



MicroRNA-200c affects bladder cancer angiogenesis by regulating the Akt2/mTOR/HIF-1 axis

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Background: Bladder cancer is one of the most frequent urologic tumours in the world. MicroRNA-200c (miR-200c) has been considered a regulator of tumour angiogenesis. Akt2/mTOR was considered a regulator of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 α (HIF-1 α). However, the mechanism by which miR-200c regulates bladder cancer angiogenesis remains unknown.

Methods: Western blotting and qRT-PCR were used to detect the expression of protein and mRNA, respectively. Cell proliferation, migration and invasion were detected using MTT, wound-healing and transwell assays, respectively. A dual luciferase reporter assay was used to identify the binding site between miR-200c and Akt2. A tube formation assay was also applied to detect the angiogenesis ability.

Results: Significantly higher expression levels of HIF-1 α and VEGF and lower levels of miR-200c were observed in three types of bladder cancer cell lines. Transfection with the miR-200c mimic markedly inhibited cell viability, angiogenesis, and the expression of VEGF and HIF-1 α . Overexpression of miR-200c remarkably suppressed the expression of Akt2, and the binding site between them was identified. Knockdown of Akt2 remarkably decreased the expression of VEGF and HIF-1 α by regulating mTOR. miR-200c influenced the expression of VEGF and HIF-1 α through the Akt2/mTOR signalling pathway and further regulated angiogenesis in bladder cancer cells.

Conclusions: We proved that miR-200c could suppress HIF-1 α /VEGF expression in bladder cancer cells and inhibit angiogenesis, and these regulations were achieved by targeting Akt2/mTOR. This study may provide new insight into the prevention and treatment of bladder cancer.

Keywords: MicroRNA-200c; bladder cancer; angiogenesis; Akt2/mTOR/HIF-1 α

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Introduction

Bladder cancer is one of the most common malignant tumours in the urinary system and has a complex pathogenesis (1). At present, the clinical incidence of bladder cancer is increasing every year (2). Bladder cancer, like other cancers, is associated with a substantial amount of angiogenesis during the growth of tumours (3). Clinical studies have shown that inhibiting angiogenesis can effectively inhibit the growth of tumours (4).

MicroRNAs (miRNAs) are small non-coding RNAs that mediate related target genes by binding to the 3' untranslated regions of the protein-coding transcripts (5). It was reported that they play an important role in tumorigenesis, progression and migration of tumours (6,7). MicroRNA-200c (miR-200c) has been considered a regulator during the progression of endometrial (8) and pancreatic cancer (9). Meanwhile, miR-200c could regulate the EMT process and further suppress the invasion and proliferation of bladder

cancer cells (10). We previously found that microRNA-200c (miR-200c) is differentially expressed in bladder cancer. Meanwhile, it is associated with the deterioration of bladder cancer (5,11) and is also involved in angiogenesis (9). However, the mechanism of its involvement in the angiogenesis of bladder cancer is unclear.

Akt, a serine/threonine kinase, plays a key role in tumorigenesis, and Akt2, a pro-survival protein, is a member of the Akt family (12,13). According to database queries and literature reports, Akt2 is viewed as a direct target of miR-200c (11,14). Akt2/mTOR has been considered an important pathway influencing the angiogenesis progression of cancers (15,16). Meanwhile, Akt2/mTOR can regulate the level of vascular endothelial growth factor (VEGF) (17), and miR-200c may regulate the angiogenesis of bladder cancer by targeting Akt2. It was demonstrated that the hypoxia-inducible factor 1 α (HIF-1 α)/VEGF signalling pathway plays a key role in tumour angiogenesis (18,19), and Akt/mTOR could influence the expression of HIF-1 α (20,21). However, the modulation of Akt/mTOR in bladder cancer has not been reported.

In this study, we found significantly lower expression of miR-200c in bladder cancer cells compared with control cells. The upregulation of miR-200c could inhibit Akt2 and influence the Akt2/mTOR pathway, resulting in the suppression of the HIF-1 α /VEGF axis. Therefore, miR-200c may inhibit the proliferation of bladder cancer cells and angiogenesis through the method described above, and the present study may provide novel insight for the prevention and treatment of bladder cancer.

Methods

Cell culture

In this study, the SV-HUC-1 (human bladder epithelial cells), 5637 (human bladder cancer cells), T24 (human bladder cancer cells), and MB49 (mouse bladder cancer cells) cell lines were obtained from American Type Culture Collection (Rockville, MA, USA). The cells were cultured normally in DMEM (Gibco, USA) with 10% FBS (Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO₂. The reagents Rapamycin (activator of mTOR) and MHY1485 (inhibitor of mTOR) used to treat cells were purchased from Sigma (St. Louis, USA).

