Tetracycline-inducible shRNA targeting antisense long non-coding RNA

HIF1A-AS2 represses the malignant phenotypes of bladder cancer

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ABSTRACT

Various studies have indicated that long non-coding RNAs (lncRNAs) play vital roles in the cancer development and progression. lncRNA hypoxia inducible factor 1alpha antisense RNA-2 (HIF1A-AS2) is upregulated in gastric carcinomas and knockdown of HIF1A-AS2 expression by siRNA could inhibit cell proliferation in vitro and tumorigenesis in vivo. Inspired by these observations, we hypothesized that HIF1A-AS2 possibly plays the analogous role in bladder cancer. In our study, we first reported that HIF1A-AS2 was up-regulated in bladder cancer tissues and cells, and HIF1A-AS2 expression level in bladder cancer tissues is positively associated with advanced clinical pathologic grade and TNM phase. Cell proliferation inhibition, cell migration suppression and apoptosis induction were observed by silencing HIF1A-AS2 in bladder cancer T24 and 5637 cells. Overexpression HIF1A-AS2 in normal SV-HUC-1 cells could promote cell proliferation, cell migration and anti-apoptosis. Besides, we utilized the emerging technology of medical synthetic biology to design tetracycline-inducible small hairpin RNA (shRNA) vector which specifically silenced HIF1A-AS2 in a dosage-dependent manner to inhibit the progression of human bladder cancer. In conclusion, our data suggested that HIF1A-AS2 plays oncogenic role and can be used as a therapeutic target for treating human bladder cancer. Synthetic “tetracycline-on” switch system that quantitatively controlled the expression of HIF1A-AS2 in bladder cancer can inhibit the progression of bladder cancer cells in a dosage-dependent manner. Our findings provide new insights into the role of the lncRNA HIF1A-AS2 in the bladder cancer.

Keywords: HIF1A-AS2; bladder cancer; lncRNAs; tetracycline-inducible; synthetic biology

1. Introduction

As the most common malignancy in urological system worldwide, bladder carcinoma brings huge economic burden and high morbidity [1, 2] and frequently causes deaths in patients with advanced-stage cancer. Transurethral resection of bladder tumor (TURBT) is often used for treating high-risk non-muscle-invasive bladder cancer and radical cystectomy is employed in high-risk muscle-invasive bladder cancer [3, 4]. It is well-known that early stage cancer is the indication of the operation, while radiation therapy and chemotherapy are considered as the main treatments for advanced-stage cancer. However, the five-year survival rate remains at a low level. The recommended maintenance schedules at present cannot prevent patients at high risk from recurrence and/or progression. Therefore, alternative treatments are urgently required [4], and it is inevitable to develop more efficient and safer therapeutic methods for curing urothelial bladder cancer.

With the rapid development of sequencing technologies [5], a series of dysregulated long non-coding RNAs (lncRNAs) have been found in many human diseases [6, 7], especially in cancers [8, 9]. Accumulating evidences suggested that long non-coding RNAs (lncRNAs) perform important or vital functions in the malignant tumors [10, 11]. LncRNA hypoxia inducible factor 1alpha antisense RNA-2 (HIF1A-AS2) is upregulated in gastric carcinomas and knockdown of HIF1A-AS2 expression by siRNA could inhibit cell proliferation in vitro and tumorigenesis in vivo [12]. However, there is no report that reveals the relationship between HIF1A-AS2 and bladder cancer.

Synthetic biology uses unique combinatorial and spatiotemporal layers of regulation to create synthetic systems of unprecedented sophistication and has biomedical and biotechnological engineering applications. Synthetic biology approaches are extended to diverse applications throughout basic biological research, biotechnology and medicine [13-18]. Medical synthetic biology is an emerging field that can combine engineering principles with

medicine [19, 20]. Based on these engineering principles, we used a standard platform for generation of tetracycline inducible vectors [21], which control the expression of LncRNAs in a dosage-dependent manner and can provide a more efficient research tool for bladder cancer.

In this research, we firstly reported that HIF1A-AS2 was up-regulated in bladder cancer tissues and cells compared to matched normal bladder tissues and normal bladder epithelial cells. So we suggested that HIF1A-AS2 acts as a potential oncogene in bladder cancer. Synthetic tetracycline-inducible shRNA targeting HIF1A-AS2 effectively arrested the expression of HIF1A-AS2 and suppressed progression of bladder cancer in a dosage-dependent manner.

