

# Knockdown of ZNF280A inhibits cell proliferation and promotes cell apoptosis of bladder cancer

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**Summary.** Objective. ZNF280A is a member of the zinc finger protein family, whose role in human cancers is little known and rarely reported. This study aimed to investigate the role of ZNF280A in bladder cancer.

**Methods.** Immunohistochemical analysis was performed to detect the expression of ZNF280A in clinical samples. ZNF280A knockdown cell models were constructed by transfection of shRNA-expressing lentivirus. MTT assay and flow cytometry were performed for detecting cell proliferation, apoptosis and cycle. Wound healing and Transwell assays were operated to detect cell migration. Western blotting and Human Apoptosis Antibody Microarray were used to measure expression of related proteins. A mouse xenograft model was constructed for *in vivo* study.

**Results.** Our study demonstrated that ZNF280A was up-regulated in bladder cancer tissues compared with normal tissues, whose high expression was significantly correlated with advanced malignant grade. Knockdown of ZNF280A inhibited cell proliferation and cell migration, promoted cell apoptosis and G1/G2 phase arrest. The tumor growth *in vivo* was also proved to be inhibited by ZNF280A. Moreover, ZNF280A may promote bladder cancer through regulation of MAPK9, Cyclin D1 and the Akt pathway.

**Conclusions.** In this study, ZNF280A was shown as a potential tumor promoter and prognosis indicator for bladder cancer. Targeting ZNF280A may be a promising strategy for the development of novel bladder cancer treatment.

**Key words:** Bladder cancer, ZNF280A, Proliferation, Apoptosis, Migration

## Introduction

Bladder cancer is one of the top ten malignant tumors worldwide, with a relatively higher incidence among men (Kaufman et al., 2009; Siegel et al., 2019, 2023; Sung et al., 2021). In China, bladder cancer is also one of the most commonly diagnosed malignancies in the urinary system (Chen et al., 2016). Epidemiology studies showed that the morbidity of bladder cancer in China is still increasing rapidly in recent years (Chen et al., 2016). Statistical data showed that, in China, 80,500 cases of newly diagnosed bladder cancer and 32,900 deaths occurred in 2015 (Chen et al., 2016). At present, the major strategy of bladder cancer treatment is surgical resection, assisted by chemotherapy, radiotherapy and immunotherapy (Smith, 2018). However, due to the strong invasiveness of bladder cancer cells, a considerable number of patients with bladder cancer suffer from local infiltration or distant metastasis upon initial diagnose or after surgery (Bhanvadia, 2018). Therefore, the long-term efficacy of the current treatment for bladder cancer is of low efficiency, and the 5-year survival rate is far from satisfactory (Smolensky et al., 2016). With the rapid development of molecular research technologies and the in-depth study of molecular mechanisms of cancer, exploring the molecular mechanism of the occurrence and progression of bladder cancer and seeking novel specific molecular targets has become a hot topic (Feber et al., 2017).

Zinc finger protein is a type of transcription factor with a special "finger-like" domain, which usually exists in various eukaryotes and possesses the function of regulating and controlling gene expression (Brayer and Segal, 2008). The most representative characteristic of zinc finger protein family members is that they can produce a short stereoscopic structure model of polypeptide according to their own folding pattern, and maintain the stability of such molecular structure by combining with zinc ions (Brayer and Segal, 2008). It has been revealed that zinc finger protein plays critical

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roles in embryonic development, cell differentiation, signal transduction and, especially, the development and progression of human cancers (Jen and Wang, 2016). For example, ZNF280B was identified as a potential mechanism of p53 suppression in prostate cancer, which promoted the development of prostate cancer. ZNF280A, which encodes a zinc finger protein with C<sub>2</sub>H<sub>2</sub> motif as well as a transcription factor, was found to be potentially involved in mantle cell lymphoma (Beà et al., 2009). However, the relationship between ZNF280A and most types of human cancers including bladder cancer remains unclear.

In this study, ZNF280A was found to be up-regulated in bladder cancer tissues compared with normal tissues, high expression of which was significantly correlated with advanced malignant grade. The role of ZNF280A in bladder cancer was also proved by the subsequent *in vitro* and *in vivo* experiments, which showed the inhibition of bladder cancer by knockdown of ZNF280A. Therefore, this study presents the first report that ZNF280A may act as a tumor promoter for bladder cancer and could be a potential therapeutic target for developing novel therapies against bladder cancer.

## Materials and methods

### Cell culture and lentivirus infection

EJ and T24 human bladder cancer cell lines were purchased from Genechem (Shanghai, China). J82 and 5637 were obtained from the BeNa Technology (Hangzhou, Zhejiang, China). EJ, 5637 and T24 cell lines were cultured in RPMI-1640 medium (Gibco, Rockville, MD, USA) containing 10% FBS (Gibco, Rockville, MD, USA) at 37°C with 5% CO<sub>2</sub>. J82 were cultured in 90% EME medium (Gibco, Rockville, MD, USA) with 10% FBS additive. HCV29 cell line was purchased from ATCC, and cultured with RPMI-1640 medium containing 10% FBS at 37°C with 5% CO<sub>2</sub>.

### Tissue microarray and immunohistochemistry analysis

A tissue microarray containing 105 formalin-fixed unpaired bladder cancer and para-carcinoma tissue samples was obtained from Shanghai Outdo Biotech Company (Shanghai, China). Tumor information of patients with bladder cancer and other related characteristics were collected as well. The tumor characteristics such as tumor grade were confirmed by at least 2 pathologists. Written informed consents were collected from all participants. Our study was approved by the Ethics Committee of General Hospital of Northern Theater Command.

The tissue samples were dewaxed and hydrated. After washing, antigen retrieval was accomplished by citric acid buffer heating at 110°C. The chip was washed three times with PBS and blocked with 3% hydrogen peroxide. Sections were incubated with ZNF280A

primary antibody (1:400, Bioss, Beijing, China) overnight at 4°C and subsequently incubated with HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA) for 2h at room temperature. 3,3'-diaminobenzidine (DAB) were used for coloring and counterstaining with hematoxylin, then dehydrated and sealed with cover slips. ZNF280A expression was observed with ImageScope and CaseViewer then quantified for analysis with IHC scores. Scoring standard for ZNF280A was graded as 0-4 (negative to +++positive). The staining extent was graded as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). The staining intensity varied from weak to strong. Specimens were classified based on the sum of the staining intensity and staining extent scores.

