expression of ERα and ERβ was down-regulated by YB-1 knock-down. Wu and colleagues (20) have reported an inverse relationship between ERα and YB-1 in breast cancer samples. In the present study, ERα expression was inversely correlated with nuclear YB-1 localization, whereas ERβ expression was positively correlated with nuclear YB-1 localization. Like ERα, ERβ expression is closely associated with the PI3K/Akt signaling cascade (32). ERβ has emerged as an important determinant in breast cancer (33) and is a useful biomarker for breast cancer independent of ERα expression (34). The close linkage of nuclear YB-1 localization with ERβ expression points to the presence of a novel signaling pathway that could be a target for anticancer therapy in breast cancer.

We examined two targets of YB-1, MVP/LRP and CXCR4, which were identified by our expression profiling analysis. MVP/LRP expression, which is involved in drug resistance, is promoted by 5-fluorouracil and other anticancer agents in response to transcriptional activation by YB-1, suggesting a direct link between YB-1– and MVP/LRP-mediated drug resistance (35–37). MVP/LRP expression was not affected by YB-1 knock-down in ovarian cancer cells in

Table 2. Univariate analysis of patient characteristics and target gene expression regarding overall survival and progression-free survival

Variables	No. of patients	Overall survival		Progression-free survival	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Nuclear YB-1					
Negative	43	1.00		1.00	
Positive	30	3.48 (1.21–10.02)	0.0139	2.41 (1.07–5.44)	0.0280
EGFR					
Negative	58	1.00		1.00	
Positive	15	0.49 (0.11–2.17)	0.3376	0.46 (0.14–1.56)	0.2021
HER2					
Negative	59	1.00		1.00	
Positive	14	1.54 (0.50–4.77)	0.4528	2.01 (0.83–4.84)	0.1137
ERα					
Negative	24	1.00		1.00	
Positive	49	0.58 (0.21–1.54)	0.2661	0.60 (0.27–1.36)	0.2114
ER β					
Negative	18	1.00		1.00	
Positive	55	0.86 (0.27–2.66)	0.7867	1.14 (0.43–3.06)	0.7909
PgR					
Negative	39	1.00		1.00	
Positive	34	0.47 (0.16–1.36)	0.1535	1.160 (0.21–1.16)	0.0980
CXCR4					
Negative	29	1.00		1.00	
Positive	44	0.63 (0.24–1.68)	0.3509	0.59 (0.26–1.31)	0.1866
p-Akt					
Negative	27	1.00		1.00	
Positive	46	1.88 (0.61–5.83)	0.2669	1.56 (0.65–3.77)	0.3171
MVP/LRP					
Negative	41	1.00		1.00	
Positive	32	0.78 (0.28–2.15)	0.6283	0.76 (0.33–1.74)	0.5109
Age					
<56	38	1.00		1.00	
≥56	35	0.83 (0.31–2.22)	0.7032	0.63 (0.27–1.43)	0.2623
Histologic grade					
I	33	1.00		1.00	
II	20	1.56 (0.48–5.12)		1.05 (0.38–2.90)	
III	20	1.41 (0.43–4.62)	0.7364	1.42 (0.56–3.60)	0.7478
Menopausal status					
Pre	31	1.00		1.00	
Post	42	0.54 (0.20–1.45)	0.2138	0.47 (0.21–1.06)	0.0629
Tumor size					
<2 cm	30	1.00		1.00	
≥2 cm	43	2.26 (0.73–7.01)	0.1476	2.44 (0.97–6.16)	0.0508
Lymph node metastasis					
Absent	39	1.00		1.00	
Present	34	6.33 (1.80–22.29)	0.0010	8.49 (2.88–25.03)	<0.0001

Abbreviation: 95% CI, 95% confidence interval.

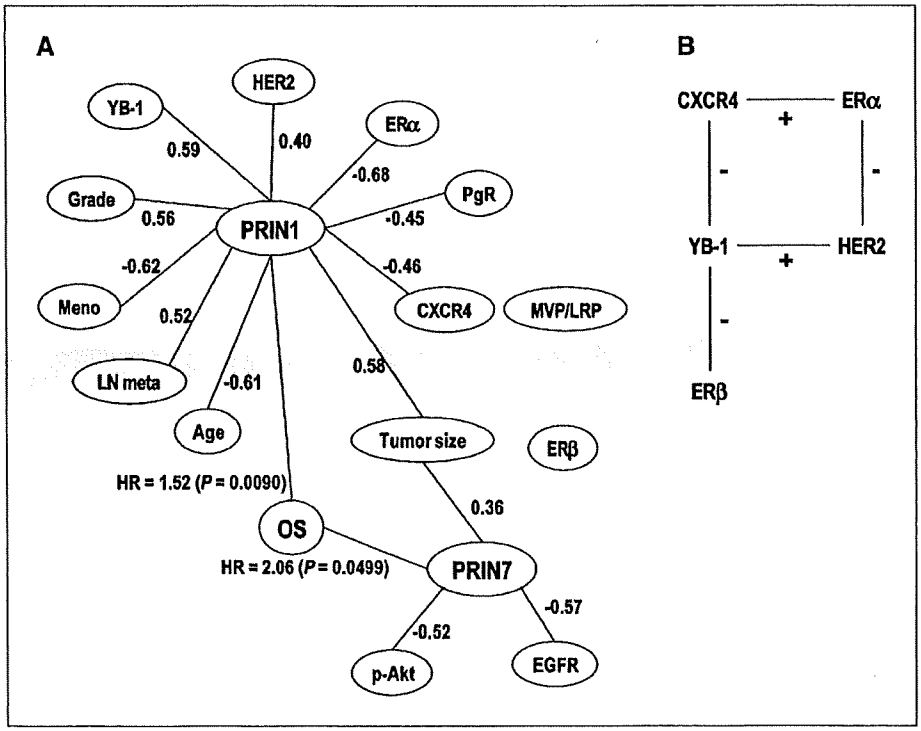

Figure 4. Statistical modeling of nuclear YB-1 localization–based network in human breast cancer. *A*, relationships among principal components, which were found significantly related to overall survival (PRIN1 and PRIN7) and clinicopathologic findings/molecular markers. Principal components and clinicopathologic findings or molecular markers are linked by a line if and only if the absolute value of correlation coefficient among them is >0.3. Each line is labeled by the correlation coefficient. *B*, relationship of molecular markers by graphical modeling incorporating with logistic regressions (+, positive correlation; −, negative correlation).

culture, although nuclear YB-1 expression and MVP/LRP expression are closely associated in patients with ovarian cancer (11, 27). CXCR4 is also known to play a critical role in the growth and metastasis of human breast cancers (38, 39). CXCR4 expression was down-regulated in YB-1 siRNA–treated ovarian cancer cells, and nuclear YB-1 expression was closely associated with CXCR4 expression in clinical samples of human ovarian cancers (11, 27). A significant positive association of nuclear YB-1 location with CXCR4 expression in breast cancer was also shown in the present study.

Nuclear localization of YB-1, in part mediated by Akt activation, thus modulates the expressions of EGFR, HER2, ERα, ERβ, and CXCR4 in breast cancer cells. YB-1–driven cell signaling of growth, survival, and hormone responses might be mainly mediated by transcriptional activation of the above-mentioned genes (1, 2); however, from our biostatistical analysis, YB-1 nuclear expression was positively associated with the expression of HER2, and negatively associated with the expressions of CXCR4 and ERβ (Fig. 4B). Moreover, ERα expression was positively correlated with CXCR4 expression and negatively correlated with HER2 expression. Although there remain inconsistencies between the data for cultured breast cancer cells and actual breast cancers with regard to the relationship between YB-1 nuclear location and the expression of other biomarkers, our biostatistical linkage map should provide important information for the development of strategies for molecular diagnosis and therapy.

In conclusion, nuclear YB-1 expression might be a prognostic marker in breast cancer. Furthermore, YB-1 plays a key role in the network annotation of genes such as HER2, CXCR4, ERα, and ERβ (Fig. 4). In addition to YB-1–mediated acquisition of multidrug resistance, the close association of nuclear YB-1 localization with HER2 expression should be considered part of the underlying mechanism. The determination of the nuclear versus cytoplasmic localization of YB-1 might provide a useful molecular indicator for personalized therapeutics of anticancer drugs targeting HER2 and/or ERα.

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Prognostic implications of the nuclear localization of Y-box-binding protein-1 and CXCR4 expression in ovarian cancer: Their correlation with activated Akt, LRP/MVP and P-glycoprotein expression

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The nuclear localization of Y-box-binding protein-1 (YB-1) is known to be a poor prognostic factor in several human malignancies, including ovarian carcinoma. Following on from our basic study dealing with microarray analyses of YB-1-associated gene expression in ovarian cancer cells, we examined whether nuclear localization of YB-1 is associated with the expression of CXCR4, a vault protein named lung resistance-related vault protein (LRP/MVP), phosphorylated Akt (p-Akt) or P-glycoprotein (P-gp) in human ovarian carcinoma. Fifty-three surgically resected ovarian carcinomas treated with paclitaxel and carboplatin were examined immunohistochemically for nuclear YB-1 expression and intrinsic expression of p-Akt, P-gp, LRP/MVP and CXCR4. Nuclear expression of YB-1 demonstrated significant correlation with p-Akt, P-gp and LRP expression, but no relationship with CXCR4 expression. By multivariate analysis, only YB-1 nuclear expression and CXCR4 expression were independent prognostic factors with regard to overall survival. These results indicate that YB-1 nuclear expression and CXCR4 expression are important prognostic factors in ovarian carcinoma. (*Cancer Sci* 2007; 98: 1020–1026)

Y-box-binding protein-1 (YB-1) has been identified as a transcription factor that binds to the promoter region of several genes involved in positive regulation of the cell cycle, such as proliferating cell nuclear antigen, DNA topoisomerase II α, and multidrug resistance 1 gene (*MDR1*) which is linked to classical multidrug resistant (*MDR*).[1–3] Nuclear expression of YB-1 has been reported to have a close relationship with MDR1/P-glycoprotein (P-gp) expression in several human malignancies.[4–7] Moreover, YB-1 has been reported to be a prognostic marker of breast cancer,[4] ovarian cancer,[6] lung cancer[7] and synovial sarcoma.[8] These clinicopathological studies consistently supported the notion that the absence or presence of YB-1 within the nucleus plays a critical role in the acquisition of malignant characteristics, including global drug resistance.

Sutherland *et al.* have also reported that YB-1 phosphorylation by Akt is required for the nuclear translocation of YB-1:[9] Akt is a signal transduction protein that plays an important role in inhibiting apoptosis, stimulating angiogenesis, and promoting tumor formation in a variety of human malignancies.[10] Taking these findings together, translocation of YB-1 into the nucleus would seem to be mediated through pleiotropic signaling pathways. Our recent study demonstrated that the nuclear translocation of YB-1 is in part stimulated through Akt activation, and also that YB-1 is involved in upregulation and downregulation of various genes including *P-gp*, lung resistance-related vault protein (*LRP/MVP*) and *CXCR4* in human ovarian cancer cells.[11]

The lung resistance-related vault protein (LRP) has been identified as the major vault protein (MVP), which is the major component of vaults, of subcellular particles that have been implicated in transmembrane transport processes.[12] YB-1 also has been reported to promote basal and 5-fluorouracil-induced expression of the *LRP/MVP* gene, the promoter of which contains the Y-box in human colon cancer.[13] Furthermore, the chemokine stroma-derived factor 1 (SDF-1)/CXCL12, and its receptor, CXCR4, have recently been shown to play an important role in metastasis of several kinds of carcinoma.[14,15] This SDF-1/CXCR4 pathway has also been implicated in the invasion and metastasis of ovarian cancer.[16,17] Our preliminary study demonstrated that a human ovarian cancer cell line treated with YB-1 knockdown by small interfering RNA showed downregulated expression of CXCR4, using oligonucleotide microarray analysis.[11]

In the present study, we focused on whether nuclear localization of YB-1 could be associated with the expression of these molecular targets, p-Akt, LRP/MVP, CXCR4 as well as P-gp in ovarian cancer patients, using immunohistochemical analysis. We also studied the various clinicopathological characteristics and the prognostic impact in ovarian carcinoma when patients were treated with a regimen containing both paclitaxel and carboplatin (CBDCA). The coupling of the nuclear localization of YB-1 with p-Akt and global drug resistance-related markers will be discussed with regard to its possible association with the therapeutic efficacy of paclitaxel and carboplatin.

