

lncRNA TINCR facilitates bladder cancer progression via regulating miR-7 and mTOR

GUOYING XU^{1*}, HONGLAN YANG^{2*}, MEICHUN LIU³,
JINTAO NIU¹, WEIDONG CHEN⁴, XIAOJING TAN² and LI SUN⁴

¹Department of Urology Surgery, Shengli Hospital of Shengli Petroleum Administration, Dongying, Shandong 257055;

²Department of Oncology, Dongying People's Hospital, Dongying, Shandong 257091;

³Clinical Laboratory and ⁴Department of Oncology, Shengli Hospital of Shengli Petroleum Administration, Dongying, Shandong 257055, P.R. China

Received June 7, 2019; Accepted March 26, 2020

DOI: 10.3892/mmr.2020.11530

Abstract. Long non-coding RNAs (lncRNAs) have been implicated in various human malignancies, but the molecular mechanism of lncRNA TINCR ubiquitin domain containing (TINCR) in bladder cancer remains unclear. The present study found that the expression of TINCR was significantly increased in bladder cancer tissues and cell lines, when compared with that in adjacent normal tissues and normal urinary tract epithelial cell line SV-HUC-1, respectively. Moreover, the high expression of TINCR was associated with tumor metastasis and advanced tumor, node, metastasis stage, as well as reduced overall survival rates of patients with bladder cancer. Further investigation revealed that microRNA (miR)-7 was negatively mediated by TINCR in bladder cancer cells. Silencing of TINCR expression significantly increased miR-7 expression and reduced bladder cancer cell proliferation, migration and invasion, while knockdown of miR-7 expression reversed the inhibitory effects of TINCR downregulation on bladder cancer cells. mTOR was then identified as a target gene of miR-7 in bladder cancer, and it was demonstrated that overexpression of mTOR reversed the inhibitory effects of miR-7 on bladder cancer cells. In conclusion, this study suggests that TINCR/miR-7/mTOR signaling may be a potential therapeutic target for bladder cancer.

Introduction

Bladder cancer is a common human malignancy worldwide, with an increasing incidence in recent years (1). Currently, the

main treatment of bladder cancer is surgery, however due to high rates of recurrence and metastasis, the overall survival rate of patients with bladder cancer is still unsatisfactory (1-3). Recently, various oncogenes and tumor suppressors have been reported to have roles in the development and progression of bladder cancer (4,5). Understanding the molecular mechanisms may be beneficial for developing effective therapeutic strategies for bladder cancer.

Long non-coding RNAs (lncRNAs) are a type of non-coding transcripts containing >200 nucleotides, they have been shown to serve important regulatory roles in different types of cancer via interactions with microRNAs (miRNAs/miRs) or proteins (6,7). Recently, the dysregulation of lncRNA TINCR ubiquitin domain containing (TINCR) has been observed in gastric (8), breast (9) and colon cancer (10), and esophageal squamous cell carcinoma (11). Moreover, it has been reported that TINCR levels are significantly increased in bladder cancer, and that the inhibition of TINCR has the ability to suppress bladder cancer cell proliferation while also promoting cell apoptosis (12). miRNAs are also a type of non-coding transcripts containing only 22-25 nucleotides. They can directly interact with the 3'-untranslated region (UTR) of their target mRNAs, and thus are important regulators in gene expression. A large number of studies have demonstrated that miRNAs participate in different cellular processes, such as cell proliferation, survival, differentiation, motility and tumorigenesis (13-15). By mediating the expression of different target genes, miR-7 serves different roles in various types of human cancer (16-19). For instance, miR-7 functions as a tumor suppressor in non-small cell lung cancer by inhibiting the expression of paired box 6 (16). Conversely, miR-7 contributes to colorectal tumorigenesis by targeting YY1 transcription factor (18). mTOR, a serine/threonine kinase, belongs to the PI3K-associated family. Previous studies have demonstrated that mTOR is essential for the survival, proliferation, migration and invasion of tumor cells (20-22). Therefore, it has been suggested that mTOR could be a potential therapeutic target in the treatment of bladder cancer (20-22).

However, until now, the exact association between TINCR, miR-7 and mTOR in bladder cancer remains unknown. The present study explored the regulatory mechanism of TINCR

Correspondence to: Professor Li Sun, Department of Oncology, Shengli Hospital of Shengli Petroleum Administration, 107 Qingzhou Road, Dongying, Shandong 257055, P.R. China
E-mail: dongyingsunli@126.com

*Contributed equally

Key words: TINCR ubiquitin domain containing, mTOR, microRNA-7, bladder cancer

underlying bladder cancer progression involving miR-7 and mTOR. The present study demonstrated that the upregulation of TINCR was associated with an aggressive tumor phenotype and poor prognosis in bladder cancer, and suggested that TINCR promotes the malignant phenotypes of bladder cancer by regulating the miR-7/mTOR axis.

Materials and methods

Clinical samples. The present study obtained approval from the Ethics Committee of Shengli Hospital of Shengli Petroleum Administration and all patients provided written informed consent. Bladder cancer tissues and their matched adjacent normal tissues were obtained from 53 patients during surgical treatment between March 2010 and March 2012 at Shengli Hospital. TNM staging for bladder cancer was used in the present study (23). No patients received chemotherapy or radiotherapy prior to surgery. The clinical characteristics of the patient cohort are summarized in Table I.

Cell culture and transfection. The normal human urinary tract epithelial SV-HUC-1 cell line and bladder cancer T24, HT-1376 and 5637 cell lines were obtained from the American Type Culture Collection. These cell lines were cultured in DMEM (Thermo Fisher Scientific, Inc.) with 10% FBS (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. Cell transfection was conducted in T24 and 5637 cells with Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In brief, T24 and 5637 cells (2 × 10⁵ cells/well) were seeded into 6-well plates, and transfected with 100 nM negative control (NC) small interfering (si)RNA (cat. no. 12935200; Thermo Fisher Scientific, Inc.), 100 nM TINCR siRNA (cat. no. AM16708; Thermo Fisher Scientific, Inc.), 100 nM NC inhibitor (cat. no. 4464076; Thermo Fisher Scientific, Inc.), 100 nM miR-7 inhibitor (cat. no. 4464084; Thermo Fisher Scientific, Inc.), 100 nM miR-NC mimic (cat. no. 4464058; Thermo Fisher Scientific, Inc.), 100 nM miR-7 mimic (cat. no. 4464066; Thermo Fisher Scientific, Inc.), 800 ng blank pcDNA3.1 vector (cat. no. V79020; Thermo Fisher Scientific, Inc.), or 800 ng mTOR expression plasmid (Yearth Biotech; www.yearthbio.com). After cell transfection for 48 h, reverse transcription-quantitative (RT-q)PCR was conducted to examine gene expression levels.

RT-qPCR. Total RNA was isolated from tissues and cell lines using TRIzol® Reagent (Thermo Fisher Scientific, Inc.), which was reverse transcribed into cDNA with a PrimeScript RT reagent kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Reverse transcription was performed at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd.) was used to examine the expression of lncRNA, miRNA and mRNA. PCR was initiated at 95°C for 3 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec; and 72°C for 15 sec. The relative gene expression was determined using the 2^{-ΔΔC_q} method (24). U6 was used as an internal reference for miRNA. GAPDH was used as an internal reference for lncRNA and mRNA. Primer sequences used were as follow: GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-ATG GTGGTGAAGACGCCAGT-3'; TINCR forward, 5'-TGTGGC

CCAAACTCAGGGATACAT-3' and reverse, 5'-AGATGA CAGTGGCTGGAGTTGTCA-3'; U6 forward, 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGGCT-3'; and mTOR forward, 5'-ATGCTTGGAAACCGGA CCTG-3' and reverse, 5'-TCTTGACTCATCTCTCGGAGT T-3'. Primers for miR-7 (cat. no. HmiRQP0785) were obtained from FugenGen, Co., Ltd.

