BMC Urology

RESEARCH

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Cytokeratin 17 activates AKT signaling to induce epithelial-mesenchymal transition and promote bladder cancer progression

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Abstract

Objective Bladder cancer is a common malignant tumor of the urinary tract as well as one of the most common cancers worldwide. Therefore, the study of key molecular targets involved in bladder carcinogenesis and progression is crucial for the prognosis of bladder cancer. Our study aims to investigate the mechanism by which cytokeratin 17 induces epithelial-mesenchymal transition and promotes bladder cancer progression.

Methods In this study, 78 bladder cancer tissue specimens were collected, the expression level of cytokeratin 17 (CK17) in bladder cancer and paracancerous tissues was detected by immunohistochemistry, and the relationship between the CK17 expression level and the prognosis of the patients was analyzed via follow-up visits. Western Blot was performed to detect the expression level of CK17 in common bladder cancer cell lines, and the CK17-silenced and overexpressed cell lines were constructed from the selected T24 cell line with high expression of CK17 and 5637 cell line with low expression of CK17. The effects of CK17 on the proliferation, migration and invasion abilities of bladder cancer cells were evaluated by flow cytometry, Cell Counting Kit-8 (CCK-8) assay, Trans-well assay, and scratch assay. The effect of CK17 on epithelial-mesenchymal transition (EMT) markers was further detected by Western Blot and immunofluorescence, and the phosphorylation levels of AKT Ser473 and Thr308 were detected by Western Blot.

Results In the clinical samples, CK17 expression was significantly up-regulated in cancer tissues compared with paracancerous tissues, and high levels of CK17 indicated shortened progression free survival and predicted a poorer clinical prognosis. By analyzing the relationship between CK17 and clinicopathological features, we found that the CK17 expression level was correlated with bladder cancer grade and TNM stage. Overexpression of CK17 promoted the proliferation, migration and invasion abilities of bladder cancer cells 5637, and silencing of CK17 inhibited the proliferation, migration and invasion abilities of bladder cancer cells T24. Further, we found that overexpression of CK17 in 5637 cells activated the AKT signaling pathway by increasing the phosphorylation level of AKT (Ser473), so as

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to up-regulate the expressions of the EMT mesenchymal markers vimentin, N-cadherin, and the transcription factors Slug and twist, while the opposite results were obtained by silencing CK17 in T24 cells.

Conclusion We found that high expression of CK17 promoted the proliferation, migration and invasion of bladder cancer cells and induced EMT through AKT-Ser473 phosphorylation. These findings suggest that CK17 is significantly associated with malignant progression and poor prognosis of bladder cancer patients, and it may become a new biological target for bladder cancer treatment.

Keywords Bladder cancer, Cytokeratin 17, Prognosis, AKT, Epithelial mesenchymal transformation

Introduction

Bladder cancer is the 10th most common cancer in the world, with approximately 573,000 new cases and 213,000 deaths each year [1]. People over 65 years old are more susceptible to bladder cancer than those who are younger [2], and the incidence of bladder cancer for men is approximately four times higher than that for women worldwide [1, 3]. In China, bladder cancer is the 13th most common tumor, and the incidence and mortality rates of men are 3.8 and 4.0 times higher than those of women. The incidence rate of bladder cancer in urban areas is 1.4 times higher than that in rural areas. The incidence rates in western and central China are close, both lower than that in eastern China. The geographical distribution of mortality rates is correlated to the incidence rate. Both incidence and mortality rates are low until the ages of 45 and 55 years, and then increase rapidly, peaking in the age groups of 80–84 years and over 85 [4]. Therefore, measures should be taken to reveal the molecular mechanisms of bladder cancer, search for more sensitive and specific molecular markers involved in the progression of bladder cancer, and explore their mechanisms of action, which can help improve the early diagnosis and prognosis of bladder cancer.

Keratins include the epidermal keratin (EK) and cytokeratin (CK) according to their locations. Keratin is a subtype of intermediate filament protein, and includes 28 acidic type I proteins and 26 basic type II proteins. Type I keratins (CK17) are mainly expressed in stem cells of the appendages of the skin, embryonic endocervical mucus, or reserve cells of the epithelium, while they are poorly expressed in mature epithelial tissues [5]. These cytoskeletal proteins have cellular and molecular functions, as well as the functions of maintaining cellular structure and morphology and regulating signaling pathways and metabolic processes. Increasing evidences suggest that keratin 17 is an oncogene with abnormal expressions in various human malignancies. For example, keratin 17 promotes the growth of gastric cancer cells and is associated with poor prognosis of gastric cancer [6]. Keratin 17 knockdown significantly inhibits the proliferation, migration and invasion of pancreatic cancer cells [7]. The high expression of keratin 17 in oral cancer tissues is associated with tissue differentiation, which has high expressions in poorly and moderately differentiated tissues [8]. Moreover, keratin 17 has also been found to be closely associated with a variety of cancers, such as colorectal cancer, breast cancer and lung cancer [9–11].

Epithelial-mesenchymal transition (EMT) is an evolutionarily preserved mechanism in tumor metastasis that promotes increased mobility, invasiveness, and resistance to apoptotic signals [12]. According to relevant studies, the expression of CK17 is closely associated with the EMT process, in which the epithelial cells lose apical-basal polarity, thus weakening cell-cell adhesion and leading to tumor cell proliferation, migration, and invasion [13]. CK17 has been shown to up-regulate and promote the activation of AKT signaling and to induce EMT, which can promote the growth, migration, and migration of esophageal squamous cell carcinoma (ESCC) cells [14]. In cervical cancer, forced overexpression of CK17 promoted lymphatic metastasis in vivo [15]. In basal-like breast cancers, CK17 knockdown generated decreased lymph node and lung metastases in vivo. Loss of CK17 moderately reduced the IC_{50} dose of doxorubicin in vitro and led to increased doxorubicin efficacy in vivo [16]. Whether the high expression of CK17 plays an oncogene role in bladder cancer and its regulatory mechanisms are still unknown.

This study aims to investigate the relationship between the expression level of cytokeratin 17 and the patient prognosis, as well as its mechanism of promoting bladder cancer progression. According to our findings, CK17 was significantly up-regulated in bladder cancer tissues, and its expression was correlated with bladder cancer grade and TNM stage, and poor prognosis. As for its mechanism, CK17 can activate the AKT signaling pathway and promote EMT. CK17 plays a crucial role in tumorigenesis and progression of bladder cancer, and may serve as a potential prognostic biomarker and therapeutic target in bladder cancer.