### Plasmid construction and lentivirus infection

shRNAs targeting ZNF280A and its flanking control sequence were designed by Shanghai Biosciences (Shanghai, China) and cloned in BR-V-108 vector and transformed into *E. coli* competent cells (Tiangen, Beijing, China). PCR was used to confirm and sequence the plasmids and the target shRNA sequence was identified as 5'-CTGTCACTATGAAGTCTTCAT-3'. The plasmid was purified by EndoFree Plasmid Mega Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and the qualified plasmids were packaged in lentivirus production. Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used for cell transfection. For lentivirus infection, the EJ and T24 cells in the logarithmic growth phase were washed with PBS, adjusted to a cell density of 2×10<sup>5</sup> cells/mL, and re-inoculated into a 6 well dish, and lentivirus (1×10<sup>7</sup> TU/mL, 400 μL) were used to infect the cells for 72h. The transfected cells were screened under Puromycin (Takara Bio, Otsu, Japan) and verified through observing fluorescence of GFP by a fluorescence microscope (Olympus, Tokyo, Japan).

### Western blotting assay

T24 and EJ cells were lysed in ice-cold RIPA buffer (Millipore, Temecula, CA, USA) and the lysates were cleared with centrifuging and the total protein concentration was detected by BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were separated by 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) with 20 μg in each lane, and were transferred onto PVDF membranes. Cells were blocked by TBST containing 5% non-fat milk at room temperature for 1h. Then the membranes were incubated with anti-ZNF280A (1:1000, Abcam, Cambridge, MA, USA), anti-GAPDH (1:3000, Bioworld, St. Louis, MN, USA), anti-MAPK9 (1:1000, Abcam, Cambridge, MA, USA), anti-Akt (1:2000, Proteintech), anti-p-Akt (1:2000, Proteintech) and anti-Cyclin D1 (1:2000, CST, Danvers, MA, USA) overnight at 4°C on a rocker.

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Afterward, the membranes were subsequently incubated with corresponding secondary antibodies (Beyotime, Beijing, China) at room temperature for 30 min. TBST containing 5% non-fat milk was used for the resuspension of the antibodies. The blots were visualized by enhanced chemiluminescence (ECL).

### *qPCR assay*

Total RNA was extracted with TRIzol reagent (Sigma, St. Louis, MO, USA) from cells and the quality of total RNA was evaluated by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The reverse transcribe of cDNA was performed using the Hiscript QRT supermix kit (Vazyme, Nanjing, Jiangsu, China). The expression of mRNA was examined by qRT-PCR with SYBR Green mastermix Kit (Vazyme, Nanjing, Jiangsu, China) and Applied Biosystems 7500 Sequence Detection system. GAPDH was used as inner control and the amplification results for qRT-PCR were calculated using the  $2^{-\Delta\Delta C_t}$  method. The PCR cycling conditions were 95°C for 6 min, 40 cycles of 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 70°C for 5 min. Primers used for PCR are shown as below: ZNF280A forward, 5'-GATCTGATCTATGT TGGGGTGGGA-3'; ZNF280A reverse, 5'-CGTGAG CAGGATATTGACGGA-3'; GAPDH forward 5'-TGACTTCAACAGCGACACCCA-3'; GAPDH reverse 5'-CACCTGTTGCTGTAGCCAAA-3'.

### *MTT assay*

Cell viability was assessed using MTT assay. T24 and EJ cells ( $2 \times 10^3$  cells/well) were seeded in a 96-well plate and incubated at 37°C for 120h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT, 20  $\mu$ L) was added into each well and incubated for another 4h at 37°C. Afterwards, the culture medium was removed and 100  $\mu$ L DMSO was added and the cells were incubated at room temperature in the dark for 20 min. The absorbance was measured using a microplate spectrophotometer (Bio-Tek instruments, Winooski, VT, USA) at 490/570 nm and the cell viability was calculated according to the following equation: Cell viability (%)=(mean absorbance in treatment group/mean absorbance in control group) $\times$ 100.

### *Flow cytometry for cell apoptosis and cycle*

EJ and T24 cells groups were inoculated in 6-well plates at a seeding density of  $1 \times 10^3$  cells/mL (2 mL/well) in triplicate and further cultured for 5 days. The cells were harvested with centrifuge (1000 $\times$ g), washed with 4°C cold D-Hanks and resuspended with binding buffer, then 5  $\mu$ L Annexin V-APC (eBioscience, San Diego, CA, USA) was added for staining without light. The percentage of apoptotic cells was measured using FACScan (Millipore, Schwalbach, Germany) to assess the apoptotic rate.

For cell cycle detection, EJ and T24 cells were seeded in a 6-well plate (5 mL/well). After 5 days of culture, cells were harvested and fixed in 70% ethanol for 24h at -20°C. Then the cells were stained by staining solution containing 40 $\times$  PI (Sigma, St Louis, MO, USA) solution (2 mg/ml), 100 $\times$  RNase A solution (10 mg/ml) and PBS (25:10:1000) and cell cycle distribution was detected and analyzed by flow cytometer (Millipore, Schwalbach, Germany) and the software provided by the manufacturer.

### *Wound healing assay*

Lentivirus infected EJ and T24 cells ( $5 \times 10^4$  cells/well) were plated onto a 96-well dish and grew until 90% confluence in serum-free medium. Scratch was made by a pipette tip across the cell layer, the floating cells were washed and cultured. Photographs were taken by fluorescence micrograph at the time points (0h, 4h and 8h). Cell migration rate of each group was calculated based on the randomly selected areas in each well.

### *Transwell assay*

Cell transwell assay was operated by Corning Transwell Kit (Corning, NT, USA) and was done in triplicate and repeated three times. EJ and T24 exponentially growing cells were trypsinized, counted and incubated in the upper chamber with 100  $\mu$ L of medium ( $5 \times 10^4$  cells/well) in 24-well plate. 600  $\mu$ L medium supplemented with 30% FBS was added in the lower chamber. Cells were incubated for 24h at 37°C with 5% CO<sub>2</sub> and non-metastatic cells were removed with a cotton swab. Cells were fixed by 4% formaldehyde and 400  $\mu$ L Giemsa was added for staining and the migration ability of cells was analyzed. Experiments were repeated three times.

### *Human apoptosis antibody array*

Human Apoptosis Antibody Array (R&D Systems, Minneapolis, MN, USA) was performed in T24 cells transfected with ZNF280A or NC following the manufacturer's instructions. Briefly, lentivirus infected EJ and T24 cells were collected and washed and then lysed by lysis buffer. Total protein was extracted and diluted with Array Diluent Buffer. Each array antibody membrane was blocked and then incubated with protein samples (0.5 mg/mL) overnight at 4°C and then HRP linked Streptavidin was added to the membranes. The spots were visualized by chemiluminescence and the signal densities were analyzed with ImageJ software (National Institute of Health, Bethesda, MD, USA).

