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RESEARCH PAPER

KRT6A expedites bladder cancer progression, regulated by miR-31-5p

Yuan Chen[a], Shiben Ji[b], Jianxin Ying[b], Yongchang Sun[b], Jun Liu[b], and Guohong Yin [b]

[a]Department of Geriatric (Urology), Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei, China; [b]Department of Urology, Wuhan Hankou Hospital, Wuhan, Hubei, China

ABSTRACT

Bladder cancer is one of the most severe life-threatening illnesses worldwide. To contribute to a solution to this public health issue, here, we sought to identify a novel biomarker for the early diagnosis of bladder tumors. We conducted RNA sequence analysis utilizing samples from tumorous tissue and adjacent healthy tissue in bladder cancer patients and found that *KRT6A* was upregulated in bladder tumor tissues, suggesting that it might be a candidate for involvement in bladder tumorigenesis. Accordingly, we performed a series of experiments to further verify the role of *KRT6A* in bladder tumor progression. Our results revealed that *KRT6A* promoted bladder tumor cell viability, proliferation, and adhesion, while diminishing bladder tumor cell apoptosis. We also focused on the role of epigenetics in bladder tumors and verified that *KRT6A* was a miR-31-5p target gene, and its positive effect on bladder tumor progression was relieved by miR-31-5p. Overall, this study sheds new light regarding a novel oncogenic regulatory axis, *KRT6A*/miR-31-5p, which is related to bladder tumor growth.

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Introduction

As one of the most common tumors of the urinary system, bladder cancer has a malignancy with high incidence, which poses a serious threat to human health [1–3]. Moreover, the incidence of bladder tumors has been increasing significantly due to environmental deterioration over recent years [4]. Bladder tumors are biologically characterized by high malignancy and high recurrence rates, thereby leading to strong invasive ability and poor prognosis [5]. Therefore, it is of great importance to explore new therapeutic targets for the early diagnosis of bladder tumors.

Keratin 6A *(KRT6A)* is a protein-coding gene and an important member of the keratin gene family [6]. KRT6A protein is a type II cytokeratin that plays important roles in the formation of nail beds, filiform papillae, and epithelial cells [6–8]. It is reported that *KRT6A* promotes lung cancer proliferation and metastasis via regulating epithelial-mesenchymal transition and cancer stem cell transformation [9]. *KRT6A* expression has been found to be significantly upregulated in colorectal cancer compared to that in normal tissue [10]. It has been observed that KRT6A promotes cell invasion and metastasis of nasopharyngeal carcinoma via the β-catenin cascade [11]. However, little is known regarding the effects of *KRT6A* on the development of bladder tumors.

MicroRNAs (miRNAs) are small non-coding RNAs that mainly regulate gene expression following the transcription process by binding to the 3'-untranslated region (UTR) of target genes [12]. In recent years, the abnormal expression of miRNAs in tumors and their roles in carcinogenesis have gradually been elucidated [13–15], including in bladder cancer [16–18]. For instance, inactivation of the TGF-β2/Smad3 pathway by miR-1305 curbs epithelial-mesenchymal transition and then mitigates bladder cancer development [17]. Usuba et al. underscored the diagnostic value of a seven miRNA panel by analyzing miRNA profiles in patients with bladder cancer [19]. The maturation of pri-miR221/222 antagonizes the tumor-suppressive PTEN protein and facilitates bladder cancer progression [20]. As for miR-31-5p, miR-31-5p increases the expression of anti-apoptotic BCL-2 proteins and suppresses prostate cancer

CONTACT Guohong Yin yinguohong9@163.com Department of Urology, Wuhan Hankou Hospital, No. 7, Erqi Side Road, Jiangan District, Wuhan, Hubei 430030, China
Supplemental data for this article can be accessed here

proliferation [21], whereas miR-31-5p silencing promotes the malignant behavior of colorectal cancer cells [22]. Therefore, the function of miR-31-5p during tumor progression is context-dependent. miR-31-5p reportedly performs its action in bladder cancer [23,24]. However, the regulatory mechanisms underlying bladder cancer progression are largely unknown.

In this study, we uncovered for the first time the role of *KRT6A* in bladder cancer *in vitro*. Subsequently, we applied bioinformatic analysis and found that *KRT6A* 3′-UTR might be targeted by miR-31-5p. Therefore, we ascertained a regulatory mechanism mediated by the miR-31-5p/*KRT6A* axis during bladder carcinogenesis. Our findings suggest the utilization of a novel target for therapeutic bladder cancer intervention.

Methods

Bioinformatic analysis

GEPIA database (http://gepia.cancer-pku.cn/index.html) for analyzing the gene expression data from TCGA was used to identify the expression of KRT6A in bladder cancer samples and normal samples. Another online database Kaplan Meier plotter (http://kmplot.com/) was used to assess the correlation between KRT6A expression and prognosis of bladder cancer with best cutoff. Besides, the miRNAs that could bind to KRT6A was predicted by miRDB algorithm (http://mirdb.org/).

Tissue sample collection

Bladder tumor tissue and adjacent healthy tissues (3 cm from malignant lesions) samples were collected from 40 patients presenting with bladder tumors. All samples were collected immediately before the transurethral resection of the bladder tumor or the cystectomy at our hospital. The Human Use Committee of our hospital approved the study, and informed consent was obtained from all patients.

