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Original Article

MicroRNA-206 acts as a tumor suppressor in bladder cancer via targeting YRDC

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Abstract: Accumulating evidence suggested that microRNA (miRNA) plays important regulatory roles in the initiation and development of various cancers. Previous study showed that microRNA-206 (miR-206) is dysregulated in human bladder cancer tissues, however, the biological function and underlying mechanisms of miR-206 in human bladder cancer remain unknown. In the present study, we aimed to investigate the clinical significance of miR-206 and its target gene YRDC in human bladder cancer, and to determine its effects on oncogenic phenotypes of this disease. Our results showed that miR-206 expression was downregulated significantly in bladder cancer tissues and cell lines compared with adjacent normal bladder tissues and human bladder epithelial immortalized SV-HUC-1 cell line, respectively. Overexpression of miR-206 reduced the expression of YRDC and inhibited bladder cancer cell proliferation, colony formation, migration, invasion and induced cell cycle arrest at G0/G1 phase. In addition, knockdown of YRDC exhibited similar effects with miR-206 overexpression in bladder cancer cells and restoration of YRDC partially reversed the effects of miR-206 in bladder cancer cells. These findings indicated that mir-206 might be a novel target for bladder cancer therapy by targeting YRDC.

Keywords: miR-206, tumor suppressor, bladder cancer, YRDC

Introduction

Bladder cancer is one of the most common cancer types worldwide. In 2016, the estimated newly diagnosed cases were 76,960 and the estimated new deaths caused by bladder cancer were 16,390 in the United States [1]. At presentation, about 75% of cases present with non-muscle-invasive bladder cancer which have a good prognosis [2, 3], and other 25% cases present with muscle-invasive bladder cancer with a poor prognosis [4]. Therefore, it is important to understand the potential molecular mechanisms involved in the tumorigenesis of bladder cancer, which may contribute to developing novel therapeutic strategies for the treatment of bladder cancer.

MicroRNAs (miRNAs), which are small (approximately 22 nucleotides in length), endogenous, non-coding RNAs, regulate gene expression post-transcriptionally by binding to the 3'-untranslated regions (UTR) of target mRNAs, inducing mRNAs degradation and/or translational repression [5, 6]. It has been reported that miRNAs are dysregulated in human tumors and are involved in various biological processes, including cell proliferation, cell cycle, differentiation, migration, invasion and apoptosis [7]. Growing evidence indicated that abnormal expression of miRNAs is associated with the progression and development of human cancers, functioning as oncogenes or tumor suppressors [8]. Recent reports have shown that miR-206 is downregulated and functions as tumor suppressor in many cancers, including lung adenocarcinoma, breast cancer and colorectal cancer [9-12]. Additionally, a previous study by Itesako et al. demonstrated that miR-206 was downregulated in bladder cancer [13]. However, the detailed biological function of miR-206 in bladder cancer has not been studied.

In this study, we confirmed that miR-206 was downregulated in human bladder cancer tis-

sues and cell lines. Overexpression of miR-206 inhibited the growth and metastasis of bladder cancer cells. Furthermore, we predicted and verified that YRDC was a target of miR-206. Restoration of YRDC significantly rescued the inhibition effect induced by miR-206. These results demonstrated that miR-206 functions as tumor suppressor in bladder cancer by targeting YRDC.

Materials and methods

Tissues samples and Cell culture

A total of 22 pairs of human bladder cancer tissues and their matched adjacent normal tissues were obtained from the Shanghai Tenth people's hospital, Tongji University School of Medicine (China). The study was approved by Shanghai Tenth people's hospital ethics committee and written informed consents were obtained from all patients.

Human bladder cancer cell lines (T24, EJ, 5637 and J82) and human bladder epithelial immortalized SV-HUC-1 cell line were obtained from the Chinese Science Institute (Shanghai, China). The SV-HUC-1 cells were cultured in F12K medium (Sigma-Aldrich, St Louis, MO, USA), the T24, EJ and 5637 cells were cultured in RPMI 1640 (Gibco), and the J82 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM). These media were supplemented with 10% fetal bovine serum (FBS; TBD, Tianjin, China) and 1% penicillin/streptomycin (Hyclone). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from frozen tissues and cultured cells using TRIzol reagent (Invitrogen Ltd) in accordance with the manufacturer's protocol. The concentration and purity of RNA were determined by ND-2000 Spectrophotometer (Thermo Fisher Scientific, USA). For miR-206 detection, cDNA was synthesized with 1 μg of total RNA by using One Step Prime script miRNA cDNA Synthesis Kit (Qiangen, Valencia, USA). Quantitative real-time PCR (qRT-PCR) assay was performed using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, USA). The expression of miR-206 was normalized to the expression level of U6.

For the detection of YRDC mRNA level, the cDNA was synthesized using PrimeScript RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. qRT-PCR was performed with KAPA SYBR FAST qPCR Kit (Kapa Biosystems, USA). The YRDC mRNA level was normalized to the β-Actin mRNA level. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

The primers for qRT-PCR analysis were as follows: miR-206 forward primer: 5'-TGGAATGTAAGGAAGTG-3'; miR-206 reverse primer: 5'-CAGTGCGTGTCGTGGAGT-3'; U6 forward primer: 5'-TGCGGGTGCTCGCTTCGCAGC-3'; U6 reverse primer: 5'-CCAGTGCAGGGTCCGAGGT-3'; YRDC forward primer: 5'-GGTTATTGATGGGGGACAAA-3'; YRDC reverse primer: 5'-TCTGTTGGAGGATGGCTGTA-3'.

