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ORIGINAL ARTICLE

Adenylate kinase 4 promotes bladder cancer cell proliferation and invasion

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Abstract

Bladder cancer is the second most common urological cancer worldwide with low early diagnosis and high mortality. Since the time of diagnosis directly affects survival rate, early detection and precise biomarkers of bladder cancer are very important. Adenylate kinase 4 (AK4) is a key enzyme involved in cellular metabolism and multiple cancer development; however, the potential role of AK4 in bladder tumorigenesis is still unclear. Immunohistochemistry assay was conducted to evaluate the expression level of AK4 in 107 human bladder cancer tissues. Overall survival and recurrence-free survival were used to assess the prognosis of patients. Colony formation and MTT assays [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] were performed to measure the proliferation capacity of tumor cells. Cell scratch assays and transwell assays were performed to measure the invasion capacity of tumor cells. The expression level of involved genes was measured by reverse transcription-polymerase chain reaction and western blot assays. The animal model was used to examine the effects of indicated protein on tumorigenesis and invasion in vivo. Herein, our study demonstrated that increased AK4 expression in patients with bladder cancer was associated with a poor prognosis. We further found that inhibition of AK4 in bladder cancer cell line T24 and 5637 can obviously inhibit the proliferation of cancer cells. Transwell assay results showed that down-regulated AK4 was related to the decreased metastasis of T24 and 5637 cells. In addition, AK4-shRNA transfected obviously inhibited tumor growth and metastasis in mice compared with the scramble group. Taken together, the results provide strong evidence of the involvement of AK4 in the progression of bladder cancer and suggest that it could have high potential as a therapeutic target of disease.

Keywords AK4 · Bladder cancer · Proliferation · Invasion · Therapeutic target

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Abbreviations

AK4	Adenylate kinase 4
DAB	Diaminobenzidine
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
PCNA	Proliferating cell nuclear antigen
RT-qPCR	Real-time quantificational polymerase chain reaction
HRP	Horseradish peroxidase
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline

Introduction

Bladder cancer is one of the most prevalent cancers globally, with about four million new cases diagnosed every year [1, 2]. The incidence rates of bladder cancer are nearly four times higher in men than in women [3]. Patients are

widely at an age of 70 years at the time of diagnosis [4]. Since the time of diagnosis directly affects early detection and survival rate of bladder cancer, life-long surveillance is very important [5]. Currently diagnostic methods, which are most widely used clinically, such as urine cytology, are limited due to its invasiveness and costliness [4]. With low early diagnosis and high mortality, to combat this disease, new markers and therapeutic targets are desperately needed [6, 7].

Adenylate kinases, which belong to the nucleoside monophosphate kinases, are involved in multiple cell process, including homeostasis of cellular adenine nucleotide composition and energy metabolism [8, 9]. AK4, a member of adenylate kinases, located in the mitochondrial matrix and has a high degree of sequence homology with AK3 [10]. AK4 has been reported to be enzymatically inactive in vitro, unlike AK3, but still retains its nucleotide-binding ability, physically interacts with the mitochondrial ADP/ATP translocator, and plays as a stress-responsive element to promote cell survival [11–13].

A microarray analysis had been published and indicated that proteins of the adenylate kinase family were abnormally up-regulated during the progress of invasive transformation [12, 14]. Meanwhile, by comparing gene expression profile, adenylate kinase-4 (AK4) was also observed to be markedly more over-expressed in highly invasive tumor [12, 15]. It has been reported that AK4 is required to promote lung cancer metastasis and recurrence in an ATF3-dependent manner [15]. AK4 plays a vital role in hypoxia tolerance, resistance to anti-tumor drug, and the regulation of mitochondrial activity [12, 16]. Also, AK4 regulates cellular ATP levels, AMPK signaling, and whose expression also significantly correlates with glioma patient survival, providing a link between adenylate kinase function and energy sensing pathways and suggesting a potential role in cancer development [12, 17]. Despite extensive studies into its fundamental, the exact correlation between AK4 and tumor has not been further explored [13, 18].

In this study, we found that AK4 was positively correlated with poor prognosis of bladder cancer patient. Knockdown of AK4 in bladder cancer cells T24 and 5637 inhibited proliferation and invasion and suppressed tumor formation and metastasis in mice. AK4 may therefore represent a potential biomarker for the prognosis of bladder cancer and a novel therapeutic target for this disease.

Materials and methods

Statement

Clinicopathological characteristics were noted in all cases. Corresponding patient clinical histories were obtained from the clinic to follow up visits, or telephone contact. We have obtained approval from the Second People's Hospital of Lianyungang.

Immunohistochemistry

Samples of human bladder tissues were obtained from patients who underwent surgical resection at the Second People's Hospital of Lianyungang. For immunohistochemistry staining, tumor tissue sections were de-paraffinized, rehydrated with xylene and graded alcohols. Then, the sections were blocked with 2% BSA and incubated with primary antibody specific for AK4 for 2 h. Subsequently, the sections were incubated with biotinylated secondary antibody for 1.5 h and HRP-conjugated streptavidin. Subsequently, diaminobenzidine (DAB) was dripped on in 3–5 min. Sections were then counterstained with hematoxylin.