Cell transfection

The miR-200c mimic, miR-200c inhibitor, pcDNA-Akt2,

and sh-Akt2 were designed and synthesized by GenePharma Co., Ltd (Shanghai, China). Cells were seeded on 60-mm dishes and cultured for 24 h. After 70% cell confluence was achieved, cell transfection and co-transfection were applied using Lipofectamine 2000 (Invitrogen). Lipofectamine 2000 suspension was prepared with 5 μ L of Lipofectamine 2000 and 250 μ L of Opti-MEM. After incubation with Lipofectamine 2000 suspension for 6 h at 37 °C in a humidified atmosphere containing 5% CO₂, the transfection efficiency was measured, and the medium was replaced. Cells were collected after 48 h of incubation at 37 °C and 5% CO₂.

mRNA extraction and quantitative real-time PCR (qRT-PCR)

First, RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, USA). A total of 500 ng of RNA was reverse-transcribed into cDNA using the Primer Script RT reagent kit (Takara Bio, China). Real-time PCR was performed using the SYBR Premix Ex Taq™ II kit (Takara Bio, China). Amplification conditions were conducted as follows: 95 °C for 10 min, followed by 45 cycles consisting of 95 °C for 15 s, 60 °C for 30 s and 68 °C for 60 s. The relative mRNA level was analysed by the 2^{- $\Delta\Delta$ Ct} method. The primers used for miR-200c, HIF-1 α , VEGF, and Akt2 were as follows: mus-miR-200c-forward 5'-GCCCCGCTAATACTGCCGGGTAAT-3', reverse 5'-GTGCAGGGTCCGAGGT-3'; mus-HIF-1 α -forward 5'-GTCCCAGCTACGAAGTTACAGC-3', reverse 5'-CAGTGCAGGATACACAAGGTTT-3'; mus-VEGF-forward 5'-GCACCCACGACAGAAGGA-3', reverse 5'-GCTTCGCTGGTAGACATCCAT-3'; hsa-miR-200c-forward 5'-TAATACTGCCGGGTAATGATGGA-3', reverse 5'-TGGTGTCGTGGAGTCG-3'; hsa-HIF-1 α -forward 5'-TCAAAGTCGGACAGCCTCA-3', reverse 5'-CCCTGCAGTAGGTTTCTGCT-3'; hsa-VEGF-forward 5'-GAAGTGGTGAAGTTCATGGATGTC-3', reverse 5'-CGATCGTTCTGTATCAGTCTTTCC-3'; Akt2-forward 5'-GAGGTCATGGAGCACAGGTT-3', reverse 5'-CTGGTCCAGCTCCAGTAAGC'; mus-U6-forward: 5'-CTCGCTTCGGCAGCACACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; hsa-U-forward: 5'-CTTCGGCAGCACATATAC-3', reverse: 5'-GAACGCTTCACGAATTTGC-3'; mus- β -actin-forward: 5'-GGCTGTATCCCCCTCCATC-3', reverse: 5'-ATGCCATGTTCAATGGGGTA-3'; hsa- β -actin-forward: 5'-TGGCACCCAGCACAAATGAAGA-3', reverse: 5'-GAAGCATTTGCGGTGGACGAT-3'. Every sample

was replicated 3 times with no RT and no template controls. Data were analysed by comparing cycle threshold values. The relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. $\Delta\Delta Ct = \Delta Ct_{\text{experiment}} - \Delta Ct_{\text{control}}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{control gene}}$. The fold change between the experimental group and the control group = $2^{-\Delta\Delta Ct}$.

Western blot analysis

Primary antibodies used in this study, including HIF-1 α , VEGF, Akt2, mTOR, and p-mTOR, were obtained from Abcam (Hong Kong, China). Cells were collected and lysed in RIPA buffer (Sigma-Aldrich, USA). Protein concentrations were measured with the BCA protein assay kit (Thermo Fisher Scientific, USA). Equal amounts of protein were loaded on an SDS-PAGE gel, and then the proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Skimmed milk (5%) in TBST was used for blocking, and then the membrane was incubated overnight at 4 °C with primary antibody (1:1000). After washing and incubation, the membrane was incubated in TBST with secondary antibody (1:2000). Immunodetection was performed using the ECL Plus detection system (Sigma, USA) according to the manufacturer's instructions. The grey of the protein bands was measured using ImageJ software.

MTT assay

Cells were collected 48 h after transfection and seeded in 96-well plates at a concentration of 5×10^4 cells per well. MTT assay was applied for measuring cell proliferation after treatments with NC, miR-200c mimic, and miR-200c inhibitor. Briefly, 20 μ L of MTT reagent was added to the cells. After 3 h of incubation, the supernatant was removed, and 200 μ L of DMSO was added. The optical density of each well at 450 nm was detected after a 2-h incubation. Each assay was performed in triplicate.

Dual luciferase reporter assay

The binding site between miR-200c and AKT2 was predicted using Targetscan (http://www.targetscan.org/vert_71/). The 3'-untranslated region (UTR) of the AKT2 gene including the miR-200c binding target was amplified by PCR. The PCR amplification product of the AKT2 3'UTR was subcloned into the plasmid PGL3 vector (Promega, Madison, USA). The successfully identified vector was named AKT2-WT. The vector

constructed from the mutant binding site sequence was named AKT2-MUT. To verify the binding site between the AKT2 and miR-200c, 5,637 cells in 96-well plates were co-transformed with miR-NC or miR-200c mimic and AKT2-WT or AKT2-MUT using Lipofectamine 2000 (Invitrogen, United States). After 36 h, the dual-luciferase reporter gene (firefly and Renilla) was measured. Firefly luciferase activity was normalized against Renilla luciferase activity.

Tubule formation assay

A tube formation assay was conducted as follows. Briefly, the 96-well plates were coated with 100 μ L of Matrigel (BD Biosciences, USA) per well and maintained at 37 °C for 45 min. Then, the cells were seeded into wells that were re-suspended in 150 μ L of complete DMEM at a concentration of 5×10^5 /mL and cultured for 24 h at 37 °C. Pictures were captured under the microscope in bright field at 100 \times magnification. The number of tube branches and junctions were measured in three fields (4 \times) using ChemiImager 5500 V2.03 software (Alpha Innotech, USA). The average was calculated by counting the branch number in three fields.