Cell culture and cell transfection

SV-HUC-1 cells (a human epithelial cell line) were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin solution (Invitrogen, USA) in a humidified atmosphere with 5% CO_2 at 37°C. T24 cells (a human bladder cancer cell line) were cultured in RPMI 1640 medium (Gibco) containing 10% FBS (Gibco) and 1% penicillin/streptomycin solution (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37°C. J82 cells (a bladder cancer cell line) were cultured in MEM-EBSS (Gibco) containing 10% FBS (Gibco) and 1% penicillin/streptomycin solution (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37°C. UMUC3 cells were cultured in MEM-EBSS (Gibco) containing 10% FBS (Gibco) and 1% penicillin/streptomycin solution (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37°C.

For transient transfections, we used Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol. For the transfection of RNA oligonucleotides, a concentration of 100 nM was used.

Oligodeoxynucleotide synthesis

Small interfering RNA (siRNA) of *KRT6A*, negative control (NC), miR-31-5p mimic, and miR-31-5p inhibitor, used in this study were synthesized by GeneCreate (Wuhan, China). A non-targeting siRNA, 5′-CCTAAGGTTAAGTCGCCCTCGCTC-3′ served as the NC. For *KRT6A* silencing, a siRNA-targeting sequence, 5′-CCAGCAGGAAGAGCUAUA-3′, was synthesized.

RNA extraction, cDNA synthesis, and quantitative real-time PCR (RT-qPCR)

Total RNA content was extracted from the sample tissues or cells using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions and then were reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Toyobo, Japan). The mRNA expression levels were quantified by RT-qPCR using a CFX96 instrument (Bio-Rad, USA) and iTAP^{TM} universal SYBR Green SuperMix (Bio-Rad). *U6* and *GAPDH* were used as reference genes for miRNA and mRNA, respectively, in RT-qPCR. Primers used for RT-qPCR listed in Table 1, were designed using Premier

Table 1. Primer sequence used in this study.

Gene		Primer Sequence
KRT6A	Forward	TCACCGTCAACCAGAGTCTC
	Reverse	GAACCTTGTTCTGCTGCTCC
ST3GAL5	Forward	CAAAGCAAGATGAGAAGG
	Reverse	AAACTTGGGACGACATTC
miR-31-5p	Forward	ACACTCCAGCTGGGTAGCAGCGGGAACAGTTC
	Reverse	CTCAACTGGTGTCGTGGA
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
GAPDH	Forward	CCATCTTCCAGGAGCGAGAT
	Reverse	TGCTGATGATCTTGAGGCTG

Primer v.5.0 software and synthesized by Sangon Biotech (Shanghai, China). Relative mRNA expression levels were calculated using the comparative $2^{-\Delta\Delta CT}$ method.

Cell Counting Kit-8 (CCK-8) assays

Approximately 5×10^4 T24 or J82 cells were seeded into 96-well culture plates and transfected with oligonucleotides using Lipofectamine 3000 (Invitrogen) at 60%–70% confluence. Cell proliferation was monitored every 24 h for 4 days (days 1, 2, 3, and 4) using the CCK-8 kit (TransGen Biotech, China). CCK-8 solution (10 μL) was added to each well of the 96-well culture plate and incubated for 1 h, and then the optical absorbance was measured at 450 nm using a Multiskan Go system (Thermo Fisher Scientific, USA).

5-bromodeoxyuridine (BrdU) enzyme linked immunosorbent assay (ELISA)

A BrdU cell proliferation ELISA kit (Beyotime, China) was used to detect the BrdU incorporation into the newly synthesized DNA of actively proliferating cells. BrdU (10 μM) was added to T24 or J82 cells cultured in microtiter plates. Fixing solution was used to immobilize antibody bound to the BrdU-incorporated cells. Then, the detector anti-BrdU monoclonal antibody was pipetted into the wells and incubated for 1 h. Goat anti-mouse antibody conjugated to horseradish peroxidase was added. The Multiskan Go system (Thermo Fisher Scientific) was used to measure optical absorbance of the solution at 450 nm.

Flow cytometry for cell apoptosis detection

Approximately 5×10^5 T24 or J82 cells were seeded into 12-well plates and transfected with si-*KRT6A*, NC, or si-*KRT6A* with miR-31-5p inhibitor using Lipofectamine 3000 (Invitrogen) at 60–70% confluence. After 48 h of transfection, the cells were collected and stained for 20 min at 25°C using an Annexin V-FITC/PI apoptosis detection kit (Beyotime) and analyzed using a CytoFLEX instrument (Beckman Coulter, USA), following the manufacturer's protocol.

Caspase 3 activation detection

Caspase 3 Activity Assay Kit (Beyotime) was used to measure caspase-3 activity in T24 or J82 cells, following the manufacturer's protocol. After 48 h of transfection, cells seeded into 6-well plates were collected after trypsin digestion (Gibco). One hundred microliters of cell lysis solution (Beyotime) was used for protein extraction. Then, 50 μL of protein supernatant was added to 96-well plates with 50 μL of Ac-DEVD-pNA (Beyotime). After a 2 h incubation at 37°C in dark conditions, caspase-3 activity was quantified at an optical absorption value of 405 nm using a Multiskan Go system (Thermo Fisher Scientific).

Cell adhesion assay

Approximately 5×10^5 cells were seeded into a 12-well plate and subjected to different treatments. A cell adhesion assay kit (BestBio, China) was used to coat a 96-well plate. After 48 h, cells were transferred to the coated plate at a concentration of 5×10^4 cells per well and incubated in a cell incubator at 37°C for 1 h. Then, the medium was replaced. Subsequently, 10 μL MTT solution (Beyotime) was added and incubated in a cell incubator at 37°C in the dark for 1 h. Finally, the optical absorption value at 450 nm was measured using a Multiskan Go system (Thermo Fisher Scientific).