The expression of AK4 protein is mainly located in the cytoplasm of tumor cells, which is brown–yellow granule. Results analysis is as follows: positive cells: the percentage of positive tumor cells < 10%, score 0; the percentage of positive tumor cells 10 to 30% score 1. The positive percentage of tumor cells was 30–70% score 2. The positive percentage of tumor cells was 70%>, score 3. At the same time, the membrane and plasma staining intensity of tumor cells with positive staining was evaluated: 0 for negative staining, 1 for weak positive staining, 2 for moderate positive staining, and 3 for strong positive staining. Results high expression (2–3) or low expression (0–1) were determined according to the score of positive cell percentage score and staining intensity score. The sections of each patient were observed with five visual fields, and two experienced pathologists read the sections without getting the pathological grade and clinical data. The results were judged by double-blind method.

Antibodies and primers

Rabbit anti-AK4 (1:200 dilution, ab131327 plc, Cambridge, UK) mouse anti-β-actin (1:1000 dilution, ab8226, Abcam plc, Cambridge, UK), rabbit anti-Ki67 (1:1000 dilution, ab16667, Abcam plc, Cambridge, UK), mouse anti-proliferating cell nuclear antigen (PCNA) (1:500 dilution, ab29, Abcam plc, Cambridge, UK), mouse anti-MMP2 (1:1000 dilution, ab37150, Abcam plc, Cambridge, UK), and mouse anti-MMP9 (1:1000 dilution, ab38898, Abcam plc, Cambridge, UK).

The qPCR primer sequences of AK4 are as follows: forward, 5′-ATGGACCGTGTGCTGCTGAAGT-3′ and reverse, 5′-TCCGAAACTTCTCTCCTGGCTC-3′; the qPCR primer sequences of GAPDH are as follows: 5′-GAGTCAACGGATTTGGTCGT-3′ and reverse, 5′-TTGATTTTGGAGGGATCTCG-3′.

The AK4 shRNA sequences were as follows: sense, 5′-AACTTTGGTCTCCAGCATCTCTC-3′.

Cell line and cell culture

The T24 and 5637 human bladder cancer cell lines were purchased from ATCC (Chicago, USA). Both cell lines were maintained in H-DMEM and RPMI 1640 culture medium, respectively, supplemented with 10% of fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator.

Transfection

The AK4 shRNA plasmids were transfected into cancer cells by Invitrogen Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.). Ready-to-package AAV shRNA clones of AK4 were purchased and transfected into cancer cell lines, respectively. Also, control cells were transfected with a control shRNA that did not match any known human coding cDNA. 100,000 cell per well in six-well plates according to the manufacturer's protocol, three groups were set, including: sh-AK4 group, which transfected with shRNA targeting AK4; Negative Control group, which transfected with scrambled sequence; and Mock group was treated without transfection (data not shown). Silence efficiency was measured by reverse transcription-polymerase chain reaction (RT-PCR) and western blot after 48 h' transfection. Then, the AK4 stable depletion cell lines were screened and used for the in vitro and in vivo assays.

Total RNA isolation and qRT-PCR

Total RNA was isolated from T24 and 5637 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Then, total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega). Quantitative real-time PCR was performed using SYBR mixture (Takara), and the relative AK4 expression was normalized to GAPDH.

Western blot analysis

Total protein was extracted from cancer cells or tumor tissues, and the procedure for western blot analysis was described before. The membranes were blocked and then incubated with the primary antibodies for detection of AK4, Ki67, PCNA, MMP2, MMP9, and β-actin was used as loading control. Subsequently, the membranes were incubated with HRP-conjugate secondary antibodies for 45 min. Signals were visualized by using an ECL kit.

Colony formation assays

For colony formation assay, nearly 5000 cells were added to a 6-well culture plate and infected with shRNA lentivirus targeting negative control or AK4. Then, the cells were cultured for 14 d at 37 °C. After this, cells were fixed in 70% ethanol and stained with 0.1% crystal violet for 25 min. Cell numbers were counted under a microscope (Olympus, Japan). Quantification of cells was performed by dissolving crystal violet with 10% acetic acid, and the optical density of each sample was read with a microplate reader at 570 nm.

Cell proliferation assay

Cells were plated in 96-well plates at a density of about 2000 cells in each well and cultured for 2 days. Cells were then incubated with MTT for 2 h and removed the medium. MTT was extracted by 150 μL DMSO, and absorbance value was quantified with a microplate reader at a wave length of 570 nm.

Cell scratch assays

The interested cells were seeded in 6-well plates and grew into full confluence overnight. A sterile pipette tip was used to introduce a scratch in the middle of each well. Next, the growth medium was discarded and replaced with fresh medium. The rate of migration toward the center of the wound was ascertained at indicated time points.

Transwell assay

T24 and 5637 cells were transfected with control or AK4-shRNA for 48 h and then trypsinized and re-suspended in serum-free medium. The upper chambers of transwell filters (8.0 μm membrane pores) were subsequently coated with 20% Matrigel and incubated at 37 °C for 30 min to form a thin gel layer. A total of 10^5 cells in 200 μL of medium were then added to the upper chambers of the inserts and were allowed to migrate toward the bottom chambers, which contained medium with 10% FBS. After 24 h, the cells that remained in the top chamber were removed, and cells on the underside were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet for 15 min. Phase contrast micrographs were obtained. Quantification of migrated cells was performed by dissolving crystal violet with 10% acetic acid, and the optical density of each sample was read with a microplate reader at 570 nm.

