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).

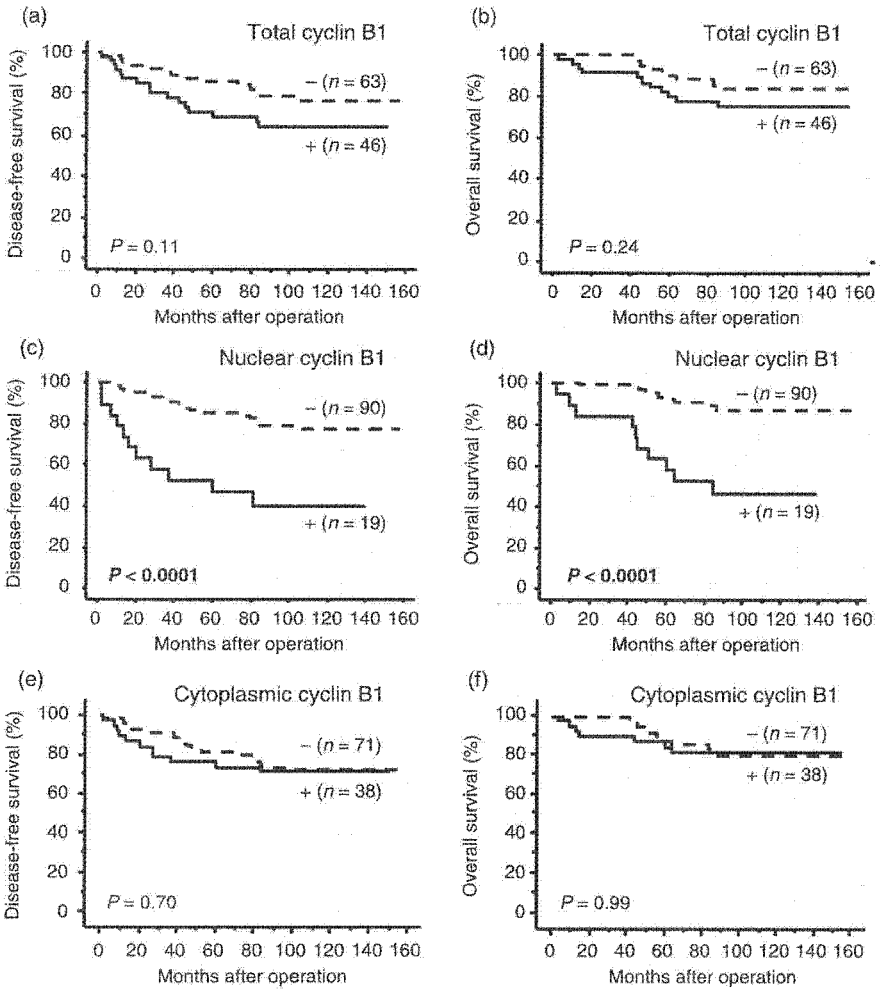

Fig. 2. Disease-free and overall survival of 109 patients with breast carcinoma according to the intracellular localization of cyclin B1 immunoreactivity (Kaplan–Meier method). Total cyclin B1 was not significantly associated with (a) disease-free or (b) overall survival. Nuclear cyclin B1 was significantly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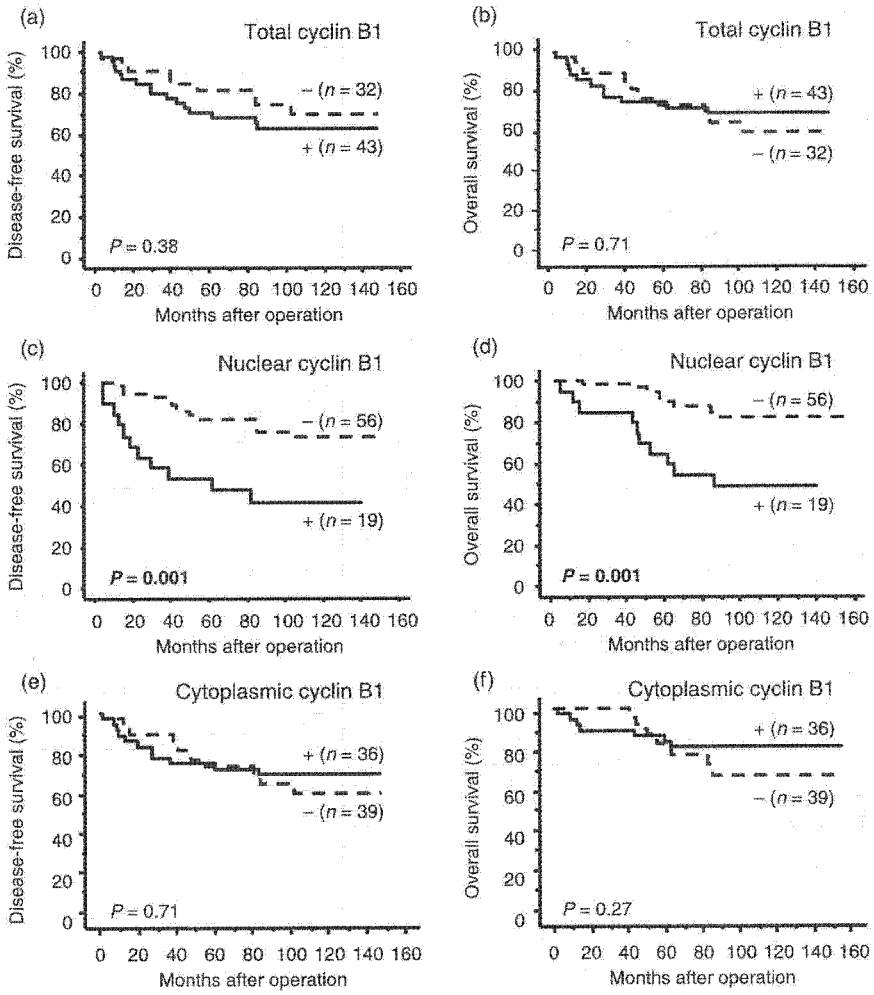