Materials and Methods

Patients. Fifty-three patients with primary ovarian carcinoma who had undergone debulking surgery at Kyushu University Hospital between 1998 and 2004 were examined. Patients were staged according to the International Federation of Obstetrics and Gynecology classification.[18] All of the patients were subjected to chemotherapy using a regimen containing both taxanes (paclitaxel for 51 patients, 180 mg/m^2 body surface/day; docetaxel for two patients, 70 mg/m^2 body surface/day) and CBDCA. The doses of CBDCA were calculated using Calvert's formula.[19] The effect of chemotherapy was evaluated 3–4 weeks after each administration of chemotherapy by ultrasonography or computed tomography. After chemotherapy, all patients were followed up every 2 months

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for the first year, every 3 months for the next year, every 4 months for the next year, every 6 months for the next 2 years, and every year thereafter.

Clinical outcome was measured by treatment-free survival, defined as the interval from the date of the end of the treatment to the date of the diagnosis of progression (drug-free interval), as well as overall survival.

Primary tumors were classified according to a recent WHO classification[20] and were graded as grade 1, 2 or 3 according to Silverberg's proposal[21] using extensively sampled paraffin-embedded samples. We obtained written informed consent from all patients. For strict privacy protection, identifying information for all samples was removed before analysis.

Antibodies. The polyclonal antibody to YB-1 was prepared against a 15-amino acid synthetic peptide (residues 299–313) in the tail domain of the YB-1 protein.[22] The working dilution of anti-YB-1 polyclonal antibody was 1:100. Polyclonal anti-pAkt (Ser473) (diluted 1:100) was obtained from Cell Signaling Technology (Beverly, MA, USA). The monoclonal antibodies 12G5 (BD PharMingen, San Diego, CA, USA; diluted 1:100) for the detection of CXCR4, LRP56 (Nichirei, Tokyo, Japan; diluted 1:50 for LRP), and JSB-1 (Sanbio, Uden, the Netherlands; diluted 1:20) for P-gp were used. Tissue from a normal kidney served as a control for LRP56 and JSB-1, whereas primary breast cancer tissue with regional lymph node metastasis was used as a control for anti-YB-1, anti-pAKt and 12G5.

Immunohistochemistry. Surgically resected specimens prior to chemotherapy were fixed with 10% formalin and embedded in paraffin. Four-micrometer-thick sections on silane-coated slides were stained using the streptavidin–biotin–peroxidase method with a Histofine Sab-Po kit (Nichirei) according to the manufacturer's instructions. At least one representative section was examined in each tumor. After deparaffinization, rehydration and inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4°C overnight. After incubation of the secondary antibody and streptavidin–biotin–peroxidase complex at room temperature, the sections were then incubated in 3,3′-diaminobenzidine, counterstained with hematoxylin, and mounted. For staining with all of the antibodies, sections were pretreated with microwave irradiation for the purpose of antigen retrieval.

Scoring of immunohistochemical results. The evaluation of immunohistochemical results was scored by two pathologists (Y. Oda and Y. Ohishi) without knowledge of the clinical data of the patients. YB-1 expression was evaluated as to whether its expression was localized in both the nucleus and the cytoplasm, or only in the cytoplasm.[6] For P-gp and LRP, when >10% of the tumor cells showed a positive reaction, either weakly or strongly, we judged the case to be positive in accordance with a previous study.[23] As for P-gp expression, only membranous staining was evaluated, whereas cytoplasmic granular staining pattern was estimated for LRP expression. A consensus judgment was adopted as to the proper immunohistochemical score of the tumors based on the strength of p-Akt and CXCR4 expression: 0, negative; 1+, weak staining; 2+, moderate staining; or 3+, strong staining. The distribution of positive cells was also recorded to portray the diffuse or focal nature of the positive cells: sporadic (positive cells <10%); focal (positive cells ≥11% but <50%); diffuse (positive cells ≥50%). Samples with immunohistochemical scores of 2+ and 3+ with focal to diffuse distributions were considered to be positive for p-Akt and CXCR4 antibodies.[24]

Statistics. Association between two dichotomous variables was evaluated by a two-sided Fisher's exact test. Differences in progression-free survival and overall survival were analyzed using log-rank statistics. Multivariate analysis was carried out with a Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

Table 1. Clinical and pathological characteristics of 53 patients

Characteristic	*n*
Age (years)	
<56	26
≥56	27
Stage (FigO)	
I/II	7
III/IV	46
Grade	
I/II	37
III	16
Histology	
Endometrioid	4
Serous	49
Residual tumor (cm)	
<2	36
≥2	11
Unknown	6
Chemotherapy	
Paclitaxel/carboplatin	51
Docetaxel/carboplatin	2

Results

Patients. Clinical and pathological characteristics at diagnosis are summarized in Table 1.

The median age of the patients was 58 years (range, 36–77 years). Four tumors were considered to be stage I, three stage II, 29 stage III, and 17 stage IV. Six tumors showed histological grade I, 31 grade II, and 16 grade III. Histologically, 49 tumors were serous adenocarcinoma and four were endometrioid adenocarcinoma. As for overall survival, follow-up data were available for 52 of the 53 patients. The median treatment-free survival of all 53 patients was 307 days (range, 2–1854 days), whereas the median survival was 858 days (range, 138–2292 days). The median treatment-free follow-up of those patients who are currently progression free is 783 days (range, 30–1854 days).

Immunohistochemistry. The results of the immunohistochemical analyses are summarized in Table 2. Of the 53 tumors, 15 (28.3%) showed intense YB-1 expression in the nucleus but weak expression in the cytoplasm of the tumor cells (Fig. 1A). These cases

Table 2. Correlation between nuclear expression of Y-box-binding protein-1 (YB-1) and phosphorylated Akt (p-Akt), P-glycoprotein (P-gp), lung resistance-related vault protein (LRP) or CXCR4 expression

Protein	Nuclear expression of YB-1		*P*-value
	+	−	
p-Akt			
+	12	10	0.0005*
−	3	28	
P-gp			
+	4	1	0.0191*
−	11	37	
LRP			
+	12	15	0.0084*
−	3	23	
CXCR4			
+	7	13	0.2963
−	8	25	

*Statistically significant.

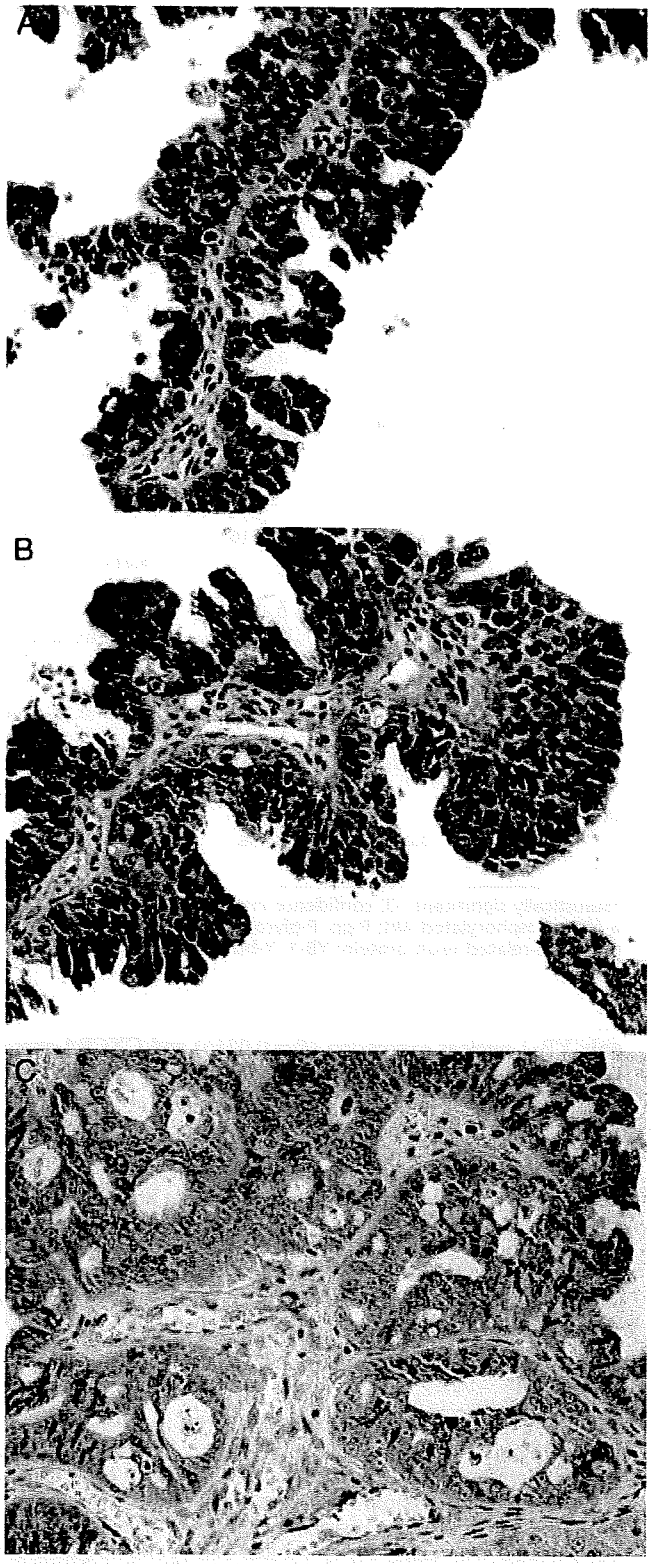

Fig. 1. Grade 2 and stage IIc serous cystadenocarcinoma of a 51-year-old woman. (A) Both nuclear and cytoplasmic expression of Y-box-binding protein-1 were observed in the tumor cells. (B) Strong and diffuse phosphorylated Akt expression was also evident in both the cytoplasm and nuclei. (C) Lung resistance-related vault protein was expressed as a granular cytoplasmic staining pattern. The patient died of disease 51 months after initial surgery.

