

Original Article

siRNA-mediated Loc554202 knockdown regulates the proliferation, invasion and apoptosis of human bladder cancer cells

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Abstract: This study is aimed to investigate the effect of small interfering RNAs (siRNAs)-mediated Loc554202 knockdown on the proliferation, invasion and apoptosis of human bladder cancer cells. The expression of long non-coding RNA (lncRNA) Loc554202 in bladder cancer and adjacent noncancerous tissue, and bladder cancer cell lines (T24, J82 and Bui87) and normal uroepithelium SV-HUC-155 cells was compared by qRT-PCR. The siRNAs targeting Loc554202 and negative control si-Scramble were transfected into these cells. Loc554202 expression was detected by qRT-PCR. Cell proliferation was examined by MTT assay. Cell apoptosis was detected by flow cytometry. The expression of key apoptotic proteins including caspase-3, caspase-9 and Bcl-2 was measured by Western blot analysis. Cell invasion was assessed by Matrixgel invasion assay. Loc554202 expression in tumor and bladder cancer cells was significantly higher than that in normal tissue and SV-HUC-155 cells, respectively. The highest inhibitory effect on Loc554202 expression was observed in T24 and J82 cells transfected with siRNA-1, which were used for subsequent experiments. Cell viability of T24 and J82 cells in siRNA-1 group was significantly lower compared with si-Scramble group at 72, 96 and 120 h after transfection ($P < 0.05$). The percentage of necrotic T24 and J82 cells in siRNA group was significantly higher compared with si-Scramble group ($P < 0.05$). The percentage of transmembrane T24 and J82 cells in siRNA-1 group was significantly decreased compared with si-Scramble group ($P < 0.05$). Caspase-3 and caspase-9 expression in T24 and J82 cells transfected with siRNA-1 was significantly higher compared with si-Scramble group ($P < 0.05$), whereas the expression of the anti-apoptotic proteins Bcl-2 was significantly lower ($P < 0.05$). The siRNA-mediated Loc554202 knockdown significantly inhibits cell proliferation and invasion of T24 and J82 cells, and promotes their apoptosis.

Keywords: Human bladder cancer cells, lncRNA, small interference RNA, Loc554202, apoptosis

Introduction

Human bladder cancer is one of the most common malignancies worldwide [1]. In the United States, it is the second most common cancer of urinary tract causing over 13,000 deaths annually [2]. In China, bladder cancer is the eighth most common malignancy with gradually increasing incidence and mortality rate in recent years [3]. Bladder cancer is characterized by a high recurrence rate, and strong cell invasion and metastasis. There are two major types of bladder cancer: low grade papillary tumors and high grade invasive tumors. The typical treatment for papillary bladder tumors is surgical removal of the tumor followed by post-operative chemotherapy [4]. However, tumor recurrence often occurs, and some patients

may even develop invasive and metastatic bladder cancer [5, 6]. Invasive bladder cancer is much more likely to spread, and thereby have a much worse prognosis with approximately a 5-year survival rate of 50% [7]. Therefore, investigation of biological characteristics of bladder cancer cells and identification of novel molecular targets are important for effective treatment and prognosis of bladder cancer.

Long non-coding RNAs (lncRNAs) are non-coding RNAs that are more than 200 nucleotides in length without protein-coding capacity [8]. lncRNAs have been known to be crucial for a wide range of biological processes, including X chromosome silencing, genomic imprinting, cell growth, and tumorigenesis [9-11]. Aberrant expression of lncRNAs has been identified in

several types of cancer including bladder cancer [12, 13]. Recent studies have demonstrated that lncRNAs play important roles in carcinogenesis and cancer metastasis [14, 15] probably through interaction with microRNAs [16]. In recent years, it has been suggested that lncRNAs may serve as a molecular therapeutic target during cancer treatment [17, 18].

Loc554202, the gene located on human chromosome 9 for a lncRNA, is also the host gene of miR-31 [19], a microRNA with oncogenic properties [20]. ACpG is located upstream of the miR-31 locus which also spans the first exon of Loc554202 has been identified. Studies have confirmed an epigenetic transcriptional down-regulation of lncRNA Loc554202 and miR-31 by hypermethylation of the CpG island in breast cancer [21], which inhibits cancer metastasis. Therefore, Loc554202 might be a promising molecular target for effective treatment of malignant cancers such as breast cancer and bladder cancer with exceptionally high rates of recurrence and metastasis.