Fig. 3. Association between intracellular localization of cyclin B1 immunoreactivity and clinical outcome of the 75 patients showed high (>5 cells) mitotic count in the breast carcinoma (Kaplan–Meier method). There was no significant association between total cyclin B1 and (a) disease-free or (b) overall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

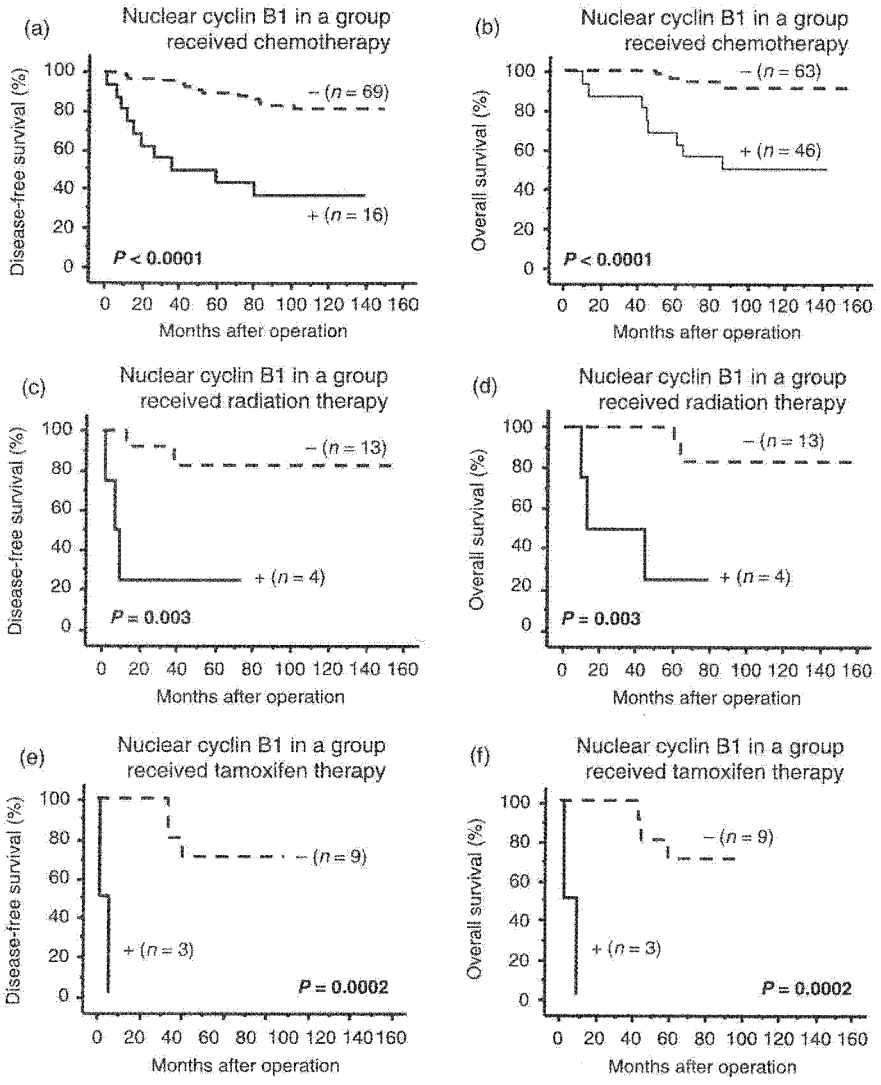

Fig. 4. Association between nuclear cyclin B1 immunoreactivity and clinical outcome of 109 breast carcinoma patients according to the adjuvant therapy (Kaplan–Meier method). Nuclear cyclin B1 immunoreactivity was significantly associated with adverse prognosis in the groups of patients receiving (a,b) 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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Association of a single nucleotide polymorphism in the WISP1 gene with spinal osteoarthritis in postmenopausal Japanese women

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Short title: Association of an SNP in WISP1 gene with spinal osteoarthritis

Abstract

Wnt-β-catenin signaling pathway that regulates bone density is also involved in cartilage development and homeostasis *in vivo*. Here, we assumed that genetic variation in Wnt-β-catenin signaling genes can affect the pathogenesis of cartilage related diseases, such as osteoarthritis. Wnt-1-induced secreted protein 1 (WISP1) is a target of the Wnt pathway and directly regulated by β-catenin. In the present study, we analyzed the association of a single nucleotide polymorphism (SNP) in the WISP1 3' UTR region with the development of radiographically observable osteoarthritis of the spine. For this purpose, we evaluated the presence of osteophytes, endplate sclerosis, and narrowing of disk spaces in 304 postmenopausal Japanese women. We compared those who carried the G allele (GG or GA, n=184) with those who did not (AA, n=120). We found that the subjects without the G allele (AA) were significantly over-represented in the subjects having higher endplate sclerosis score (P = 0.0069; odds ratio 2.91; 95% confidence interval 1.34-6.30 by logistic regression analysis). On the other hand, the occurrence of disk narrowing and osteophyte formation did not significantly differ between those with and without at least one G allele. Thus, we suggest that a genetic variation in the WISP1 gene locus is associated with spinal osteoarthritis, in line with the involvement of the Wnt-β-catenin regulated gene in the bone and cartilage metabolism.

