Increased cyclin-dependent kinase 6 expression in bladder cancer

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Abstract. Cyclin-dependent kinase 6 (Cdk6) controls the cell cycle and aberrant expression of Cdk6 is involved in cancer progression. The aim of this study was to investigate the role of Cdk6 in bladder cancer development. Cdk6 expression was examined in 31 cases of bladder cancer and 29 tissues adjacent to bladder transitional cell carcinoma (TCC) using an immunohistochemistry assay. The correlation between Cdk6 expression and clinical characteristics was also analyzed. Compared with the adjacent tissues, cytoplasmic and nuclear Cdk6 expression levels were significantly increased in the invasive bladder cancer cases (P=0.005 and P<0.001, respectively), but not in the non-invasive superficial cases of bladder cancer (P>0.05 for both). Cytoplasmic and nuclear Cdk6 expression levels were correlated with bladder cancer stage (superficial vs. invasive, P=0.026 and P=0.006, respectively). The results therefore indicate that increased Cdk6 expression contributes to bladder cancer development and may serve as a biomarker for bladder cancer.

Introduction

Bladder cancer is an increasingly significant international public health problem. In the USA, bladder cancer is the second most common genitourinary malignant disease, with 69,250 new cases and 14,990 mortalities estimated in 2011 (1). The incidence of bladder cancer increases with age, peaking between 50 and 70 years, and the disease is approximately three times more common in males than females (2). The established risk factors for bladder cancer include tobacco smoke, exposure to industry-related aromatic amines and the uptake of drugs such as phenacetin, chlornaphazine and cyclophosphamide (3). Exposure to these chemical carcinogens may lead to direct and indirect DNA damage, genome instability and carcinogenesis (4). However, the precise mechanism of bladder cancer development remains unclear.

The cell cycle and cell proliferation are controlled by cyclins, cyclin-dependent protein kinases (Cdks) and Cdk inhibitors (5). The alteration of various components of the cell cycle regulatory mechanism that controls the progression of cells from a quiescent to a growing state contributes to the development of numerous types of human cancer (6). Cdk6, in cooperation with cyclin D, drives cell cycle progression from G1 to S phase through the phosphorylation and subsequent inactivation of the retinoblastoma 1 protein (7). Aberrant Cdk6 expression has been reported in pancreatic cancer (8), T-cell lymphoma (9), malignant glioma (10) and medulloblastoma (11), suggesting the involvement of Cdk6 in cancer. However, the expression of Cdk6 in bladder cancer has not been reported, although the roles of Cdk6 partner proteins such as p16 in bladder cancer development have been investigated previously (12,13). To investigate the role of Cdk6 in bladder cancer development, we examined the Cdk6 expression in cases of bladder cancer and their adjacent tissues using an immunohistochemistry (IHC) assay and evaluated the correlation between Cdk6 expression and bladder cancer progression.

Materials and methods

Tissue microarray and ethics statement. The tissue microarray (TMA) for the bladder cancer was obtained from Shanghai Outdo Biotech Company (Shanghai, China). The use of human bladder transitional cell carcinomas (TCCs) and their adjacent tissues in this study was approved by the Clinical Research Ethics Board of the First Hangzhou People Hospital

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Abbreviations: Cdk6, cyclin-dependent kinase 6; IHC, immunohistochemistry; TMA, tissue microarray

Key words: bladder cancer, cyclin-dependent kinase 6, immunohistochemistry, tissue microarray

(Hangzhou, China). The study was conducted in accordance with the Declaration of Helsinki guidelines.

IHC. The IHC assay was carried out as previously described (14). The monoclonal mouse anti-Cdk6 antibody (1:50 dilution; Millipore, Billerica, MA, USA) was used for primary antibody incubation at 4˚C overnight. A slide incubated without the primary antibody was used as a negative control.

Evaluation of immunostaining. The Cdk6 staining was blindly and independently examined by two pathologists. In certain cases where discrepancy between the two observers occurred, the immunostained slides were reviewed in a double viewing microscope to resolve the discrepancy. Cdk6 staining intensity was scored as 0, 1+, 2+ or 3+. The percentage of Cdk6-positive cells was scored as: 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The level of Cdk6 staining was evaluated by the immunoreactive score (IRS) (15), which is calculated by multiplying the scores of the staining intensity and the percentage of positive cells.

Statistical analysis. The paired Student's t-test was applied to evaluate the differences in Cdk6 expression levels between the bladder cancer cases and the adjacent tissues. The independent t-test was used to evaluate the differences in Cdk6 expression levels between different stages of bladder cancer. The differences in clinical characteristics and the expression levels of Cdk6 were evaluated using the χ^2 test between patient subgroups. $P<0.05$ was considered statistically significant and all tests were two-sided. SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) software was used for all analyses.