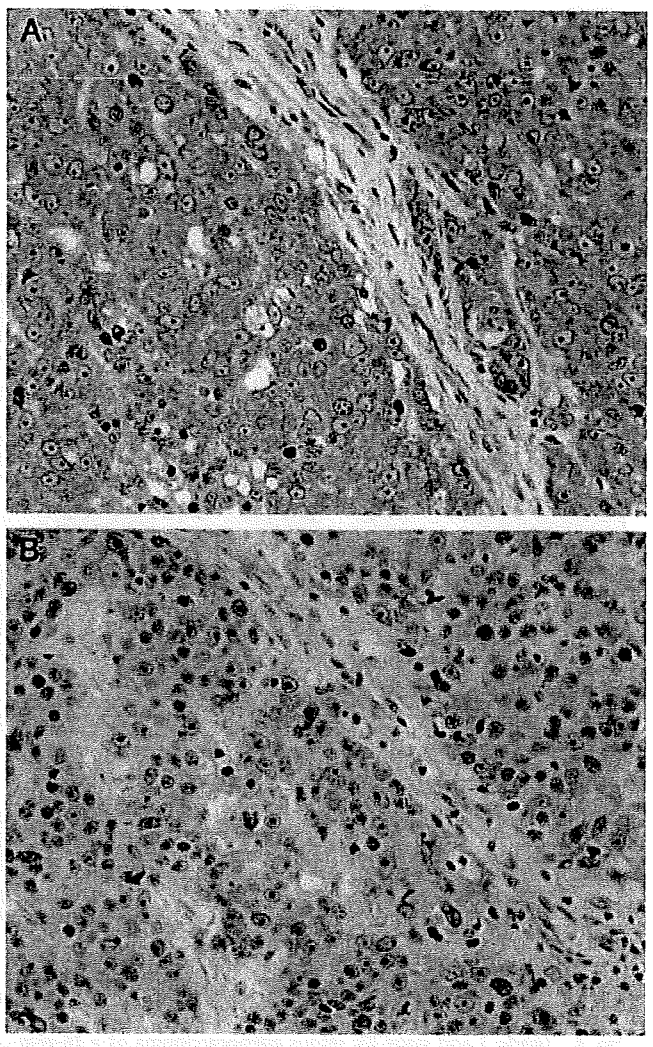

Fig. 2. Grade 3 and stage IIIc serous adenocarcinoma of a 66-year-old woman. Y-box-binding protein-1 expression was observed only in the cytoplasm (A), whereas immunoreactivity for phosphorylated Akt was recognized very faintly in a few tumor cells and was interpreted as negative (B). The patient currently shows no evidence of disease 22 months after surgery.

were interpreted as nuclear expression of YB-1-positive cases. The remaining 38 tumors (71.7%) revealed YB-1 expression only in the cytoplasm, and were interpreted as nuclear expression of YB-1-negative cases. Positive immunostaining for p-Akt was found in 22 tumors (41.5%) with it being predominantly cytoplasmic staining. In 5 of these 22 tumors, immunoreactivity was also recognized in the nucleus (Fig. 1B). Of the 15 tumors in which YB-1 expression was observed in the nucleus, 12 (80%) showed positive immunoreaction for p-Akt, and there was a significant correlation between the nuclear expression of YB-1 and p-Akt expression ($P = 0.0005$) (Fig. 1A,B,2). The membranous expression of P-gp was detected in only five tumors (9.4%) (Fig. 3A). A statistical significance was found between P-gp and YB-1 nuclear expression ($P = 0.0191$). LRP immunostaining was positive in 27 (50.9%) tumors with a granular cytoplasmic staining pattern. There was a significant correlation between LRP expression and YB-1 nuclear expression ($P = 0.0084$) (Fig. 1A,C). Positive immunoreactivity for CXCR4 was

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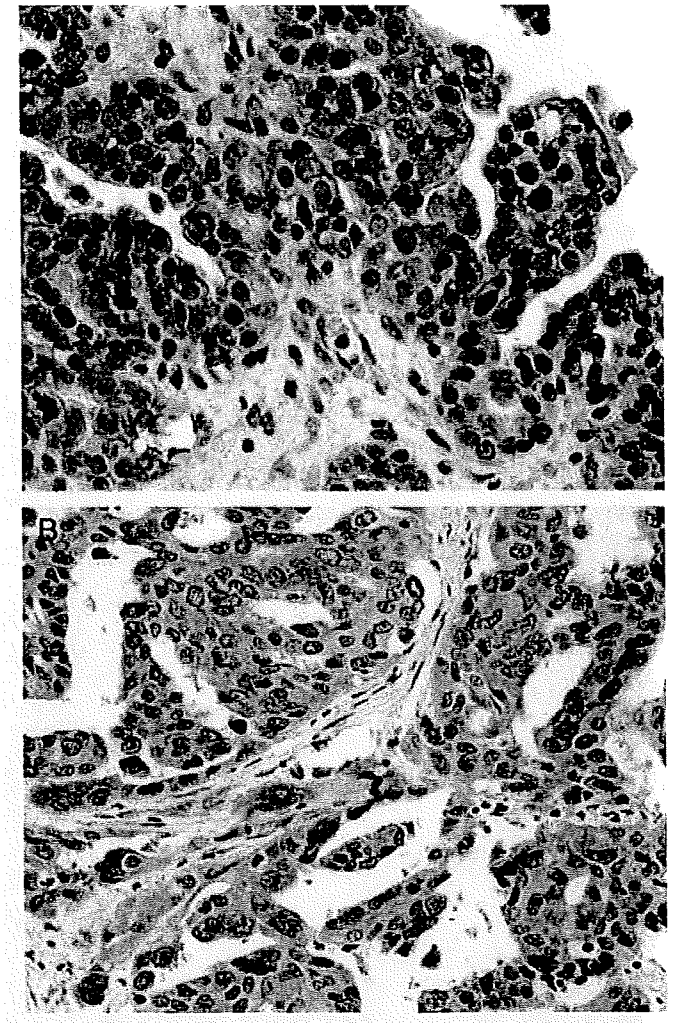

Fig. 3. Grade 3 and stage IIIc serous adenocarcinoma of a 76-year-old woman. P-glycoprotein was expressed in this case as a diffuse membranous staining pattern (A). CXCR4 expression was diffusely visible in the cytoplasm as well as in a few nuclei (B). Y-box-binding protein-1 nuclear expression was also recognized in this case and the patient died of disease 18 months after surgery.

observed in 20 tumors (37.7%) (Fig. 3B); however, it showed no significant relationship with YB-1 nuclear expression.

p-Akt expression was also related to P-gp ($P = 0.0092$), LRP ($P < 0.0001$) and CXCR4 ($P = 0.0078$) expression. Moreover, a significant correlation was found between LRP immunostaining and P-gp ($P = 0.0281$) or CXCR4 ($P = 0.0001$) expression. As for the correlation between clinicopathological parameters and immunohistochemical results, LRP expression was significantly correlated with an age higher than 56 years ($P = 0.0363$). No association was found between any other clinicopathological characteristics and immunostaining for YB-1, p-Akt, P-gp, LRP or CXCR4.

Survival analysis. The results of overall survival analysis and treatment-free (drug-free) survival analysis are summarized in Tables 3 and 4, respectively. As for overall survival, immunohistochemical YB-1 nuclear expression ($P = 0.0126$), p-Akt expression ($P = 0.0167$) and CXCR4 expression ($P = 0.0077$) were adverse prognostic factors, using univariate analysis (Table 3; Fig. 4). No clinicopathological parameters demonstrated a predictive value for overall survival. By multivariate analysis including clinicopathological and immunohistochemical parameters,

Table 3. Overall survival in 52 cases of ovarian carcinoma

Variable	*n*	*P*-value in survival analysis		HR (95% CI)
		Univariate	Multivariate	
Clinicopathological				
Age (years)				
<56	26	0.8903	0.5488	1
≥56	26			1.582 (0.36–6.98)
Stage				
I/II	7	0.1577	0.2087	1
III/IV	45			4.064 (0.46–36.19)
Grade				
I/II	36	0.7422	0.6	1
III	16			1.553 (0.3–8.06)
Residual tumor (cm)				
<2	35	0.82	0.2039	1
≥2	11			2.714 (0.58–12.67)
Immunohistochemical				
YB-1 nuclear expression				
–	37	0.0126*	0.0216*	1
+	15			6.014 (1.3–27.81)
P-gp				
–	47	0.8995	0.6383	1
+	5			0.619 (0.08–4.57)
p-Akt				
–	30	0.0167*	0.5195	1
+	22			1.866 (0.28–12.46)
CXCR4				
–	32	0.0077*	0.0316*	1
+	20			9.007 (1.21–66.88)
LRP				
–	25	0.0897	0.458	1
+	27			0.44 (0.05–3.85)

*Statistically significant. CI, confidence interval; HR, hazard ratio; p-Akt, phosphorylated Akt; P-gp, P-glycoprotein; LRP, lung resistance-related vault protein; YB-1, Y-box-binding protein-1.

only YB-1 nuclear expression ($P = 0.0216$) and CXCR4 expression ($P = 0.0316$) were found to be independent prognostic factors with regard to overall survival (Table 3).

As for treatment-free survival, high-stage tumors ($P = 0.0102$) and cases with p-Akt expression ($P = 0.0133$) and LRP expression ($P = 0.0199$) showed adverse prognosis, whereas CXCR4 expression had no impact on prognosis by univariate analysis (Table 4; Fig. 5). Although the cases with YB-1 nuclear expression tended to have worse prognosis, the difference was not statistically significant ($P = 0.0537$; Fig. 5). By multivariate analysis, tumor stage ($P = 0.0428$) and CXCR4 expression ($P = 0.0373$) were poor prognostic factors for treatment-free survival (Table 4).

Discussion

Nuclear expression of YB-1 is reported to be associated with poor prognosis in malignant solid tumors.[7,8] As for ovarian cancer, Kamura *et al.* first demonstrated the prognostic value of YB-1 nuclear expression on disease-free survival in a group of advanced (stage III) serous adenocarcinoma patients who had been treated with cisplatin, epirubicin and cyclophosphamide.[6] In contrast, Huang *et al.* could detect no significant difference in overall survival between patients with YB-1 nuclear expression and those without such expression among patients with epithelial ovarian cancers that consisted of several histological subtypes.[25] These studies help us to further understand why the nuclear localization of YB-1 is associated with poor prognosis in patients

Table 4. Treatment-free survival in 53 cases of ovarian carcinoma

Variable	*n*	*P*-value in survival analysis		HR (95% CI)
		Univariate	Multivariate	
Clinicopathological				
Age (years)				
<56	26	0.7085	0.3508	1
≥56	27			1.536 (0.62–3.79)
Stage				
I/II	7	0.0102*	0.0428*	1
III/IV	46			4.869 (1.05–22.51)
Grade				
I/II	37	0.237	0.2335	1
III	16			0.577 (0.23–1.43)
Residual tumor (cm)				
<2	36	0.8	0.4657	1
≥2	11			1.424 (0.55–3.68)
Immunohistochemical				
YB-1 nuclear expression				
–	38	0.0537	0.6326	1
+	15			1.236 (0.52–2.95)
P-gp				
–	48	0.1768	0.1859	1
+	5			2.415 (0.65–8.92)
p-Akt				
–	31	0.0133*	0.7813	1
+	22			1.149 (0.43–3.07)
CXCR4				
–	33	0.0824	0.0373*	1
+	20			3.102 (1.07–9.00)
LRP				
–	26	0.0199*	0.7685	1
+	27			0.844 (0.27–2.61)

*Statistically significant. CI, confidence interval; HR, hazard ratio; p-Akt, phosphorylated Akt; P-gp, P-glycoprotein; LRP, lung resistance-related vault protein; YB-1, Y-box-binding protein-1.

with various malignancies, including ovarian cancers. In the current study, all of the ovarian cancer patients were treated with taxanes and carboplatin and YB-1 nuclear expression was found to be a poor prognostic marker with regard to overall survival by univariate analysis. As for treatment-free survival, the patients with YB-1 nuclear expression tended to show worse prognosis compared with the patients without YB-1 nuclear expression. Moreover, multivariate analysis revealed that the nuclear expression of YB-1 was an independent adverse prognostic marker with regard to overall survival.

We then asked ourselves how YB-1 could affect the prognosis of patients with ovarian cancer and other malignancies. One representative ATP-binding cassette superfamily protein, P-gp, is often overexpressed in various types of human tumors including ovarian cancer, breast cancer, osteosarcoma and synovial sarcoma.[4–6,8] YB-1 has been identified as a transcription factor that binds to the Y-box of the *MDR1* promoter.[22] Some investigators have shown the prognostic value of intrinsic P-gp expression in ovarian carcinoma,[26] whereas others have failed to demonstrate its predictive value for survival.[23,25] In the current study, we could detect P-gp expression in only 9.4% of the examined cases. Although a statistically significant correlation between P-gp expression and nuclear YB-1 expression was observed, P-gp expression did not affect the patient's prognosis because of the small number of P-gp-positive cases. Further studies with an increased number of patients with P-gp-positive ovarian cancer are required to clarify the notion that the close association of YB-1 with P-gp could play a clinically significant role in the acquisition of drug resistance in ovarian cancer when patients are treated with paclitaxel and cisplatin.