Key Words

single nucleotide polymorphism (SNP), Wnt-β-catenin signaling, WISP1, osteoarthritis, endplate sclerosis

Introduction

Spinal osteoarthritis is a highly prevalent musculoskeletal disorder and a major cause of back symptoms [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 physical loading and other environmental factors [2,3]. Moreover, spinal osteoarthritis has been shown to have a familial component and in some studies to be influenced by specific genetic risk factors, 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, insulin-like growth factor-I, and estrogen receptor α) [4,5].

The Wnt (wingless-type MMTV integration site family) represents a large group of secreted signaling proteins that are involved in cell proliferation, differentiation and morphogenesis [6]. The name of 'Wnt' is derived from *wingless* gene in Drosophila melanogaster [7] and murine *int-1* oncogene identified in tumors induced by mouse mammary tumor virus [8]. It is also known that Wnt and bone morphogenetic protein (BMP) signals control apical ectodermal ridge (AER) formation and dorsal-ventral patterning during limb development [9,10]. Wnt proteins activate signal transduction through Frizzled which act as receptors for Wnt proteins [11] and induce stabilization of cytoplasmic β-catenin protein, which also regulates target gene expression as a transcriptional co-activator. The physiological role of the Wnt in the regulation of osteoblastogenesis has been studied in experimental models. Mice expressing Wnt10b transgene in bone marrow have shown high bone mass by simulating osteoblastgenesis [12]. It is also shown that activated β-catenin stimulate osteoblast differentiation [13]. Meanwhile, LDL receptor-related protein 5 and 6 (LRP5/6) were also found to be required for Wnt co-receptors [14,15]. Recent reports demonstrated that the Wnt/β-catenin signaling pathway regulates bone mineral density (BMD) through LRP5 [16-19]. Moreover, we and several groups reported that single nucleotide polymorphisms (SNPs) in the LRP5 gene predicted the bone mass [20-23]. These findings indicate that the Wnt-β-catenin signaling pathway plays important roles in the skeletal biology.