Results

Clinicopathological features of TMAs. A total of 62 bladder tissues (31 pairs of bladder cancer cases and adjacent tissues) were used for TMA construction. The distributions of selected demographic characteristics of bladder cancer patients are listed in Table I. Of the 31 bladder cancer patients, 26 were male and 5 were female. Patient age ranged between 48 and 83 years (median, 67). Due to the loss of biopsy cores or insufficient tumor cells being present in the cores, only 29 pairs of bladder cancer cases (29 adjacent tissues and 31 bladder cancer cases) could be evaluated for Cdk6 staining. The 31 bladder cancer tissues included seven cases of non-invasive, low-grade papillary lesions (excluding carcinomas *in situ*) and 24 cases of invasive bladder cancer.

Cdk6 expression is increased in bladder cancer. The Cdk6 staining was detected in the cytoplasm and nuclei (Fig. 1A-H). Cytoplasmic and nuclear Cdk6 staining was increased in 38% (11/29) and 65% (19/29) of all bladder cancer cases compared with their adjacent tissues (Fig. 1A and B) and the differences were significant ($P=0.005$ and $P<0.001$ for cytoplasmic and nuclear staining, respectively; paired-samples t-test; Table II). No significant difference was found in either cytoplasmic or nuclear Cdk6 staining between the superficial bladder cancer cases and their adjacent tissues ($P=0.086$ and $P=0.172$, respectively; paired-samples t-test; Table II). However, significant differences in cytoplasmic and nuclear Cdk6 staining were observed between the invasive bladder cancer cases and their adjacent tissues ($P=0.005$ and $P<0.001$, respectively; paired-samples t-test; Fig. 1A and B; Table II).

Table I. Clinical characteristics of bladder cancer patients.

Clinical characteristics	N (%)
Total	31 (100)
Age (years)	
≤67	16 (51.6)
>67	15 (48.4)
Gender	
Female	5 (16.1)
Male	26 (83.9)
Stage of bladder cancer	
Superficial	7 (22.6)
Invasive	24 (77.4)

Correlation between Cdk6 expression and clinicopathological parameters. In the bladder cancer patients, we did not find any significant correlations between either cytoplasmic or nuclear Cdk6 expression and the clinicopathological characteristics, including age and gender (data not shown). However, cytoplasmic and nuclear Cdk6 staining were increased in invasive bladder cancer cases compared with that in the superficial bladder cancer cases ($P=0.026$ and $P=0.006$, respectively; independent-samples t-test; Fig. 1C and D; Table III), suggesting that cytoplasmic and nuclear Cdk6 expression correlates with bladder cancer progression.

Discussion

In the present study, we examined Cdk6 expression in cases of bladder cancer and their adjacent tissues and evaluated the correlation between Cdk6 expression and the clinical characteristics of bladder cancer patients. Similar to other types of cancer, Cdk6 expression was increased in bladder cancer. DNA amplification is a common mechanism found in numerous types of human tumors and may result in the overexpression of genes whose products are involved in cell proliferation. However, Cdk6 overexpression was not restricted to cases with gene amplification and previous studies have reported that the aberrant post-transcriptional regulation of Cdk6 resulted in Cdk6 overexpression. For example, miR-9 was found to be methylated in acute lymphoblastic leukemia patients and the methylation of miR-9 was associated with its downregulation (16). The epigenetic downregulation of miR-9 induced the upregulation of its target, Cdk6 (16). The elucidation of the mechanism of increased Cdk6 expression in bladder cancer requires further investigation.

At the initiation of cell cycle progression, cyclin D enhances the cell transition through the G1 phase of the cell cycle by binding to and activating Cdk6 (7). The specific binding of p16 to Cdk6 inhibits the catalytic activity of the cyclin D-Cdk6 complex and consequently arrests the cell cycle at the G1 phase (17). Alteration of this pathway

Table II. Cdk6 staining in bladder lesions.

Stages	Subcellular	Tissue	Cdk6 staining (mean ± SD)	P-value[a]
Superficial (n=7)	Cytoplasmic	Adjacent	6.29±2.43	0.086
		Bladder cancer	8.29±2.93	
	Nuclear	Adjacent	3.57±1.99	0.172
		Bladder cancer	5.57±3.31	
Invasive (n=22)	Cytoplasmic	Adjacent	8.82±2.81	0.005
		Bladder cancer	10.64±2.08	
	Nuclear	Adjacent	6.73±3.14	<0.001
		Bladder cancer	9.36±3.14	

[a]Paired-samples t-test. SD, standard deviation; Cdk6, cyclin-dependent kinase 6.