2. Materials and Methods

2.1 Patient samples

44 bladder cancer patients who received radical cystectomy were included in this research. Bladder cancer tissues and matched normal peritumoral tissues were snap-frozen in liquid nitrogen quickly after resection. Written informed consent was also obtained from all the patients. The study was approved by the institutional research ethics committee of Shenzhen Second People's Hospital.

2.2 Cell lines and cell culture

Human bladder cancer 5637, T24 and human normal bladder epithelial cells(SV-HUC-1) were purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The 5637 cells were cultured in RPMI-1640 (1640) (Invitrogen, Carlsbad, CA, USA). The T24 cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA, USA). The SV-HUC-1 cells were cultured in F12K medium (Invitrogen, Carlsbad, CA, USA). 1640,DMEM and F12K mediums were mixed with 1% antibiotics (100 U/ml penicillin and 100 µg /ml streptomycin sulfates) and 10% fetal bovine serum (FBS). Then plates were placed in incubator at 37°C with an atmosphere of 5% CO₂.

2.3 Construction of synthetic tetracycline-inducible shRNA vector targeting HIF1A-AS2

The specific sequences of the related tetracycline-inducible shRNA targeting HIF1A-AS2 and the negative control were constructed and inserted into plasmid vector psi-LVRInU6TGP which was purchased from FulenGen firm, Guangzhou, China. The sequences of tetracycline-inducible shRNA were “GAGUUGGAGGUGUUGAAGCAAAUUAU” [12].

2.4 Short hairpin RNA(shRNA)

The sequences were 5'- GAGUUGGAGGUGUUGAAGCAAUAU -3' [12]. HIF1A-AS2 shRNA was ordered from GenePharma (Suzhou,China).Negative Control 1 shRNA was also purchased from GenePharma (Suzhou, China).

2.5 PcDNA3.1-HIF1A-AS2

The pcDNA3.1-HIF1A-AS2 was ordered from GenePharma (Suzhou,China),. This vector overexpressed HIF1A-AS2 in cells. Negative Control 2 was also purchased from GenePharma (Suzhou, China).

2.6 Transfection of cell lines

5637 and T24 cells were transiently transfected with specific siRNA oligonucleotides.The HIF1A-AS2 siRNA sequences were 'GAGUUGGAGGUGUUGAAGCAAUAU' [12]. Negative Control siRNA (si-NC) and si-HIF1A-AS2 were purchased from GenePharma (Suzhou, China). Bladder cancer cells were cultured in 6-well plates at optimum density until transfection.The plasmid vectors(shRNA,pcDNA3.1-HIF1A-AS2, tetracycline-inducible HIF1A-AS2 shRNA and NC shRNA) were constructed using Plasmid Midiprep kits (Promega, Madison, USA). Cells were transiently transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

2.7 Real-time quantitative PCR

Total RNA was extracted from the tissues or the transfected cells using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The cDNA was converted from total RNA by using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the instructions. The primer sequences were as follows: HIF1A-AS2 primers sense: 5'-TCTGTGGCTCAGTTCCTTTTGT-3' and reverse: 5'-ATGTAGGAAGTGCCAGAGCC-3' [12]. GAPDH primers forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTT CAC-3'. We use a standard SYBR Green PCR kit (Takara, Dalian, China) for performing quantitative PCR (qPCR), and the final reaction volume was 20 μ l according to the manufacturer's instructions, including 1 μ l of First-Strand cDNA, 0.4 μ l of sense primer, 0.4 μ l of reverse primer, 10 μ l of 2 $\times$ All-in-OneTM qPCR Mix (Takara, Dalian, China), 0.4 μ l of 50 $\times$ ROX Reference Dye and 7.8 μ l of double-distilled water. The reactions were performed in triplicate by using the ABI PRISM 7500 Fluorescent Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was chosen as the internal control. The average value in each triplicate was used to calculate the relative amount of HIF1A-AS2 using the comparative Δ Ct method.