In addition to the regulation of the limb development and bone metabolism, Wnt/β-catenin signaling may be involved in the maintenance and pathophysiology of cartilage. This possibility is indirectly supported by the observation that several Wnt proteins and Frizzled receptors are expressed in the synovial tissue of arthritic cartilage [24]. In addition, a secreted Frizzled-related protein (FrzB-2) that act as an antagonist for Frizzled receptor is strongly expressed in osteoarthritic cartilage and may regulate chondrocyte apoptosis [25]. It is also shown that chondrocytes express β-catenin at a low level and that an accumulation of β-catenin is sufficient to cause dedifferentiation of chondrocytes, suggesting that Wnt signaling is involved in cartilage metabolism [26].

Wnt-1-induced secreted protein 1 (WISP1) is a member of the CCN family growth factors, which includes connective tissue growth factor (CTGF), cysteine-rich 61 (Cyr61), nephroblastoma overexpressed (NOV), WISP2 and WISP3 [27-30]. WISP1 is a target of the Wnt/β-catenin pathway and its expression is regulated by β-catenin [30,31]. WISP1 activity and availability is modulated by its interaction with decorin and biglycan, two extracellular matrix-associated proteoglycans abundantly found in bone and cartilage [32]. In mouse chondrocytic cell lines, WISP1 increased proliferation and saturation density but repressed chondrocytic representation [33]. These data suggest that WISP1 could play an important regulatory role in the bone and cartilage homeostasis. In the present study, we examine an association between a polymorphism in WISP1 gene and radiographic feautures of spinal osteoarthritis including osteophyte formation, endplate sclerosis and disc space narrowing number to investigate a possible contribution of WISP1 to human bone and cartilage metabolism.

Materials and methods

Subjects

Genotypes were analyzed in DNA sample obtained from 304 healthy postmenopausal Japanese women (mean age + SD; 66.3 + 9.0) 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 the 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 spinal osteoarthritis

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 L5 vertebrae by using the grading scale of Genant [34]. 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. These semi-quantitaive grading on radiographics were performed by two expert medical doctors.

Determination of a SNP in the WISP1 gene

We extracted a polymorphic variation in the WISP1 gene exon5 3' UTR 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 2364A/G. We determined the 2364A/G polymorphism of the WISP1 gene using the TaqMan (Applied Biosystems) polymerase chain reaction (PCR) method [35]. To determine the WISP1 SNP we used Assays-on-Demand SNP Genotyping Products C_9086661_10(Applied BioSystems) (rs2929970), which contains sequence-specific forward and reverse primers and two TaqMan 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

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 WISP1 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, WISP 1 SNP genotypes) on endplate sclerosis score. We also divided subjects into those having one or two allele(s) of the minor G allele (AG+GG) and those with only the major A allele (AA) 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 endplate sclerosis. Analyses for the association of WISP1 2364A/G 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 WISP1 gene at 3' UTR region (2364A>G) in 304 subjects, using the TaqMan method. Among these postmenopausal Japanese women, 120 were AA homozygotes, 149 were AG heterozygotes, and 35 were GG homozygotes (Table1). 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 ANOVA analysis, we found significant associations between WISP1 2364A/G genotype and endplate sclerosis score (Table1, P=0.0062). On Kruskal-Wallis analysis, we also found significant associations between WISP1 2364A/G genotype and endplate sclerosis score (Table1, P=0.024). Women with

the AA allele had a significantly higher endplate sclerosis score than did subjects bearing at least one G allele (AG + GG). On the other hand, the occurrence of disc narrowing and osteophyte 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 WISP1 SNP genotypes on endplate sclerosis score. Among these factors, only age and WISP1 SNP genotypes correlated significantly with spinal endplate sclerosis score (Table 2). The standard regression coefficients were 0.261 for age and –0.166 for WISP1 SNP genotypes.

We finally analyzed the association between the alleic frequency of WISP1 SNP genotypes and endplate sclerosis score after stratification by age. In these analyses, we divided subjects into two groups, those who carried the G allele (GG or GA, n=184) and with those who did not (AA, n=120). We found that the subjects without the G allele (AA) were significantly over-represented in the subjects having one or more endplate sclerosis score compared in the subjects having no endplate sclerosis after age-adjusted (P = 0.044; odds ratio 1.78; 95% confidence interval 1.01-3.13 by logistic regression analysis). We also found that the subjects with the genotype AA were significantly over-represented in the subjects having higher (two or more) endplate sclerosis score compared in the subjects having lower (one or no) endplate sclerosis score after age-adjusted (P = 0.0069; odds ratio 2.91; 95% confidence interval 1.34-6.30 by logistic regression analysis). Thus, we suggest that a genetic variation at the WISP1 gene locus is associated with spinal osteoarthritis, especially with endplate sclerosis, independently with background parameters.