Cell proliferation, migration and invasion assays. Transfected cells (5,000 cells/well) were seeded in 96-well plates, and a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) was used to detect cell proliferation, according to the manufacturer's instruction. The abundance at 450 nm was measured. Cell migration and invasion were detected using Transwell chambers with or without Matrigel (BD Biosciences). The precoating was conducted at room temperature for 1 h. The re-suspended transfected cells (1 × 10⁵ cells) in serum-free DMEM were added to the upper chamber, and the lower chamber contained DMEM with 10% FBS. Cells were then incubated at 37°C for 48 h. The migratory and invading cells through the membrane were fixed using 70% methanol at room temperature for 30 min, stained with 0.1% crystal violet at room temperature for 10 min, and images were captured under a light microscope (magnification, ×200).

Bioinformatics analysis and luciferase reporter assay. TargetScan software version 7.1 (www.targetscan.org) was used for bioinformatics analysis. To clarify the association between TINCR and miR-7, a QuikChange Site-Directed Mutagenesis kit (Stratagene; Agilent) was applied to generate the mutated miR-7-binding sites of TINCR. Then, the wild or mutated TINCR was sub-cloned into a Dual-luciferase Target Vector (Promega Corporation), generating the wild-type (WT) or mutated (MT) TINCR luciferase reporter plasmid, respectively. To clarify the association between miR-7 and mTOR, a QuikChange Site-Directed Mutagenesis kit (Stratagene; Agilent) was applied to generate the mutated miR-7-binding sites of mTOR 3'UTR. The WT or MT mTOR luciferase reporter plasmid was then generated. Cells were co-transfected as aforementioned with miR-7 mimic or negative control miR mimic (miR-NC), WT (or MT) TINCR reporter plasmid, WT (or MT) mTOR 3'UTR reporter plasmid. At 48 h after cell transfection, a Dual-Luciferase Reporter Assay System (Promega Corporation) was used to examine the luciferase activities. The ratio of firefly luciferase activity to *Renilla* luciferase activity was determined.

Western blotting. The cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology). Protein concentration was determined using a BCA kit (Abcam). Proteins (60 μg per lane) were separated using 12% SDS-PAGE, and then transferred onto PVDF membranes (Thermo Fisher Scientific, Inc.). The membranes were blocked with 5% dry milk in Tris-buffered saline with 0.2% Tween-20 at 4°C overnight, and then incubated with primary antibodies against mTOR (1:500; cat. no. ab134903) and GAPDH (1:500; cat. no. ab8245) at room temperature for 3 h, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6721) at room temperature for 1 h. All antibodies were purchased from Abcam. Then, the signals were visualized with an enhanced chemiluminescence kit

Table I. Association between TINCR expression and clinicopathological characteristics in bladder cancer.

Variables	Cases (n=53)	TINCR expression		P-value
		High (n=23)	Low (n=30)	
Age				0.775
<55	19	9	10	
≥55	34	14	20	
Sex				0.779
Male	33	15	18	
Female	20	8	12	
Grade				0.052
Well and moderately	40	14	26	
Poor	13	9	4	
Lymph node metastasis				0.009 ^a
Negative	34	10	24	
Positive	19	13	6	
Distant metastasis				0.012 ^a
Present	5	5	0	
Absent	48	18	30	
TNM stage				0.002 ^a
I-II	27	6	21	
III-IV	26	17	9	

TINCR, TINCR ubiquitin domain containing; TNM, Tumor Node Metastasis. ^aP<0.05 was considered to indicate a statistically significant difference.

(GE Healthcare), and quantified using ImageJ software v1.46 (National Institutes of Health).

Statistical analysis. Statistical analyses were conducted using SPSS 20.0 software (IBM Corp). Data in this study are expressed as the mean ± SD. A Student's t-test was used for comparisons between two groups, and a one-way ANOVA followed by a Turkey's post hoc test was used for comparisons among >2 groups. A χ^2 test was used to analyze the association between gene expression and clinical characteristics of patients. A Kaplan-Meier method followed by a log-rank test was applied for the survival analyses. Spearman's correlation analysis was also performed. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of TINCR is associated with aggressive phenotypes and poor prognosis in bladder cancer. To reveal the function of TINCR in bladder cancer progression, the expression levels of TINCR in clinical tissue samples were examined. The results of the RT-qPCR assays showed that the expression of TINCR was significantly increased in bladder cancer tissues compared to matched adjacent normal tissues (Fig. 1A). Moreover, the TINCR levels were also increased in the bladder cancer cell lines compared with those in the SV-HUC-1 cells (Fig. 1B). Based on the median expression value (1.97) of TINCR as a cut-off value, the patients with bladder cancer were divided into high and low TINCR expression groups.

Moreover, the patients with median expression values were included in high TINCR expression group. It was identified that a high expression of TINCR was significantly associated with advanced TNM stage and metastasis (Table I). Moreover, the patients with a high TINCR expression exhibited poorer survival rates compared with those with a low TINCR expression (Fig. 1C).

miR-7 is negatively regulated by TINCR in bladder cancer. The function of TINCR in bladder cancer was then examined, as well as the underlying molecular mechanism. Bioinformatics analysis data showed that miR-7 could potentially bind to TINCR, suggesting a possible interaction between TINCR and miR-7 (Fig. 2A). T24 and 5637 cells were used in further *in vitro* experiments. As TINCR was upregulated in bladder cancer, a TINCR siRNA was transfected into T24 and 5637 cells to knock down TINCR. Following transfection, TINCR expression levels were significantly downregulated in the TINCR siRNA group compared with the negative control (NC) siRNA group (Fig. 2B). Further investigation indicated that the expression levels of miR-7 were significantly increased in the TINCR siRNA group compared with the NC siRNA group, suggesting that the miR-7 levels were negatively affected by TINCR in bladder cancer cells (Fig. 2C). Moreover, the luciferase reporter gene assay data showed that co-transfection of miR-7 and WT TINCR luciferase reporter plasmid significantly decreased luciferase activity (Fig. 2D and E). These results indicated the association between TINCR and miR-7 in bladder cancer cells. It was then identified that miR-7 was

