mean ± SE.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

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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

Kuniko Horie-Inoue · Satoshi Inoue

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

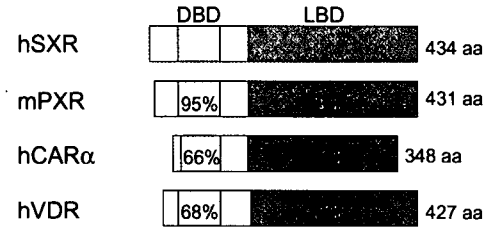

Fig. 1. Schematic comparisons among the nuclear receptor steroid and xenobiotic receptor (SXR) and its related receptors. All the receptors belong to nuclear receptor subfamily 1, group I (NR1I), and form heterodimers with their common partner retinoid X receptor (RXR). The similarity between SXR and other receptors is expressed as percent amino acid identity [1]. *DBD*, DNA-binding domain; *LBD*, ligand-binding domain; *hSXR*, human SXR; *mPXR*, mouse pregnane X receptor; *hCARα*, human constitutive androstane receptor-α; *hVDR*, human vitamin D receptor

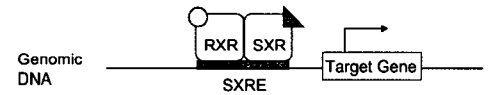

Fig. 2. Transcriptional regulatory mechanism of SXR. The ligand-activated SXR forms heterodimers with RXR and regulates the transcription of adjacent target genes by binding to SXR response elements (SXREs) in the genome

(UGT) 1A gene, and phase II drug transporters including multidrug resistance protein 1 are SXR-targeted drug-metabolizing enzymes, and they coordinately function in the catabolic processes [3].

Regarding SXR agonists, the antibiotic rifampicin, the calcium blocker nifedipine, and a constituent of St. John's wort, hyperforin, are known as prototypic human SXR ligands. As already described, there is a difference in ligand specificity between human SXR and its orthologue mouse PXR. For example, a potent agonist for human SXR rifampicin is not reactive to mouse PXR, whereas a synthetic steroid pregnenolone 16α-carbonitrile is an effective agonist for mouse PXR [2].

Physiological roles of SXR and vitamin K in bone metabolism

Recently, our group and collaborators have demonstrated that SXR is expressed in osteoblastic cells and that vitamin K_2 modulates the expression of bone-related genes via the SXR signaling pathway [4]. It has been revealed that vitamin K_2 activates SXR by binding to the LBD of SXR and that the active form of SXR promotes the transcription of a prototypic SXR target gene CYP3A4 by conjugating to the SXR in the regulatory region of CYP3A4 gene. Furthermore, it has been also shown that osteoprotegerin (OPG) and alkaline phosphatese (ALP), which are related to the maintenance of bone-building function, are induced in osteoblastic cells by vitamin K_2 and other SXR ligands such as rifampicin and hyperfolin. Because vitamin K_2-dependent induction of the OPG gene was not observed in osteoblasts derived from PXR-deficient mice, it has been suggested that this vitamin K_2-induced gene expression is mediated through SXR-dependent transcriptional regulation.

Vitamin K has been known as a critical cofactor of γ-glutamyl carboxylase (GGCX) as well as a potent stimulator of the bone-building process. Vitamin K_2, or menaquinones, is one of the natural forms of vitamin K derived from animal sources and the Japanese fermented soybean food, *natto*, as well as being produced by intestinal bacteria. Vitamin K_2 is particularly more active in both promoting bone formation and reducing bone loss compared with vitamin K_1, which is also a natural vitamin K originated from plants [5–9]. Thus, vitamin K_2 is clinically important for bone tissues and it is shown to be effective for osteoporosis treatment as well as the prevention of bone fractures [10,11]. Interestingly, we have previously shown that there was a significant inverse correlation between the incidence of hip fracture and *natto* intake in Japanese postmenopausal women. Serum MK-7 levels were much higher in Japanese women compared with British women and were significantly associated with *natto* consumption, suggesting that *natto* is a good resource of vitamin K_2 that reduces the risk of bone fracture [12].

In regard to the functions of vitamin K, the posttranslational modification of vitamin K-dependent proteins containing γ-carboxyglutamic acid (Gla) residues is the best known action, which is particularly important in blood coagulation [13]. In vitamin K-dependent carboxylation reactions, the reduced form of vitamin K deprotonates glutamate via the GGCX and the reduced vitamin K is converted to vitamin K epoxide. Osteocalcin (bone Gla protein, BGP) and matrix Gla protein (MGP) are known as bone-related Gla proteins that play roles in bone homeostasis. Osteocalcin is a protein that is only synthesized in osteoblasts and odontoblasts and thus it serves as a good biochemical marker of the metabolic turnover of bone. The expression of human and rat osteocalcins is positively regulated by 1,25-dihydroxyvitamin D_3 [14–17], whereas mouse osteocalcin genes OG1 and OG2 are indirectly downregulated by 1,25-dihydroxyvitamin D_3 [18]. Osteocalcin contains three Gla residues at positions 17, 21, and 24 of the amino acid sequence, which are responsible for its high affinity for hydroxyapatite, one of the major components of bone matrix [19,20]. Thus, the level of undercarboxylated osteocalcin reflects vitamin K function, and it has been reported to increase during aging and to significantly correlate with fracture risk [21]. On the other hand, MGP is predominantly expressed in chondrocytes and vascular smooth muscle cells. *Mgp*-deficient mice exhibited inappropriate calcification of various cartilages as well as arterial walls, indicating that MGP is a modulator of extracellular matrix mineralization [22,23]. As considered by the functions of these bone-related Gla proteins, it is plausible that one of the major vitamin K actions is the modulation of bone matrix mineralization. Yet, it has been speculated that vitamin K may

have another critical mechanism that is involved in the modulation of bone quality.

