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.

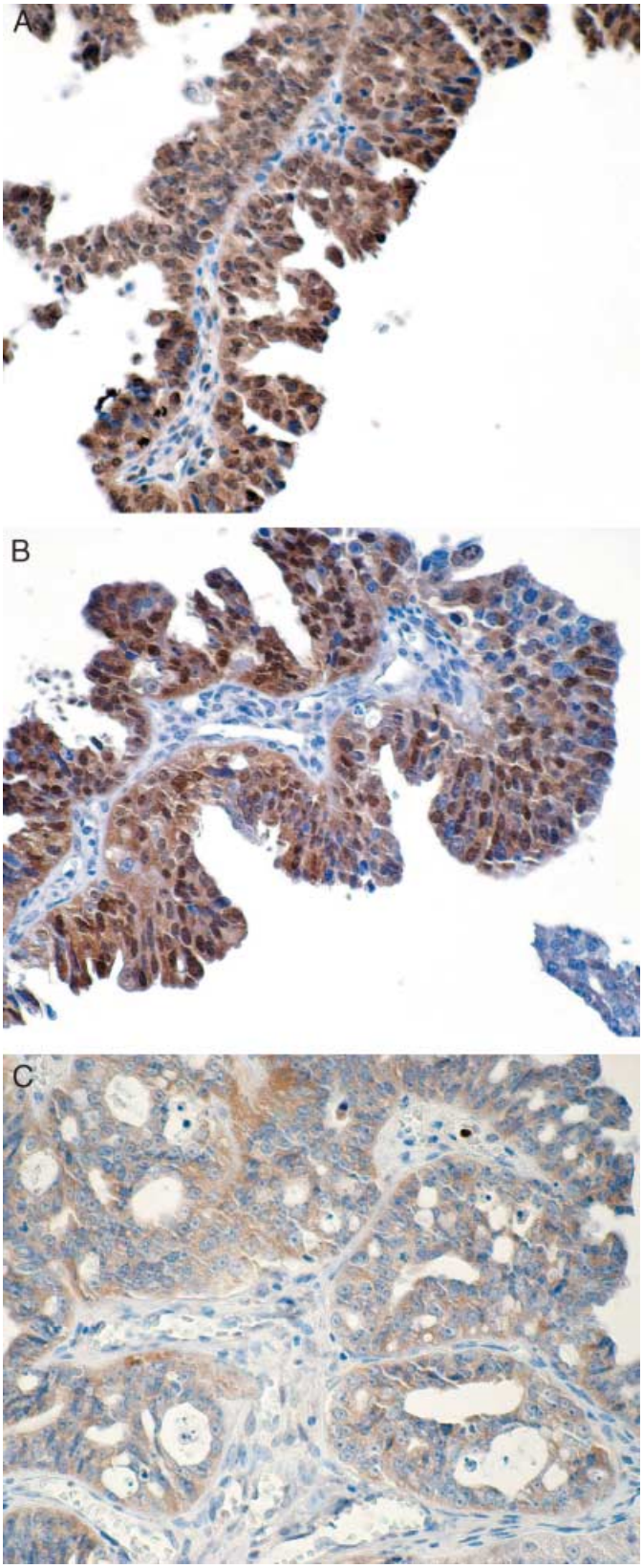

Fig. 1. Grade 2 and stage IIc serous cystadenocarcinoma of a 51-year-old woman. (A) Both nuclear and cytoplasmic expression of Y-box-binding protein-1 were observed in the tumor cells. (B) Strong and diffuse phosphorylated Akt expression was also evident in both the cytoplasm and nuclei. (C) Lung resistance-related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

