Original Article
Expression of miR-149-3p inhibits proliferation, migration, and invasion of bladder cancer by targeting S100A4

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Abstract: MicroRNAs play key roles during various crucial cell processes, such as proliferation, migration, and invasion. In addition, microRNAs have been shown to possess oncogenic and tumor suppressive functions in human cancers. Increasing evidence has clarified that miR-149-3p, a novel cancer-related microRNA, plays an important role in suppression of proliferation, migration, and invasion; however, the effect and mechanisms underlying the miR-149-3p effect in bladder cancer (BCa) remain unclear. In the current study we found that the increased expression of miR-149-3p significantly suppressed cell proliferation, migration, and invasion ability in BCa. The suppressive effect was related to S100A4. A further investigation showed that miR-149-3p negatively regulated S100A4, as verified by the luciferase reporter assay. Furthermore, our study showed that S100A4 mediated the anti-metastatic effects of miR-149-3p on proliferation, migration, and invasion of BCa cells. Analysis of a xenograft mouse model showed that miR-149-3p expression significantly decreased tumor growth by targeting S100A4. Taken together, these data indicate that S100A4 promotes cell growth, migration, and invasion and can by reversed by miR-149-3p in BCa.

Keywords: Bladder cancer, S100A4, miR-149-3p, proliferation, migration

Introduction

Urinary bladder cancer (BCa) is a prevalent urogenital malignant tumor which causes an estimated 380,000 new cases and 150,000 deaths annually. BCa is among the fifth most common malignancies worldwide [1, 2]. The annual incidence of new BC cases has declined over the last 10 years, but the fatality rate has remained stable, especially for transitional cell carcinoma (TCC) [3]. Conventional cytotoxic chemotherapy, such as platinum-based combination chemotherapy, is commonly used as a main regimen for advanced stages of TCC, which currently provides a potential cure only in select patients and is underpowered [4, 5]. In addition, the frequent emergence of drug resistance due to tumor heterogeneity and serious side effects from current treatment regimens make treatment outcomes unsatisfactory [6]. Increasing evidence has shown that chemotherapy resistance is related to tumor metasta-sis [7-9]. Thus, it is of great significance to understand the molecular mechanisms underlying the metastasis and proliferation of BCa.

MicroRNA (miR) is a short non-coding RNA sequence that plays an important role in adjusting gene expression. MicroRNA binds to the 3' untranslated region (UTR) of target messenger RNAs (mRNAs) and affects the corresponding transcription process [10-12]. Emerging evidence has established that miRNAs are actively involved in a number of different cellular processes, such as cell proliferation, invasion, and migration [13-16]. Recent studies have suggested that miR-149-3p may play an important role in tumor suppression in various cancer types, such as pancreatic and gastric cancer [17, 18]. The role of miR-149-3p in BCa remains unclear.

S100A4 is a member of the S100 protein family, which is associated with cell differentiation,

cell motility, and transcriptional regulation [19, 20]. Additional studies showed that S100A4 is highly expressed in metastatic tumor cell lines and the expression of S100A4 relative to several malignancies, including bladder, colorectal, breast, and thyroid cancers, and plays a role in tumor aggressiveness [21-26]; however, the exact pathologic role of S100A4 in bladder cancer remains unclear. Bioinformatics analysis showed that miR-149-3p can target the 3'UTR of S100A4. Thus, this study aimed to clarify the regulatory effect of miR-149-3p in S100A4-mediated bladder cancer progression.

The present study demonstrated that up-regulation of the expression of miR-149-3p can significantly suppress the proliferation, invasion, and migration *in vitro* and suppresses tumor growth of BCa cells *in vivo*. Additionally, miR-149-3p is capable of down-regulating the expression by targeting S100A4 and binding to the 3'-UTRs. Over-expression of S100A4 can restore the phenotypes of BCa cell lines and inhibit the suppressive effect of miR-149-3p. Finally, the present study demonstrated that miR-149-3p has a tumor suppressive role in BCa progression.

Materials and methods

Ethics statement

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals, and all experiments were approved and performed according to the guidelines of the Ethics Committee of Tongren Hospital of Shanghai Jiaotong University School of Medicine. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg).

Reagents

Cell lines and cell culture: Two human bladder cancer cell lines (UM-UC-3) were purchased from the American Type Culture Collection (Manassas, VA, USA). All of the cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37°C.

In vivo tumor model

Male BALB/C nude mice at 6 weeks of age were randomly divided into two groups (five mice per group). miR-149-3p mimics or miR-control stable transfection UM-UC-3 cells were suspended (1×10^6 cells/mL) in 100 μL of PBS were subcutaneously injected into the flanks of nude mice. Tumor growth was examined every 5 days. After 30 days, tumor samples were carefully removed and weighed.