In the present study, we determined the expression of lncRNA Loc554202 in both bladder cancer tissues and cells. We further explored the effect of small interfering RNAs (siRNAs)-mediated lncRNA Loc554202 knockdown on the proliferation, invasion and apoptosis of bladder cancer cells in order to investigate whether Loc554202 represents a novel therapeutic target in bladder cancer. The study provides a theoretical basis for molecular targeting therapy for bladder cancer.

Materials and methods

Cells and reagents

The cell lines used in this study including one normal uroepithelium cell line, SV-HUC-155, two muscle invasive (T24 and J82) bladder cancer cell lines, one superficial transitional bladder cancer cell lines (Biu87) were purchased from RPMI culture media, fetal bovine serum (FBS) and penicillin-streptomycin double-resistance were purchased from Sigma (Gibco, St. Louis, MO, USA). RNA extraction kit, reverse transcription kit, DreamTaq Green PCR Master Mix (2x) were purchased from Takara (Japan). qRT-PCR β -actin was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The siRNAs targeting Loc554202 and negative control (si-Scramble) were synthesized by GenePharma Biotech. (Shanghai, China). The sequences were as follows: of

siRNA-1 sequence, siRNA 2-sequence, siRNA-3 sequence, and si-Scramble. Lipofectamine 2000 transfection reagent was purchased from Shanghai Bioleaf Technology Co (Shanghai, China). Tetrazolium reagent (MTT) was purchased from Sigma (St. Louis, MO, USA). AnnexinV-FITC/PI apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). Protein extraction kit and BCA kit were purchased from Beyotime Institute of Biotechnology. Rabbit anti-caspase-3, caspase-9, Bcl-2 and β -actin antibody were purchased from Abcam (Cambridge, MA, USA). Transwell chambers were purchased from Corning (Corning, NY, USA). Matrigel and crystal violet were purchased from BD Biosciences. All other reagents were purchased from Sigma unless otherwise specified. The collection of human tissues was approved by the Research Ethics Committee at Tangshan Gongren Hospital Affiliated to Hebei Medical University and performed in strict accordance with international standards. All patients were required to sign the informed consent.

Culture of cells

Cells were cultured in RPMI 1640 medium containing 10% FBS at 37°C in an incubator with 5% CO₂. After 48 h of incubation, cells at exponential phase were subjected to subsequent experiments.

RNA extraction and real-time reverse transcription-PCR (qRT-PCR) analysis

Cells in each group were collected and total RNA was extracted using RNA extraction kit according to the manufacturer's instruction. RNA concentration was determined using a spectrometer under a wavelength of 260 nm. Total RNA was reverse transcribed into cDNA. The level of lncRNA Loc554202 and β -actin mRNA were measured by qRT-PCR using cDNA as template and reverse transcription kit following the manufacturer's instructions. The reaction condition: 95°C 15 s; followed by 30 cycles of 95°C 5 s, 60°C 60 s. After amplification, melting curve analysis was performed: 95°C 15 s, 60°C 30 s, 72°C 30 s. The sequences of primers: β -actin 5'-ACGTGGACATCCGCAAG-3' (forward) 5'-AGGGCAGTGATCTCCTTCTG-3' (reverse); Loc554202 5'-GCTTTTCAGAGGCAATTAGA-3' (forward) 5'-CAGTGGCATTGGATTCTC-3' (reverse). The experiment was repeated three times. Data was analyzed using 2^{- Δ Ct} method. Bladder cancer tissues and adjacent

Down-regulated Loc554202 inhibits bladder cancer cell

non-cancerous tissues were also collected and subjected to qRT-PCR analysis.

Transfection of T24 and J82 cells

Aliquots of 2 ml of T24 and J82 cells were inoculated into each well on 6-well plates and cultured at 37°C in a 5% CO₂ incubator. Cells at 70% confluent were transfected respectively with 0.1 nmol siRNA-1, -2, -3 and 0.1 nmol si-Scramble using Lipofectamine 2000 according to the manufacturer's instruction. The sequences of siRNAs were as follows:

si-1: 5'-GCAGGTAGAGATGGATTCTGGAAA-3'; si-2: 5'-CAGGTAGAGATGGATTCTGGAAAT-3'; si-3: 5'-TGGATTCTGGAAATACCTCCTCAA-3'; si-Scramble: 5'-GCAAGATGTAGTTAGGTCCGGGAAA-3'.

