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MiRNA-616 aggravates the progression of bladder cancer by regulating cell proliferation, migration and apoptosis through downregulating SOX7

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Abstract. – OBJECTIVE: To investigate the regulatory effect of microRNA-616 (miRNA-616) on cellular behaviors of bladder cancer and the potential mechanism.

PATIENTS AND METHODS: The expressions of miRNA-616 and SOX7 in bladder cancer tissues and cell lines were examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between miRNA-616 and SOX7 was assessed through Dual-Luciferase Reporter Gene Assay. The regulatory of miRNA-616 and SOX7 on cellular behaviors of bladder cancer were evaluated through cell counting kit-8 (CCK-8), colony formation, transwell migration assay, and flow cytometry.

RESULTS: MiRNA-616 was upregulated, whereas SOX7 was downregulated in bladder cancer tissues and cell lines. The silence of miRNA-616 attenuated the proliferation and migratory abilities, arrested cell cycle progression in G0/G1 phase, and stimulated apoptosis of BIU-87, EJ-JMUC3 and T24 cells. SOX7 was the target gene of miRNA-616, and its level was negatively regulated by miRNA-616. The knockdown of SOX7 enhanced the proliferation and migratory abilities, and attenuated apoptosis of bladder cancer cells.

CONCLUSIONS: MiRNA-616 accelerates bladder cancer cells to proliferate and migrate and inhibits apoptosis by downregulating SOX7. MiRNA-616/SOX7 may be potential therapeutic targets for bladder cancer.

Key words:
MiRNA-616, SOX7, Bladder cancer.

Introduction

Bladder cancer is the second most common urological malignancy in the United States, which ranks first in our country. In 2012, the International Agency for Research on Cancer (IARC) reported 330,956 new cases and 26,820 death cases of bladder cancer in China. In 2015, 740,000 new cases and 16,000 deaths of bladder cancer were reported in the United States[2]. The genetic polymorphisms, chromosomal abnormalities, and genetic changes are responsible for the occurrence and progression of bladder cancer[3]. The urothelial transitional cell carcinoma is the most prevalent type of bladder cancer[4]. For non-metastatic bladder cancer, surgery with post-operative chemical instillation is the preferred therapeutic strategy[5]. Although great strides have been made in the surgical procedures and adjuvant chemotherapy, the prognosis of bladder cancer remains unsatisfactory[6]. Hence, it is of significance to develop new sensitive biomarkers and therapeutic targets for bladder cancer.

MicroRNAs (miRNAs) are a group of single-stranded, endogenous, non-coding short RNAs[7]. They are post-transcriptional regulators that degrade target genes or inhibit their translation by complementary base pairing with the 3′-untranslated region (3′-UTR) of the target genes[8,9]. Currently, a great number of miRNAs have been identified in human genome[10,11]. They widely participate in physiological and pathological processes[12,13]. Abnormally expressed miRNAs are involved in the development and progression of a variety of human diseases, such as atherosclerosis, diabetes mellitus, migraine, and cancer diseases[14-16]. Serving as oncogenes or tumor-suppressor genes, miRNAs could influence tumor progression[17]. It is believed that miRNAs are capable of diagnosing and treating malignant diseases.

MiRNA-616 is a newly discovered cancer-related miRNA. It is upregulated in serum samples of patients with gastric cancer[18], lung cancer[19], and prostate cancer[20]. In addition, miRNA-616

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upregulates TFPI-2 in androgen-independent prostate cancer[20]. Our previous discovery found that miRNA-616 was upregulated in bladder cancer cells. In this paper, we mainly explored the biological role of miRNA-616 in the progression of bladder cancer.

Patients and Methods

Sample Collection

40 paired bladder cancer tissues and adjacent normal tissues were surgically resected, immediately placed in liquid nitrogen, and preserved at -80°C. None of the enrolled bladder cancer patients received preoperative anti-tumor therapies. Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of Shanghai Tongren Hospital.