Cell viability assay

A Cell Counting Kit-8 (CCK8) was used to assess cell viability. Cells (1×10^4) were seeded into a 96-well plate and incubated overnight in the previously described conditions. The cells were pre-treated with sterile water or 10 mM NAC for 2 h, then with 0.1% DMSO or 40 μM wogonin for 24 h. Then, the medium was removed and the cells were washed three times with PBS. DMEM (90 μl) and CCK8 (10 μl) were subsequently added to each well and incubated for 1.5 h at 37°C. A microplate reader was used to measure the optical density (OD) at 450 nm.

Transfection of miR-149-3p mimics and western blotting analyses

The miR-149-3p mimics and corresponding negative control (miR-NC) were purchased from GenePharma (Shanghai, China). The mimics or control oligonucleotides were transfected into UM-UC-3 cells at a final concentration of 100 nM. The transfected cells were collected 48 h post-transfection. Proteins were resolved in an SDS/PAGE gel and subjected to immunoblot analysis using monoclonal antibodies against S100A4, E-cadherin, vimentin, or GAPDH (Abcam, Cambridge, MA, USA). All antibodies were used at 1 μg/ml in PBS with 5% dried milk. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol.

Luciferase reporter assay

To construct luciferase reporter vectors, the 3'-UTR of S100A4 cDNA fragments containing the predicted potential miR-149-3p binding sites were amplified by PCR and sub-cloned downstream of the luciferase gene in the PYr-MirTarget luciferase vector (Ambion, Inc., Austin, TX, USA). The 3'-UTR of S100A4 con-

taining the binding sites for miR-149-3p was amplified from a cDNA library with the following primers: forward, 5'-CTCGAGGTCTGCCAGCTGG-GGCCCTCCCT-3' and reverse, 5'-GCGGCCGC-CTTCCAAGAATCTTTATTG-3'. The mutant 3'-UTR of S100A4, in which 8 nucleotides were mutated in the binding sites, was amplified using the following primer sequences: forward, 5'-CTCGA-GGTCTGCCGAGTGGGGCGGCTGCT-3' and reverse, 5'-GCGGCCGCCTTCCAAGAATCTTTATTG-3'.

For luciferase assays, HEK293T cells were cultured in 24-well plates and co-transfected with 50 ng of the corresponding vectors containing firefly luciferase together with 25 ng of miR-149-3p or control. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). At 48 h post-transfection, relative luciferase activity was calculated by normalizing the Firefly luminescence to the Renilla luminescence using a Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Real-time quantitative PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription was carried out using a Rt-PCR system (Promega, Shanghai, China). Real-time quantitative Rt-PCR analysis was performed in a 20 µl final reaction volume using SYBR Green I Supermix (Takara, Dalian, China) according to the manufacturer's protocol. All reactions were performed in triplicate on an iCycler IQ multicolor Detection System (BioRad, Hercules, CA, USA) with the following cycling parameters: 95°C for 10 s, followed by 40 cycles of 94°C for 15 s, annealing at 55°C for 30 s, and final extension at 70°C for 30 s. All quantifications were normalized to the level of human U6 snRNA in the reaction. The comparative threshold cycle (CT [DDCT]) method, which compares differences in CT values between common reference RNA and target gene RNA, was used to obtain the relative fold changes in gene expression. miR-149-3p and S100A4 primers for PCR were designed by GenePharma Co., Ltd. (Shanghai, China). The results are expressed as the mean ± SE.

Lentiviral vector construction and infection

Human S100A4 full-length cDNA was generated by PCR using forward primer (5'-CCACCG-GTATGGCGTGCCCTCTGGAG-3') and reverse pri-

mer (5'-CGGCTAACTCATTTCTTCCTGGGCTG-3'). The amplified fragment was then sub-cloned into pGC-FU-EGFP-3FLAG plasmid (GeneChem, Shanghai, China) after *AgeI/NheI* restriction enzyme digestion to produce pGC-FU-NEDD4-1-3FLAG.

siRNA specifically targeted to S100A4 (forward, 5'-GCCAUGAUGUGUAACGAAUUU-3'; reverse, AUUCGUUACACAUCAUGGCUU) were purchased from GenePharma Co., Ltd. Cells were plated in six-well plates and grown to ~80% confluence before transient transfections with siRNAs (100 pmol per well) were performed using Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. After a 48-h transfection, cells were collected for other experiments.

Colony formation assay

UM-UC-3 cells were harvested 24 h after transfection with RNA duplexes (50 nM of NC or miR-149-3p mimics). All of the cells were resuspended in DMEM medium supplemented with 10% FBS and seeded in six-well plates at a density of 400 cells per well. After 10 days of culture under standard conditions, the colonies on the plates were fixed with absolute methanol for 15 min and stained with 0.1% crystal violet for 20 min. The colonies with diameters > 2 mm were counted.