Discussion

The present study is the first report that shows the influence of a SNP of the WISP1 gene on spinal osteoarthritis. The WISP1 is an osteogenic potentiating factor promoting mesenchymal cell proliferation and osteoblastic differentiation while repressing chondrocytic differentiation [33]. We demonstrated that the Japanese postmenopausal women who had AA genotype at the WISP1 2364A/G SNP showed significantly higher endplate sclerosis score of spine. Our finding might also be supported by genetic linkage scan for early-onset osteoarthritis and chondrocalcinosis susceptibility loci that showed a linkage to chromosome 8q [36], which includes the WISP1 gene locus on 8q24.

It has been recently shown that haplotype analysis in LRP5 gene revealed that there was a common haplotype that provided a 1.6-fold increased risk of knee osteoarthritis [37]. We have revealed that a SNP (Q89R) in the LRP5 gene is associated with spinal osteoarthritis [38]. It is also reported that there was a significant association of a functional genetic variant of secreted frizzled-related protein 3 (sFRP3), which

antagonizes Wnt signaling, with hip osteoarthritis in women [39]. Taken together, our results and the recent evidence suggest that the Wnt-β-catenin signaling pathway including WISP1 is important in the pathogenesis of skeletal abnormality including osteoarthritis.

WISP1 is a member of the CCN family of connective tissue growth factors, which also includes WISP2 and WISP3. Members of the CCN family have been implicated in the developmental processes such as chondrogenesis, osteogenesis and angiogenesis [27-29]. Specifically, mutations of WISP3 cause the rare skeletal syndrome, progressive pseudorheumatoid dysplasia (PPD) [40]. In affected individuals, symptoms develop between the age of 3 years and 8 years and consist of stiffness and swelling of multiple joints, motor weakness, and joint contractures. It has been also reported that WISP3 polymorphisms were associated with susceptibility to juvenile idiopathic arthritis [41]. Moreover, the WISP3 was shown to be expressed in chondrocytes derived from human cartilage and be able to regulate type II collagen and aggrecan expression [42]. On the other hand, the expression of the WISP2 was preferentially detected in rheumatoid arthritis synovum [43]. These data suggest that CCN family members play a critical role in cartilage homeostasis. In the present study, we investigated a possible contribution of WISP1 polymorphism to spinal osteoarthritis in Japanese women. Taken together, the CCN family gene polymorphisms may affect the pathogenesis of cartilage disease.

In the present study, we excluded the subjects with severe hip or knee arthritis, because these joint diseases themselves may induce spinal deformity or mal-alignment. Therefore, we could not assess these joint arthritis here. Recent studies have shown that some SNPs in the sFRP3 and LRP5 genes, involved in Wnt signaling, were associated with hip and knee osteoarthritis, respectively [37,39]. Moreover, WISP3 polymorphisms are associated with juvenile idiopathic arthritis which affect multiple joints [41]. In this regard, it may be important to examine the association of the SNPs in the WISP1 gene with hip and knee arthritis in the future. Meanwhile, it would be better if we had evaluated also facet joint, since spinal osteoarthritis is represented not only by the anterior elements such as disc narrowing, osteophytosis, or endplate sclerosis but also by the posterior elements such especially as facet joint lesion. However, we here evaluated only the anterior elements of thoraco-lumbar vertebral bodies, because a reproducible semi-quantitative assessment for facet joint using A-P and lateral X-ray radiographs has not been well established.

In conclusion, we have shown an association of the polymorphism in the WISP1 gene with a radiographic feauture of spinal endplate sclerosis in postmenopausal Japanese women. The women with AA genotypes had significantly higher endplate sclerosis scores. The WISP1 genotyping may be benefical in the prevention and management of spinal osteoarthritis. Thus, the WISP1 would be a useful molecular target for the development of new diagnostic markers as well as therapeutic options in the osteoarthritis.

Acknowledgments

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Table 1. Comparison of background and clinical characteristics among subjects with single nucleotide polymorphism (SNP) genotypes (AA genotype, AG genotype and GG genotype) in the WISP1 gene 3'UTR region (2364A/G).