Cell Culture

The human bladder immortalized epithelium cells (SV-HUC-1) and bladder cancer cells (UMUC3 and T24) were provided by A... can Type Culture Collection (ATCC) (Ma... VA, USA). The cells were cultured in D... co's Modified Eagle's Medium (DMEM; G... Rockville, MD, USA) containing 10% fetal ... vine serum (FBS; Life Techno... Gaither... burg, MD, USA), 100 UI/mL ... and 100 µg/mL streptomycin. The ... were ...intained at 37°C, in a 5% CO_2 inc... The ... replaced every 2-3 da...

Transfection

The transfe... ids were ...ided by GenePharma (Shangha... ina). The cells were pre-seede... the 6-well ... and transfected using L... ectamine 2000 (I... ogen, Carlsbad, CA, ...) at 70... f confluence. At 24-48 h, the cel... re ha... ted for subsequent experiments.

RNA Ex...ion ... qRT-PCR

RNA ex... from cells was performed usi... TRIzol ... ent (Invitrogen, Carlsbad, CA, U... RNA was purified by DNase I treat..., ... versely transcribed into comple...entary deoxyribose nucleic acid (cDNA) us... PrimeScript RT Reagent (TaKaRa, Otsu, S... Japan). The obtained cDNA underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references. Each sample was performed in triplicate, and the relative level was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: miRNA-61... F: 5'-CTGCTCAAACCCTCCAATGACT... 5'-GTGTAACACGTCTATACGCC... ; SOX... F: 5'-CAAGATGCTGGGAAA... GT-3'; R: 5'-CCGGTACTTGTAGTTGGGGTA... GAPDH, F: 5'-GGTGGTCTCCTCT... CTTC... R: 5'-GTTGCTGTAGCCAA... CGTTGT-... F: 5'-AGAGAAGATT... CATG... CCCCT... R: 5'-ATCCAGTGC... TCCC... G-3'.

Cell Counting ...8 (C...

The cells w... eeded in t... ...plate with 2×10^3 cells ... At the appo... ime points, the abso... e va... t 450 nm of each sample was recorded using t... K-8 kit (Dojindo Laborat... Kumamoto, ...) for depicting the v... ty curves.

...ony Forma... Assay

... cells wer... oculated in a 6-well plate with 1×1... ...ll and incubated for 2 weeks. Subseq... e cells were fixed in 100% methanol and dyed with 0.5% crystal violet for 20 min. ...onies were finally captured and calculated ...ber.

Cell Cycle Distribution Determination

The cells were fixed in 70% ethanol overnight, incubated with RNase A, and dyed with Propidium Iodide (PI) for 25 min. Subsequently, cell cycle distribution was analyzed on the BD FACSCalibur™ flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Cell Apoptosis Determination

The cells were collected, centrifuged at 1000 r/min for 3 min, and resuspended in 800 µL of phosphate-buffered saline (PBS). After dying with Annexin V and PI in the dark for 15 min, the distribution of normal, early-stage apoptotic, late-stage apoptotic, and necrotic cells were analyzed using flow cytometry.

Transwell Migration Assay

The cells were inoculated in the upper side of the transwell chamber (Corning, Corning, NY, USA). In the bottom side, 700 µL of medium containing 10% FBS was applied. After 48 h of incubation, the cells migrated to the bottom side were subjected to fixation in methanol for 15 min, crystal violet staining for

20 min, and cell counting using a microscope. The number of migratory cells was counted in 5 randomly selected fields per sample (magnification 200×).

Dual-Luciferase Reporter Gene Assay

The potential binding sites between miRNA-616 and SOX7 were predicted by TargetScan. The cells were co-transfected with miRNA-616 mimics/NC and wt SOX7 3'UTR/mut SOX7 3'UTR using Lipofectamine 2000. After 24 h, the co-transfected cells were collected to determine the luciferase activity using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 (La Jolla, CA, USA) was used for the data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. The Spearman regression test was performed to evaluate the relationship between the two genes. $p<0.05$ was considered as statistically significant.