Dual-Luciferase assay

To better understand the role of *KRT6A* in the development of bladder tumors, we identified

that *KRT6A* could represent a target gene of miR-31-5p through TargetScan Human v.7.2. miR-31-5p was predicted to bind to position 262–268 bp ("TCTTGCC") of *KRT6A* 3′-UTR.

PCR was used to amplify the segment sequence of *KRT6A*-3′-UTR encompassing the miR-31-5p binding site, which was then subcloned into the XbaI and XhoI restriction sites of the pmirGLO dual-luciferase reporter vector (Promega, USA) to generate a wild-type reporter (WT) vector. PCR mutagenesis was performed to generate a mutant-type plasmid (MUT). The binding site of miR-31-5p was changed from "TCTTGCC" to "TCTGACC" and DpnI digestion was used to remove the parental DNA. After obtaining the pmirGLO dual-luciferase reporter vector, co-transfection of WT or MUT plasmids, or miR-31-5p mimic and mimic NC, were performed. After 48 h of co-transfection, firefly and Renilla luciferase activities were measured using a Dual-GLO Luciferase Assay System Kit (Promega), following the manufacturer's instructions. Luminescence was measured using a fluorescence/multi-detection microplate reader (Bio-Tek, USA).

Western blotting

Approximately 1.2×10^5 T24 or J82 cells were seeded into 6-well plates. After 48 h of transfection, the cells were harvested and used to extract total proteins with Protein Extraction Kit (Solarbio, China). Protein concentrations were determined using the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific). Then, the protein samples were subjected to electrophoresis by 12% SDS-PAGE (MultiSciences Biotech, China), initially at 120 V for 20 min and then 100 V for 1 h. Subsequently, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad), and then probed using antibodies according to standard procedures. The antibodies used in this study were anti-KRT6A (Cat# ab238013, 1:10,000, Abcam, USA) and anti-GAPDH (Cat# ab8245, 1:10,000, Abcam) as equal sample loading controls. The secondary antibody used was goat anti-mouse IgG (H&L)-HRP (Cat# ab6728, 1:10,000, Abcam). The gray values of bands were determined using ImageJ software.

RNA pull-down assay

miR-31-5p mimic-biotin and miR-31-5p mimic-biotin with mutated seed sequences were synthesized by GeneCreate. After 48 h of T24 or J82 cell transfection, the cells were lysed using lysis buffer (Millipore, USA). Proteinase K (Millipore) and DNase I (Millipore) were used to remove proteins and DNA. Streptavidin magnetic beads (Invitrogen) were added and incubated at 4°C for 4 h. A magnetic grate (Thermo Fisher Scientific) was used to absorb streptavidin magnetic beads. After washing thrice, RNA was extracted and stored at −80°C. *KRT6A* mRNA levels were measured using RT-qPCR.

Data analysis

Each experiment was repeated at least thrice. All data are presented as means ± standard deviation. Statistical significance was tested using paired *t*-tests and one-way analysis of variance (ANOVA). Differences were considered statistically significant when the p-value was <0.05 (*) or $p < 0.01$ (**).

Results

KRT6A as gene of interest in bladder cancer

By comparing the gene expression profiles of all noninvasive samples and all invasive tumor samples in the GSE120736 data series, we obtained 25 significantly differentially expressed genes (DEGs, criteria: adjusted $p < 0.05$, $|\log FC| \geq 1.5$, Supplementary Table 1). Among them, *KRT6A* was the only upregulated gene. Data from the GEPIA database (http://gepia.cancer-pku.cn/index.html) showed that *KRT6A* was significantly upregulated in bladder cancer (Figure 1(a)). The overall survival prognosis analysis results also demonstrated that higher *KRT6A* levels predicted poorer survival outcomes (Figure 1(b)). *KRT6A* was also detected in tumor tissues by RT-qPCR and western blotting (Figures 1(c,d)). These results suggested that