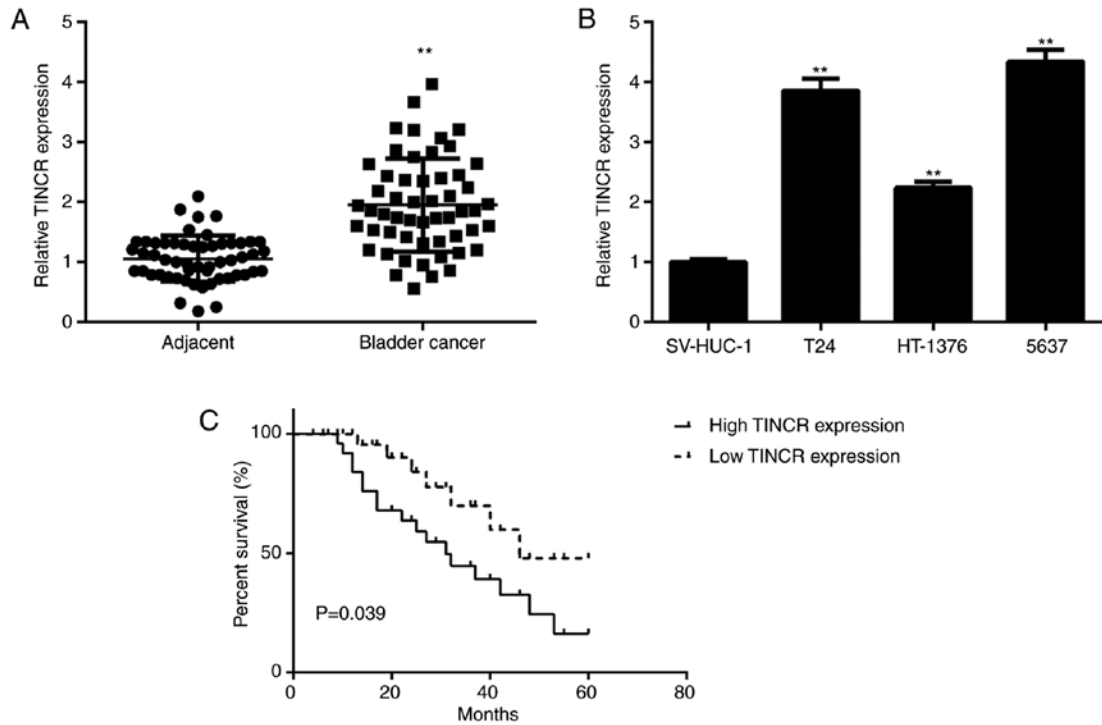


Figure 1. Upregulation of TINCR in bladder cancer. (A) Expression of TINCR is increased in bladder cancer tissues compared with adjacent normal tissues. **P<0.01 vs. Adjacent. (B) Expression of TINCR is increased in bladder cancer cell lines compared with SV-HUC-1 cells. **P<0.01 vs. SV-HUC-1. (C) Patients with bladder cancer who had high TINCR expression exhibited poorer survival rates compared to those with a low TINCR expression. TINCR, TINCR ubiquitin domain containing.

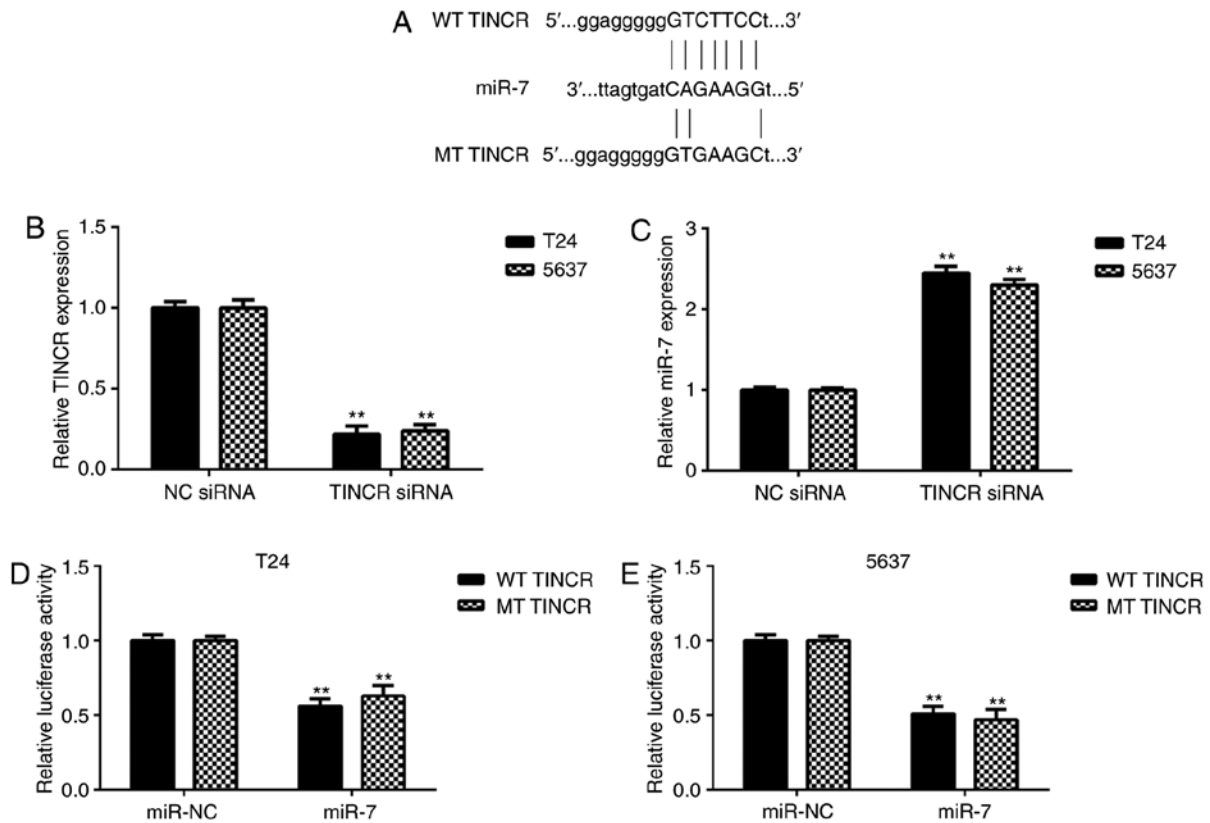


Figure 2. TINCR negatively mediates the expression of miR-7 in bladder cancer cells. (A) Bioinformatics analysis identified that miR-7 may have a potential binding site with TINCR. (B) Transfection with TINCR siRNA decreased the expression of TINCR in the T24 and 5637 cells. **P<0.01 vs. NC siRNA. (C) Transfection with TINCR siRNA increased the expression of miR-7 in T24 and 5637 cells. **P<0.01 vs. NC siRNA. (D and E) Transfection of miR-7 mimics inhibited the luciferase activity of the WT TINCR luciferase reporter gene plasmid, without affecting that of the MT TINCR plasmid in (D) T24 and (E) 5637 cells. **P<0.01 vs. miR-NC. TINCR, TINCR ubiquitin domain containing; miR, microRNA; siRNA, small interfering RNA; NC, negative control; WT, wild-type; MT, mutated.

Table II. Association between miR-7 expression and clinicopathological characteristics in bladder cancer.

Variables	Cases (n=53)	miR-7 expression		P-value
		High (n=26)	Low (n=27)	
Age				0.398
<55	19	11	8	
≥55	34	15	19	
Sex				0.779
Male	33	17	16	
Female	20	9	11	
Grade				0.054
Well and moderately	40	23	17	
Poor	13	3	10	
Lymph node metastasis				0.021 ^a
Negative	34	21	13	
Positive	19	5	14	
Distant metastasis				0.051
Present	5	0	5	
Absent	48	26	22	
TNM stage				0.014 ^a
I-II	27	18	9	
III-IV	26	8	18	

miR, microRNA; TNM, Tumor Node Metastasis. ^aP<0.05 was considered to indicate a statistically significant difference.

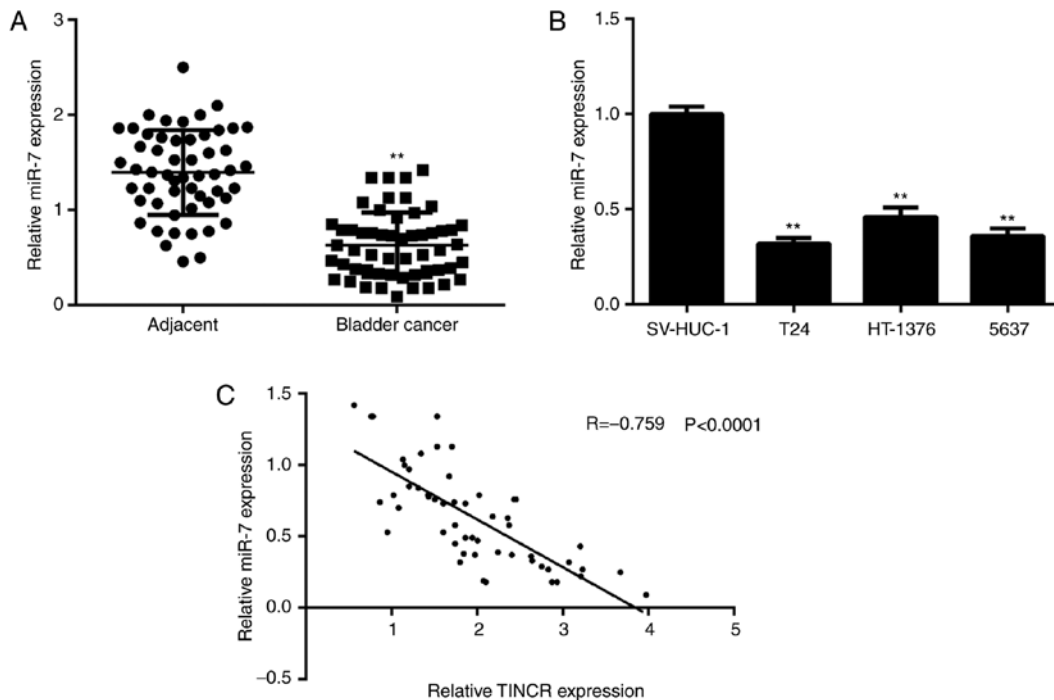


Figure 3. miR-7 expression is downregulated in bladder cancer. (A) miR-7 levels are lower in bladder cancer tissues compared with adjacent normal tissues. **P<0.01 vs. Adjacent. (B) Expression of miR-7 is downregulated in bladder cancer cell lines compared with SV-HUC-1 cells. **P<0.01 vs. SV-HUC-1. (C) The TINCR expression is inversely correlated with the miR-7 expression in bladder cancer tissues. TINCR, TINCR ubiquitin domain containing; miR, microRNA.