Results

MiRNA-616 Was Upregulated in Bladder Cancer

By analyzing the colle bladder ca sues and adjacent norm nes, i found miRNA-616 was up ated i dder cancer (Figure 1A). Simil rly, 616 wa ghly expressed in the der c cells t that of the bladder ep al cells (F o uncover the biolo tion of mi 616 in bladder canc e n A-616 inhibitor was constructed. Transfectio iRNA-616 inhibitor in UM d T24 cells dly downregulated m A-616 level (Figures C, 1D). It is indicated

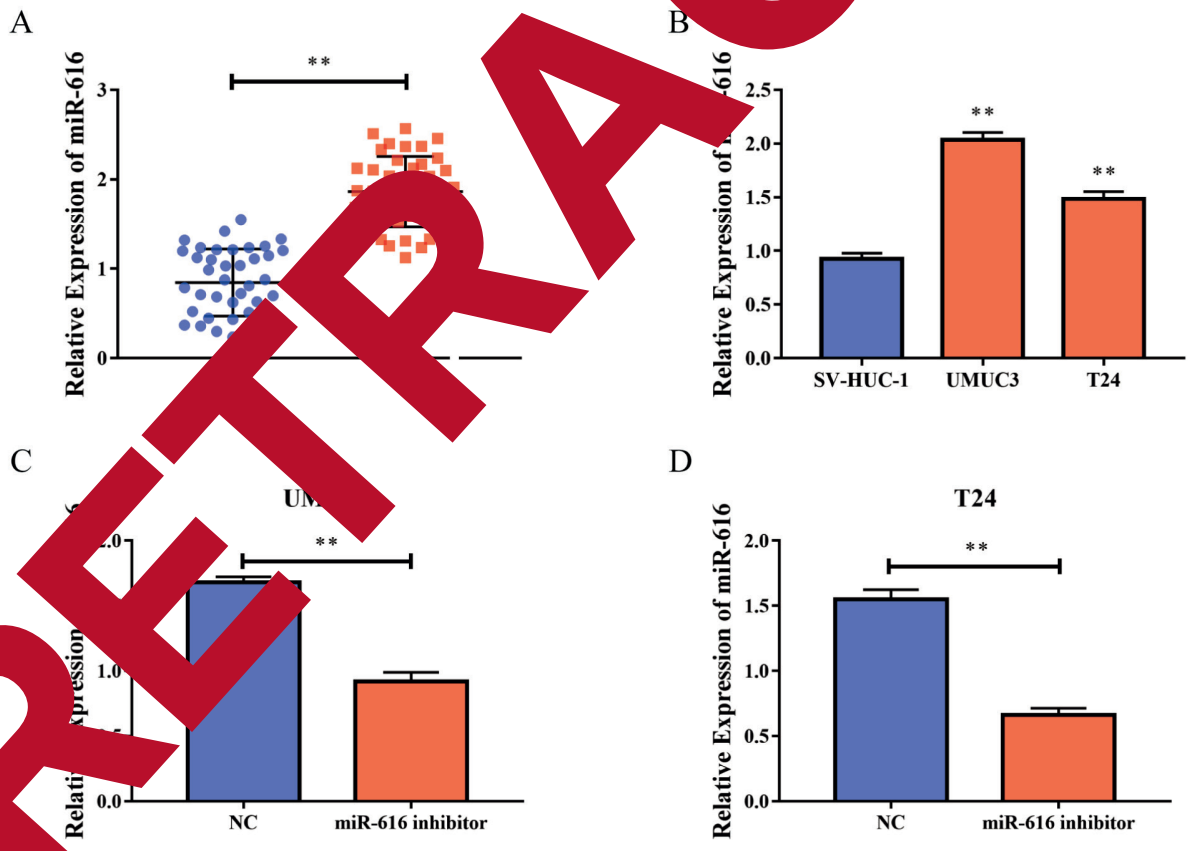


Figure 1. MiR-616 was upregulated in bladder cancer. **A,** The relative level of miR-616 in bladder cancer tissues and adjacent normal tissues. **B,** The relative level of miR-616 in human bladder immortalized epithelium cells (SV-HUC-1) and bladder cancer cells (UMUC3 and T24). **C,** The transfection efficacy of miR-616 inhibitor in UMUC3 cells. **D,** The transfection efficacy of miR-616 inhibitor in T24 cells.

that miRNA-616 may exert a carcinogenic role in bladder cancer.

