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Expression of Cell-Cycle Markers in Bladder Cancer : Superiority of Cyclin A and Ki-67 as Indicators of Poor Prognosis

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ABSTRACT

Aims : The aim of this study was to investigate the proliferative activity of cancer cells on the basis of cell-cycle proteins and to find biomarkers indicating the malignant potential and prognosis of bladder cancer.

Methods and results : Immunohistochemical examinations for Ki-67, cyclin A, cyclin B1, and cyclin D1 were carried out in 78 patients with primary bladder cancer. The expression of cyclin A, cyclin B1, and Ki-67 was significantly higher in high-grade, high-stage tumors. Conversely, expression of cyclin D1 was negatively correlated with tumor grade and stage. Analysis of overall survival revealed that overexpression of cyclin A, cyclin B1, or Ki-67 was associated with significantly shorter overall survival and that overexpression of cyclin D1 was associated with significantly longer overall survival. Univariate Cox regression analysis revealed that cyclin A overexpression and Ki-67 overexpression were significant prognostic variables for survival.

Conclusion : Our results suggest that both Ki-67 and cyclin A can provide objective information for determining malignant potential and for selecting treatment in cases of primary bladder cancer.

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Key words : cyclin A, cyclin B1, cyclin D1, Ki-67, transitional cell carcinoma

INTRODUCTION

Bladder cancer is the most common malignancy of the genitourinary tract, and its optimal management requires that a tumor's biological potential be accurately assessed. To select an appropriate treatment, superficial tumors likely to recur or progress should be identified and the various bladder cancers and their true biological potential should be better characterized. At present, histologic tumor grade and stage are the main prognostic variables that dictate treatment. Although tumor grade and stage do provide some information on tumor biological potential, tumor aggressiveness is difficult to predict accurately and reliably.

An important step in the growth of cancer cells is the alteration of cell-cycle control. The cell cycle is a series of enzymatic reactions involving cell-cycle proteins, the most important of which are cyclins, which vary in abundance and associated with different stages of the cell cycle. Therefore, cyclins might be used as markers for tissue proliferation. In this

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study, we performed immunostaining for Ki-67, cyclin A, cyclin B1, and cyclin D1 to assess the proliferative activity and malignant potential of bladder cancers.

Immunostaining for Ki-67, a nuclear protein expressed by proliferating cells, provides objective information for determining malignant potential and for selecting treatment in bladder cancer. Although previous studies have found that Ki-67 immunoreactivity is significantly correlated with tumor grade, stage, and morphology of bladder cancer[1–3], they but did not evaluate the usefulness of Ki-67 immunoreactivity as a prognostic factor.

Overexpression of cyclin D1 has been reported in low-grade, low-stage bladder cancers. Lee et al. (1997) reported no cyclin D1 overexpression in bladders cancers of grade 3 or T2 and higher[4].

To the best of our knowledge, no previous immunohistochemical studies have analyzed cyclin B1 expression in bladder cancer. In esophageal cancer, overexpression of cyclin B1 has been observed in high-stage tumors.

During cell cycle progression, cyclin A is involved in the onset of DNA replication and is required for DNA replication. Overexpression of cyclin A has been reported to be an independent poor prognostic factor in non-small-cell lung carcinoma, hepatocellular carcinoma, and colorectal carcinoma.

In the present study, we assessed the relationship of the expression of these cell-cycle proteins to clinicopathologic factors and prognosis in bladder cancer.

We conclude that overexpression of cyclin A, cyclin B1, or Ki-67 correlates with high-grade, high-stage tumors and that cyclin D1 overexpression correlates with low-grade, low-stage tumors. Univariate analysis revealed that overexpression of cyclin A or Ki-67 was a superior indicator of poor prognosis.

Materials and Methods

Tissue samples

We examined 78 patients (65 men and 13 women ;

Table 1. Relationship between cyclin A, B1, D1, and Ki-67 expression and clinicopathologic valiables in 78 bladder cancers.

