

LncRNA ITGB1 promotes the development of bladder cancer through regulating microRNA-10a expression

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Abstract. – **OBJECTIVE:** This study aims to investigate the expression level of lncRNA ITGB1 both in bladder cancer (BCa) tissue and cell lines, as well as to evaluate its function and potential mechanism in the progression of BCa.

PATIENTS AND METHODS: The expressions of lncRNA ITGB1 in 36 BCa tissues samples (and corresponding adjacent normal ones) and cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). After transfection of sh-ITGB1 in BCa cell lines, the effect of ITGB1 on the proliferation of BCa cells was examined by cell counting kit-8 (CCK-8) assay and colony formation assay. Subsequently, qRT-PCR was used to examine microRNA-10a expression in BCa tissues and cells after ITGB1 was silenced. At the same time, the correlation between ITGB1 and microRNA-10a expression was analyzed. Finally, cell recovery experiment was applied for the in-depth study of the interaction between ITGB1 and microRNA-10a and its underlying mechanism.

RESULTS: LncRNA ITGB1 was found upregulated in BCa tissues and cell lines. Knockdown of lncRNA ITGB1 remarkably inhibited cell proliferation. The expression levels of ITGB1 and microRNA-10a in BCa tissues were negatively correlated. ITGB1 downregulation was found to be able to enhance microRNA-10a expression, suggesting that microRNA-10a may be a potential target for ITGB1 in BCa. In addition, cell reverse experiment also verified that ITGB1 could regulate the expression of microRNA-10a, and their interaction affected the malignant progression of BCa.

CONCLUSIONS: LncRNA ITGB1 level is up-regulated in BCa tissues and associated with the pathological stage of BCa, which could be

used as a new predictor of BCa patients' prognosis. In addition, ITGB1 might promote BCa cell proliferation *via* regulating microRNA-10a expression.

Key Words:

ITGB1, MicroRNA-10a, BCa, Proliferation.

Introduction

Bladder cancer (BCa) is one of the most common malignancies in the world, with about 74,000 new cases each year, and its incidence in males is three times that in females^{1,2}. Histologically, BCa includes urothelial carcinoma, squamous cell carcinoma, and adenocarcinoma, among which urothelial carcinoma is the most important histological type, accounting for 95%^{3,4}. Although the treatment for BCa has made progress year by year, the 5-year survival rate, morbidity, and mortality of BCa patients have not been well improved in the past decade⁵. The complex pathogenesis of BCa is one of the most important factors for its high mortality rate. Risk factors for the occurrence and development of BCa include three aspects: Genetic and molecular abnormalities, chemical or environmental exposure, and chronic inflammation. Molecular abnormality has been considered as a very important cause of BCa in recent years, which has been well concerned⁵. Currently, molecular diagnosis and targeted therapy have become an important part for the diag-

nosis and treatment of many cancers^{6,7}. Therefore, it is very urgent to find new biological indicators related to early diagnosis and prognosis of BCa⁸⁻¹⁰. Genome-wide data showed that only 2% of the human genome could encode proteins and most of which are non-coding RNAs¹¹⁻¹³. Although these non-coding RNA (ncRNAs) do not encode proteins, they play a very pivotal role in transcription, chromosome modification, nuclear transport, and other aspects¹⁴. Long non-coding RNAs (lncRNAs), defined as a type of RNA molecules with a transcription length of more than 200 nucleotides. They do not possess an open reading frame. So far, over 3000 lncRNAs have been discovered, while only 1% of them are well known for their functions^{15,16}. Although the potential function of lncRNA is still mysterious. Some researchers have found that lncRNA can regulate various cell progression, such as proliferation, cell growth, and apoptosis¹⁷. Currently, many studies have reported that lncRNA expression is abnormal in many cancers, playing the role of oncogenes or tumor-suppressor genes, and some of them are related to cancer recurrence, metastasis, and poor prognosis¹⁷. However, lncRNA ITGB1 in the development of tumors is rarely reported. One report indicated that lncRNA ITGB1 can promote the migration and invasion of clear cell renal cell carcinoma by down-regulating McL-1¹⁸. Therefore, we comprehensively analyzed the expression and biological effect of ITGB1 in BCa and preliminarily discussed the molecular mechanism of its tumor regulatory effect. In general, lncRNA can regulate target genes at the level of transcription and post-transcription^{19,20}. In recent years, Dumas et al²¹ have found that microRNA-10a has been analyzed in a variety of tumors. This study investigated the potential function of lncRNA ITGB1 in influencing the proliferation of BCa through targeting microRNA-10a, thus providing an experimental basis for its clinical application.