After 48 h of incubation, cells were collected and Loc554202 expression was quantified as described in 2.3.

MTT assay

Aliquots of 100 µl of T24 and J82 cells were inoculated into each well of 96-well plates. Cells at 70% confluence were transfected with siRNAs and si-Scramble as described earlier. A total of 20 µl of 5 mg/ml MTT was added to the appropriate well after 24, 48, 72, 96 and 120 h, respectively. Medium was discarded after 4 h, and 150 µl of DMSO was added to each well. The optical density of dissolved MTT crystals was measured by a plate reader (Bio-Rad Laboratories) at 490 nm.

Detection of cell apoptosis by Annexin V-FITC/PI double staining

T24 and J82 cells in exponential phase in all groups were digested with trypsin, collected, washed twice with cold PBS, and resuspended in 100 µl of Annexin V binding buffer. A total of 5 µl of Annexin V-FITC solution were added and the mixture was incubated at room temperature in the dark for 15 min. Next, 5 µl of PI staining solution were added and the mixture was incubated for another 5 min. A total of 400 µl of Annexin V binding buffer was added and cell apoptosis was detected by flow cytometry within 1 h. The experiment was repeated three times and results were analyzed using Cellquest Pro software. The left lower quadrant (Q3) represents normal viable cells with low staining intensity of both AnnexinV and PI, whereas the upper left quadrant (Q1) represents necrotic cells. Q2 and Q3 represent late and early apoptotic cells, respectively.

Western blot analysis

The expression of caspase-3, caspase-9, Bcl-2 in T24 and J82 cells and normal uroepithelium SV-HUC-155 cells was compared by Western blot analysis. Briefly, after transfection, cells at 70% confluence were collected. Total protein was extracted using protein extraction kits and quantified using a BCA kit according to the manufacturer's instruction. Equal amounts of total protein (20 µg) were separated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes. The membrane was blocked in TBS buffer containing 5% skim milk and 0.1% Tween 20 at room temperature for 1 h, and incubated with the appropriate primary antibody (1:200) overnight at pH 7.6 at 4°C with gentle shaking. The peroxidase-labeled secondary antibodies (1:200) were added and the membranes were incubated at 37°C for 1 h. The membranes were washed 3 times with TBST for 5 min each and subjected to ECL detection. The intensity of bands was detected by a Molecular Imager® ChemiDoc-TMXRS System (Bio-Rad Laboratories). The gray value of bands was analyzed by Image Lab 2.0 software (Bio-Rad Laboratories).

Cell invasion assay

Matrigel (BD Biosciences) was spread evenly on the micro-film of a transwell chamber. Cells were transfected with siRNA with the highest inhibitory effect and si-Scramble as described in 2.4. After 72 h, cells were digested and added into the upper chamber of transwell. DMEM medium containing 5% FBS was added into the lower chamber. After 24 h of culture, transmembrane cells were washed, fixed and stained with crystal violet. The cells were counted under an inverted microscopy. Mean values were obtained from five randomly selected fields for each well.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS 11.0. Difference between groups was analyzed by t tests. P<0.05 is considered statistically significant.

Results

Increased Loc554202 expression in tumor tissue and bladder cancer cell lines

The expression of Loc554202 in tumor and adjacent noncancerous tissue was compared

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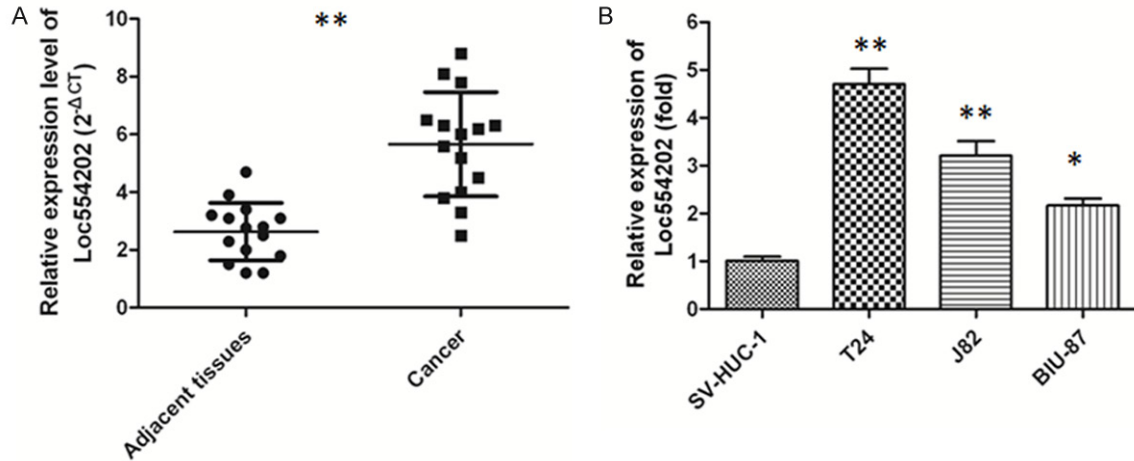