Materials and methods

Collection of clinical specimens

Seventy-eight paraffin specimens of bladder cancer tissues were selected from Affiliated Hospital of Hebei University from 2019 to 2022, and CK17 immunohistochemical staining was performed on the cancerous

tissues and adjacent non-cancerous tissues. The results were examined by a diagnostic pathologist, with the following criteria: the intensity of the immunostaining was defined as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), and according to the proportion of the positive cells, it was classified into 0 (< 5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (> 75%). The value obtained by multiplying these two scores is the final score for each case. The cases with a score less than 6 were considered to have low CK17 expression, and those with a score greater than or equal to 6 were considered to have high CK17 expression, and the correlation between the clinicopathological features and the CK17 expression was analyzed statistically in conjunction with clinical follow-up records. All bladder cancer patients were primary bladder cancer patients, none of them had received any other surgery, radiotherapy and other treatments before surgery, these patients had no special underlying diseases, and none of them had been taking oral immunosuppressants for a long time. This study was approved by the Ethics Committee of the Affiliated Hospital of Hebei University(Ethics Approval Number: 2019 – 0115). Written informed consent was obtained from all patients for the usage and publication of their clinical data.

Cell culture

Human Bladder cancer cell lines (including BIU87, HT1376, T24, 5637, J82, and RT112) were purchased from the Type Culture Collection (Shanghai, China) at the Chinese Academy of Sciences. All cell lines are authenticated by STR profiling and tested for mycoplasma contamination. The 5637 and RT112 cells were cultured in 1640 medium 10% fetal bovine serum (FBS) (Gibco, USA), and other cells were cultured using modified Eagle medium (DMEM) of Dulbecco (Thermo, USA) with 10% fetal bovine serum (Biological Industries, Israel) and 1% penicillin-streptomycin-neomycin (Gibco-BRL Life Technologies, Grand Island), and incubated in a 5% CO_2 incubator at 37 °C.

Table 1 Antibody information

Protein	Primary antibody	Manufacturer / Item No.
CK17	Recombinant Anti-Cytokeratin 17 antibody [EPR1624Y]	Abcam; ab51056
vimentin	Recombinant Anti-Vimentin antibody [EPR3776]	Abcam; ab92547
N-cadherin	Anti-N Cadherin antibody [5D5]	Abcam; ab98952
twist	Anti-Twist antibody [10E4E6]	Abcam; ab175430
slug	Recombinant Anti-SLUG antibody [EPR25113-46]	Abcam; ab302780
P-AKT-T308	Anti-AKT (phospho T308) antibody	Abcam; ab38449
P-AKT-S473	Recombinant Anti-AKT1 (phospho S473) antibody [EP2109Y]	Abcam; ab81283

Immunohistochemistry (IHC)

The expression levels of CK17 in bladder cancer tissues and paracancerous tissues were detected by immunohistochemistry. The selected pathological tissues of bladder cancer were fixed in formalin for 48 h, and then prepared into paraformaldehyde-fixed paraffin-embedded 4-μm sections, infiltrated with xylene for deparaffinization, and had the xylene washed off. The sections were placed in buffer, heated, and then infiltrated in 3% hydrogen peroxide solution for antigen repair and quenching. Next, the specimens were incubated with primary antibody (Cytokeratin 17 antibody, Gene Tex, GTX103765, USA), and secondary antibody (goat anti-rabbit IgG H&L (HRP), Abcam, ab205718, UK). Finally, staining and observation were performed.

Western blot (WB)

Whole cell lysates were extracted using RIPA (P0013B, Beyotime, China) lysis buffer. The BCA protein assay kit (71285-M, Thermo, USA) was used to measure the protein concentration. The specimens were diluted in 5× SDS uploading buffer, separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, USA), and then, they were closed in 5% skimmed milk powder at room temperature. Next, the membranes were incubated with primary antibody at 4 °C overnight, and then incubated with secondary antibody (Goat Anti-Rabbit IgG H&L (HRP), ab6721, Abcam, UK). See Table 1 for the information of primary antibodies. The ECL chemiluminescent chromogenic solution was used for developmental exposure of PVDF membranes. After scanning by the Invitrogen iBright gel imaging system (iBright CL1500, Thermo, USA), grayscale analysis of the imprinted areas was performed using the ImageJ image analysis software.

Immunofluorescence

Cells were fixed using 4% paraformaldehyde and deparaffinized. Then, the cells were repermeabilized with 0.1% TritonX-100 for 10 min, sealed for 1 h using 10% goat serum at room temperature, and incubated with primary antibody. After washing with PBS, the cells were incubated with secondary antibody for 1 h at room temperature in the dark. Finally, the cell nuclei were stained with DAPI solution for 10 min, and the images were captured using a laser confocal fluorescence microscope (Olympus, Tokyo, Japan). Primary antibodies (Abcam, UK): Vimentin: Anti-Vimentin antibody (ab137321); N-Cadherin: Anti-N-Cadherin antibody (ab18203); Twist: Anti-Twist antibody [10E4E6] (ab175430); Slug: Anti-SLUG antibody [GTX32879], (GeneTex, USA). Secondary antibody: goat anti-rabbit IgG H&L (Alexa Fluor® 488), (ab150077, UK).

Wound-healing assay

The bladder cancer cells of the logarithmic phase were taken, inoculated in the 6-well plate, with about 5×105 cells per well, and divided into the OE-Control group, OE-CK17 group, sh-Control group and sh-CK17 group. On the next day, a straight line of uniform thickness was lightly scratched on the bottom of the 6-well plate with a 10 μL sterile tip using uniform strength with uniform angle (3 replicates were set up for each group). The width of the scratch in the same field of view was observed and photographed under an inverted phase contrast microscope at 0 h and 24 h, respectively, the healing distance of the scratch was calculated [△d (μm) = (width of the scratch at 0 h - width of the scratch at 24 h)/ width of the scratch at 0 h], and the healing distance of the scratch was used to represent the migration ability of the cells.

Trans-well

The pre-cooled matrigel at 4 ℃ was diluted in 1:5, then added to the upper chamber of Trans-well, spread evenly and dried at 37 ℃ for 70 min; 5 replicate wells were set up in each group, and the cell concentration was adjusted to 5×10^5 cells per ml; 200 μL of cell suspension was added to the upper chamber, and 500 μL of medium containing 20% FBS was added to the lower chamber as chemokines. The small chambers were cultured in 5% CO_2 at 37 ℃ for 24 h. The unmetastasized cells in the upper chamber were wiped off using cotton swabs, and the chambers were rinsed in PBS; the cells were fixed in methanol, and stained with 0.1% crystal violet. The cells in 3 fields of view per well were selected and photographed under an optical microscope, and the number of cells in each group was counted.

Cell counting Kit-8 (CCK-8)

The bladder cancer cells at logarithmic phase were taken, the cell density was adjusted to 2×10^4 cells/mL, and the cells were inoculated in the 96-well plate, with 100 μL per well. Then, the cells were incubated in the incubator of 5% CO_2 at 37 °C for 24 h, 48 h, and 72 h, respectively. Next, the CCK-8 solution of concentration 10 μL (MBS2557034, MyBioSource, USA) was added to each well, and the plate was incubated at 37 °C for 2 h. The absorbance value at 450 nm was measured by ELIASA (TECAN, infinite M200 pro).