Wound healing assay

UM-UC-3 cells transfected with corresponding vectors were seeded in six-well plates to form the single confluent cell layer. The wounds were made with 100 µl tips in the confluent cell layer. Zero and 12 h after wound scratching, the width of the wound was photographed with a phase contrast microscope.

Transwell migration and invasion assay

The migratory and invasive ability of bladder cancer cells were evaluated with Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA). Cells (5-10×10^4) were suspended in 100 µl of medium without serum and seeded into the upper chamber, and the lower chamber was filled with 20% FBS to induce bladder cancer cell migration or invasion through the membrane. Matrigel (1:6 dilution; BD Biosciences)
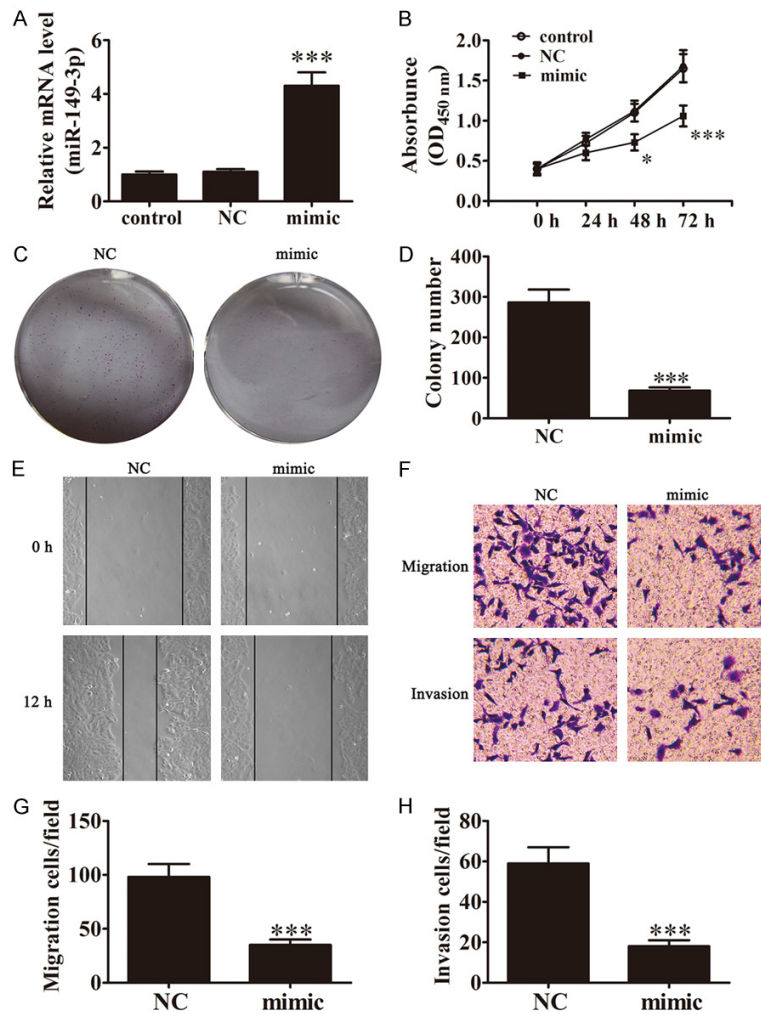
Figure 1. Expression of miR-149-3p inhibits the proliferation, invasion, and migration of BCa cells. A. Expression of miR-149-3p in UM-UC-3 cells was measured by Rt-PCR after transfection with miR-149-3p mimics for 48 h (n=3). Data are presented as the mean ± SD (***P < 0.001 versus control group). B. The CCK-8 assay showed that the relative viability of UM-UC-3 cells treated with miR-149-3p mimics was significantly lower than NC-treated cells (n=5). Data are presented as the mean ± SD (*P < 0.05, ***P < 0.001 versus control group). C and D. Colony formation assay show that the colony formation rates of miR-149-3p mimic-transfected UM-UC-3 cells were lower in contrast with NC-transfected cells (n=5). Data are presented as the mean ± SD (***P < 0.001 versus NC group). E. Wound healing assays indicated that miR-149-3p over0expression reverses the migration of UM-UC-3 cells. F-H. Transwell assays confirmed that miR-149-3p over-expression inhibited migration and invasion of UM-UC-3 cells (n=5). Data are presented as the mean ± SD (***P < 0.001 versus NC group).

was added on the upper chamber for the invasion assay. Twenty-four hours later, cells with crystal violet (MedChem Express, Shanghai, China) staining that migrated or invaded across the Transwell membrane were counted under optical microscope.

