Urol Res (2006) 34:321–328
DOI 10.1007/s00240-006-0063-3

ORIGINAL PAPER

Deregulation of the p16-cyclin D1/cyclin-dependent kinase 4–retinoblastoma pathway involved in the rat bladder carcinogenesis induced by terephthalic acid-calculi

Lunbiao Cui · Yuan Shi · Jie Qian · Guidong Dai · Yubang Wang · Yankai Xia · Jianfeng Chen · Ling Song · Shouling Wang · Xinru Wang

Received: 28 March 2006 / Accepted: 12 June 2006 / Published online: 29 July 2006

Abstract Prolonged cell proliferation in response to irritation by calculi may itself evoke malignant transformation of the urothelium. However, the molecular mechanisms underlying this process are still unknown. The aim of the present study was to investigate cell cycle regulatory mechanisms in bladder carcinogenesis induced by bladder calculi. Six-week-old Wistar rats were consecutively fed a diet containing 5% terephthalic acid (TPA), 5% TPA plus 4% sodium bicarbonate ($NaHCO_3$), 4% $NaHCO_3$, or basal diet for 48 weeks. Animals were killed at weeks 12, 24, and 48. Treatment with 5% TPA caused high incidences of bladder calculi, preneoplastic lesions, and neoplastic lesions. Immunohistochemical examination revealed overexpression of cyclin D1, cyclin-dependent kinase 4 (Cdk4), retinoblastoma (Rb), and proliferating cell nuclear antigen (PCNA) in bladder preneoplastic and neoplastic lesions. In contrast, p16 expression was reduced or absent. These results were confirmed by immunoblotting analysis. Quantitation of mRNA by real-time reverse transcription-polymerase chain reaction (RT-PCR) showed a significant increase in cyclin D1 and PCNA mRNA in tumor cells. None of the 16 transitional cell carcinomas (TCCs) had ras mutations as examined by PCR-single strand conformational polymorphism (PCR-SSCP) analysis. These results suggested that deregulation of p16-cyclin D1/Cdk4-Rb pathway, but not oncogenic activation of ras, plays a crucial role in bladder tumorigenesis induced by bladder calculi.

Keywords Urinary bladder · Calculus · Carcinogenesis · Cell cycle · Cyclin D1

L. Cui · Y. Xia · J. Chen · X. Wang
Key Laboratory of Reproductive Medicine,
Nanjing Medical University, Nanjing 210029, China

L. Cui · Y. Shi · J. Qian · G. Dai · Y. Wang · Y. Xia · J. Chen · L. Song · S. Wang · X. Wang (✉)
Key Laboratory of Modern Toxicology,
Institute of Toxicology, Nanjing Medical University,
140 Hanzhong Road, Nanjing 210029, China
e-mail: xrwang@njmu.edu.cn

Introduction

Bladder cancer is the fourth most common cancer in men and the eighth most common in women, accounting for 8% of adult cancers. It is estimated that 54,300 new cases and 12,400 deaths were reported in the USA in 2001 [1]. Bladder cancer is the second most common malignancy in the genitourinary tract in the USA and is the most common in China [2]. A number of risk factors have been identified for bladder cancer, such as carcinogens derived from occupational exposures, cigarette smoking, calculi, catheter indwelling, and schistosomal infections [3]. Bladder calculi can cause irritation and urothelial cell proliferation [4]. Epidemiological evidence indicated that urinary tract cancer is associated with a history of calculi in the bladder [5–7]. Depending on the extent of irritation, the histological changes underwent focal or diffuse simple hyperplasia of the transitional cell epithelium, followed by papillary or nodular hyperplasia (PN hyperplasia), then papillomas or diffuse papillomatosis, and ultimately transitional cell carcinoma (TCC) [8]. However, the molecular mechanisms underlying the processes remain unclear.

High incidence of bladder calculi can be induced in rats in less than 2 weeks by exposure to 3–5% tere-

phthalic acid (TPA) in the diet. TPA has been considered to be a non-genotoxic chemical compound and TPA-induced bladder urothelial hyperplasias and tumors in rats were considered as a result of calculi formation rather than TPA itself [9]. Thus, the animal model of bladder lesions induced by TPA can serve as a good tool to study molecular mechanisms of irritation-driven bladder carcinogenesis.