MiRNA-616 Regulated Proliferative, Migratory, and Apoptotic Abilities, and Cell Cycle Progression of Bladder Cancer

The cellular behavior changes of bladder cancer influenced by miRNA-616 were explored through a series of functional experiments. CCK-8 assay showed a viability reduction in UMUC3 and T24 cells transfected with miRNA-616 inhibitor (Figures 2A, 2B). The silence of miRNA-616 also reduced the colony formation number in bladder cancer cells, suggesting the inhibited proliferative ability (Figures 2C, 2D). The apoptotic rate was found to be markedly elevated in UMUC3 and T24 cells transfected with miRNA-616 inhibitor (Figure 2E). The flow cytometry was conducted to assess cell cycle progression. The ratio of the cells distributed in the G2 phase significantly increased, and that in G1 phase decreased after the knockdown of miRNA-616 (Figures 2F, 2G). Moreover, the number of migratory cells was reduced after the transfection of miRNA-616 inhibitor (Figure 2H).

MiRNA-616 Bound to SOX7 3'UTR

Through bioinformatics prediction, the b ing sites between SOX7 and miRNA-616 w identified (Figure 3A). Based c sites, w constructed wt SOX7 3'UT nd t SOX7 3'UTR vectors for perfor the D Lucifer- ase Reporter Gene Assay lucif markedly decreased r t sfecti of miRNA-616 mimi nd wt 7 3'UTR in UMUC3 and T24 verifying nding between miRNA OX7 (Figu B, 3C). The transfecti of mi 616 inhibitor remarkably upre ed the SOX el in bladder cancer cell gure 3D). The tra ction efficacy of si-SO was v ed in both UMUC3 and T24 cel gure . The miR-66 level was upregulated sfectio si-SOX7, suggesting a ative onshi tween the expressions of A-61 7 (Figure 3F).