Transwell assay

Cell activity was measured through polycarbonate membrane Boyden chambers in a transwell apparatus (Costar, USA). Cells were first suspended in serum-free medium. Then, the suspended cells (10^5) were added to the top chamber. The lower chamber was filled with 1 mL of DMEM containing 10% FBS. The cells on the top surface of the membrane were removed by a cotton swab after incubation (24 h, 37 °C). The migrated cells on the bottom surface of the membrane were fixed with methanol (100%) and stained with crystal violet (0.5%) for 2 min. Then, the number of invasive cells was counted. The values for this experiment were obtained after counting three different fields in each membrane.

Wound-healing assay

After different treatments, cells were plated in a 6-well plate and cultured at 37 °C. When the cells grew to 70% confluence, a wound was conducted along the centre of every well by a sterile 1-mL pipette tip. Images were acquired after different incubation times (0 h, 24 h) at 37 °C (0 h) with an inverted microscope (Olympus CKX31, Japan). Six fields for each group were chosen, and the cells

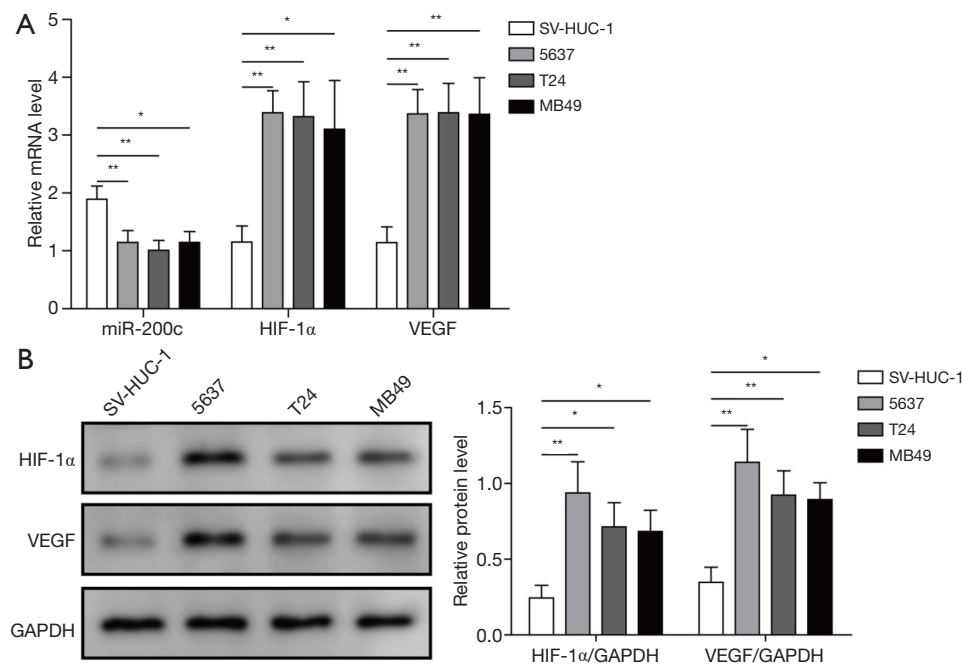


Figure 1 Expression of VEGF and HIF-1 α in bladder cancer cells. The expression of miR-200c, HIF-1 α , and VEGF in SV-HUC-1, 5637, T24, and MB49, as measured by qPCR (A). The expression of HIF-1 α , and VEGF in SV-HUC-1, 5637, T24, and MB49, as measured by western blotting (B). Values are displayed as the mean \pm SD from 3 independent experiments. * $P < 0.05$ or ** $P < 0.01$, compared with SV-HUC-1.

in the wound area were counted and analysed by ImageJ software.

Statistical analysis

Assays were performed in at least three independent experiments with one representative picture presented. Data are presented as the mean \pm standard deviation (SD). Statistical analysis was conducted using SPSS 24.0. A two-tailed Student's t-test was applied to assess the statistical significance of the difference between two independent groups. P values < 0.05 were considered statistically significant.

Results

Expression of HIF-1 α and VEGF in bladder cancer cells

We measured the mRNA expression of miR-200c, VEGF, and HIF-1 α in SV-HUC-1, 5637, T24, and MB49 cells by qPCR, and the protein expression of VEGF and HIF-1 α were also detected using western blotting. The results indicated that both the mRNA and protein expression of HIF-1 α and VEGF in bladder cancer cells (5637, T24, and MB49) was significantly higher than that in SV-HUC-1,

a type of normal bladder epithelial cell line (Figure 1). However, the mRNA expression of miR-200c was markedly downregulated in bladder cancer cells compared with normal bladder epithelial cells (Figures 1A). These findings suggest that the expression of miR-200c in bladder cancer may be associated with abnormal expression of angiogenesis-related proteins.

miR-200c affects bladder cancer cells by inhibiting angiogenesis

Two bladder cancer cell lines, T24 and 5637, were chosen to study the influence of miR-200c on angiogenesis in bladder cancer cells. The mRNA expression of miR-200c in both cell lines was significantly higher after transfecting miR-200c mimic compared with the control group, and transfection with miR-200c inhibitor notably reduced the level of miR-200c (Figure 2A), indicating effective transfection in bladder cancer cell lines. Cell viability was measured after treatments with NC, miR-200c mimic, and miR-200c inhibitor. The results indicated that the cell viability of bladder cancer cells, 5637 and T24, increased markedly after miR-200c inhibition, but transfection with miR-200c mimic markedly reduced the proliferation