Cell transfection

miR-206 mimic (miR-206) and corresponding miRNA negative control (miR-NC), the siNRAs targeting human YRDC (si-YRDC) and corresponding negative control (si-NC) were brought from GenePharma (Shanghai, China). YRDC overexpressed plasmids were obtained from Ribobio Co. (Guangzhou China). These molecular products were transfected into T24 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell proliferation and colony formation assay

Cells proliferation was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). Briefly, the transfected cells were seeded into 96-well plates at a density of 1000 cells per well, 10 μl of CCK-8 were added into each well at indicated time (24 h, 48 h, 72 h and 96 h) and incubated for 2 hours at 37°C. The absorbance at 450 nm was detected on a microplate spectrophotometer (BioTek, USA).

For colony formation assay, the 1000 transfected cells were seeded into 6-well plates and cultured for 14 days at RMPI 1640 medium containing 10% FBS. The colonies were imaged and counted after fixing with 75% ethanol and staining with 0.1% crystal violet solution.

Wound healing and invasion assays

Cell migration was detected by wound healing assays, the transfected cells were seeded into

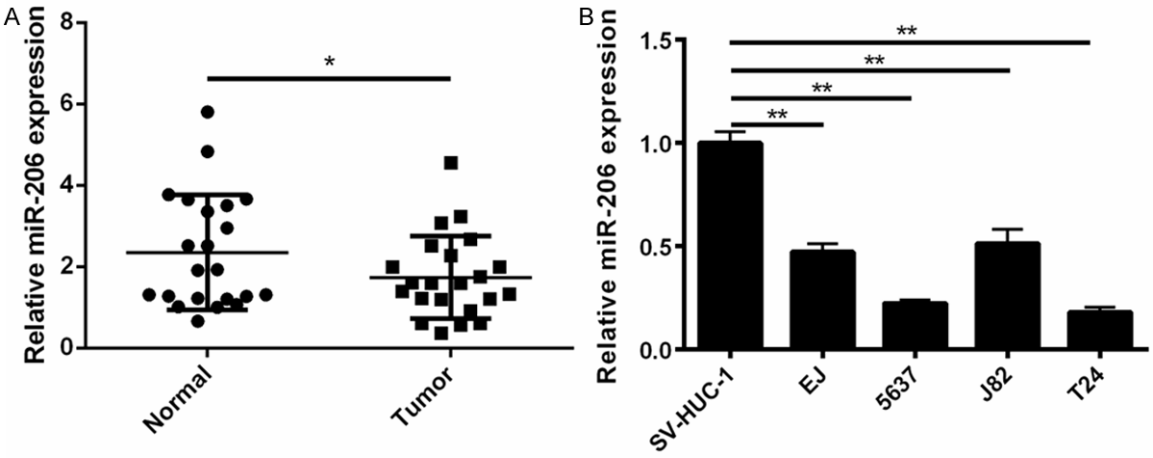


Figure 1. miR-206 expression was downregulated in bladder cancer tissues and cell lines. A. The expression of miR-206 was determined in tumor tissues and their matched adjacent normal tissues by qRT-PCR. $^{**}P<0.05$ vs adjacent normal tissues. B. The expression of miR-206 was determined in four human bladder cancer cell lines (EJ, 5637, J82 and T24) as well as in one normal human bladder transitional cell line SV-HUC-1. $^{**}P<0.01$ vs SV-HUC-1.

6-well plates and cultured until they reached 95% confluence. A sterile 200 μl pipette tip was used to generate a scratch through each well. The wound closure was observed after 24 h and photographed under a microscope (Olympus, Tokyo, Japan).

For invasion assays, 5×10^4 transfected cells in serum-free medium were plated in the upper well of the matrigel-coated invasion chamber, and 600 μl of RPMI 1640 medium containing 10% FBS were added into the lower chamber as chemoattractant. After incubated at 37°C for 16 hours, the non-invading cells were removed with a cotton tip, the cells migrating to the lower surface of the chamber were fixed with 95% ethanol for 20 min, stained with 0.1% crystal violet solution for 10 min, washed for three times, air dried, photographed and counted in five randomly selected fields for each well by a light microscope (Olympus).

Cell cycle assay

Forty-eight hours after transfection, bladder cancer cells were detached, washed by PBS, centrifuged, and fixed in 75% ethanol at 4°C overnight. Then the cells were washed with PBS, centrifuged and resuspended in 1 ml of PBS containing 1 mg/ml RNase-A and 50 μg/ml propidium iodide. Cells were incubated for 30 minutes at room temperature in the dark, and analyzed immediately using flow cytometer (BD Biosciences, USA).

Luciferase reporter assays

A wild-type 3'-UTR segment and a mutant 3'-UTR segment of YRDC were amplified using PCR and inserted into downstream of the luciferase gene in the psiCHECK-2 vector (Promega, USA). For luciferase assay, 293T cells were plated to 24-well plates, and then transfected with 100 ng of luciferase reporter vectors and 100 nM of miR-206 or miR-NC. The luciferase activity was measured using a luciferase reporter assay system (Promega, USA) after 48 h of incubation, according to the manufacturer's instructions.