Recently, Stein *et al.* showed an increased expression of endogenous LRP protein by transduction of YB-1 cDNA *in vivo*, and a strong coexpression of LRP and YB-1 in human colon cancer specimens.[13] The prognostic value of LRP expression in ovarian carcinoma is also controversial. LRP has been shown to be a predictor of poor response to chemotherapy and prognosis in ovarian cancer patients,[23] whereas other authors have

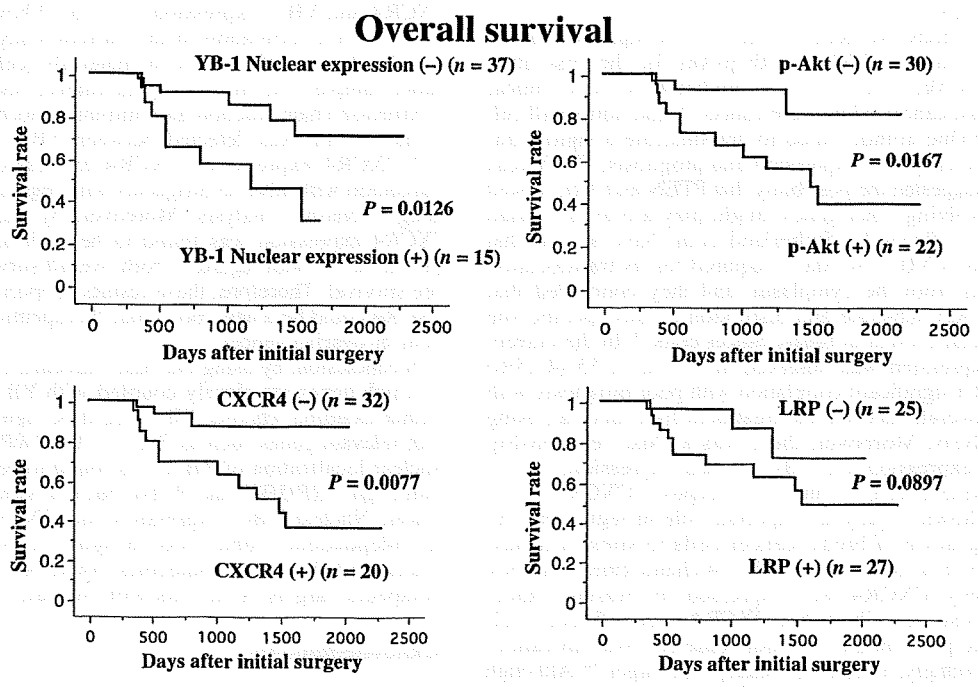

Fig. 4. Overall survival according to immunohistochemical expression in 52 patients with ovarian carcinoma. Y-box-binding protein-1 nuclear expression, and phosphorylated Akt and CXCR4 expression have a significant predictive value for survival.

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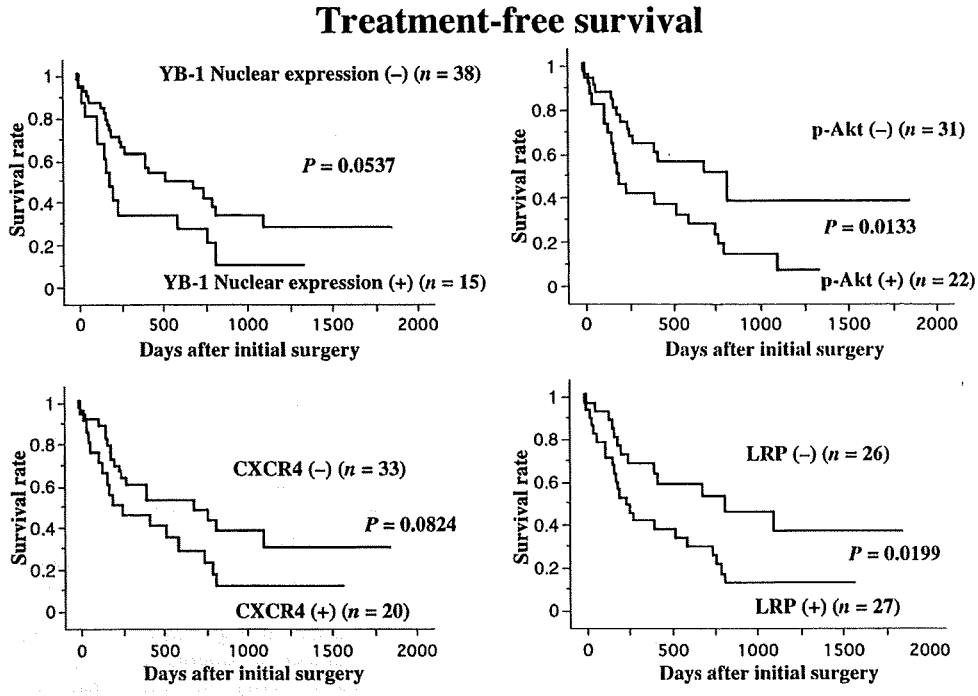

Fig. 5. Treatment-free survival. Cases with Y-box-binding protein-1 nuclear expression tended to show poor prognosis. Cases with phosphorylated Akt and lung resistance-related vault protein expression showed adverse prognosis, whereas CXCR4 expression had no impact on prognosis.

demonstrated no association between LRP expression and clinical outcome.[26] In contrast, the present study demonstrated a close correlation between YB-1 nuclear expression and LRP expression in ovarian carcinoma, as has also been reported in colon cancer. Moreover, LRP expression in untreated ovarian carcinoma was an unfavorable prognostic factor with regard to treatment-free survival. This YB-1-LRP/MVP network may also play a role in global drug resistance in ovarian cancer treated with chemotherapy.

In the present study, we demonstrated a very high association of nuclear localization of YB-1 with p-Akt for the first time. Activated Akt (p-Akt) is known to be predictive of poor clinical outcome in breast cancer,[27] prostate cancer[28] and non-small cell lung cancer.[29] One author failed to demonstrate a significant correlation between p-Akt expression and prognosis,[30] whereas another author suggested the possibility that PTEN and Akt, as well as pathways involving other genes, might play a role in ovarian carcinogenesis.[31] Recently, Sutherland *et al.* have shown that phosphorylation of YB-1 by Akt is required for its translocation into the nucleus from the cytoplasm, and they concluded that YB-1 is a new Akt substrate and disruption of this specific site inhibits tumor cell growth in breast cancer cells.[9] In the current study, p-Akt expression was observed in 22 out of 53 (41.5%) cases and it had a significant correlation with poor prognosis with regard to both overall survival and treatment-free survival, using univariate analysis. Moreover, there was a close relationship between p-Akt expression and YB-1 nuclear expression.

The chemokine-CXCL12 and its receptor, CXCR4, have recently been shown to play an important role in regulating the directional migration of breast cancer cells to sites of metastasis.[14] Scotton *et al.* found that of the 14 chemokines that they investigated, only CXCR4 was expressed in ovarian cancer cells.[16] They also described that CXCR4 may influence cell migration in the peritoneum, a major route for ovarian cancer spread, and accordingly, it could be a therapeutic target.[16] Although CXCR4 is a seven-domain membrane G-protein-coupled receptor, cytoplasmic CXCR4 expression has been described in many human cancers.[17,24] Engl *et al.* demonstrated distinct CXCR4 expression at the intercellular boundaries and strong intracellular accumulation, using confocal laser scanning microscopic analysis.[32] In the current study ovarian cancer cells mainly showed cytoplasmic CXCR4 staining, as previously reported.[17] Jiang *et al.* demonstrated that CXCR4 expression was one of the independent prognostic factors in clinical samples of ovarian cancer.[17] In our recent study we demonstrated the close correlation between CXCR4 and YB-1 expression *in vitro*;[11] however, we failed to reveal such a correlation in the current study. This discrepancy may be due to differences in materials (cell line and clinical tumor sample) and methods (quantitative reverse transcription–polymerase chain reaction and immunohistochemistry). Although no association was detected between YB-1 nuclear expression and CXCR4 expression, CXCR4 expression demonstrated a correlation with adverse prognosis with regard to overall survival, using univariate analysis. Moreover, by multivariate analysis, CXCR4 expression was found to be an independent poor prognostic factor with regard to both overall survival and treatment-free survival. Therefore, these results support the possibility that CXCR4 could be a new molecular therapeutic target in the treatment of ovarian cancer.

In conclusion, by using our basic information on the expression of which genes are closely coupled with YB-1, we were able to further examine whether YB-1 could be significantly associated with relevant genes such as *P-gp*, *p-Akt*, *LRP/MVP* and *CXCR4*. Nuclear localization of YB-1 was found to be closely associated with *P-gp*, *LRP/MVP* and *P-Akt*, but not with *CXCR4* in ovarian cancer. Nuclear YB-1 expression and CXCR4 expression may be independent global poor prognostic markers in ovarian cancer, and these two molecules could be novel candidates as therapeutic targets in patients with ovarian cancer.

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ORIGINAL ARTICLE

Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells

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Y-box-binding protein 1 (YB-1), which is a member of the DNA-binding protein family containing a cold-shock domain, has pleiotropic functions in response to various environmental stimuli. As we previously showed that YB-1 is a global marker of multidrug resistance in ovarian cancer and other tumor types. To identify YB-1-regulated genes in ovarian cancers, we investigated the expression profile of YB-1 small-interfering RNA (siRNA)-transfected ovarian cancer cells using a high-density oligonucleotide array. YB-1 knockdown by siRNA upregulated 344 genes, including *MDR1*, *thymidylate synthetase*, *S100 calcium binding protein* and *cyclin B*, and downregulated 534 genes, including *CXCR4*, *N-myc downstream regulated gene 1*, *E-cadherin* and *phospholipase C*. Exogenous serum addition stimulated YB-1 translocation from the cytoplasm to the nucleus, and treatment with Akt inhibitors as well as Akt siRNA and integrin-linked kinase (ILK) siRNA specifically blocked YB-1 nuclear localization. Inhibition of Akt activation downregulated *CXCR4* and upregulated *MDR1* (*ABCB1*) gene expression. Administration of Akt inhibitor resulted in decrease in nuclear YB-1-positive cancer cells in a xenograft animal model. Akt activation thus regulates the nuclear translocation of YB-1, affecting the expression of drug-resistance genes and other genes associated with the malignant characteristics in ovarian cancer cells. Therefore, the Akt pathway could be a novel target of disrupting the nuclear translocation of YB-1 that has important implications for further development of therapeutic strategy against ovarian cancers.

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Introduction

The Y-box-binding protein 1 (YB-1), which is a DNA/RNA-binding protein also known as dbpB, regulates transcription, translation, DNA damage repair and other biological processes in both the nucleus and cytoplasm (Matsumoto and Wolffe, 1998; Kohno *et al.*, 2003). In the cytoplasm, YB-1 regulates mRNA stability and translational regulation (Evdokimova *et al.*, 2001; Ashizuka *et al.*, 2002; Fukuda *et al.*, 2004), while in the nucleus, it plays a pivotal role in transcriptional regulation through specific recognition of the Y-box promoter element (Ladomery and Sommerville, 1995; Kohno *et al.*, 2003). Interaction of YB-1 with its cognate Y-box-binding site (inverted CCAAT box) is promoted by cytotoxic stimuli, including actinomycin D, cisplatin, etoposide, ultraviolet (UV) and heat shock, leading to the activation of a representative ABC transporter *MDR1/ABCB1* and DNA topoisomerase IIα genes (Asakuno *et al.*, 1994; Furukawa *et al.*, 1998; Ohga *et al.*, 1998). YB-1 also selectively interacts with damaged DNA or RNA, and protects from cytotoxic effects following cellular exposure to cisplatin, mitomycin C, UV and oxygen radicals (Ohga *et al.*, 1996; Ise *et al.*, 1999).

Royer and co-workers were the first to report that nuclear localization of YB-1 is associated with intrinsic *MDR1* expression in human primary breast cancer (Bargou *et al.*, 1997). Immunostaining analysis of various human cancers also supported this result, and showed that nuclear expression of activated YB-1 was closely associated with the acquisition of P-glycoprotein-mediated multidrug resistance (Kuwano *et al.*, 2004). YB-1 has also been shown to induce basal and 5-fluorouracil-induced expression of the major vault protein (*MVP/LRP*) gene, the promoter of which contains a Y-box (Stein *et al.*, 2005). In human malignancies, vault proteins are involved in acquiring drug resistance (Mossink *et al.*, 2003). Taken together, these findings suggest that nuclear localization of YB-1 might play a key role in the acquisition of global drug resistance through transcriptional activation of relevant genes and the repair of damaged DNA (Kuwano *et al.*, 2004).