Figure 1. A. qRT-PCR analysis comparing lncRNA Loc554202 expression in bladder cancer tissues and adjacent non-cancerous tissues (control). *, P<0.05 compared with the control. B. qRT-PCR analysis of lncRNA Loc554202 expression in bladder cancer cells and normal SV-HUC-1 cells, *, P<0.05 compared with the control.

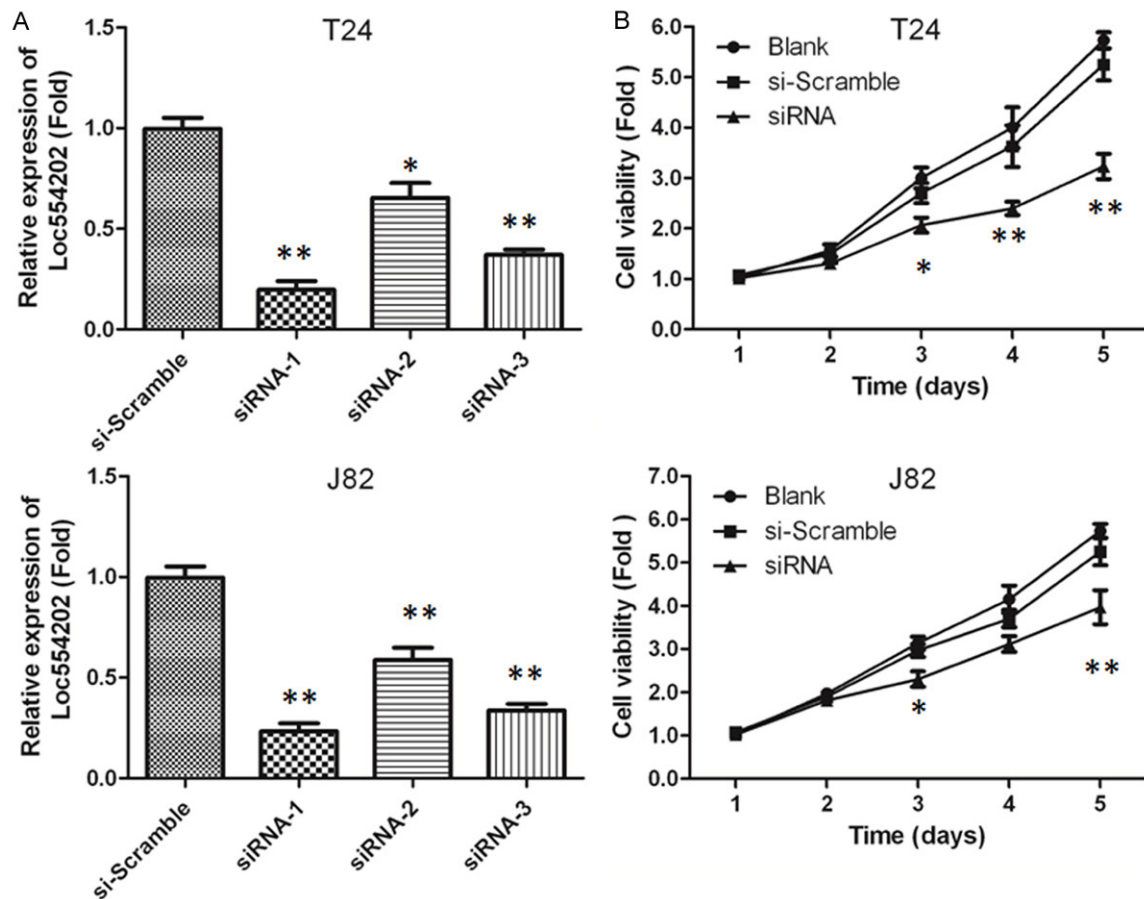


Figure 2. A. The relative Loc554202 expression in T24 and J82 cells. B. MTT assay of T24 and J82 cells transfected with siRNA targeting Loc554202. *, P<0.05 compared with si-Scramble group.