Flow cytometry

The cells in the logarithmic phase were inoculated into the 6-well plate with three replicates, after digestion with trypsin, the cells were collected and counted, and approximately 1×10^5 cells were collected. Then, the cells were resuspended after centrifugation. Next, 5 μL of Annexin V-FITC and 5 μL of propidium iodide were added, and after mixing gently, the cells were placed at room temperature away from light for 15 min. Apoptosis was detected by flow cytometry (BD, BD Canto, USA), the analyzed data were recorded, and the apoptosis rate was calculated.

Hematoxylin and Eosin (H&E) staining

The bladder tissues were embedded in paraffin, made into paraffin blocks, and cut into 5 μm sections for deparaffinating and rehydration; then, the cells were stained with hematoxylin solution and re-stained with eosin solution. The stained specimens were observed under an optical microscope (Nikon. Japan), and then analyzed histopathologically.

Xenograft mouse model experiment

The female BALB/c SPF-grade nude mice ($n = 22$) used in this experiment were purchased from Sipeifu (Beijing) Biotechnology Co., Ltd., with an average weight of 22 g ± 2 g. The mice were maintained under standard conditions in an animal facility: temperature of 22 ± 1 °C, relative humidity of 50–70%, a 12-hour light/dark cycle, and bedding changed 2–3 times per week. The mice had free access to food and water. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. After a 1-week acclimatization period, the mice were randomly divided into 4 groups ($n = 5$). Cell suspensions of OE CK17, OE NC, sh NC, and sh CK17 were prepared. The dorsal skin of the nude mice was sterilized, and the cell suspension (approximately 1×10^7 cells) was injected subcutaneously into the axillary region. Seven days post-injection, palpable nodules were observed at the injection sites. After 28 days, the mice were euthanized by cervical dislocation, the tumors were excised and weighed, and the longest (a) and shortest (b) diameters of the tumors were measured. Tumor volume was calculated using the formula: $V = a^*b^2/2$.

Statistical analysis

The collected data were analyzed for significance and plotted using the GraphPad Prism 8 (San Diego, CA, USA) software. All data are expressed in terms of mean ± standard deviation. The statistical differences between the two groups were compared via the t-test, and one-way ANOVA for multiple group analyses. The relationship between the high expression of CK17 and the clinicopathologic features was compared by the Chi-Squared Test. The survival analysis was performed using the Kaplan-Meier and log-rank tests. When $P<0.05$, it is considered statistically significant.

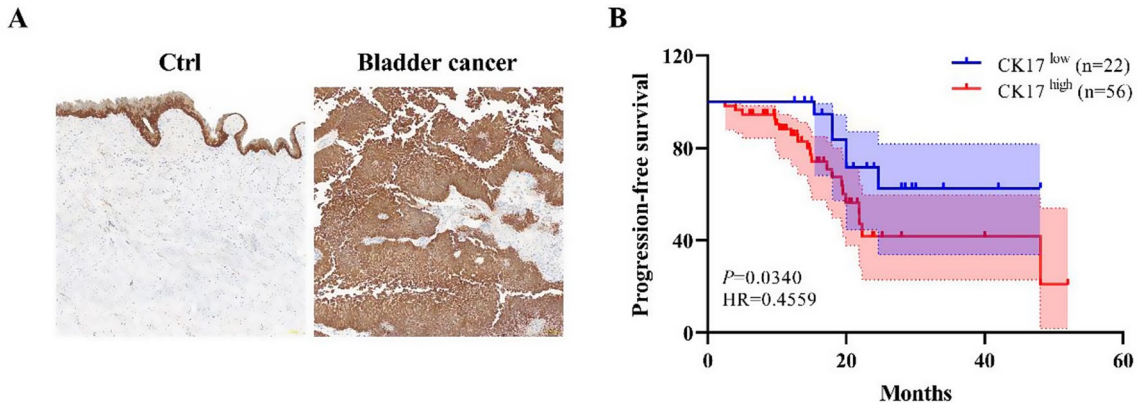

Fig. 1 High expression of CK17 in bladder cancer tissues and associated with poor prognosis. **A**: Immunohistochemical staining using anti-CK17 antibody, amplification, 10 ×; **B**: Difference in progression-free survival (PFS) between patients with high and low CK17 expressions. *P*<0.05, significance

Table 2 Clinicopathologic feature analysis of CK17 expression in bladder cancer patients

Characteristics	CK17 expression level		*p*
	High expression	Low expression	
Age			
<65	9(11.5%)	29(37.2%)	0.3871
≥65	13(16.7%)	27(34.6%)	
Gender			
Male	11(14.1%)	27(34.6%)	0.8871
Female	11(14.1%)	29(37.2%)	
T stage			
T1	7(9%)	4(5.1%)	0.0251
T2	3(3.8%)	12(15.4%)	
T3	11(14.1%)	30(38.5%)	
T4	1(1.3%)	10(12.8%)	
N stage			
N0	8(10.3%)	6(7.7%)	0.0391
N1	9(11.5%)	30(38.5%)	
N2	2(2.6%)	14(17.9%)	
N3	3(3.8%)	6(7.7%)	
M stage			
M0	10(12.8%)	12(15.4%)	0.0338
M1	12(15.4%)	44(56.4%)	
Tumor grade			
Grade I	5(6.4%)	3(3.8%)	0.0412
Grade II	8(9%)	17(24.4%)	
Grade III	5(11.5%)	29(37.2%)	
Grade IV	4(1.3%)	7(6.4%)	

Results

Up-regulated expression of CK17 in bladder cancer tissues is positively correlated with poor prognosis of patients

First, we evaluated the differences in the expression levels of CK17 in cancerous tissues and corresponding paracancerous tissues of bladder cancer patients. As shown in Fig. 1A, more intact tissues could be seen in the paracancerous tissues, and it was observed that CK17 was expressed in the epithelial tissues of paracancerous tissues, but its expression was extremely low in the cells of the other tissues; compared with the paracancerous tissues, the expression level of CK17 was obviously higher in the bladder cancer tissues. To determine the prognostic impact of CH17 on bladder cancer patients, the Kaplan-Meier curve was used to analyze the relationship between the CK17 expression level evaluated by immunohistochemistry and the patient survival. As shown in Fig. 1B, the progression-free survival of bladder cancer patients with high CK17 expression was significantly shorter than that of patients with low CK17 expression ($p = 0.0340$). To sum it up, these results suggest that the CK17 expression was upregulated in bladder cancer tissues, CK17 may be a tumor promoter, and its increased expression may contribute to the malignant progression of bladder cancer.

Correlation between high expression of CK17 and clinicopathologic features

To demonstrate the clinical significance of CK17 upregulation in bladder cancer, we analyzed the clinicopathologic feature of tumor samples from 78 patients with bladder cancer. Of the 78 patients, 38 (48.7%) were male and 40 (51.3%) were female; 38 cases (48.7%) were <65 years old, and 40 cases (51.3%) were ≥65 years old. The results of clinicopathological analysis are shown in Table 2. We found that high CK17 expression was correlated with the clinical grading stages of bladder cancer patients, and the high CK17 expression was significantly correlated with the extent and size of the primary tumor T stage, ($p = 0.0107$), metastasis (M stage, $p = 0.0338$), lymph node metastasis (N stage, $p = 0.0391$), and tumor grade ($p = 0.0412$), while the CK17 expression level was not associated with gender ($p = 0.8871$) or age ($p = 0.3871$).