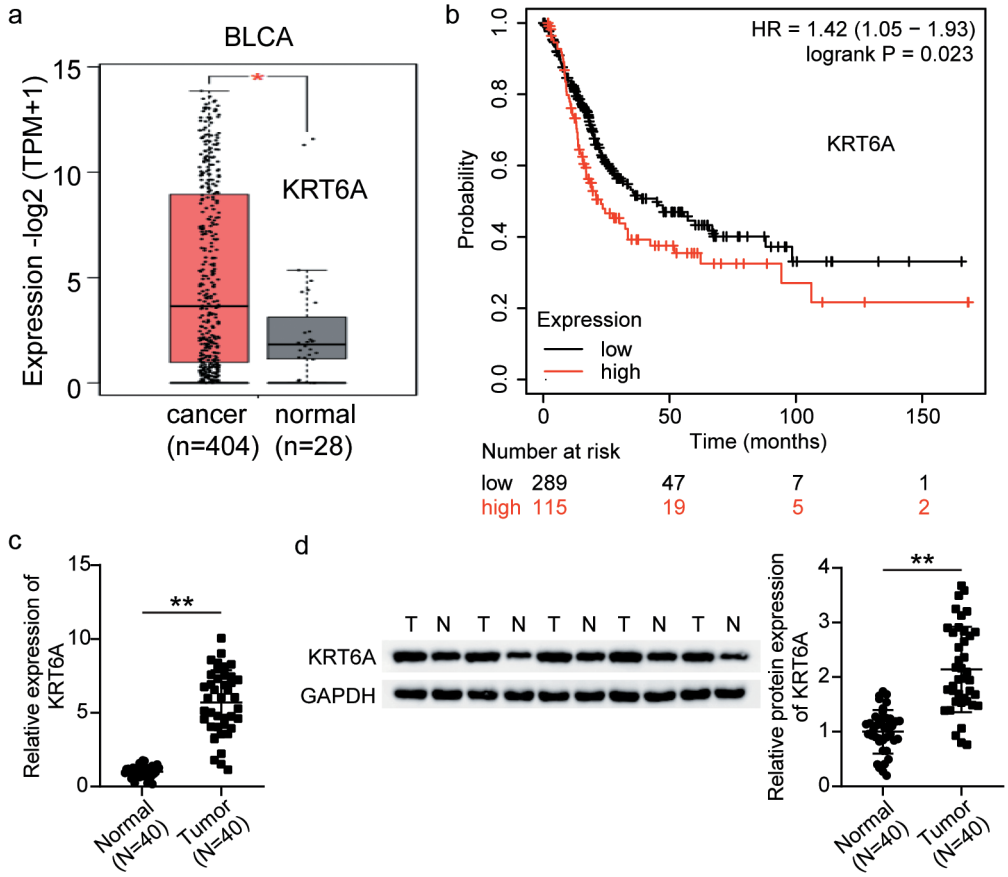

Figure 1. The potential significance of *KRT6A* in bladder cancer. (a) The expression of *KRT6A* in bladder cancer obtained from GEPIA database. BLCA, bladder cancer. *P < 0.01 (b) The overall survival prognosis outcome of *KRT6A* in bladder cancer calculated by Kaplan Meier plotter algorithm (http://kmplot.com). (c). RT-qPCR analysis of *KRT6A* mRNA levels in bladder cancer tissues and corresponding normal tissues, **P < 0.01 vs normal. (d). Western blots analysis of KRT6A proteins in bladder cancer tissues and corresponding normal tissues, **P < 0.01 vs normal.

KRT6A could be a possible tumor promoter in bladder cancer. The miRDB algorithm (http://mirdb.org/) was used to predict candidate miRNAs complementary to *KRT6A* mRNA. A total of 31 candidates were identified (Supplementary Table 2). Among the top-five ranked candidates, we noticed that miR-31-5p has been reported to be a tumor suppressor in bladder cancer [23,25,26]. Whether miR-31-3p could also suppress bladder cancer by targeting *KRT6A* has aroused our interest. Thus, we selected *KRT6A* as a gene of interest.

KRT6A knockdown relieves bladder tumor progression

Based on the aberrant, elevated expression of *KRT6A* in bladder tumor tissues, we also carried out RT-qPCR to measure *KRT6A* mRNA expression at the cellular level. The results showed that *KRT6A* was upregulated in bladder tumor cell lines including T24, J82, and UMUC3 (Figure 2(a)), whereas *KRT6A* was more dramatically upregulated in T24 and J82 cells. Accordingly, we used the T24 and J82 cell lines for further investigation. To explore the definite effect of KRT6A, we knocked down *KRT6A* via siRNA (si-*KRT6A*), and si-*KRT6A* efficiently reduced the levels of *KRT6A* mRNA and protein by > 50% in both T24 and J82 cells (Figure 2(b,c)), which meant that it could be used for further verification.

Tumor cell viability, proliferation, and apoptosis can affect tumorigenesis and cancer progression [27,28]; therefore, we performed CCK-8 and BrdU ELISA assays to verify the effect of

