MicroRNA-335 inhibits bladder cancer cell growth and migration by targeting mitogen-activated protein kinase 1

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Abstract. The abnormal expression of microRNAs (miRs) as oncogenes or tumor-suppressor genes has been widely investigated in various tumor types. However, the roles of miR-335 in bladder cancer cells have remained elusive. The aim of the present study was to assess the expression of miR-335 in bladder cancer as well as the effects of miR-335 on bladder cancer cell proliferation, metastasis and apoptosis. PCR and western blot analyses revealed that miR-335 was significantly downregulated in bladder cancer tissues, and low levels of miR-335 were associated with more aggressive phenotypes of bladder cancer. Overexpression of miR-335 in T24 cells inhibited cell proliferation and induced apoptosis as indicated by an MTT assay and flow cytometric analysis, respectively. Furthermore, overexpression of miR-335 significantly suppressed cell migration, as indicated by a Transwell assay. The expression of mitogen-activated protein kinase (MAPK)1 was decreased after overexpression of miR-335, indicating that MAPK1 may be a target gene of miR-335. In addition, silencing of MAPK1 inhibited the proliferation and migration of bladder cancer cells. In conclusion, the results of the present study demonstrated that miR-335 was significantly downregulated in bladder cancer, and may act as a tumor suppressor through repression of MAPK1.

Introduction

Bladder cancer (BC) is one the most common urological malignancies worldwide. In China, BC is the most common genitourinary malignancy and its incidence has increased over the last decades (1). Following surgery and adjuvant chemotherapy, bladder cancer frequently recurs in a more aggressive form (2,3). Therefore, it is necessary to develop novel treatment strategies to improve the poor prognosis of bladder cancer patients.

MicroRNAs (miRs) are small non-coding RNAs which regulate gene expression at transcriptional and post-transcriptional levels (4). Increasing evidence has indicated that miRs have important roles in various biological processes (5-7). Aberrant expression of miRs is known to be correlated with cancer progression (8,9). Upregulation of certain oncogenic miRs, including miR-145, miR-150, miR-19a and miR-155, in bladder cancer cells has been documented (10-12). However, certain tumor-suppressive miRs, including miR-320c and miR-24-1, inhibit tumor invasion and metastasis (13,14).

miR-335 expression has been observed to be deregulated in several types of cancer, and its implication in tumorigenesis and metastasis of has also been reported (15,16). In pediatric acute lymphoblastic leukemia, deregulated miR-335 that targets mitogen-activated protein kinase (MAPK)1 has been implicated in poor outcome (17). However, the clinical significance and biological roles of miR-335 in bladder cancer have yet to be fully elucidated. Therefore, the present study assessed the expression of miR-335 in bladder cancer tissues and adjacent non-tumor tissues, as well as bladder cancer cell lines. Furthermore, the effects of miR-335 on the proliferation and migration of bladder cancer cells, in addition to the underlying mechanisms, were investigated.

Materials and methods

Clinical specimens. Tissue samples were collected from surgical specimens of 50 bladder cancer patients who underwent surgery at Nantong Tumor Hospital (Nantong, China) between May 2010 and July 2014. The corresponding adjacent non-neoplastic tissues were obtained at the same time and used as controls. All samples were immediately snap-frozen in liquid nitrogen and stored at -80˚C prior to RNA extraction. Patient demographics are displayed in Table I. The Clinical Research Ethics Committee of Nantong Tumor Hospital (Nantong, China) approved the study protocol and written informed consent was obtained from all patients.

