miR-200a-3p Facilitates Bladder Cancer Cell Proliferation by Targeting A20

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Research

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Abstract

Background

MicroRNAs (miRs) are endogenous, single-stranded, non-coding RNAs that are involved in various physiological processes, development and the progression of various types of cancer. The role of miR-200a-3p in various types of cancer has been previously reported. The present study aimed to investigate the expression levels of miR-200a-3p in human bladder cancer, as well as its potential role in disease pathogenesis.

Methods

In vitro: Agilent miRNA microarray and QPCR analysis of miR-200a-3p expression in bladder cancer. Gene overexpression and interference technology analysis of the effects of miR-200a-3p and de-ubiquituation enzyme TNFα induced protein 3 (A20) on the function of bladder cancer cells; dual luciferase technology for promoter efficiency analysis;

Animal experiments: Nude mice are used for tumor formation experiments, and the effects of genes on tumors are directly analyzed on animals;

Results

Compared with adjacent non-tumor tissues, miR-200a-3p expression levels were significantly upregulated in bladder cancer tissues. Moreover, increased miR-200a-3p expression was significantly associated with distant metastasis and advanced stage. In addition, compared with the miR-Negative control (NC) group, miR-200a-3p overexpression promoted bladder cancer cell proliferation, migration, invasion, cell cycle and release of inflammatory cytokines, but inhibited cell apoptosis. Mechanistically, de-ubiquituation enzyme TNFα induced protein 3 (A20) was identified as a target gene of miR-200a-3p in bladder cancer cell lines. An inverse association between miR-200a-3p expression and A20 expression in bladder cancer tissues and cell lines was also identified. A20 overexpression in miR-200a-3p-overexpression bladder cancer cells attenuated miR-200a-3p overexpression-mediated effects on cell proliferation, migration, apoptosis and cytokine production. Moreover, compared with the miR-NC group, miR-200a-3p overexpression significantly promoted tumor growth in vivo, and A20 overexpression blocked the promoting effect of miR-200a-3p on bladder cancer.

Conclusions

The results of the present study indicated that miR-200a-3p might serve as an oncogene in human bladder cancer by targeting a novel gene A20; therefore, miR-200a-3p and A20 might serve as novel therapeutic targets for bladder cancer.

Introduction
In the urinary system, bladder cancer is one of the most common malignant tumors[1]. Bladder cancer displays a higher morbidity rate in males, with a three to ten times higher occurrence rate in males compared with females[2]. Several risk factors are associated with bladder cancer, including industrial chemical contamination, bad diet habits and smoking[3]. Surgery remains the primary treatment strategy for bladder cancer; however, an increasing rate in its morbidity has been identified[4]. In addition, bladder cancer displays a high recurrence rate with high levels of invasion and metastasis, which directly lead to poor patient prognosis[5]. Therefore, novel treatments and potential molecular mechanisms underlying bladder cancer require further investigation.

MicroRNAs (miRNAs/miRs) are non-coding, short, single-stranded RNAs[6]. Target protein expression is commonly regulated by miRNAs via binding to the 3’untranslated region (UTR) of substrates[7–8]. Specific pathological features result in altered microRNA expression, which indicates oncogenic or antioncogenic properties. A number of studies have revealed that miRNAs could regulate various gene expression levels, which in turn regulates the cellular signaling pathway that is associated with controlling tumor proliferation, invasion, inflammatory responses and apoptosis[9–11].