### *In vivo tumorigenicity assay*

Our animal study was reviewed, approved and carried out according to the guidelines provided by the

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Institutional Animal Care and Use Committee of General Hospital of Northern Theater Command. 4-week-old BALB/c female nude mice were purchased from Shanghai Lingchang Experimental Animals Co., Ltd (Shanghai, China) and randomly divided into shZNF280A group and shCtrl group, with 5 mice in each group. 0.2 mL T24 cells ( $2.5 \times 10^7$  cells/mL) suspension was injected into the mice for the construction of tumor model. The tumor size was observed and recorded every 2 days (volume of tumor =  $\pi/6 \times L \times W \times W$ , L represent longest dimension and W means dimension perpendicular to length). Mice were sacrificed 25 days post injection and tumor weight was measured. The removed tissues were stored at  $-80^\circ\text{C}$  for subsequent measurement.

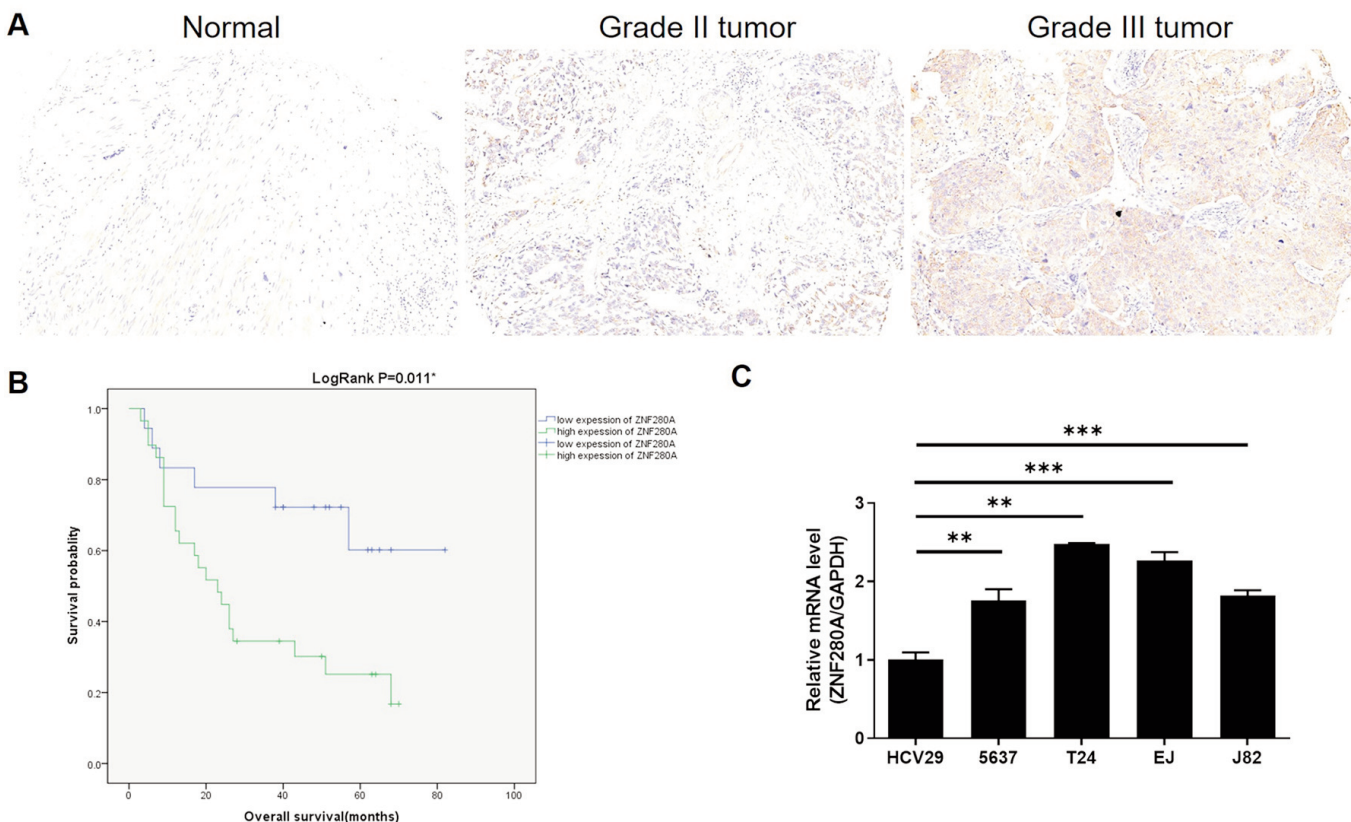
### Ki-67 immunostaining

Tumor tissues were fixed with formalin and embedded using paraffin, then 2  $\mu\text{m}$  sections were immersed in xylene and 100% ethanol for deparaffinization and rehydration, then blocked with PBS- $\text{H}_2\text{O}_2$  and washed. Next, Ki-67 primary antibody (1/200)

was added and incubated with all slides at  $4^\circ\text{C}$  overnight, then incubated with 1:400 HRP-conjugated secondary antibody. Slides were stained by Hematoxylin and Eosin. Stained slides were examined with a microscopic.

### Statistical Analysis

The statistical analysis was performed using SPSS 17 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (Graphpad Software, La Jolla, CA). Continuous variables were shown as the mean  $\pm$  SD from and Student's T-Test was used to analyze the statistical significance. Otherwise, significant differences between different experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. All experiments were repeated at least 3 times.  $P < 0.05$  was considered statistically significant. Chi-square analysis was utilized to analyze the expression difference of ZNF280A gene in bladder cancer tissues and para-carcinoma tissues. Mann-Whitney U analysis and Spearman Rank correlation analysis were used while analyzing the relationship between ZNF280A expression



**Fig. 1.** ZNF280A was up-regulated in bladder cancer. **A.** Expression of ZNF280A in bladder cancer and normal tissues was detected by IHC. **B.** Kaplan-Meier survival analysis was performed to construct the relationship between ZNF280A expression and prognosis. **C.** Background expression of ZNF280A in normal bladder epithelial cells and bladder cancer cells was detected by qPCR. Results are presented as mean  $\pm$  SD. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . A,  $\times 200$ .

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and tumor characteristics in patients with bladder cancer.