Statistical analysis

Continuous variables are presented as the mean ± standard deviation (SD). One-way ANOVA was carried out for multiple comparisons using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). P-values ≤ 0.05 indicate a statistically significant difference.

Results

miR-149-3p inhibits the proliferation and mobility of BCa cells

Increased cancer cell proliferation and mobility is an important reason for the metastasis and recurrence of human cancers [27, 28]. Previous results have demonstrated that the expression of miR-149-3p has tumor suppression effects in many human cancers, including pancreatic and gastric cancers [17, 18]. The effect of miR-149-3p in regulating the progress of BCa cells is unclear. To identify the regulatory effect of miR-149-3p, UM-UC-3 cells were used with or without transfection with miR-149-3p-NC or miR-149-3p mimics vector. After a 48-h transfection, the expression of miR-149-3p in UM-UC-3 cells was measured by Rt-PCR. The results showed that miR-149-3p expression was increased significantly (**Figure 1A**). CCK-8 assays showed that the expression of miR-149-3p significantly suppressed the growth of cultured BCa cells from 48 h (**Figure 1B**). The colony formation rates of BCa cells after the overexpression of miR-149-3p were clearly low in contrast with cells transfected with NC (**Figure 1C** and **1D**). The wound healing assays showed that miR-149-3p overexpression nota-
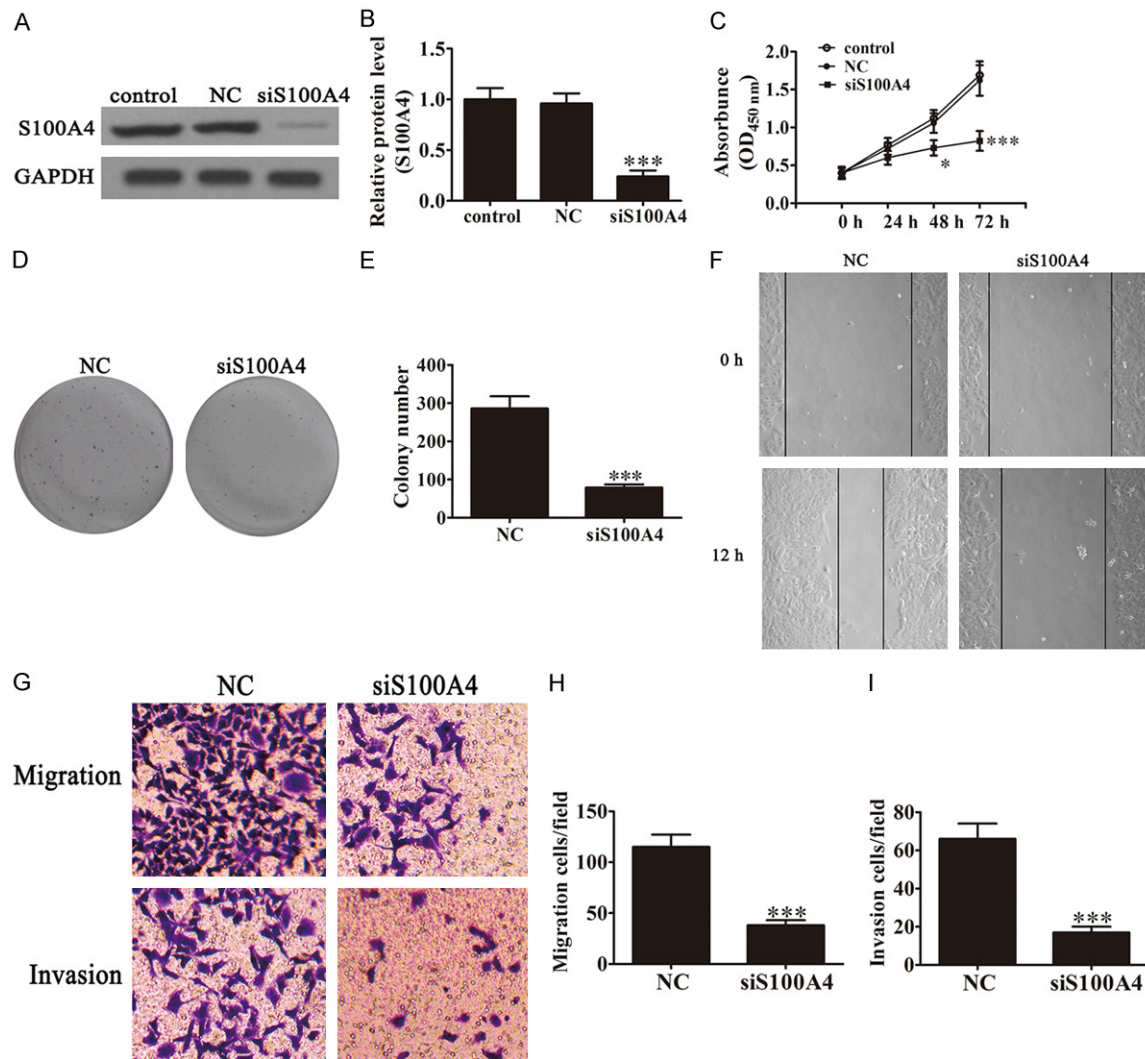
Figure 2. Down-regulation of S100A4 phenocopied the effect of miR-149-3p. (A and B) Expression of S100A4 in UM-UC-3 cells was measured by western blot (A) and Rt-PCR (B) after transfection with siRNA against S100A4 (n=5). Data are presented as the mean ± SD (***P < 0.001 versus control group). (C) The CCK-8 assay showed that the relative viabilities of UM-UC-3 cells were significantly decreased after down-regulating the expression of S100A4 (n=5). Data are presented as the mean ± SD (*P < 0.05, ***P < 0.001 versus control group). (D and E) Colony formation assay showed that the colony formation rates of UM-UC-3 cells were decreased after transfection with siS100A4 (n=5). Data are presented as the mean ± SD (***P < 0.001 versus NC group). (F) Wound healing assays indicated that suppression of S100A4 expression reverses the migration of UM-UC-3 cells. (G-I) Transwell assays confirmed that down-regulation of S100A4 expression inhibited the migration and invasion of UM-UC-3 cells (n=5). Data are presented as the mean ± SD (***P < 0.001 versus NC group).