Patients and Methods

Patients and BCa Samples

36 pairs of BCa tissues and adjacent normal ones were surgically resected from BCa patients, and then collected at -80°C . The collection of clinical specimens was approved by the Ethics Monitoring Committee. Patients and their families had been fully informed that their specimens would be used for scientific research.

Cell Lines and Reagents

Six human HCC cell lines (EJ, 253j, 5637, J82, RT4, UMUC3) and one human bladder epithelial immortalized cell line (SV-HUC-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). BCa cell lines were cultured in high-glucose DMEM (Dulbecco's Modified MEM Medium) high glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g/mL}$). All cells were cultured in a 37°C incubator with 5% CO_2 . Cells were passaged using 1% trypsin + EDTA (ethylenediaminetetraacetic acid) at 80%-90% confluence.

Transfection

The negative control group (sh-NC) and lentivirus containing the ITGB1 knockdown sequence (sh-ITGB1) were purchased from GenePharma, Shanghai, China. Cells were inoculated in 6-well plates and grown to cell density of 40%, and then transfection was performed according to the manufacturer's instructions. After that, cells were collected 48 h later for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assay

After 48 h of transfection, cells were harvested and inoculated into 96-well plates with 2000 cells per well. Cells were cultured for 24 h, 48 h, 72 h, and 96 h respectively, and then cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) reagent was applied. After incubation for 2 h, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Colony Formation Assay

After 48 h of transfection, 200 cells were seeded in each well of a 6-well plate and cultured in complete medium for 2 weeks. The medium was changed once after one week, and then twice a week. The medium should not be replaced as much as possible in the previous week to avoid cell adhesion. After 2 weeks, visible colonies were washed twice with phosphate-buffered saline (PBS) and fixed in 2 ml of methanol for 20 min. After the methanol was aspirated, colonies were stained with 0.1% crystal violet staining solution for 20 min, washed 3 times with PBS, photographed and counted under a light-selective environment.

QRT-PCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and purify the RNA. Reverse transcription of RNA was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions. Real-time PCR was performed according to the SYBR® Premix Ex Taq™ kit instructions (TaKaRa, Otsu, Shiga, Japan), and the PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) system. The following primers were used for qRT-PCR reaction: ITGB1: forward: 5'-AAC-CAGGCCCTCCTTACTC-3', reverse: 5'-GAT-GTGTCCGAAGGCTAGGA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-CGCTCTCTGCTCCTCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'; miRNA-10a: forward: 5'-CGCTAGAAGCTTTTG-GGTTA-3', reverse: 5'-GCCCTAGACCATG-GATTT-3'; U6: forward: 5'-CGCTTCGGCAG-CACATATAC-3', reverse: 5'-TTCACGAATTTG-CGTGTCAT-3'. Three replicate wells were set for each sample, and the assay was repeated twice. The Bio-Rad PCR instrument was used to analyze and process the data with the software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). GAPDH and U6 genes were used as internal references. Gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was made using One-way ANOVA test, followed by Post-Hoc Test (Least Significant Difference). Independent experiments were repeated at least three times. Data were expressed as mean $\pm$ standard deviation (mean $\pm$ SD). $p < 0.05$ was considered statistically significant (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

Results

ITGB1 Was Highly Expressed in BCa Tissues and Cell Lines

The expression of ITGB1 in 36 BCa tissue specimens and adjacent normal ones was de-

tected by qRT-PCR. The results indicated that ITGB1 expression was remarkably upregulated in BCa tissues (Figure 1A). In addition, ITGB1 expression was found remarkably higher in bladder tumor cell lines, especially EGB and 253j cell lines, than that in epithelial immortalized cells (SV-HUC-1) (Figure 1B).