### Results

#### ZNF280A was up-regulated in bladder cancer

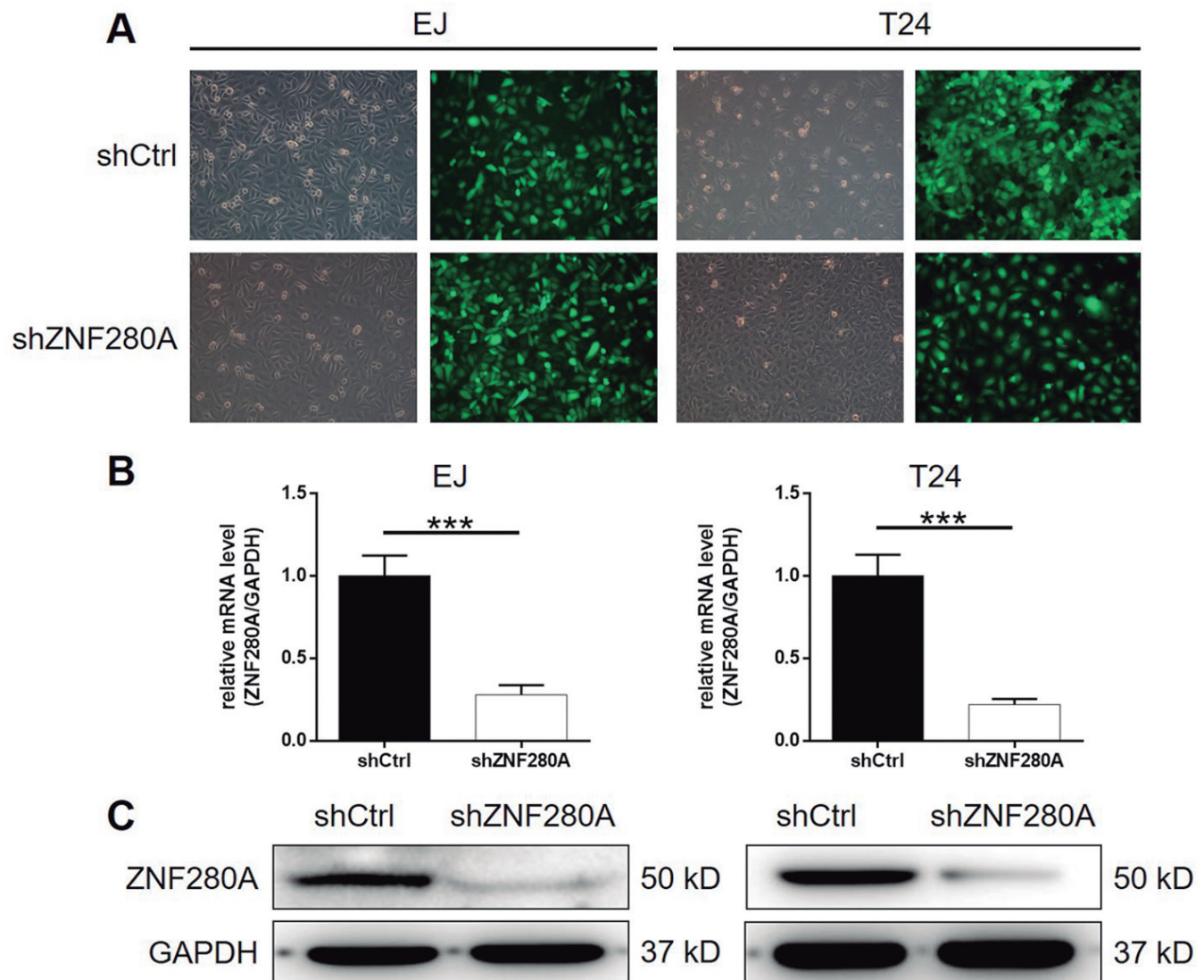
In order to reveal the role of ZNF280A in bladder cancer, its expression levels in bladder cancer tissues were detected by immunohistochemical (IHC) analysis. Based on the median of IHC scores, all tissue samples were divided into high/low groups. The results demonstrated significant up-regulation of ZNF280A in tumor tissues compared with normal tissues, and in grade III tumor tissues compared to grade II ones (Fig.

1A,  $P < 0.001$ , Table 1). Accordingly, the statistical analysis of ZNF280A expression and characteristics of

**Table 1.** Expression patterns of ZNF280A in bladder tissues and normal tissues revealed in immunohistochemistry analysis.

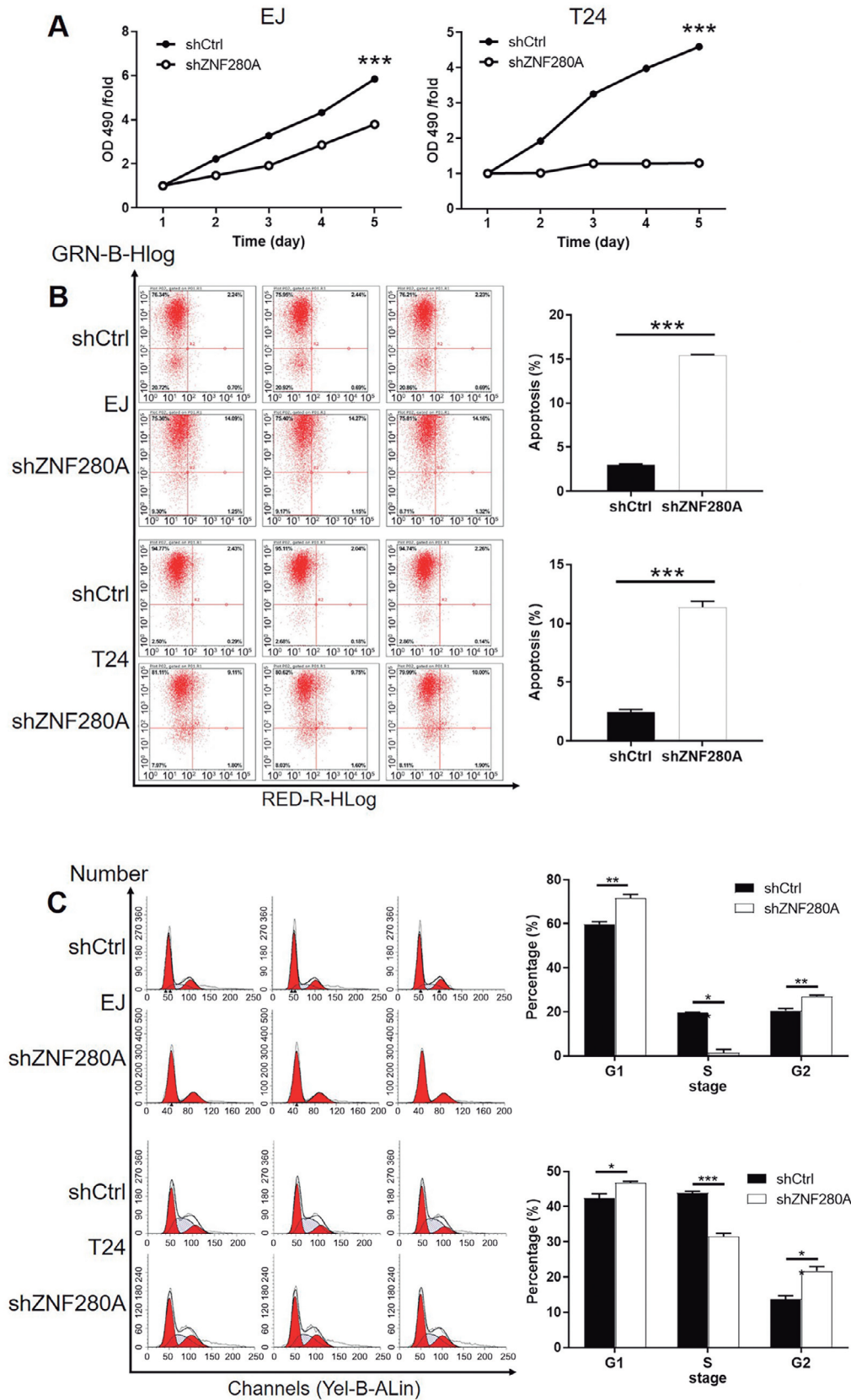
ZNF280A expression	Tumor tissue		Normal tissue	
	Cases	Percentage	Cases	Percentage
Low	26	45.6%	43	100%
High	31	54.4%	0	-

$P < 0.001$ .