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Cell culture. The T24 and 5637 human bladder cancer cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) as was the TCHu169 immortalized human bladder epithelial cell line, which was also used. The bladder cancer cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS; Biological Industries, Cromwell, CT, USA) and 100 μg/ml penicillin-streptomycin (Gibco; Thermo Fisher Scientific), while the TCHu169 cells were propagated in F-12K medium (Gibco; Thermo Fisher Scientific) containing 10% FBS. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Prior to RNA extraction, tissues were immersed in RNAlater (Ambion; Thermo Fisher Scientific) and stored at 20°C. Subsequently, total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific) and quantified using a Synergy HT Multi-Mode Microplate Reader (Biotek Instruments, Inc., Winooski, VT, USA). Total RNA, including miR, was extracted using the mirVana miRNA isolation kit (Ambion). RNA (1 μg) was reverse transcribed to cDNA using random primers and a Reverse Transcription kit (Takara Biotechnology Co., Ltd., Dalian, China), in a final volume of 20 μl. RT was performed at 37°C for 15 min, then at 85°C for 5 sec. qPCR was performed using the Power SYBR Green PCR (Takara), according to the manufacturer's protocol. RT-qPCR was performed using a SYBR Green Premix Ex Taq (Takara Bio Inc., Otsu, Japan) in a Light Cycler 480 (Roche, Basel, Switzerland). U6 small nuclear RNA and β-actin mRNA were used as internal controls. Primers were synthesized by Shanghai Sangon Biological Engineering Technology Services (Shanghai, China). All reactions were run in triplicate. The primers had the following sequences: miR-335 forward, 5'-TCAAGA GCAATAACGAAAAATGT-3' and reverse, 5'-GCTGTC AACGATACGCTACGT-3'; U6 forward, 5'-CGCTTCGGC AGCACATATAC-3' and reverse, 5'-TTCACGAATTTGCG TGTCAT-3'; β-actin forward, 5'-AGTGTGACGTGGACAT CCGCAAAG-3' and reverse, 5'-ATCCACATCTGCTGG AAGGTGGAC-3'; miR-335 mimics forward, 5'-GCUAGA AGAACUAUUUGCUUU-3' and reverse, 5'-AAAGCAAAT AGTTCTTCTAGC-3'; miR-NC forward, 5'-UUCUCCGAA CGUGUCACGUTT-3' and reverse, 5'-AAAGCTGACACG TTCGGAGAA-3'. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (18), in which values were normalized to those of β-actin.

miRNA mimics, small interfering (si)RNAs and transfection. miR-335 mimic and siRNAs specific for MAPK1 were synthesized by RiboBio (Guangzhou, China). miR-335 mimics and negative control miR (miR-NC) were used for gain-of-function experiments, whereas MAPK1 siRNA and control siRNA were used in the loss-of-function experiments. Transfection with miRNAs or siRNAs was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions and the cycling conditions were as follows: 94°C for 2 min for 2 cycles, followed by 40 cycles of 94°C for 15 sec, 58°C for 25 sec,

Table I. Clinical features and tumor characteristics of patients in the present study.

Clinical characteristic	ACCs
Age, years	
≥65	27
<65	23
Gender	
Male	42
Female	8
pT stage	
Ta, Tis, T1	21
T2-T4	29
Tumor grade	
Low grade	19
High grade	31
Lymph node status	
N0	17
N1, N2	33

ACCs, adenoid cystic carcinomas; pT, primary tumor.

and 72°C for 30 sec. Cells in the logarithmic growth phase were seeded in a 10 cm dish for RNA and protein extraction, in a six-well plate for protein extraction and apoptosis assay, and in a 96-well plate for MTT assay and luciferase reporter assay. Small interfering RNAs (siRNAs) against MAPK1 were also obtained from RiboBio. The two sets of siRNA sequences were as follows: siRNA-1 (negative control) forward, 5'-CUCUACGUAAGAUCCAGCUUU-3' and reverse, 5'-AGCUGGAUCUUACGUAGAGUU-3'; siRNA-2 forward, 5'-AGCAAAUAGUUCCUAGCUUUU-3' and reverse, 5'-AAGCUAGGAACUAUUUGCUUU-3'.

Cell proliferation assay. Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and seeded in 96-well plates at $3x10^3$ cells per well. After 72 h of incubation, cell proliferation was determined using an MTT assay (Invitrogen) according to the manufacturer's protocol. The number of cells per well was determined by measuring the absorbance at 540 nm. All experiments were performed in triplicate.