Urothelium showed extensive proliferation as a response to mechanical irritation. Cell proliferation is controlled by a group of cell cycle regulation proteins. The major regulatory events occur in the G1-S phase of the cell cycle. G1-S transition is mainly controlled through retinoblastoma (Rb) and p53 pathway. The Rb pathway, comprising p16, cyclin D1, cyclin-dependent kinase (Cdk4), and Rb, plays a pivotal role in tumorigenesis [10]. The current study was conducted to determine the timing of expression and the interrelationship among key proteins in the Rb pathway, relative to cell proliferation, at different stages of bladder cancer development and progression induced by TPA-calculi. The finding from this study provided evidence for deregulation of the p16-cyclin D1/Cdk4-Rb pathway in bladder cancer induced by calculi.

Materials and methods

Animals and chemicals

A total of 78 male Wistar rats, 5 weeks old (Shanghai Laboratory Animal Center of Chinese Academy of Sciences, Shanhai, China), were quarantined for 1 week before the start of the experiment. They were housed two per cage in an animal facility at a temperature of 22 ± 3°C and relative humidity of 60 ± 10%. Fluorescent lighting was provided in a 12 h light/dark cycle. Diet (Xie-Tong Biotech Co. Ltd, Nanjing, China) and tap water were given ad libitum. TPA with a purity of > 99.9% (Yi Zheng Chemical Fiber Co., China) was added to the basal diet or 4% sodium bicarbonate ($NaHCO_3$) containing diet at a final concentration of 5%. Body weights and food consumptions were measured once a week for the first 13 weeks, then once every 2 weeks.

Experimental design

Rats were randomly divided into four groups. Group 1 (38), group 2 (10), and group 3 (10) animals were fed diet containing 5% TPA, 5% TPA plus 4% $NaHCO_3$, 4% $NaHCO_3$, respectively. Group 4 (control group, 20 animals) remained untreated throughout. The total period of observation was 48 weeks. At weeks 12 and 24, eight rats from group 1 and four rats from group 4 were killed, respectively. The urinary bladders were excised and opened longitudinally. After the calculi were washed away with saline solution, half the bladder was fixed in 10% phosphate-buffered formalin. At week 48, end-point tumor incidence analysis was performed on the remaining animals. The largest tumor from each bladder was removed and stored at − 80°C for molecular assessment. Bladder epithelium of control rat was prepared with the aid of a blade.

Histopathology

The bladder was routinely embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined for histopathological assessment. Histological changes of the urinary bladder epithelium were classified into PN hyperplasia, papilloma, and TCC, as described previously [11, 12].

Immunohistochemistry

Serial sections of 4 μm thickness were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched by applying 3% hydrogen peroxide for 10 min. Sections were immersed in buffer (pH 6.0) and heated in a microwave oven (92–98°C, 2×10 min) for antigen retrieval. Non-specific binding was blocked by incubating sections with non-immune serum at room temperature for 20 min. Sections were then incubated overnight at 4°C in a humidified chamber with respective primary antibodies: monoclonal mouse anti-PCNA (proliferating cell nuclear antigen) antibody at 1:100 dilution, mouse anti-p16, cyclin D1 antibody at 1:50 dilution, goat anti-Cdk4 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and Rb polyclonal antibody (Boster Co., Wuhan, China) at 1:100 dilution. Immunoreactivity was detected using an SP kit (Zymed Laboratories, Inc., South San Francisco, CA, USA) and 3,3′-diaminobenzidine tetrahydrochloride followed by light hematoxylin counterstain. Negative control tissues were prepared in the same manner as described above, except for the omission of primary antibodies. Incidences of positive cells were determined by counting the positive cells among at least 1,000 cells in representative areas, and indicated as a percentage.