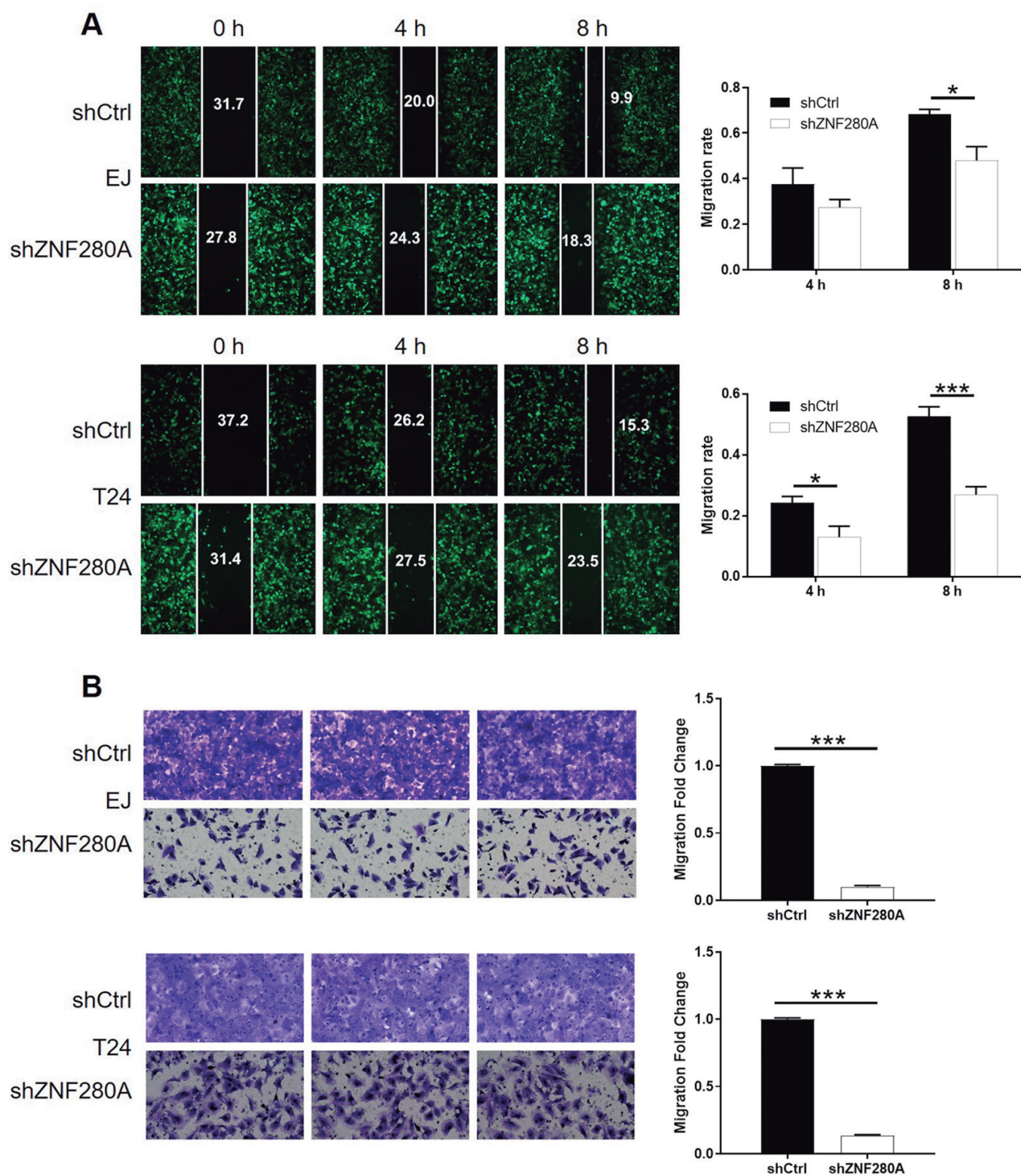
**Fig. 2.** Construction of ZNF280A knockdown cell lines. **A.** The efficiency of lentivirus transfection was evaluated through observing fluorescence of the GFP tag. **B.** The efficiency of ZNF280A knockdown was detected by qPCR. **C.** The efficiency of ZNF280A knockdown was estimated by western blotting. Results are presented as mean  $\pm$  SD. \*\*\* $P < 0.001$ .