We have recently found a novel mechanism of vitamin K action in bone cells. Based on microarray analysis, we showed that vitamin K_2 regulated the transcription of genes encoding extracellular matrix (ECM) proteins in an SXR-dependent manner and increased the accumulation of collagen in osteoblastic cells [24]. In the experimental system of the human osteoblastic cell line MG63 that stably expresses SXR, ECM-related genes tsukushi (TSK) and matrilin-2 (MATN2) have been identified as genes inducible by vitamin K_2 and rifampicin. Tsukushi is a novel gene that has been found to belong to the small leucine-rich repeat (SLRR) proteoglycan family [25]. It has been shown that the SLRR family plays a critical role in bone formation, as significant bone phenotypes have been observed in mice deficient for biglycan and decorin, other known members of this family [26,27]. The matrilins form a four-member family of modular, multisubunit matrix proteins that are expressed in cartilage but also in many other forms of extracellular matrix. They participate in the formation of fibrillar or filamentous structures and are often associated with collagens [28]. The interaction between the SLRR proteins and matrilins has been reported previously as biglycan and decorin form complexes with collagen fibrils together with the matrilin-1 [29]. We generated MG63 cells stably expressing TSK and showed that collagen accumulation was significantly increased in the TSK-expressing cells compared with the control MG63 cells expressing empty vector. Vitamin K_2 itself augments collagen accumulation in MG63 cells, and this reaction can be repressed by gene knockdown using a small interfering RNA specific to TSK or SXR [24]. Moreover, we confirmed, by performing immunoprecipitation and immunohistochemistry studies, that TSK and MATN2 could colocalize and interact with each other on the cell surface. Taken together, our results demonstrate that SXR has a collagen-accumulating action in osteoblastic cells by regulating the transcription of ECM-related genes and suggest that vitamin K_2 potentially contributes to the improvement of bone quality using the SXR-dependent pathway (Fig. 3).

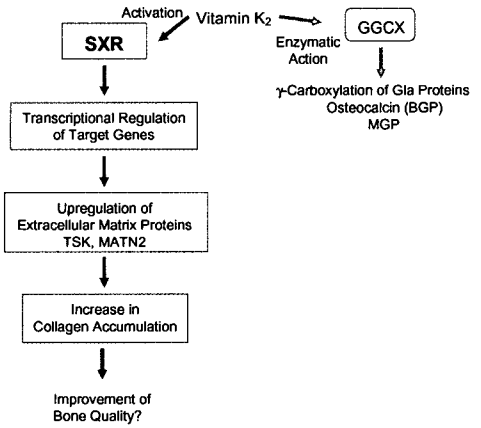

Fig. 3. SXR- and vitamin K_2-dependent regulatory mechanisms of bone metabolism in osteoblastic cells. SXR promotes collagen accumulation in osteoblastic cells by regulating the transcription of its target genes including those encode extracellular matrix proteins. Vitamin K_2 plays a role in the posttranslational modification of Gla proteins by functioning as a coenzyme of γ-glutamyl carboxylase (GGCX) and also acts as a potent SXR ligand in bone metabolism

SXR and its surrounding molecules

It remains to be studied how SXR exerts its physiological functions, including osteogenic differentiation and collagen accumulation in bone tissues. Although SXR seems to have a relatively low specificity for ligands compared with other nuclear receptors such as steroid hormone receptors and vitamin D receptor, it has also been reported that the types of SXR target genes as well as the cofactors involved in the transcriptional regulation may vary depending on the ligands [30]. In hepatocytes, it has been shown that the transcription of CYP3A4 gene is activated by SXR in collaboration with another transcriptional factor, hepatocyte nuclear factor 4α [31], suggesting that SXR signaling may be modulated by the interaction with other surrounding molecules such as nuclear receptors, transcriptional factors, and cofactors. Interestingly, SXR has been recently revealed to compete with the NF-κB signaling pathways that can be activated by inflammatory status [32]. This reciprocal repression between SXR and NF-κB is physiologically important as it is known that inflammatory stimuli suppress the expression of hepatic CYP genes and that various xenobiotics and SXR-responsive drugs exert immunosuppressant effects. Whether this mutual inhibitory mechanism of SXR and NF-κB is critical also in the bone system remains to be investigated. If the activation of SXR represses the NF-κB pathways in bones, some of the SXR agonists could be beneficial to suppress inflammation caused by bone fracture or osteoarthritis.

In conclusion, the effectiveness of vitamin K in bone metabolism has been known for years, but its molecular mechanisms have not been precisely clarified except the function related to γ-carboxylation. Recent advances in vitamin K study has revealed the novel signaling pathway of vitamin K that activates the transcriptional factor SXR and regulates its target genes including collagen-interacting genes. Further investigations of SXR and vitamin K mechanisms will provide new therapeutic options for the treatment of bone disorders including osteoporosis, osteoarthritis, and bone fracture.

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Spine (in press)

Association of a single nucleotide polymorphism in the insulin-like growth factor-1 receptor gene with spinal osteoarthritis in postmenopausal Japanese women

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STUDY DESIGN. An association study investigating the genetic etiology for spinal osteoarthritis.

OBJECTIVE. To determine the association of single-nucleotide polymorphism (SNP) in the insulin-like growth factor-1 receptor (IGF1R) with spinal osteoarthritis.