Protein extraction and Western blotting

Total protein of bladder epithelia and tumor specimens was isolated using Tripure reagent (Roche Applied

Science, Inc.) following the manufacturer's instructions. Equal amounts of proteins were separated by 15% (for p16), 12.5% (for PCNA, cyclin D1, and Cdk4), and 7.5% (for Rb) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane. Membranes were individually blocked with 5% non-fat dry milk and probed with antibodies against p16, cyclin D1, Cdk4, Rb, PCNA (the same antibodies as used for immunohistochemistry), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, KangChen Biotechnology, Shanghai, China). Secondary horseradish peroxidase conjugated sheep anti-mouse or anti-rabbit IgG antibodies were employed and protein signals were analyzed by chemiluminescence (ECL, Cell Signaling Technology Co.).

RNA extraction and real-time RT-PCR analysis

Total RNA was also isolated using Tripure reagent (Roche Applied Science, Inc.) simultaneously when isolating protein. Reverse transcriptions were carried out with MLV reverse transcriptase and oligo-dT primers. Resultant cDNA (1 μl) was amplified in a total volume of 30 μl reaction mixture solution that consisted of 1.25 U Taq polymerase, 1 × Taq buffer, 2.5 mM $MgCl_2$, 1.5 μl SYBR green, 0.3 mM of each dNTP mixture, and 1 μM of each primer. PCR amplification began with a 3 min pre-incubation at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and elongation at 72°C for 30 s. The sequences of forward and reverse primers for selected genes were as follows: 5′-CCG TAT CGG ACG CCT GGT TA-3′ and 5′-TGA ACT TGC CGT GGG TAG AG-3′ for GAPDH, 5′-TGA CAA CTC TAT CCG CCC CGA-3′ and 5′-GAA AGT GCG TTG TGC GGT AGC-3′ for cyclin D1, 5′-GCC CTC AAA GAC CTC ATC AA-3′and 5′-GCT CCC CAC TCG CAG AAA AC-3′ for PCNA. A standard curve for each run was constructed by plotting the crossover point against the log(number of starting molecules). The number of target molecules in each sample was then calculated with reference to this curve. Results were expressed relative to the number of GAPDH transcripts that was used as an internal control. Relative differences between groups were expressed as percentage increases versus the control.

DNA isolation and PCR-SSCP analysis

Genomic DNA from bladder tumor and normal bladder tissue was isolated from frozen tissue by phenol–chloroform extraction. Mutations of K-ras and H-ras were analyzed by the PCR-single strand conformational polymorphism (PCR-SSCP) method [13], where 100 ng of genomic DNA was amplified in a reaction volume of 30 μl, and the cycling parameters were as follows: pre-incubation at 95°C for 5 min, then 35 cycles of 95°C for 30 s, 54°C for K-ras and H-ras for 45 s, 72°C for 45 s. The sequences of PCR primers used were 5′-GCC TGC TGA AAA TGA CTG AG-3′ and 5′-CTC TAT CGT AGG ATC ATA TTC-3′ for K-ras exon1 (122 bp), 5′-CTC CTA CAG GAA ACA AGT AG-3′ and 5′-CAC AAA GAA AGC CCT CCC CA-3′ for K-ras exon2 (128 bp), 5′-AAG CGA TGA CAG AAT ACA AG-3′ and 5′-AGC TCA CCT CTA TAG TGG GA-3′ for H-ras exon1 (123 bp), 5′-AGG ACC CTT AAG CTG TGT TC-3′ and 5′-GAC TTG GTG TTG TTG ATG GC-3′ for H-ras exon2 (183 bp). PCR products were mixed with 15 μl gel-loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF), denatured at 100°C for 10 min, immediately loaded onto 8% polyacrylamide gel containing 5% glycerol, and electrophoresed at 500 V for 4–5 h at 4°C. The gel was dyed with silver nitrate. If shifted bands were observed, they were cut out from the polyacrylamide gel to be sequenced.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by least significance difference (LSD) tests with SPSS software. $P < 0.05$ was accepted as statistically significant.