High expression of CK17 promotes proliferation of bladder cancer cells

Given the correlation between high CK17 expression levels and tumor grading & TMN staging, to further understand the cell biological functions of CK17, we verified the effects of overexpressing and silencing CK17 on apoptosis and proliferation. First of all, we analyzed the differences in CK17 expression levels in different cell lines (Fig. 2A and B), selected the bladder cancer cell line T24 with high CK17 expression for CK17 gene silencing, and overexpressed the CK17 low-expressing cell line 5637, the validations of overexpression and silence efficiency are shown in Figure S1. Then, apoptosis was detected by flow cytometry, as shown in Fig. 2C and D. Compared with the control group, overexpression of CK17 significantly increased the apoptosis rate of 5637 cells, and silencing CK17 inhibited apoptosis of cell line T24. Further, we detected the cell viability using the CCK-8 kit at 0 h, 24 h, 48 h, 72 h and 96 h, respectively (Fig. 2E and F), and found that overexpression of CK17 significantly increased the cell viability of bladder cancer cells 5637 and silencing of CK17 inhibited the cell viability of bladder cancer cells T24. The results indicate that overexpression of CK17 in bladder cancer cells could promote the proliferation of bladder cancer cells.

High expression of CK17 promotes migration and invasion of bladder cancer cells

Next, we explored the effect of CK17 expression level on the migration and invasive of bladder cancer cells, and we performed scratch assay and Trans-well assay. The results of the scratch assay are shown in Fig. 3A. Compared to the control group, the migration ability of 5637 cells with overexpressed CK17 significantly increased, while the migration ability of T24 cells with silenced CK17 was significantly reduced (Fig. 3B). Detection of cell invasion by Trans-well showed that the overexpression of CK17 in 5637 cells significantly enhanced cell invasion, and silencing of CK17 in T24 cells significantly reduced cell invasion (Fig. 3C and D). The above results indicate that

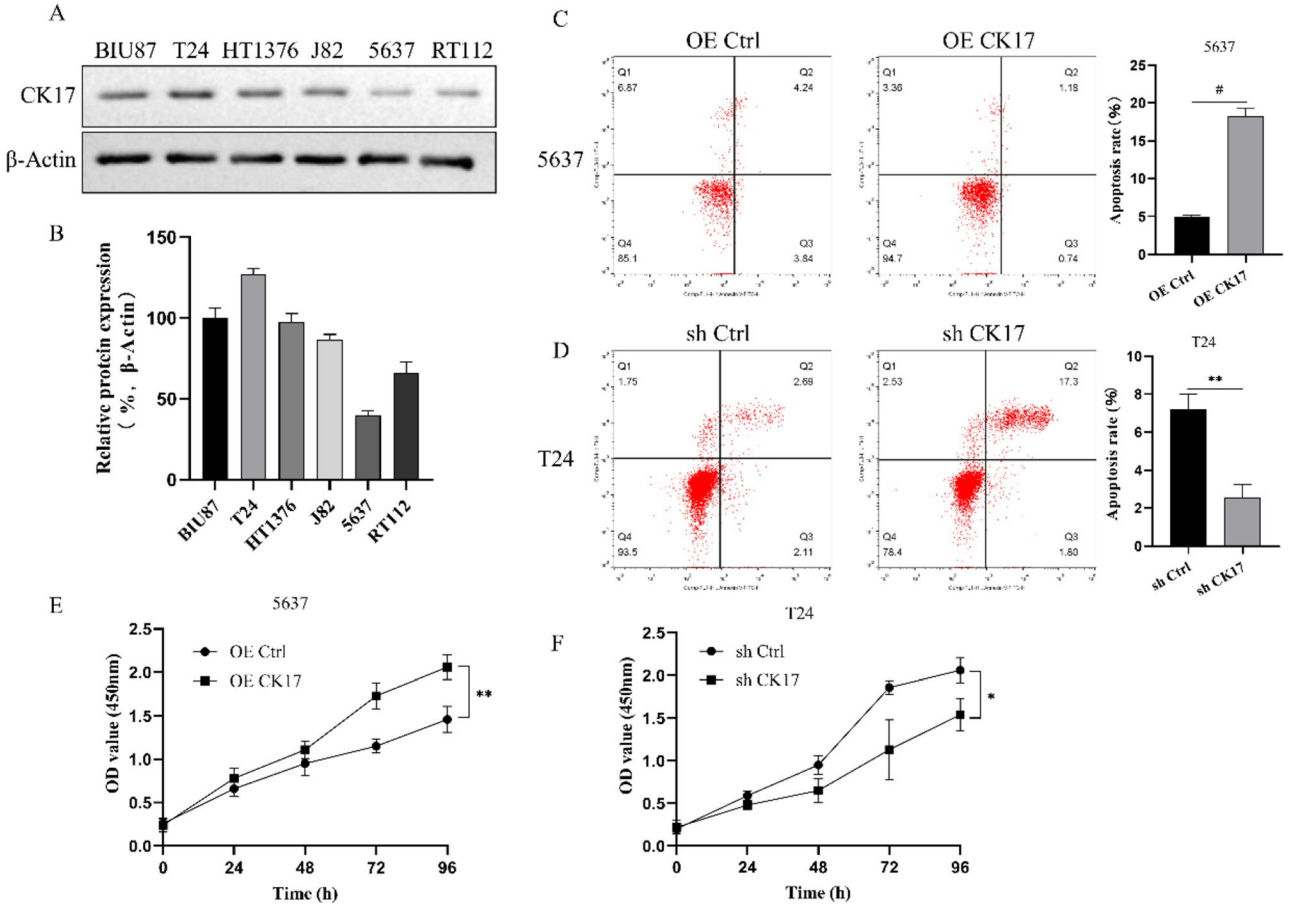

Fig. 2 High expression of CK17 promotes cell proliferation. **A**: Western Blot detection of CK17 protein expression bands in common bladder cancer cell lines; **B**: Gray scale values of protein expression of common bladder cancer cell line CK17; **C**、**D**: Overexpressing CK17 in bladder cancer cells 5637, silencing CK17 in T24 cells, and apoptosis detected by flow cytometry; **E**、**F**: CCK8 detection of cell viability changes after overexpression and silencing of CK17. *: $p<0.05$; **: $p<0.01$; #: $p<0.0001$