SUMMARY OF BACKGROUND DATA. Insulin-like growth factor-1 (IGF-1) signaling pathway is involved in cartilage development and homeostasis, suggesting that genetic variations of genes involved in this pathway may affect the pathogenesis of cartilage-related diseases, such as osteoarthritis.

METHODS. We evaluated the presence of endplate sclerosis, osteophytes, and narrowing of disc spaces in 434 Japanese postmenopausal women. A SNP in the IGF1R gene at intron 1 was determined using TaqMan polymerase chain reaction (PCR) method.

RESULTS. We compared those who carried the G allele (GG or GC, n=290) with those who did not (CC, n=144). We found that the subjects with the G allele (GG or GC) were significantly over-represented in the subjects having higher disc narrowing score (P=0.0033; odds ratio, 2.04; 95% confidence interval, 1.27-3.29 by logistic regression analysis).

CONCLUSIONS. We suggest that a genetic variation at the *IGF1R* gene locus is associated with spinal osteoarthritis, in line with the involvement of the *IGF1R* gene in the cartilage metabolism.

Key Words: Single-nucleotide polymorphism (SNP), insulin-like growth factor 1 receptor, spinal osteoarthritis, disc narrowing

Key Points

Insulin-like growth factor 1 (IGF-1) signaling pathway regulates cartilage metabolism.

The single-nucleotide polymorphism in insulin-like growth factor-1 receptor (IGF1R) gene at intron 1 was associated with spinal disc degeneration in Japanese postmenopausal women.

We suggest that a genetic variation at the IGF1R gene locus is associated with spinal osteoarthritis.

Mini Abstract

Insulin-like growth factor 1 (IGF-1) and its receptor regulate cartilage metabolism. The single nucleotide polymorphism in insulin-like growth factor 1 receptor gene at intorn 1 was associated with spinal disc narrowing in Japanese postmenopausal women. We suggest that a genetic variation at the IGF1R gene locus is associated with spinal osteoarthritis.

Introduction

Osteoarthritis of the spine is a very common condition in the axial skeletons of aged people [1]. Vertebral osteophytes, endplate sclerosis and intervertebral disc narrowing are recognized as characteristic features of spinal degeneration. Recent studies indicate that the appearance of these radiographical features is influenced by genetic factors, physical loading and other environmental factors [2,3]. Genetic association studies using various definitions of osteoarthritis have been performed, mainly investigating genes encoding structural proteins of the extracellular matrix of cartilage (e.g. collagen type II α1, cartilage matrix protein, and aminoguanidine) or genes playing a role in the regulation of bone density and mass (e.g. vitamin D receptor and estrogen receptor α) [4,5]. Many studies have shown that the Tt and the tt genotypes of Taq I polymorphism of the vitamin D receptor gene was associated with disc degeneration in various population [5 citation].

The insulin-like growth factor 1 (IGF-1) is a small 70-amino acid polypeptide mediator with a potent anabolic impact on cartilage homeostasis [6]. IGF-1 is expressed in cartilage, where it can act in a paracrine and autocrine manner to stimulate cartilage matrix synthesis as well as inhibit matrix degradation [7]. IGF-1 stimulates the production of both proteoglycans and collagen, the two major constituents of the cartilage matrix, and it also stimulates production of integrins, which are cell receptors that bind extracellular matrix proteins and are important for tissue repair [8, 9]. An age related decline in the ability of IGF-1 to stimulate chondrocytes to produce articular matrix components has also been demonstrated [10]. Recent study reported that chronic growth hormone and IGF-1 deficiency caused an increased severity of articular cartilage lesions of osteoarthritis [11]. Moreover, two reports have shown that a polymorphism in the promoter region of the IGF-1 gene associated with an increased prevalence of radiographical osteoarthritis in the subset data on the Rotterdam study [12, 13]. These reports have highlighted the importance of IGF-1 in promoting cartilage growth and development, implying a potential role of IGF-1 in the etiology of osteoarthritis. The first step in IGF-1 activation of chondrocytes is interaction with insulin-like growth factor 1 receptor (IGF1R), which cause auto-phosphorylation of the receptor intracellular β-subunit, triggering phosphorylation of downstream signal molecules, and initiating an array of anabolic effects. Thus, it is assumed that IGF1R modulates IGF-1 signaling pathway in the cartilage homeostasis. In the present study, we examine an association between a polymorphism in the IGF1R gene and radiographic feautures of spinal osteoarthritis including osteophyte formation, endplate sclerosis and disc space narrowing number to investigate a possible contribution of IGF1R to human cartilage metabolism.

Materials and methods

Subjects.

Genotypes were analyzed in DNA sample obtained from 434 healthy postmenopausal Japanese women (mean age + SD; 66.5 + 8.4) living in central area of Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin sodium), or unusual gynecologic history. Patients with severe hip and knee arthritis were excluded from the present study. The eligibility of subjects was determined by taking history-physical examination. All were non-related volunteers and provided informed consent before this study. Ethical approval for the study was obtained from appropriate ethics committees.

Radiographic grading of osteoarthritis of the spine.

Conventional thoracic and lumbar spinal plain roentgenograms in lateral and anteroposterior projection were obtained from all participants. The severities of spinal degeneration including osteophyte formation, endplate sclerosis and disc space narrowing were assessed semi-quantitatively from Th4/5 to L4/5 disc level or from Th4 to L4 vertebrae by using the grading scale of Genant [14]. Then we assessed radiographical spinal osteoarthritis using scoring system as previously reported [15]. Briefly, osteophyte formation at a given disc was graded 0-3 degrees, endplate sclerosis at given vertebra was graded 0-2 degrees, and disc space narrowing was graded 0-1 degrees. Then we defined sum of each degree from Th4/5 to L4/5 disc level for osteophyte formation on anteroposterior radiographs as a score of osteophyte formation. We also defined sum of each degree from Th4 to L4 vertebra for endplate sclerosis and that from Th4/5 to L4/5 disc level for disc space narrowing on lateral radiographs as a score of endplate sclerosis and disc narrowing, respectively.