2.8 Cell proliferation assays

Cell Counting Kit-8, CCK-8 (Beyotime Institute of Biotechnology, Shanghai, China) and 5-ethynyl-20-deoxyuridine (EdU) assay kit (Ribobio, Guangzhou, China) were used for cell proliferation according to the manufacturer's instructions. For CCK-8 assay, cells were incubated in a 96-well plate for 24 hours, and then transiently transfected with siRNAs or plasmids. The absorbance in each well was measured at 0, 24, 48 and 72 h after transfection by a microplate reader (Bio-Rad, Hercules, CA, USA).

2.9 Cell migration assay

Cell migration was detected by scratch assay according to the reported methods[22]. Cells were seeded in 6-well plates and incubated in incubator to get 100% confluence before transfection. The cells were transfected with siRNAs or plasmids. A sterile 200 μ l pipette tip was used to generate a clear line in the wells. Pictures were taken from each well quickly using a digital camera system. After one day, pictures were taken again. Migration distance was counted at the time of 0 h and 24 h.

2.10 Caspase 3 ELISA assay

Bladder cancer 5637 and T24 cells were transfected with siRNA or plasmids

in 12-well plates. 48 hours after transfection of siRNA or plasmids, apoptosis were detected by measuring the activity of caspase 3 using the caspase 3 enzyme-linked immunosorbent assay (ELISA) assay kit (Hcusabio, Wuhan, China) according to the manufacturer's instructions. OD values were measured by using a microplate reader (Bio-Rad).

2.11 Flow cytometry assay

Bladder cancer 5637 and T24 cells were transiently transfected with siRNAs or plasmid vectors. 48 hours after transfection, cells were harvested and resuspended in fixation fluid 5 μ l Annexin V - FIFC and 2 μ l propidium iodide were added to 500 μ l cell suspension. Cell apoptosis was then determined by using flow cytometry (EPICS, XL-4, Beckman, CA, USA). In the graphs, the quadrant respectively stands for dead cells, living cells, early apoptotic cells and late apoptotic cells.

2.12 Statistical analysis

All experimental assays were performed in triplicate. All data were presented as mean $\pm$ standard deviation (SD). All statistical analyses were executed by using SPSS 20.0 software (IBM, Chicago, IL, USA). The HIF1A-AS2 RNA expression differences between bladder cancer tissues and matched normal tissues were analyzed using paired samples t-test. CCK-8 assay data were

analyzed by ANOVA. The independent samples t-test was used to analyze other data. P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 Up-regulation of HIF1A-AS2 in bladder cancer tissues, cells and its correlation with clinical pathologic factors

The relative expression level of HIF1A-AS2 was detected by using Real-Time qPCR in a total of 44 patients with bladder cancer. Compared to matched normal peritumoral tissues, the HIF1A-AS2 expression was up-regulated remarkably in 68.2% (30 of 44) of cancer tissues ($P = 0.0056$) (Figure 1 A and B). Compared with the SV-HUC-1, the HIF1A-AS2 expression was increased remarkably in both bladder cancer cells, 5637 ($P < 0.001$) and T24 ($P = 0.00016$) (Figure 1 C and D). As shown in Table 1, up-regulated HIF1A-AS2 was positively associated with bladder cancer histological grade ($P = 0.003$), tumor invasion depth ($p < 0.001$) and TNM stage ($P < 0.001$). But gender, age, tumor size and lymph node metastasis had no relation with HIF1A-AS2 expression level. These results indicated that long non-coding HIF1A-AS2 should play oncogenic role in bladder cancer.