bly reduced cell migration in UM-UC-3 cells (**Figure 1E**). Transwell assays revealed that ectopic expression of miR-149-3p significantly reduced the number of migrated and invaded UM-UC-3 cells (**Figure 1F-H**). Thus, miR-149-3p has an anti-metastatic and proliferation role in BCa cells.

Down-regulation of S100A4 expression phenocopied the effect of miR-149-3p

To identify if S100A4 expression has an anti-tumor effect in BCa, we transfected UMUC-3

cells with siS100A4, a small interfering RNA against S100A4, to specifically knock down the expression of S100A4. After a 48-h transfection, the expression of S100A4 was measured by western blot and Rt-PCR. The results showed that transfection of siS100A4 remarkably suppressed expression of S100A4 (**Figure 2A** and **2B**). CCK-8 assays showed that siS100A4 significantly suppressed the growth of cultured UMUC-3 cells after 48 h as the result of overexpression of miR-149-3p (**Figure 1C**). The colony formation rates of BCa cells after down-

regulation of S100A4 were obviously low in contrast with cells transfected with NC (**Figure 1D** and **1E**). The wound healing assays showed that siS100A4 significantly reduced cell migration in UM-UC-3 cells (**Figure 1F**). Transwell assays showed that knock-down of S100A4 significantly reduced the number of migrated and invaded UM-UC-3 cells (**Figure 1G-I**). Thus, knock-down of S100A4 expression exerts a similar anti-metastatic and proliferation effect in BCa cells as over-expression of miR-149-3p, suggesting some internal relationships between miR-149-3p and S100A4.

Ectopic expression of S100A4 reversed the anti-tumorous effect of miR-149-3p

In order to identify the effect of S100A4 to miR-149-3p-induced growth inhibition of BCa cells, we transfected UM-UC-3 cells with miR-149-3p mimics and S100A4 over-expression vector alone or in combination. Western blot showed that the expression of miR-149-3p suppressed S100A4 expression. After transfection, S100A4 over-expression vector reversed the inhibitory effect of miR-149-3p to S100A4 expression. The results also showed that over-expression of miR-149-3p decreased vimentin expression, and increased E-cadherin expression in UM-UC-3 cells, which play an important role in mediating BCa migration and invasion. Over-expression of S100A4 reversed miR-149-3p-mediated migration and invasion of protein expression (**Figure 3A**). CCK-8 assays showed that over-expression of S100A4 significantly removed the inhibitory effect of miR-149-3p to the growth suppression of UMUC-3 cells (**Figure 3B**). The results of colony formation showed that over-expression of S100A4 reversed the inhibitory effect of miR-149-3p to UMUC-3 cell proliferation *in vitro* (**Figure 3C** and **3D**). The wound healing assays (**Figure 1E**) and Transwell assays (**Figure 1F-H**) also showed that over-expression of S100A4 reversed the inhibitory effect of miR-149-3p to cell migration and invasion in UM-UC-3 cells. Taken together, we found that over-expression of S100A4 reversed the suppression of cell proliferation and metastasis caused by miR-149-3p.