Determination of a single nucleotide polymorphism in the IGF1R gene.

We extracted a polymorphic variation in the IGF1R gene intron 1 region from the Assays-on-Demand SNP Genotyping Products database (Applied Biosystems, Foster City, CA) and, according to its localization on the gene, denoted it IVS1+14488C>G (rs11247361). We determined the IVS1+14488C>G polymorphism of the IGF1R gene using the TaqMan (Applied Biosystems) polymerase chain reaction (PCR) method [16]. To determine the IGF1R SNP, we used Assays-on-Demand SNP Genotyping Products C_11527385_10 (Applied BioSystems), which contains sequence-specific forward and reverse primers and two TaqMan MGB probes for detecting alleles. TaqMan PCR method utilizes two kinds of TaqMan probes that correspond to a DNA fragment including the target SNP site with different alleles and the 5'-3' nuclease activity of Taq polymerase that is essential for PCR. TaqMan probes include fluorescence dyes at their 5' ends and a quencher at their 3' ends. During PCR cycles, TaqMan probes will anneal

to target DNA and will be excised by the 5'-3' nuclease activity of Taq polymerase if there is no mismatch between the probes and target sequences. Then the fluorescence dyes will be released from the probes and the intensity of fluorescence can be monitered by using ABI PRISM 7000 (Applied Biosystems) as a fluorescence detector. The allele frequencies of IVS1+14488C>G polymorphism were confirmed as they were not significantly deviated from Hardy-Weinberg equilibrium. Since Hardy-Weinberg equilibrium is based on the following assumptions including no genetic drift, no gene flow, no natural selection, negligible mutations, and random mating, the population under the equilibrium is not evolving and its genotype and allele frequencies are predicted to remain unchanged over successive generations. Thus, we considered that our subjects were eligible for the correlation study.

Statistical analysis.

Age, height, body weight, body mass index (BMI) and osteoarthritis parameters (number of osteophyte, endoplate sclerosis and disc narrowing) in the groups of subjects classified by the IGF1R SNP genotypes were compared by ANOVA and Kruscal-Wallis test. Stepwise regression analysis was carried out to assess the independent effect of 4 variables (age, height, body weight, IGF1R SNP genotypes) on disc narrowing score. We also divided subjects into those having one or two allele(s) of the minor G allele (GC+GG) and those with only the major C allele (CC) encoded at the same locus. Multivariate logistic regression was used to estimate odds ratios and 95% confidence intervals (95% CIs) for these two groups and the risk of disc narrowing. Analyses for the association of IGF1R genotypes and radiographic spinal endplate sclerosis were performed with adjustment for age. *P* values less than 0.05 were considered significant. Analysis was performed using StatView-J 4.5 software (SAS Institute Inc., Cary, NC).

Results

We analyzed the genotypes for the SNP of IGF1R gene at intron 1 (IVS1+14488C>G, rs11247361) in 434 subjects, using the TaqMan method. Among these postmenopausal Japanese women, 144 were CC homozygotes, 229 were GC heterozygotes, and 61 were GG homozygotes (Table 1). The allelic frequencies of this SNP in the present study were in Hardy-Weinberg equilibrium.

The background data (age, height, body weight, BMI) were not statistically different among these groups (Table 1). On Kruskal-Wallis analysis, we found significant associations between IGF1R genotype and disc narrowing score (Fig 1, Table1, P=0.0051). On ANOVA analysis, we also found significant associations between IGF1R genotype and disc narrowing score (Table1, P=0.015). On the other hand, the occurrence of endplate sclerosis and osteophyte formation did not significantly differ among those SNP genotypes (Table 1).

Recent studies have shown that the physical and constitutional factors contribute to spinal osteoarthritis. Therefore, we carried out stepwise regression analysis to assess the independent effect of age, height, body weight, and IGF1R SNP genotypes on disc narrowing score. Among these factors, age and IGF1R SNP genotypes correlated significantly with spinal disc narrowing score (Table 2). The standard regression coefficients were 0.291 for age and 0.110 for IGF1R SNP genotypes.

We finally analyzed the association between the alleic frequency of IGF1R SNP genotypes and disc narrowing score after stratification by age. In these analyses, we divided subjects into two groups, those who carried the G allele (GG or GC, n=290) and with those who did not (CC, n=144). We found that the subjects with the G allele (GG or GC) were significantly over-represented in the subjects having two or more disc narrowing score compared in the subjects having no or one disc narrowing after age-adjusted (P=0.0042; odds ratio 1.84; 95% confidence interval 1.21-2.79 by logistic regression analysis). We also found that the subjects with the genotype GG or GC were significantly over-represented in the subjects having higher (three or more) disc narrowing score compared in the subjects having lower (no, one or two) disc narrowing score after age-adjusted (P=0.0033; odds ratio 2.04; 95% confidence interval 1.27-3.28 by logistic regression analysis). Thus, we suggest that a genetic variation at the IGF1R gene locus is associated with spinal osteoarthritis, especially with disc narrowing, independently with background parameters.