Previous studies have indicated that miR-200a-3p was upregulated in various types of cancer, including non-small cell lung, pancreatic and breast cancer, as well as hepatitis B virus-related hepatocellular carcinoma[12–15]. By targeting the regulator of PCDH9, miR-200a-3p suppresses gastric carcinoma cell proliferation and invasion[16]. In addition, other studies have shown that miR-200a-3p can directly target KLF12 and p21 to inhibit the growth of gastric cancer and lung cancer[17]. However, the functional roles and specific molecular mechanisms underlying miR-200a-3p in bladder cancer cells are not completely understood. TNFα induced protein 3 (A20) is an important regulator of inflammation and immunity[18, 19]. A20 has been reported to negatively regulate inflammatory responses via de-ubiquitination enzymatic activity and ubiquitin binding activity[20]. A20 has also been reported to serve as an oncogene[21]. Additionally, increased A20 expression is associated with a poor survival rate in patients with breast cancer[22]. The association between miRNAs and A20 has been reported in numerous types of human cancer via altering the NF-κB signaling pathway. For example, miR-19b-3p functioned as a tumor suppressor and inhibited nasopharyngeal carcinoma growth by regulating A20[23]. A20 is a potential target gene of miR-19b-3p, however, the relationship between miR-200a-3p and A20 in bladder cancer has not been previously reported[24–26]. Therefore, the present study aimed to investigate the relationship between miR-200a-3p and A20 in bladder cancer. In addition, the present study explored the potential molecular mechanisms underlying miR-200a-3p in bladder cancer. The results were first to demonstrated that miR-200a-3p expression was significantly increased expressed in bladder cancer tissues and cell lines compared with adjacent non-tumor tissues and a normal bladder cell line, respectively, which facilitated bladder cancer cell proliferation, migration and inflammatory cytokine production, but suppressed cell apoptosis via downregulating A20. The results of the present study indicated an important role of miR-200a-3p in regulating A20 expression, providing a potential novel therapeutic target for bladder cancer.
Materials And Methods

Patient samples. In the present study, 40 bladder cancer tissues and 40 adjacent non-tumor tissues were collected from Meizhou People's Hospital. Patients had not received radiation therapy or chemotherapy prior to surgery treatment. The clinical stage of patients with bladder cancer was determined using the World Health Organization criteria. Tumor tissues were stored in liquid nitrogen or at -80˚C. The present study was approved by The Institutional Review Board of Meizhou People's Hospital. Written informed consent was obtained from all patients.

Cell culture and transfection. A normal bladder cell line (SV-HUC-1) and bladder cancer cell lines (5637, J82, ECV-304, BIU-87, T24, HCV29 and H/RB-CL2) were purchased from American Type Culture Collection. Cells were cultured in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 2 mM glutamine and 100 µg/ml streptomycin/penicillin (Sangon Biotech Co., Ltd.) at 37˚C with 5% CO2.

Western blotting. Western blotting was performed as previously described. Briefly, total protein was extracted using NP40 buffer (Beijing Solarbio Science & Technology Co., Ltd.) at 4˚C for 20 min, followed by centrifugation at 12,000 x g at 4˚C for 10 min. Proteins were separated via 12% SDS-PAGE and transferred to PVDF membranes (Beijing Solarbio Science & Technology Co., Ltd.). Following blocking with 5% BSA in PBST (0.1% Tween 20) at room temperature for 1 h, the membranes were incubated with primary antibodies at 4˚C overnight. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody at 37˚C for 1 h. Protein bands were visualized using ECL reagent. GAPDH was used as the loading control.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (1 mg) was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). Subsequently, qPCR was performed using an RT-qPCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). miRNA and mRNA expression levels were normalized to the internal reference genes U6 and GAPDH, respectively.

Apoptosis analysis and Cell cycle analysis. Collected different groups of cells, each group has no less than 1×105 cells. Wash the cells with PBS twice. According to the instructions of the apoptotic kit (KeyGEN Biotech China), add the dyes in turn, protect from light for 10min, flow Instrument (ACEA Biosciences, USA) detection and analysis. Collect different groups of cells, and wash the cells with PBS twice. Then cells were fixed by 70% ethanol overnight. According to the cell cycle kit (KeyGEN Biotech, China), dyes were added sequentially, protected from light for 30min, and analyzed by flow cytometry (ACEA Biosciences, USA).