Feature	Number of cases	Cyclin A		Cyclin B1		Cyclin D1		Ki-67	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Grade									
Grade 1, 2	47	4	43	2	45	44	3	14	33
Grade 3	31	29	3	19	12	12	19	27	4
P-value		<0.0001		<0.0001		<0.0001		<0.0001	
pTstage									
pTa, 1	58	13	45	6	52	48	10	23	35
pT2, 3	20	20	0	15	5	8	12	18	2
P-value		<0.0001		<0.0002		<0.0001		<0.0001	
Tumor Size									
<2.0 (cm)	48	19	29	37	11	37	11	21	27
≧2.0	30	14	19	10	20	19	11	20	10
P-value		0.5379		0.3130		0.1892		0.0486	
Age									
<70 (years)	45	17	31	38	10	36	12	22	26
≧70	33	16	14	11	19	20	10	19	11
P-value		0.1192		0.1251		0.4262		0.1321	
Sex									
male	65	25	40	17	48	50	15	34	31
female	13	8	5	4	9	6	7	7	6
P-value		0.1241		0.7320		0.0244		0.9192	

*$p<0.01$ by χ^2-test

mean age, 64.8 years ; age range, 28–84 years ; Table 1) with primary bladder cancer diagnosed and treated with transurethral resection at the Jikei University Hospital or the Fuji City General Hospital from 1991 through 1997. The 78 tumors were transitional cell carcinomas (21 grade 1, 26 grade 2, and 31 grade 3) staged according to the TNM system (Union Internationale Contre le Cancer, 1977). The depth of tumor invasion was classified as noninvasive papillary carcinoma (pTa, n=36), invasion of subepithelial tissue (pT1, n=22), and invasion of muscle or perivesical tissue (pT2,3, n=20). In addition, samples of normal bladder tissue were taken from patients with nonneoplastic diseases (n=3). The survival rate of the patients from the date of resection of the bladder cancers to the date of death was calculated on April 2001.

Immunohistochemistry

Immunohistochemical studies were performed with the avidin-biotin complex immunoperoxidase technique. Paraffin-embedded tissues were cut into 4-μm-thick sections and mounted on slides. After deparaffinization in xylene, the slides were immersed in 10 mmol/l citrate buffer (pH 6.0), and heated in a microwave oven for 23 minutes at 500 W to retrieve the antigenicity. The slides were then placed in methanol containing 3% hydrogen peroxidase for 30 minute at 4°C to block endogenous peroxidase activity. After the sections were incubated with goat serum for 10 minutes to block nonspecific antibody-binding sites, the primary antibody was applied and the sections were incubated overnight at 4°C in a chamber. The primary antibodies used for the immunochemical examinations were monoclonal antibodies (MAb) against Ki-67 (MIB-1 ; Immunotech, Marseilles, France), cyclin A (6E6 ; Novocastra, Newcastle-Upon-Tyne, UK), cyclin B1 (7A9 ; Novocastra), and cyclin D1 (PD211F11 ; Novocastra). The conventional streptavidin-biotinylated horseradish peroxidase complex method (LSAB kit, DAKO Japan, Kyoto) was used according to the manufacturer's instructions. Colorization was performed with the peroxidase-diaminobenzidine method. A vermiform appendix was used as a positive control for Ki-67, cyclin A, and cyclin B1, and mantle cell lymphoma were used as a positive control for cyclin D1. Negative controls were performed by replacing the primary mouse MAbs with mouse myeloma proteins of the same subclasses (IgG1 and IgG2a ; DAKO, Copenhagen, Denmark) at the same concentration.

Scoring of immunoreactivity

The numbers of positively immunostained tumor cells were independently evaluated by 2 observers, who counted a total of 1000 tumor cells in a high-power field ($\times$400) in each case. The percentages of positively stained cells among counted tumor cells were then calculated (LI : Labeling Index). Expression of cell-cycle proteins was graded as positive at the following percentages of positively stained cells : cyclin A, $\geq$20%[5] ; cyclin B1, $\geq$15%[6] ; cyclin D1, $\geq$20%[5] ; and Ki-67, $\geq$25%[6].