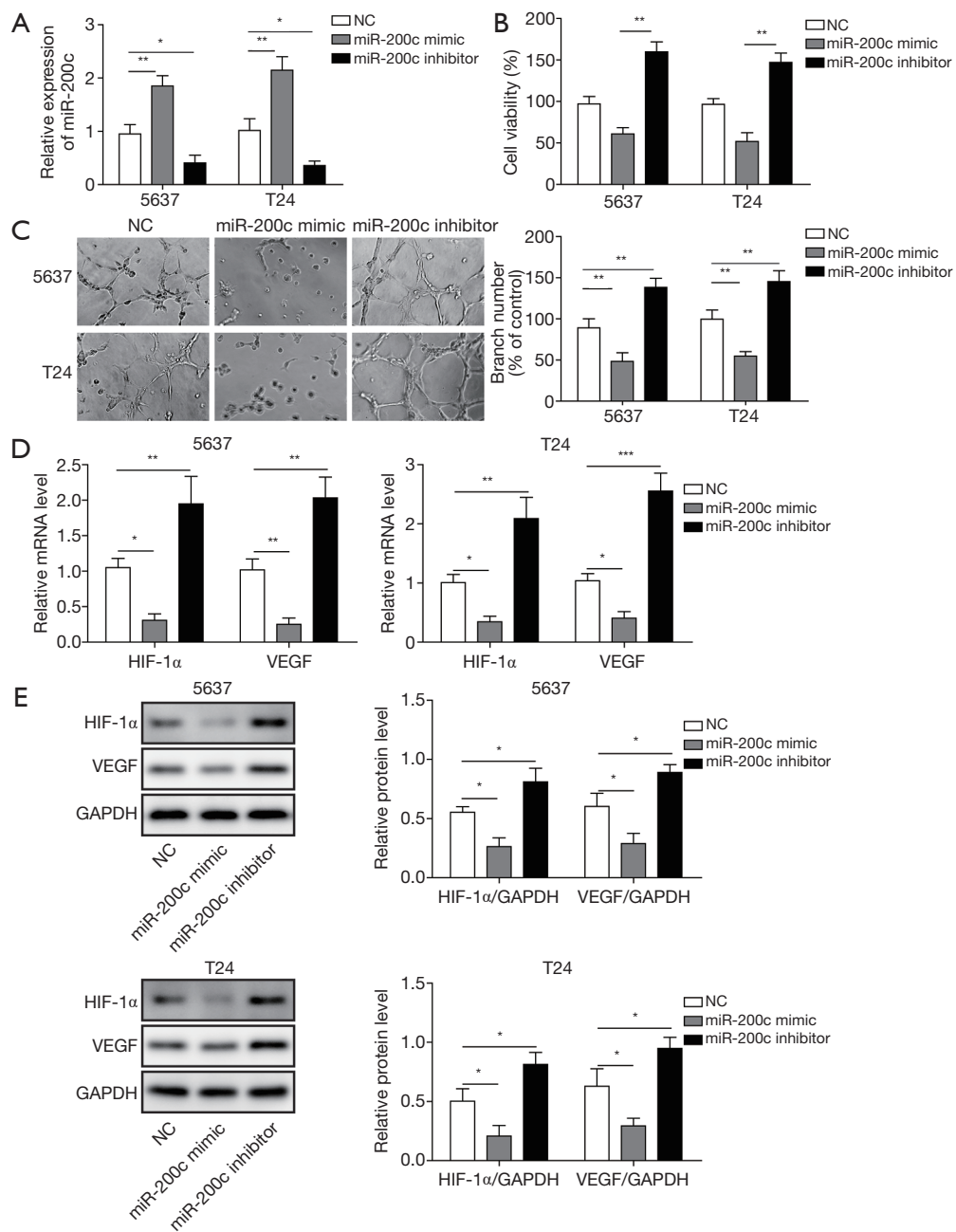


Figure 2 miR-200c affects bladder cancer cells by inhibiting angiogenesis. Transfection with miR-200c mimic or miR-200c inhibitor in 5637 and T24 cell lines (A); Measurement of cell viability after transfection with miR-200c mimic or miR-200c inhibitor (B); The angiogenesis was measured through tube formation assay after transfection with miR-200c inhibitor or miR-200c mimic (100x) (C); mRNA measurement of HIF-1α and VEGF by qPCR after transfecting miR-200c mimic or miR-200c inhibitor (D); Protein measurement of HIF-1α and VEGF by western blotting after transfecting miR-200c mimic or miR-200c inhibitor (E). Values are displayed as the mean ± SD from 3 independent experiments. *P<0.05 or **P<0.01, ***P<0.001 compared with the blank control and NC.