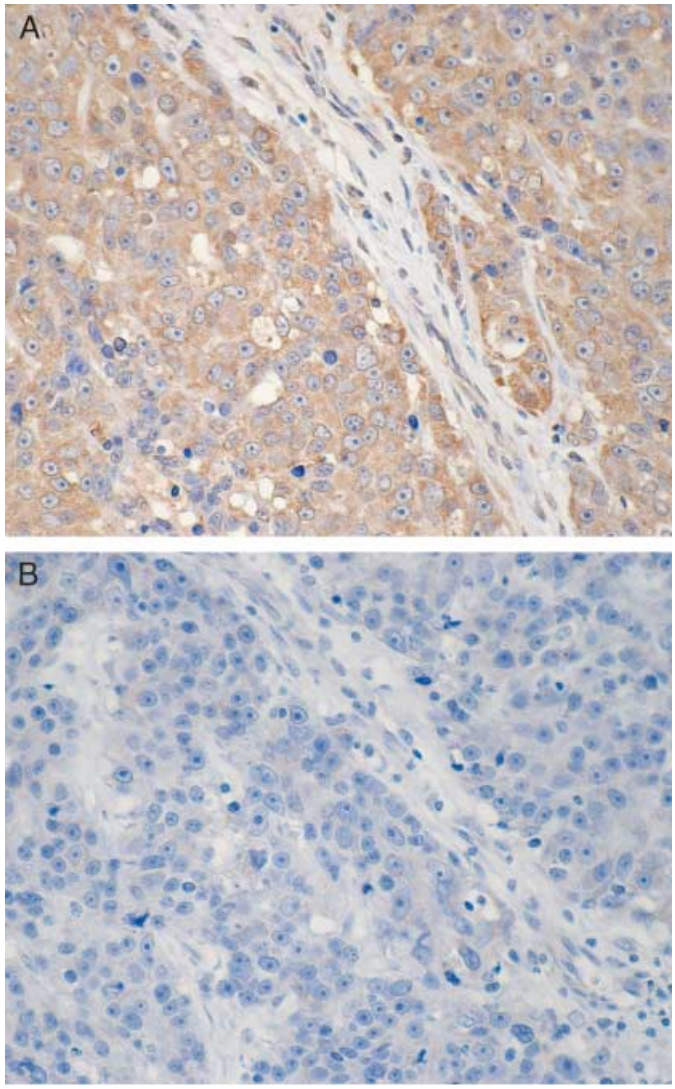

Fig. 2. Grade 3 and stage IIIc serous adenocarcinoma of a 66-year-old woman. Y-box-binding protein-1 expression was observed only in the cytoplasm (A), whereas immunoreactivity for phosphorylated Akt was recognized very faintly in a few tumor cells and was interpreted as negative (B). The patient currently 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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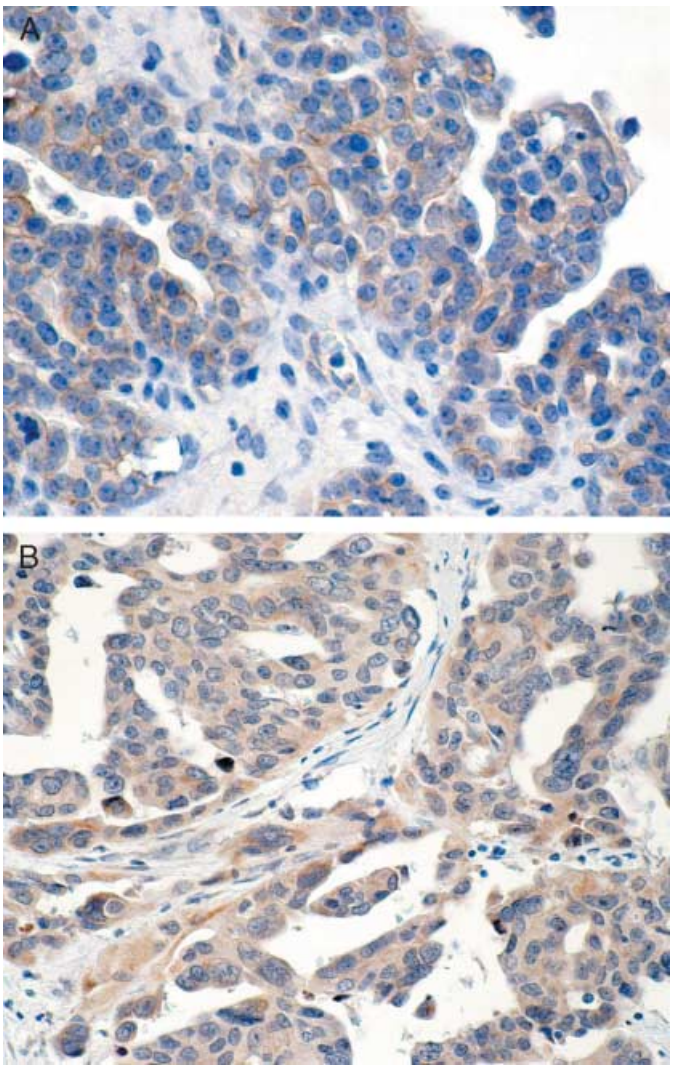

Fig. 3. Grade 3 and stage IIIc serous adenocarcinoma of a 76-year-old woman. P-glycoprotein was expressed in this case as a diffuse membranous staining pattern (A). CXCR4 expression was diffusely visible in the cytoplasm as well as in a few nuclei (B). Y-box-binding protein-1 nuclear expression was also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

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.