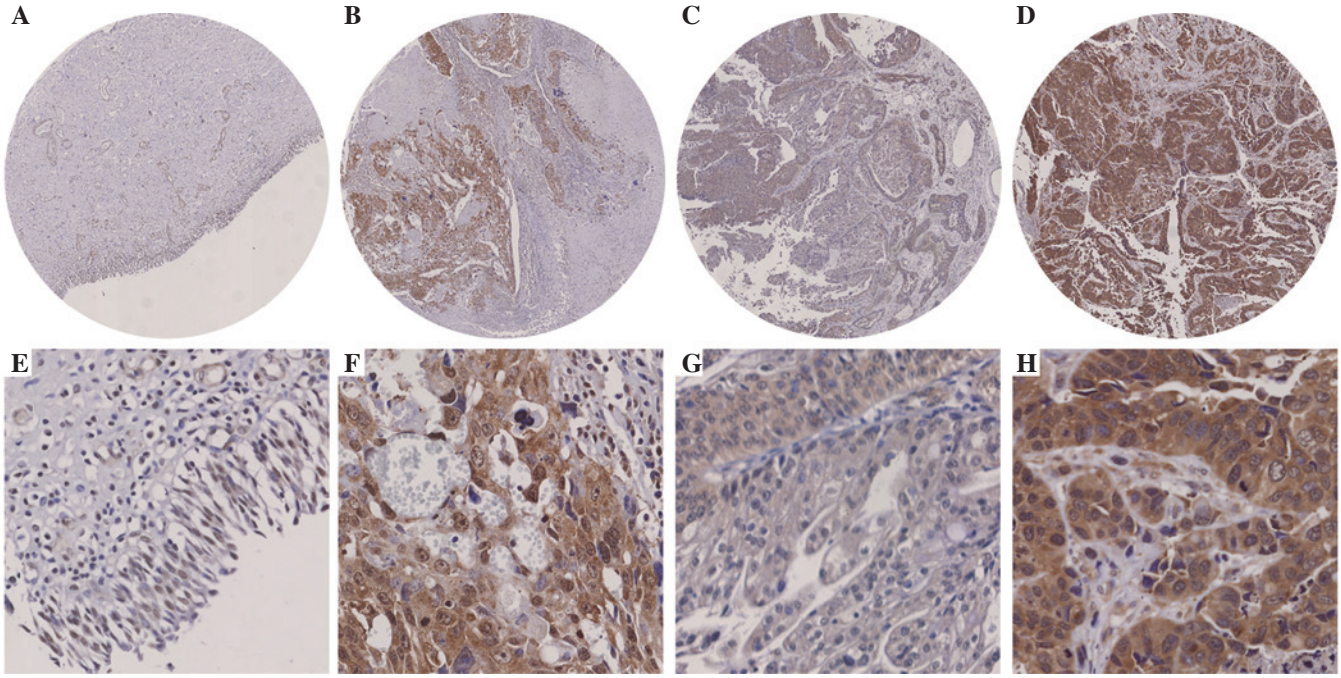

Figure 1. Representative Cdk6 staining in bladder tissues. Cdk6 staining in tissue adjacent to bladder cancer (A and E) and bladder cancer (B and F); Cdk6 staining in superficical (C and G) and invasive (D and H) cases of bladder cancer. Magnification: A-D, x25; and E-H, x200. Cdk6, cyclin-dependent kinase 6.

Table III. Cdk6 staining in bladder lesions.

Subcellular	Stages	Cdk6 staining (mean ± SD)	P-value[a]
Cytoplasmic	Superficial (n=7)	8.29±2.93	0.026
	Invasive (n=24)	10.58±2.08	
Nuclear	Superficial (n=7)	5.57±3.31	0.006
	Invasive (n=24)	9.56±3.09	

[a]Independent-samples t-test. SD, standard deviation; Cdk6, cyclin-dependent kinase 6.

results in the onset of cancer cell cycle progression and tumor development. The p16 gene deletion is reportedly an early event in bladder cancer (18,19). Our data have shown that Cdk6 expression was increased in the cases of invasive bladder cancer, suggesting that the overexpression of Cdk6 is a subsequent effect of the dysfunction of p16 contributing to bladder cancer development.

Identifying biomarkers for bladder cancer in conjunction with traditional cancer stages may improve early diagnosis and patient care. However, reliable biomarkers, particularly for advanced bladder cancer, are lacking. As Cdk6 expression may be determined by means of IHC on formalin-fixed paraffin-embedded sections, this marker is well suited for the

routine diagnostic setting as well as evaluation in controlled clinical studies.

A major limitation of the present study was the relatively small number of cases of bladder cancer tissues for IHC study. Nevertheless, the potential of Cdk6 as a biomarker for bladder cancer identified in this investigation may be useful for distinguishing between non-invasive superficial and invasive cases of bladder cancer. To confirm this hypothesis, more studies should be performed with an independent large cohort of patients. To the best of our knowledge, this is the first study to demonstrate the involvement of Cdk6 in bladder cancer development.

In conclusion, findings of our study showed that Cdk6 expression was increased in cases of invasive bladder cancer and that an increased Cdk6 expression may contribute to bladder cancer development and serve as a biomarker for bladder cancer.

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