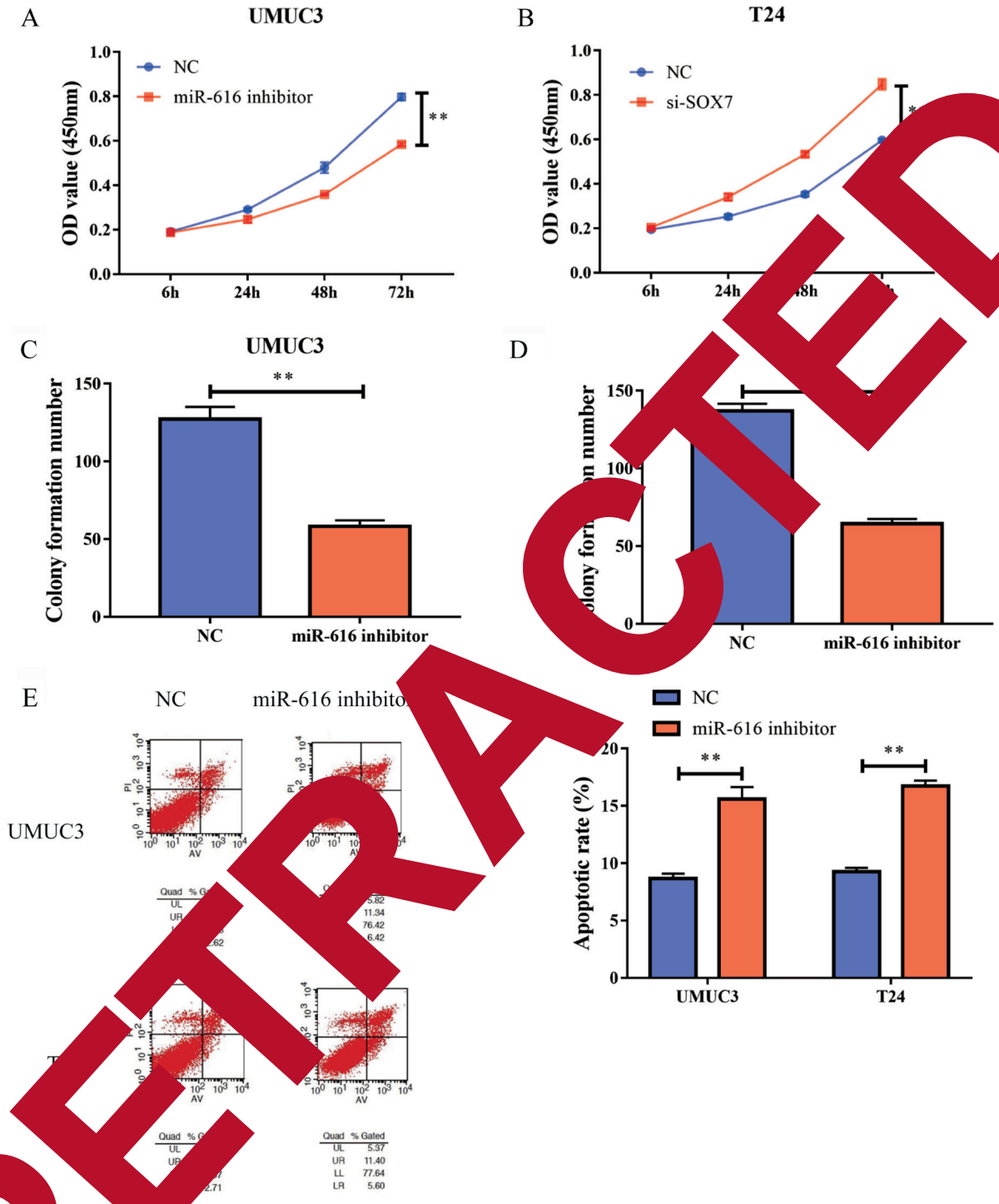
SO Was Downregulated in Bladder

Compared with the adjacent normal tissues, 7 was downregulated in bladder cancer tis- igure 4A). A negative correlation was observed between SOX7 and miRNA-616 in bladder cancer tissues (Figure 4B). Subsequently, we explored the influence of SOX7 on cellular behaviors of bladder cancer. As the viability curves revealed, the transfection of si-SOX7 elevated the viability in both UMUC3 and T24 cells (Figures 4C, 4D). The colony formation numb as enhanced after the knockdown of SO strating the improved proliferative a y (Figur 4E). Meanwhile, the migratory umber per field was markedly elevated after t sfection of si-SOX7 (Figure 4G). On cont e si- lence of SOX7 attenuated tosis of the cancer cells (Figure 4F

Discus

Bladder one of the m mmon malignancie ma rldwide[21]. So far, radical cystectomy is the sta l treatment for bladder can eoadjuvant therapy effectively p gs the survival in patients with muscu- nvasive bladd r cancer (MIBC) undergoing al cystecto . However, the prognosis of b r cancer still poor due to high local rec a etastasis rates[24]. The identification y molecules involved in bladder ancer progression is particularly important to therapeutic efficacy. Many upregulated nregulated miRNAs in bladder cancer have been identified, which are closely related to the changes in cellular behaviors and signaling pathways in bladder cancer[25].