Cell Counting Kit-8 (CCK-8) assay. Cells (1×10^4 cells/well) were seeded into 96-well plates. Following culture for 48 or 72 h, cell proliferation was analyzed by performing the CCK-8 assay (Dojindo Molecular
Technologies, Inc.) according to the manufacturer's protocol. Absorbance was measured at a wavelength of 450 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.).

**Cell migration assay.** Cell migration was measured using 6.5-mm Transwell inserts with 8.0-mm pore polycarbonate membranes (Costar; Corning Inc.). Cell invasion was assessed using 6.5-mm Transwell inserts with 8.0-mm pore polycarbonate membranes (Costar; Corning Inc.). Cell migration and invasion were determined as previously described. Subsequently, the average number of migratory/invading cells was counted.

**Wound healing assay.** At 24 or 36 h post-transfection, cells were harvested and cultured for a further 24 h. Subsequently, a single scratch in the cell monolayer was made using a 300-µl sterile pipette. The wounds were observed at 24 h. The intersection of the bottom line and the cell scratch line was considered as the observation point.

**Dual-luciferase reporter assay.** The wild-type (WT) A20 3’UTR sequence was amplified via PCR and cloned into the pmirGLO vector (Promega Corporation). To construct the mutant (Mut) plasmid, the complementary sequences for miR-200a-3p in the 3’UTR of A20 were mutated. J82 and T24 cells were co-transfected with A20-WT or A20-Mut and miR-200a-3p mimic or miR-NC. At 36h post-transfection, luciferase activities were measured using the DLR dual luciferase reporter assay system (Promega Corporation).

**Tumor growth in vivo.** The function of miR-200a-3p in bladder cancer growth was assessed by evaluating tumor growth in vivo. Male nude mice (age, 6 weeks; n = 6 per group) were used in the present study to assess tumor growth and metastasis. To assess tumor growth, mice were subcutaneously injected with miR-200a-3p-overexpression or control cells (1x10^5). Tumor volume was calculated at 1, 2, 3 and 4 weeks post-injection. At 4 weeks post-injection, all mice were euthanized and the tumors were isolated. All animal experiments were approved by the ethics committee of Meizhou People's Hospital.

**Western blotting.** Proteins were separated via 12% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). Following blocking with 5% BSA and washing three times with PBST (1% Tween-20) the membranes were incubated with primary antibodies. Subsequently, the membranes were incubated with a HRP-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich; Merck KGaA). Protein bands were visualized using ECL detection reagents (cat. no. E412-02; Vazyme Biotech Co., Ltd.) and scanned using the ChemiDoc XRS + Imaging System (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Data are presented as the mean ± SD from at least three independent repeats. Statistical analyses were performed using SPSS software (version 19.0; IBM Corp.). Comparisons among multiple groups were analyzed using one-way or two-way ANOVA followed by Bonferroni’s post hoc test. Comparisons between two groups were analyzed using a paired or unpaired Student’s t-test. P < 0.05 was considered to indicate a statistically significant difference.

**Results**
miR-200a-3p expression levels are significantly increased in bladder cancer tissues. To investigate the role of miR-200a-3p in bladder cancer, three bladder cancer tissues and adjacent non-tumor tissues were sent to Guangzhou Sage Bioscience for Agilent miRNA microarray analysis. Sequencing results showed that the expression of miR-200a-3p in cancer tissues was higher than that in non-tumor tissues, and the comprehensive score of differential expression of miR-200a-3p was among the top ten in the results of microarray (Fig. 1A). To further clarify the expression of miR-200a-3p in bladder cancer, miR-200a-3p expression levels in bladder tumor tissues (n = 40) and adjacent non-tumor tissues (n = 40) were determined via RT-qPCR. miR-200a-3p expression was significantly upregulated in bladder cancer tissues compared with adjacent non-tumor tissues (Fig. 1B). Moreover, miR-200a-3p expression levels were also significantly upregulated in advanced clinical stage bladder cancer tissues (n = 20 per stage) compared with earlier clinical stage bladder cancer tissues (Fig. 1C), with the highest expression levels observed in stage III and IV metastatic bladder cancer tissues. Moreover, miR-200a-3p expression was also significantly increased in the various bladder cancer cell lines compared with the normal bladder cell line (Fig. 1D). Collectively, the results suggested that miR-200a-3p was upregulated in bladder cancer tissues compared with adjacent non-tumor tissues, indicating that miR-200a-3p may serve an stimulative role in bladder cancer development.