In vivo tumorigenesis and metastasis

All procedures carried out in mice were approved by the Institutional Animal Care and Use Committee at Tianjin

medical university general hospital. Nude BalB/c mice (6–8 weeks, 18–22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). For tumorigenesis assay, T24 cells were stable transfected with AK4 shRNA or shRNA scramble. 3×10^6 cells were subcutaneously inoculated into 6–8 weeks old male athymic nude mice. After tumors (100–150 mm^3) had established, the tumor volume was measured every 7 days with a caliper and calculated in length × (width 2)/2.

For metastasis assay, 5×10^5 T24/shControl, T24/shAK4 cells in 80 μL PBS were, respectively, injected through the tail vein. One mouse from each group was sacrificed at 30 days after injection to evaluate metastasis formation. 7 weeks later, all mice were sacrificed. Pulmonary metastases were then checked.

Statistics

Data are presented as the mean ± standard error of mean (SEM), and the Student's *t* test was used for comparison between two groups. Connection between AK4 expression level and patients' survival time after surgery was performed by Kaplan–Meier method. The categorical data were analyzed by Chi-square test. The value of statistical significant was considered as $P < 0.05$.

Results

AK4 was associated with prognosis of bladder cancer

To identify the role of AK4 in bladder tumorigenesis and explore the clinical relevance, 107 bladder cancer tissues from patients who underwent surgical resection were collected. We then conducted immunohistochemistry assay to detect the expression of AK4, these tumor tissues were then divided into two groups according the expression level of AK4: AK4-low and AK4-high (Fig. 1a). The potential association between AK4 expression and clinical features of bladder cancer was analyzed. We found that AK4-high patients had significant low overall survival rate and progression-free survival rate compared with low-expressed of AK4 group patients (Fig. 1b). These data indicate that the patients in the AK4-high expression group had poor prognosis.

High expression of AK4 predicted the poor clinical outcomes of patients with BC

One hundred seven patients with bladder cancer were collected to investigate the associations between the expression of AK4 and clinical-pathological characters. Immunohistochemistry was performed to evaluate the expression of AK4

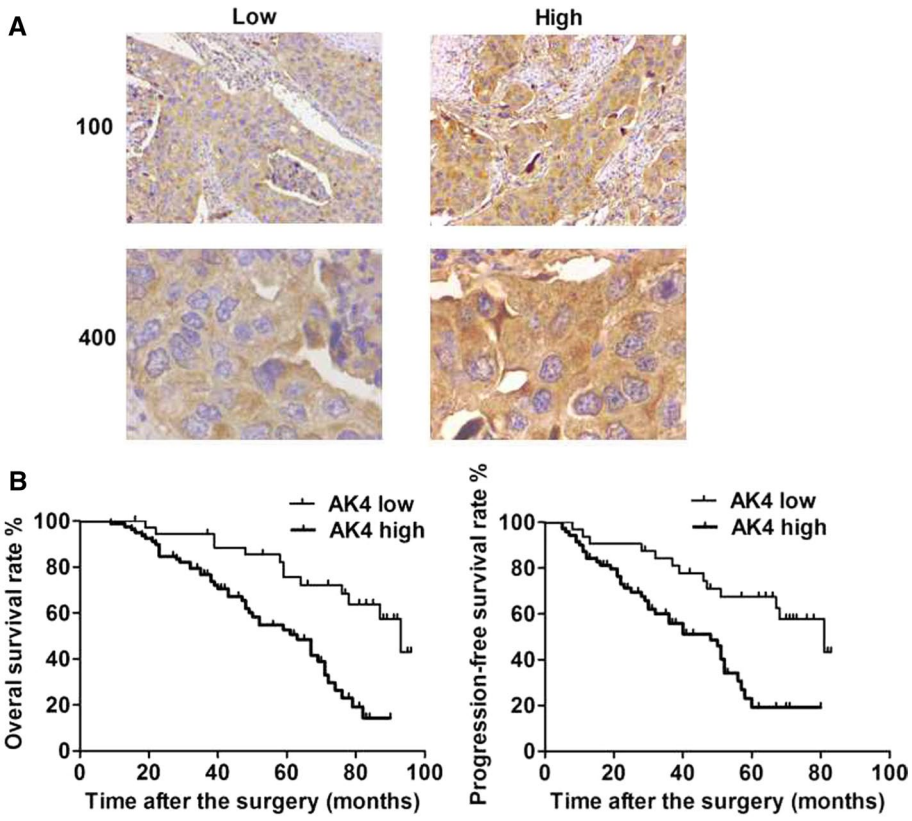

Fig. 1 AK4 expression in tumor tissues was corrected with poor prognosis of patients with bladder cancer. **a** Immunohistochemical staining analysis of AK4 protein expression in bladder cancer tissues (×100 and ×400 magnification, respectively). **b** The difference analysis of overall survival (OS) rate and progression-free survival (PFS) rate between AK4 low and high expression groups

protein in tumor tissues. The results found that AK4 was significantly associated with tumor stage ($P < 0.05$), distant metastasis ($P < 0.05$) and vascular invasion ($P < 0.05$) of BC patients. However, no associations were found between AK4 and other common clinical features such as patients' ages, genders, tumor pathological stages, grades and lymph node metastasis (Table 1). In all, we concluded that AK4 was more likely to be an unfavorable factor, which was associated with the poor clinical outcomes of BC patients.