significantly downregulated in bladder cancer tissues and cell lines (Fig. 3A and B). It was then demonstrated that there was

an inverse correlation between the expression of miR-7 and TINCR in bladder cancer tissues (Fig. 3C), which further

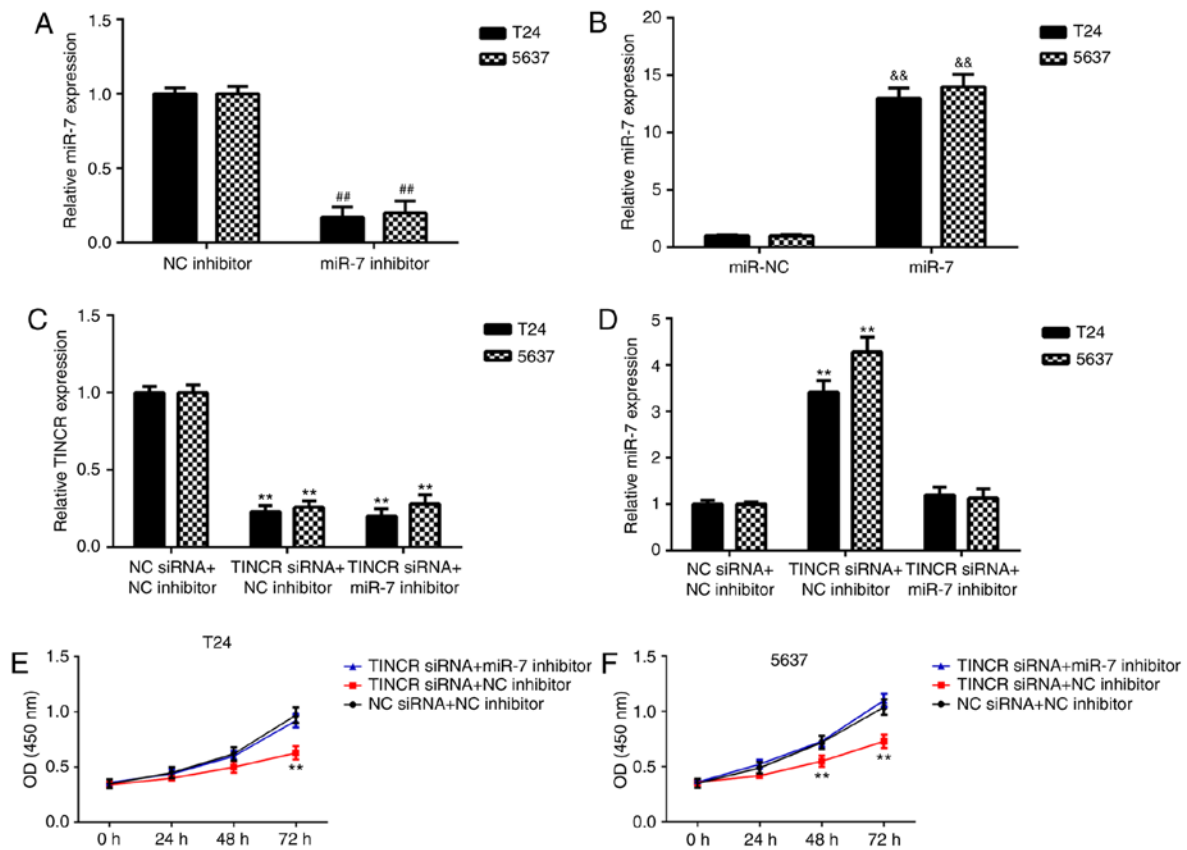


Figure 4. Knockdown of miR-7 attenuates the inhibitory effects of TINCR knockdown on bladder cancer cell proliferation. (A) T24 and (B) 5637 cells were transfected with NC inhibitor, miR-7 inhibitor, miR-NC mimic or miR-7 mimic. Following transfection, the expression of miR-7 was examined. ^{##}P<0.01 vs. NC inhibitor; ^{&&}P<0.01 vs. miR-NC. (C and D) T24 and 5637 cells were transfected with NC siRNA + NC inhibitor, TINCR siRNA + NC inhibitor, TINCR siRNA + miR-7 inhibitor, following which the expression of (C) TINCR and (D) miR-7 was measured. (E and F) A CKK-8 assay was conducted to study cell proliferation in (E) T24 and (F) 5637 cells. ^{**}P<0.01 vs. NC siRNA + NC inhibitor. TINCR, TINCR ubiquitin domain containing; miR, microRNA; NC, negative control; siRNA, small interfering RNA.

confirms their targeting relationship. In addition, low miR-7 expression was associated with advanced TNM stage and lymph node metastasis (Table II).

miR-7 is involved in the TINCR-mediated bladder cancer cell proliferation, migration and invasion. The regulatory mechanism of TINCR and miR-7 underlying bladder cancer cell proliferation and invasion was further studied. Firstly, bladder cancer cells were transfected with an NC inhibitor, miR-7 inhibitor, miR-NC mimic or miR-7 mimic. As shown in Fig. 4A and B, transfection with a miR-7 inhibitor significantly decreased miR-7 expression, and transfection with a miR-7 mimic significantly increased miR-7 expression in bladder cancer cells. Then, bladder cancer cells were transfected with NC siRNA + NC inhibitor, TINCR siRNA + NC inhibitor, TINCR siRNA + miR-7 inhibitor. Following transfection, the expression levels of TINCR were significantly downregulated in the TINCR siRNA + NC inhibitor and TINCR siRNA + miR-7 inhibitor groups, compared with the NC siRNA + NC inhibitor group (Fig. 4C). Whereas, miR-7 was upregulated in the TINCR siRNA + NC inhibitor group compared with the NC siRNA + NC inhibitor group, which was reversed by transfection with a miR-7 inhibitor (Fig. 4D). It was identified that inhibition of TINCR repressed bladder cancer cell proliferation, which was reversed by miR-7 inhibition (Fig. 4E and F). Moreover, knockdown of TINCR also suppressed cell

migration and invasion, which was reversed by knockdown of miR-7 (Fig. 5). For cell migration, the number of migratory cells were as follows: In Fig. 5A, NC siRNA + NC inhibitor (45.2 ± 3.7), TINCR siRNA + NC inhibitor (23.4 ± 1.8) and TINCR siRNA + miR-7 inhibitor (49.9 ± 5.1); and in Fig. 5B, NC siRNA + NC inhibitor (46.3 ± 3.5), TINCR siRNA + NC inhibitor (27.2 ± 3.6) and TINCR siRNA + miR-7 inhibitor (50.4 ± 3.4). For cell invasion, the number of invaded cells were as follows: In Fig. 5C, NC siRNA + NC inhibitor (77.2 ± 3.1), TINCR siRNA + NC inhibitor (45.1 ± 2.6) and TINCR siRNA + miR-7 inhibitor (79.4 ± 2.9); and in Fig. 5D, NC siRNA + NC inhibitor (82.4 ± 2.9), TINCR siRNA + NC inhibitor (52.1 ± 1.7) and TINCR siRNA + miR-7 inhibitor (84.3 ± 2.5). These data suggest that miR-7 participates in the TINCR-mediated bladder cancer cell proliferation, migration and invasion.