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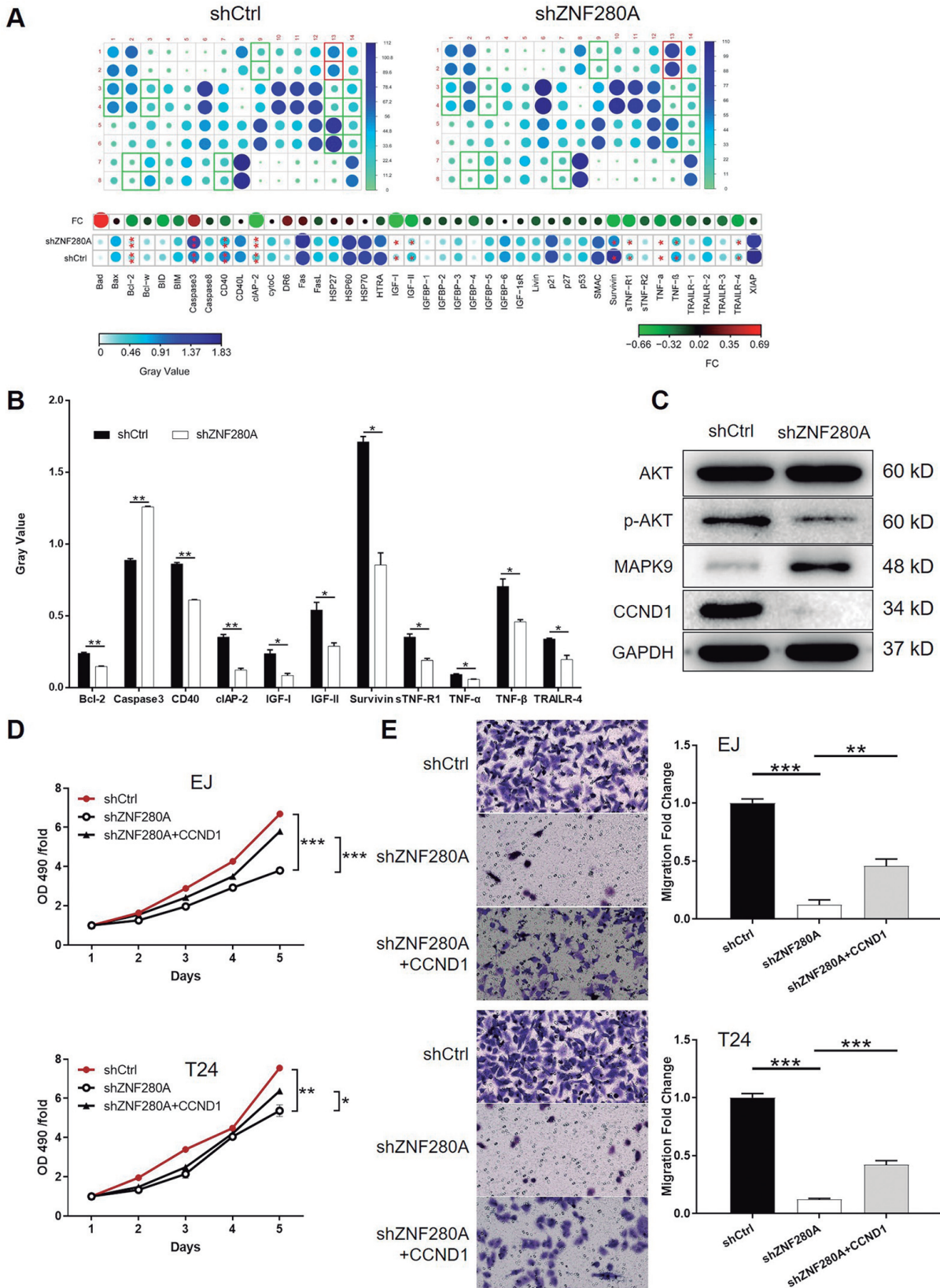


**Fig. 3.** ZNF280A knockdown inhibited cell proliferation and induced cell apoptosis as well as cell cycle arrest of bladder cancer cells. **A.** The effects of ZNF280A knockdown on cell proliferation were detected by MTT assay. **B.** The change of cell apoptosis induced by ZNF280A knockdown was examined by flow cytometry. Results are presented as mean  $\pm$  SD. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

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**Fig. 4.** ZNF280A knockdown inhibited cell migration of bladder cancer cells. **A.** The influence of ZNF280A knockdown was estimated by wound-healing assay. **B.** The effects of ZNF280A knockdown were detected by Transwell assay. Results are presented as mean  $\pm$  SD. \* $P$ <0.05, \*\*\* $P$ <0.001.



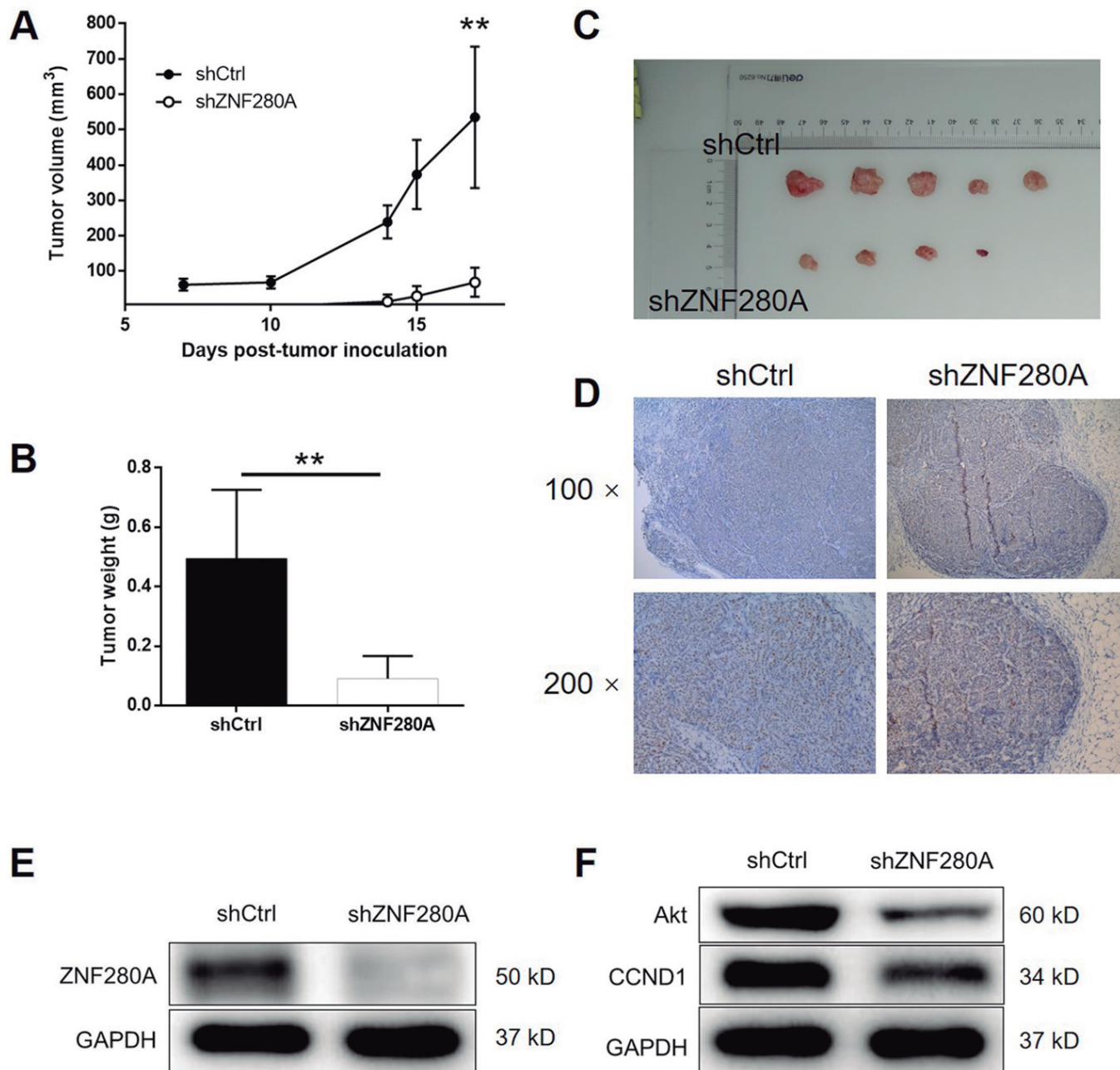
**Fig. 5.** The exploration of regulatory mechanism of ZNF280A on bladder cancer. **A.** Human apoptosis antibody microarray was performed to identify differentially expressed proteins in T24 cells of shCtrl and shZNF280A groups. **B.** The grey value analysis of the differentially expressed proteins. **C.** The expression of Akt, p-Akt, MAPK9 and Cyclin D1 was detected by western blotting in T24 cells of shCtrl and shZNF280A groups. **D.** MTT assay was used to evaluate cell proliferation of EJ and T24 cells with ZNF280A knockdown or simultaneous ZNF280A knockdown and Cyclin D1 overexpression. **E.** Transwell assay was used to evaluate cell migration of EJ and T24 cells with ZNF280A knockdown or simultaneous ZNF280A knockdown and Cyclin D1 overexpression. Results are presented as mean  $\pm$  SD. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.