Items	Genotype (mean ± SD)			*P* value	*P* value
	AA	AG	GG	(ANOVA)	(Kruskal-Wallis)
Number of subjects	120	149	35		
Age (years)	66.1 ± 9.2	66.3 ± 8.5	67.1 ± 10.6	NS	NS
Height (cm)	150.7 ± 5.6	150.2 ± 6.8	150.0 ± 5.0	NS	NS
Body weight (kg)	50.3 ± 7.6	50.2 ± 8.3	48.0 ± 5.4	NS	NS
BMI	22.1 ± 2.9	22.2 ± 2.9	21.3 ± 3.3	NS	NS
Endplate sclerosis	0.58 ± 1.09	0.34 ± 0.74	0.09 ± 0.28	0.0062	0.024
Osteophyte	5.89 ± 3.93	5.72 ± 3.40	5.57 ± 4.08	NS	NS
Disk narrowing	2.21 ± 1.79	2.09 ± 2.00	2.03 ± 1.86	NS	NS

BMI; body mass index, NS; not significant

Table 2. Results of stepwise regression analysis of four factors for endplate sclerosis score.

Factors	F value			r.c.	s.r.c.
	Step 0	Step 1	Step 2	Step 2 (R^2=0.094)	
Intercept	63.7	12.8	9.4	-1.106	-1.106
WISP1 SNP genotypes (AA=0, AG, GG=1)			9.1	-0.297	-0.166
Age		21.5	22.7	0.025	0.261
Weight			not selected		
Height			not selected		

r.c.; regression coefficient. s.r.c.; standard regression coefficient.

Table 3. Association of the WISP1 SNP genotype (2364A/G) in the subjects with spinal endplate sclerosis after stratifying age.

Group compared	AA vs. AG+GG		
	OR	*P* value	95%CI
Endplate sclerosis (≥1)(n=235) versus no endplate sclerosis (=0)(n=69)	1.78	0.044	1.01-3.13
Higher endplate sclerosis (≥2)(n=271) versus Lower endplate sclerosis (≤0)(n=33)	2.91	0.0069	1.34-6.30

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

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Increased expression of estrogen-related receptor α (ERRα) is a negative prognostic predictor in human prostate cancer

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The nuclear receptor ERRα (estrogen-related receptor α) is known to modulate the estrogen-signaling pathway, but the biological significance of ERRα in the prostate remains unclear. We investigated the expression of ERRα in human prostate tissues and cancer cell lines to evaluate the potential roles of the receptor in prostate cancer (PC). Western blot analysis of ERRα was performed in three cell lines of human PC (LNCaP, DU145 and PC-3). The expressions of ERRα in cancerous lesions (n = 106) and benign foci (n = 99) of 106 surgically obtained prostate specimens were evaluated by immunohistochemistry. The relationships between the ERRα expression and clinicopathological features were evaluated. Western blot analysis using the polyclonal anti-ERRα antibody detected a 52 kD band in all three PC cell lines. Positive immunostaining of ERRα in the nuclei was found in 73 (69%) cancerous and 47 (47.5%) benign epithelium, whereas the stromal tissues were negative for ERRα. The mean immunoreactivity score (IR score) of the cancerous lesions (3.5 ± 2.6) was significantly higher than that of the benign foci (1.8 ± 2.1) (p < 0.0001). The IR score of the cancerous lesions significantly correlated with the Gleason score (p = 0.0135). Univariate and multivariate hazard analyses revealed significant correlations between elevated ERRα expression and poor cancer-specific survival (p = 0.0141 and 0.0367, respectively). The enhanced expression of ERRα might play a role in the development of human PC and serve as a significant prognostic factor for the disease.
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Key words: estrogen related receptor α (ERRα); prognostic predictor; prostate cancer

Estrogens have been widely used for the treatment of advanced prostate cancer (PC).[1] The direct effect of estrogens on normal prostate and PC is assumed to be mediated through estrogen receptors (ERs) α and β.[2,3] ERα is predominantly localized in the stromal cells of the prostate.[3–6] In light of this, the ERα-mediated effects of estrogens on the prostate epithelium are thought to be conferred via paracrine pathways. ERβ, on the other hand, is localized predominantly in the epithelial cell compartment of the normal human prostate, and the expression of ERβ is decreased in PC compared with benign epithelium.[2,3] Thus, ERβ may exert a protective effect against aberrant cell proliferation and carcinogenesis.[7–10]

Recent studies have focused on an additional estrogen signaling pathway mediated by estrogen-related receptors (ERRs) in the estrogen-targeted organs.[11–13] ERRs belong to the nuclear receptor super family and consist of three closely related members (α, β and γ).[11–13] The cDNA for ERRα was isolated by screening cDNA libraries using probes corresponding to the DNA-binding domain of human ERα.[11] Evidence suggests that there may be an overlap between ERRα and ER biology. While ERRα shows no direct response to 17β-estradiol, it has been found to bind to functional estrogen response elements (EREs) in ER target genes such as lactoferrin and aromatase.[12–14] ERRα may participate in processes such as bone development, skeleton formation and fat metabolism.[15–18] Further, ERRα is now established to be associated with unfavorable biomarkers in human breast cancer.[19–21] Much less is known, however, about the extent or pattern of ERRα expression in the prostate.[22] The present study evaluated ERRα expression in human prostate tissues and PC cell lines by immunohistochemistry and Western blot analysis to assess its clinical significance.