mTOR is a target gene of miR-7 in bladder cancer cells. Recently, an increasing number of lncRNAs have been reported to competitively inhibit the expression levels of miRs by acting as ceRNAs, which could further mediate the expression of the downstream target genes (7). Bioinformatics analyses data have shown that the 3'UTR of mTOR mRNA contained putative binding sites of miR-7 (Fig. 6A). The results of the luciferase reporter gene assays showed that transfection of miR-7 mimics significantly inhibited the luciferase activity of WT of 3'UTR of mTOR, without affecting the

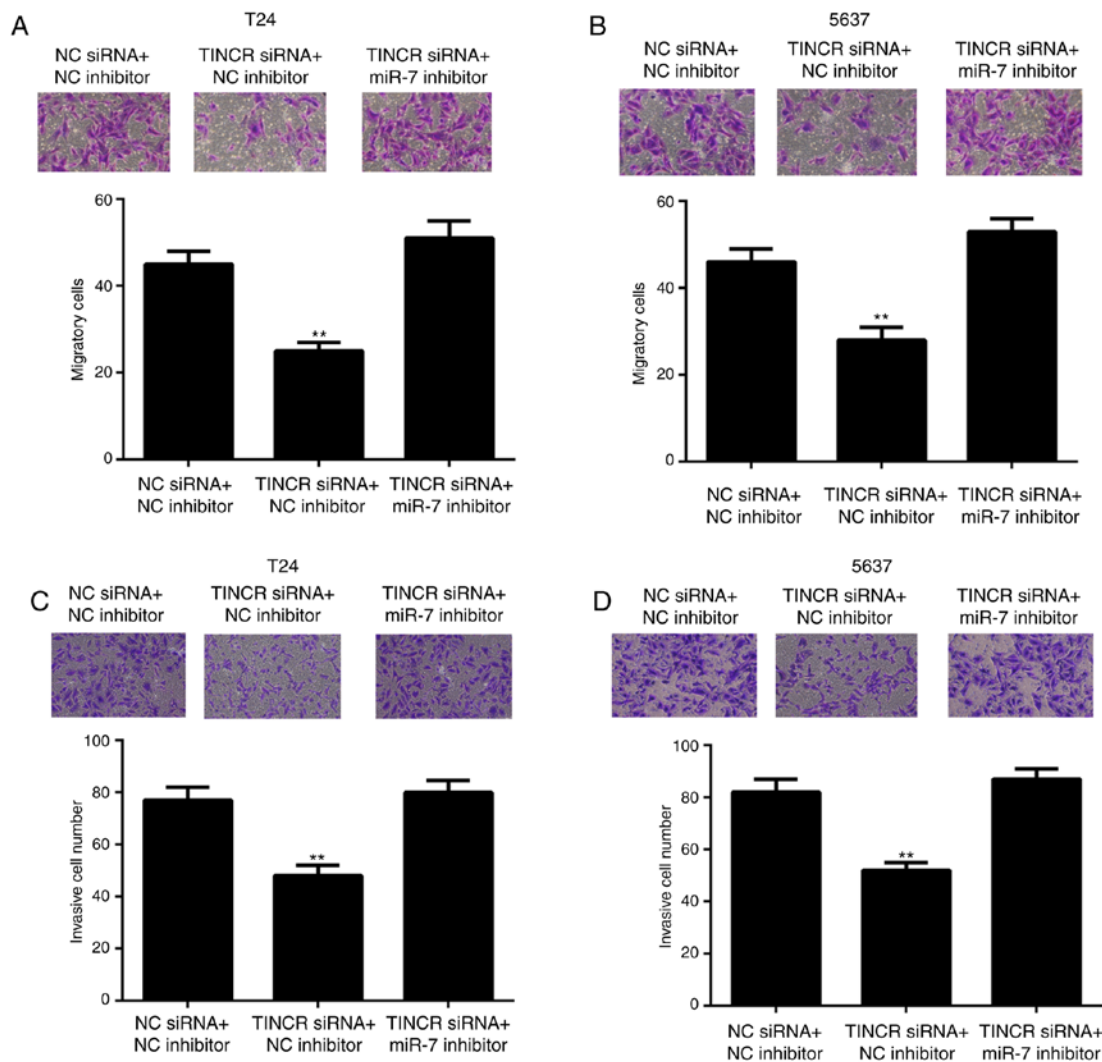


Figure 5. Knockdown of miR-7 attenuates the inhibitory effects of TINCR knockdown on bladder cancer cell migration and invasion. T24 and 5637 cells were transfected with NC siRNA + NC inhibitor, TINCR siRNA + NC inhibitor, TINCR siRNA + miR-7 inhibitor. Following transfection, (A and B) cell migration in (A) T24 and (B) 5637 cells, and (C and D) invasion capabilities in (C) T24 and (D) 5637 cells were examined using Transwell assays. Magnification for cell migration and invasion, x200. **P<0.01 vs. NC siRNA + NC inhibitor. TINCR, TINCR ubiquitin domain containing; miR, microRNA; NC, negative control; siRNA, small interfering RNA.

luciferase activity of MT of 3'UTR of mTOR (Fig. 6B and C). Accordingly, mTOR is a direct target gene of miR-7 in bladder cancer cells. Consistently, miR-7 overexpression led to the downregulation of mTOR expression in bladder cancer cells, while silencing of miR-7 expression increased mTOR expression (Fig. 6D-G).

mTOR participates in the miR-7-mediated malignant phenotypes of bladder cancer cells. It was then studied whether mTOR was a downstream effector in the miR-7-mediated proliferation and invasion of bladder cells. Firstly, bladder cancer cells were transfected with a blank vector or mTOR expression plasmid. As shown in Fig. 7A and B, transfection with an mTOR expression plasmid significantly increased the mRNA and protein levels of mTOR in bladder cancer cells compared with the blank group. Bladder cancer cells were co-transfected with miR-NC + blank vector, miR-7 mimic + blank vector, or miR-7 mimic + mTOR plasmid. As shown in Fig. 7C and D, the expression of mTOR was significantly reduced after miR-7 overexpression, which was reversed by

transfection with the mTOR plasmid. Following this, it was identified that cell proliferation was decreased by miR-7 overexpression, which was reversed by mTOR overexpression (Fig. 7E and F). Moreover, overexpression of mTOR also reversed the miR-7-induced inhibition of migration and invasion of bladder cancer cells (Fig. 8). For cell migration, the number of migratory cells were as follows: In Fig. 8A, miR-NC + blank vector (40.1±3.0), miR-7 mimic + blank vector (23.3±1.9) and miR-7 mimic + mTOR plasmid (56.6±2.9); and in Fig. 8B, miR-NC + blank vector (45.3±3.9), miR-7 mimic + blank vector (23.8±1.8) and miR-7 mimic + mTOR plasmid (49.6±3.8). For cell invasion, the number of invaded cells were as follows: In Fig. 8C, miR-NC + blank vector (55.2±2.2), miR-7 mimic + blank vector (37.6±1.1) and miR-7 mimic + mTOR plasmid (83.4±4.4); and in Fig. 8D, miR-NC + blank vector (62.1±3.2), miR-7 mimic + blank vector (39.5±2.1) and miR-7 mimic + mTOR plasmid (87.4±4.5). Therefore, the present findings suggest that miR-7 plays an inhibitory role in the malignant phenotypes of bladder cancer cells by directly targeting mTOR.