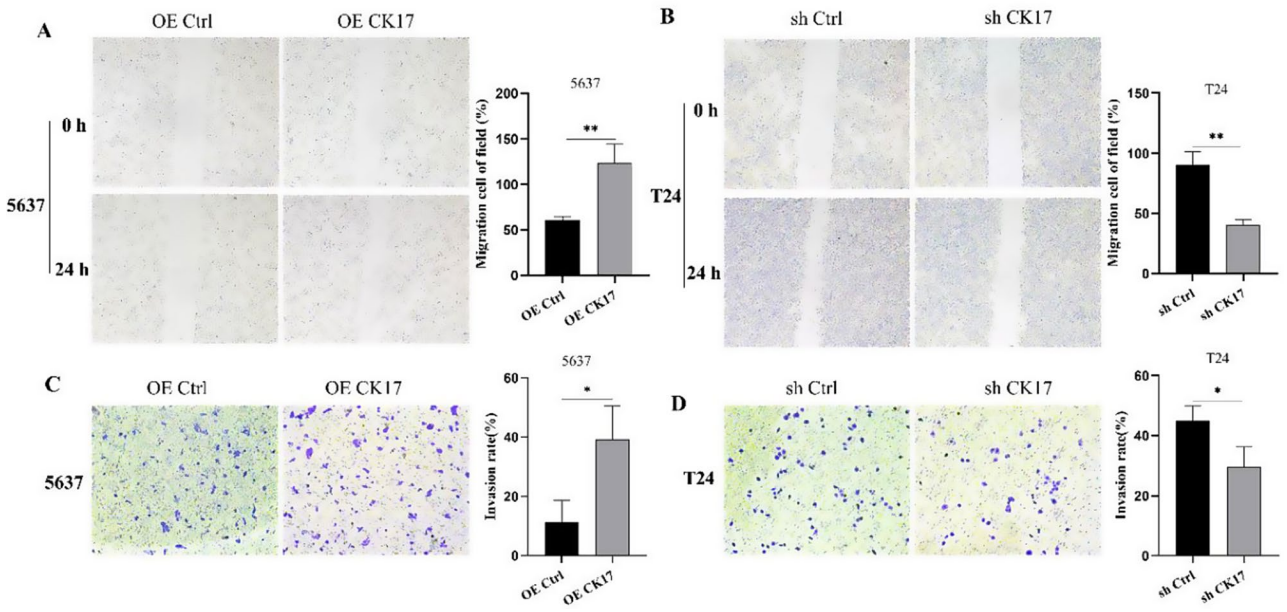

Fig. 3 Effects of CK17 on migration and invasion of bladder cancer cells. **A**: Effect of overexpressing CK17 in 5637 cells on the migration ability of bladder cancer cells detected by scratch assay, amplification, 10 ×; **B**: Effect of silencing CK17 in T24 cells on the migration ability of bladder cancer cells detected by scratch assay, amplification, 10 ×; **C**: Effects of overexpressing CK17 in 5637 cells on cell invasion ability detected by Trans-well assay, amplification, 10 ×; **D**: Effect of silencing CK17 in T24 cells on cell invasion ability detected by Trans-well assay, amplification, 10 ×. *: $p<0.05$; **: $p<0.01$

the high expression of CK17 could promote the migration and invasion of bladder cancer cells.

CK17 induces EMT by activating the AKT signaling pathway

EMT is a key step in the tumor migration process, which provides the migratory and invasive properties of tumor cells. Therefore, we tried to determine whether CK17 is also involved in the regulation of EMT in bladder cancer cells, so we examined the expression levels of EMT marker proteins by immunofluorescence and Western Blot. The results of the Western Blot assay are shown in Fig. 4A and B. The mesenchymal stromal cell markers vimentin and N-cadherin and the protein levels of EMT transcription factors Slug and twist were significantly increased in 5637 cells with overexpressed CK17. On the other hand, the protein levels of mesenchymal cell markers vimentin and N-cadherin and EMT transcription factors Slug and twist were significantly decreased in T24 cells with silenced CK17 (Fig. 4A and C). The results indicate that overexpression of CK17 promoted EMT in bladder cancer cells, while silencing of CK17 inhibited EMT.

To further explore the molecular mechanism of CK17-mediated EMT, we verified the molecular signaling pathway AKT that plays an important role in cancer cell metastasis. According to the results of Western blot analysis, compared with the control group, the phosphorylation level of AKT-Ser473 was significantly increased in 5637 cells with overexpressed CK17, and there was no significant difference in the phosphorylation level of AKT-Thr308 (Fig. 4D and E). The phosphorylation level of pAKT-S473 in T24 cells with silenced CK17 decreased, and there was no significant difference in the phosphorylation level of AKT-Thr308 (Fig. 4D and E). These results suggest that CK17 could induce EMT by activating AKT-S473 phosphorylation in bladder cancer cells.

We have demonstrated that CK17 could induce EMT by activating phosphorylation of AKT-S473. To further prove the reliability of our results, we observed the histopathological situation of the control group and bladder cancer tissues by H&E staining, and the results are shown in Fig. 5 (left). According to Fig. 5, the normal tissues of the control group were well-layered, with uniform distribution of nuclei, whereas the cancerous tissues were tightly packed and with damaged nuclei in an irregular state. Further we detected the protein expression levels of mesenchymal cell markers vimentin and N-cadherin and the EMT process transcription factors Slug and Twist in normal tissues and bladder cancer tissues by immunofluorescence. The results were shown in Fig. 5, in which, the protein levels of vimentin (red), N-cadherin (green), Slug (red) and Twist (green) in cancer tissues were significantly higher than those in normal tissues. Twist is a highly conserved transcription factor, which can activate the mesenchymal cell markers and promote EMT; Slug can inhibit the expressions of E-calmodulin, tight junction proteins and epithelial markers, promote EMT and enhance cell metastasis; Vimentin and N-cadherin are