Western blot analysis

Cells were washed with cold PBS and lysed in ice-cold RIPA buffer containing protease inhibitor. BCA protein assay kit was used to determine the concentration of total cellular protein according to the manufacturer's instructions. Then equal amounts of protein were load into 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat milk for one hour and then incubated with primary antibodies: anti-YRDC (1:1000, Santa Cruz, USA) and anti-β-Actin (1:2000, Santa Cruz, USA) overnight at 4°C. After washing with PBST three times, the membranes were incubated with the corresponding secondary antibodies at room temperature for one hour. The protein bland was visualized using the Odyssey scanner (LI-COR Biosciences, USA).

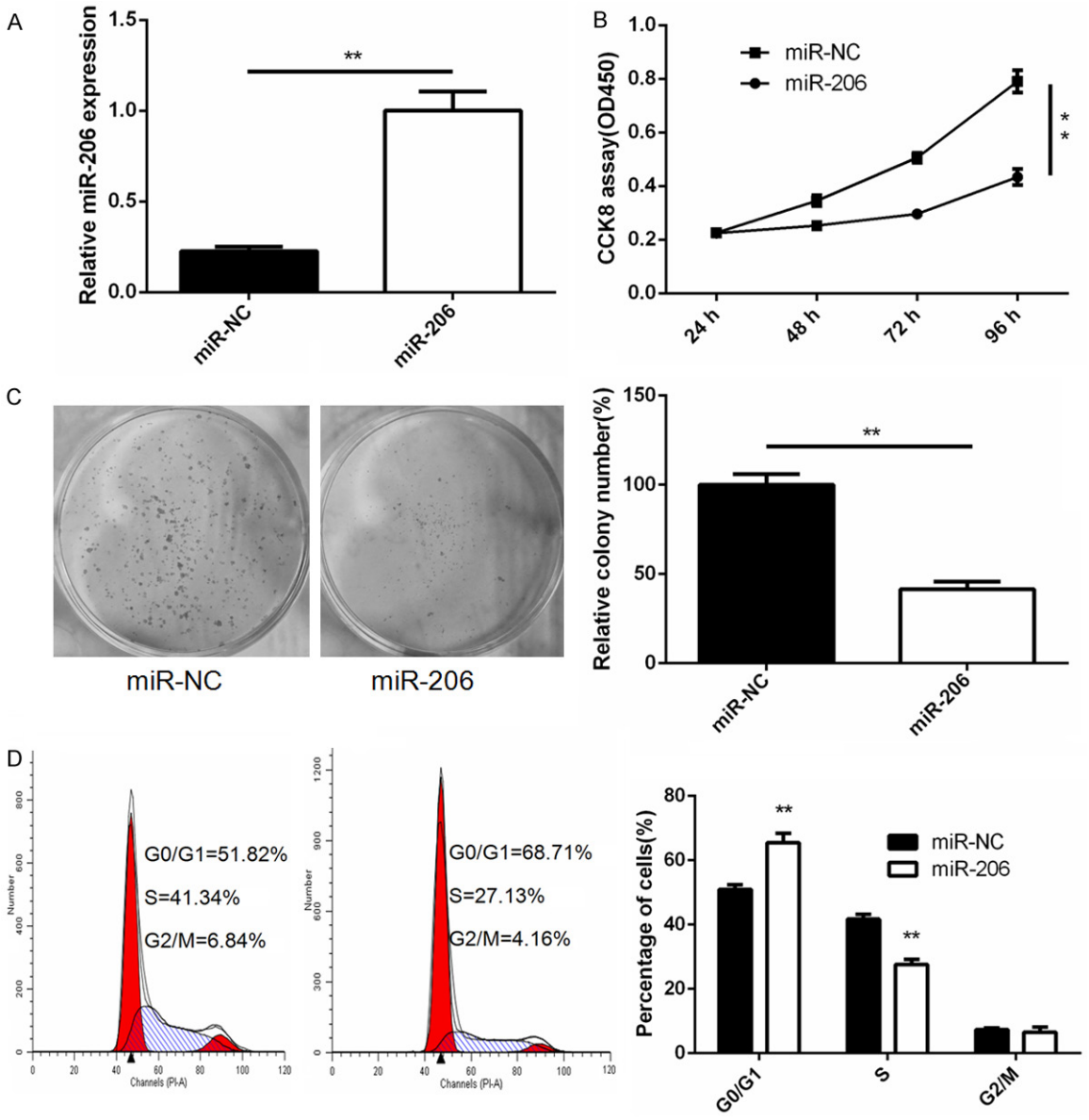


Figure 2. miR-206 inhibited cell growth in human bladder cancer cells. A. The expression of miR-206 was determined by qRT-PCR in T24 cells transfected with miR-206 mimic or miR-NC. B. Cell proliferation was determined in T24 cells transfected with miR-206 mimic or miR-NC by CCK8 assay. C. Colony formation was determined inT24 cells transfected with miR-206 mimic or miR-NC. D. Cell cycle distribution was determined in T24 cells transfected with miR-206 mimic or miR-NC by flow cytometer assay. $^{**}P<0.01$ vs miR-NC.

Immunohistochemical (IHC) analysis

The tissue expression levels of candidate target gene YRDC were detected with IHC testing technology. The paraffin-embedded tissue samples were dewaxed and incubated with 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. The sections were infiltrated in citrate buffer and then heated in a microwave for 10 min to carry out antigen retrieval. Then the sections were incubated with the primary antibody anti-YRDC (1:100, Santa Cruz, USA) overnight at 4°C. After that, the sections were washed with PBS, the peroxidase-labeled goat anti-mouse secondary antibody was applied at room temperature for 1 h. Next, the slides were stained with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and hematoxylin for visualization.