3.2 Knockdown of HIF1A-AS2 inhibited cell proliferation of bladder cancer

We further determined whether HIF1A-AS2 promotes cell proliferation in bladder cancer. The relative expression level of HIF1A-AS2 was analyzed by qRT-PCR at 48 hours after transfection of siRNA in 5637 and T24 cell lines. The relative expression levels of HIF1A-AS2 in 5637($p=0.0057$) and T24($P=0.0027$) cells were down-regulated significantly by siRNA at 48 hours post transfection (Figure 2 A). In the 5637 cell lines, the relative expression of HIF1A-AS2 was decreased by 72.82%. In T24 cell lines, the relative expression was decreased by 65.53%. And the relative expression levels of HIF1A-AS2 were down-regulated significantly at 48 hours post transfection of shRNA in 5637($p = 0.001164$) and T24($P= 0.000476$) cells (Figure 2 B). In the 5637 cell lines, the relative expression of HIF1A-AS2 was decreased by 54%. In T24 cell lines, the relative expression was decreased by 48%. CCK-8 assay was carried out to detect whether si-HIF1A-AS2 or shRNA inhibited the proliferation in 5637 and T24 bladder cancer cells. The results demonstrated that si-HIF1A-AS2 and shRNA inhibited cell proliferation remarkably in both bladder cancer cells ($p < 0.01$ in two cell lines) (Figure 2 C and D). EdU assay was further illustrated to detect cell proliferation. As shown in Figure 4 E and Figure F, compared to si-NC or negative control 1 group, EdU positive 5637 and T24 cells in si-HIF1A-AS2 or shRNA group were reduced after transfection. EdU assay illustrated that the quantity of EdU positive cells in si-HIF1A-AS2

group was reduced by 43% in 5637 ($P = 0.010655$) and decreased by 66% in T24 ($P = 0.00659$) (Figure 2 E). The quantity of EdU positive cells in shRNA group was reduced by 34.86% in 5637 ($P = 0.010014$) and decreased by 40.33% in T24 ($P = 0.007229$) (Figure 2 F). These results demonstrated that knockdown of HIF1A-AS2 inhibited cell proliferation in bladder cancer.

3.3 Knockdown of HIF1A-AS2 inhibited cell migration of bladder cancer

Cells were transfected with si-RNA or shRNA plasmids in 6-well plates. The cell scratch assay was utilized to detect the role of specific si-RNA or plasmids in cell migration. Compared with si-NC and negative control 1 group, cell migration of bladder cancer cells was significantly suppressed by si-HIF1A-AS2 and shRNA (Figure 3 A,B,C,D,E and F). Scratch assay illustrated that the ratio of the relative migration in si-HIF1A-AS2 group was reduced by 42.54% in 5637 ($P = 0.007607$) and decreased by 37.10% in T24 ($P = 0.00932$) (Figures 3 A,B and C). The ratio of the relative migration in shRNA group was reduced by 39.89% in 5637 ($P = 0.001834$) and decreased by 37.69% in T24 ($P = 0.002286$) (Figures 3 D,E and F). These data suggested that knockdown of HIF1A-AS2 suppressed cell migration of bladder cancer.

3.4 Knockdown of HIF1A-AS2 promoted cell apoptosis of bladder cancer

Furthermore, we asked whether knockdown of HIF1A-AS2 can promote cell apoptosis in bladder cancer after transfection of si-RNA or shRNA plasmids and cell apoptosis was detected by flow cytometry assay and the caspase 3 enzyme-linked immunosorbent assay (ELISA). The apoptotic cells were increased remarkably (Figures 4 A,B,C and D) in both cell lines after transfection with the si-HIF1A-AS2 and shRNA as revealed by flow cytometry assay. Compared with control groups, the activities of caspase 3 were increased significantly in cells transfected with the si-HIF1A-AS2 ($P = 0.0085$ in 5637 cells; $P = 0.0053$ in T24 cells) (Figure 4 E) . In the 5637 cell lines ,the Caspase 3 activity was increased by 81.72%. In T24 cell lines, the relative activity was increased by 64.67%.And the activities of caspase 3 were also increased dramatically after transfection with the shRNA ($P = 0.000632$ in 5637 cells; $P = 0.010082$ in T24 cells) (Figure 4 F). In the 5637 cell lines, the Caspase 3 activity was increased by 67.18%. In T24 cell lines, the relative activity was increased by 56.07%. These results indicated that knockdown of HIF1A-AS2 promoted cell apoptosis in bladder cancer.