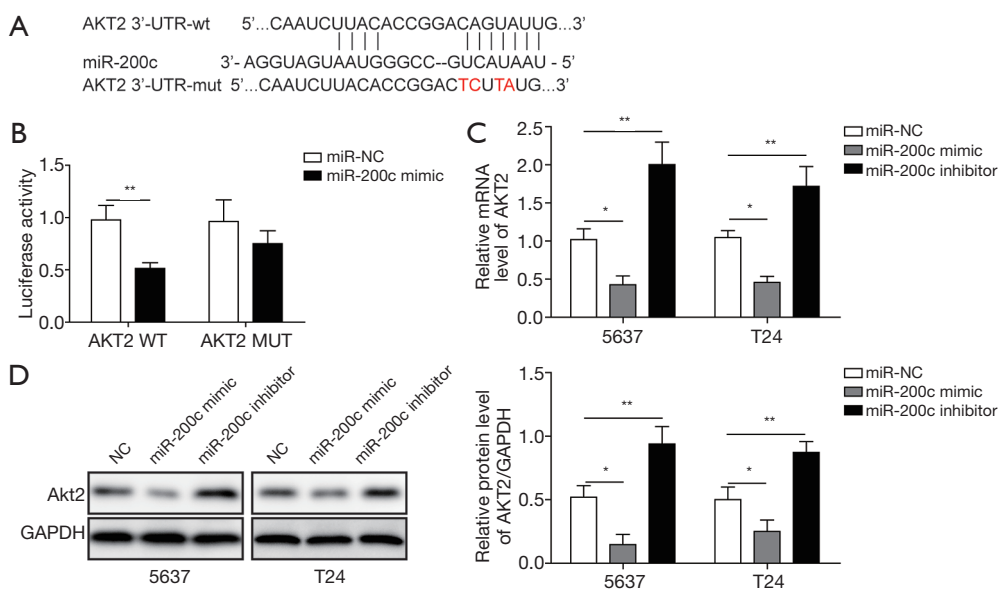


Figure 3 miR-200c affects the expression of Akt2. Binding sequence between miR-200c and the 3'-UTR of Akt2 transcripts (A); The dual luciferase activity assay showed binding affinity between miR-200c and Akt2 (B); mRNA measurement of Akt2 by qPCR after transfecting miR-200c mimic or miR-200c inhibitor (D); Protein measurement of Akt2 by western blotting after transfecting miR-200c mimic or miR-200c inhibitor (E). Values are displayed as the mean \pm SD from 3 independent experiments. * $P < 0.05$ or ** $P < 0.01$, compared with the blank control and NC.

ability of bladder cancer cells (5637 and T24) (Figure 2B). We further performed a tubule formation test to detect the influence of different treatments on angiogenesis ability. After calculating the branch number, we found that transfecting the miR-200c mimic markedly inhibited angiogenesis (Figure 2C), but transfection with the miR-200c inhibitor produced the opposite effect. We also determined that transfection with miR-200c inhibitor significantly upregulated the protein and mRNA expression of HIF-1 α and VEGF, but miR-200c mimic markedly reduced their levels (Figure 2D,E). Therefore, miR-200c can negatively regulate angiogenesis in bladder cancer and further negatively regulate the expression of angiogenesis-related proteins, such as HIF-1 α and VEGF.

miR-200c affects the expression of Akt2

Akt2 was considered a downstream target of miR-200c, and a luciferase assay was used to unfold the binding site between them. We predicted the binding sites between miR-200c and WT-Akt2 by bioinformatics. The wild-type 3'-UTR of Akt2 (WT-Akt2) contained the predicted miR-200c target sites, and the mutant-type 3'-UTR of Akt2 (MUT-Akt2) lacked the miR-200c binding sites.

WT-Akt2 and MUT-Akt2 were constructed (Figure 3A). Subsequently, we validated the binding site between miR-200c and Akt2 using a dual luciferase reporter assay. The findings suggested that miR-200c mimics significantly suppressed the luciferase activity of the reporter gene, which contained the 3'-UTR of Akt2, compared with the miR-NC group. The suppression was stopped when miR-200c binding sites were absent in the MUT-Akt2 group (Figure 3B). The expression of Akt2 was measured by qPCR and western blotting after transfection with miR-200c mimic or miR-200c inhibitor. We found that overexpression of miR-200c induced by transfecting miR-200c mimic markedly suppressed the mRNA and protein expression of Akt2 compared with the controls (Figure 3C,D). However, transfection with miR-200c inhibitor markedly upregulated the level of Akt2 (Figure 3C,D). Therefore, we believe that Akt2 is a target of miR-200c in bladder cancer and that miR-200c can modulate the expression of Akt2.

Akt2 influences the expression of HIF-1 α /VEGF through mTOR

Additionally, we further investigated if Akt2 could influence HIF-1 α /VEGF through mTOR. First, we transfected