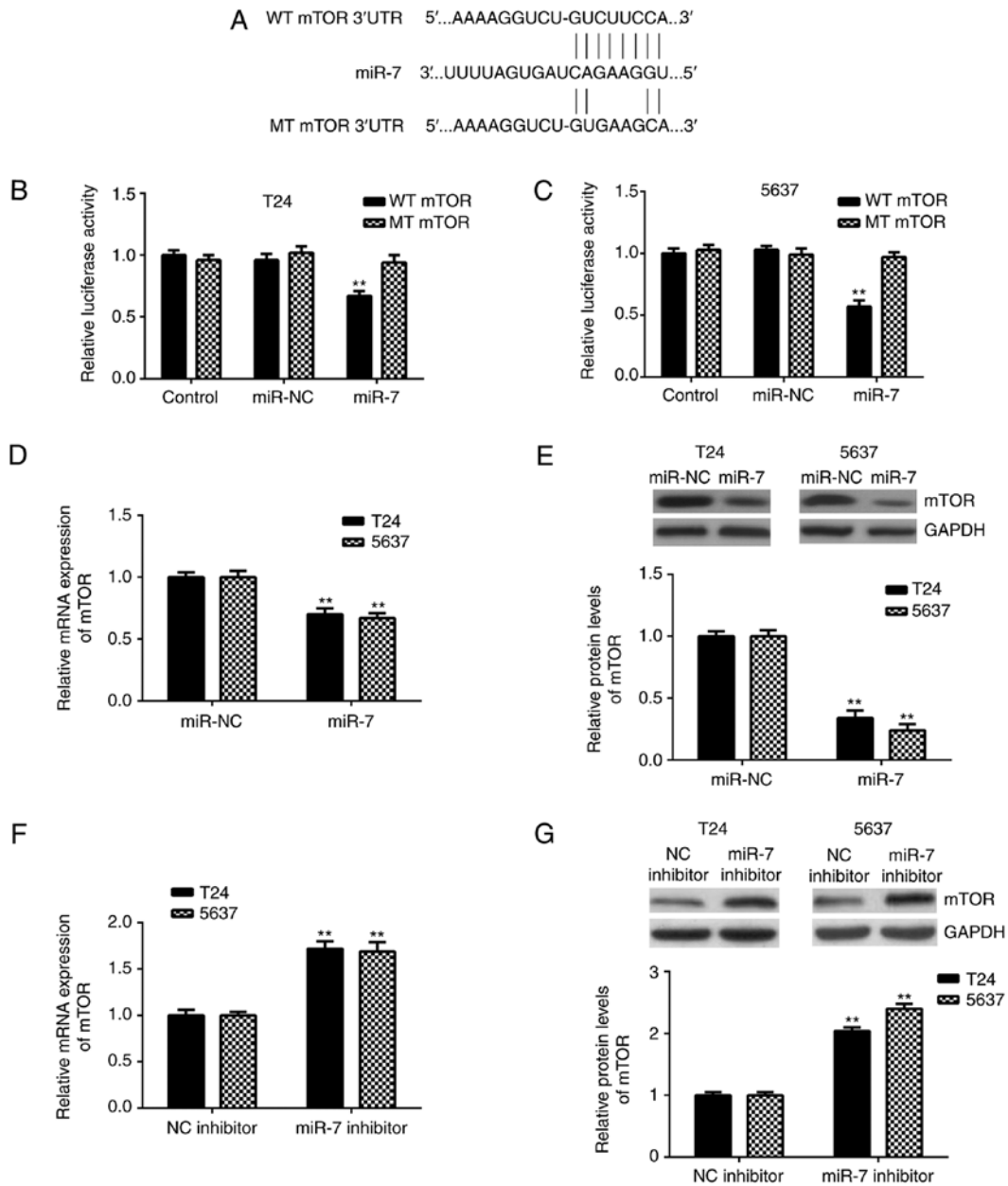


Figure 6. mTOR is a target gene of miR-7 in bladder cancer cells. (A) miR-7 has a potential binding site with mTOR. Transfection with miR-7 mimics inhibits the luciferase activity of the WT mTOR luciferase reporter gene plasmid, without affecting that of the MT mTOR plasmid in (B) T24 and (C) 5637 cells. **P<0.01 vs. miR-NC. Transfection with miR-7 mimics decreases the (D) mRNA and (E) protein expression levels of mTOR in T24 and 5637 cells. **P<0.01 vs. miR-NC. Transfection with miR-7 inhibitor increases the (F) mRNA and (G) protein expression levels of mTOR in T24 and 5637 cells. **P<0.01 vs. NC inhibitor. miR, microRNA; NC, negative control; siRNA; WT, wild-type; MT, mutated.

Discussion

The regulatory mechanism of TINCR during bladder cancer progression is complicated. In the present study, it was identified that the expression of TINCR was significantly increased in bladder cancer tissues and cell lines, when compared with that in adjacent normal tissues and normal urinary tract epithelial cell line SV-HUC-1, respectively. Moreover, the high expression of TINCR was associated with tumor metastasis and advanced TNM stage, as well as decreased survival rates of patients with bladder cancer. Further investigation revealed that miR-7 was negatively mediated by TINCR in bladder cancer cells. Silencing of TINCR expression significantly increased the expression of

miR-7 and reduced bladder cancer cell proliferation, migration and invasion, while knockdown of miR-7 expression eliminated the inhibitory effects of TINCR downregulation on bladder cancer cells. mTOR was then identified as a target gene of miR-7 in bladder cancer, and it was demonstrated that overexpression of mTOR abolished the inhibitory effects of miR-7 on bladder cancer cells.

In recent years, an increasing number of lncRNAs, including TINCR, have been reported to serve critical roles in different types of human cancer (25,26). For example, SP1-induced upregulation of TINCR regulates gastric cancer cell proliferation and apoptosis (27). Loss of TINCR expression promotes colorectal cancer cell proliferation and metastasis (28). The present study identified that TINCR was upregulated in bladder