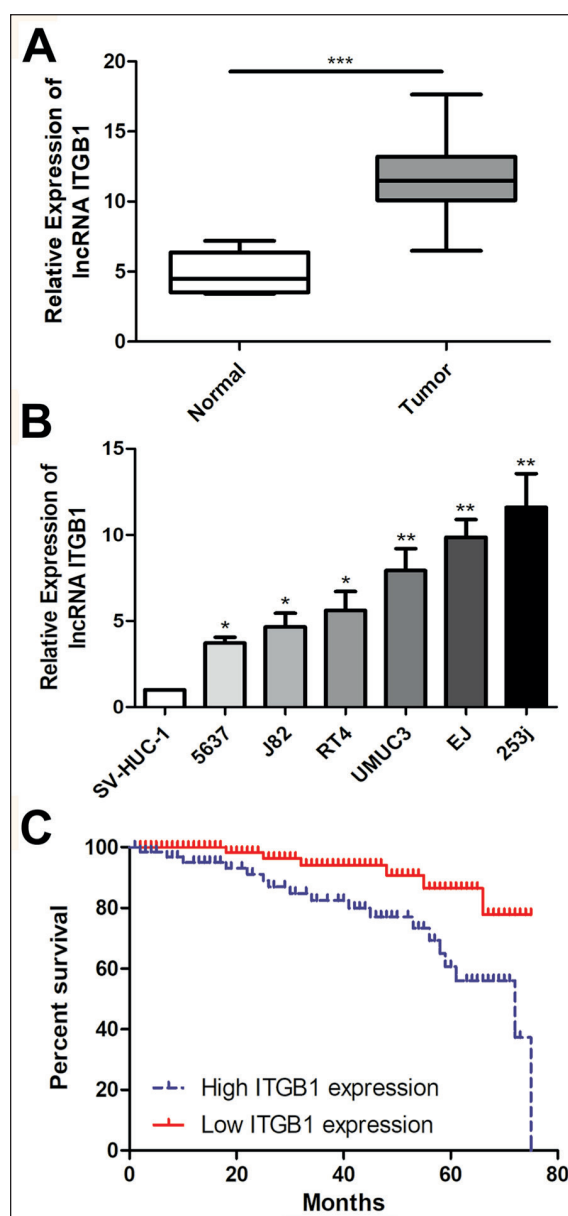


Figure 1. ITGB1 was highly expressed in bladder cancer tissues and cell lines. **A**, QRT-PCR was used to detect the difference in expression of ITGB1 in bladder cancer tissues and adjacent tissues. **B**, QRT-PCR was used to detect the expression level of ITGB1 in bladder cancer cell lines. **C**, Kaplan Meier survival curve of bladder cancer patients based on ITGB1 expression. Data are expressed as mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ITGB1 Expression Was Correlated with Pathological Staging and Overall Survival in BCa Patients

Based on the mRNA expression of ITGB1, the collected bladder tissue samples were divided into high expression group and low expression one, and the correlation between ITGB1 expression and age, sex, pathological stage, lymph node or distant metastasis of patients with BCa was analyzed. As shown in Table I, highly expressed ITGB1 was positively correlated with the pathological stage of BCa, but not with age, gender, distant metastasis, and lymph node metastasis. In addition, Kaplan-Meier survival curves revealed that high expression of ITGB1 was remarkably associated with poor prognosis of BCa patients ($p < 0.05$; Figure 1C).

Knockdown of ITGB1 Inhibited Cell Proliferation

To investigate the function of ITGB1 in BCa, a knockdown model of ITGB1 was constructed. After transfection of the ITGB1 lentiviral vector in the EJ and 253j cell lines, qRT-PCR was performed to verify the interference efficiency, and the difference was statistically significant (Figure 2A). After knockdown of ITGB1 in EJ and 253j cell lines, CCK-8 and colony formation assay were performed to detect the proliferation of BCa cells. The results showed that compared with those transfected with sh-NC, the proliferation of BCa cells transfected with sh-ITGB1 was remarkably reduced (Figure 2B-2D).

MicroRNA-10a was Lowly Expressed in BCa Tissues and Cell Lines

TargetScan (http://www.targetscan.org/vert_71/), miRBase (<http://www.mirbase.org/>) and MiRcode (<http://www.mircode.org/>) databases were used to evaluate the association between lncRNAs, miRNAs, and mRNA. As a result, bioinformatics suggested a link between ITGB1 and three potential miRNAs (Figure 3A). In addition, qRT-PCR was applied to detect expression changes of the three potential miRNAs in cells transfected with sh-NC or sh-ITGB1. MicroRNA-10a was found to be the most differentially expressed one (Figure 3B). Therefore, it is considered that there could be an interaction between ITGB1 and microRNA-10a. In this study, the expression of microRNA-10a in 36 BCa tissue specimens and adjacent normal ones was detected by qRT-PCR. The results indicated that microRNA-10a was remarkably down-regulated in BCa tissues compared with the normal ones (Figure 3C). Similarly, ITGB1 level was found remarkably lower in BCa cells than in normal bladder epithelial immortalized cells (SV-HUC-1), and the difference was statistically significant (Figure 3D). Subsequently, the expressions of ITGB1 and microRNA-10a were both detected by qRT-PCR and found they were negatively correlated in BCa tissues (Figure 3E). At the same time, we analyzed the clinical indicators and prognosis of microRNA-10a in BCa. As shown in Table I, low expression of microRNA-10a was positively relevant to the pathological stage of BCa, but not with age,

Table I. Association of ITGB1 and miR-10a expression with clinicopathologic characteristics of bladder cancer.