3.5 Overexpression of HIF1A-AS2 promoted cell proliferation in SV-HUC-1.

We further investigated the function of HIF1A-AS2 by overexpression of HIF1A-AS2 in SV-HUC-1. The relative expression level of HIF1A-AS2 was up-regulated significantly by pcDNA3.1-HIF1A-AS2 in SV-HUC-1 cells at 48

hours post-transfection ($p=0.004506$, Figure 5 A). The relative expression of HIF1A-AS2 was up-regulated about 2.33-fold over the control. CCK-8 assay was carried out to detect whether pcDNA3.1-HIF1A-AS2 promoted the proliferation of SV-HUC-1. The results demonstrated that pcDNA3.1-HIF1A-AS2 promoted cell growth in SV-HUC-1 ($p < 0.05$) (Figure 5 B). EdU assay was further illustrated to detect cell growth. As shown in Figures 5 C and D, compared to negative control 2 group, EdU positive cells in pcDNA3.1-HIF1A-AS2 group was increased after transfection. EdU assay illustrated that the quantity of EdU positive cells was increased by 1.5-fold in 5637 ($P = 0.010655$).

3.6 Overexpression of HIF1A-AS2 promoted cell migration in SV-HUC-1

Compared to negative control 2 group, cell migration of SV-HUC-1 was promoted by pcDNA3.1-HIF1A-AS2. Scratch assay illustrated that the ratio of the relative migration in PcDNA3.1-HIF1A-AS2 group was increased by 52.04% in SV-HUC-1 ($p=0.011703$, Figure 6 A and B). These data suggested that HIF1A-AS2 increased cell migration in SV-HUC-1.

3.7 Overexpression of HIF1A-AS2 inhibited cell apoptosis in SV-HUC-1

We investigated whether HIF1A-AS2 can promote cell anti-apoptosis in

SV-HUC-1 after transfection with pcDNA3.1-HIF1A-AS2 and cell apoptosis was detected by flow cytometry assay and the caspase 3 enzyme-linked immunosorbent assay (ELISA). The apoptotic cells were decreased (Figure 7 A and B) after transfection with the pcDNA3.1-HIF1A-AS2 by flow cytometry assay. Compared to control groups, the activity of caspase 3 was decreased by 43.47% ($P = 0.010941$) (Figure 7 C) after transfection with the pcDNA3.1-HIF1A-AS2. These results indicated that HIF1A-AS2 suppressed cell apoptosis in SV-HUC-1.

3.8 Tetracycline-inducible HIF1A-AS2 shRNA down-regulated expression of HIF1A-AS2

Bladder cancer 5637 and T24 cells were cultured in 6-well plates and transfected with plasmids (2 μ g) expressing either the corresponding tetracycline-inducible HIF1A-AS2 shRNA or NC shRNA. The relative expression level of HIF1A-AS2 was analyzed by qRT-PCR at 48h post transfection. When cells harboring HIF1A-AS2 shRNA were treated with different concentrations of doxycycline, the relative expression level of HIF1A-AS2 in both 5637 and T24 cells were down-regulated significantly ($P < 0.01$ in two cell lines) (Figure 8 A and B). When 2 μ l/ml doxycycline was added into cells transfected with HIF1A-AS2 shRNA plasmids, the expression level of HIF1A-AS2 was decreased by 51% in 5637 ($P < 0.01$) and decreased by 55%

in T24 ($P < 0.01$). And it showed that doxycycline promotes the expression of HIF1A-AS2 shRNA to suppress the expression of HIF1A-AS2 in a dosage-dependent manner. Therefore, we chose the concentration of the 2 $\mu\text{g/ml}$ for further study because 2 $\mu\text{g/ml}$ doxycycline induced the expression of HIF1A-AS2 shRNA to inhibit HIF1A-AS2 maximumly. These results showed substantial dose-dependent repression upon induction with doxycycline.

3.9 Tetracycline-inducible HIF1A-AS2 shRNA inhibited cell proliferation

We transfected the plasmids expressing either the corresponding tetracycline-inducible HIF1A-AS2 shRNA or the negative control into bladder cancer cells, and CCK8 and EdU assays were performed to detect cell proliferation. ANOVA was used to analyze CCK8 assays. Compared with the negative control groups, CCK8 assay demonstrated that tetracycline-inducible HIF1A-AS2 shRNA induced by 2 $\mu\text{g/ml}$ doxycycline efficiently inhibited cell proliferation in both bladder cancer 5637 and T24 cells ($P < 0.01$ in two cell lines) (Figure 9 A and B). Compared with the negative control, EdU assay showed that lesser EdU positive cells were observed when 5637 and T24 cells were treated with 2 $\mu\text{g/ml}$ tetracycline-inducible HIF1A-AS2 shRNA (Figure 9 C). The number of EdU positive was decreased by 59.4% in 5637($P = 0.0075$) and decreased by 69.1% in T24 ($P = 0.0157$) (Figure 9 D and E). The results showed that tetracycline-inducible HIF1A-AS2 shRNA induced by doxycycline could inhibit the cancer cell growth remarkably.