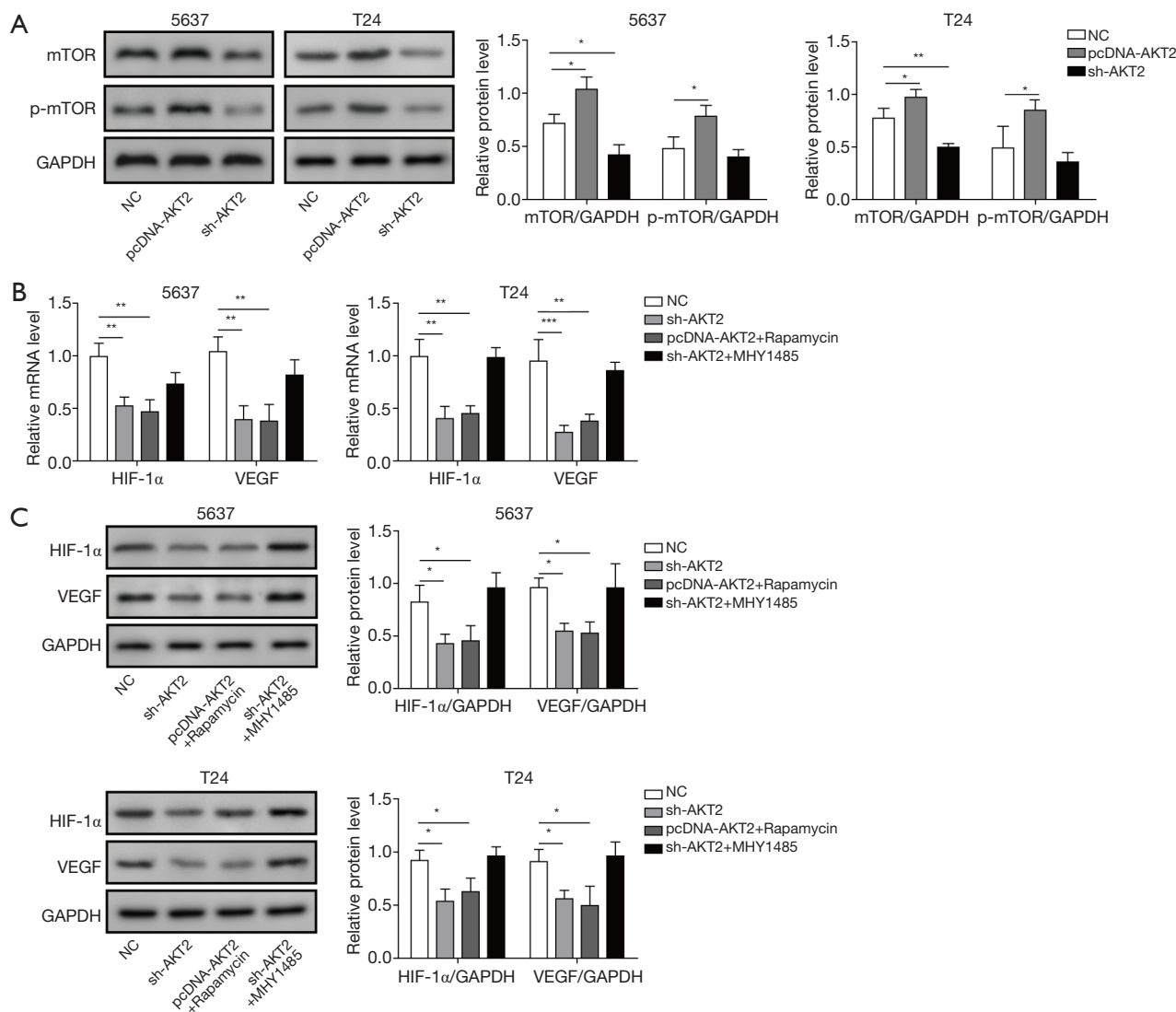


Figure 4 Akt2 influences the expression of HIF-1α/VEGF through mTOR. Protein measurement of mTOR and p-mTOR by western blotting after transfecting pcDNA-Akt2 and sh-Akt2 (A); mRNA measurement of VEGF and HIF-1α by qPCR after treatment with sh-Akt2, pcDNA-Akt2 + Rapamycin, or sh-Akt2 + MHY1485 (B); Protein measurement of VEGF and HIF-1α by western blotting after treatment with sh-Akt2, pcDNA-Akt2 + Rapamycin, or sh-Akt2 + MHY1485 (C). Values are displayed as the mean ± SD from 3 independent experiments. *P<0.05 or **P<0.01, ***P<0.001 compared with the blank control and NC.

pcDNA-Akt2 and sh-Akt2 in both the 5627 and T24 cell lines and proved that overexpression of Akt2 significantly increased the expression of mTOR and p-mTOR, but sh-Akt2 exerted the opposite effect (Figure 4A). Therefore, Akt2 could influence the expression of mTOR. Then, we treated both cell lines with sh-Akt2, pcDNA-Akt2 + Rapamycin, and sh-Akt2 + MHY1485 and measured the protein and mRNA levels of HIF-1α and VEGF. Rapamycin is the specific inhibitor of mTOR, and MHY1485 is

the activator of mTOR. The expression of mTOR was markedly decreased after treatment with sh-Akt2 (Figure 4A). Compared with group NC, the protein and mRNA expression of HIF-1α/VEGF were remarkably decreased in sh-Akt2 and pcDNA-Akt2 + Rapamycin. However, no significant difference was observed between group sh-Akt2 + MHY1485 and group NC (Figure 4B,C). These findings indicate that Akt2 might influence the expression of VEGF and HIF-1α through mTOR.

miR-200c affects angiogenesis by regulating Akt2/mTOR/HIF-1 α in bladder cancer cells

To determine whether miR-200c influences the expression of HIF-1 α and VEGF by regulating Akt2/mTOR, we treated two cell lines with miR-200c mimic, miR-200c mimic + pcDNA-Akt2, and miR-200c mimic + MHY1485. We proved that transfection with miR-200c mimic markedly decreased the expression of VEGF and HIF-1 α (Figure 5A,B), but overexpression of Akt2 or activation of mTOR could reverse the expression inhibition of HIF-1 α and VEGF induced by miR-200c (Figure 5A,B). These results indicate that miR-200c could influence the expression of VEGF and HIF-1 α through Akt2/mTOR.

Then, we investigated whether miR-200c influences the angiogenesis of HUVEC induced by bladder cancer through Akt2/mTOR. Compared with the inhibition effect of miR-200c mimic on branch numbers (Figure 5C), treatment with miR-200c mimic + pcDNA-Akt2 or miR-200c mimic+MHY1485 markedly promoted angiogenesis (Figure 5C). Therefore, miR-200c could influence angiogenesis through Akt2/mTOR. Additionally, we detected the influence of miR-200c/Akt2/mTOR on cell invasion and migration ability. The results indicated that transfection with miR-200c mimic markedly suppressed the invasion and migration of cells (Figure 5D,E), but miR-200c mimic + pcDNA-Akt2 or miR-200c mimic + MHY1485 significantly promoted migration and invasion of bladder cancer cells (Figure 5D,E), which was consistent with the results of both angiogenesis factor expression and angiogenesis tests. Therefore, miR-200c in bladder cancer affects the expression of HIF-1 α and VEGF by regulating Akt2 and further influences angiogenesis, and the migration and invasion of bladder cancer cells.