Parameters	No. of cases	ITGB1 expression			MiR-10a expression		
		Low (%)	High (%)	<i>p</i> -value	High (%)	Low (%)	<i>p</i> -value
Age (years)				0.083			0.221
< 60	13	9	4		3	10	
≥ 60	23	9	14		10	13	
Gender				0.182			0.298
Male	18	11	7		5	13	
Female	18	7	11		8	10	
T stage				0.002			0.002
T1-T2	23	16	7		4	19	
T3-T4	13	2	11		9	4	
Lymph node metastasis				0.457			0.064
No	26	14	12		7	19	
Yes	10	4	6		6	4	
Distance metastasis				0.729			0.096
No	23	12	11		6	17	
Yes	13	6	7		7	6	

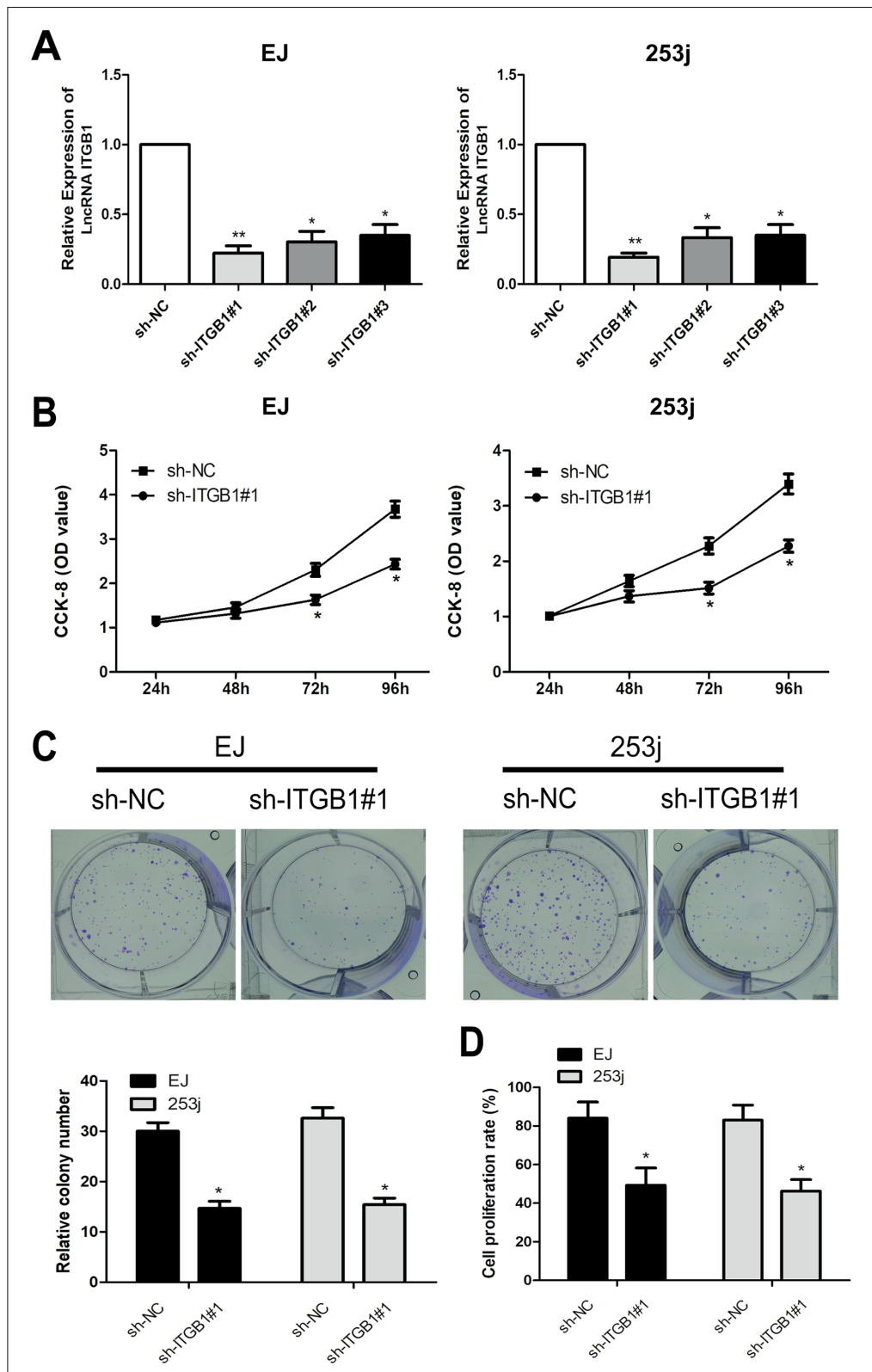


Figure 2. Silencing ITGB1 inhibited the proliferation of bladder cancer cells. **A**, QRT-PCR verified the interference efficiency of sh-ITGB1 in EJ and 253j cell lines. **B**, CCK-8 assay detected proliferation of EJ and 253j cells after transfection of sh-ITGB1. **C**, **D**, Colony formation assay detected the clonality in EJ and 253j cells after transfection of sh-ITGB1 (magnification $\times 20$). Data are expressed as mean $\pm$ SD, $*p < 0.05$.