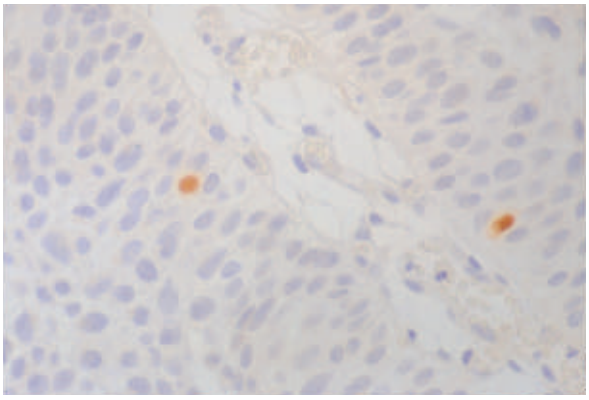
Statistical analysis

Statistical analysis was performed with the StatView 5.0 software package (SAS Institute, Inc., Gary, NC). The χ^2 test was used to compare groups. Correlation between the indexes was determined with simple linear regression analysis. Survival curves were calculated with the Kaplan-Meier method and analyzed with the log-rank test. The effect of each variable on survival was assessed with the Cox proportional hazards model.

Results

Cyclin A staining and clinicopathologic factors

Of the 78 tumors, 33 (42.3%) expressed cyclin A. Staining for cyclin A was confined to the nuclei of neoplastic cells. The distribution of cyclin A-positive cells was homogeneous, and staining was more diffuse in high-grade, high-stage cancers (Fig. 1a, b). Cyclin A was more frequently expressed by high-grade, high-stage cancers (grade 3 and pT2, 3) than by low-grade, low-stage cancers (grade 1, 2 and pTa, 1 ; $p<0.01$; Table 1). However, cyclin A expression



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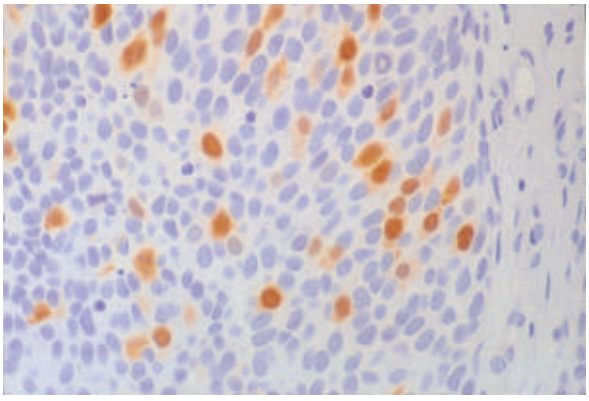
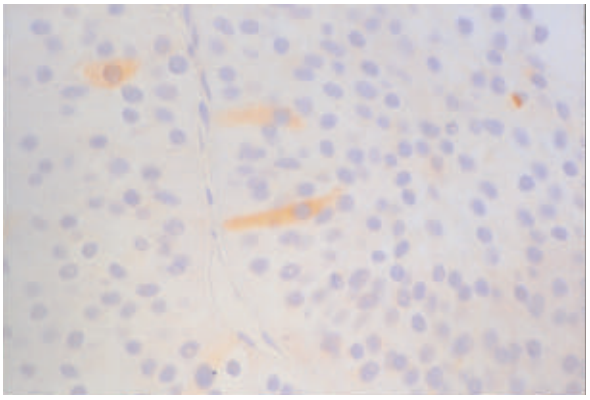


(b)

Fig. 1. Immunostaining for cyclin A (a), low-grade, low-stage tumor (TCC G1 pT1) (b), high-grade, high-stage tumor (TCC G3 pT2)



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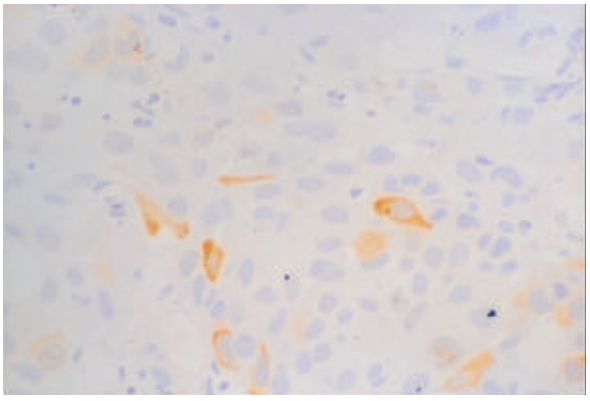