Down-regulation of AK4 regressed the proliferation of bladder cancer cells

To gain mechanistic insight into the function of AK4 in bladder tumorigenesis, we down-regulated the level of AK4 in the bladder cell line T24 and 5637. The transfection efficiency was detected by the qRT-PCR and western blot analysis. We found that the transfection of AK4 shRNA effectively reduced its mRNA expression level (both $P < 0.05$, Fig. 2a) and protein level (both $P < 0.05$, Fig. 2b) in both T24 and 5637 cells.

On this basis, we explore the effects of AK4 on bladder cancer cell proliferation performing colony formation assays, which confirmed that the proliferation degree was markedly inhibited by AK4 ablation, with decreased cell numbers (both $P < 0.05$, Fig. 3a). Also, we observed a significant dropped OD value of 570 nm in both T24 and 5637 cells (both $P < 0.05$, Fig. 3b).

Finally, to further assess the effects of AK4 depletion on cell proliferation in T24 and 5637 cells, we detected the expression level of Ki67 and PCNA, respectively. We found a markedly decreased expression level of ki67 and PCNA in AK4 shRNA-treated group, compared with control (both $P < 0.05$, Fig. 3c, d).

Table 1 Relationships of AK4 and clinicopathological characteristics in 107 patients with bladder cancer

Feature	All n = 107	AK4 expression		χ^2	P
		Low n = 32	High n = 75		
Age (year)				3.685	0.055
< 65	42	17	25		
≥ 65	65	15	50		
Gender				0.765	0.382
Male	60	20	40		
Female	47	12	35		
Tumor stage				8.793	0.003*
T2	32	16	16		
T3/T4	75	16	59		
Tumor grade				1.299	0.254
Low	35	13	22		
High	72	19	53		
Lymph node metastasis				0.234	0.629
Yes	30	10	20		
No	77	22	55		
Recurrence				0.750	0.386
Yes	57	15	42		
No	50	17	33		
Distant metastasis				4.901	0.027*
Yes	44	8	36		
No	63	24	39		
Vascular invasion				4.424	0.035*
Yes	60	13	47		
No	47	19	28		

*$P < 0.05$

The inhibition of AK4 reduces cell motility of bladder cancer

Since cancer cell motility is requirement for tumor metastasis, we wanted to explore the effects of AK4 ablation on bladder cancer cell migration and invasion. Cell scratch assay was used to study the cell migration changes. Comparing with non-effective scrambled shRNA-treated cells, the AK4 silence cells showed bigger scratch space in T24 and 5637 cell lines, respectively (both $P < 0.05$, Fig. 5a). To investigate the possible role of regulating the cancer cells invasive capacity in stable knockdown ITGB6 cells, cell invasion assays were performed. T24 and 5637 cells showed an obvious low invasive property through the matrigel-coated membranes when transfected with AK4-shRNA, and cell numbers and the OD value of 570 nm were reduced markedly after AK4 ablation (both $P < 0.05$, Fig. 4b). Moreover, to further confirm the decreased cell invasion, we measured the expression levels of migration markers. The expression of matrix metalloproteinase (MMP)-2 and MMP-9 was obviously decreased by knockdown of AK4 in the T24 and 5637 cells, suggesting the inhibition of invasion capacity (both $P < 0.05$, Fig. 4c, d). Taken together, these results have amply demonstrated that AK4 plays a pivotal role in bladder cancer cell invasion.

AK4 ablation inhibited proliferation and metastasis of bladder cancer in vivo

Our in vitro data indicated an important role of AK4 in the proliferation and invasion of bladder cancer cells. To further determine whether inhibition of AK4 could suppress the progression of bladder cancer, an in vivo tumor model injected with T24 cells infected with shControl and shAK4 lentivirus was used. Tumor volumes were measured every 7 days after 2 weeks following cells injection. Six representative tumors from each group were photographed and shown