Statistical analysis

The SPSS version 16.0 for windows (SPSS Inc., USA) was used for statistical analysis. All data

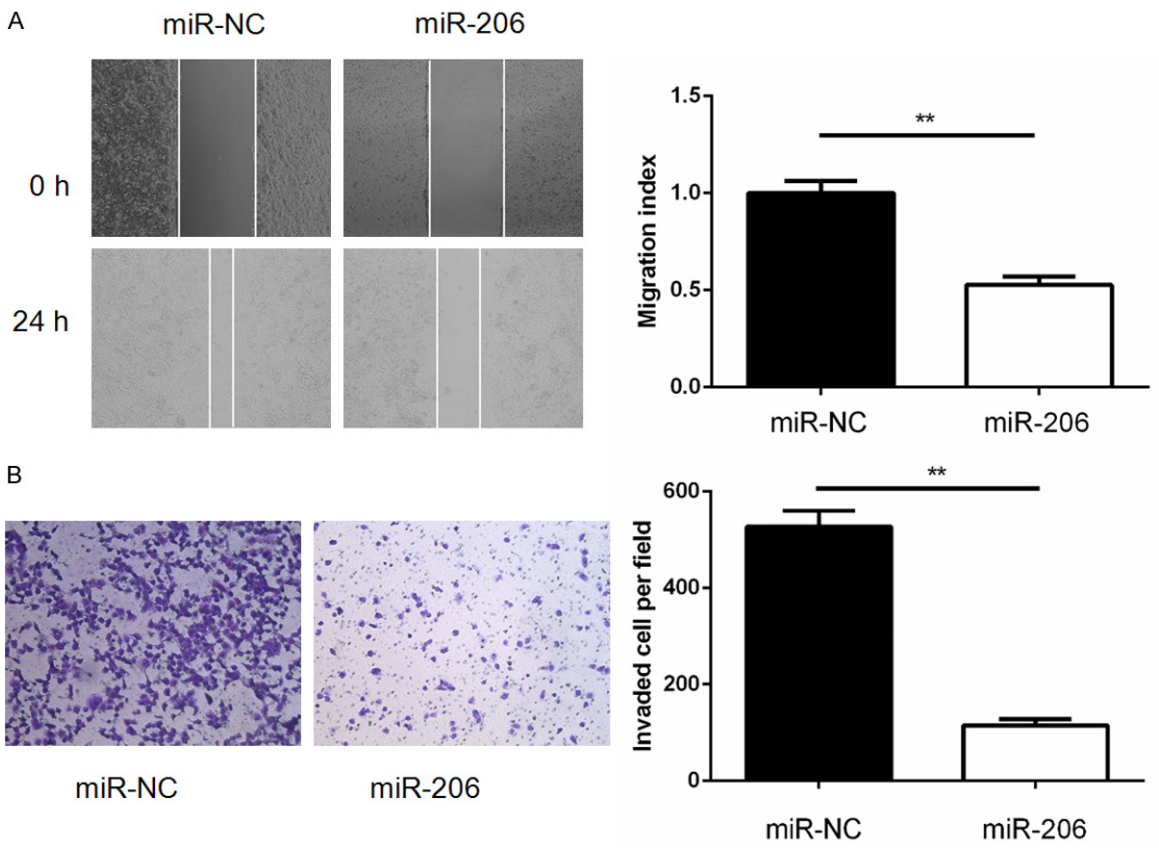


Figure 3. miR-206 inhibited cell migration and invasion in human bladder cancer cells. A. Cell migration was determined in T24 cells transfected with miR-206 mimic or miR-NC by wound healing assay. B. Cell invasion was determined in T24 cells transfected with miR-206 mimic or miR-NC by transwell invasion assay. $^{**}P<0.01$ vs miR-NC.

are presented as means ± standard deviation (SD) from at least three independent experiments. The experiment results were calculated using Student's t-test and one-way ANOVA. *P* value less than 0.05 were considered to be statistically significant.

Result

miR-206 expression is downregulated in bladder cancer tissues and cell lines

To determine the expression levels of miR-206 in human bladder cancer specimens, qRT-PCR was performed in 22 pairs of tumor tissues and matched adjacent normal tissues. The results showed that miR-206 expression levels in tumor tissues were significantly downregulated compared to adjacent normal tissues ($P<0.05$) (**Figure 1A**). We also examined miR-206 expression in four bladder cancer cell lines (EJ, 5637, J82 and T24) and one normal human bladder transitional cell line SV-HUC-1 by qRT-PCR. We found that miR-206 was significantly decreased in four bladder cancer cell lines compared with normal human bladder transitional cell line (**Figure 1B**). The lowest expression level of miR-206 was detected in T24 cell line, which was selected for next study.

miR-206 inhibits cell proliferation, colony formation and induces cell cycle arrest in bladder cancer cells

To assess the role of miR-206 in the growth of bladder cancer cells, miR-206 mimic or miR-NC were transfected into T24 cells, respectively. We confirmed that the expression of miR-206 was upregulated in T24 cells after transfected with miR-206 mimic compared to cells transfected with miR-NC by qRT-PCR (**Figure 2A**). CCK8 assay showed that overexpression of miR-206 significantly inhibited cell proliferation (**Figure 2B**). Colony formation assay showed that overexpression of miR-206 significantly decreased colony formation (**Figure 2C**). To fur-