The nuclear localization of YB-1 is required for transcription and DNA repair in response to various environmental stimuli, such as adenovirus infection (Holm *et al.*, 2002), DNA-damaging agents, UV irradiation, hyperthermia (Stein *et al.*, 2001) and serum stimulation (En-Nia *et al.*, 2005). However, as a nucleocytoplasmic shuttling protein, it is important to understand which signalling molecules are involved in the translocation of YB-1 into the nucleus. Koike *et al.* (1997) first reported the possible role of protein kinase C in YB-1 nuclear translocation in cancer cells exposed to UV irradiation, and highlighted the importance of the YB-1 C-terminal region in cytoplasmic retention. Other studies have suggested the involvement of additional molecules: thrombin-mediated YB-1 nuclear translocation was shown to be inhibited by protein tyrosine phosphatase inhibitor in endothelial cells (Stenina *et al.*, 2000), while Dooley *et al.* (2006) demonstrated the involvement of Jak1 in YB-1 nuclear translocation. Sutherland *et al.* (2005) recently reported that phosphorylation of YB-1 by Akt at serine 102 in the cold-shock domain is required for YB-1 nuclear translocation in cancer cells. Another mechanism for nuclear translocation of YB-1 was shown to be promoted by various cytotoxic anticancer agents, which trigger the proteolytic cleavage by the 20S proteasome of the YB-1 C-terminal fragment containing the cytoplasmic retention signal (Sorokin *et al.*, 2005). In our present study, we have provided evidence that Akt activation is one of the mechanisms for nuclear translocalization of YB-1, and also that YB-1 regulates expression of various cell growth and malignant progression-related genes as well as global drug resistance-related genes including *MDR1*.

Results

Suppression of YB-1 leads to an enhancement of MDR-1 expression and decrease of CXCR-4 expression

We previously reported that YB-1 was expressed in the nucleus in almost 30% of serous ovarian cancers, and that YB-1 nuclear-positive patients had a poor prognosis (Kamura *et al.*, 1999). As nuclear translocation of YB-1 is highly susceptible to environmental stimuli, we first examined whether the stress-inducing exogenous addition of serum could stimulate nuclear translocation of YB-1 in seven serum-deprived human ovarian cancer cell lines. Among the seven cell lines, nuclear YB-1 translocation was stimulated more than twofold in two: RMG-III and SKOV-3 (Figure 1a). In these two lines, serum incubation markedly enhanced Akt phosphorylation and increased translocation of YB-1 into the

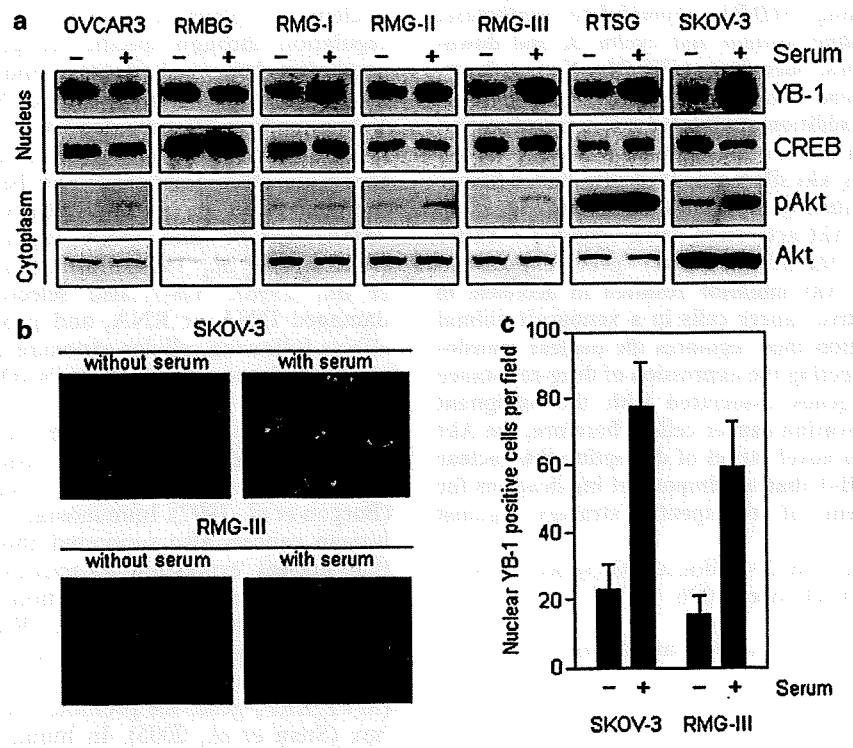

Figure 1 Levels of Akt phosphorylation and nuclear localization of YB-1 in ovarian cancer cell lines with or without serum stimulation. (**a**) Cytoplasmic and nuclear extracts were prepared 1 h after 10% serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed on nuclear extracts, and anti-pAkt and anti-Akt immunoblots were performed with cytoplasmic extracts. CREB and Akt are shown as a loading control. (**b**) Immunofluorescent staining of YB-1 in ovarian cancer cells. Cells stimulated with or without serum for 1 h were fixed and permeabilized, incubated at 4°C with the primary YB-1 antibody, then with the Alexa Flour 546-labelled secondary antibody. (**c**) Quantitative analysis of YB-1 nuclear localization as shown in Figure 1b. Data are mean of three independent experiments; bars ±s.d.

nucleus, as shown by immunofluorescense analysis (Figure 1b and c).

Although YB-1 is known to regulate the expression of several genes at the transcriptional level, the complete network of genes associated with YB-1 has not been elucidated. We therefore, explored the expression profile of YB-1 siRNA-treated SKOV-3 cells and mock-treated SKOV-3 cells using a high-density oligonucleotide microarray. We transfectd YB-1 siRNA into SKOV-3 cells at a concentration of 200 and 400 nM. Transfection of 200 nM YB-1 siRNA decreased expression of YB-1 mRNA by only 45%, whereas 400 nM YB-1 siRNA decreased by 70% (Figure 2). Of the 54 675 RNA transcripts and variants in the microarray, we identified 344 genes that were increased more than twofold and 534 genes that were decreased 0.5-fold or less in both 200 and 400 nM YB-1 siRNA-transfected cells (Supplementary Table S1). Upregulated genes were classified into 'cell cycle' ($P<0.0001$), 'cytoskeleton organization and biogenesis' ($P=0.0003$), 'cell growth and/or maintenance' ($P=0.0005$), and GO SLIMS Biological Process' ($P=0.0013$). Downregulated genes were classified into 'catalytic activity' ($P=0.0007$) and 'transferase' ($P=0.0010$). We selected 46 genes that we expected to be associated with drug resistance, cell growth, cancer malignant progression and cell signalling (Table 1), and chose three of these for further study: *MDR1*, *MVP/LRP* and chemokine (C-X-C motif) receptor 4 (*CXCR4*).

We used quantitative real-time PCR (QRT-PCR) to confirm whether expression of these three genes was modulated in YB-1 siRNA-transfected cells. Expression of *CXCR4* decreased by 67%, whereas expression of *MVP/LRP* was unaffected by the siRNA (Figure 2). *MDR1* expression was increased approximately 30-fold in 400 nM YB-1 siRNA-transfected cells compared with control siRNA-transfected cells. The results of QRT-PCR are broadly consistent with those of the microarray analysis.

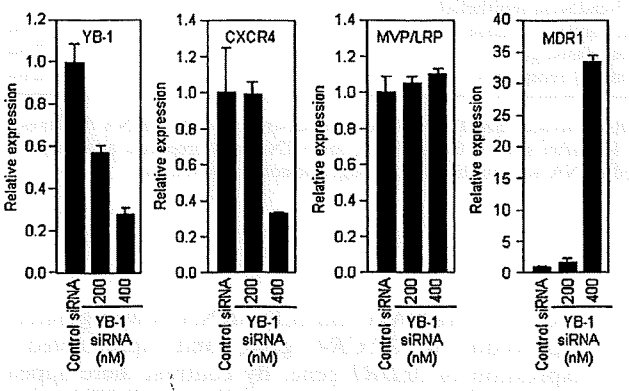

Figure 2 Effect of YB-1 knock down on expression of MDR1, MVP/LRP and CXCR4. SKOV-3 cells were treated with YB-1 siRNA for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\mathrm{GAPDH}) - C_t(\mathrm{target}))}$ which reflected target gene expression normalized to GAPDH levels. Data were mean of three independent experiments; bars ± s.d.

Pearson correlation and hierarchical cluster analysis of selected NCI-60 genes

We next examined a database containing the expression profile of the National Cancer Institute (NCI)-60 panel from the Developmental Therapeutics Program (http://www.dtp. nci.nih.gov/), shown as a log of mRNA expression level in the NCI screen. When the Pearson correlation coefficients were calculated, YB-1 was negatively correlated with *MDR1* expression, positively correlated with *CXCR4* expression and showed little correlation with *MVP/LRP* (Figure 3). Moreover, the hierarchical dendrogram of gene expression revealed that *YB-1* and *CXCR4* belong to the same cluster, whereas *MDR1* and *MVP/LRP* are clustered in a separate group (Figure 4). Together, these NCI-60 panels suggest that cellular levels of YB-1 negatively modulate expression of *MDR1* and positively regulate expression of *CXCR4*. In this cluster analysis, six ovarian cancer cell lines including SKOV-3 showed various correlation coefficiencies with YB-1 expression. Our oligonucleotide array analysis was performed only with SKOV-3, and correlation coefficiencies among ovarian cancer cell lines would depend upon which cell line was analysed.

Akt activity is prerequisite for nuclear translocation of YB-1and transcriptional regulation by YB-1

Phosphorylation of YB-1 by Akt is a necessary requirement for its translocation from the cytoplasm into the nucleus (Sutherland *et al.*, 2005). We therefore investigated the effect of two inhibitors of Akt activation (LY294002 and 1L-6-hydroxymethyl-*chiro*-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate) on serum-stimulated SKOV-3 cells. Both Akt inhibitors markedly blocked the nuclear accumulation of YB-1, whereas treatment with inhibitors of MEK (U0126), p38MAPK (SB203580) and JNK (SP600125) had no effect on nuclear translocation (Figure 5a). In addition, phosphorylation of Akt was inhibited by LY294002 and octadecylcarbonate, but not by U0126, SB203580 and SP600125. Immunofluorescence analysis with a YB-1 antibody also demonstrated the predominant accumulation of YB-1 in the cytoplasm when treated with LY294002 and octadecylcarbonate (Figure 5b and c). As Akt inhibitors blocked the nuclear translocation of YB-1, we examined whether they could also affect expression of YB-1-regulated genes. *CXCR4* expression was found to be downregulated in a dose-dependent manner following treatment with the Akt inhibitors when determined by QRT-PCR analysis (Figure 5d). Treatment with Akt inhibitors upregulated the expression of *MDR1*, but not *MVP/LRP*.