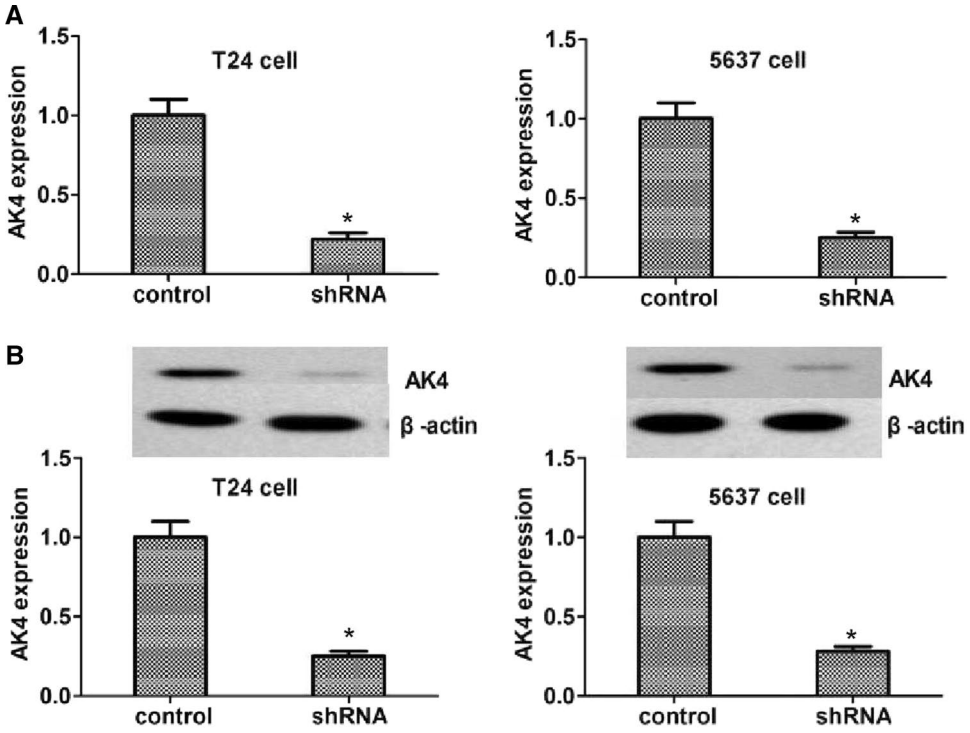

Fig. 2 AK4 was efficiently knockdown in human bladder cancer cells. **a** Quantitative RT-PCR analysis revealed the AK4 expression was efficiently knockdown in the T24 and 5637 cells. **b** Western blot analysis revealed the AK4 expression was efficiently knockdown in the T24 and 5637 cells. The expression of AK4 was dramatically inhibited in shRNA group in both mRNA and protein levels. Results are presented as mean ± SEM, $*P < 0.05$

in Fig. 5a, and the tumor volumes in shAK4 group were significantly smaller than that in control group ($P < 0.05$, Fig. 5a). In addition, we performed lung metastasis assay in mice and found that the incidence of lung metastasis for T24-shAK4 cells was obviously decreased compared with control ($P < 0.05$, Fig. 5b). We then measured the expression of AK4 protein in the tumors, and the western blot and the immunohistochemistry results both proved that the expression of AK4 in sh-AK4 group was markedly reduced compared with control tumor tissues ($P < 0.05$, Fig. 5c). Moreover, we detected the protein expression of Ki67 and MMP9 in the tumors, and the results proved that their expression in sh-AK4 group was markedly reduced compared with control tumor tissues ($P < 0.05$, Fig. 5d). These data confirm that AK4 plays an important role in bladder tumorigenesis by affecting Ki67 and MMP9 in vivo.

Discussion

The precise mechanism of AK4 in cancer development is still unclear. AK4 was reported to promote tumor growth and metastasis in lung cancer in an ATF3-dependent manner [15]. In addition, recent studies have indicated an intricate role for ATF3 in different types of cancer. It has been reported that the expression of ATF3 was detected to be inhibited in prostate cancer [19, 20]. Meanwhile, ATF3 could promote cell migration in breast cancer [21, 22]. On the contrary, the down-regulation of ATF3 in colon cancer promotes tumorigenesis [23, 24]. In this study, we found that AK4 could promote the proliferation of bladder cancer cells in vitro, and the AK4-shRNA-treated nude mice exhibited restrained tumor growth in vivo. We suspect that AK4 promotes proliferation of bladder cancer likely in an ATF3-dependent manner.

Cancer metabolism is considered a sign of cancer because cancer cells could produce and use energy from multiple sources even under anaerobic conditions more efficiently than other cells, so that cancer cells can use energy to maintain an invasive phenotype [25, 26]. In recent studies, AK4 is known to be involved in the regulation of metabolism, and the expression of AK4 was obviously associated with transcriptional regulation and several metabolic processes [27]. It is reported that MMP2 was up-regulated when AK4 was over-expression in lung cancer cells, suggesting an increase in tumor invasion [15]. Consistent with previous studies, we found that AK4 ablation inhibited bladder cancer cell invasion, as well as the remarkably decreased expression of MMP2 and MMP9.

Although AK4 is located in the mitochondrial matrix, whereas, adenylate kinase activity has not been detected in AK4 [28]. It is hard to explore the function, and the enzymatically inactive AK4 is a stress-responsive protein critical to cell survival and proliferation [12]. We also demonstrated that AK4 also plays the similar role in promoting cell proliferation in bladder cancer. It is reported that the interaction with the mitochondrial inner membrane protein ANT is requirement for several functions of AK4, and the