Material and methods

Tissue selections and patient characteristics

Formalin-fixed, paraffin-embedded sections were obtained from 106 patients who underwent radical prostatectomy for prostatic adenocarcinoma between 1987 and 2001. We obtained informed consent from all the patients. The age of the patients ranged from 52 to 78 years (mean 66.8 ± 6.0), and pretreatment serum PSA level ranged from 2.2 to 136 ng/ml (mean 16.9 ± 19.5). The pathological stages included B (n = 33), C (n = 59) and D_1 (n = 14). Prostate tissue sections submitted for this study contained 99 benign and 106 cancerous foci. The cancerous lesions consisted of tumors with Gleason score (GS) 6 (n = 22), 7 (n = 41), 8 (n = 20), 9 (n = 22) and 10 (n = 1), which was evaluated by 2 trained pathologists. Thirty-five patients (33%) were treated with surgery alone, whereas the remaining patients received adjuvant antiandrogen therapy. Patients were followed postoperatively by their surgeons at 3-month intervals to 5 years and yearly thereafter. Mean patient follow-up period was 82 ± 39 months (range 10–192). During the follow-up period, 77 patients (73%) are alive with no evidence of the disease, and 12 (11%) are alive with biochemical or clinical recurrence. Eleven patients (10%) died of PC, and 6 (6%) died of other diseases during the follow-up period.

Cell culture

The human prostatic cancer cell lines (PC-3, DU145 and LNCaP) and COS7 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in the RPMI 1640 with 10% FBS. All cell lines were maintained at 37°C in 5% CO_2. Transfections of hERRα were performed using 3.5 × 10^6 COS7 cells, 5 μg of pcDNA3-hERRα vector and FuGENE® 6 transfection kit (Roche Applied Science, Indianapolis, IN) according to the manufacture's protocol. After 48 hr cell extracts were analyzed.

Antibodies

Rabbit polyclonal antibody for ERR1 (PA1-314) was purchased from Affinity Bio Reagents (Golden, CO). The characterization of this antibody was confirmed by Western blot analysis in hERRα-transfected COS7 cells.

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Western blot analyses

Western blot analysis was performed using total cell extracts obtained from 3 strains of LNCaP, DU145, PC-3 and hERRα-transfected COS7 cells as previously described.[23] Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 5 mM EGTA, 2 mM sodium vanadate, 0.5% sodium deoxycholate, 1 mM diethiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mg/ml aprotinin and 0.1% no diet P-40), and the lysates were cleared by centrifugation at 15,000 for 15 min at 4°C. Total protein lysate (20 μg) of each cell line was fractionated on sodium dodecyl sulfate (SDS)-12.5% polyacryl-amide gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilin, Millipore, Bedford, MA). The membranes were blocked in Tris-buffered saline (TBS) with 5% skim milk before incubating with the anti-ERRα antibodies diluted (1:500), followed by a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin IgG (Amersham-Pharmacia Biotech, Arlington Heights, IL). Bands were visualized with the chemiluminescence's -based ECL plus detection system (Amersham-Pharmacia Biotech).

Immunohistochemistry

We performed immunohistochemical analysis of ERRα employing the streptavidin-biotin amplification method using a peroxidase catalyzed signal amplification system: CSA system (DAKO, Carpentaria, CA) following the manufacturer-supplied protocol. Tissue-sections (6 μm) were deparaffinized, dehydrated through a graded ethanol series and rinsed in phosphate-buffered saline (PBS). For antigen retrieval, the sections were autoclaved at 120°C for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). After blocking endogenous peroxidase with 0.3% H_2O_2, the sections were incubated in 10% bovine serum for 10 min. Application of the polyclonal antibody for ERRα (1:100 dilution) followed by sequential 15-min incubations with biotinylated link antibody, streptavidin-biotin-peroxidase complex, amplification reagent and streptavidin-peroxidase. 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).

As positive controls, sections of human heart tissues (BioChain Institute, Inc, Hayward, CA) were immunoassayed with the primary antibodies in the same manner as described above.

In addition to the standard negative controls with rabbit IgG, we performed peptide blocking of anti-ERRα antibody using its neutralizing peptide purchased from Affinity Bio Reagents (Golden, CO) in order to confirm the specificity of the antibody.