miR-200a-3p overexpression facilitates cell proliferation via suppressing apoptosis, but promotes inflammatory cytokine production. To assess the role of miR-200a-3p in bladder cancer development, miR-200a-3p was overexpressed in J82 and T24 cell lines. miR-200a-3p was successfully overexpressed in J82 and T24 cells (Fig. 2A). To further explore the role of miR-200a-3p in bladder cancer, cell proliferation assays were performed. Cell proliferation was significantly increased at 48 and 72 h following transfection with miR-200a-3p mimic in J82 and T24 cells compared with transfection with miR-Ctr (Fig. 2B). Cell migration was also significantly increased in miR-200a-3p mimic-transfected cells compared with miR-NC-transfected cells, which was consistent with the CCK-8 proliferation assay results (Fig. 2C). In addition, miR-200a-3p overexpression significantly inhibited J82 and T24 cell apoptosis compared with the miR-NC group (Fig. 2D). The wound healing assay results demonstrated that miR-200a-3p overexpression remarkably facilitated J82 and T24 cell migration compared with miR-NC (Fig. 2E). Furthermore, miR-200a-3p overexpression greatly promoted cell cycle progression with supporting G1 phase compared with miR-NC transfection (Fig. 2F). It has been reported that cellular inflammatory responses may be associated with apoptosis. Compared with the miR-NC group, miR-200a-3p overexpression significantly promoted the release of inflammatory cytokines IL-6 and TNFα in J82 and T24 cells (Fig. 2G and H). Collectively, the results demonstrated that miR-200a-3p overexpression promoted bladder cancer progression.

A20 is a target of miR-200a-3p. miRNAs have been reported to recognize and regulate gene expression via attaching to the 3'UTRs of their target genes. The results demonstrated that miR-200a-3p possessed a complementary sequence with A20 mRNA (Fig. 3A). To further verify A20 as a target of miR-200a-3p, the complementary sequence was mutated and dual-luciferase reporter assays were performed. The dual-luciferase reporter assay results indicated that miR-200a-3p overexpression significantly inhibited the luciferase activity of A20-WT compared with miR-NC, but miR-200a-3p overexpression did not
significantly alter the luciferase activity of A20-Mut compared with miR-NC (Fig. 3B), which suggested that miR-200a-3p bound to the 3'UTR of A20. Compared with the miR-NC group, miR-200a-3p overexpression significantly decreased A20 mRNA expression levels and notably decreased A20 protein expression levels in J82 and T24 cells (Fig. 3C). To further elucidate the function of A20 in bladder cancer, the expression level of A20 in various bladder cancer cell lines was assessed. Compared with the normal bladder cell line, A20 mRNA expression levels were significantly decreased and A20 protein expression levels were notably decreased in the seven bladder cancer cell lines (Fig. 3D). Moreover, A20 mRNA expression levels were significantly reduced in bladder tumor tissues (Fig. 3E) and different stage bladder tumor tissues (Fig. 3F) compared with adjacent non-tumor tissues and the control group, respectively. A20 protein expression levels displayed a similar trend. Therefore, in contrast to the expression levels of miR-200a-3p in bladder cancer, A20 expression was significantly decreased in bladder cancer tissues compared with adjacent non-tumor tissues (Fig. 1B). The aforementioned results demonstrated that A20 was a target of miR-200a-3p, indicating that miR-200a-3p downregulated A20 expression by binding to its 3'UTR.