SKOV-3 cells expressed high level of Akt1 protein, very low level of Akt2 protein, and no Akt3 protein when assayed by immunoblotting analysis (Figure 6a). We introduced siRNA targeting Akt or ILK into SKOV-3 cells at a concentration of 100 and 10 nM,

Table 1 List of genes differentially expressed in YB-1 siRNA-transfected SKOV-3 cells

Unigene	*Accession*	*Symbol*	*Description*	*Mean fold change*
Hs.489033	NM_000927	ABCB1	MDR1, ATP-binding cassette, sub-family B (MDR/TAP), member 1	2.46
Hs.369762	AB077208	TYMS	Thymidylate synthetase	1.71
Hs.198363	NM_018518	MCM10	MCM10 minichromosome maintenance deficient 10	1.70
Hs.405958	U77949	CDC6	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	1.66
Hs.442658	AB011446	AURKB	Aurora kinase B	1.65
Hs.516484	NM_005978	S100A2	S100 calcium-binding protein A2	1.48
Hs.23960	NM_031966	CCNB1	Cyclin B1	1.40
Hs.460184	AA604621	MCM4	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	1.40
Hs.438720	AF279900	MCM7	MCM7 minichromosome maintenance deficient 7 (*S. cerevisiae*)	1.36
Hs.433168	NM_002960	S100A3	S100 calcium binding protein A3	1.33
Hs.115474	NM_002915	RFC3	Replication factor C (activator 1) 3, 38 kDa	1.28
Hs.122908	NM_030928	CDT1	DNA replication factor	1.28
Hs.329989	NM_005030	PLK1	Polo-like kinase 1 (*Drosophila*)	1.21
Hs.334562	NM_001786	CDC2	Cell division cycle 2, G1 to S and G2 to M	1.21
Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22 kDa	1.19
Hs.477481	NM_004526	MCM2	MCM2 minichromosome maintenance deficient 2, mitotin	1.16
Hs.284244	M27968	FGF2	Fibroblast growth factor 2 (basic)	1.10
Hs.179565	NM_002388	MCM3	MCM3 minichromosome maintenance deficient 3 (*S. cerevisiae*)	1.08
Hs.194698	NM_004701	CCNB2	Cyclin B2	1.04
Hs.506989	BC001866	RFC5	Replication factor C (activator 1) 5, 36.5 kDa	1.02
Hs.171596	NM_004431	EPHA2	EPH receptor A2	1.01
Hs.194143	NM_007294	BRCA1	Breast cancer 1, early onset	0.75
Hs.156346	NM_001067	TOP2A	Topoisomerase (DNA) II alpha 170 kDa	0.64
Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	0.54
Hs.391464	NM_004996	ABCC1	MRP-1, ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.20
Hs.256301	NM_199249	MGC13170	Multidrug resistance-related protein	0.15
Hs.513488	NM_017458	MVP	Major vault protein	−0.05
Hs.482526	NM_014886	TINP1	TGF beta-inducible nuclear protein 1	−0.23
Hs.525557	NM_000295	SERPINA1	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member	−1.01
Hs.500466	BG403361	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	−1.05
Hs.25292	NM_002229	JUNB	Jun B proto-oncogene	−1.06
Hs.132225	AI934473	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	−1.16
Hs.83169	NM_002421	MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	−1.22
Hs.508999	NM_002742	PRKCM	Protein kinase C, mu	−1.29
Hs.326035	NM_001964	EGR1	Early growth response 1	−1.29
Hs.2256	NM_002423	MMP7	Matrix metalloproteinase 7 (matrilysin, uterine)	−1.32
Hs.197922	NM_018584	CaMKIINalpha	Calcium/calmodulin-dependent protein kinase II	−1.36
Hs.132966	AA005141	MET	Met proto-oncogene (hepatocyte growth factor receptor)	−1.39
Hs.208124	NM_000125	ESR1	Estrogen receptor 1	−1.50
Hs.73793	M27281	VEGF	Vascular endothelial growth factor	−1.53
Hs.381167	AW512196	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	−1.70
Hs.413111	NM_002661	PLCG2	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	−1.75
Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	−1.92
Hs.472793	AI631895	SGK2	Serum/glucocorticoid regulated kinase 2	−2.04
Hs.372914	NM_006096	NDRG1	*N-myc* downstream regulated gene 1	−2.34
Hs.421986	NM_001008540	CXCR4	Chemokine (C-X-C motif) receptor 4	−2.64

High-density oligonucleotide array was performed on 400 nM YB-1 siRNA-treated SKOV-3 cells and mock-treated cells. siRNA duplexes were transfected using LipofectAMINE2000 with Opti-MEM mediums. At 48 h after siRNA transfection, total RNA was prepared, and subjected to double-stranded cDNA synthesis and *in vitro* transcription. The labeled cRNA was applied to the oligonucleotide microarray.

respectively, and silencing effects of siRNA were analysed by immunoblotting (Figure 6a). In Akt siRNA almost completely silenced both Akt1 and Akt2, and siRNA for ILK, the upstream kinase for Akt, silenced ILK on protein level. Treatment with Akt siRNA and ILK siRNA resulted in a marked decrease in both pAkt expression and nuclear accumulation of YB-1 (Figure 6a). As both Akt and ILK siRNA blocked the nuclear translocation of YB-1, we examined their effects on expression of YB-1-regulated genes (Figure 6b). Treatment with Akt and ILK siRNA downregulated the expression of *CXCR4* gene, and upregulated the expression of *MDR1* gene. By contrast there appeared no marked effect on the expression of *MVP/LRP* and *YB-1* genes when treated with both siRNAs (Figure 6b).

Effect of LY294002 treatment on Akt phosphorylation and YB-1 nuclear localization in SKOV-3 xenograft

To further investigate the involvement of Akt in tumoural YB-1 nuclear localization, an *in vivo* xenograft

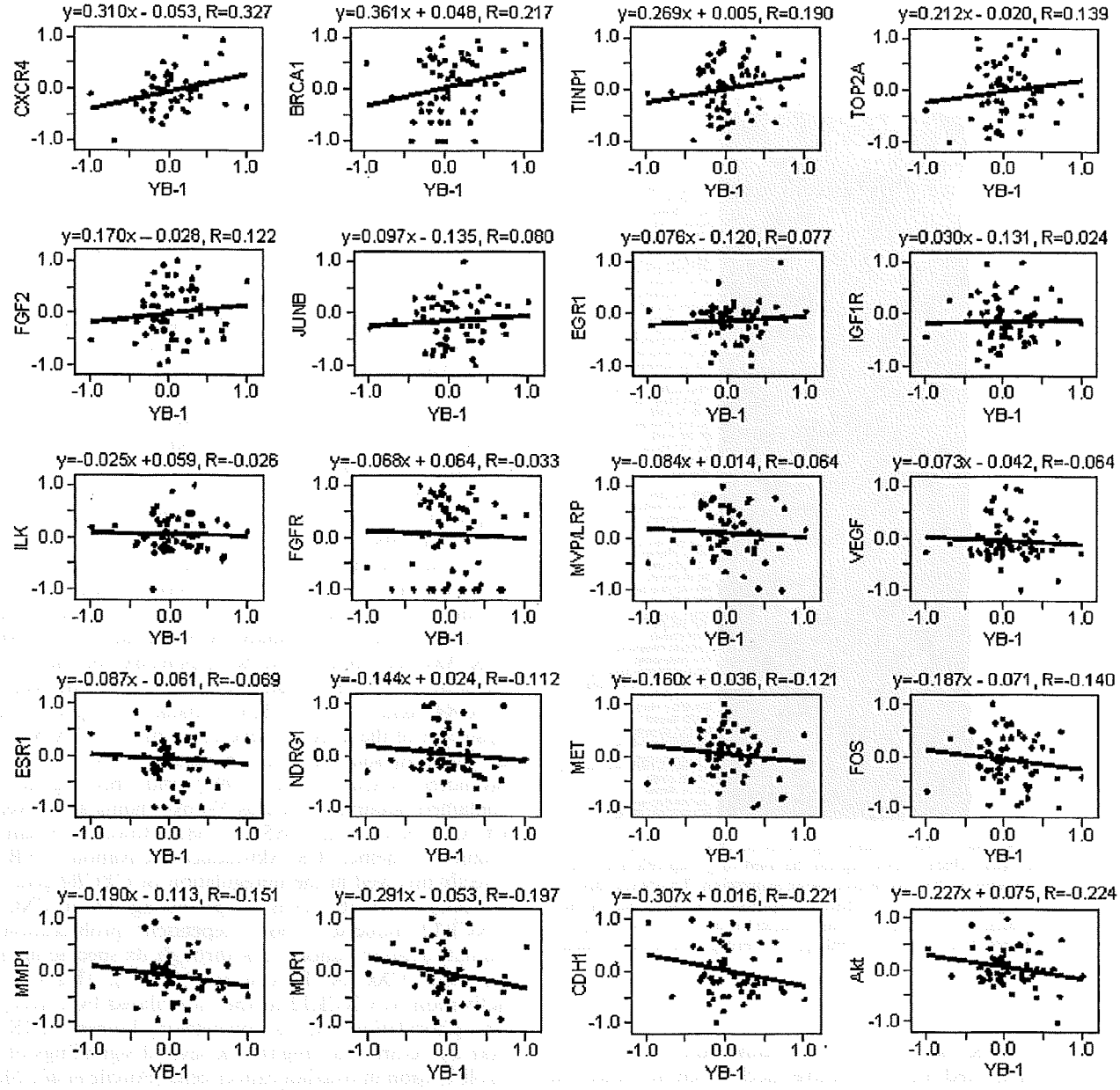

Figure 3 Correlation analysis of gene expression in NCI-60 screen. Gene expression data for the 60 human tumor cell lines were obtained from the Developmental Therapeutics Program (http://www.dtp.nci.nih.gov/), expressed as log of the mRNA levels in cell line/mRNA levels in reference pool in the NCI screen. Pearson correlation coefficients were calculated for each gene–gene pair.

assay was performed. Administration of LY294002 (i.p.) to mice carrying SKOV-3 cell tumors inhibited the phosphorylation of Akt (Figure 7a and b). Akt phosphorylation and YB-1 nuclear localization were also evaluated by immunohistochemical analysis. Tumors in the LY294002-treated group displayed a lower level of pAkt staining (3.3 ± 0.5) than those in the control group, where the mean number of nuclear YB-1-positive cells was 24.7 ± 3.4 (Figure 7c and d). Taken together, these results suggest that nuclear localization of YB-1 in ovarian cancer cells is closely associated with Akt phosphorylation activity *in vitro* and *in vivo*.

Discussion

The nuclear localization of YB-1 is essential process for YB-1-driven transcription of various genes and DNA repair in cancer cells in response to various environmental stimuli. One should understand which signalling pathway specifically controls the translocation of YB-1 from cytoplasm into nucleus. Our previous study has demonstrated that PKC activates the nuclear localization of YB-1 in cancer cells treated with UV irradiation or cisplatin, and also that the C-terminal region of YB-1 was important for its cytoplasmic

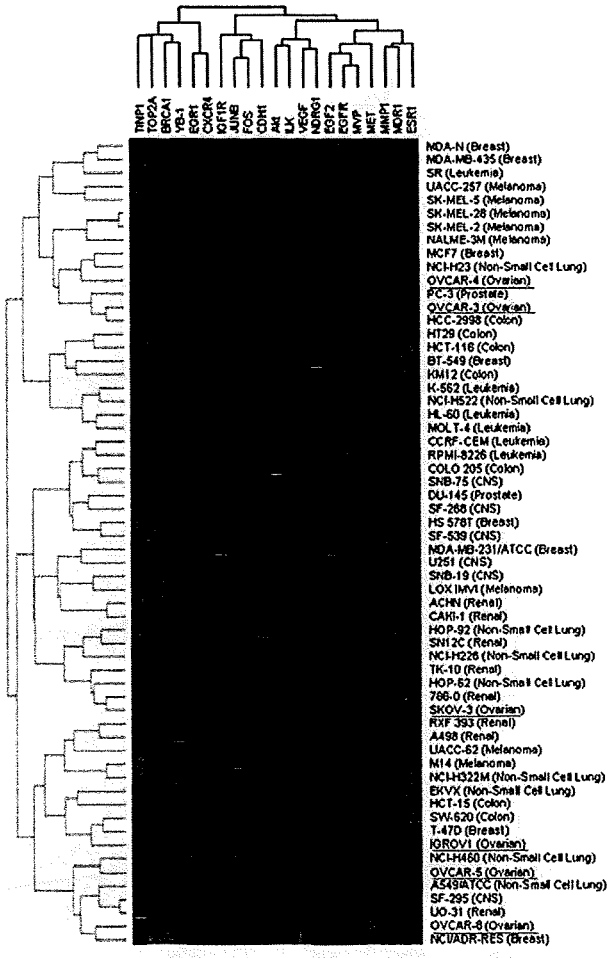

Figure 4 Hierarchical clustering of gene expression in NCI-60 screen. Hierarchical clustering can be used to group cell lines and genes in term of their patterns of gene expression. To obtain cluster trees for genes that showed distinct expression patterns across the 60 cell lines, we used the program 'Cluster' and 'Tree View' (http://rana.lbl.gov/) with average linkage clustering and a correlation metric.

retention (Koike *et al.*, 1997). Sutherland *et al.* (2005) have presented more definitive mechanism at molecular basis that phosphorylation of serine 102 at cold-shock domain of YB-1 by Akt is essential for the nuclear YB-1 localization in breast cancer cells, and also that ILK phosphorylate its downstream Akt, resulting in activation of YB-1 and it nuclear localization. Consistent with this study, our present study also demonstrated that Akt as well as ILK played a critical role in the nuclear YB-1 localization and YB-1-driven-transcriptional control of various genes including *CXCR4* and *MDR1* in human ovarian cancer cells.