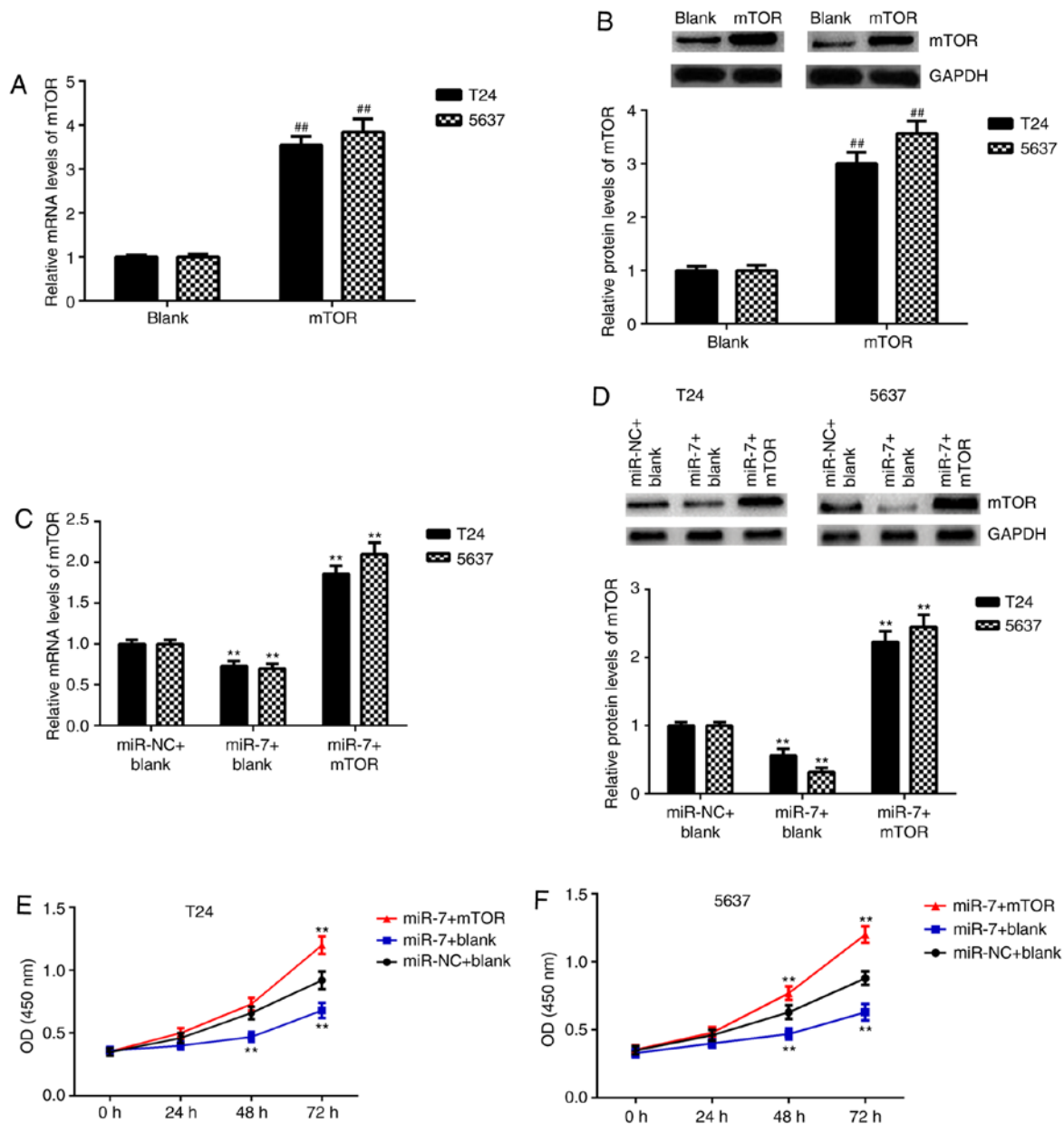


Figure 7. mTOR is involved in miR-7-mediated bladder cancer cell proliferation. T24 and 5637 cells were transfected with a blank vector or mTOR expression plasmid, following which the (A) mRNA and (B) protein levels of mTOR were measured. $^{##}P < 0.01$ vs. blank. T24 and 5637 cells were co-transfected with miR-NC + blank vector, miR-7 mimic + blank vector, or miR-7 mimic + mTOR plasmid, following which the (C) mRNA and (D) protein levels of mTOR were measured. Cell proliferation was measured in (E) T24 and (F) 5637 cells. $^{**}P < 0.01$ vs. miR-NC + blank. miR, microRNA; NC, negative control.

cancer, and that the increased expression of TINCR was correlated with metastasis and advanced TNM stage, consistent with the results from a previous study (12). Therefore, the upregulation of TINCR may contribute to bladder cancer progression. Moreover, it was found that the patients with bladder cancer who had higher TINCR expression showed shorter survival time, when compared with those with low TINCR expression, suggesting that TINCR may be used as a potential prognostic maker for bladder cancer. Functionally, it was demonstrated that knockdown of TINCR inhibited the malignant phenotypes of bladder cancer cells, which indicates that TINCR has an important role in bladder cancer. In future experiments it would be useful to study the function of TINCR in bladder cancer *in vivo* using animal experiments. A limitation of this study is that only samples of the tumor tissue and adjacent

tissue from the same patient were collected during surgical resection. Thus, further studies comparing the expression levels of TINCR in the normal population with tumor tissue from patients would be beneficial.

It has been well-established that lncRNAs can interact with miRNAs and thus affect their function (6,7). In the present study, it was demonstrated that TINCR sponges miR-7 in order to negatively regulate its expression in bladder cancer cells. Moreover, miR-7 was identified to be significantly downregulated in bladder cancer, which may be due to the upregulation of TINCR, as it was observed that TINCR expression was inversely correlated to the miR-7 expression in bladder cancer tissues. Further investigation indicated that the inhibition of miR-7 reversed the inhibitory effects of TINCR knockdown on bladder cancer cells, suggesting that TINCR serves a role

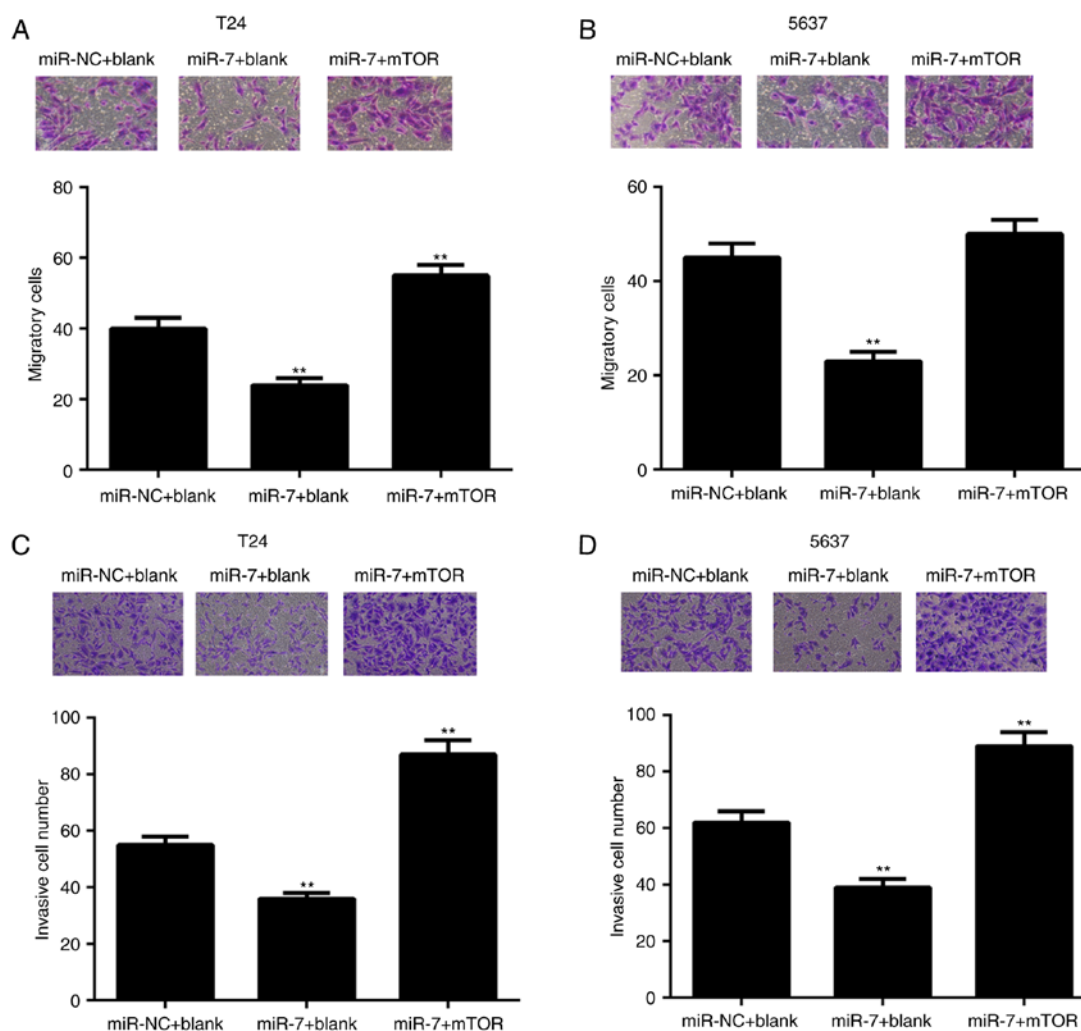


Figure 8. mTOR is involved in miR-7-mediated bladder cancer cell migration and invasion. T24 and 5637 cells were co-transfected with miR-NC + blank vector, miR-7 mimic + blank vector, or miR-7 mimic + mTOR plasmid, following which cell migration in (A) T24 and (B) 5637 cells, and cell invasion in (C) T24 and (D) 5637 cells were measured using Transwell assays. Magnification for cell migration and invasion, x200. ** $P < 0.01$ vs. miR-NC + blank vector. miR, microRNA; NC, negative control.

in promoting the malignant phenotypes of bladder cancer cells by negatively regulating the expression of miR-7. In addition to miR-7, miR-375 was also recently identified to interact with TINCR in gastric cancer (8).