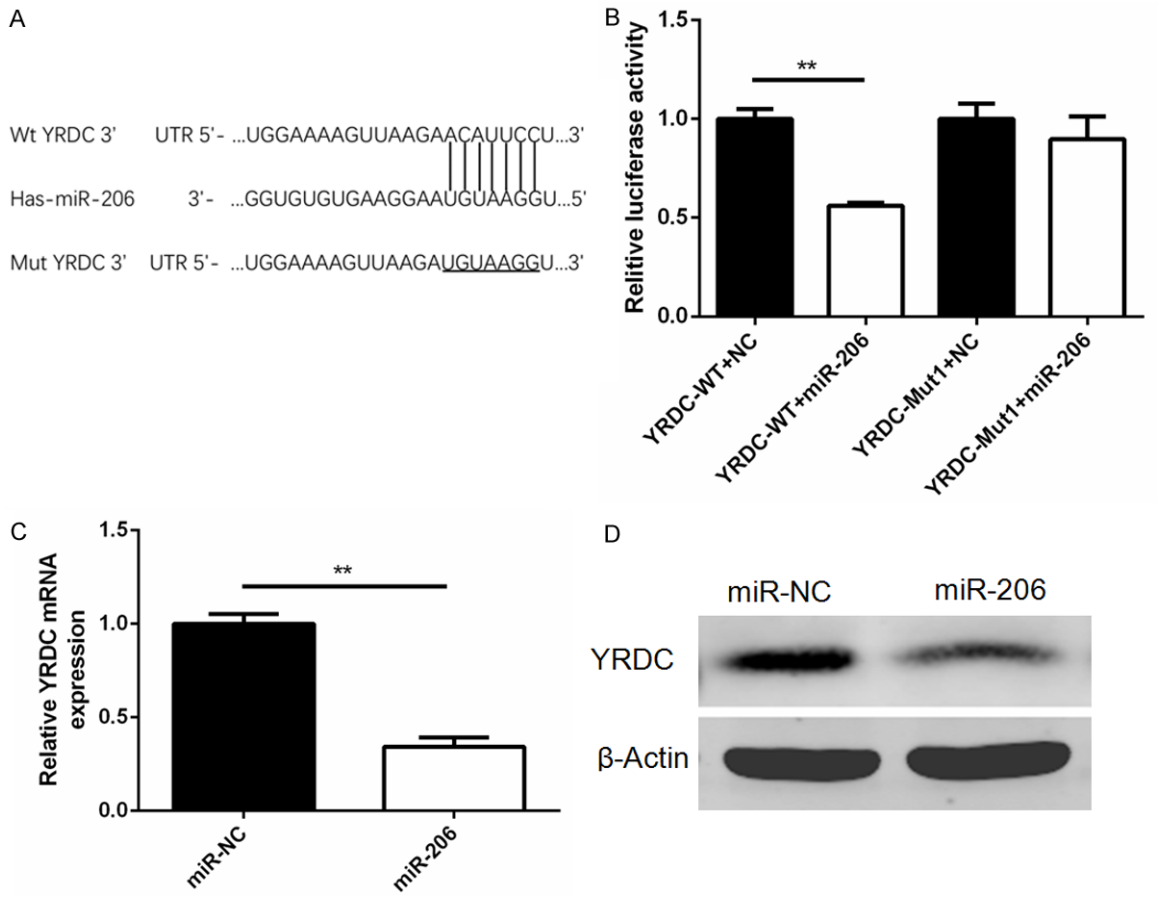


Figure 4. YRDC was a target of miR-206. A. Schematic construction of wild-type (WT) and mutant (Mut) 3'-UTR of YRDC were shown. B. The luciferase activities of WT and mutant YRDC 3'-UTR reporter constructs in the presence of miR-206 or miR-NC were determined in 293T cells. C. Relative YRDC mRNA expression was measured in T24 cells transfected with miR-206 mimic or miR-NC by qRT-PCR. D. YRDC protein expression was detected in T24 cells transfected with miR-206 mimic or miR-NC by western blot, respectively. β-actin was used as internal control. $^{**}P<0.01$ vs miR-NC.

ther investigate the possible mechanism underlying the cell growth inhibition effect by upregulation of miR-206, cell cycle analysis was performed. The results showed that overexpression of miR-206 induced G0/G1 arrest and decreased the percentage of cells in S phase compared to negative control (**Figure 2D**).

miR-206 inhibits cell migration and invasion in bladder cancer cells

To determine the effect of miR-206 on metastasis of bladder cancer cells, T24 cells were transfected with miR-206 or miR-NC, then wound healing and transwell invasion assay were performed in indicated time. The results showed that overexpression of miR-206 markedly inhibited migration (**Figure 3A**) and invasion (**Figure 3B**) of T24 cells.

YRDC is a target of miR-206 in bladder cancer cells

Three bioinformatics soft (TargetScan, miRTarBase and miRanda) were used to identify the potential downstream target of miR-206, and we found that there was a miR-206 binding site in YRDC 3'-UTR at position 249-255 (**Figure 4A**). To verify whether YRDC was a direct target of miR-206, luciferase reporter assay was performed. The results showed that overexpression of miR-206 dramatically reduced the wild-type YRDC reporter luciferase activity, while had no inhibition effect on the mutant-type YRDC reporter luciferase activity (**Figure 4B**). In addition, overexpression of miR-206 significantly decreased YRDC expression on mRNA level (**Figure 4C**) and protein level (**Figure 4D**) in T24 cells. These results indicated that YRDC is a direct target of miR-206.