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patients with bladder cancer showed a positive correlation between high expression of ZNF280A and advanced malignant grade ( $P=0.007$ , Table 2), which was further verified by Spearman rank correlation analysis ( $P=0.006$ ). Furthermore, the Kaplan-Meier survival analysis also indicated that ZNF280A high

expression was associated with poor prognosis ( $P=0.011$ , Fig. 1B). On the other hand, the background expression of ZNF280A in human bladder epithelial cell line HCV29 and bladder cancer cell lines, including 5637, J82, EJ and T24, also showed up-regulation of ZNF280A in bladder cancer cells compared with normal



**Fig. 6.** ZNF280A knockdown inhibited tumor growth of bladder cancer *in vivo*. **A.** The volume of tumors was measured and calculated during experiments. **B.** The weight of tumors was measured after the sacrifice of the mice. **C.** The photos of xenograft tumors removed from the mice in shCtrl and shZNF280A groups were collected after the sacrifice of the mice. **D.** The Ki-67 index of the removed tumors was evaluated by IHC analysis. **E.** Protein level of ZNF280A was detected in tumor tissues collected from both groups of mice. **F.** Protein level of Akt and Cyclin D1 was detected in tumor tissues collected from both groups of mice. Results are presented as mean  $\pm$  SD. \*\* $P<0.01$ . D, x 100 or x 200.

cells (Fig. 1C). All these results indicated the potential role of ZNF280A as a promoter in development and progression of bladder cancer.

#### ZNF280A knockdown inhibited development of bladder cancer *in vitro*

To further investigate the promotion effect of ZNF280A on the development of bladder cancer, ZNF280A knockdown cell models (shZNF280A) were constructed based on EJ and T24 cells through the transfection of lentivirus expressing shRNA for silencing ZNF280A, while cells transfected with lentivirus vector were used as negative control (shCtrl). The efficiencies of transfection were verified to be >80% by fluorescence imaging (Fig. 2A). The >70% knockdown of ZNF280A was clarified by qPCR ( $P<0.001$ , Fig. 2B) and western blotting (Fig. 2C), respectively, indicating the successful construction of ZNF280A knockdown cell models. Subsequently, the results of MTT assay showed that knockdown of ZNF280A slowed down bladder cancer cell proliferation (inhibition rate 42.3% for EJ cells and 91.8% for T24 cells) ( $P<0.001$ , Fig. 3A). Conversely, cell apoptosis was significantly promoted in shZNF280A groups compared with shCtrl groups (5.1-fold for EJ cells and 4.7-fold for T24 cells) ( $P<0.001$ , Fig. 3B). Cell cycle of EJ and T24 cells in shZNF280A and shCtrl groups was also detected, which showed that, compared with shCtrl groups, knockdown of ZNF280A promoted G1 and G2 phase arrest and decreased the percentage of cells in S phase (30% enhancement in G2 phase for EJ

cells and 58% enhancement in G2 phase for T24 cells) ( $P<0.01$ , Fig. 3C). Moreover, the effects of ZNF280A knockdown on cell migration ability were also evaluated by wound-healing and Transwell assays, which demonstrated a significantly suppressed migration rate of cells by ZNF280A knockdown (inhibition rate 27.4% in wound-healing assay and 90% in transwell assay for EJ cells, and inhibition rate 48.7% in wound-healing assay and 86.3% in transwell assay for T24 cells) ( $P<0.05$ , Fig. 4A,B). Collectively, the *in vitro* studies suggested that knockdown of ZNF280A was able to inhibit the development and progression of bladder cancer.

#### Exploration of the regulation mechanism of ZNF280A on bladder cancer

Given the promotion effects of ZNF280A on bladder cancer proved by the above results, the underlying mechanism was next explored by a Human Apoptosis Antibody Array (Fig. 5A). Through the detection of protein expression in T24 cells of both shCtrl and shZNF280A groups, 11 differentially expressed proteins were identified, among which there were 1 up-regulated protein Caspase-3, and 10 down-regulated proteins including Bcl-2, CD40, cIAP-2, IGF-I, IGF-II, Survivin, sTNF-R1, TNF- $\alpha$ , TNF- $\beta$ , TRAILR-4 in shZNF280A group ( $P<0.05$ , Fig. 5B). These results suggested the involvement of CD40 and IGF signaling pathway in ZNF280A induced regulation of bladder cancer. Moreover, the elevated expression of MAPK9, and descended activation of Akt pathway as well as expression of Cyclin D1 (CCND1) by ZNF280A knockdown were also detected in T24 cells (Fig. 5C). Moreover, functional rescue assays showed that the inhibitory effects of ZNF280A knockdown on bladder cancer cell proliferation and cell migration could be partially reversed by the overexpression of Cyclin D1 (Fig. 5D,E).

#### ZNF280A knockdown inhibited growth of bladder cancer *in vivo*

Finally, the role of ZNF280A in the development of bladder cancer was investigated *in vivo*. A mouse xenograft model was constructed through the subcutaneous injection of T24 cells of shCtrl and shZNF280A groups. The calculation of tumor volume during animal culture revealed a much slower growth rate of tumors formed by T24 cells of shZNF280A group than shCtrl group ( $P<0.01$ , Fig. 6A). After sacrificing the mice, the measurement of tumor weight also showed much smaller tumors formed by T24 cells with ZNF280A knockdown ( $P<0.01$ , Fig. 6B), which could be further visualized by the images of the removed tumors (Fig. 6C). Furthermore, the suppressed tumor growth by knockdown of ZNF280A was also proved by the lower Ki-67 index in tumors of the shZNF280A group (Fig. 6D). Moreover, it was also demonstrated

**Table 2.** Relationship between ZNF280A expression and tumor characteristics in patients with bladder cancer.

Features	No. of patients	ZNF280A expression		P value
		low	high	
All patients	57	26	31	
Age (years)				0.904
$\leq 71$	29	13	16	
$> 71$	28	13	15	
Gender				0.128
Male	48	24	24	
Female	9	2	7	
Tumor size				0.852
$< 4$ cm	24	11	13	
$\geq 4$ cm	31	15	16	
Lymphadenopathy				0.901
no	36	19	17	
yes	6	3	3	
Grade				0.007
II	18	13	5	
III	39	13	26	
AJCC Stage				0.787
I	6	3	3	
II	11	4	7	
III	16	9	7	
IV	7	3	4	

that, with the lower expression of ZNF280A in tumors of the shZNF280A group, the expression of Akt and Cyclin D1 was also downregulated, which is consistent with the aforementioned results. Therefore, the inhibition of bladder cancer by ZNF280A knockdown was proved *in vivo*, which was in accordance with the results of *in vitro* studies.