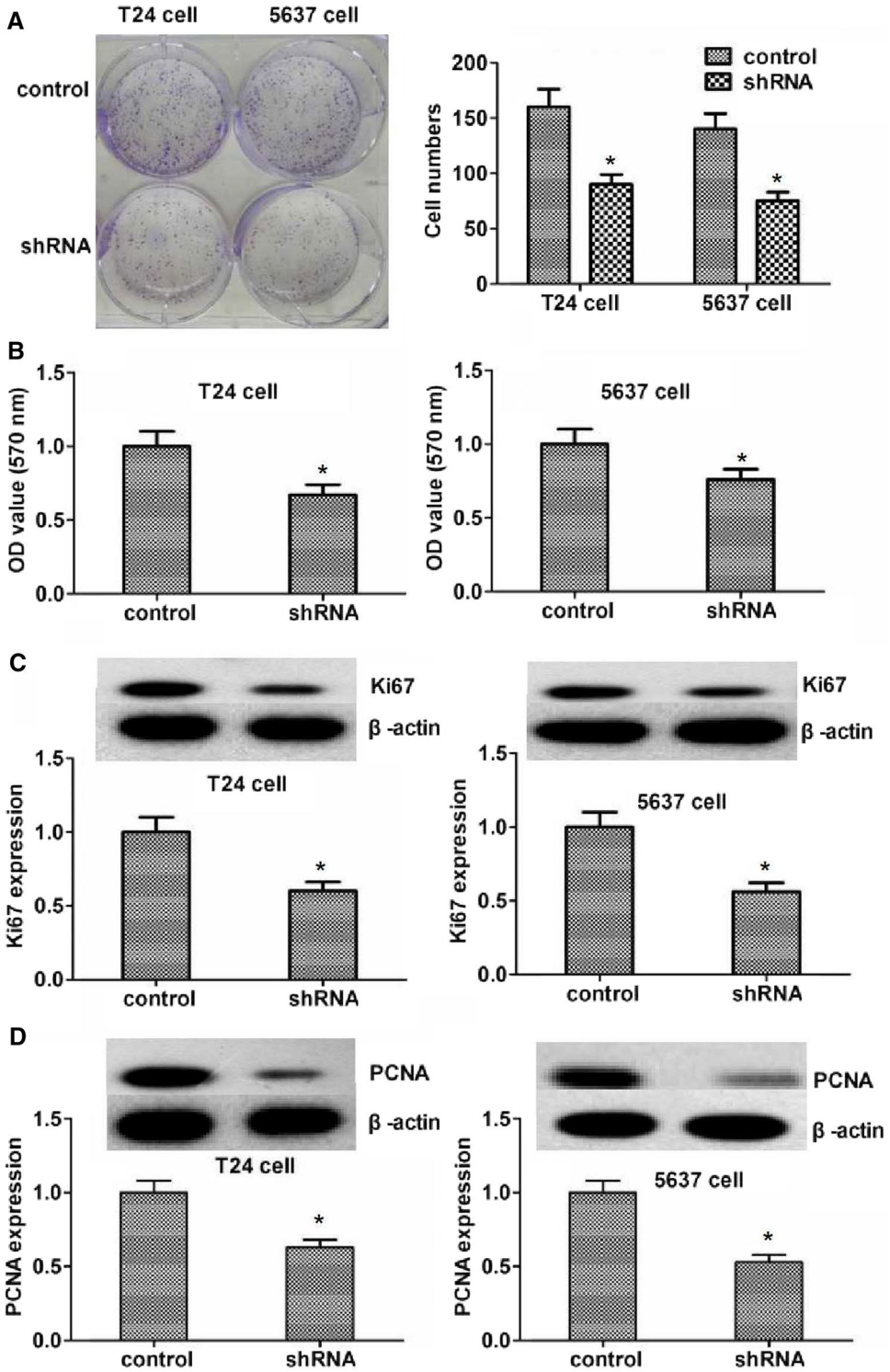

Fig. 3 AK4 depletion significantly reduced colony growth in T24 and 5637 cell lines. **a**, **b** Colony formation and MTT arrays were performed to investigated the proliferation of tumor cells. The numbers of colonies and cells in shAK4 group were significantly decreased compared to controls. **a** Colony formation assay measured T24 and 5637 cell lines that were infected with shControl or shAK4 lentivirus. Cell seed concentration was 500 cells. Cultivation lasted 14 days, respectively. Cell colony numbers were calculated. The numbers of colonies and cells in shAK4 group were significantly decreased compared to controls. **b** MTT arrays were performed to investigate the proliferation of tumor cells. Cell seed concentration was 5000 cells. Cultivation lasted 2 days, respectively. The numbers of colonies and cells in shAK4 group were significantly decreased compared to controls. **c** Western blot analysis of Ki67 and β-actin shRNA-transfected T24 cells and 5637 cells. Western blot analysis revealed the Ki67 expression was obviously down-regulated in T24 and 5637 cells. **d** Western blot analysis of PCNA and β-actin shRNA-transfected T24 cells and 5637 cells. Western blot analysis revealed the PCNA expression was obviously down-regulated in T24 and 5637 cells. Results are presented as mean ± SEM, $^{*}P < 0.05$

underlying mechanism needs further study [12]. Another report demonstrated that Ki67 could be served to be a significant prognostic factor in human bladder carcinoma [29], and our results indicated AK4 could promote cell proliferation and affecting the expression of Ki67 in bladder cancer. Of course, it was better to use the KO of AK4 in cell lines, but now we could not do that in our group. And we could prove our conclusion by our ways. It was difficult for us to have the results of AK4 over-expression in bladder cell lines, which were our future study. In bladder cancer, there have been many published articles related to the genetic polymorphisms of glutathione S-transferase T1 [30, 31], and the