Discussion

The present study is the first report that shows the influence of a single-nucleotide polymorphism of IGF1R gene on spinal osteoarthritis as far as we know. Targeting the pathogenesis of low back pain, we have previously investigated associations of genetic factors with osteoporosis. Our group and several other groups have reported that the IGF-1 gene polymorphism was correlated with bone mineral density and risk of fracture [17-21]. Spinal osteoarthritis is another major reason for low back pain and IGF-1 polymorphism has been shown to be associated with osteoarthritis [12, 13]. Thus, we have extended our study to the association of a polymorphism in the IGF1R gene, the receptor of IGF-1 signaling, with spinal osteoarthritis. We demonstrated that Japanese postmenopausal women who had one or two allele(s) of G allele in the IGF1R gene at intron 1 showed significantly higher disc narrowing score of spine. These data suggest that IGF-1 signaling-related genes may affect the pathogenesis of osteoarthritis. Our finding is in line with the genome-wide scan for osteoarthritis-susceptibility loci that showed a linkage to chromosome 15q21.3-26.1 [22], which includes the IGF1R gene locus on 15q25-q26.

Recently, mutations of the IGF1R gene have been described to be associated with

both intrauterine and postnatal growth retardation [23]. This suggests that the variant of IGF1R gene may have an important role in the pathogenesis of the human diseases. Actually, some reports have shown that the SNPs in the IGF1R gene were associated with human diseases. Common polymorphism in exon16 of the IGF1R gene was reported [Abu-Amero S, Preece M, Wakeling E, et al. A common polymorphism in exon 16 of the human insulin-like growth factor-1 receptor gene. Mol. Cellular Probes. 1997,11, 381-383]. Bonafe et al. studied a polymorphism 1013G>A presented in the exon 16, codon 1013, whose variant is predicted to generate no change of amino acid, E1013E [24]. This polymorphism has been reported to be more represented among people with a long lifespan [24]. Moreover, this 1043G>A polymorphism was associated with vascular dementia [25].

Osteoarthritis occurs as results of both mechanical and biological events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix as well as subchondral bone [1, 26]. Cartilage destruction during osteoarthritis involves the loss of differentiated phenotype and apoptotic death of chondrocytes [27]. IGF-1 has been shown to regulate differentiation, mainteinance of the differentiated phenotype, and apoptosis of articular chondrocytes [28-30]. One of the essential pathomechanisms of age-related degeneration of intervertebral discs is a loss of proteoglycan, which account for the reversible resistance to the compressive force of intervertebral discs [31, 32]. IGF-1 is a peptide known to activate matrix metabolism, particularly proteoglycan synthesis [33-35]. It was also reported that the expression and downstream signaling of IGF1R in cultured nucleus pulposus cells from aged rat interval discs decreased compared with in the cells from young rat interval discs, suggesting that a loss of proteoglycan synthesis during the late stage of aging is caused by the down regulation of IGF-1 and IGF1R pathway [36]. The interaction of IGF-1 and IGF1R is modulated by a set of high-affinity binding proteins, IGF binding proteins (IGFBPs) 1-6 (Le Roith et al. 2001). IGFBPs 2-5 have been detected in articular cartilage from multiple species (Olney et al. 1993, Tardif et al. 1997). Increased levels of IGFBPs, particularly IGFBP-3, in arthritic cartilage most likely contribute to IGF-1 hyposensitiveness (Chevalier and Tyler, 1996, Olney et al. 1996, Tardif et al. 1996, Morales 2002). Taken together, our results and the recent reports suggest that the IGF-1, IGFBPs and IGF1R signaling pathway is critical role in the pathogenesis of cartilage diseases, such as osteoarthritis and disc degeneration. The intronic IVS1+14488C>G polymorphism may influence the IGF1 and IGFBPs signaling by alteration in gene transcription or mRNA stability. Further studies will be required to clarify the role of this variant of the IGF1R gene in the pathogenesis of disc space narrowing.

In this study, we have evaluated not only disc space narrowing but also endplate screlosis and osteophyte formation. Among them, only disc space narrowing was associated

with IGF1R polymorphism. We have previously shown that the LRP5 polymorphism was associated with osteophyte formation and the WISP1 polymorphism was associated with ebdplate screlosis among these parameters (Urano et al. Spine 2007, Urano et al. J. Bone Miner. Metab. 2007). These data suggest that these 3 parameters of disk degeneration may have each genetic background in some part independently. Based on our data, the IGF1R polymorphism has specific effect on the disk space homeostasis in spine. It is important to study the IGF1R polymorphism in patients with disc space narrowing in other tissues, especially with hip and knee osteoarthritis.

LRP5 is a co-receptor of Wnt signaling pathway and its polymorphism associate with spinal osteophyte formation. Why the SNP of the IGF1R gene influence the disc narrowing scores while the SNP of the LRP5 gene associate with the osteophyte formation scores? It will be important to study how signals from Wnt and IGF-1 can be delivered to the regulation of this machinery.

Conclusion

We have shown an association of the SNP in the IGF1R gene at intron 1 with a radiographic feauture of spinal disc narrowing in postmenopausal Japanese women. The women with G allele had significantly higher disc narrowing scores. The IGF1R genotyping could be beneficial in the prevention and management of spinal disc degeneration. The present findings regarding the correlation of IGF1R polymorphism with spinal osteoarthritis provide a new promising direction for the clinical medicine of the spine disease, which leads us to the development of new diagnostic markers as well as therapeutic options based on the molecular target.

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Table 1.

Comparison of background and clinical characteristics among subjects with single nucleotide polymorphism (SNP) genotypes (GG genotype, GC genotype and CC genotype) in the IGF1R gene at intron 1.