by qRT-PCR analysis. It was shown that relative Loc554202 expression in tumor was signifi-

cantly higher than that in adjacent noncancerous tissue (P<0.01, **Figure 1A**). The expression

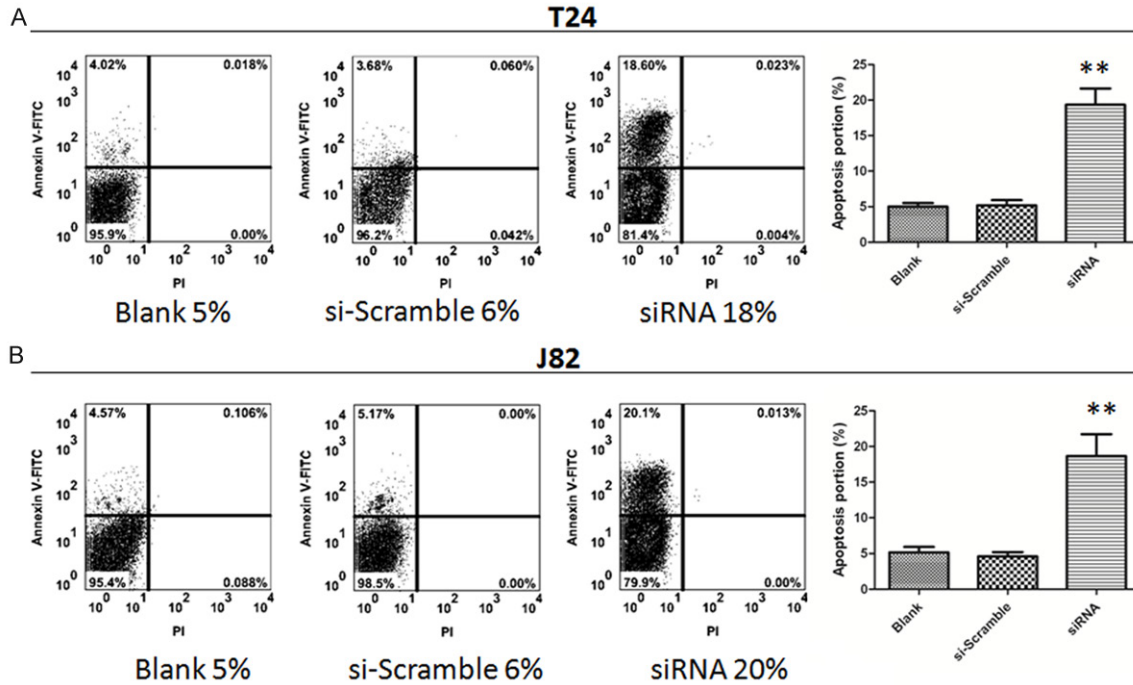


Figure 3. Flow cytometry analysis of cells transfected with siRNA targeting Loc554202. Cell apoptosis in siRNA group was significantly higher compared with si-Scramble group. *, P<0.05 compared with si-Scramble group.

of Loc554202 in bladder cancer cell lines T24, J82 and BIU-87, and normal uroepithelium SV-HUC-155 cells was also compared. As shown in **Figure 1B**, relative Loc554202 expression in T24, J82 and BIU-87 cells was significantly higher compared with SV-HUC-155 cells (P<0.05). Moreover, relative Loc554202 expression in T24, J82 cells (4.5±0.1 and 3.0±0.1, respectively) was higher than that in BIU-87 cells, which were used for subsequent experiments.

Inhibitory effect of siRNA targeting Loc554202 on cell viability of T24 and J82 cells

The expression of Loc554202 in T24 and J82 cells transfected with siRNA-1, -2 and -3 was quantified by qRT-PCR analysis. As shown in **Figure 2A**, the relative Loc554202 expression in these cells was significantly reduced compared with the si-Scramble group (P<0.05). The highest inhibitory effect was observed in siRNA-1 group, which was used for the transfection in subsequent experiments. Further, the proliferation of T24 and J82 cells transfected with siRNA-1 was detected by MTT assay. As shown in **Figure 2B**, cell viability of T24 and J82 cells

in siRNA-1 group was significantly lower compared with si-Scramble group at 72, 96 and 120 h after transfection (P<0.05), suggesting that the siRNA-mediated inhibition of Loc554202 expression markedly reduced the proliferation of T24 and J82 cells.