Results

General observations

Hematuria was found in some rats after 2 weeks of 5% TPA administration. Two rats from group 1 died because of urethral obstruction. The mean body weights of rats given TPA were consistently lower than that of controls. The mean absolute and relative urinary bladder weights (excluding bladder calculi) of rats fed with 5% TPA were significantly higher than that of the control rats (data not shown).

Bladder calculi formation and histopathological changes

Histopathological findings and data on calculus formation in the urinary bladder are summarized in Table 1. Almost all animals in group 1 had calculi at weeks 12–48 except for one rat at week 12. The calculi in the urinary bladder were whitish or darkish and varied in

Table 1 Sequential changes in the formation of calculi and in the histology of the urinary bladder

Group	Treatment	12 weeks			24 weeks				48 weeks				
		No. of rats	Calculi	PN hyperplasia	No. of rats	Calculi	PN hyperplasia	Papilloma	No. of rats	Calculi	PN hyperplasia	Papilloma	TCC
1	5% TPA	8	7	7	8	8	8	8	20	20	20	18	16
2	5% TPA + 4% $NaHCO_3$								10	0	0	0	0
3	4% $NaHCO_3$								10	0	0	0	0
4	Untreated	4	0	0	4	0	0	0	12	0	0	0	0

shape and size (with the diameter about 0.1–6 mm). Histopathologically, PN hyperplasia was noted at week 12 in 5% TPA-treated rats. At week 24, papillomas appeared in all animals given 5% TPA. TCCs of urinary bladder were observed at high incidence (80%) at week 48. No bladder calculi and obvious bladder lesions were found in groups 2–4.

Urothelium cell proliferation determined by quantitative PCNA immunohistochemistry

Proliferating cell nuclear antigen positive indices at three time points are presented in Fig. 1. At all time points, three types of bladder lesions showed a higher PCNA-positive index than their corresponding control urothelia. Progressive increase in labeling index (LI) from PN hyperplasia to papilloma to TCC was also noted. However, no significant difference was observed in the same type of lesion during different time points.

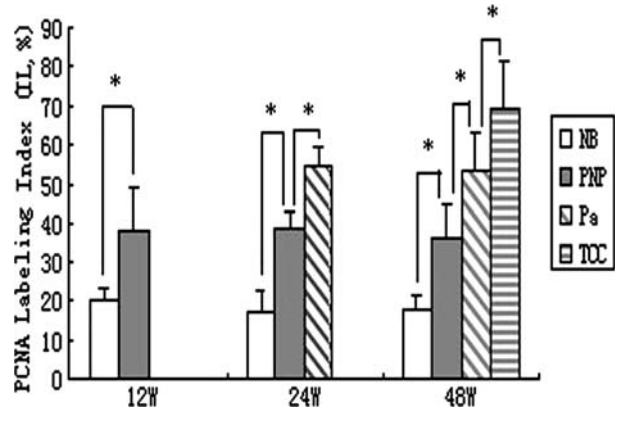

Fig. 1 Assessment of cell proliferation in rat urinary bladder lesions induced by TPA-calculi as determined by PCNA immunohistochemistry. Epithelia of lesions showed higher PCNA-positive indices than their respective control bladder mucosa. Progressive increase of PCNA labeling index from PN hyperplasia to papilloma to TCC was also observed. However, no significant difference was observed in the same type of lesions between three time points. Data are means ± SD. *$P < 0.01$. *NB* normal bladder mucosa, *PNP* papillary or nodular hyperplasia, *Pa* papilloma, *TCC* transitional cell carcinoma