(b)

Fig. 2. Immunostaining for cyclin B1 (a), low-grade, low-stage tumor (TCC G1 pT1) (b), high-grade, high-stage tumor (TCC G3 pT2)

was not correlated with patient sex or age or with tumor size.

Cyclin B1 staining and clinicopathological factors

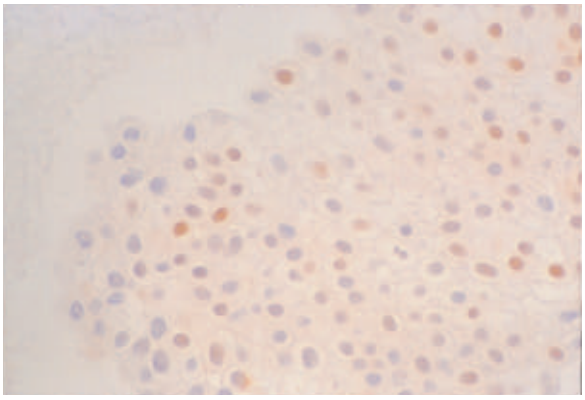
Of the 78 tumors, 21 (26.9%) expressed cyclin B1. Cyclin B1 staining was confined to the nucleus and cytoplasm of neoplastic cells. In high-stage and high-grade tumors, the distribution of cyclin B1-positive cells was diffuse and homogeneous. However, the distribution was heterogeneous in low-grade, low-stage tumors (Fig. 2a, b). Cyclin B1 was more frequently expressed by high-grade, high-stage cancers (grade 3 and pT2, 3) than by low-grade, low-stage cancers (grade 1, 2 and pTa, 1 ; $p<0.01$). However expresssion of cyclin B1 was not correlated with patient sex or age or with tumor size.

Cyclin D1 staining and clinicopathological factors

Of the 78 tumors, 56 (71.8%) expressed cyclin D1. Cyclin D1 staining was confined to the nucleus. Staining intensity varied, and the distribution of cyclin D1-positive cancer cells was markedly heterogenous, even within an individual tumor. Staining in low-grade, low-stage tumors was more homogenous than in high-grade, high-stage tumors (Fig. 3a, b). Cyclin D1 was more frequently expressed by low-grade, low-stage cancers (grade 3 and pT2, 3) than by high-grade, high-stage cancers (grade 1, 2 and pTa, 1 ; $p<0.01$). However, cyclin D1 expression was not correlated with patient sex or age or with tumor size.

Ki-67 staining and clinicopathological factors

Of the 78 tumors, (52.6%) expressed Ki-67. Ki-



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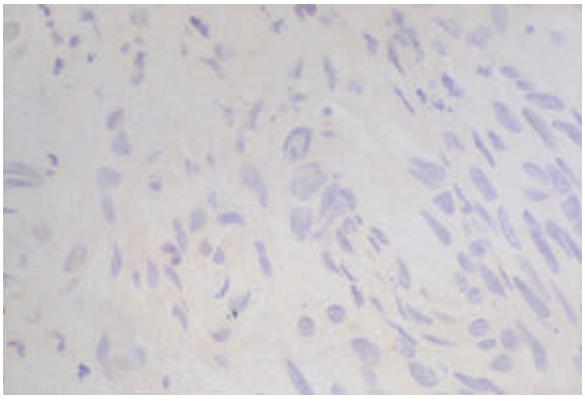
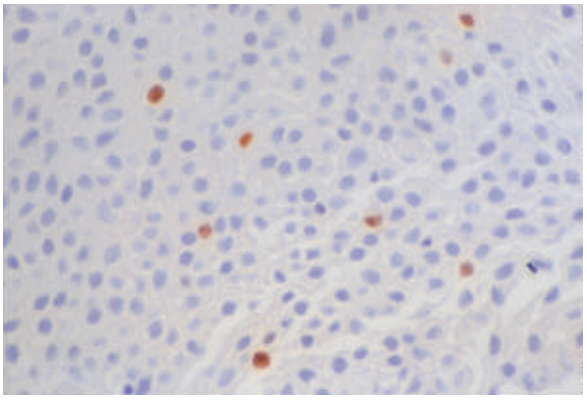