## Discussion

The occurrence and development of bladder cancer is a multi-factor and multi-step process (Harris and McCormick, 2010). Current studies have confirmed that multiple oncogenes and tumor suppressor genes play very important roles in this process (Kang et al., 2014). The abnormally high expression of oncogenes such as MDM2 (Shinohara et al., 2009) and CCND1 (Seiler et al., 2014), and abnormal deletion or mutation of tumor suppressor genes such as TP53 (Kelsey et al., 2005) and PTEN (Yang et al., 2018) have been revealed. However, the 5-year survival of bladder cancer remains unchanged in recent years. Therefore, the exploration of novel molecular targets in bladder cancer is of profound significance for improving prognosis of bladder cancer patients. Recently, Zhuo et al. identified CSTP1 as a tumor suppressor in bladder cancer, which executes a suppressive function through the inhibition of IL-6 expression by targeting the Akt/FoxO3a signaling pathway (Zhuo et al., 2019). On the other hand, OGT was reported to be a tumor promoter in bladder cancer through promoting cell proliferation and inhibiting cell apoptosis (Wang et al., 2018).

Zinc finger proteins are a group of transcription factors with special finger-like domains. They can generate finger-like structures by self-folding and bind to zinc ions to maintain stability (Hamed and Arya, 2016). In the past decades, accumulating evidence indicated that zinc finger proteins were involved in the development and progression of various human cancers (Jen and Wang, 2016). For example, a study of Li et al. indicated that ZNF677 could transcriptionally suppress the expression of CDKN3 and HSPB1, thus inhibiting the activation, as well as phosphorylation of Akt and the tumorigenesis of thyroid cancer (Li et al., 2018). Nie et al. identified ZNF139 as a target of miR-195-5p in the multi-drug resistance of gastric cancer (Nie et al., 2018). ZNF668 was reported to be capable of suppressing the invasion and migration of non-small cell lung cancer through regulation of EMT related factors (Zhang et al., 2018). Ying et al. indicated a tumor-promoting role of ZNF280C in colorectal cancer by maintaining epigenetic repression at H3K27me3-marked loci (Ying et al., 2022). In bladder cancer, a member of the zinc finger protein family ZNF224 was found to form a complex with DEPDC1, inhibition of which could potentially repress bladder carcinogenesis (Harada et al., 2010). Previously, ZNF280A has also been reported to play some roles in the development of human cancers such as lung adenocarcinoma (Liu et al., 2021) and colorectal cancer

(Wang et al., 2019; Tian et al., 2022). In this study, the relationship between bladder cancer and ZNF280A, which is rarely investigated in the development of cancer, was studied. The IHC analysis of clinical specimens clarified the significantly elevated expression of ZNF280A in tumor tissues in comparison with normal tissues. The potential role as tumor promoter of ZNF280A was also elucidated by the positive correlation between high ZNF280A expression and advanced malignant grade. Accordingly, human bladder cancer cells with ZNF280A knockdown were constructed for the *in vitro* study, which showed that the down-regulation of ZNF280A in bladder cancer cells could inhibit cell proliferation, migration and induce cell apoptosis. The regulation of apoptosis related proteins by ZNF280A knockdown was consistent with the results of cell apoptosis. Moreover, cell cycle analysis demonstrated that the knockdown of ZNF280A significantly promoted G1/G2 phase arrest and decreased the S phase percentage. Furthermore, the effects of ZNF280A on bladder cancer were also verified in a mouse xenograft model, which was subcutaneously injected with T24 cells with or without ZNF280A knockdown. The *in vivo* study displayed slower growth rate, smaller tumors and lower Ki-67 index in the shZNF280A group. All the above results indicated that ZNF280A could promote the development and progression of bladder cancer, and may act as a therapeutic target for bladder cancer treatment.

Given the role of ZNF280A in bladder cancer, its regulatory mechanism was also studied in this study. MAPK9, which is also known as JNK2 and is one of the three isozymes of c-Jun N-Terminal kinase (JNK), has been previously proved to be involved in human cancer (Hamed and Arya, 2016). For example, Pang et al. reported that JNK2 acted as the target to mediate the miR-146a induced increase of cisplatin resistance in non-small cell lung cancer (Pang et al., 2017). More importantly, in bladder cancer, JNK2 was also reported to be the target of miR-200c in the RhoGDI $\beta$  induced up-regulation of Sp1/MMP2 and promotion of bladder cancer invasion (Huang et al., 2017). In this study, we also found that the expression of JNK2 was enhanced in the shZNF280A group, indicating JNK2 as the potential downstream mechanism. Cyclin D1, which is a positive regulator in the cell cycle, has been proved to be over-expressed in urinary tumors (Hong et al., 2018). It was also identified as a key mediator during the metabotropic glutamate receptors (mGluRs) induced regulation of cell proliferation and cell apoptosis of bladder cancer cells (Zhang et al., 2019). Herein, our study also revealed the significantly down-regulated expression of Cyclin D1 by ZNF280A knockdown, which was in agreement with the cell cycle analysis. The Akt pathway is a well-known tumor-regulating axis which plays important roles in several types of malignant tumors such as gastric cancer (Fattahi et al., 2020), ovarian cancer (Ediriweera et al., 2019), lung cancer (Tan, 2020), and notably, bladder cancer (Liu et al., 2020). Moreover, the Akt pathway

was also shown to be a potential downstream pathway of ZNF280A in this study as represented by the down-regulated expression in the shZNF280A group. Notably, as potential downstream targets of ZNF280A, RPS14 and p53 pathway were reported in previously reported studies, and the well-known tumor-promoting Akt pathway was identified. These results indicate the silencing of ZNF280A as a promising strategy in inhibiting tumor growth or as adjuvant therapy for other therapies.

In conclusion, ZNF280A was identified as a tumor promoter in the development and progression of bladder cancer through *in vitro* and *in vivo* studies, which may execute its role through the regulation of the Akt pathway, MAPK9, Cyclin D1 and apoptosis-related proteins such as Bcl-2 and Caspase-3. Therefore, ZNF280A may be an effective therapeutic target in the treatment of bladder cancer.

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**Conflict of interests.** The authors declare no conflict of interests.

**Data Availability.** The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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