SOX genes (SOXs) exert an important role in the occurrence, migration and invasion of malignant tumors[26]. SOX7 locates on chromosome 8p23.1. It consists of two exons and an intron with a gene length of about 7.7 kbp. The SOX7 gene is composed of 388 amino acids. Its C-terminus is a transactivation domain that regulates the transcriptional activity and the N-terminus is an HMG-box DNA binding domain that specifically recognizes 5'-(A/T) (A/T) CAA (A/T)-3' sequence[27]. The transcriptional regulators encoded by the SOX gene are involved in the nervous system development, cartilage development, cardiovascular formation, and blood cell formation[28]. Meanwhile, these transcriptional factors are also involved in tumorigenesis and tumor development[29]. It is reported that SOX7 can inhibit the activation of the catenin and block catenin-induced transcription of the downstream genes, suggesting that SOX7 may be a potential checkpoint for bladder cancer[30]. In addition, the tumor proliferative and differentiation rates in SOX7$^{-/-}$ mice are significantly accelerated. The immunohistochem-



Fi 616 regulated proliferative, migratory and apoptotic abilities, and cell cycle progression of bladder cancer. **A,** UMUC3 cells transfected with NC or miR-616 inhibitor. **B,** The viability in T24 cells transfected with NC miR-616 inhibitor. **C,** The colony formation number in UMUC3 cells transfected with NC or miR-616 inhibitor. **D,** The ny formation number in T24 cells transfected with NC or miR-616 inhibitor. **E,** Apoptotic rate in UMUC3 and T24 cells cted with NC or miR-616 inhibitor. **F,** The cell cycle distribution in UMUC3 cells transfected with NC or miR-616 in r. **G,** The cell cycle distribution in T24 cells transfected with NC or miR-616 inhibitor. **H,** The migratory cell number per field in UMUC3 and T24 cells transfected with NC or miR-616 inhibitor (magnification: 40×).