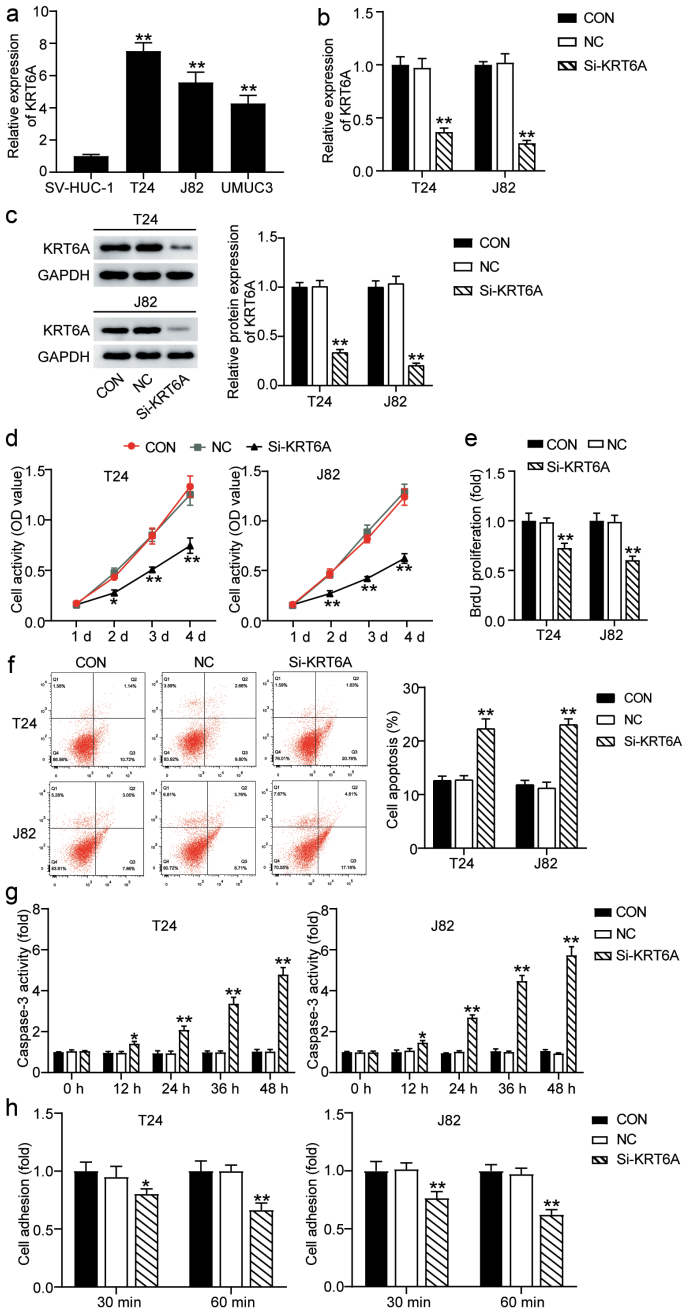

Figure 2. The knockdown of *KRT6A* relieves bladder tumor progression.(a) Relative mRNA expression level of *KRT6A* in normal bladder cell line (SV-HUC-1) and bladder cancer cell line (T24, J82, UMUC3) showed by RT-qPCR. (b) T24 or J82 cells were transfected with si-*KRT6A* or si-NC, and then the relative mRNA expression level of KRT6A was tested by RT-qPCR. (d) T24 or J82 cells were transfected with si-*KRT6A* or si-NC, and then the KRT6A protein was tested by Western blotting. (c) Cell viability was monitored by CCK-8 assay in T24 or J82 cells after transfection with si-*KRT6A* or si-NC. (d) BrdU assay was carried out to monitor the T24 or J82 cell proliferation after transfection with si-*KRT6A* or si-NC. (e) Flow Cytometry was used to detect cell apoptosis in T24 or J82 cells after transfection with si-*KRT6A* or si-NC. (f) Caspase 3 activation was detected in T24 or J82 cells after transfection with si-*KRT6A* or si-NC. (g) Cell adhesion detection was performed to observe the effect of KRT6A on tumor cell adhesion in T24 or J82 cells after transfection with si-*KRT6A* or si-NC. Data are presented as mean ± SD, *P < 0.05, **P < 0.01.

KRT6A on the viability and proliferation of bladder tumor cells, whereas flow cytometry was performed to verify the effect of KRT6A on apoptosis of bladder tumor cells. Compared to the control group, knockdown of *KRT6A* caused by si-*KRT6A* resulted in a visible decrease in the cell viability rates of T24 and J82 cells (Figure 2(d)). Similarly, BrdU ELISA assay results also indicated that knockdown of *KRT6A* significantly suppressed the proliferation of T24 and J82 cells by approximately 30% (Figure 2(e)). These results demonstrated that *KRT6A* knockdown had an inhibitory effect on bladder tumor cell viability and proliferation. After Annexin V-FITC/PI dual staining of T24 and J82 cells, flow cytometry was used to detect cell apoptosis. The results indicated that transfection with si-*KRT6A* increased the apoptosis rate (Q2+ Q3) of T24 and J82 cells by almost twice the baseline level (Figure 2(f)). As a crucial factor in cell apoptosis progression, caspase 3 activation levels reflect the degree of cell apoptosis. Thus, we also detected caspase 3 activation levels. The results showed that *KRT6A* knockdown increased caspase 3 activation levels in both T24 and J82 cells, with a peak level at 48 h, which increased almost six times (Figure 2(g)). These results indicated that *KRT6A* knockdown promoted apoptosis in bladder tumor cells. We also performed cell adhesion detection to observe the effect of *KRT6A* on tumor cell adhesion. The results showed that knockdown of *KRT6A* weakened adhesion of bladder tumor cells, and this weakening trend became more obvious over time (Figure 2(h)). In general, *KRT6A* knockdown is capable of relieving bladder tumor progression by regulating bladder tumor cell viability, proliferation, apoptosis, and adhesion.

KRT6A is a target of miR-31-5p

In view of the abnormal expression of *KRT6A* in bladder cancer cells, we attempted to determine its upregulation mechanism. We paid specific attention to the role of epigenetics in bladder tumors. In another RT-qPCR experiment, we found that miR-31-5p was significantly downregulated in bladder tumor tissues (n = 40) (Figure 3(a)), in contrast to the effect of *KRT6A*. Subsequently, we conducted correlation analysis between the expression of *KRT6A* and miR-31-5p to more clearly observe any relationship between their expression levels. This showed that miR-31-5p expression was negatively correlated with *KRT6A* mRNA expression (Figure 3(b)). Moreover, we also tested miR-31-5p expression at the cellular level. RT-qPCR results demonstrated that miR-31-5p was downregulated in bladder tumor cells by approximately 50% compared to SV-HUC-1 cells (Figure 3(c)).