(b)

Fig. 3. Immunostaining for cyclin D1 (a), low-grade, low-stage tumor (TCC G1 pT1) (b), high-grade, high-stage tumor (TCC G3 pT2)



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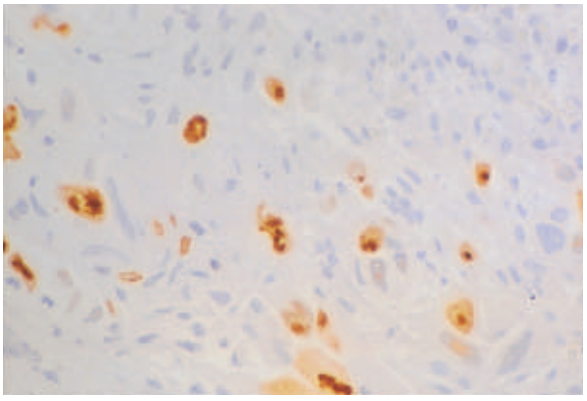

(b)

Fig. 4. Immunostaining for cyclin Ki-67 (a), low-grade, low-stage tumor (TCC G2 pT1) (b), high-grade, high-stage tumor (TCC G3 pT2)

67 immunostaining was confined to the nuclei of neoplastic cells. As was staining for cyclin A, Ki-67 for staining was more diffuse in high-grade, high-stage tumors (Fig. 4a, b). The expression of Ki-67 was not correlated with patient sex or age but was strongly correlated with tumor grade and stage. High-grade, high-stage tumors had more diffuse and finer staining for Ki-67 ($p<0.01$).

Survival analysis

Patient outcome data were collected from hospital charts. Informative charts were available for 64 patients. The mean follow-up period was 74.8 months (range, 3-136 months). Fifteen of the 64 patients had died by April 1, 2001. Univariate Cox regression analysis revealed that cyclin A expression, Ki-67 expression, high grade (baseline: grade 1, 2), and high stage (baseline: pTa, pT1) were significant prognostic variables for survival ($p<0.01$; Table 2). The Kaplan-Meier survival curves for subgroups of patients divided by marker status are shown in Fig. 5. Patients with tumors that expressed cyclin A or overexpressed Ki-67 had significantly poorer survival ($p<0.01$). In contrast, patients with tumors that overexpressed cyclin D1 had significantly better survival ($p<0.01$).

Discussion

In the present study, immunoreactivity for cyclin A was associated with high-grade, high-stage tumors and poor prognosis in cases of primary bladder cancer. Overexpression of cyclin A has been reported in primary lung cancer, hepatocellular carcinoma, and colorectal carcinoma[7-9]. Expression of cyclin A is

Table 2. Univariate analysis of the relationship between survival and variables tested.

Predictive variables	Hazard ratio	95% confidence interval	*p*
Cyclin A(LI) Positive (baseline Negative)	10.729	2.411–47.743	0.0018
Cyclin B1 (LI) Positive (baseline Negative)	3.234	1.170–8.940	0.2370
Cyclin D1 (LI) Negative (baseline Positive)	4.527	1.635–12.535	0.0037
Ki-67 (L1) Positive	22.761	2.983–173.691	0.0026
Grade Grade 1, 2 (baseline"Grade 3)	9.312	2.941–29.486	0.0007
pTstage pT2, pT3 (baseline pTa, pT1)	12.754	4.021–40.449	<0.0001
Tumor size (cm) $\geqq 2.0$ (baseline<2.0)	3.543	1.209–10.385	0.0211
Sex female (baseline male)	0.881	0.425–1.829	0.7347
Age (year) $\geqq 70$ (baseline<70)	0.690	0.236–2.018	0.4795