3.10 Tetracycline-inducible HIF1A-AS2 shRNA inhibited cell migration of bladder cancer

To detect whether cell migration was suppressed by tetracycline-inducible HIF1A-AS2 shRNA, cell migration assay was performed. Compared with the negative control, tetracycline-inducible HIF1A-AS2 shRNA inhibited cell migration significantly when 2 μ g/ml doxycycline was added into bladder cancer 5637($P=0.0095$) and T24 cells ($P=0.0049$) (Figure 10 A,B,C and D). In the 5637 cell lines, the relative migration was decreased by 35.41%. In T24 cell lines, the relative migration was decreased by 45.45%. These results demonstrated that tetracycline-inducible HIF1A-AS2 shRNA suppressed cell migration in bladder cancer.

3.11 Tetracycline-inducible HIF1A-AS2 shRNA induced apoptosis of bladder cancer

Lastly, we transfected the plasmids expressing either the corresponding tetracycline-inducible HIF1A-AS2 shRNA or the negative control into bladder cancer cells to detect apoptosis. The relative activity of caspase 3 was tested by ELISA assay. The apoptosis was detected using flow cytometry assay. Compared with the negative control group, the number of early apoptotic cells was dramatically elevated in tetracycline-inducible HIF1A-AS2 shRNA group by 2 μ g/ml doxycycline(Figure 11 A and B). The relative activity of caspase-3

was remarkably elevated when cells were transfected with the HIF1A-AS2 shRNA induced by 2 µg/ml doxycycline ($P < 0.01$ in two cell lines) (Figure 11 C and D); In the 5637 cell lines, the relative activity was increased by 63.38%. In T24 cell lines, the relative activity was decreased by 82.36% . These results manifested that tetracycline-inducible HIF1A-AS2 shRNA suppressed cell apoptosis in bladder cancer.

4. Discussion

Accumulating evidences suggest that long non-coding RNAs (lncRNAs) perform important functions in regulation of genes which control tumor proliferation, apoptosis, and migration, and stretched our understanding of the biological behavior in diseases especially in carcinomas including bladder cancer[23-29].Furthermore, these previous study indicate that lncRNAs are valuable biomarkers and therapeutic targets[28, 30-33]. The antisense Long non-coding RNA HIF1A-AS2 was located in chromosome 14q23.2, and has been found to be overexpressed in tumor tissues, such as chronic myeloid leukemia and neuroblastoma[34].HIF1A-AS2 is upregulated in gastric cancer and knockdown of HIF1A-AS2 expression by siRNA could inhibit cell proliferation in vitro and tumorigenesis in vivo[12]. However, the relation between HIF1A-AS2 and bladder cancer is still unknown and mysterious.

This is the first report to illustrate the function of HIF1A-AS2 in bladder

cancer. In this research, we evaluated the expression patterns and clinical significances of HIF1A-AS2 in bladder cancer, and furthermore explored its possible function in bladder cancer cells. Our study validated that HIF1A-AS2 can be considered as a promising biomarker for the diagnosis of bladder cancer. Expression of HIF1A-AS2 was remarkably higher in the bladder cancer, and its up-regulation was positively correlated with histological grade and TNM stage in bladder cancer. Furthermore, we explored the function of HIF1A-AS2 in bladder cancer cells and found that inhibition of HIF1A-AS2 expression through HIF1A-AS2 siRNAs and shRNA could suppress tumor cell proliferation, inhibited cell migration and induced apoptosis. To get a better insight to the role of HIF1A-AS2, we over-expressed HIF1A-AS2 in SV-HUC-1 cells, and it is found that overexpression of HIF1A-AS2 could promote cell proliferation, migration and anti-apoptosis. Targeting HIF1A-AS2 may present a favorable therapeutic strategy for treating bladder cancer in the clinic. Proudly, it is the the first study to implicate that HIF1A-AS2 plays oncogenic role in bladder cancer and plays a critical role in the regulatory process of bladder cancer. Further study is required to elucidate the exact molecular regulatory mechanisms of HIF1A-AS2.