Items	Genotype (mean ± SD)			*P* value	*P* value
	CC	GC	GG	(ANOVA)	(Kruskal-Wallis)
Number of subjects	144	229	81		
Age (years)	65.0 ± 9.2	66.3 ± 8.5	67.1 ± 10.6	NS	NS
Height (cm)	150.8 ± 6.1	150.1 ± 6.3	150.3 ± 6.1	NS	NS
Body weight (kg)	50.6 ± 7.4	49.5 ± 8.1	50.6 ± 8.7	NS	NS
BMI	22.3 ± 2.8	21.8 ± 3.0	22.6 ± 3.2	NS	NS
Endplate sclerosis	0.40 ± 0.86	0.35 ± 0.83	0.43 ± 1.04	NS	NS
Osteophyte	5.69 ± 3.68	5.31 ± 3.44	6.10 ± 3.58	NS	NS
Disc narrowing	1.63 ± 1.70	2.13 ± 1.82	2.26 ± 1.89	0.015	0.0051

BMI; body mass index, NS; not significant

Table 2.

Results of stepwise regression analysis of four factors for disc narrowing score.

Factors	*F* value			s.r.c.
	Step 0	Step 1	Step 2	Step2 (R^2=0.103)
Intercept	523	11.7	13.2	-2.323
IGF1R SNP genotypes (CC=0, GC, GG=1)			5.7	0.110
Age		43.1	40.4	0.291
Weight		not selected		
Height		not selected		

s.r.c.; standard regression coefficient

Table 3.

Association of the IGF1R SNP genotype (GG and GC (n=290) vs. CC (n=144)) in the subjects with disc narrowing score after stratifying age.

Severity of disc narrowing	OR	95%CI	*P* value
One or more disc narrowing (n=342) versus no disc narrowing (n=92)	1.36	0.83-2.23	0.21
Two or more disc narrowing (n=223) versus less (≤1) disc narrowing (n=211)	1.84	1.21-2.79	0.0042
Three or more disc narrowing (n=140) versus less (≤2) disc narrowing (n=294)	2.04	1.27-3.29	0.0033

OR; odds ratio, 95%CI; 95% confidence intervals

Fig. 1.

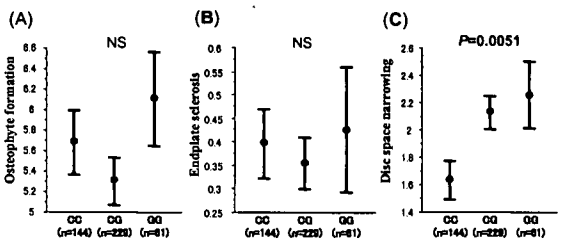

Figure 1. Scores of spinal osteoarthritis between the single nucleotide polymorphism (SNP) genotypes (CC genotype, CG genotype, GG genotype) in the IGF1R gene at intron1. A, Scores of osteophyte formation are shown for CC, CG and GG genotype. Scores are expressed as mean ± SE. Number of subjects are shown in parentheses. B, Scores of endplate sclerosis. C, Disc space narrowing scores. The association of the three genotype groups with osteoarthritis parameters was determined by Kruskal-Wallis test.

Splicing potentiation by growth factor signals via estrogen receptor phosphorylation

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Mitogen-activated protein kinase-mediated growth factor signals are known to augment the ligand-induced transactivation function of nuclear estrogen receptor α (ERα) through phosphorylation of Ser-118 within the ERα N-terminal transactivation (activation function-1) domain. We identified the spliceosome component splicing factor (SF)3a p120 as a coactivator specific for human ERα (hERα) activation function-1 that physically associated with ERα dependent on the phosphorylation state of Ser-118. SF3a p120 potentiated hERα-mediated RNA splicing, and notably, the potentiation of RNA splicing by SF3a p120 depended on hER Ser-118 phosphorylation. Thus, our findings suggest a mechanism by which growth factor signaling can regulate gene expression through the modulation of RNA splicing efficiency via phosphorylation of sequence-specific activators, after association between such activators and the spliceosome.

nuclear receptor | estrogen | coactivator | mitogen-activated protein kinase | RNA splicing

Most of the actions of estrogen are thought to be mediated via the transcriptional control of target genes by nuclear estrogen receptors (ER) α and β, members of the steroid hormone receptor gene superfamily that act as ligand-inducible transcription factors. ERs bind as dimers to specific estrogen response elements in the promoters of some target genes (1). However, most ER target promoters appear to recruit ERs without specific DNA binding, presumably through associations with sequence-specific factors bound to the promoters (2). ERs contain two transactivation functions (AFs), AF-1 in the N-terminal A/B domain and AF-2 in the C-terminal ligand-binding E/F domain. Ligand-induced transactivation by ERs requires multiple distinct classes of coactivator complexes, as well as a number of coregulators. The best-characterized complex contains p160/SRC family proteins (3, 4) and CBP/p300 histone acetyltransferases (5, 6), along with the RNA coactivator steroid receptor RNA activator (7) and presumably other known and unknown coactivators (8, 9). Another histone acetyltransferases-containing complex, the TBP-free TAF_{II}-containing (TFTC)-like complex (10), can also coactivate ER transactivation, as can the nonhistone acetyltransferases DRIP (VDR interacting protein)/TRAP (thyroid hormone receptor-associated protein)/SMCC (SRB/MED cofactor complexes)/ARC (activator-recruited cofactor) complex (11–13).