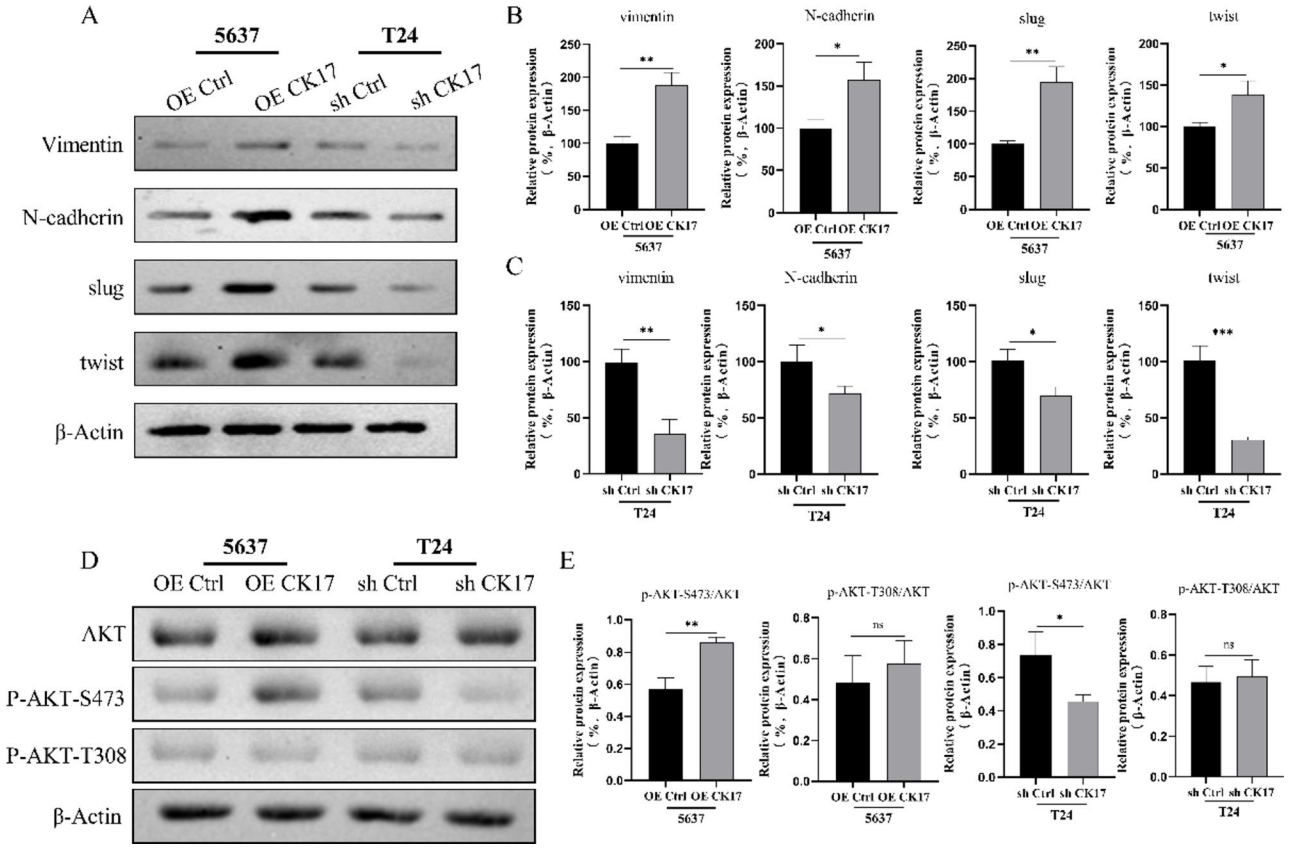

Fig. 4 CK17 induces EMT by activating the AKT signaling pathway. **A**, **B** & **C**: Western blot detection of the expression levels of EMT marker proteins in 5637 cells with overexpressed CK17 and T24 cells with silenced CK17; **D** & **E**: Western blot detection of the phosphorylation levels of Ser473 and AKT-Thr308 in 5637 cells with overexpressed CK17 and T24 cells with silenced CK17. ns: no significance, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

mesenchymal cell markers, all of which imply the EMT process. The results indicate that EMT was activated in bladder cancer tissues, and also further suggest that high CK17 expression was associated with the induction of EMT and the malignant progression of tumor.

Silencing AKT could inhibit EMT caused by overexpressed CK17

To further prove that CK17 could induce EMT by activating the AKT signaling pathway, we silenced AKT on the basis of overexpressing CK17 in bladder cancer cells 5637. According to the results, overexpression of CK17 in 5637 cells significantly increased the protein expression levels of the mesenchymal cell markers vimentin and N-cadherin and the transcription factors Slug and Twist. However, in the 5637 cells with overexpressed CK17 and silenced AKT, the protein expression levels of mesenchymal cell markers vimentin and N-cadherin and the transcription factors Slug and Twist were significantly reduced (Fig. 6A and B). The results suggest that overexpression of CK17 in 5637 cells promoted EMT, but silencing of AKT inhibited EMT induction by overexpressed CK17. Therefore, we believe that CK17 plays an important role in the EMT process by activating the AKT pathway.

We evaluated the impact of CK17 on tumorigenesis in vivo through a xenograft mouse model. As shown in Fig. 7, the tumor weight of subcutaneous grafts in the CK17 OE group was significantly higher than that of the control group (OE NC), $p<0.01$. In contrast, the tumor weight of subcutaneous grafts in the sh CK17 group was significantly lower than that of the control group (sh NC), $p<0.05$. A similar result was observed in tumor volume measurements, where the tumor volume of subcutaneous grafts in the CK17 OE group was significantly higher than that of the control group (OE Ctrl), $p<0.001$. In contrast, the tumor volume of subcutaneous grafts in the sh CK17 group was significantly lower than that of the control group (sh NC), $p<0.05$. These results suggest that CK17 promotes the growth of subcutaneous grafts in mice.

Discussion

With the development of modern medicine and molecular biology, searching for specific molecular targets has become hot research in cancer treatment or evaluating

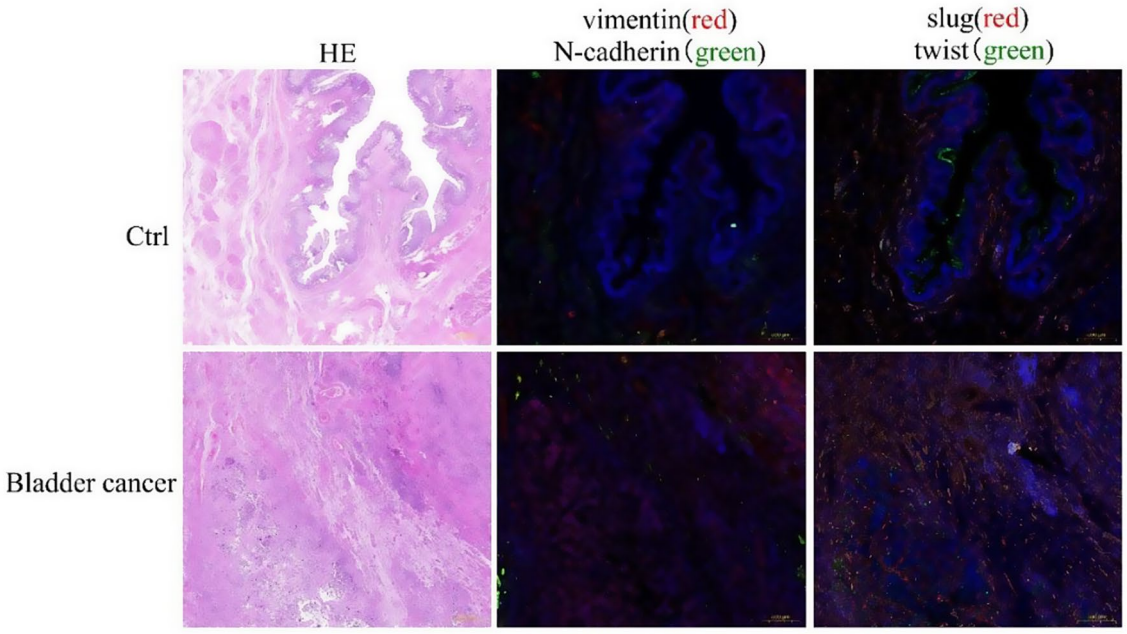

Fig. 5 Analysis of different expression levels of EMT marker proteins in normal paracancerous tissues and bladder cancer tissues. H&E staining was performed to analyze the pathological conditions of paracancerous tissues and normal tissues; immunofluorescence was performed to detect the protein expression levels of Vimentin (red), N-cadherin (green), Slug (red) and Twist (green) in normal paracancerous tissues and bladder cancer tissues. Amplification, 2 ×