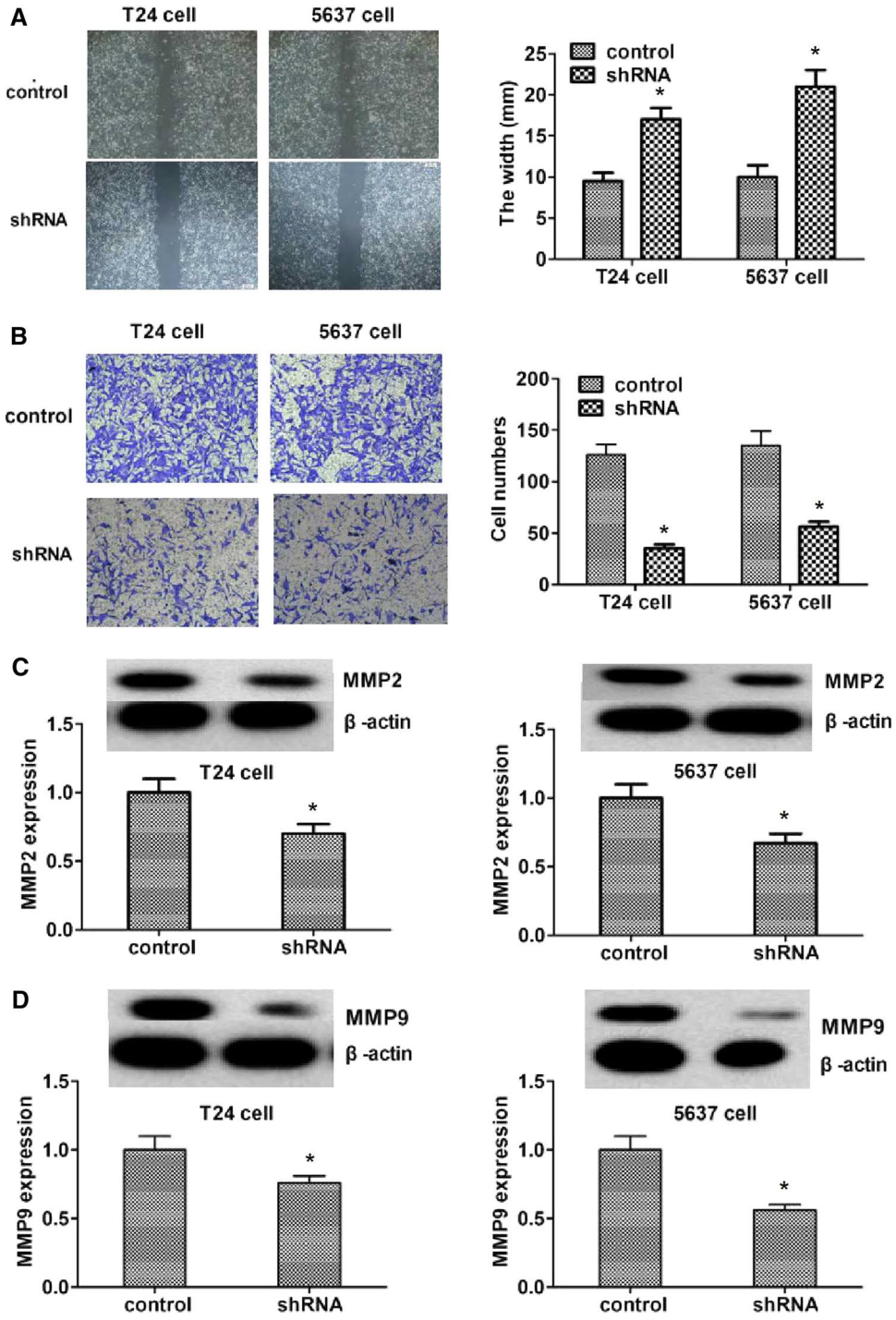

Fig. 4 AK4 knockdown inhibits the invasion of T24 and 5637 cells. **a** Wound healing array showed that AK4 depletion led to bigger width (scratch space) in T24 and 5637 cells (×200 magnification). **b** Transwell migration assays using T24 and 5637 cells transfected with control or AK4 shRNA, and the extent of transwell migration was quantified by cell numbers and the absorbance at the wavelength value of 570 nm. Comparing to controls, the cells of low chamber in shAK4 group were reduced significantly. **c** Western blot analysis of MMP2 and β-actin shRNA-transfected T24 cells and 5637 cells. Western blot analysis revealed the MMP2 expression was obviously down-regulated in T24 and 5637 cells. **d** Western blot analysis of MMP9 and β-actin shRNA-transfected T24 cells and 5637 cells. Western blot analysis revealed the MMP9 expression was obviously down-regulated in T24 and 5637 cells. Results are presented as mean ± SEM, $^*P < 0.05$

polymorphisms of methylenetetrahydrofolate reductase and methionine synthase genes [32], which could be the future study in the mechanism in our study.

In conclusion, the increased AK4 expression in patients with bladder cancer was associated with a poor prognosis. We further found that inhibition of AK4 obviously inhibited bladder cancer growth and metastasis in vitro and in vivo compared with the scramble group. Thus, the results provide strong evidence of the involvement of AK4 in the progression of bladder cancer and suggest that it might serve as a therapeutic target of bladder cancer.