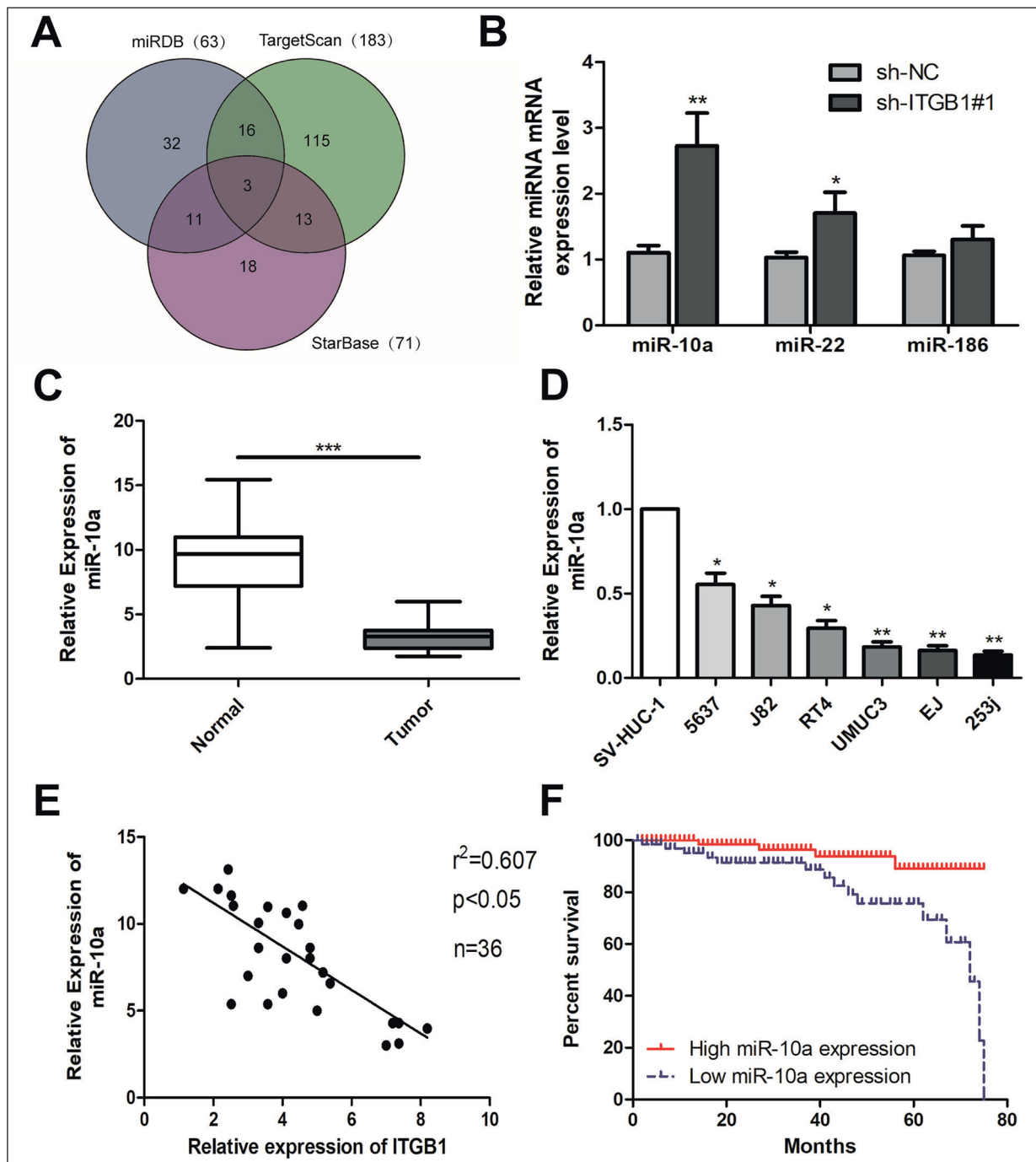


Figure 3. ITGB1 directly targeted miR-10a. **A**, TargetScan, miRbase, and MiRcode suggest that ITGB1 can target miR-10a. **B**, After silencing ITGB1, miR-10a level was found significantly elevated. **C**, qRT-PCR was used to detect the differential expression of miR-10a in bladder cancer tissues and adjacent tissues. **D**, QRT-PCR verified the mRNA expression level of miR-10a after transfection of ITGB1 in bladder cancer cell lines. **E**, There was a significant negative correlation between the expression levels of ITGB1 and miR-10a in bladder cancer tissues. **F**, Kaplan-Meier survival curves of bladder cancer patients based on miR-10a expression. Data are expressed as mean $\pm$ SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

gender, lymph node metastasis, and distant metastasis. In addition, according to the expression of microRNA-10a, the tissue samples were divided into high and low expression group.

Kaplan-Meier survival curves demonstrated that low expression of microRNA-10a was significantly associated with poor prognosis of BCa ($p<0.05$; Figure 3F).

Over-Expression of MicroRNA-10a Inhibited Cell Proliferation

To investigate the function of microRNA-10a in BCa, microRNA-10a mimics was constructed. After transfection of microRNA-10a mimics in EJ and 253j cell lines, the qRT-PCR experiment was performed to verify the interference efficiency (Figure 4A). After overexpression of microRNA-10a in EJ and 253j cell lines, CCK-8 and colony formation assays showed that compared with those transfected with miR-NC, cell proliferation ability was remarkably attenuated after overexpression of microRNA-10a (Figure 4B-4D).

ITGB1 Modulated MicroRNA-10a Expression in BCa

To further explore the interaction between ITGB1 and microRNA-10a in BCa cells, rescue experiments were conducted by co-transfection of microRNA-10a inhibitor and si-ITGB1 in BCa cells. QRT-PCR assay detected ITGB1 level after cell co-transfection (Figure 5A). Subsequently, our results showed that microRNA-10a could abolish the regulatory effect of ITGB1 on the proliferative ability in BCa (Figure 5B and 5C).

Discussion