ER-mediated estrogen signaling is known to involve cross-talk with other signaling pathways (14). For instance, growth factors potentiate estrogen-induced cellular proliferation in female reproductive tissues (15). Phosphorylation of the Ser-118 residue in the human ERα (hERα) A/B domain by mitogen-activated protein kinase (MAPK) activated by growth factors (16, 17) and cyclin-dependent kinase-7 (18) results in the potentiation of AF-1 function (16). However, the molecular basis of ERα AF-1 potentiation by MAPK-mediated phosphorylation remains unclear. In a previous study, we identified the DEAD-box RNA helicase subfamily member p68/p72 as a hERα AF-1 coactivator that associated with steroid receptor RNA activator as a component in the p160/CBP histone acetyltransferases complex (19, 20). However, it appeared that the p68/p72-mediated facilitation of hERα AF-1 did not fully depend on Ser-118 phosphorylation, which suggested the presence of another, unknown factor. In the present study, we identified the spliceosome component splicing factor (SF)3a p120 as a coactivator specific for hERα AF-1 through Ser-118 phosphorylation-dependent interaction. Moreover, we found that this interaction potentiated hERα-mediated RNA splicing. Thus, our study indicated that MAPK-mediated cross-talk between growth factor and estrogen-signaling pathways modulates RNA splicing control through the phosphorylation-dependent interaction between hERα and a component of the spliceosome complex.

Materials and Methods

Plasmids and Constructs. The Pinpoint hERα (A/B) vector was prepared by inserting hERα (A/B) cDNA encoding amino acids 1–180 in-frame into HindIII/BamHI digested pinpoint Xa-3 vector (Promega). For Flag-SF3a p120 and Myc-SF3b p49 fusion constructs, respective cDNA fragments were amplified by PCR from a Marathon-Ready cDNA library (Clontech) by using pyrobest DNA polymerase (Takara, Tokyo). Myc-p68 and U1 70K cDNA were amplified by PCR, and the resultant products were digested and subcloned into pcDNA3-Flag (10), pcDNA3, and pCMV-Myc. pcDNA3-His-p72, pCMVβ-p300, pcDNA3-hERβ, pSG1-hERα, and mutants were as described (16, 20, 21). The complete coding sequences of all constructs used were verified by sequencing.

***In Vitro* Phosphorylation and Western Blotting of Biotin-Tagged hERα (A/B) Proteins.** Biotin-tagged hERα (A/B) and hERα (A/B)[S118A] proteins were expressed from Pinpoint Xa-3 vectors in *Escherichia coli* HB101, purified on avidin resin, and eluted with free biotin according to the manufacturer's protocol (Promega). To determine the efficiency of hERα (A/B) domain Ser-118 residue phosphorylation by MAPK *in vitro*, purified proteins and 50 ng of myelin basic protein (MBP) (Sigma) were coincubated for 30 min with MAPK (Erk2, New England Biolabs) in a total volume of 25 μl. Phosphorylated proteins were analyzed by 12.5% SDS/PAGE and autoradiography. Biotinylation of purified hERα (A/B) proteins was confirmed by Western blotting by using conjugated streptavidin-alkaline phosphatase (Promega).

Abbreviations: ERα, estrogen receptor α; hER, human ER; E2, 17β-estradiol; AF, transactivation function; TAM, 4-hydroxy-tamoxifen; SF, splicing factor; snRNP, small nuclear ribonucleoprotein particle; MAPK, mitogen-activated protein kinase; siRNA, short interfering RNA.

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Far-Western Blotting and Expression Cloning. To detect endogenous interactants for phosphorylated Ser-118 hERα (A/B) domains, nuclear extracts from HeLa, COS-1, and MCF7 cells were prepared, and 10-μg aliquots of each nuclear extract were boiled and loaded onto 7.5% SDS/PAGE gels. Proteins were transferred onto poly(vinylidene difluoride) membranes and denatured in 6 M guanidine hydrochloride, 50 mM Tris·HCl (pH 8.0), 5 mM 2-mercaptoethanol, and 0.05% Tween 20 for 1 h at room temperature. Immobilized proteins were renatured overnight at 4°C. Membranes were rinsed and incubated with phosphorylated and biotin-tagged hERα (A/B) probe (0.1 μg/ml) for 4 h at room temperature. Membranes were washed and hybridization products detected by using conjugated streptavidin-alkaline phosphatase.

For expression cloning, a human kidney cDNA library in the Zap II vector was infected into bacteria, plated, and incubated at 42°C until minute plaques were seen. Recombinant plaques were grown at 37°C for 4 h and then induced with 1 mM isopropyl β-D-thiogalactoside-impregnated nitrocellulose filters for 3.5 h. Filters were then treated under the same conditions as for Far-Western blotting for denaturation and detection. Positive plaques were isolated, amplified, and processed, with the procedure repeated until 100% of the plaques were positive after plating. Four rounds of screening were performed after which cDNA inserts from plaques were obtained as pBluescript plasmids by *in vivo* excision. Isolated clones were sequenced by using T7 primers on an Applied Biosystems automatic sequencer.

Protein Identification by MALDI-TOF MS. Protein bands in SDS/PAGE gels were excised and digested in-gel with trypsin. Eluted peptides were then loaded onto the sampling plate for MALDI-TOF MS (Voyager DE-STR, Perseptive Biosystems). After analysis of each protein fragment mass, results were compared by using the MS-FIT program (University of California, San Francisco Mass Spectrometry Facility).

Pull-Down Assay. GST-fused proteins were expressed in *E. coli* and purified on glutathione-Sepharose beads (Amersham Pharmacia). Pull-down assays were performed as described (10, 16, 19–21). Immobilized glutathione-Sepharose and avidin-resin were incubated with ^{35}S-methionine-labeled SF3a p120 protein. Bound proteins were eluted and analyzed by 7.5% SDS/PAGE.