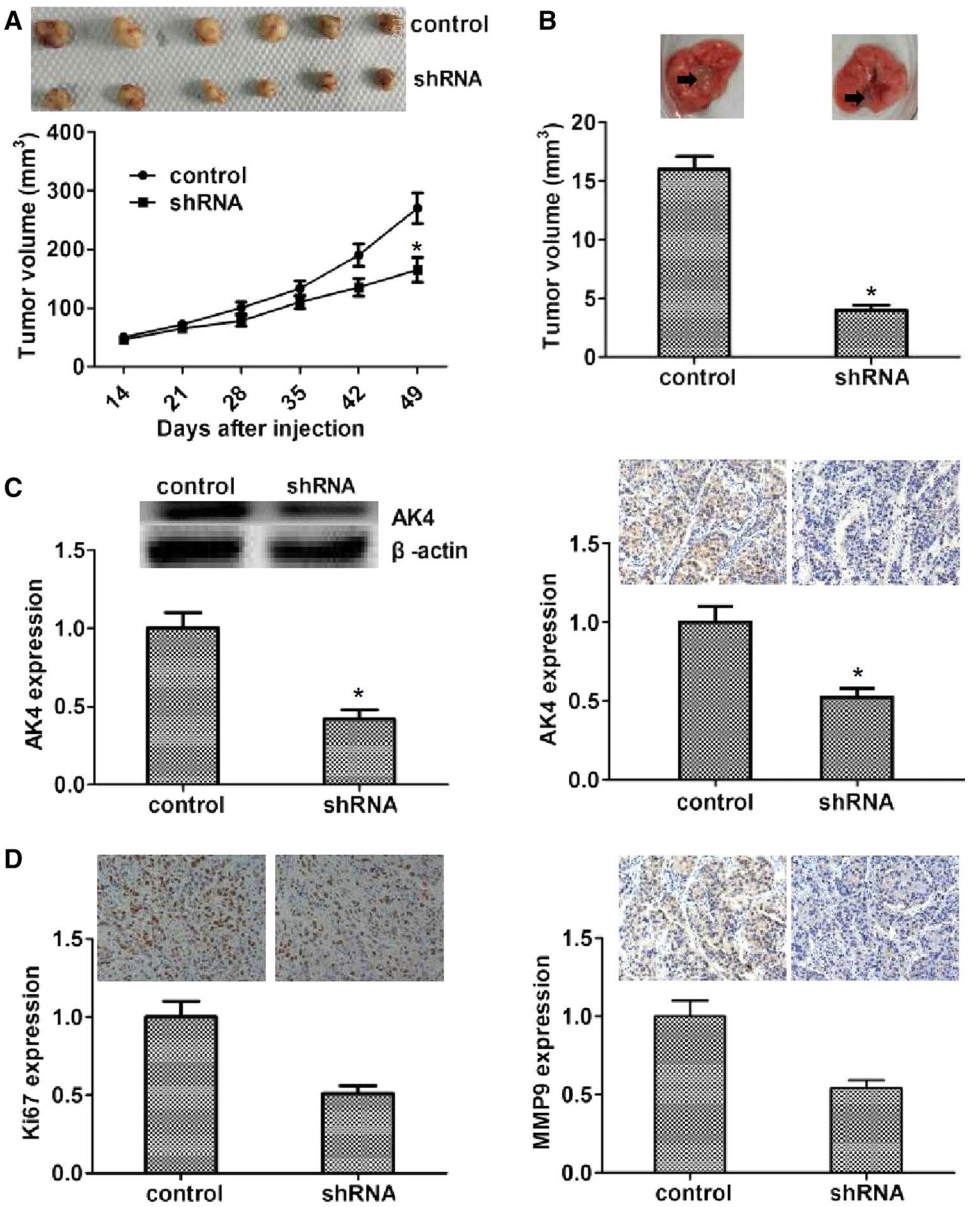

Fig. 5 AK4 ablation represses tumor formation of T24 cells in mice. **a** Subcutaneous tumors in nude mice and isolated tumors after 2 weeks and every 1 week formed by T24 cells infected with shControl and shAK4 lentivirus (n = 6 in each group). The tumors in shAK4 group grew slowly, and tumor sizes were smaller than controls. **b** The images of lung metastasis of lung in the groups of T24 cells transfected with shAK4 or shControl were shown. Tumor growth curves were measured by average volume of 3 tumors for each group. The lung metastatic tumors in different groups were harvested after 4 weeks of feeding. Tumors in shAK4 group were obviously smaller than controls. **c** Western blot analysis and quantified of AK4 and β-actin in shAK4 and shControl groups, and the immunohistochemistry analysis and quantified of AK4 in shAK4 and shControl groups. **d** Immunohistochemistry analysis and quantified of Ki67 and MMP9 in shAK4 and shControl groups. Results are presented as mean ± SEM, $*P < 0.05$

Author's contributions FX and X-DL carried out the experiment and drafted the manuscript. D-WY and LF participated in the design of the study and performed the statistical analysis. J-HL and X-DL participated in its design and coordination and helped to draft the manuscript. All authors have read and approved the final manuscript.

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Availability of data and material The dataset supporting the conclusions of this article is included within the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent for publication All of the authors have agreed to publish this article in your journal if it is accepted.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (include name of committee + reference number) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent Informed consent was obtained from all individual participants included in the study.

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