Effects on of siRNA targeting Loc554202 on cell apoptosis

As shown in **Figure 3**, the percentage of necrotic T24 cells in siRNA group was 18%, which was significantly higher compared with both the blank control (5%) and si-Scramble group (6%, P<0.05). The percentage of necrotic J82 cells in siRNA group (20%) was also significantly higher compared with si-Scramble group (6%, P<0.05), indicating that siRNA-mediated inhibition of Loc554202 expression significantly promoted apoptosis of T24 and J82 cells.

Effects of siRNA on the expression of proteins of the mitochondrial apoptosis pathway

The expression of proteins of the mitochondrial apoptosis pathway including caspase-3, caspase-9, Bcl-2 in T24 and J82 cells in each group was compared by Western blot analysis. As

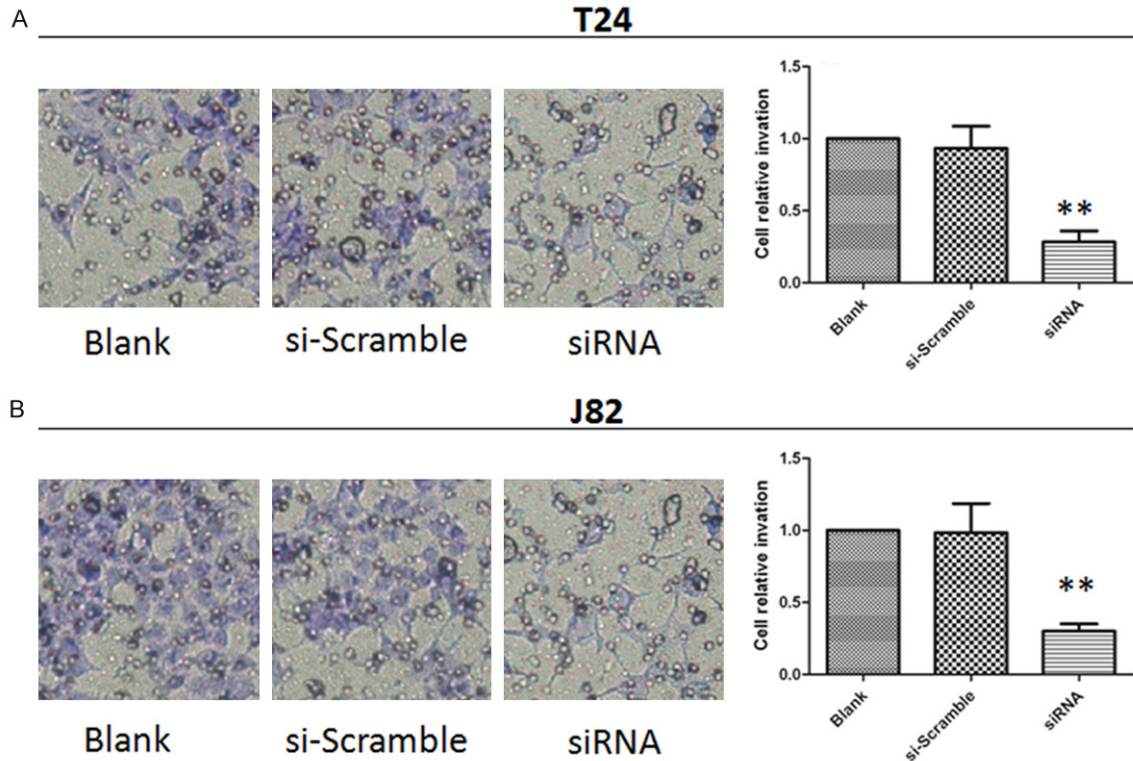


Figure 4. Transwell matrigel invasion assay of cells transfected with siRNA targeting Loc554202. The number of invasive cells in siRNA group was significantly lower compared with si-Scramble group. *, $P < 0.05$ compared with si-Scramble group.

shown in **Figure 3**, caspase-3 and caspase-9 expression in T24 and J82 cells transfected with siRNA-1 was significantly higher compared with si-Scramble group ($P < 0.05$), whereas the expression of the anti-apoptotic proteins Bcl-2 was significantly lower ($P < 0.05$), suggesting that siRNA-mediated inhibition of Loc554202 expression induced cell apoptosis by activating the mitochondrial apoptosis pathway.