Discussion

Angiogenesis has been considered a key factor in the growth and progression of tumours (22,23), and angiogenesis inhibition has been viewed as an important therapeutic strategy for the prevention of tumour expansion and metastasis (24). It was reported that miRNAs can regulate angiogenesis by affecting the expression of angiogenesis-related genes (25,26). However, the specific mechanism of angiogenesis in bladder cancer remains unknown.

Some miRNAs have been demonstrated to be involved in the development of bladder cancer. Downregulation of miR-430 enhanced the development of bladder cancer by

upregulating C-X-C chemokine receptor type 7 (CXCR7) (27). miR-430 interacted with the X-inactive specific transcript (XIST) to modulate the growth, migration, and invasion of bladder cancer (28). Meanwhile, miRNAs participate in angiogenesis in many types of tumours (29). It was reported that miR-200c could modulate pancreatic cancer endothelial cell proliferation, migration, and angiogenesis and that it was closely related to the tumorigenesis of other cancers (9). Therefore, we investigated the influence of miR-200c on the angiogenesis of bladder cancer. We proved that the expression of miR-200c was downregulated in bladder cancer cells, and miR-200c can negatively regulate the expression of the angiogenesis-related proteins VEGF and HIF-1 α and then affect the process of tubule formation. Therefore, miR-200c regulates angiogenesis in bladder cancer and is a potential therapeutic target.

Subsequently, we further demonstrated that miR-200c regulated angiogenesis in bladder cancer through Akt2. Activation of the Akt pathway is frequently reported in tumorigenesis. Overexpression of Akt2 has been observed in prostate and breast cancer cells (30,31), and it could be regulated by several miRNAs, including miR-708, miR-203, and miR-200c (8). miR-200c plays a key role in osteosarcoma tumour growth and chemosensitivity by targeting Akt2 (32). In this study, we identified the binding site between miR-200c and Akt2 using a dual luciferase reporter assay, and we demonstrated that miR-200c influenced angiogenesis by negatively regulating Akt2. PI3K/AKT/mTOR pathway has been believed to be closely linked with bladder cancer (33), and PI3K could be activated by cytokine receptors through a tyrosine-kinase-dependent mechanism consisting of PI3KCA (PI3K- α) and PI3KCB (PI3K- β) (34). Meanwhile, it was reported that NMIBC (Non-muscle invasive bladder cancer) patients with a PI3KCA mutation could have a better prognosis in the recurrence and progression of several cancers, including bladder cancer (35,36). Therefore, whether patients with some alteration in PI3KCA have a better prognosis through Akt2/mTOR is a promising research point. PI3KCA could activate the PI3K pathway through AKT1 phosphorylation and then block the methylation that EZH2 exerts on its targets (37,38). EZH2 is a major molecule in the progression of bladder cancer and can regulate the levels of members of the miR-200 family, including miR-200c and miR-141 (39). Meanwhile, the EZH2/miR-200 axis was considered to regulate anti-tumour effects through STAT3 signalling, which has been believed to be closely linked with muscle invasive tumours (39). This evidence indicates that there