regulated in the cell cycle and peaks during the late G1 and S phases in both normal and transformed cells[10]. Cyclin A-associated enzymes are key promoters of progression through the S phase of the cell cycle[11]. In normal human fibroblasts, cyclin A-Cdk2 is part of a quaternary complex that includes p21 and proliferating cell nuclear antigen, but in many transformed cells cyclin A-Cdk2 forms complexes with p19Skp1 and p45Skp2[12]. Injection of cyclin A antisense inhibits DNA synthesis in fibroblasts[13]. Overexpression of cyclin A mediates the adhesion-independent cell cycle progression typical of oncogenic transformation[14] and is thought to contribute to high proliferative activity in bladder cancer cells. Cox regression analysis indicated that cyclin A overexpression is an independent negative prognostic factor for bladder cancer.

Ki-67 recognizes nuclear proteins in proliferating cells (G1, S, G2, and M phases)[15]. In the present study, we found immunoreactivity for Ki-67 was correlated with tumor grade and stage. High-grade, high-stage tumors showed increased immunoreactivity for Ki-67. Cox regression analysis indicated that Ki-67 overexpression is an independent negative prognostic factor for bladder cancer. The Ki-67 antibody has been used to predict biological behavior in some human neoplasms. Previous studies have indicated that Ki-67 immunoreactivity significantly correlates with the grade and stage of bladder cancer[1–3]. These studies, however, did not evaluate the usefulness of Ki-67 immunoreactivity as a prognostic factor.

Cyclin D1 is involved in regulating the transition through the G1 phase. Overexpression of cyclin D1 has been reported in many malignant tumors[4,16–18]. In the present study, intense immunoreactive staining for cyclin D1 was noted in low-grade, low-stage tumors. However, overexpression of cyclin D1 was observed in almost no high-grade, high-stage tumors. The cyclin D1 gene is consistently amplified in certain neoplasms as a part of the 11q13 amplicon region. Several studies have also reported amplification at chromosome 11q13 in bladder cancer[19,20] but did not perform correlative analysis of clinicopathologic variables. However, Lee et al. (1993) have reported overexpression of cyclin D1 in 47 grade 1 and 20 grade 2 transitional cell carcinomas but in no grade 3 carcinomas or carcinomas staged higher than T2[4]. The deregulated expression of cyclin D1 in some tumors disrupts cell-cycle control and increases genomic instability, thus contributing to tumor progression[21]. Previous studies have reported that cyclin D1 overexpression is associated with a poor prognosis in esophageal, head and neck, prostate, and breast cancers.