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, 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 l *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

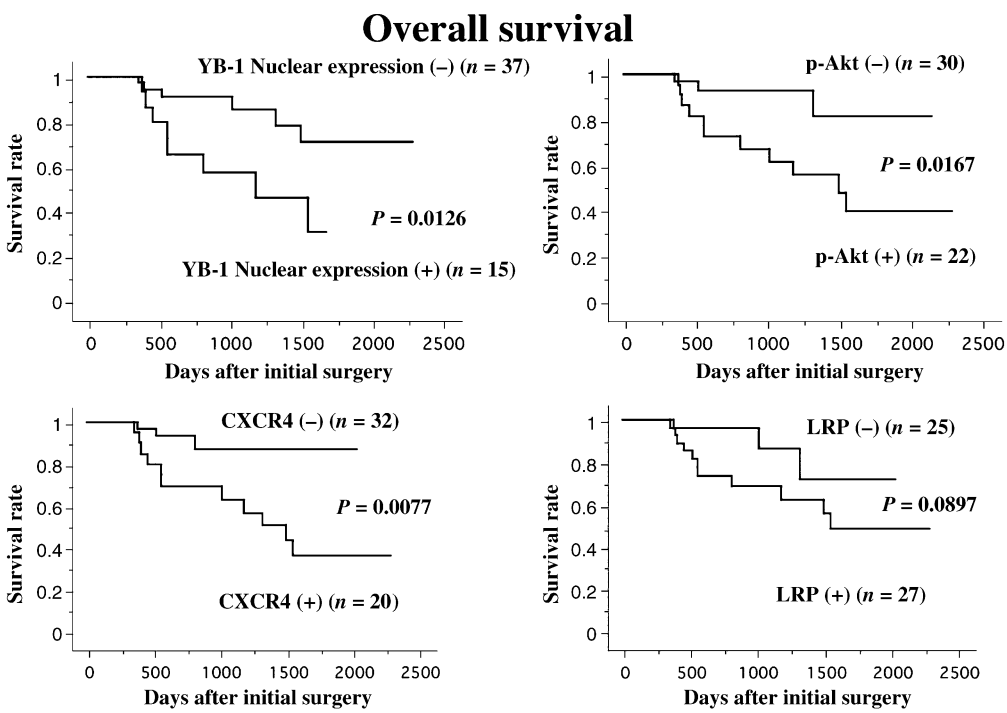

Fig. 4. Overall survival according to immunohistochemical expression in 52 patients with ovarian carcinoma. Y-box-binding protein-1 nuclear expression, and phosphorylated Akt and CXCR4 expression have a significant predictive value for survival.

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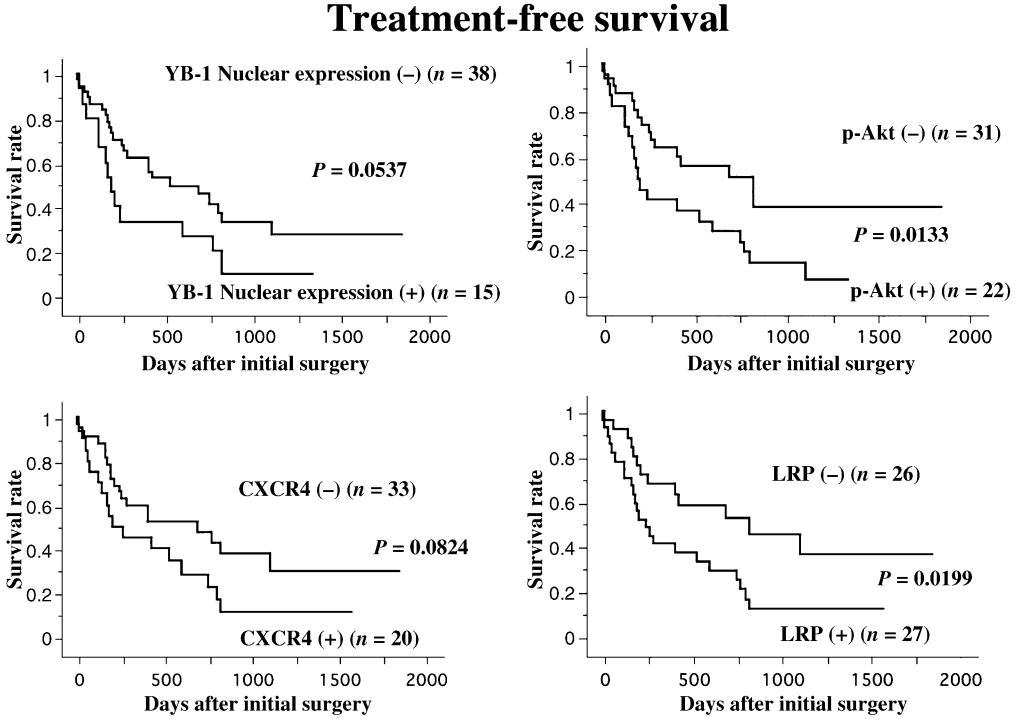

Fig. 5. Treatment-free survival. Cases with Y-box-binding protein-1 nuclear expression tended to show poor prognosis. Cases with phosphorylated Akt and lung resistance-related vault protein expression showed adverse prognosis, whereas CXCR4 expression had no impact on prognosis.

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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