Expression of cell cycle proteins in bladder tumor progression

According to the widely accepted criteria of p16 staining [14], only distinct nuclear immunostaining was considered positive. At all time points, normal urothelia from control animals showed strong nuclear staining for p16 (Fig. 2a). In contrast, hyperplastic and neoplastic epithelia typically showed absent or reduced staining for p16 (Fig. 2b). Positive staining for cyclin D1 was very low in normal urothelium (Figs. 2e, 3), but significantly higher in preneoplasm and tumor (Figs. 2f, 3). Moreover, the positive indices of cyclin D1 were increased in the order PN hyperplasia, papilloma, and carcinoma. The same type of lesions at different time points showed similar cyclin D1-positive indices (Fig. 3). Normal bladder epithelia exhibited weak Rb and Cdk4 nuclear immunostaining with LI < 10% (Fig. 2c, g). Rb and Cdk4-positive staining was observed in 38–67% of cells in various lesions of the urinary bladder (Fig. 2d, h). No significant difference was observed among PN hyperplasia, papilloma, or TCC. Remarkably, there was an inverse relationship between Rb and p16 staining. Hyperplastic or tumor areas that lack p16 expression always displayed a strong nuclear staining for Rb (Fig. 2b, d).

Immunoblotting results are consistent with those obtained using immunohistochemistry. The amounts of cyclin D1, Cdk4, Rb, and PCNA increased in TCCs, whereas the total amounts of p16 decreased (Fig. 4). In this study, the Rb antibody recognizes the hypophosphorylated (pRb), phosphorylated, and hyperphosphorylated (ppRb) forms of Rb. The hyperphosphorylated form of Rb was also observed in TCCs induced by TPA-calculi (Fig. 4E).