Figure continued

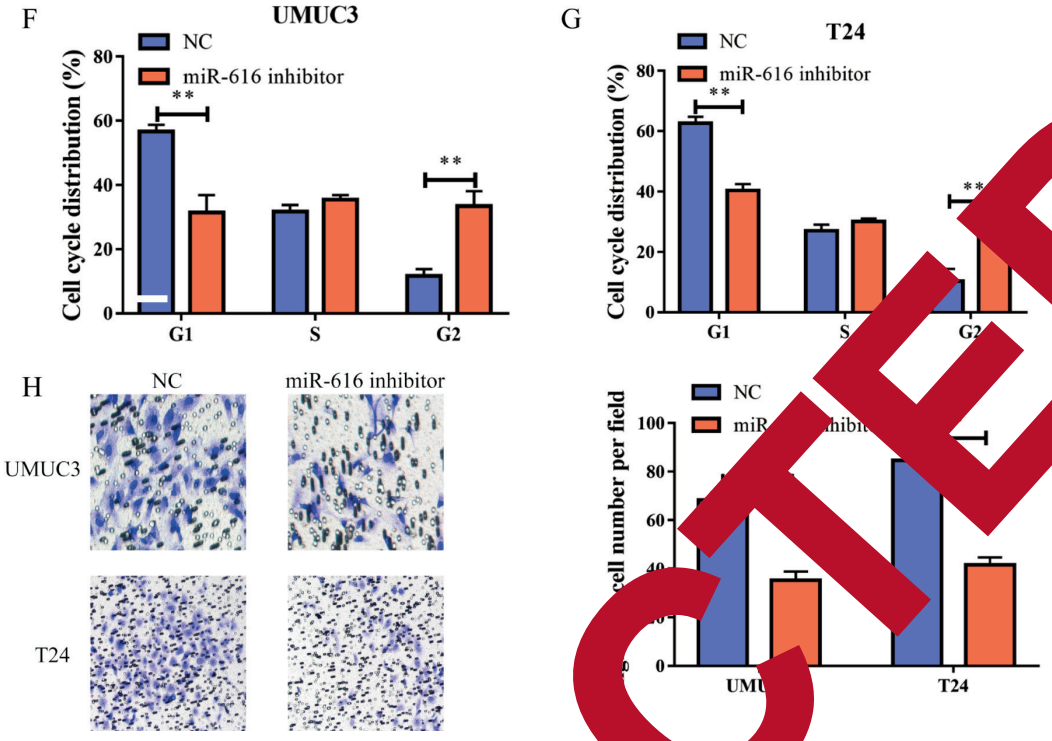
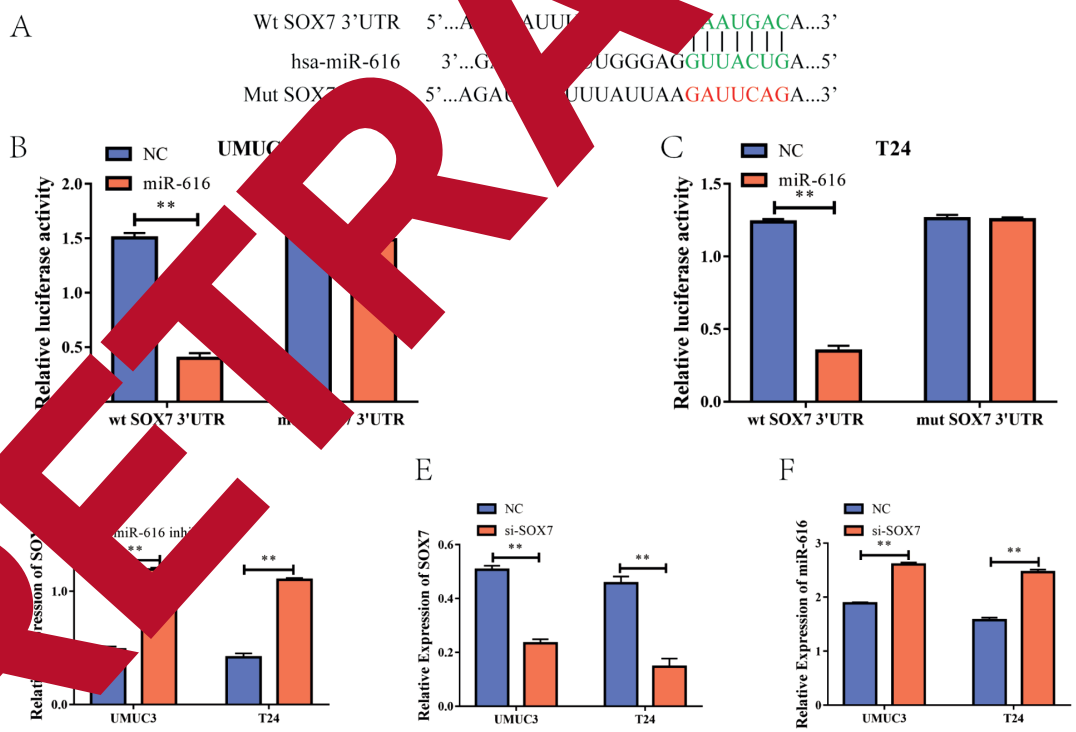