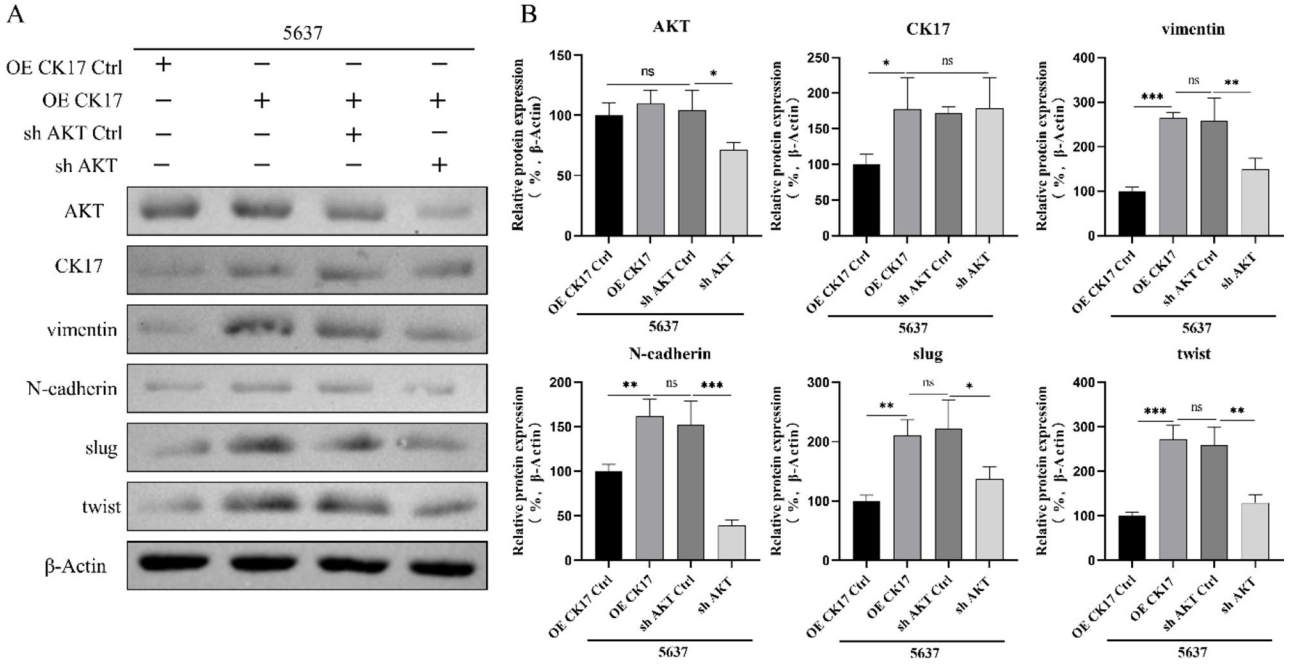

Fig. 6 Effect of silencing AKT on the expression level of EMT markers. **A**: Detection of relevant protein expression bands by Western Blot in the 5637 cells with overexpressed CK17 and the 5637 cells with overexpressed CK17 and silenced AKT; **B**: Protein band gray values of the 5637 cells with overexpressed CK17 and the 5637 cells with overexpressed CK17 and silenced AKT. ns: no significance, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

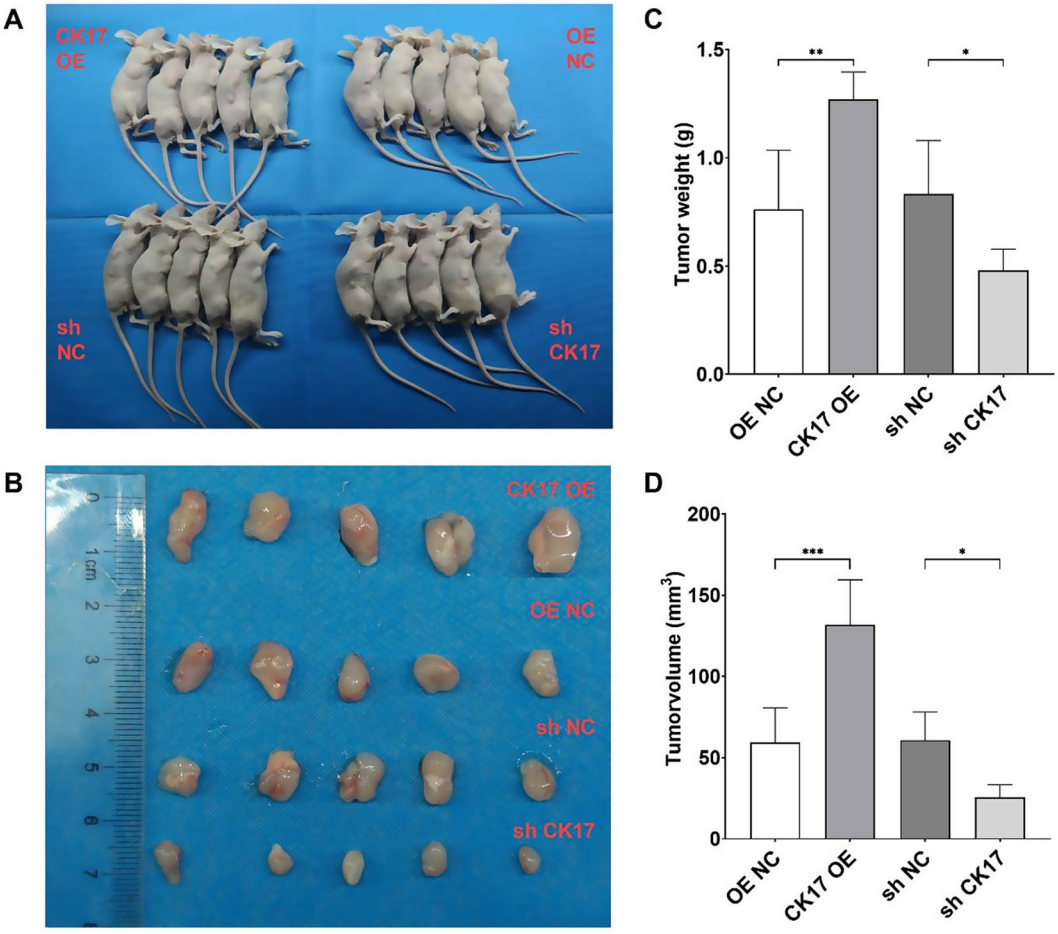

Fig. 7 CK17 promotes tumor growth in mice. **A**: Photographs of nude mice; **B**: Photographs of tumor in vivo; C: Statistical results of tumor weight; Statistical results of tumor volume; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$

cancer prognosis, and remarkable results have been achieved. Such as the prognostic value of HPV DNA positivity in urothelial carcinoma of the bladder [17]. Despite significant progress in investigating the biological targets of bladder cancer, the prognosis for patients with this aggressive tumor remains poor, and it is difficult to achieve substantial progress in its treatment.