Based on the negative correlation between miR-31-5p and *KRT6A*, we hypothesized that *KRT6A* could be a target gene of miR-31-5p, and upregulation of *KRT6A* in bladder tumors might be due to low expression of miR-31-5p. To confirm our hypothesis, we performed a prediction of the target relationship between miR-31-5p and *KRT6A* using TargetScan Human v.7.2 software. The results revealed that miR-31-5p perfectly matched the *KRT6A* 3′-UTR (Figure 3(d)). To verify binding involving miR-31-5p and *KRT6A* 3′-UTR, a dual-luciferase reporter assay was performed in T24 and J82 cells. Compared with other groups, the co-transfection of miR-31-5p mimic and pmirGLO-WT plasmid led to a 50% lower fluorescence activity (Figure 3(e)), indicating that miR-31-5p was able to bind to the *KRT6A* 3'-UTR and thereby reduced fluorescence activity. In addition, we synthesized an miR-31-5p mimic modified with biotin and synthesized an miR-31-5p mimic with a mutated seed sequence and biotin modification. RNA pull-down assays were carried out and we could clearly observe that the miR-31-5p mimic effectively pulled-down *KRT6A* (Figure 3(f)). These results demonstrated that *KRT6A* is a target of miR-31-5p.

Low miR-31-5p expression exacerbates bladder tumor progression by relieving KRT6A inhibition

These results confirmed the target relationship between miR-31-5p and *KRT6A*, and the low expression of miR-31-5p in bladder tumors. Given the aberrantly high expression of *KRT6A* in bladder tumors, we further investigated whether

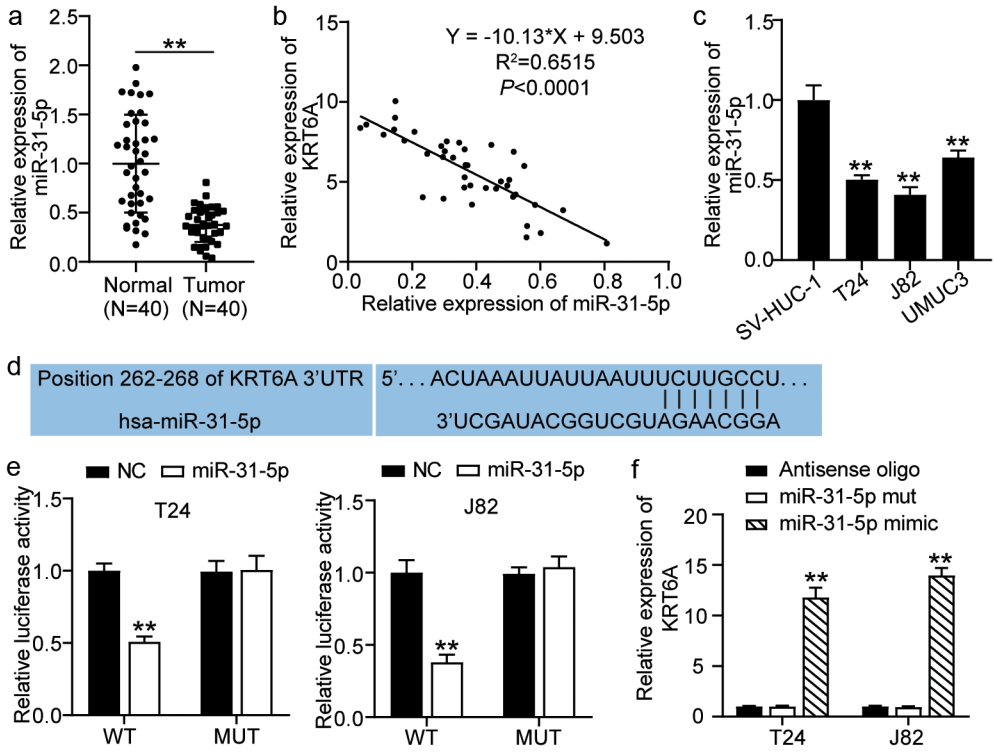

Figure 3. *KRT6A* is a target of miR-31-5p.(a) Relative mRNA expression level of miR-31-5p in bladder tumorous tissues and adjacent healthy tissues showed by RT-qPCR. (b) Correlation analysis of mRNA expression level between *KRT6A* and miR-31-5p. (c) Relative expression level of miR-31-5p in normal bladder cell line (SV-HUC-1) and bladder cancer cell line (T24, J82, UMUC3) showed by RT-qPCR. (d) TargetScan Human 7.2 was used to detect the target relationship between *KRT6A* and miR-31-5p. (e) Dual-luciferase report assay was performed after co-transfecting the wild type or mutant type of *KRT6A* with miR-31-5p mimic or mimic NC in T24 or J82 cells. (f) RNA Pull Down was performed to verify the target relationship between *KRT6A* and miR-31-5p in T24 or J82 cells. Data are presented as mean ± SD, *P < 0.05, **P < 0.01.

the low expression of miR-31-5p could exacerbate bladder cancer by inhibiting KRT6A. First, we synthesized an miR-31-5p inhibitor to achieve low expression of miR-31-5p in T24 and J82 cells. We performed co-transfection of si-*KRT6A* and inhibitor to observe whether the inhibitor could restore *KRT6A* expression downregulation at the mRNA and protein levels caused by si-*KRT6A*. The results showed that si-*KRT6A* was able to downregulate *KRT6A* mRNA expression by > 50%, while the miR-31-5p inhibitor increased *KRT6A* mRNA expression to the levels observed in the control and NC groups (Figure 4(a)). In addition, the miR-31-5p inhibitor increased KRT6A protein abundance, generating approximately 1.5-times the usual level, and knockdown of *KRT6A* restored this increase to normal levels (Figure 4(b)).