Figure 2. *(Continued).* **F,** The cell cycle distribution in UMUC3 cells [illegible] NC or miR-616 inhibitor. **G,** The cell cycle distribution in T24 cells transfected with NC [illegible] 616 inhibitor. [illegible] gratory cell number per field in UMUC3 and T24 cells transfected with NC or miR-616 inh[illegible]fication: 40×).



[illegible]3. MiR-616 bound to SOX7 3'UTR. **A,** The binding sites between miR-616 and SOX7. **B,** The luciferase activity in UM[illegible]3 cells co-transfected with wt SOX7 3'UTR/mut SOX7 3'UTR and miR-616 mimics/NC. **C,** The luciferase activity in T24 cells co-transfected with wt SOX7 3'UTR/mut SOX7 3'UTR and miR-616 mimics/NC. **D,** The relative level of SOX7 in UMUC3 and T24 cells transfected with NC or miR-616 inhibitor. **E,** The transfection efficacy of si-SOX7 in UMUC3 and T24 cells. **F,** The relative level of miR-616 in UMUC3 and T24 cells transfected with NC or si-SOX7.

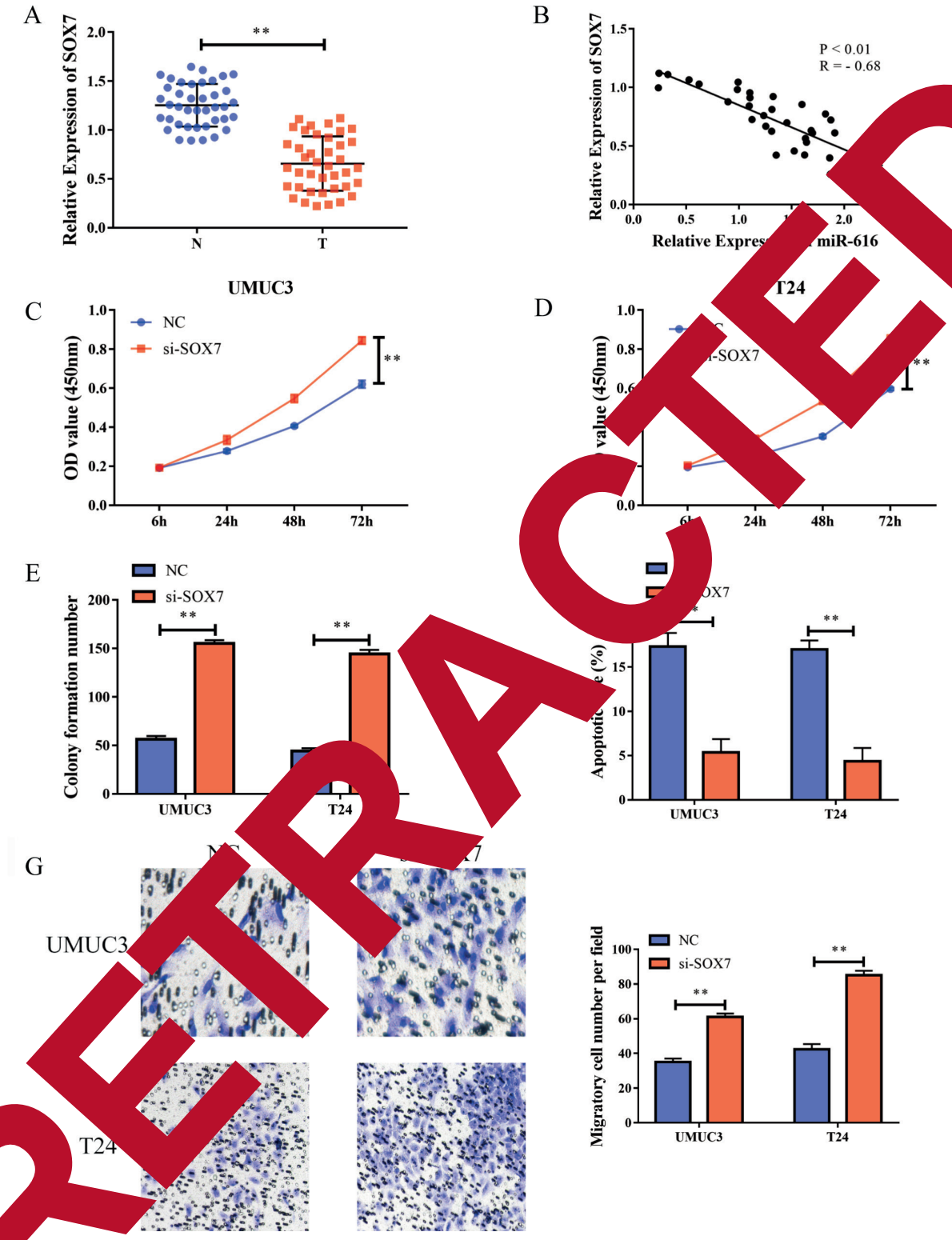


Figure 4. SOX7 was downregulated in bladder cancer. **A,** The relative level of SOX7 in bladder cancer tissues and adjacent normal tissues. **B,** A negative correlation between the expression levels of miR-616 and SOX7. **C,** The viability in UMUC3 cells transfected with NC or si-SOX7. **D,** The viability in T24 cells transfected with NC or si-SOX7. **E,** The colony formation number in UMUC3 and T24 cells transfected with NC or si-SOX7. **F,** The apoptotic rate in UMUC3 and T24 cells transfected with NC or si-SOX7. **G,** Migratory cell number per field in UMUC3 and T24 cells transfected with NC or si-SOX7 (magnification: 40×).

ical examination has found that SOX7 expression in prostate cancer is markedly downregulated[31]. SOX7 can be used as a tumor suppressor of prostate cancer. The downregulation of SOX7 in prostate cancer is associated with high methylation of promoters. It is found that SOX7 regulates the decomposition of active S33Yβ-catenin through APC-independent mechanism and thus exerts an anti-tumor effect[32]. In liver cancer, the upregulated SOX7 can impair the carcinogenic effect of microRNA-24[33]. A survival analysis showed that SOX7 is an independent prognostic factor for assessing overall survival and recurrence-free survival in lung adenocarcinoma[34].

In this paper, miRNA-616 was upregulated, whereas SOX7 was downregulated in bladder cancer tissues and cell lines. The silence of miRNA-616 attenuated proliferative and migratory abilities, arrested cell cycle progression in G2 phase, and stimulated apoptosis in UMUC3 and T24 cells. SOX7 was the target gene of miRNA-616, and its level was negatively regulated by miRNA-616. The knockdown of SOX7 enhanced the proliferative and migratory abilities, and attenuated apoptosis of the bladder cancer cel

Conclusions

MiRNA-616 accelerates bladd er cells proliferate and migrate and i tosis by downregulating SOX7. MiR -616/SO may be potential therapeutic targe bladd

Conflict of Inter

The Authors decl have no confl interests.

Referenc

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