Our study intends to explore the possibility of using keratin 17 as a biological marker of bladder cancer and its effective diagnostic and therapeutic target. First, we analyzed the different expression levels of CK17 in cancerous tissues and corresponding paracancerous tissues of bladder cancer patients, and the results show that the expression level of CK17 in bladder cancer tissues was significantly higher than that in paracancerous tissues, which are consistent with the findings of Chen Li et al. [18]. Sruthi Babu et al. have also reported that keratin 17 can be used as a novel cytological biomarker for diagnosis of uroepithelial carcinoma [19]. Furthermore, our data confirmed that the up-regulation of CK17 in bladder cancer tissues was positively correlated with tumor grade and TMN stage. According to the survival analysis, the bladder cancer patients with elevated CK17 expression had significantly shorter progression-free survival than those with low CK17 expression levels, implying that CK17 may be involved in bladder cancer progression. CK17 plays a critical role in cell viability and a variety of biological processes, and abnormal up-regulation of CK17 has been detected in many diseases, ranging from traumatic injuries to malignant tumors [11, 20–22]. Numerous studies have shown that proliferation, migration and invasion of tumor cells indicate poor prognosis for cancer patients[19]. The proliferation, migration and invasion of bladder cancer cells into surrounding tissues and lymph are the main factors causing the progression of bladder cancer [23]. The regulation of many upstream factors in the core signaling pathways of bladder cancer will ultimately affect the genes that play important roles in cell migration and invasion [24, 25]. According to relevant reports, keratin 17 is overexpressed in malignant lesions of a variety of tumors [11, 14, 26], suggesting that keratin 17 may be a factor mediating tumor proliferation,

migration, and invasion. To further confirm the role of CK17 in bladder cancer cells, we established stable CK17 silencing and overexpression in bladder cancer cells for analysis. We found that the 5637 cells with overexpressed CK17 had reduced apoptosis, increased cell viability, and significantly increased migration and invasion ability. Silencing CK17 would result in opposite results. Therefore, CK17 plays a crucial role in promoting proliferation, migration and invasion of bladder cancer cells.

Several pathways are known to play important roles in tumor progression. For example, the AKT signaling pathway is one of the major growth regulatory pathways of normal and cancer cells, and AKT is involved in various oncogenic processes such as cell proliferation, survival, cell cycle progression, metabolism, and EMT [14, 27, 28]. AKT is highly activated through its phosphorylation at Thy308 and Ser473 sites and participates in EMT, leading to cell migration and invasion [29]. EMT is a complex multistage process of primary invasion during cancer progression and is regulated through multiple signaling pathways, involving various transcription factors such as Snail, Slug, Twist, and ZEB1/ZEB2 [30]. These transcription factors are core regulators of the EMT process. They drive EMT by suppressing epithelial markers (such as E-cadherin) and activating mesenchymal markers (such as N-cadherin and vimentin) [31] In accordance with our study, the expression levels of the transcription factors (Slug and Twist) and the mesenchymal markers (vimentin, N-cadherin) that drive EMT were increased in CK17 up-regulated 5637 cells and decreased in CK17 silenced cells. These results suggest that CK17 could mediate EMT. In addition, in the CK17 up-regulated 5637 cells, we found that the AKT-Ser473 site was significantly enhanced. Opposite results were obtained in the CK17 silenced cells. Chen Li et al. found that keratin 17 knockdown inhibited the proliferation of malignant tumor in bladder cancer cells through the AKT and ERK pathways [18]. Our findings are consistent with their results. To further confirm that CK17 induced EMT by activating the AKT signaling pathway, we silenced AKT on the basis of overexpressing CK17 in bladder cancer cells 5637, and the results indicate that the protein expression levels of the mesenchymal markers (vimentin and N-cadherin) and the transcription factors (Slug and Twist) were significantly reduced after silencing AKT. It is noteworthy that EMT is not only associated with enhanced migration and invasiveness of tumor cells, but is also closely linked to drug resistance. Certain EMT-related transcription factors, such as Snail, can help tumor cells evade chemotherapy and targeted therapy by downregulating drug efflux proteins (such as P-glycoprotein) or by influencing apoptotic pathways [32]. In conjunction with our research findings, we believe that CK17 may play a critical role in bladder cancer cell proliferation and EMT process by activating the AKT pathway. These findings support the results that CK17 is associated with poor prognosis of bladder cancer.

Although our study demonstrated that CK17 overexpression could promote the proliferation, migration, and invasion of bladder cancer cells and be associated with a poor prognosis of bladder cancer patients, it still has some shortcomings. First, it is worth noting that the limited number of patients in our study (78 cases) affected the accuracy of the study. Second, although the biological functions of CK17 in bladder cancer have been verified its specific mechanisms also need to be fully explored. Therefore, the action mechanism of CK17 in bladder cancer cells needs to be verified by further studies, which may involve more other complex processes. In subsequent study, we will further assess the potential impact of CK17 on tumor cell resistance. To provide a research foundation for confirming the possibility of CK17 as a critical molecular target for bladder cancer.

Conclusion

CK17 is highly expressed in bladder cancer tissues, and its high expression is associated with lower survival in patients. Based on cell experiments, we demonstrated that high CK17 expression promoted the proliferation, migration and invasion of bladder cancer cells. Finally, our study shows that CK17 activates the AKT pathway and promotes EMT, which explains that high CK17 expression promotes the malignant progression of bladder cancer.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12894-025-01760-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Acknowledgements

The authors thank the patients who participated in this study.

Author contributions

P. Z., M.L.: Writing–original draft, Conceptualization, Data curation. S.Z.: Visualization, Writing– review & editing. C.L.: Project administration, Methodology. Q.Z.: Methodology, software. Y.L.: Data curation. Z.C.: Methodology. J.L.: validation, supervision. Y.W.: Resources, formal analysis. C.B.: Formal analysis, supervision. All authors read and approved the final manuscript.

Funding

Hospital Fund of the Affiliated Hospital of Hebei University, Fund number: 2022QA09. Baoding Science and Technology Plan Project, Fund number: 2241ZF322.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the Affiliated Hospital of Hebei University(Ethics Approval Number: 2019 – 0115).

Informed consent statement
The study was conducted according to the guidelines of the Declaration of Helsinki. Written informed consent has been obtained from the patient(s) to publish this paper.

Competing interests
The authors declare no competing interests.

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Received: 27 September 2024 / Accepted: 25 March 2025
Published online: 07 April 2025

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