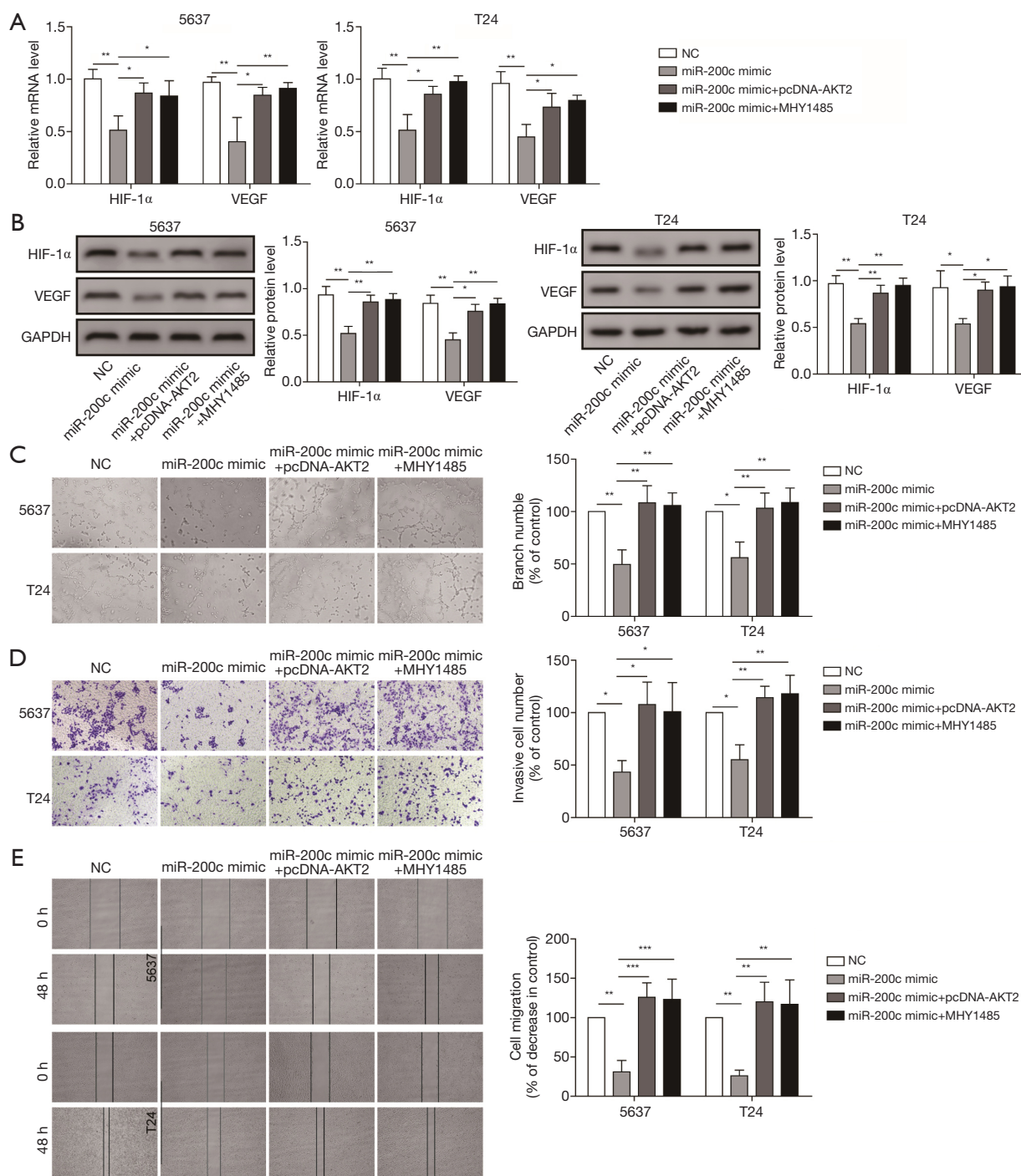


Figure 5 miR-200c affects angiogenesis by regulating Akt2/mTOR/HIF-1 α in bladder cancer cells. mRNA measurement of HIF-1 α and VEGF by qPCR after treatment with miR-200c mimic, miR-200c mimic + pcDNA-Akt2, or miR-200c mimic + MHY1485 (A); Protein measurement of HIF-1 α and VEGF by western blotting after treatment with miR-200c mimic, miR-200c mimic + pcDNA-Akt2, or miR-200c mimic + MHY1485 (B); The angiogenesis was measured using tube formation test after treatment with miR-200c mimic, miR-200c mimic + pcDNA-Akt2, or miR-200c mimic + MHY1485 (100 \times) (C); Measurement of cell invasion by transwell assay after treatment with miR-200c mimic, miR-200c mimic + pcDNA-Akt2, or miR-200c mimic + MHY1485, cells stained with crystal violet (100 \times) (D); Measurement of cell migration by wound healing assay after treatment with miR-200c mimic, miR-200c mimic + pcDNA-Akt2, or miR-200c mimic + MHY1485 (100 \times) (E). Values are displayed as the mean \pm SD from 3 independent experiments. *P<0.05 or **P<0.01, ***P<0.001 compared with the blank control and NC.

may be a relationship of mutual regulation between AKT2 and EZH2. Therefore, the Akt2/mTOR/HIF-1 α pathway may also be involved in the development of bladder cancer through the STAT3 pathway. Therefore, investigating the influence of Akt2/mTOR/HIF-1 α /STAT3 on the processes of drug resistance and intratumoural heterogeneity should be a promising research direction. Notch pathway has been viewed as a regulator of tumor angiogenesis, and activated STAT3 transfers from cytoplasm to nucleus and induces transcriptional activation of HIF-1 α (40). This study proved that HIF-1 α was up-regulated in bladder cancer cells, and the angiogenesis ability of bladder cancer cells was significantly higher in group HIF-1 α high expression, which is in line with previous study. Additionally, AKT2 is an important molecule of the insulin signalling pathway (41). Increased miR-200c expression in arteries from diabetic mice and patients with diabetes was observed, and miR-200c has been considered a new mediator of diabetic endothelial dysfunction (42). It has been found that miR-200c could mediate diabetic vascular dysfunction by up-regulating COX-2 expression (42). Therefore, this study may provide a novel treatment for bladder cancer patients combined with diabetes (type II), and AKT2 may also influence the muscle-invasive bladder carcinoma patients with diabetes (type II). The limitation of this study is that the cell lines we used were all invasive. Investigating other types of cell lines, such as high-risk or grade non-muscle-invasive cell lines, may help to confirm the conclusion of this study.

We explored the mechanism by which miR-200c influenced angiogenesis by regulating Akt2. The role of mTOR in tumour angiogenesis has been reported and related to the activity of the mTORC1 complex (43,44). It was reported that blocking mTOR with rapamycin inhibits tumour angiogenesis by suppressing VEGF (45) and HIF-1 α (46). Meanwhile, Akt2 could affect angiogenesis by regulating mTOR (47). In this study, we found that miR-200c could influence the expression of HIF-1 α and VEGF by regulating Akt2/mTOR and could affect tubule formation and cell migration. Therefore, miR-200c may regulate the angiogenesis of bladder cancer through this mechanism described above.

In the present study, we demonstrated *in vitro* that miR-200c could affect bladder cancer angiogenesis by regulating Akt2/mTOR/HIF-1 α . This study provides new insight into the progression, deterioration, and migration of bladder cancer. Meanwhile, therapeutic strategies targeting the Akt2/mTOR/HIF-1 α pathway may be a promising treatment for bladder cancer.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.10.23>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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