miR-149-3p regulates the expression of S100A4 by directly targeting the 3'-UTR

Knowing the basic mechanism by which miR-NAs regulate mRNA expression, we determined the exact targets of miR-149-3p responsible for the anti-tumor effect. By using an online bioinformatics database (TargetScan, http://www.targetscan.org/), we found a broadly-conserved binding site between 3'UTR of S100A4 and miR-149-3p (**Figure 4A**). A mutated version of the S100A4 3'-UTR was generated in which 10 complementary nucleotides in the binding site were altered (**Figure 4B**) and this mutated construct was fused to the luciferase coding region (PYr-RGS-17 3'-UTR) and co-transfected into HEK293T cells along with miR-149-3p mimics (**Figure 4C**). Determination of the relative luciferase activity revealed that when the wild-type S100A4 3'-UTR was co-transfected with miR-149-3p mimics, S100A4 expression was significantly decreased compared to co-transfection with control miRNA. This effect was not observed, however, when the mutant S100A4 3'-UTR construct was used for co-transfection, indicating the specific targeting and suppression of the 3'-UTR of S100A4 by miR-149-3p. Western blot was used to check the levels of S100A4 expression after transfection with a miR-149-3p mimic or miR-203-NC. The expression of S100A4 were significantly suppressed in UM-UC-3 cells with miR-149-3p overexpression (**Figure 4D**).

miR-149-3p expression inhibits tumor growth in vivo

To determine whether or not miR-149-3p is involved in tumorigenesis *in vivo*, a xenograft tumor model was used in nude mice. One hundred microliters ($1×10^6$ cells/mL) of UM-UC-3 cells transfected with miR-149-3p mimics or miR-149-3p-NC were subcutaneously injected into the flanks of nude mice. Compared with the miR-149-3p-NC group, the miR-149-3p over-expression group showed dramatically reduced tumor volume and weight during the 30 days after the tumors became palpable (**Figure 5A-C**). Western blot showed that S100A4 expression in tumor tissue from the miR-149-3p over-expression group was significantly decreased compared with the miR-149-3p-NC group (**Figure 5D**), suggesting that miR-149-3p inhibits BCa proliferation by targeting S100A4 *in vivo*.

Discussion

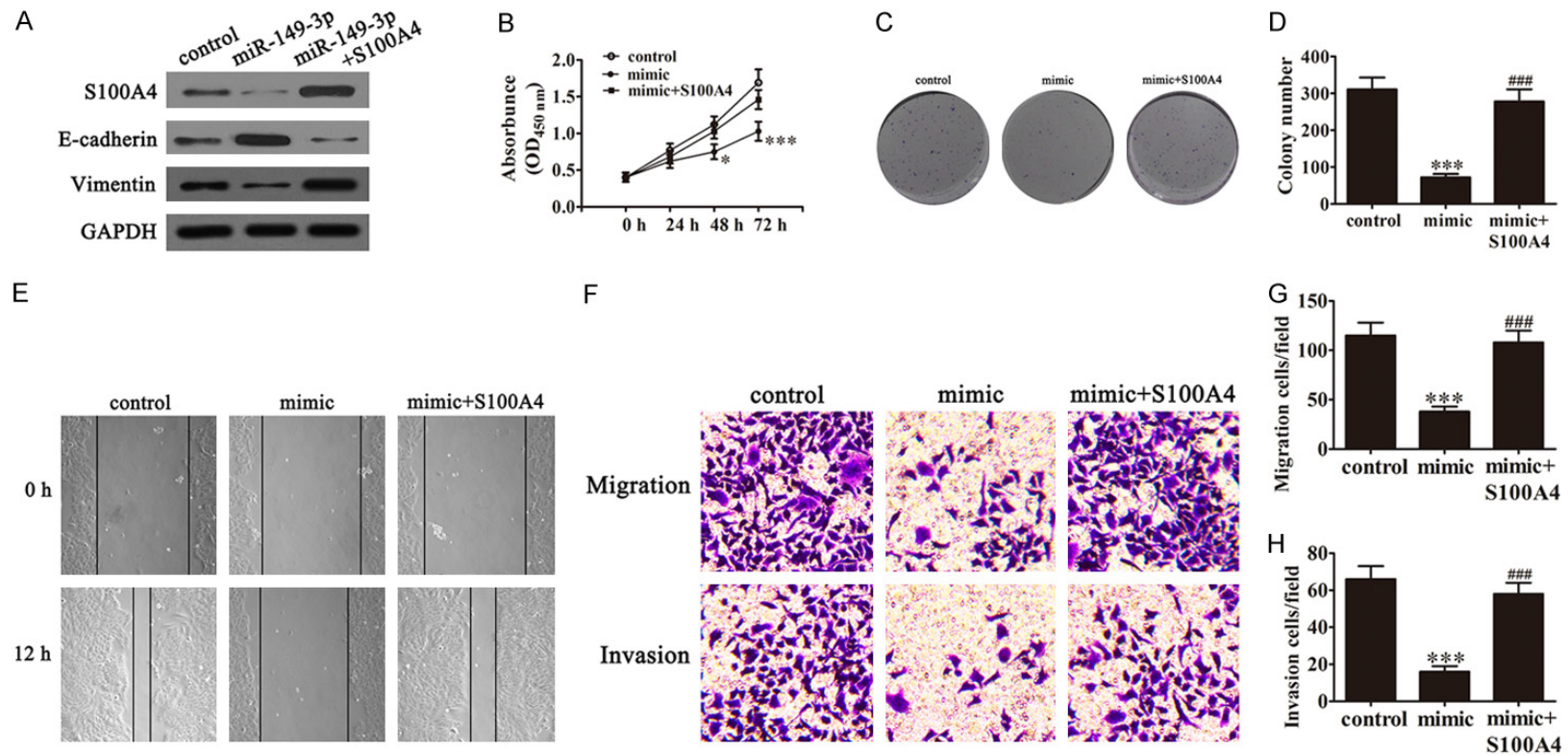Emerging evidence has confirmed that miRNAs play a critical role in carcinogenesis and cancer