BCa is one of the most common malignant tumors of the urogenital system, and its incidence increases year by year¹⁻³. However, despite the rapid development of immunology, biochemistry, and other technologies, highly sensitive and specific diagnostic markers are lacked. Therefore, cystoscopy combined with pathological biopsy is still the gold standard for the diagnosis of BCa^{4,5}. For high-risk, non-invasive BCa and invasive BCa, radical resection combined with pelvic lymph node dissection is often required, which often involves urinary diversion or reconstruction. Long-term life quality of BCa patients is seriously affected. Specific targeted therapy drugs to prevent the progression and metastasis of BCa are limited⁵⁻⁷. Effective and sensitive hallmarks for predicting, diagnosing, and treating BCa are urgently required, so as to improve their clinical outcomes⁸⁻¹¹. In recent years, many studies have shown the critical functions of lncRNAs in tumor diseases. Many lncRNAs

are differentially expressed in carcinoma tissues and adjacent normal tissues¹²⁻¹⁵. BCa-associated lncRNAs have been discovered, such as HOTAIR, H19, MALAT-1, etc²²⁻²⁴. Through high-throughput sequencing technology, ITGB1 was selected as a candidate lncRNA related to the malignant progression of BCa, and the relationship between ITGB1 and the development of BCa was finally determined. The upregulated ITGB1 was observed in BCa, which was able to promote the development of the malignant progression of BCa. ITGB1 expression was positively correlated with pathological staging and poor prognosis of BCa patients. Therefore, we suggested that ITGB1 may promote the progression of BCa. To further study the molecular mechanism of the occurrence and development of ITGB1, *in vitro* experiments were conducted. The silence of ITGB1 in EJ and 253j cells significantly attenuated proliferation. Currently, lncRNAs are identified to regulate downstream gene expressions and functions by absorbing corresponding microRNAs¹⁸⁻²⁰. To further explore the downstream target genes of lncRNA ITGB1 in BCa tissues and its related regulatory network, bioinformatics was conducted to predict potential microRNAs binding ITGB1. MicroRNA-10a was found to be involved in the occurrence and development of BCa. Subsequently, we verified the expression of microRNA-10a in BCa tissues through qRT-PCR assay, and found that microRNA-10a was significantly highly expressed in BCa tissues compared with the paired normal bladder epithelial tissues. Further investigation revealed that the expression levels of ITGB1 and microRNA-10a were negatively correlated. Notably, miRNA-10a was able to abolish the regulatory effect of ITGB1 on the proliferation of BCa cells.