In our present study, we examined whether expression of two multidrug resistance relevant genes, *MVP/LRP* and *MDR1/ABCB1*, was affected by knockdown of YB-1. Stein *et al.* (2005) have reported that the *MVP/LRP* gene is transcriptionally activated by YB-1 in response to cytotoxic anticancer agents including doxorubicin and 5-fluorouracil: *MVP/LRP* is an essential vault protein involving acquirement of multidrug resistance. However, in ovarian cancer cells, there was no causative association between the two genes when assayed by microarray and QRT-PCR. YB-1 might not regulate *MVP/LRP* expression in ovarian cancer cells used in our present study. In contrast, in human breast cancer cells, treatment with YB-1 siRNA markedly upregulated *MVP/LRP* expression (Shimoyama T, Nishio K, Basaki Y, Ono M and Kuwano M, unpublished data), suggesting that YB-1-induced regulation of *MVP/LRP* gene expression depends upon cancer cell types and/or types of stimuli. In contrast, knockdown or nuclear translocation inhibition of YB-1 upregulated expression of another drug resistance *MDR1* gene in ovarian cancer cells. Various environmental stimuli often upregulated *MDR1* gene in various human cancer cells through pleiotropic transcriptional regulations (Kuwano *et al.*, 2004). Our present study further presented a novel regulation of YB-1-induced negative control of *MDR1* gene in ovarian cancer cells, and further study should be required to understand its underlying mechanism at molecular basis.

In our present study, we first observed that the knockdown of YB-1, ILK and Akt as well as an Akt inhibitor all downregulated expression of *CXCR4* gene. Consistent with recent study by Sutherland *et al.* (2005), ILK-Akt activation could be responsible for the nuclear localization of YB-1, resulting in enhanced expression of *CXCR4* gene. The 2.6 Kb 5′-flanking region located upstream of the *CXCR4* gene contains a TATA box and the transcription start site characteristic of a functional promoter (Caruz *et al.*, 1998) and this region also contained putative consensus Y-box-binding site (inverted CCAAT box) form −685 to −681. However, it remains unknown whether ILK-Akt-induced activation of YB-1 is directly involved in the upregulation of *CXCR4* gene.

CXCL12 (SDF-1α) is a specific ligand of CXCR4. CXCL12 induced a dose dependent proliferation of human ovarian cancer cells through its specific interaction with CXCR4 (Porcile *et al.*, 2005). This CXCR4 activation by CXCL12 further stimulated EGF receptor phosphorylation and its downstream kinases, ERK1/2, Akt and c-Src that might link several signallings of cell proliferation in ovarian cancer cells (Porcile *et al.*, 2005). On the other hand, VEGF, a potent angiogenic factor, induced upregulation of *CXCR4* gene expression in vascular endothelial cells, and expression of both VEGF and CXCL12 was very high in ascites of patients with advanced ovarian cancers (Kryczek *et al.*, 2005). The cross-talk of CXCL12/CXCR4 with EGF/EGF receptor and/or VEGF/VEGF receptor might thus provide important signallings for both cell proliferation and angiogenesis in ovarian cancers.

CXCL12/CXCR4 pathway is also expected to be clinically involved in acquirement of malignant characteristics of human ovarian cancers. Of 14 chemokine receptors, only CXCR4 protein was found to be expressed in ovarian cancer cell lines and in ascites from patients with ovarian cancers (Scotton *et al.*, 2001). The CXCL12/CXCR4 pathway has been implicated in

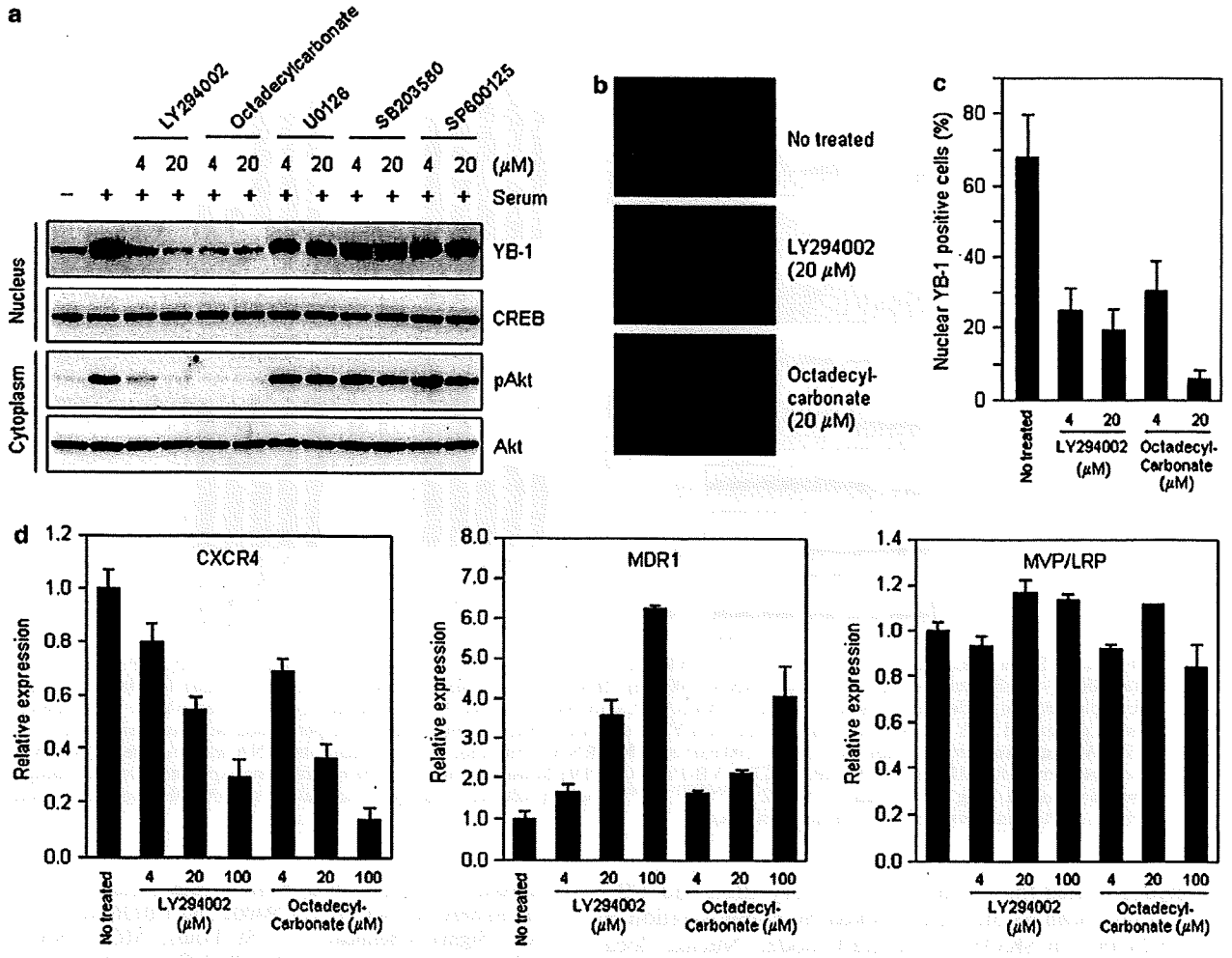

Figure 5 Akt activity is required for YB-1 nuclear accumulation and transcriptional regulation by YB-1. (**a**) The effect of kinase inhibitors on the nuclear accumulation of YB-1 in SKOV-3 cells. Inhibitors were added 3 h before serum stimulation and nuclear extracts were prepared 1 h after serum stimulation. Anti-YB-1 and anti-CREB immunoblots were performed with nuclear extracts, and anti-pAkt and anti-Akt immunoblots were performed on cytoplasmic extracts. CREB and Akt are shown as a loading control. (**b**) Immunofluorescent staining for YB-1. SKOV-3 cells were treated with LY294002 or octadecylcarbonate for 24 h and then stained with YB-1. Cells were fixed and permeabilized, incubated at 4°C with the primary YB-1 antibody, then with the Alexa Flour 546-labelled secondary antibody. (**c**) Quantitative analysis of YB-1 nuclear localization in SKOV-3 cells as shown in Figure 2b. Data are mean of three independent experiments; bars ±s.d. (**d**) QRT-PCR for MDR1, MVP/LRP, CXCR4 and housekeeping gene GAPDH. The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\text{GAPDH}) - C_t(\text{target}))}$ which reflected target gene expression normalized to GAPDH levels. Data were mean of three independent experiments; bars ±s.d.

the development of tumor growth, angiogenesis and metastasis not only in ovarian cancer (Scotton *et al.*, 2002) but also in other tumor types including breast cancer (Muller *et al.*, 2001), melanoma (Robledo *et al.*, 2001; Murakami *et al.*, 2002) and prostate cancer (Darash-Yahana *et al.*, 2004). Jiang *et al.* (2006) further demonstrated that CXCR4 expression could be an important prognostic marker for ovarian cancers: the rate of CXCR4 expression in refractory and recurrent group was significantly higher than that in non-recurrent group. Our previous studies showed a significant association of nuclear localization of YB-1 with unfavorable prognosis of patients with ovarian cancers (Kamura *et al.*, 1999; Huang *et al.*, 2004). Clinicopathological analysis whether nuclear expression of YB-1 can be associated with CXCR4 expression or CXCL12 (SDF-1α) in patients with ovarian cancers is now in progress.