Figure 3. Ectopic expression of S100A4 restores the phenotypes of BCa cell lines transfected with miR-149-3p. A. Western blot shows the expression of S100A4, E-cadherin, and vimentin in UM-UC-3 cells after ectopic expression of S100A4. B. The CCK-8 assay shows that S100A4 rescues the capacity of relative viabilities decreased in UM-UC-3 cells after up-regulation of miR-149-3p expression (n=5). Data are presented as the mean ± SD. *P < 0.05 (***P < 0.001 versus control group). C and D. Colony formation assay showed that the colony formation rates of UM-UC-3 cells recovered with up-regulation of S100A4 expression after transfection with miR-149-3p mimics (n=5). Data are presented as the mean ± SD (***P < 0.001 versus control group; ###P < 0.001 versus mimic group). E. Wound healing assays indicated that the expression of S100A4 reversed the inhibitory effect of miR-149-3p on UM-UC-3 cell migration. F-H. Transwell assays confirmed that up-regulation of S100A4 expression reverses the inhibitory effect of miR-149-3p on UM-UC-3 cell migration (F and G) and invasion (F and H).
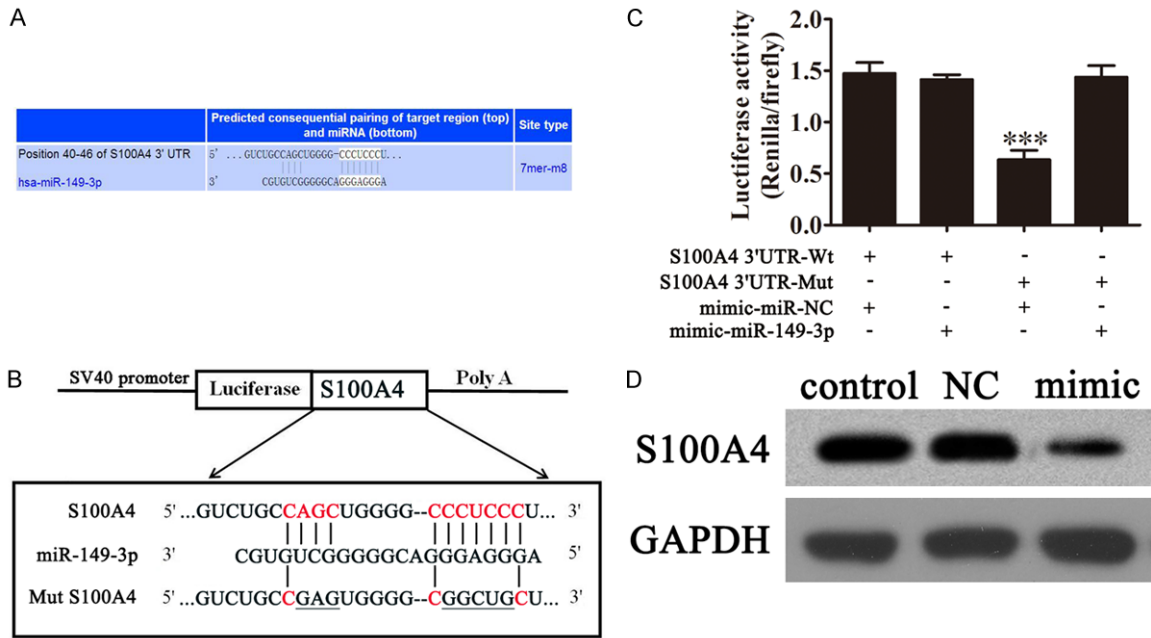
Figure 4. S100A4 is a potential target of miR-149-3p. A, B. Complementary sequences between miR-149-3p and 3'-UTR of S100A4 mRNA were obtained using publicly available algorithms. The mutated version of the USP22 3'-UTR is also shown. C. The 3'-UTR of S100A4 was fused to the luciferase coding region (PYr-RGS-17 3'-UTR) and co-transfected into HEK293T cells with miR-149-3p mimics to confirm that S100A4 is the target of miR-149-3p. The PYr-RGS-17 3'-UTR and miR-149-3p mimic constructs were co-transfected into HEK293T cells with a control vector and the relative luciferase activity was determined 48 h after transfection. The data are presented as the mean ± SD (***P<0.001 versus the control). D. Western blot analysis of the effect of S100A4 expression on UM-UC-3 cells after transfection with miR-149-3p mimics (n=5). Levels of GAPDH expression were detected as an endogenous control.
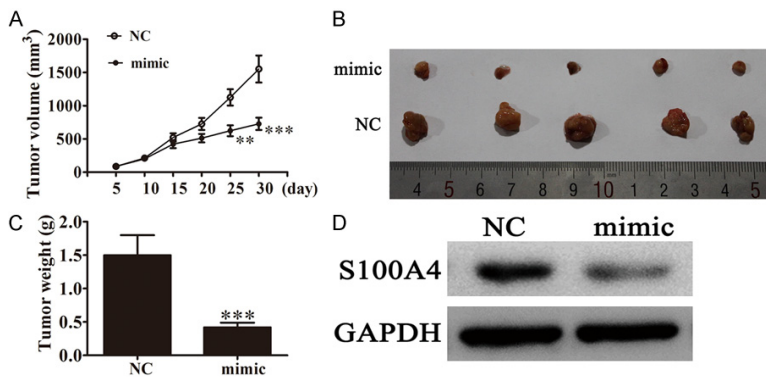