A20 reverses the effects of miR-200a-3p overexpression on bladder cancer cells. Compared with adjacent non-tumor tissues, miR-200a-3p was upregulated in bladder cancer tissues, whereas A20 was downregulated in bladder cancer tissues, and miR-200a-3p downregulated A20 expression. Therefore, whether miR-200a-3p regulated bladder cancer development via targeting A20 was investigated. A20 overexpression restored A20 expression levels in miR-200a-3p-overexpression J82 and T24 cells (Fig. 4A), which resulted in inhibition of miR-200a-3p overexpression-mediated effects on cell proliferation (Fig. 4B), migration (Fig. 4C) and apoptosis (Fig. 4D).

Effects of A20 on bladder cancer cells in vivo. A20 overexpression significantly promoted IL-6 (Fig. 5A) and TNFα (Fig. 5B) production in miR-200a-3p-overexpression J82 and T24 cells. The aforementioned results suggested that miR-200a-3p promoted bladder cancer progression via A20 in vitro. To further investigate the role of miR-200a-3p in promoting bladder cancer progression, miR-200a-3p mimic-transfected or miR-200a-3p mimic + A20 overexpression-transfected T24 cells were injected into nude mice, and the rate of tumor growth was assessed (Fig. 5C). A20 overexpression reversed miR-200a-3p-mediated promotion of tumor growth, as indicated by alterations in tumor volume (Fig. 5C), and A20 expression in vivo (Fig. 5D). Therefore, the results demonstrated that miR-200a-3p promoted bladder cancer progression in vivo. Collectively, the aforementioned results demonstrated that miR-200a-3p facilitated bladder cancer progression via A20.

Discussion

Bladder cancer has become one of the most common malignant tumors of the urinary tract; therefore, identifying oncogenes associated with bladder cancer is important for the development of efficient therapeutic strategies[27]. Although advances in diagnosis and treatment have improved long-term survival for patients with early bladder cancer, the prognosis of advanced stage bladder cancer remains poor[28]. Early bladder cancer has few symptoms, which makes it difficult to diagnose, thus bladder
cancer is typically diagnosed during the advanced stages[29]. An increasing number of studies have identified certain molecular mechanisms underlying bladder cancer progression. As we all known that tumor cells often rely on onco-proteins to continue to proliferate and survive and cyclin-dependent kinases (CDKs) directly had essential effects in cell-cycle transitions of all eukaryotic organisms. Both Chen W et al. and Tang Y et al. had demonstrated that miR-200a-3p suppresses cell invasion and migration by directly targeting ZEB1 and p21 in bladder cancer and lung cancer[29]. however, there is insufficient understanding for the development of effective therapeutic strategies for the disease. Increasing evidence demonstrates that miRNAs contribute to bladder cancer development and progression[32]. However, the role of A20 in bladder cancer progression is not completely understood. A20 has been reported to regulate type I interferon production, apoptosis, autophagy and inflammatory responses[33]. A key approach for analyzing the mechanisms underlying bladder cancer development is gene expression profile analysis. In the present study, the expression levels of miR-200a-3p and A20 in tumor tissues and adjacent non-tumor tissues isolated from patients with bladder cancer were assessed. The results demonstrated that miR-200a-3p was significantly upregulated, whereas A20 was significantly downregulated in bladder cancer tissues compared with adjacent non-tumor tissues. Previous studies have suggested that altered miRNA expression is closely associated with various types of cancer. Several miRNAs can function as oncogenes and other miRNAs can function as tumor suppressor genes. In the present study, miR-200a-3p was significantly upregulated in bladder cancer tissues and cell lines compared with adjacent non-tumor tissues and a normal bladder cell line, respectively. Compared with the miR-NC group, miR-200a-3p overexpression facilitated bladder cancer cell migration and proliferation, which suggested that miR-200a-3p functioned as an oncogene in bladder cancer. The results of the present study were consistent with a previous study that reported an oncogenic role of miR-200a-3p in other types of cancer[34]. Therefore, miR-200a-3p may serve as a universal oncogene, which suggested that it might serve as a putative target for developing therapeutics for bladder cancer.