Conclusions

We demonstrated that lncRNA ITGB1 expression was remarkably upregulated in BCa tissue, which was related to the pathological stage of BCa. ITGB1 might promote BCa cell proliferation *via* regulating microRNA-10a. As a result, ITGB1 may serve as a new prognostic indicator of BCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

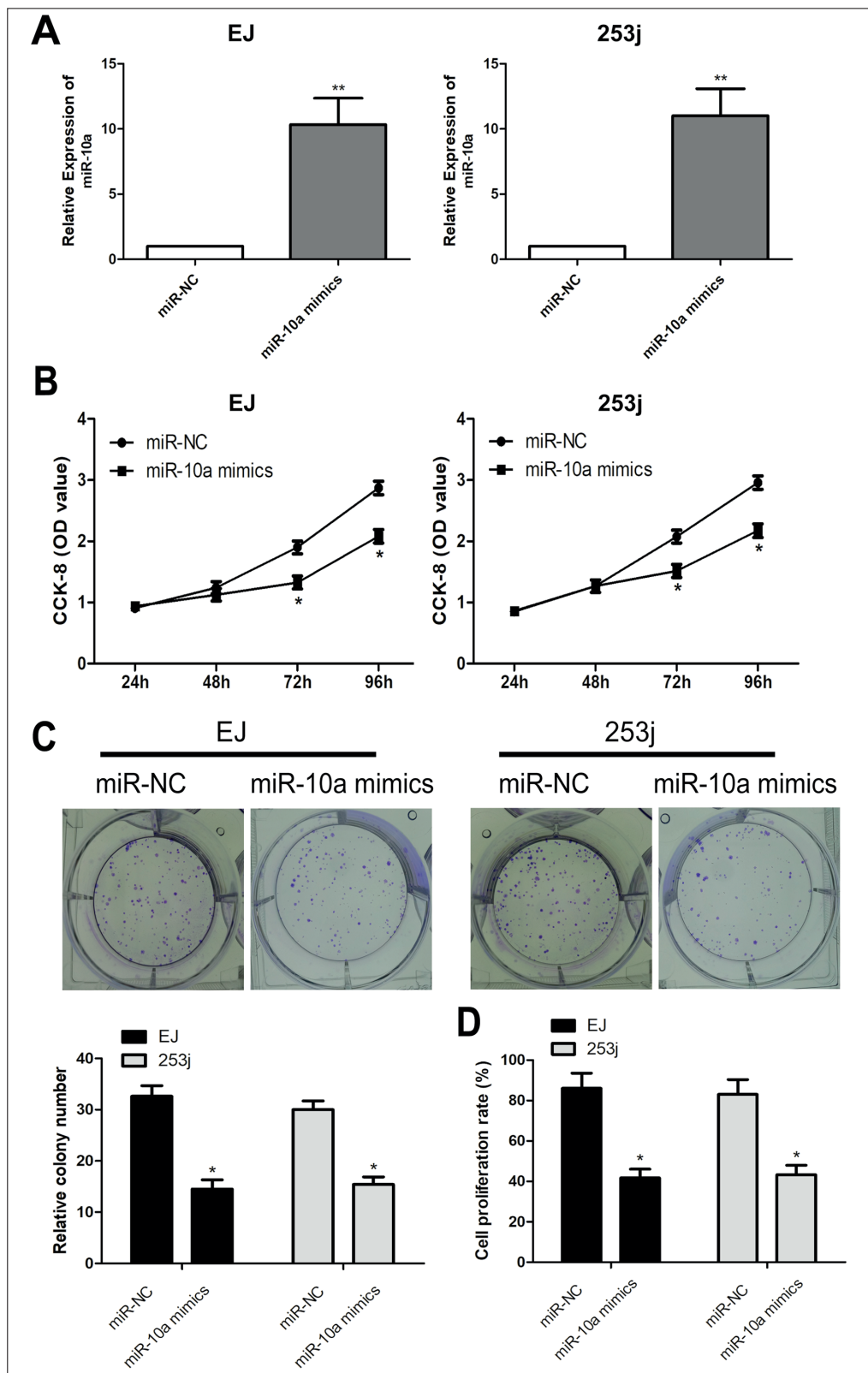


Figure 4. Overexpression of miR-10a inhibited the proliferation of bladder cancer cells. **A**, qRT-PCR verified the interference efficiency of miR-10a mimics in EJ and 253j cell lines. **B**, CCK-8 assay detected the proliferation of EJ and 253j cells after transfection of miR-10a mimics. **C**, **D**, Colony formation assay detected the clonality in EJ and 253j cells after transfection of miR-10a mimics. Data are expressed as mean $\pm$ SD, * p <0.05.