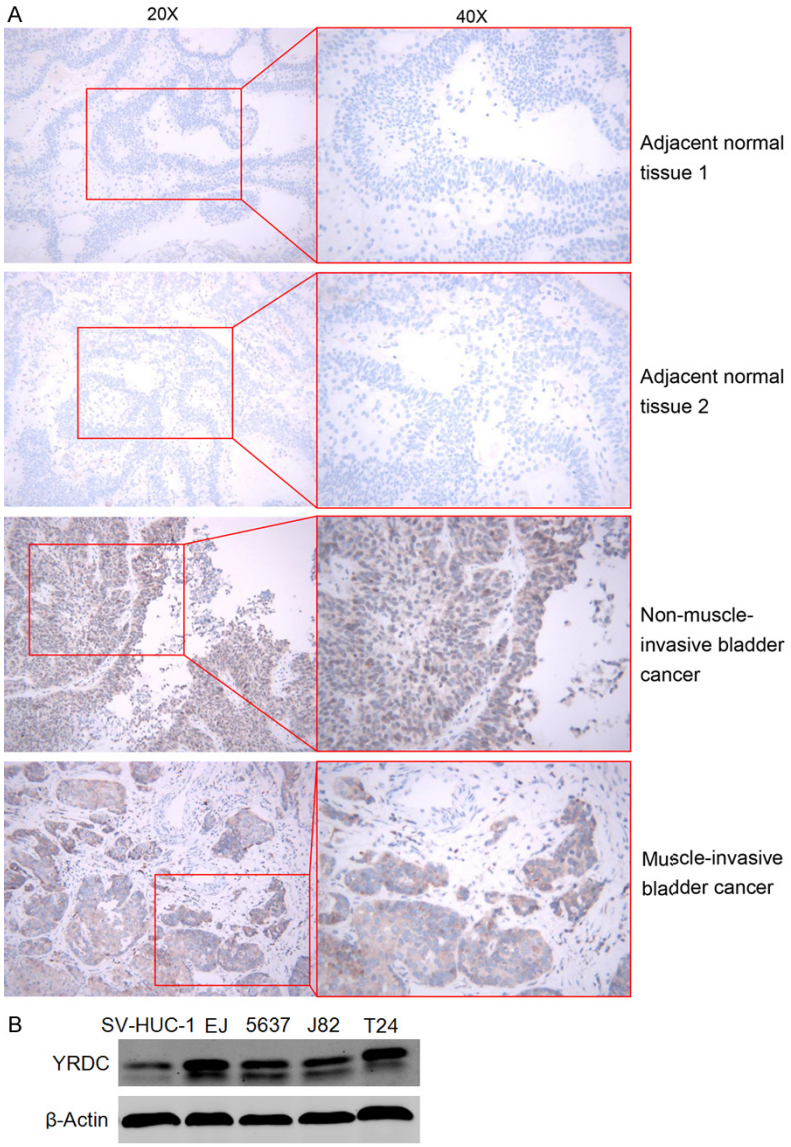


Figure 5. YRDC expression was upregulated in bladder cancer tissues and cell lines. A. Immunochemical analysis of the expression of YRDC in bladder cancer tissues and adjacent normal tissues. B. The expression of YRDC was detected in four bladder cancer cell lines and one normal human bladder transitional cell line by western blot. β-actin was used as internal control.