Cell proliferation and migration are signs of cancer. Advanced stages of cancer involve proliferation, resistance to apoptosis and inflammatory cytokines production. A20 has been reported to serve a role in hepatocellular carcinoma, pancreatic cancer and breast cancer[35]. In the present study, A20 expression was significantly decreased in bladder cancer tissues compared with adjacent non-tumor tissues, and A20 overexpression attenuated miR-200a-3p overexpression-induced development and progression of bladder cancer. The results also demonstrated that miR-200a-3p targeted the 3'UTR of A20. Moreover, compared with the miR-NC group, miR-200a-3p overexpression increased cell proliferation and inflammatory cytokine release, but decreased cell apoptosis in bladder cancer cell lines. The results of the present study suggested that A20 overexpression in miR-200a-3p-overexpression cell lines attenuated the progression of bladder cancer phenotypes, indicating a tumor suppressor function of A20 in bladder cancer.

The results of the present study also suggested that miR-200a-3p suppressed bladder cancer progression via targeting A20. Compared with the miR-NC group, miR-200a-3p overexpression facilitated bladder cancer development, whereas A20 overexpression inhibited miR-200a-3p-induced promotion of bladder cancer. In summary, to the best of our knowledge, the present study indicated for the first time that miR-
200a-3p might serve as an oncogene via A20 in bladder cancer. miR-200a-3p downregulated A20 expression by binding to its 3'-UTR, resulting in enhanced bladder cancer proliferation and migration via promoting cell apoptosis and inhibiting inflammatory cytokine production.

Declarations

Ethics approval and consent to participate

The present study was approved by The Institutional Review Board of Meizhou People's Hospital. Written informed consent was obtained from all patients. All animal experiments were approved by the ethics committee of Meizhou People's Hospital. All experiments were conducted in Meizhou People's Hospital. Patient consent for publication

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

PW designed the study. PW, ZLC and MZH performed data collection. MZH and ZLC performed molecular analyses. ZLC and HMJ analyzed the data and performed statistical analyses. MZH, HJW, KHZ, GDD and BW completes animal feeding and related experiments. All authors contributed to the writing of the proposal and manuscript, MZH and ZLC compiled and finalized the manuscript. All authors read and approved the final manuscript.

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Not applicable

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Figures
Figure 1

miR-200a-3p is upregulated in bladder cancer tissues. (A) Heat-map of Agilent miRNA microarray analysis data of bladder cancer tissues and adjacent non-tumor tissues. (A) miR-200a-3p expression levels in GC tissues and adjacent non-tumor tissues. ***P<0.001 by a paired Student’s t-test. (B) miR-200a-3p expression levels in patients with four different clinical stages of bladder cancer. **P<0.01 by one-way ANOVA followed by Bonferroni’s post hoc test. (C) miR-200a-3p expression levels in bladder
cancer cell lines and a normal human bladder cell line. *P<0.5 vs. SV-HUC-1 by one-way ANOVA followed by Bonferroni’s post hoc test. Data are presented as the mean ± SD of three independent experiments. miR, microRNA.

Figure 2

miR-200a-3p overexpression facilitates cell proliferation via suppressing apoptosis and inflammatory cytokine production. (A) Transfection efficiency of miR-200a-3p mimic in J82 and T24 cells. ***P<0.001
vs. miR-NC by an unpaired Student's t-test. (B) Effect of miR-200a-3p overexpression on J82 and T24 cell proliferation as determined by performing Cell Counting Kit-8 assays. *P<0.5 and **P<0.01 vs. miR-NC by two-way ANOVA followed by Bonferroni's post hoc test. (C) Effect of miR-200a-3p overexpression on J82 and T24 cell migration. *P<0.05 vs. miR-NC by an unpaired Student's t-test. (D) Effect of miR-200a-3p overexpression on J82 and T24 cell apoptosis. *P<0.05 vs. miR-NC by an unpaired Student's t-test. (E) Effect of miR-200a-3p overexpression on J82 cell migration. (F) Effect of miR-200a-3p overexpression on the cell cycle distribution in J82 cells. Effect of miR-200a-3p overexpression on (G) IL-6 and (H) TNFα levels in J82 and T24 cells. **P<0.01 vs. miR-NC by an unpaired Student's t-test. Data are presented as the mean ± SD of three independent experiments. miR, microRNA; NC, control.
Figure 3