In the past several years, medical synthetic biology utilizing the principles of engineering has made many remarkable achievements for treating human diseases including bladder cancer[16, 30, 35-37]. Inspired by the engineering principles of synthetic biology and these previously productive works, we

designed the tetracycline-inducible switch and measured its cancer inhibition effects in bladder cancer. Our study indicated that the tetracycline-inducible shRNA targeting HIF1A-AS2 effectively inhibited the expression of HIF1A-AS2 in a dosage-dependent manner, and obviously suppressed cell growth, inhibited cell migration and induced apoptosis in bladder cancer cells. The efficient of this tetracycline-induced device may be further improved by increasing the transfection efficiency[30, 36].

In summary, the present study elucidated that HIF1A-AS2 may participate in the bladder cancer tumorigenesis by affecting the progression of bladder cancer. Based on the engineering principles and methodologies of synthetic biology, we utilize a standard synthetic biology platform to construct synthetic tetracycline-inducible switch to control the expression of shRNA targeting oncogene HIF1A-AS2. Our results indicate this device could quantitatively suppress the progression of bladder cancer. This work provides a new approach for quantitatively controlling specific targets in human cancer. Maybe, it can be converted into a new and more effective therapeutic method for bladder cancer. It may be bright prospect to radically cure bladder cancer that could change the present dilemma of treatment for the advanced bladder cancer.

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Conflicts of interests

The authors declare that they have no conflicts of interests.

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Figure legends

Figure 1. The expression of HIF1A-AS2 in bladder cancer. 44 patients samples were included in the research. GAPDH was the internal control gene in qPCR, which detected relative expression of HIF1A-AS2 in bladder cancer tissues and cells. The relative expression patterns of HIF1A-AS2 in paired bladder cancer tissues and normal tissues (A and B) were shown. We also compared its expression level in bladder cancer cells (5637 and T24) and SV-HUC-1 (C and D). Assays were performed in triplicate, and data were shown as mean $\pm$ SD. (**P < 0.01).

Figure 2. Knockdown of HIF1A-AS2 inhibited cell proliferation. The relative expression level of HIF1A-AS2 was significantly down-regulated by si-HIF1A-AS2 (A) and shRNA (B). ANOVA was used for the comparison of curves of cell proliferation. Cell proliferation was detected in both bladder cancer cells after transfection of siRNA and shRNA (C and D). Representative images of EdU assay and the relative fold changes of EdU positive cells were detected by siRNA and shRNA (E and F). Assays were performed in triplicate, and data were shown as mean $\pm$ SD (*P < 0.05, **P < 0.01).

Figure 3. Knockdown of HIF1A-AS2 inhibited cell migration. The relative cell migration was suppressed after transfection of siRNA and shRNA (A, B,

C,D,E and F) .Representative images of 5637 and T24 transfected with siRNA and shRNA (A,B,D and E) were shown. Assays were performed in triplicate ,and data were shown as mean $\pm$ SD (**P < 0.01).

Figure 4. Knockdown of HIF1A-AS2 promoted cell apoptosis. Apoptotic cells were measured after transfection of siRNA and shRNA(A,B,C and D) by flow cytometry analysis. The activity of caspase-3 was higher after transfection as revealed by ELISA assay (E and F). Assays were performed in triplicate ,and data were shown as mean $\pm$ SD (*P < 0.05, **P < 0.01).

Figure 5. Overexpression of HIF1A-AS2 promoted cell proliferation. The relative expression of HIF1A-AS2 was up-regulated by pcDNA3.1-HIF1A-AS2 (A). ANOVA was used for the comparison of curves of cell proliferation. Cell proliferation was detected in both bladder cancer cell lines after transfection of pcDNA3.1-HIF1A-AS2 (B). Representative images of EdU assay were shown and the relative fold changes of EdU positive cells were detected (C and D). Assays were performed in triplicate ,and data were shown as mean $\pm$ SD (*P < 0.05, **P < 0.01).