YRDC expression was upregulated in bladder cancer tissues and cell lines

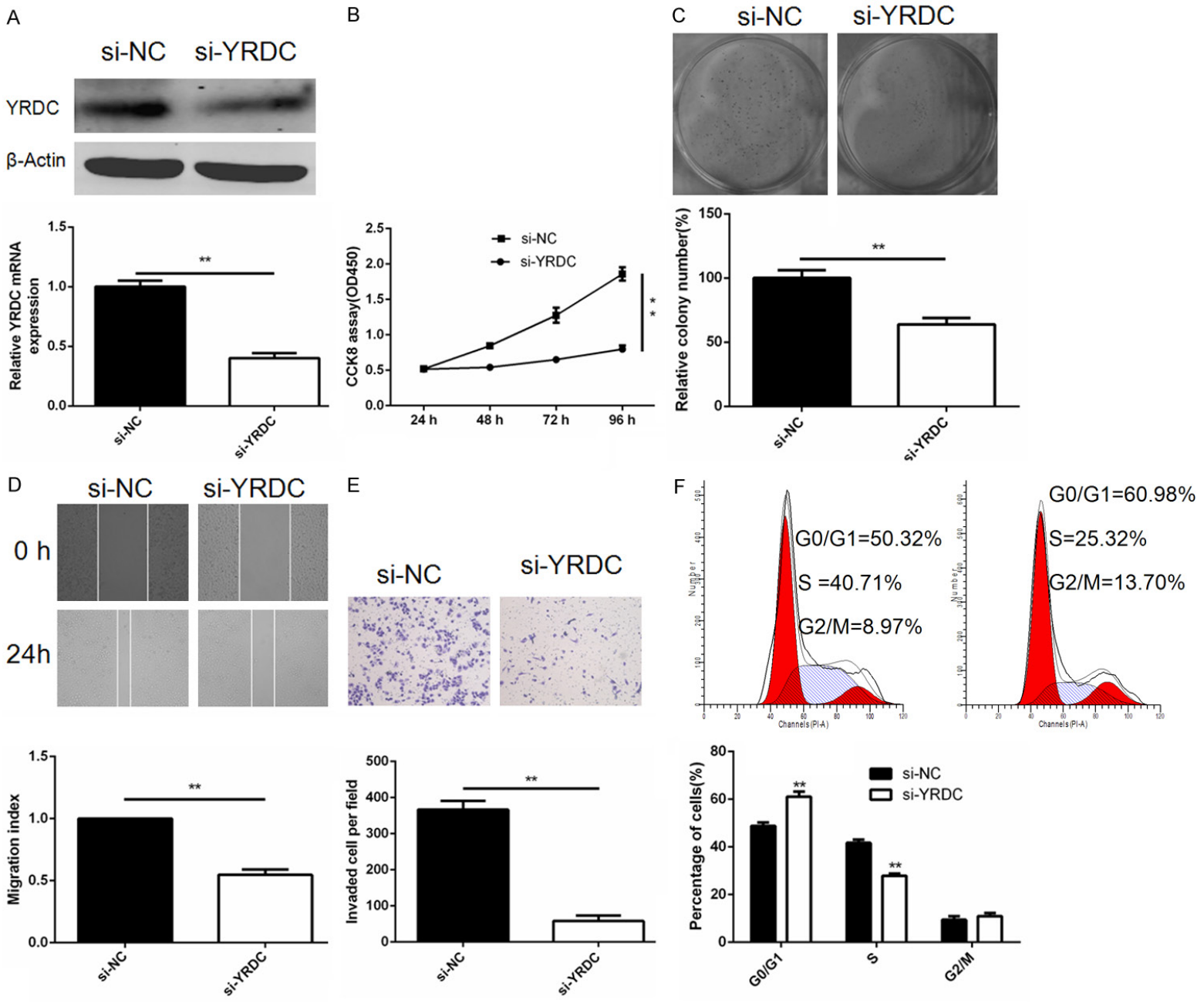
To determine the expression of YRDC protein in bladder cancer tissues, immunohistochemical analysis was performed in bladder cancer tissues and matched adjacent normal tissues. We found that there was a significant increase in YRDC expression in bladder cancer tissues compared with that in adjacent normal tissues (**Figure 5A**). In addition, we detected the expression of YRDC protein in bladder cancer cell lines by western blot. Compared with human bladder epithelial immortalized SV-HUC-1 cell line, significantly decreased YRDC levels were detected in four bladder cancer cell lines (**Figure 5B**).

Inhibition of YRDC exhibited similar effect with miR-206 overexpression in T24 cells

To investigate the biological function of YRDC in bladder cancer cells, T24 cells were transfected with si-YRDC or si-NC, and found that knockdown of YRDC by si-YRDC significantly decreased the YRDC expression on mRNA level and protein level (**Figure 6A**) in T24 cells. We also found that downregulation of YRDC in T24 cells significantly inhibited cell proliferation, colony formation, migration, migration, invasion, and induced cell cycle arrest at G0/G1 phase (**Figure 6B-F**). These results indicated that inhibition of YRDC exerts similar effect with miR-206 overexpression in bladder cancer cells.

Restoration of YRDC rescues the effects of miR-206 in bladder cancer cells

To better verify YRDC was a direct target of miR-206, T24 cells were simultaneously co-transfected with miR-206 mimic or miR-NC and overexpression of YRDC plasmids. We found that overexpression of miR-206 decreased the YRDC expression, while co-transfection of YRDC-overexpression plasmid significantly restored the YRDC expression on both mRNA level and protein level (**Figure 7A**) in T24 cells. In addition, we also found that restoration of YRDC in T24 cells could partially reverse the


A
si-NC
si-YRDC
YRDC
β-Actin
Relative YRDC mRNA expression
si-NC
si-YRDC
B
si-NC
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CCK8 assay(OD450)
24 h
48 h
72 h
96 h
C
si-NC
si-YRDC
Relative colony number(%)
si-NC
si-YRDC
D
si-NC
si-YRDC
0 h
24h
Migration index
si-NC
si-YRDC
E
si-NC
si-YRDC
Invaded cell per field
si-NC
si-YRDC
F
G0/G1=50.32%
S =40.71%
G2/M=8.97%
G0/G1=60.98%
S=25.32%
G2/M=13.70%
Channels (PI-A)
Percentage of cells(%)
si-NC
si-YRDC
G0/G1
S
G2/M

Figure 6. Inhibition of YRDC exhibited similar effect with miR-206 overexpression in human bladder cancer cells. A. YRDC expression was measured in T24 cells transfected with si-YRDC or si-NC using western blot and qRT-PCR. β-actin was used as internal control. B-F. Cell proliferation, colony formation, migration, invasion and cell cycle distribution was determined in T24 cells transfected with si-YRDC or si-NC. $^{**}P<0.01$ vs si-NC.