A20 is a target of miR-200a-3p. (A) Binding sites between the 3’UTR of A20 and miR-200a-3p. (B) Effect of miR-200a-3p overexpression on the luciferase activity of A20-WT and A20-Mut in J82 and T24 cells. **P<0.01 vs. miR-NC by two-way ANOVA followed by Bonferroni’s post hoc test. (C) Effect of miR-200a-3p overexpression on A20 mRNA and protein expression levels in J82 and T24 cells. **P<0.01 vs. miR-NC by an unpaired Student’s t-test. (D) A20 mRNA and protein expression levels in bladder cancer cell lines.
*P<0.05 and **P<0.01 vs. miR-NC by one-way ANOVA followed by Bonferroni’s post hoc test. (E) A20 expression levels in bladder cancer tissues and adjacent non-tumor tissues. **P<0.01 vs. non-tumor by a paired Student’s t-test. (F) A20 expression levels in different clinical stage bladder cancer tissues. *P<0.05 vs. NC by one-way ANOVA followed by Bonferroni’s post hoc test. Data are presented as the mean ± SD of three independent experiments. A20, TNFα induced protein 3; miR, microRNA; UTR, untranslated region; WT, wild-type; Mut, mutant; NC, control.

Figure 4
A20 reverses miR-200a-3p overexpression-mediated effects on bladder cancer cells. (A) Effect of A20 overexpression on A20 expression levels in miR-200a-3p-overexpression J82 and T24 cells. **P<0.01 vs. miR-NC by one-way ANOVA followed by Bonferroni’s post hoc test. (B) Effect of A20 overexpression on cell proliferation in miR-200a-3p-overexpression J82 and T24 cells. *P<0.05 vs. miR-NC by one-way ANOVA followed by Bonferroni’s post hoc test. (C) Effect of A20 overexpression on cell migration in miR-200a-3p-overexpression J82 and T24 cells. *P<0.05 and **P<0.01 vs. miR-NC by one-way ANOVA followed by Bonferroni’s post hoc test. (D) Effect of A20 overexpression on cell apoptosis in miR-200a-3p-overexpression J82 and T24 cells. *P<0.05 and **P<0.01 vs. miR-NC by one-way ANOVA followed by Bonferroni’s post hoc test. Data are presented as the mean ± SD of three independent experiments. A20, TNFα induced protein 3; miR, microRNA; NC, Negative-control.
Figure 5

Effect of A20 on bladder cancer cells in vivo. Effect of A20 overexpression on the production of inflammation cytokines (A) IL-6 and (B) TNFα by miR-200a-3p-overexpression J82 and T24 cells. **P<0.01 vs. miR-NC by one-way ANOVA followed by Bonferroni’s post hoc test. (C) Tumor growth curves and representative images of tumors isolated from nude mice. Transfected J82 cells were subcutaneously injected into nude mice (n=6 per group). *P<0.05 and **P<0.01 vs. miR-NC by one-way
ANOVA followed by Bonferroni’s post hoc test. (D) A20 protein expression levels was measured at the endpoint of the experiment. *P<0.05 vs. miR-Ctr by one-way ANOVA followed by Bonferroni’s post hoc test. Data are presented as the mean ± SD of three independent experiments. A20, TNFα induced protein 3; miR, microRNA; NC, Negative-control; ns, not significant.

Supplementary Files

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