Figure 5. The expression of miR-149-3p suppressed tumor growth of bladder cancer in a xenograft model. A. Growth curves for tumor volumes in xenografts of nude mice were established based on the tumor volume measured every 5 days until 30 days (n=5; *P < 0.05, **P < 0.01, ***P < 0.001 versus NC group). B. Photographs of tumor tissue with different groups on day 30. C. Tumor weights of different groups on day 30 (n=5; ***P < 0.001 versus NC group). D. S100A4 level in tumor tissues of different groups was determined by western blot (n=5). Level of GAPDH expression was detected as an endogenous control.

progression [29, 30]. Individual miRNAs have been ascribed oncogenic and tumor suppressor functions [31-33], and aberrant miRNA expression has been implicated in many malignancies, including BCa [34-37]. Profiling and functional studies have demonstrated potential novel prognostic value for a number of miRNAs in BCa. Previous research has shown that the role of miR-149-3p (also known as miR-149) is different with respect to the progression of different types of tumors, include colonic carcinoma, myeloid leukemia, hepatocellular carcinoma, and renal cell carcinoma [38-41]. Ectopic expression of miR-149 represses metastasis of hepatocellular carcinoma by targeting the actin-regulatory protein, PPM1F [42]. In colorectal cancer, miR-149 significantly suppresses cell migration and

invasion by directly targeting forkhead box transcription factor FOXM1 [43]. Here, we report that miR-149-3p expression significantly inhibits cell proliferation, migration, and invasion in BCa cells. Furthermore, we also verified that S100A4 is a direct target gene of miR-149-3p in BCa cells.

S100A4 is a member of the S100 family of calcium-binding proteins that is directly involved in tumor metastasis. S100A4 is over-expressed in most malignancies, such as breast [44], thyroid [26], pancreatic [45], gastric [46] and bladder cancers [22, 47]. S100A4 expression is correlated with metastasis and patient outcome, suggesting that enhanced S100A4 expression contributes to the manifestation of a metastatic phenotype. Based on the experimental and clinicopathologic evidence, S100A4 has emerged as a promising therapeutic target for BCa [21, 48, 49].

In summary, our research revealed that miR-149-3p may function as a tumor suppressor in the UM-UC-3 cell line by targeting S100A4. Therefore, up-regulation of S100A4 promotes BCa cell proliferation, invasion, and migration. Silencing of S100A4 significantly reduces cell invasiveness. Moreover, our results showed that S100A4 may function as an oncogene in the development of BCa. Therefore, miR-149-3p may serve as a therapeutic target for BCa treatment; however, because each miRNA may regulate many target genes that can affect carcinogenesis in different ways, more detailed molecular and cellular mechanisms underlying the action of miR-149-3p targets are warranted.

Acknowledgements

Disclosure of conflict of interest

None.

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