In addition, it was identified that mTOR was a direct target gene of miR-7 in bladder cancer cells, and that the overexpression of mTOR abolished the miR-7-induced inhibition of bladder cancer cell proliferation, migration and invasion. In fact, this targeting relationship has also been reported in several other types of human cancer (29,30). For example, miR-7 increases cisplatin sensitivity of gastric cancer cells by suppressing the expression of mTOR (29). Glover *et al* (30) reported that miR-7 functioned as a tumor suppressor and novel therapeutic agent for adrenocortical carcinoma by targeting mTOR and RAF1. Therefore, the results of the present study improved the understanding of the function of miR-7/mTOR axis in human cancer.

In summary, the present study demonstrated that the upregulation of TINCR was associated with an aggressive tumor phenotype and poor prognosis in bladder cancer, and suggested that TINCR promotes the malignant phenotypes of bladder cancer by regulating the miR-7/mTOR axis. Therefore,

the TINCR/miR-7/mTOR signaling pathway may be a potential therapeutic target for bladder cancer.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GX, HY and LS designed the study and wrote the manuscript. GX, HY, ML, JN and XT collected tissue samples and performed the experiments. WC analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shengli Hospital of Shengli Petroleum Administration. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. *CA Cancer J Clin* 65: 5-29, 2015.
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. *CA Cancer J Clin* 65: 87-108, 2015.
- Yoshino H, Seki N, Itesako T, Chiyomaru T, Nakagawa M and Enokida H: Aberrant expression of microRNAs in bladder cancer. *Nat Rev Urol* 10: 396-404, 2013.
- Jiang Z, Zhang Y, Cao R, Li L, Zhong K, Chen Q and Xiao J: miR-5195-3p inhibits proliferation and invasion of human bladder cancer cells by directly targeting oncogene KLF5. *Oncol Res* 25: 1081-1087, 2017.
- Long Y, Wu Z, Yang X, Chen L, Han Z, Zhang Y, Liu J, Liu W and Liu X: MicroRNA-101 inhibits the proliferation and invasion of bladder cancer cells via targeting c-FOS. *Mol Med Rep* 14: 2651-2656, 2016.
- Zhang Y, Dai Q, Zeng F and Liu H: MALAT1 promotes the proliferation and metastasis of osteosarcoma cells by activating the Rac1/JNK pathway via targeting MiR-509. *Oncol Res*: Apr 27, 2018 (Epub ahead of print).
- Zhang JJ, Wang DD, Du CX and Wang Y: Long Noncoding RNA ANRIL promotes cervical cancer development by acting as a sponge of miR-186. *Oncol Res* 26: 345-352, 2018.
- Chen Z, Liu H, Yang H, Gao Y, Zhang G and Hu J: The long noncoding RNA, TINCR, functions as a competing endogenous RNA to regulate PDK1 expression by sponging miR-375 in gastric cancer. *Onco Targets Ther* 10: 3353-3362, 2017.
- Xu S, Kong D, Chen Q, Ping Y and Pang D: Oncogenic long noncoding RNA landscape in breast cancer. *Mol Cancer* 16: 129, 2017.
- Zheng Y, Yang C, Tong S, Ding Y, Deng W, Song D and Xiao K: Genetic variation of long non-coding RNA TINCR contribute to the susceptibility and progression of colorectal cancer. *Oncotarget* 8: 33536-33543, 2017.
- Xu Y, Qiu M, Chen Y, Wang J, Xia W, Mao Q, Yang L, Li M, Jiang F, Xu L and Yin R: Long noncoding RNA, tissue differentiation-inducing nonprotein coding RNA is upregulated and promotes development of esophageal squamous cell carcinoma. *Dis Esophagus* 29: 950-958, 2016.
- Chen Z, Liu Y, He A, Li J, Chen M, Zhan Y, Lin J, Zhuang C, Liu L, Zhao G, *et al*: Theophylline controllable RNAi-based genetic switches regulate expression of lncRNA TINCR and malignant phenotypes in bladder cancer cells. *Sci Rep* 6: 30798, 2016.
- Ambros V: The functions of animal microRNAs. *Nature* 431: 350-355, 2004.
- Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297, 2004.
- Zhou Y, Yang C, Wang K, Liu X and Liu Q: MicroRNA-33b inhibits the proliferation and migration of osteosarcoma cells via targeting hypoxia-inducible factor-1 α . *Oncol Res* 25: 397-405, 2017.
- Luo J, Li H and Zhang C: MicroRNA-7 inhibits the malignant phenotypes of nonsmall cell lung cancer *in vitro* by targeting Pax6. *Mol Med Rep* 12: 5443-5448, 2015.
- Xu K, Chen Z, Qin C and Song X: miR-7 inhibits colorectal cancer cell proliferation and induces apoptosis by targeting XRCC2. *Onco Targets Ther* 7: 325-332, 2014.
- Zhang N, Li X, Wu CW, Dong Y, Cai M, Mok MT, Wang H, Chen J, Ng SS, Chen M, *et al*: microRNA-7 is a novel inhibitor of YY1 contributing to colorectal tumorigenesis. *Oncogene* 32: 5078-5088, 2013.
- Xiong S, Zheng Y, Jiang P, Liu R, Liu X and Chu Y: MicroRNA-7 inhibits the growth of human non-small cell lung cancer A549 cells through targeting BCL-2. *Int J Biol Sci* 7: 805-814, 2011.
- Thiel A and Ristimäki A: Targeted therapy in gastric cancer. *APMIS* 123: 365-372, 2015.
- Latacz A, Russell JA, Ocfon E, Zubeł-Łojek J and Pierzchała-Koziec K: mTOR pathway-novel modulator of astrocyte activity. *Folia Biol (Krakow)* 63: 95-105, 2015.
- Sathe A and Nawroth R: Targeting the PI3K/AKT/mTOR pathway in bladder cancer. *Methods Mol Biol* 1655: 335-350, 2018.
- Magers MJ, Lopez-Beltran A, Montironi R, Williamson SR, Kaimakliotis HZ and Cheng L: Staging of bladder cancer. *Histopathology* 74: 112-134, 2019.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
- Liu L, Chen X, Zhang Y, Hu Y, Shen X and Zhu W: Long non-coding RNA TUG1 promotes endometrial cancer development via inhibiting miR-299 and miR-34a-5p. *Oncotarget* 8: 31386-31394, 2017.
- Hua F, Li CH, Chen XG and Liu XP: Long noncoding RNA CCAT2 knockdown suppresses tumorous progression by sponging miR-424 in epithelial ovarian cancer. *Oncol Res* 26: 241-247, 2018.
- Xu TP, Liu XX, Xia R, Yin L, Kong R, Chen WM, Huang MD and Shu YQ: SP1-induced upregulation of the long noncoding RNA TINCR regulates cell proliferation and apoptosis by affecting KLF2 mRNA stability in gastric cancer. *Oncogene* 34: 5648-5661, 2015.
- Zhang ZY, Lu YX, Chang YY, Chang YY, Zheng L, Yuan L, Zhang F, Hu YH, Zhang WJ and Li XN: Loss of TINCR expression promotes proliferation, metastasis through activating EpCAM cleavage in colorectal cancer. *Oncotarget* 7: 22639-22649, 2016.
- Xu N, Lian YJ, Dai X and Wang YJ: miR-7 increases cisplatin sensitivity of gastric cancer cells through suppressing mTOR. *Technol Cancer Res Treat* 16: 1022-1030, 2017.
- Glover AR, Zhao JT, Gill AJ, Weiss J, Mugridge N, Kim E, Feeney AL, Ip JC, Reid G, Clarke S, *et al*: MicroRNA-7 as a tumor suppressor and novel therapeutic for adrenocortical carcinoma. *Oncotarget* 6: 36675-36688, 2015.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.