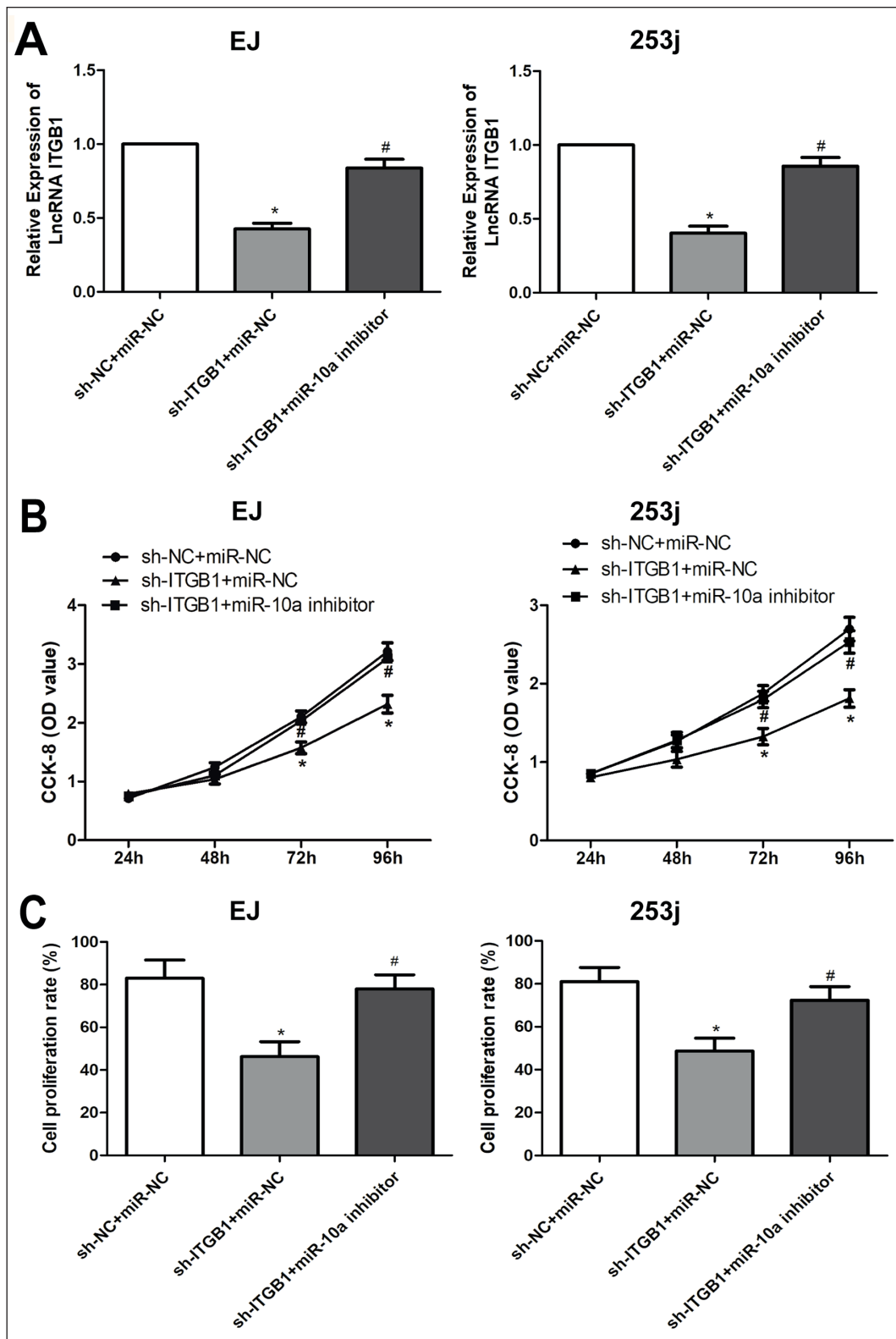


Figure 5. ITGB1 regulated the expression of miR-10a in bladder cancer. **A**, ITGB1 expression levels in co-transfected cells detected by qRT-PCR. **B**, CCK-8 assay detected the effect of ITGB1 and miR-10a on the proliferation of bladder cancer cells after co-transfection. **C**, Cell proliferation assay was used to detect the proliferation of bladder cancer cells after co-transfection of ITGB1 and miR-10a. Data are expressed as mean $\pm$ SD, $^{*}p < 0.05$.

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