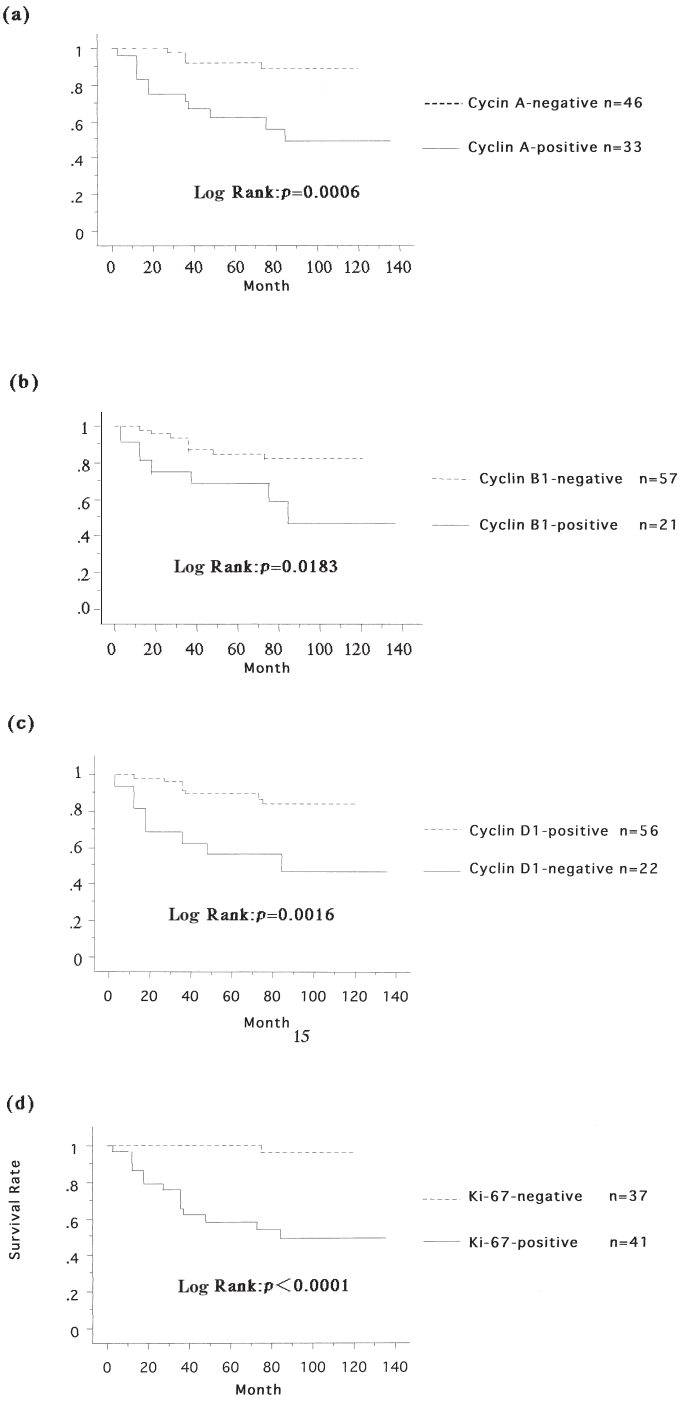

Fig. 5. Kaplan-Meier survival curves of patients with bladder cancer.
(a) Subdivided according to cyclin A status. Overall survival was significantly lower with cyclin A overexpression.
(b) Subdivided according to cyclin B1 status. Patients with tumors that overexpressed cyclin B1 had lower, but not statistically lower, survival.
(c) Subdivided according to cyclin D1 status. Overall survival was significantly higher with cyclin D1 overexpression.
(d) Subdivided according to Ki-67 status. Overall survival was significantly lower with Ki-67 overexpression.

In contrast, increased expression of cyclin D1 was associated with a good prognosis in bladder cancer.

Cyclin B1 is localized in the cytoplasm during G2 and undergoes translocation to the nucleus during prophase. Its expression peaks in late G2/M. Cyclin B1 is synthesized during G2 and is required for G2 traverse and cell entrance to mitosis. Overexpression of cyclin B1 has been reported in colorectal, esophageal, head and neck, and non-small-cell lung cancer[22-24]. In esophageal cancer, cyclin B1 overexpression is correlated with the depth of tumor invasion and is thought to affect length of survival. In the present study, survival in patients with tumors that overexpressed cyclin B1 was slightly, but not significantly, shorter. How overexpression of cyclin B1 contributes to tumor progression remains unclear; however, B1 overexpression may cause continued activation of partner kinases (Cdks) and, thus, the unscheduled phosphorylation of a variety of proteins. A recent study has reported on the down-regulation of cyclin B1 by p53 through the G2 checkpoint[25]. The overriding of p53-mediated G2-M arrest caused by the constitutive activation of cyclin B1 might be involved in tumor progression.

In conclusion, to our knowledge our study is the first to identify cyclin A overexpression as a significant independent prognostic factor for bladder cancer. We found strong correlations between overexpression of cyclin A and Ki-67 and unfavorable pathologic variables. Conversely, cyclin D1 overexpression indicated a good prognosis. The immunohistochemical assessment of cyclin A and Ki-67 overexpression more precisely indicates the biological aggressiveness of the tumor. If our findings are confirmed in larger studies, the evaluation of cyclin A and Ki-67 will provide objective information for determining malignant potential and selecting treatment in cases of bladder cancer.

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