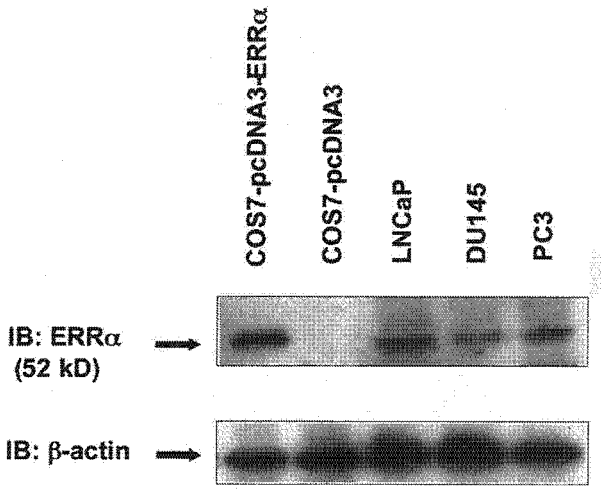

FIGURE 1 – Western blot analysis of the ERRα proteins in pcDNA3-ERRα and PC cell lines (LNCaP, DU145 and PC-3). Total cell extracts were subjected to immunoblotting with both the anti-ERRα and β-actin antibody. Anti-ERRα antibody detected a 52 kD band in COS7-pcDNA3-ERRα and human PC cell lines (LNCaP, DU145 and PC-3).

Immunohistochemical assessment

Immunostained slides were evaluated for the proportion (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3) and the intensity (0, none; 1, weak; 2, moderate; and 3, strong) of positively stained cells.[24] The total scores of immunoreactivity (0–8) were obtained as the sum of the proportion and the intensity. For immunohistochemical assessment, two investigators (T. F. and J. K.) evaluated the tissue sections independently. If IR score was different between two investigators, third investigator (S.T.) counted and we adopted the average IR score. To determine potential correlation between expression of ERRα in the malignant epithelium and clinicopathological characteristics, we considered the sections with 2 of IR score as positive for ERRα immunoreactivity. Since almost all benign foci showed <5 of IR scores for ERRα, we defined IR score 5 as a cutoff for strong immunoreactivity of ERRα.

Statistical analysis

Correlations between the immunoreactivity score (IR score) and clinicopathological characteristics (age, pretreatment serum PSA level, pathological stage and the Gleason score) were evaluated using the *t*-test or chi-square test. Cancer-specific survival curves were obtained by the Kaplan-Meier method and verified by the Log rank (Mantel-Cox) test. We analyzed statistical assessment by Stat View-J 5.0 software (SAS Institute, Cary, NC), and regarded *p*-values < 0.05 as statistically significant.

Results

Western blot analysis

Using the polyclonal anti-ERRα antibody, a 52 kD band, which corresponded to the molecular weight of ERRα, was detected in COS7-pcDNA3-ERRα. Positive signals were also observed in all three PC cell lines (LNCaP, DU145 and PC-3) (Fig. 1). F1

Immunoreactivity of ERRα in benign and malignant prostate tissues

Table I shows a summary of ERRα immunoreactivities in surgically obtained human prostate tissues. Strong immunoreactivity of T1

TABLE I – EXPRESSION OF ESTROGEN RELATED RECEPTOR α (ERRα) PROTEIN IN HUMAN PROSTATE (*N* = 106)

	Immunoreactive (IR) score[1] (%)									
	0	2	3	4	5	6	7	8	Mean ± S.D.	*p*-value
Benign (*n* = 99)	52 (52.5)	3 (3)	10 (10.1)	23 (23.2)	8 (8.1)	3 (3)	0	0	1.8 ± 2.1	<0.0001
Malignant (*n* = 106)										
Low Grade[2] (*n* = 63)	23 (36.5)	0	8 (12.7)	12 (19.0)	6 (9.5)	10 (15.9)	3 (4.8)	1 (1.6)	3.0 ± 2.6	0.0194
High Grade[2] (*n* = 43)	10 (23.2)	0	2 (4.7)	8 (18.6)	5 (11.6)	8 (18.6)	8 (18.6)	2 (4.7)	4.3 ± 2.71	

[1]Immunoreactivity (IR) score (0 to 8) was obtained as the sum of the proportion and the intensity of immunoreactivity. Proportion (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 113; 4, 1/3 to 2/3; and 5, >213), Intensity (0, none; I, weak; 2, moderate; and 3, strong).–[2]Low Grade: Gleason score; 2–7, High Grade: Gleason score; 8–10.