Similar to the verification of tumor cell viability, proliferation, apoptosis, and adhesion, we also performed a series of experiments to explore the effects of low miR-31-5p expression on bladder tumors. CCK-8 assay results showed that *KRT6A* knockdown reduced cell viability in T24 and J82 cells, while miR-31-5p inhibitor restored this decrease (Figure 4(c)). Moreover, BrdU ELISA assay results also revealed a similar effect on the proliferation of T24 and J82 cells (Figure 4(d)). These results demonstrated that the upregulation of *KRT6A* caused by low miR-31-5p expression could promote bladder tumor cell viability and proliferation. Flow cytometry assay results showed that *KRT6A* knockdown increased the bladder tumor cell apoptosis rate, which was caused by low miR-31-5p expression (Figure 4(e)). The activation level of caspase 3 in T24 and J82 gradually increased after knocking down KRT6A, whereas the activation level of caspase 3 did not increase after inhibiting miR-31-5p, although it was also treated with si-*KRT6A* (Figure 4(f)). Cell adhesion

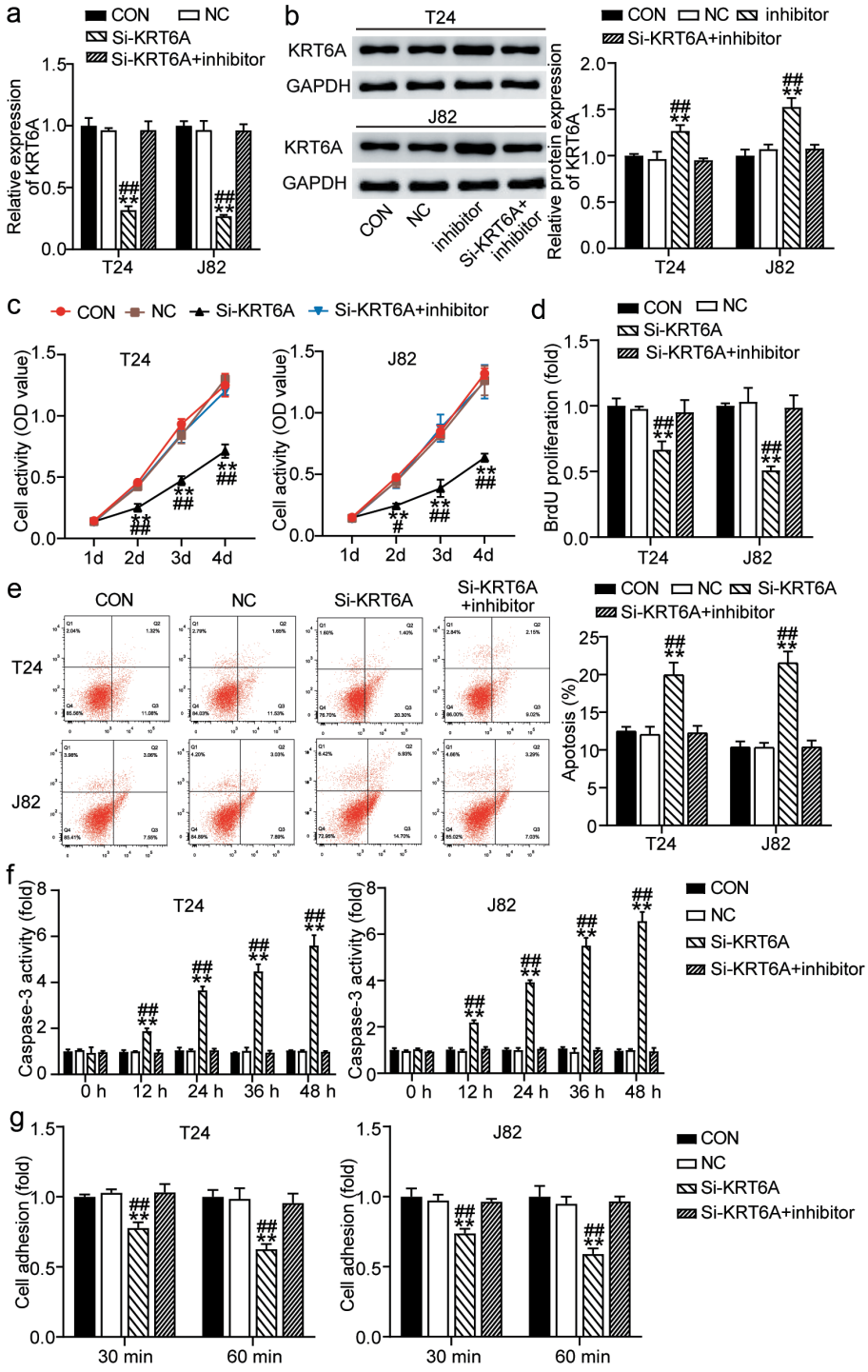

Figure 4. The low expression of miR-31-5p exacerbates bladder tumor progression by releasing the inhibition on KRT6A. (a) Relative mRNA expression level of *KRT6A* in T24 or J82 cells after transfections with si-*KRT6A* or si-*KRT6A* and miR-31-5p inhibitor or si-NC showed by RT-qPCR. (b) The protein expression level of KRT6A was detected by Western Blot. (c) Cell viability was monitored by CCK-8 assay in T24 or J82 cells after transfection with si-*KRT6A* or si-*KRT6A* and miR-31-5p inhibitor or si-NC. (d) BrdU assay was carried out to monitor the T24 or J82 cell proliferation after transfection with si-*KRT6A* or si-*KRT6A* and miR-31-5p inhibitor or si-NC. (e) Flow cytometry was used to detect cell apoptosis in T24 or J82 cells after transfection with si-*KRT6A* or si-*KRT6A* and miR-31-5p inhibitor or si-NC. (f) Caspase 3 activation was detected in T24 or J82 cells after transfection with si-*KRT6A* or si-*KRT6A* and miR-31-5p inhibitor or si-NC. (g) Cell adhesion detection was performed to observe the effect of KRT6A on tumor cell adhesion in T24 or J82 cells after transfection with si-*KRT6A* or si-*KRT6A* and miR-31-5p inhibitor or si-NC. Data are presented as mean ± SD, *P < 0.05, **P < 0.01.

detection results also indicated that low miR-31-5p expression reversed the weakening effect of si-*KRT6A* on tumor cell adhesion (Figure 4(g)). These results indicated that low miR-31-5p expression inhibited bladder tumor cell apoptosis and strengthened bladder tumor cell adhesion. In short, low expression of miR-31-5p in bladder tumors can lead to upregulation of KRT6A, which in turn aggravates bladder tumors.

Discussion

In this study, for the first time, we examined the bio-function of *KRT6A* in bladder cancer and uncovered new, underlying mechanisms. Using GEPIA database analysis, we found *KRT6A* to be strongly expressed in bladder cancer, and highlighted its prognostic potential. *In vitro*, we found that KRT6A silencing caused defective bladder cancer cell proliferation and apoptosis, suggesting a tumor-promoting role of *KRT6A* in bladder cancer. More importantly, we found that miR-31-5p targeting *KRT6A* led to KRT6A deregulation, which curbed bladder cancer progression. Our findings suggested that the miR-31-5p/*KRT6A* axis might be a candidate therapeutic target to combat bladder cancer.