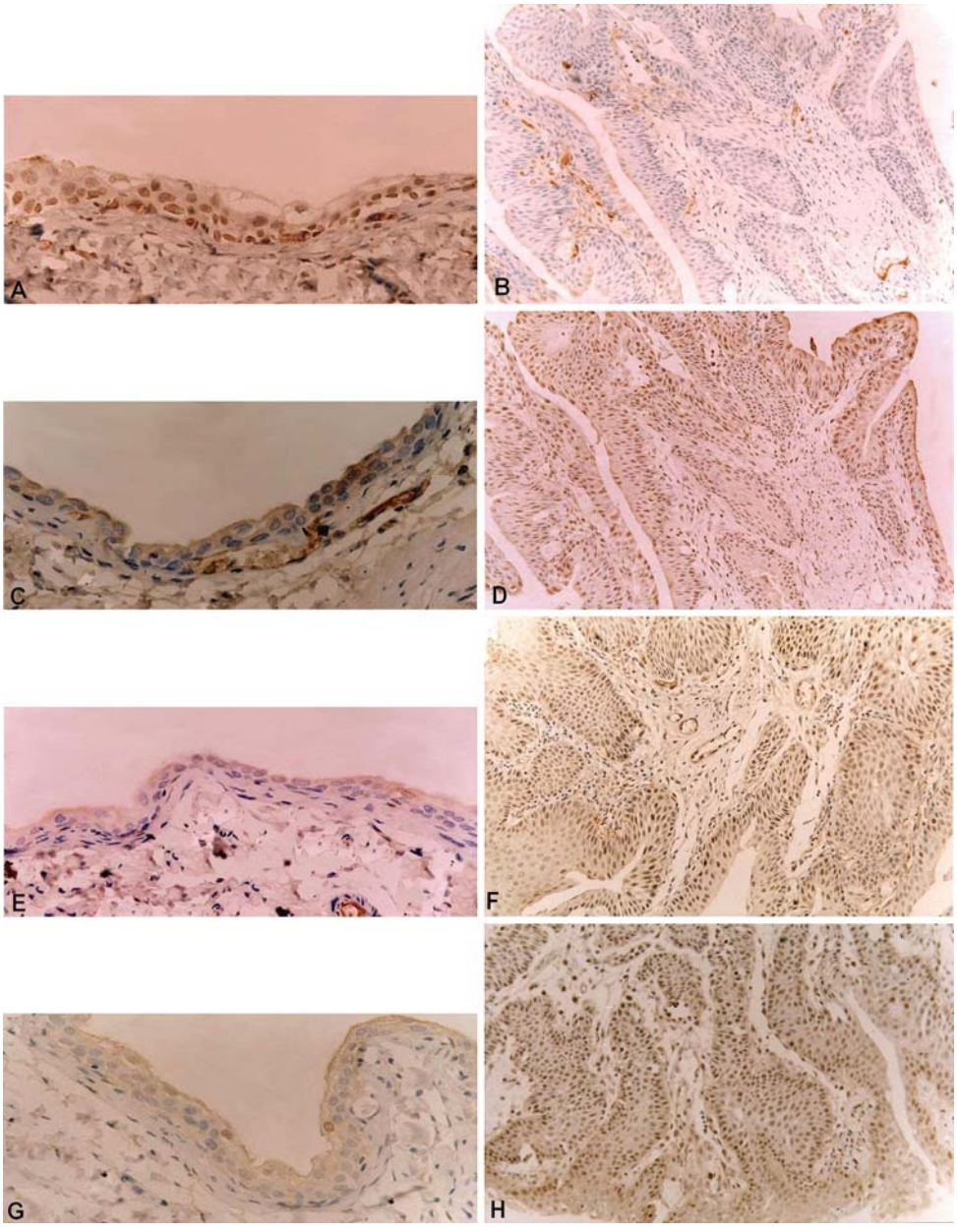

Fig. 2 Immunohistochemical assessment of cell cycle proteins in rat urinary bladder lesions induced by TPA-calculi. Representative staining is shown for p16, Rb, cyclin D1, and Cdk4 in normal bladder mucosa (*left*) and TCC (*right*) at week 48. **a** Normal bladder epithelium displayed strong nuclear staining for p16. **b** TCC showed reduced or absent staining for p16. **c**, **e**, **g** Normal bladder mucosa exhibited a low level of Rb, cyclin D1, and Cdk4 staining, respectively. **d** Intensive Rb staining in a serial section corresponding to **b**. **f**, **h** Strong and diffuse staining for cyclin D1 and Cdk4 in TCC, respectively. Magnification: **a**, **c**, **e**, and **g**: × 400; **b**, **d**, **f**, and **h**: × 200

Quantitation of cyclin D1 and PCNA mRNA expression in bladder tumors

In the present study, the mRNA levels of PCNA and cyclin D1 in normal urothelium were set at 1.00, and the mRNA expression of PCNA and cyclin D1 in TCC was 1.9- and 1.84-fold higher, respectively (Fig. 5).

Mutation analysis of ras in bladder tumors

Sixteen TCCs were examined for the presence of ras mutation by PCR-SSCP analysis. None of the tumors were found to harbor mutations within exons 1 and 2 of the K-ras and H-ras gene.

Discussion

There have been many reports of simultaneous occurrence of bladder calculi and bladder tumors in rodents given some non-genotoxic chemical compounds for long periods [8]. These studies indicated that the prolonged cell proliferation in response to irritation by calculi may itself evoke malignant transformation of the urothelium. No bladder calculi and bladder epithelial cell proliferation were found in rats treated with TPA by simultaneous administration of $NaHCO_3$ in our present and previous studies [15, 16]. The results suggested that prevention of the long-term proliferative responses and/or calculi formation with $NaHCO_3$

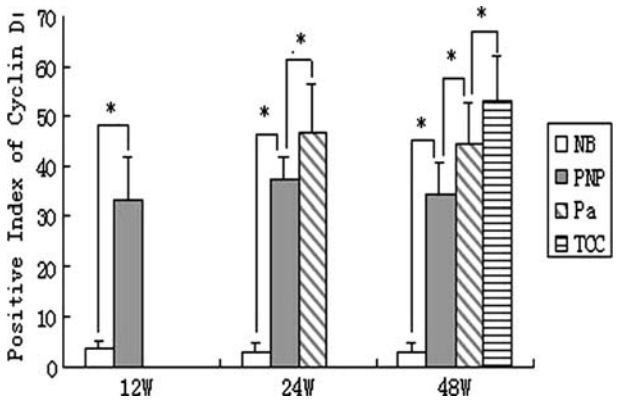

Fig. 3 Cyclin D1-positive index in rat urinary bladder lesions induced by TPA-calculi as determined by PCNA immunohistochemistry. Positive staining for cyclin D1 was very low in normal urothelium, but significantly higher in preneoplasm and tumor. Moreover, the positive indices of cyclin D1 were increased in the order PN hyperplasia, papilloma, and carcinoma. Same type of lesions at different time points showed similar cyclin D1-positive indices. Data are means ± SD. *$P < 0.01$. *NB* normal bladder mucosa, *PNP* papillary or nodular hyperplasia, *Pa* papilloma, *TCC* transitional cell carcinoma