Figure 6. Overexpression of HIF1A-AS2 promoted cell migration. Representative images of SV-HUC-1 migration were shown (A). The relative cell migration rate was increased after transfection of pcDNA3.1-HIF1A-AS2

(B). Assays were performed in triplicate ,and data were shown as mean $\pm$ SD (*P < 0.05).

Figure 7. Overexpression of HIF1A-AS2 inhibited cell apoptosis.

Representative images of flow cytometry assay in SV-HUC-1 transfected with pcDNA3.1-HIF1A-AS2 (A and B) were shown. The relative caspase-3 activity was suppressed after transfection(C). Assays were performed in triplicate , and data were shown as mean $\pm$ SD(*P < 0.05).

Figure 8. “Tet-on” shRNA switch quantitatively inhibited HIF1A-AS2 expression.

The relative expression level of HIF1A-AS2 was suppressed by tetracycline-inducible HIF1A-AS2 shRNA in 5637(A) and T24 (B). Assays were performed in triplicate ,and data were shown as mean $\pm$ SD (*P < 0.05, **P < 0.01).

Figure 9. “Tet-on” shRNA switch quantitatively inhibited cell proliferation.

ANOVA was used for the comparison of curves of cell proliferation. Cell proliferation was detected in both bladder cancer cell lines after transfection of Tetracycline-inducible HIF1A-AS2 shRNA (A and B). Representative images of EdU assay and the relative fold changes of EdU positive cells were detected in Tetracycline-inducible HIF1A-AS2 shRNA group and control group (C). The EDU positive cells were decreased by Tetracycline-inducible HIF1A-AS2 shRNA (D and E) (*P < 0.05, **P < 0.01).

Figure 10. “Tet-on” shRNA switch quantitatively inhibited cell migration.

The relative cell migration was suppressed after transfection with Tetracycline-inducible HIF1A-AS2 (A and B). Representative images of cell migration (5637 and T24) were shown (C and D). Assays were performed in triplicate, and data were shown as mean $\pm$ SD. (**P < 0.01).

Figure 11. “Tet-on” shRNA switch quantitatively promoted cell apoptosis.

Apoptotic cells were measured by flow cytometry analysis after transfection of tetracycline inducible shRNA (A and B). The activity of caspase-3 was higher after transfection as revealed by ELISA assay (C and D). Assays were performed in triplicate, and data were shown as mean $\pm$ SD (**P < 0.01).

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Figure(s)

C

D

Figure(s)

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A

B

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Table1 Correlation between HIF1A-AS2 expression and clinicopathological characteristics of bladder cancer patients

Characteristics	Total	Expression of HIF1A-AS2		P value
		High (n=30)	Low (n=14)	
Gender				
Male	30	22(73.3%)	8(26.7%)	0.091
Female	14	8(57.1%)	6(42.9%)	
Tumor size (cm)				
<3cm	15	9(60.0%)	6(40.0%)	0.501
≥3cm	29	21(72.4%)	8(27.6%)	
Age				
≤60	18	13(72.2%)	5(27.8%)	0.748
>60	26	17(65.4%)	9(34.6%)	
Histological grade				
PUNLMP/Low-grade	23	11(47.8%)	12(52.2%)	0.003**
High-grade	21	19(90.5%)	2(9.50%)	
Tumor invasion depth (T)				
Tis, Ta, T1	21	8(38.1%)	13(61.9%)	<0.001**
T2,T3 or above	23	22(95.7%)	1(4.30%)	
Lymph node metastasis(N)				
N0	41	27(65.9%)	14(34.1%)	0.540
N1 or above	3	3(100%)	0(0.00%)	
TNM stage				
0/I	20	7(35.0%)	13(65.0%)	<0.001**
II/III/IV	24	23(95.8%)	1(4.20%)	

PUNLMP,Papillary Urothelial Neoplasm of Low Malignant Potential; Low-grade, Low-gradePapillary Urothelial Carcinoma;High-grade,High-grade Papillary Urothelial Carcinoma(<http://www.pathology.jhu.edu/bladder>);

TNM according to the seventh edition of staging TNM of Union Internationale Contre Le Cancer(UICC) in 2009.