KRT6A is a gene expressed in epithelium that expresses basal cell markers. Research has shown that KRT6A is related to a characteristic feature of aggressive basal subtypes of urothelial cell carcinoma [29]. Most bladder tumors are classified as urothelial cell carcinoma [30]. Indeed, we found that *KRT6A* was robustly expressed in bladder cancer cells, and that *KRT6A*-high patients had a poor five-year survival rate. KRT6A has been found to be involved in the regulation of cell-matrix formation and cell-cell adhesion [31]. Congruently, our data showed that *KRT6A* silencing blocked the proliferation and adhesion of T24 and J82 cells. Furthermore, *KRT6A* silencing induced apoptosis in bladder tumor cells. Thus, KRT6A exerts an oncogenic action in bladder cancer. Our findings are consistent with previous investigations showing that KRT6A promotes tumor progression in lung cancer [9], nasopharyngeal carcinoma [11], and oral squamous cell carcinoma [32]. Therefore, our findings offer further evidence for the pro-tumorigenic role of *KRT6A* in cancers.

It has been widely reported that miRNAs play essential roles in regulating bladder tumorigenesis [33]. In this study, we identified that *KRT6A* was targeted by miR-31-5p. To date, miR-31-5p has been reported to be involved in the progression of different types of cancer, including colon adenocarcinoma [34], oral cancer [35], colorectal cancer [36] and hepatocellular carcinoma [37] acting as a tumor suppressor or promoter. Tang et al. showed that miR-31-5p promotes the migratory and invasive capacity of T24 and J82 cells [24], while Bi et al. found that elevated miR-31-5p expression abrogates the uncontrolled proliferative, migratory, and invasive potential of T24 and UM-UC-3 cells [23]. These findings suggest a controversial role for miR-31-5p in bladder cancer. Our data showed that miR-31-5 was deregulated in bladder cancer, and anti-miR -31-5p treatment compromised the suppressed proliferation and adhesion of T24 and J82 cells along with the abrogation of apoptosis activation by *KRT6A* silencing, suggesting that the miR-31-5 mimic abrogated the oncogenic action and then played a tumor suppressor role in bladder cancer. Our findings further support the inhibitory effect of miR-31-5 on bladder cancer cells by targeting *KRT6A*.

However, we only sampled 40 cases of bladder tumors for detecting the mRNA expression levels of *KRT6A* in tumorous tissues and adjacent healthy tissues. It would be better if there were more cases. Here, we did not overexpress *KRT6A* and carried out a corresponding assay to verify the role of KRT6A in bladder tumors. As for subsequent exploration, we will complete these and further study pathways related to the genes *KRT6A* and miR-31-5p.

Conclusions

In summary, our results, for the first time, highlight the target relationship between *KRT6A* and miR-31-5p in bladder tumorigenesis, which could represent a novel potential therapeutic approach to address the diagnosis problem for bladder tumors.

Disclosure statement

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Author contributions

SBJ and JXY performed the experiments and data analysis. YC conceived and designed the study. YCS and JL made the acquisition of data. GHY did the analysis and interpretation of data. All authors read and approved the manuscript.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The present study was approved by the Ethics Committee of Tongji Hospital Affiliated to Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). The processing of clinical tissue samples is in strict compliance with the ethical standards of the Declaration of Helsinki.

Informed consent from participants

All patients signed written informed consent.

Consent to publish

Consent for publication was obtained from the participants.

ORCID

Guohong Yin http://orcid.org/0000-0002-4799-4116

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