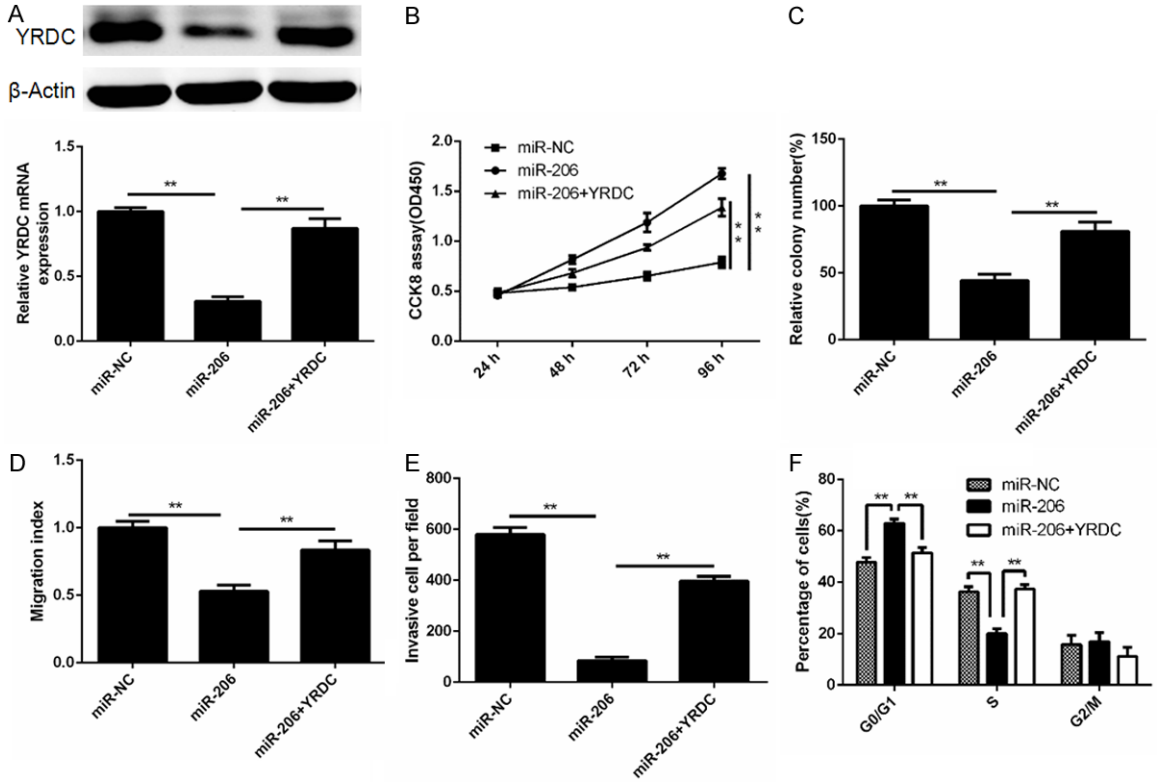

Figure 7. Restoration of YRDC rescues the effects of miR-206 in bladder cancer cells. A. YRDC expression on mRNA level and protein level was determined in T24 cells co-transfected with YRDC over expression plasmid and miR-206 mimic or miR-NC by qRT-PCR and western blot, respectively. β-actin was used as internal control. B-F. Cell proliferation, colony formation, migration, invasion and cell cycle distribution were determined in T24 cells co-transfected with YRDC over expression plasmid and miR-206 mimic or miR-NC. $^{**}P<0.01$ vs miR-206.

suppressive effect of miR-206 on cell proliferation, colony formation, migration, invasion, migration and cell cycle (**Figure 7B-F**). These data indicated that miR-206 exerts tumor suppressive effects on bladder cancer cells partially through inhibition of YRDC expression.

Discussion

Accumulating evidence showed that aberrant expression of miRNAs contributes to initiation and progression of human cancer, including bladder cancer [14, 15]. To date, numerous miRNAs have been identified to be involved in the development and metastasis of bladder cancer. For instance, miR-497 could significantly inhibit bladder cancer cell proliferation, migration, migration and invasion by targeting E2F3 [16]. miR-193a-3p regulated the multidrug resistance of bladder cancer by targeting LOXL4, PSEN1, HOXC9 or ING5 and activated DNA damage response pathway [17-20]. Overexpression of miR-429 reversed epithelial-mesenchymal transition in bladder cancer by restoring the expression of E-cadherin [21]. Here, we found that miR-206 expression was downregulated in bladder cancer tissues and cell lines which is consistent with the previous report that miR-206 was down-regulated in bladder cancer tissues [13]. In addition, we also found that overexpression of miR-206 inhibited cell proliferation, colony formation, migration, invasion and induced cell cycle arrest at G0/G1 phase by targeting YRDC. These results indicated that miR-206 may function as a tumor suppressor in bladder cancer.

miR-206 has been reported to downregulated and to act as a tumor suppressor in many human cancers, including clear cell renal cell carcinoma [22], breast cancer [10], colorectal cancer [12] and hepatocellular carcinoma [23], lung adenocarcinoma [9]. The results showed that it can inhibit cell proliferation of clear cell renal carcinoma by targeting VEGFA [24], cell migration of breast cancer via targeting PFKFB3 [25], induce colorectal cancer cell cycle arrest at G1/G0 phase by targeting FMNL2 [12], and reverse cisplatin resistance and EMT of lung adenocarcinoma cells by targeting MET [26]. To understand the possible molecular mechanism of the tumor suppressor of miR-206 in bladder cancer, we used three bioinformatics tools (TargetScan, miRTarBase and miRanda) to identify the target of miR-206 and we found that YRDC was a potential target of miR-206.

YRDC has been reported to function as oncogene promoting cell proliferation in colon cancer [27] and gastric carcinoma [28]. To test whether miR-206 inhibits proliferation, colony formation, migration, invasion and induced cell cycle arrest at G0/G1 phase via targeting YRDC, the expression of YRDC was knocked down using specific siRNAs. We found that si-YRDC could simulate the suppressive effects of miR-206 overexpression on bladder cancer cell. Luciferase reporter assay and rescue experiment were employed to further confirmed that YRDC is a direct target of miR-206.

Taken together, our results revealed that miR-206 acts as a tumor suppressor by targeting YRDC in bladder cancer, it might be a novel target for bladder cancer therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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