Several studies have focused on the role of Akt/PI3K inhibitors as potential tumor suppressor agents. It has been reported that phosphorylation of Akt and mTOR, an Akt substrate, was frequently detected in ovarian cancer (Altomare *et al.*, 2004). In animal model of ovarian cancer, LY294002, a potent inhibitor of Akt activation, could inhibit cancer growth and ascites formation (Hu *et al.*, 2000). Our study also

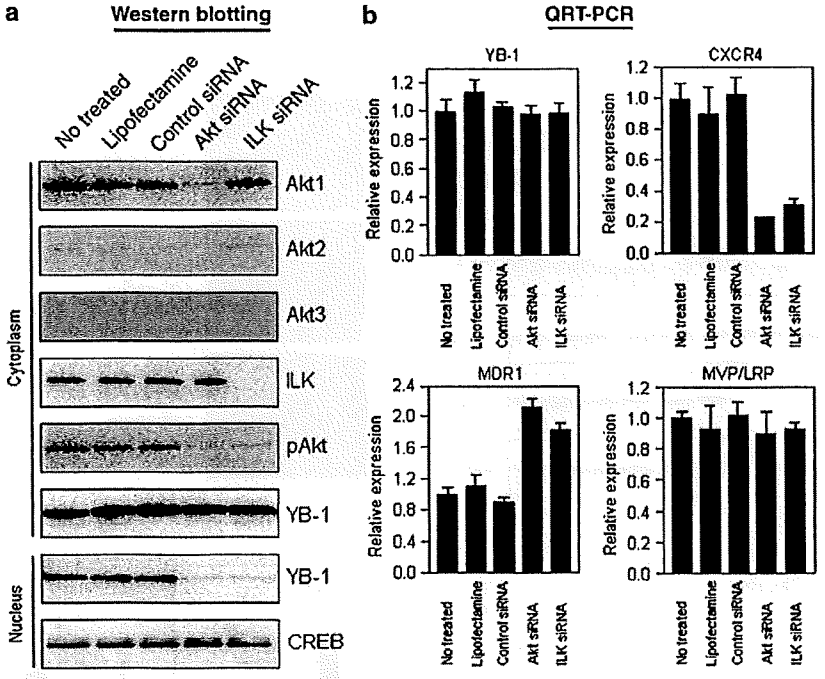

Figure 6 Effect of knock down of Akt and ILK on YB-1 nuclear accumulation, and expression of MDR1, MVP/LRP and CXCR4. (**a**) SKOV-3 cells were treated with Akt siRNA (100 nM), ILK siRNA (10 nM) or control siRNA (100 nM) for 48 h, and then cytoplasmic and nuclear extracts were prepared. Anti-Akt1, anti-Akt2, anti-Akt3, anti-ILK, anti-pAkt, and anti-YB-1 immunoblots were performed with cytoplasmic extracts, and anti-YB-1 and anti-CREB immunoblots were performed with nuclear extracts. (**b**) SKOV-3 cells were treated with Akt siRNA (100 nM) or ILK siRNA (10 nM) for 48 h and then total RNA was prepared. QRT-PCR was performed for MDR1, MVP/LRP, CXCR4, YB-1 and GAPDH housekeeping gene. The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\text{GAPDH}) - C_t(\text{target}))}$ which reflected target genes normalized to GAPDH levels. Data were mean of three independent experiments; bars ±s.d.

demonstrated that both Akt phosphorylation and YB-1 nuclear localization were blocked by administration of LY294002 in SKOV-3 xenograft model. Nuclear localization of YB-1 is induced through various pathways including Akt (see Introduction). The Akt-dependent pathway for YB-1 nuclear localization would provide further insight how Akt-targeting anticancer therapeutic strategy could be developed.

In conclusion, we have identified several genes that are regulated by YB-1 and/or its nuclear localization. Further immunohistochemical analysis should be required to elucidate the role of YB-1 in the expression of *CXCR4* and other relevant genes that are associated with the clinicopathological characteristics in human ovarian cancers. Based on our present experimental results, we aim to present YB-1 and YB-1-dependent gene networks as molecular targets for the further development of novel anticancer therapeutic strategies.

Materials and methods

Cell culture and reagents

OVCAR-3 and SKOV-3 were purchased from American Type Culture Collection (Manassas, VA, USA). RMG-I, RMG-II, RMG-III, RMBG and RTSG were kindly provided by Dr S Nozawa, Department of Obstetrics and Gynecology, Keio University. These cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2. LY294002 and U0126 were purchased from Sigma Chemical Co. (St Louis, MO, USA). 1L-6-hydroxymethyl-*chiro*-inositol 2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (Hu *et al*., 2000), SB203580 (Cuenda *et al*., 1995), and SP600125 (Bennett *et al*., 2001) were obtained from Calbiochem (San Diego, CA, USA). Anti-YB-1 was generated as described previously (Ohga *et al*., 1996). Anti-CREB, anti-PKB/Akt, anti-phospho-PKB/Akt, anti-ILK, Akt siRNA and ILK siRNA were obtained from Cell Signaling Technology (Beverly, MA, USA).

Western blotting

Western blotting was performed as previously described (Kaneko *et al*., 2004). Cells were lysed in buffer A (10 mM HEPES (pH7.9), 10 mM KCl, 10 mM EDTA, 1 mM DTT, 0.4% v/v IGEPAL, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml aprotinin and leupeptin) for 10 min on ice, and then centrifuged for 3 min at 15 000 r.p.m. The supernatant fractions (cytoplasmic soluble proteins) were collected. The nuclear pellet was then washed and then lysed in buffer C (20 mM HEPES (pH7.9), 200 mM NaCl, 1 mM EDTA, 5% v/v glycerol, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF and 10 μg/ml aprotinin and leupeptin). Lysates were incubated on ice for 2 h, and then centrifuged 15 000 r.p.m. for 5 min. The lysates were separated by sodium dodecyl sulfate–polyacryl amide gel electrophoresis (SDS–PAGE), and then were transferred to a nitrocellulose membrane. The membrane were incubated with the primary antibody and visualized with secondary antibody coupled to horseradish peroxidase (Cell Signaling Technology)

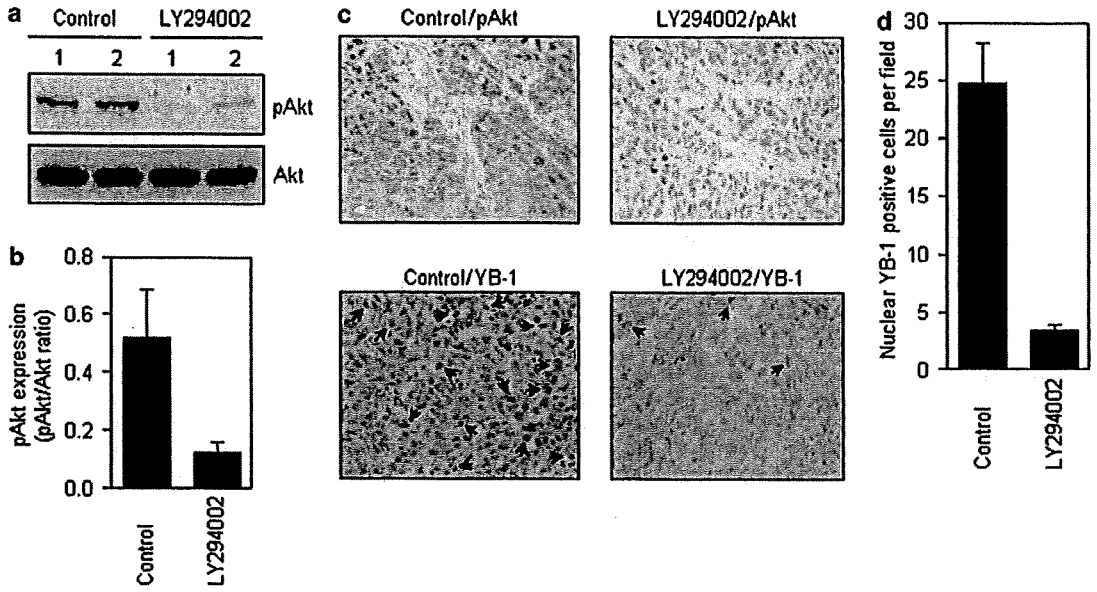

Figure 7 Effect of LY294002 on Akt phosphorylation and YB-1 nuclear localization in SKOV-3 xenograft. (**a**) Effect of LY294002 on Akt phosphorylation in SKOV-3 xenograft. SKOV-3 cells were injected subcutaneously (5.0×10^6 cells/0.1 ml/mouse). When tumors reached approximately 1000–2000 mm^3, animals were randomly assigned to two groups of five. The first group received i.p. injections of DMSO as a control. The second group received i.p. injections of 50 mg/kg LY294002. One hour after LY294002 injection, mice were killed humanely (while anesthetized) by cervical dislocation and tumors were excised. Western blot analysis was carried out using cytosolic extracts prepared from tumor tissues from two animals treated with or without drug. (**b**) Quantitative analysis of Akt phosphorylation in SKOV-3 tumor xenograft. Levels of Akt phosphorylation were normalized to their nonphosphorylated form as shown in Figure 7a. Data are expressed as mean ± s.d. of three to five mice. (**c**) Immunohistochemical staining was carried out using conventional protocols. The arrows indicate positive cell nuclei staining for YB-1 (×200 magnification). (**d**) Quantitative analysis of YB-1 nuclear localization in SKOV-3 tumor xenograft. YB-1 nuclear localization was determined by counting the number of positive YB-1 nuclear cells in high-power fields as shown in Figure 7b. Data were mean of each section (five sections per mouse). Columns, mean; bars ±s.d.

and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Bands on Western blots were analysed densitometrically using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD, USA).

Oligonucleotide microarray analysis

The siRNA corresponding to nucleotide sequences of the YB-1 (5′-GGU UCC CAC CUU ACU ACA U-3′) was purchased from QIAGEN Inc. (Valencia, CA, USA). A negative control siRNA was obtained from Invitrogen (Carlsbad, CA, USA). siRNA duplexes were transfected using LipofectAMINE2000 and Opti-MEM medium (Invitrogen) according to the manufacturer's recommendations. Duplicate samples were prepared for microarray hybridization. At 48 h after siRNA transfection, total RNA was extracted from cell cultures using ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan). Total RNA (2 μg) was reverse-transcribed using GeneChip 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix Inc., Santa Clara, CA, USA) and then labeled with Cy5 or Cy3. The labeled cRNA was applied to the oligonucleotide microarray (Human Genome U133 Plus 2.0 Array, Affymetrix). The microarray was scanned on a GeneChip Scanner3000 and the image was analysed using a GeneChip Operating Software ver1.

Correlation analysis of gene expression, and clustering of cell lines and genes expression

Gene expression data for the 60 human tumor cell lines were obtained from the Developmental Therapeutics Program (http://www.dtp. nci.nih.gov/), expressed as log of the mRNA levels in cell line/mRNA levels in reference pool in the NCI screen. Pearson correlation coefficients were calculated for each gene–gene pair. Hierarchical clustering can be used to group cell lines and genes in term of their patterns of gene expression. To obtain cluster trees for genes that showed distinct expression patterns across the 60 cell lines, we used the program 'Cluster' and 'Tree View' (http://rana.lbl.gov/) with average linkage clustering and a correlation metric (Eisen *et al.*, 1998).

Quantitative real-time polymerase chain reaction

RNA was reverse transcribed from random hexamers using AMV reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR was performed using the Real-Time PCR system 7300 (Applied Biosystems, Foster City, CA, USA) as described previously (Maruyama *et al.*, 2006). In brief, the PCR amplification reaction mixtures (20 μl) contained cDNA, primer pairs, the dual-labeled fluorogenic probe, and TaqMan Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and at 95°C for 10 min, and then alternating for 40 cycles between 95°C for 15 s and 60°C for 1 min. The primer pairs and the probe were obtained from Applied Biosystems. The relative gene expression for each sample was determined using the formula $2^{(-\Delta C_t)} = 2^{(C_t(\text{GAPDH}) - C_t(\text{target}))}$ which reflected target gene expression normalized to GAPDH levels.

Immunofluorescence

Cells were plated on glass coverslips in six-well plates and allowed to attach overnight. Then, cells were rinsed with PBS

and then fixed in 4% paraformaldehyde/PBS for 30 min. Cells were rinsed twice with PBS and then permeabilized with 0.5 ml of solution containing 5% BSA, 0.2% Triton X-100 in PBS for 90 min. After 1 h of blocking with 2% goat serum, the cells were incubated overnight with primary antibody at 4°C in 1% BSA in PBS. Cells were then rinsed three times with PBS and incubated with 1 μg/ml of Alexa Flour 546-labeled secondary antibody (Molecular Probe, Eugene, OR, USA) in 1% BSA in PBS for 60 min. Coverslips were mounted on slide glasses using gel mount and viewed using an Olympus BX51 florescence microscope (Tokyo, Japan) and photographed with Olympus DP-70 digital camera.

Tumor xenograft study

Male BALB/c nude mice were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). SKOV-3 cells were harvested and resuspended in PBS. The suspension was injected subcutaneously in the mice (5.0×10^6 cells/0.1 ml/mouse). When tumors reached about 1000–2000 mm^3, animals were randomly assigned to two groups of five mice each. The first group received i.p. injections of DMSO as control. The second group received i.p. injection of LY294002 at 50 mg/kg. At 1 h after LY294002 injection, mice were killed humanly (mice still anesthetized) by cervical dislocation and tumors were excised. For immunohistchemistry, one part of the tumor tissue was fixed in formalin and embed in paraffin.

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