Splicing Assay. The 293T cells were transfected with reporter CD44 minigene (22), pCH110 internal control plasmid containing β-galactosidase (10 ng), short-interfering RNA (siRNA), and expression plasmids as indicated. Total RNA was isolated by Isogen (Nippon Gene, Toyama, Japan), and RT-PCR for CD44 was performed as described (22). For oxytocin, total RNA (0.1 μg) was reverse-transcribed in 50-μl reaction mixtures by using the Access RT-PCR system (Promega) with primers specific for oxytocin (reverse, 5′-CAGGTAGTTCTCCTCCTGGCAGC-3′) or β-galactosidase (reverse, 5′-CCGCCGATACTGACGGGCTCC-3′). PCR was then performed by using 3 μl of this mixture in a 50-μl reaction volume containing 0.5 unit of ExTaqDNA polymerase (Takara) and gene-specific primers for oxytocin (forward, 5′-CAGCCTCGCTTGCTGTCTGCTC-3′; reverse, 5′-CAGGTAGTTCTCCTCCTGGCAGC-3′) or β-galactosidase (forward, 5′-CGACCGCTCACGCGTGGCAGC-3′; reverse, 5′-CCGCCGATACTGACGGGCTCC-3′). PCR conditions were optimized to allow semiquantitative measurement of oxytocin (denaturation at 96°C for 1 min followed by 25–30 cycles of 96°C for 20 s, 70°C for 20 s, and 72°C for 1 min) and β-galactosidase (denaturation at 96°C for 1 min followed by 20–25 cycles of 96°C for 20 s, 70°C for 20 s, and 72°C for 1 min) mRNA levels. PCR products were verified by sequencing and visualized on 2% agarose/Tris-acetate-EDTA gels. Quantitative measurement of splicing efficiency was analyzed by NIH IMAGE (National Institutes of Health, Bethesda).

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation assays for oxytocin were performed as described (10, 23). Soluble chromatin from MCF7 cells was immunoprecipitated with Abs against the indicated proteins. Specific primer pairs used to PCR amplify oxytocin were 5′-CACCCTAGTGGCCCAGGCCACC-3′ and 5′-GCTCTGTTTAAGAGGTTGGTAGTATG-3′. PCR conditions were optimized to allow semiquantitative measurement. Conditions used were 21–25 cycles of 20 s at 96°C, 20 s at 70°C, and 1 min at 72°C. PCR products were visualized on 2% agarose/Tris-acetate-EDTA gels.

Results and Discussion

Identification of SF3a p120 as a Direct Interactant for hERα Phosphorylated by MAPK at Ser-118. To identify the factor fully responsible for the phosphorylation-dependent potentiation of hERα AF-1, we performed Far-Western blot analysis on nuclear extracts by using bacterially expressed biotinylated hERα (A/B) domains as a probe. hERα (A/B) domains, phosphorylated *in vitro* by MAPK, detected three endogenous proteins with approximate M_r of 120, 72, and 68 kDa in nuclear extracts from the HeLa, COS-1, and MCF7 cell lines (Fig. 1*A Center*, lanes 5–7). As the 68- and 72-kDa bands corresponded to p68/p72 (19, 20) by Western blotting with specific Abs (data not shown), we attempted to clone the p120 factor by Far-Western screening of a human kidney cDNA library because ERα AF-1 activity is high in the kidney. Screening of 8×10^6 independent clones yielded 72 positives, three of which encoded overlapping cDNA sequences that corresponded to the C-terminal domain of SF3a p120 (24, 25). Biochemical purification of interactants from stably expressed hERα (Fig. 1*B Right*) and phosphorylated hERα A/B domains fused to GST [GST-*p*-Ser-118 hERα (A/B)] (Fig. 1*B Left*, lane 5) followed by MS (MALDI-TOF MS) confirmed the identity of the three isolated proteins as SF3a p120 and p68/p72. No such association was detected by using a hERα mutant that cannot be phosphorylated by MAPK because of the substitution of the Ser-118 residue with Ala [S118A (16) (Fig. 1*B*, *Right*)].

We then tested whether SF3a p120 directly bound the A/B or E/F domains of hERα or hERβ by using an *in vitro* GST pull-down assay (10). Unlike p68/p72 (19, 20), SF3a p120 exhibited a strict association only with A/B domains from phosphorylated Ser-118 hERα and Glu-118 hERα (S118E), a transcriptionally dominant active mutant with Ser-118 substituted with Glu to mimic the negative charge of phosphorylated Ser-118 (Fig. 1*C*, lanes 4 and 5). In contrast, SF3a p120 did not interact with the Ser-118–Ala mutant (Fig. 1*C*, lane 3), the E/F domain of ERα, or ERβ A/B or D/E/F domains (see Fig. 1*C*, lanes 7–11). We then tested whether SF3a p120 directly associated with hERα *in vivo* by using immunoprecipitation assays on 293T cells. The *in vivo* association between SF3a p120 and full-length hERα depended on ligand binding and was abrogated by both the MAPK inhibitor U0126 (Fig. 2*A*, compare lanes 6 and 7) and the Ser-118–Ala mutant (Fig. 2*A*, lanes 8–10). As expected from the coimmunoprecipitation assay results (Fig. 2*A*), activation of MAPK signaling by EGF treatment potentiated the association of hERα with endogenous SF3a p120 in MCF7 cells (Fig. 2*B*, lane 3). As the hERα AF-1 domain (hERα A/B/C) alone appeared to be sufficient for association with SF3a p120 (Fig. 2*A*, lane 12), it is possible that the structural alterations in ERα induced by ligand binding expose the functional AF-1 domain, rendering it accessible to SF3a p120.

SF3a p120 Is a Coactivator for Ser-118 Phosphorylated hERα AF-1. As SF3a p120 appeared to physically interact with the hERα AF-1 domain, we tested the coregulatory function of SF3a p120 in a