Effects of siRNA on the invasion of T24 and J82 cells

As shown in **Figure 4**, cell invasion assay demonstrated that the percentage of transmembrane T24 and J82 cells in siRNA-1 group was significantly decreased (approximately 30%) compared with the blank control and si-Scramble group ($P < 0.05$), which suggested that the invasion ability of T24 and J82 cells was significantly reduced after the downregulation of Loc554202 expression by siRNA-1.

Discussion

In recent years, many studies have suggested that lncRNAs may serve as a novel target for

cancer treatment. As a host gene of miR-31, Loc554202 controls its transcription. It has been known that hypermethylation of the CpG island in the promoter region of Loc554202 is the primary mechanism for miR-31 expression, which plays an important regulatory role in the development and metastasis of breast cancer [22]. Studies have shown that Loc554202 expression is markedly increased in breast cancer, and its expression level is positively correlated with the size and clinical stage of tumors [22]. Moreover, Loc554202 expression in triple-negative breast cancer (TNBC) cells was significantly higher compared with normal breast cells. Consistent with these findings, our results showed that Loc554202 expression in bladder cancer tissues and cells was significantly higher than that in normal tissues and SV-HUC-155 cells, respectively.

siRNA silencing has drawn increasingly more attention in the field of functional genomics research, and has been widely used in cancer research [23, 24]. Shi et al. have found that siRNA-mediated Loc554202 knockdown prohibited the proliferation, colony formation, inva-

sion and migration of TNBC cells as well as the breast tumor in vivo [25]. In this study, Loc554202 in bladder cancer cells was silenced by siRNAs to explore its effects on cell proliferation and apoptosis. Results demonstrated that lncRNA Loc554202 expression in all three siRNA groups was significantly reduced compared with control group, suggesting that siRNA could be used to establish an in vitro model for function analysis of Loc554202.

Infinite division and proliferation of tumor cells is an important feature of malignancy, and invasive ability of tumor cells is a key factor affecting the prognosis and survival rate of cancer. Studies have shown that lncRNA Loc554202 regulates the proliferation, invasion and metastasis of breast cancer cells mainly through the regulation of miR-31 [21]. Consistent with previous studies, our results showed that the cell viability of T24 and J82 cells transfected with siRNA-1 was significantly lower compared with control group at 72 h after transfection as indicated by MTT assay, suggesting the inhibitory effect of Loc554202 knockdown on the proliferation of these cancer cells. Results in the transwell matrigel invasion assay showed that the number of invasive cells in siRNA group was substantially decreased compared with control group, indicating a reduced invasion capacity of bladder cancer cells with siRNA-mediated Loc554202 knockdown. Furthermore, flow cytometry analysis demonstrated that the proportion of apoptotic cells in siRNA group was significantly higher than that in control group, suggesting an induction effect of Loc554202 silencing on apoptosis of T24 and J82 cells.

Caspase-3 and caspase-9 are important proteins of mitochondrial apoptotic pathway, increased expression of which has been shown to inhibit tumor cell proliferation, and induce cell cycle arrest and apoptosis [26]. Bcl-2, a key anti-apoptosis protein, is an important indicator for the prognosis of cancer. It has been shown that increased expression of Bcl-2 significantly inhibits apoptosis of tumor cells [27]. This study also investigated the regulatory mechanism of the induced apoptosis by Loc554202 silencing. The expression of caspase-3, caspase-9 in siRNA group was significantly increased compared with control group; whereas bcl-2 expression was significantly reduced, suggesting that siRNA-mediated Loc554202 knockdown might induce apopto-

sis through the activation of mitochondrial apoptotic pathway.

In summary, Loc554202 is closely associated with cell viability and apoptosis of bladder cancer T24 and J82 cells. siRNA-mediated Loc554202 knockdown inhibits the proliferation and invasion of these cancer cells, and promote their apoptosis.

This study provides insights into the regulatory mechanisms Loc554202 knockdown on invasion and metastasis of bladder cancer cells. Loc554202 might be an effective therapeutic target for the treatment of bladder cancers, especially invasive bladder cancers.

Disclosure of conflict of interest

None.

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