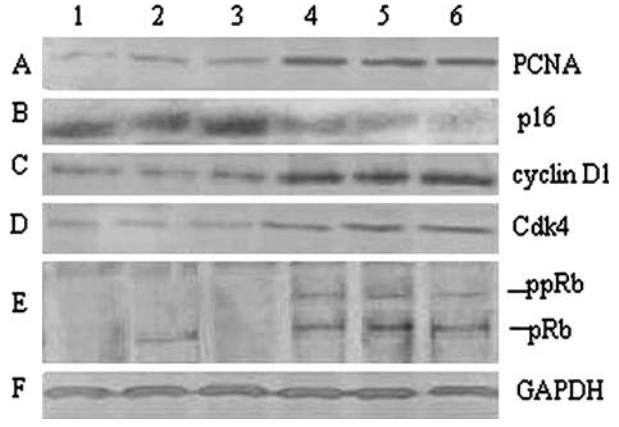

Fig. 4 Representative immunoblots for PCNA, p16, cyclin D1, Cdk4, Rb, and GAPDH in normal urothelia (*lanes 1–3*) and TCCs (*lanes 4–6*) at week 48. The amounts of PCNA (*A*), cyclin D1 (*C*), Cdk4 (*D*), Rb (*E*) were increased in TCCs, whereas total amounts of p16 were low (*B*). GAPDH (*F*) was used as an internal control. The hyperphosphorylated forms of Rb also occurred in TCCs (*E*)

would prevent bladder cancer formation and that TPA-induced bladder lesions were directly due to the irritation of the calculi. The high incidence of bladder calculi, PN hyperplasia, papillomas, and TCC developed after treatment with 5% TPA for 48 weeks. Thus, the animal model of bladder carcinogenesis induced by TPA-calculi is very useful for studying specific molecular events even in the very early stage of bladder tumorigenesis caused by prolonged cell proliferation.

Overexpression of the cyclin D1 gene has been reported in many human tumors and preneoplastic lesions including bladder TCC [17, 18]. In several animal model systems, deregulated expression of cyclin D1 has been shown to contribute to tumorigenesis [19–21]. These studies indicated that cyclin D1 functioned like an oncogene. Data from the present study showed that the levels of cyclin D1 expression in both PN hyperplasia and neoplasms were significantly higher than that of control epithelia. Moreover, the degree of overexpression of cyclin D1 was higher in the order PN hyperplasia, papilloma, and carcinoma. These observations suggested that overexpression of cyclin D1 is an early event of, and plays an important role in, bladder tumorigenesis. Overexpression of cyclin D1 may be the result of one or more of four following reasons: chromosomal rearrangement, chromosomal translocation, activation by retroviral insertion, and DNA amplification. The direct relation between cyclin D1 overexpression and chromosome 11q13 amplification in human bladder tumor has been reported [22]. The mechanisms of cyclin D1 overexpression in rat bladder tumors induced by TPA-calculi warrant further study.

The PCNA expression showed a strong correlation with cell proliferative activity [23]. Cellular proliferation was involved in all carcinogenesis processes including initiation, progression, and metastasis [24]. Similar to the expression of cyclin D1, the percentage of PCNA staining increased progressively from PN hyperplasia to papilloma to TCC. As overexpression of cyclin D1 can cause abnormalities in growth control and cell cycle progression, we postulated that the increased PCNA expression may be the result of cyclin D1 overexpression. In line with cyclin D1 expression, the increased mRNA expression of PCNA supported our hypothesis.

Cdk4, as the main partner of cyclin D1, has been shown to be overexpressed in human hepatoblastoma, acute lymphoblastic leukemia, sarcoma, and malignant glial tumors [25–28]. However, few studies have been conducted to learn the role of Cdk4 in human bladder tumorigenesis. High expression of Cdk4 in early lesions of bladder induced by chemical carcinogen in rats has been reported [29]. In this study, we observed that overexpression of Cdk4 persistently existed in each stage of bladder lesions (from PN hyperplasia to papilloma to TCC). Cdk4/cyclin D1 complex is first activated in the early G1 phase and operates to phosphorylate the Rb protein. Upon phosphorylation, Rb releases and activates the transcription factor E2F. The increase in E2F transcriptional activity results in the transactivation of genes whose products are essential for DNA replication and cell cycle progression. The

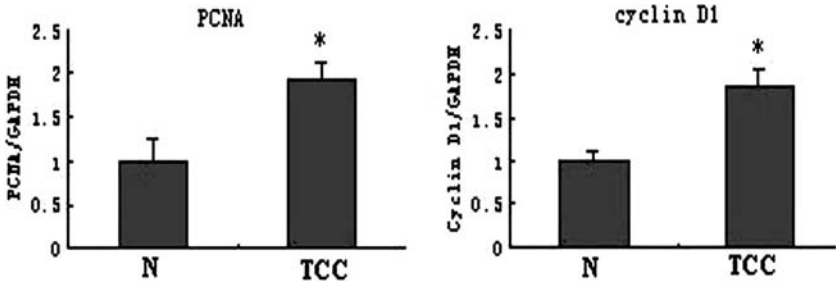

Fig. 5 Quantitation of mRNA levels of PCNA (**a**) and cyclin D1 (**b**) in normal urothelia and TCCs at week 48. Results were expressed relative to the number of GAPDH transcripts. The relative expression of PCNA and cyclin D1 mRNA was 1.9- and 1.84-fold higher in TCC than in normal urothelium, respectively. *N* normal urothelium, *TCC* transitional cell carcinoma. Data are means ± SD. $^*P < 0.05$

co-overexpression of cyclin D1 and Cdk4 suggested that urothelial proliferation induced by TPA-calculi was through cell cycle progression.

It is generally known that reverse regulation of cyclin D1/Cdk4 complex activity relies on the Cdk inhibitory protein (Cdki). P16, as an important Cdki, can bind and inactivate cyclin D1/Cdk4 complex. Accumulating evidence implicated that p16 acts as a tumor suppressor [30, 31]. Our study found urothelium to show reduced or absent p16 staining throughout the experimental period. Moreover, an inverse correlation between the expression of Rb and p16 was observed. This phenomenon has been observed in many tumor types, including human bladder cancer [32]. So, our results suggested that high Rb expression did not show tumor suppressor effects and it may be the phosphorylated form that allowed cell proliferation. Immunoblotting results supported this interpretation. The inactivation of p16 may be caused by gene deletion, mutation, or promoter hypermethylation. The mechanism of p16 inactivation will be our future research focus.

Abundant evidence indicated oncogenic ras was the upstream regulator of Cdk. Activated ras or ras transformation induces cyclin D1 overexpression [33]. High frequency of ras mutation concomitant with cyclin D1 overexpression has been observed in some animal and human carcinomas [34–37]. However, our study showed that none of the examined TCCs contained mutations of K-ras or H-ras. In two other rat bladder carcinogenesis models, no K-ras and H-ras mutations were found in any urinary bladder tumors [38, 39]. Together with these studies, we postulated that alterations of ras oncogene are not likely to be a common genetic event in rat bladder tumorigenesis.

In summary, alterations in the components of the Rb pathway, but not oncogenic activation of ras, play an important role in the initiation and progression of bladder carcinogenesis induced by TPA-calculi. Further studies about the genetic basis for the deregulation of the Rb pathway are warranted.

Acknowledgments The authors gratefully acknowledge the financial assistance from the National Natural Science Foundation of China (no. 30170798), National Key Basic Research and Development Program of China (no. 2002 CB512908), Specific Grant of National Key Basic Research Program of China (NO. 200150). We also appreciate Dr. Haitao Luo for his kindly discussion.

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