Apoptosis analysis. Following transfection for 48 h, cells were harvested and re-suspended in 1X binding buffer (BioVision, Inc., Milpitas, CA, USA) at a density of $1x10^6$ cells/ml. Double staining with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (PI; Annexin V-Phycoerythrin Apoptosis Detection kit; BD Biosciences, Franklin Lakes, NJ, USA) was used to evaluate the percentage of apoptotic cells. In the results of the apoptosis analysis, the left upper, right upper, left lower and right lower quadrants represent necrotic cells, late apoptotic cells, normal cells and early apoptotic cells, respectively. The right lower quadrant was selected for evaluation of the levels of apoptosis in cells. The cells were immediately analyzed

using a FACScan flow cytometer (BD Biosciences), where Annexin V-positive and PI-negative cells were designated as apoptotic cells. Finally, optical density was determined at 540 nm by a POLARstar+Optima (BMG Labtech GmbH, Ortenberg, Germany). The aforementioned experiment was repeated three times, and the results were analyzed using Cell Quest software (version 2.7; BD Biosciences) where the left upper, right upper, left lower and right lower quadrants represent necrotic cells, late apoptotic cells, normal cells and early apoptotic cells, respectively. The right lower quadrant was selected for evaluation of the levels of apoptosis in cells.

Cell migration assay. Transwell inserts with a pore size of 8 μm (Corning, Inc., Corning, NY, USA) were used to determine the migratory capacity of the tumor cells. After transfection for 24 h, $1x10^6$ cells were starved in medium without FBS for 24 h, then re-suspended in the FBS-free medium and placed in the top chambers of the Transwell inserts with each experimental condition performed in triplicate. The cells remaining on the upper membrane were removed with cotton wool, while the cells that had migrated to the bottom of the membrane were then fixed with 95% ethanol and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) Five fields of view for each insert were randomly selected and images were captured under a light microscope (CKX41SF; Olympus Corporation, Tokyo, Japan) at x200 magnification. All experiments were performed in triplicate.

Protein extraction and western blot analysis. Bladder cancer cells were seeded onto six-well plates ($1x10^7$ cells/well) and were transfected for 48 h. Cells were lysed with radioimmunopre-cipitation assay buffer containing protease inhibitors (Thermo Fisher Scientific). The protein concentration was determined using a bicinchoninic protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The respective tissue proteins (30 μg) were loaded onto 10% sodium dodecyl sulfate-poly-acrylamide gels and separated by electrophoresis, followed by transfer onto a polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The blots were probed with primary rabbit anti-MAPK11 monoclonal antibody (cat no. ab117949; Abcam, Cambridge, MA, USA) diluted 1:500 or mouse monoclonal anti-β-actin antibody diluted 1:2,000 (cat no. 0869100; MP Biomedicals, Santa Ana, CA, USA) in Tris-buffered saline with 0.05% Tween 20 containing 5% non-fat milk at 4°C overnight. The membranes were washed 3 times for 10 min each using Tris-buffered saline comprising 50 mM Tris (pH 7.4), 0.9% NaCl and 0.05% Tween-20, and were then incubated with horseradish peroxidase-conjugated secondary antibodies: Anti-MAPK11 [1C2] monclonal mouse antibody (cat no. ab117949; Abcam) and anti-actin monoclonal antibody (1:10,000; cat no. 0869100; MP Biomedicals, LLC, Santa Ana, CA, USA). Blots were visualized using enhanced chemiluminescence substrates (Pierce Biotechnology, Inc.). β-Actin was used as an endogenous control.

Statistical analysis. Data analyses were performed using the SPSS statistical package 15.0 (SPSS Inc., Chicago, IL, USA). Values are expressed as the mean ± standard deviation. Data were analyzed using Pearson's χ^2 test and Fisher's exact test. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

miR-335 is downregulated in bladder cancer tissues. To the best of our knowledge, the present study was the first to assess the expression of miR-335 in human bladder cancer. RT-qPCR analysis was employed to quantify the expression levels of miR-335 in 50 pairs of bladder cancer tissues and the adjacent non-neoplastic tissues. The results revealed that miR-335 expression levels in bladder cancer tissues were significantly lower compared with those in healthy tissues, and 29/50 samples displayed a reduction of ≤50% (Fig. 1A). To further investigate the association between the expression of miR-335 and the clini-copathological characteristics, the levels of miR-335 between muscle invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC) were compared. The results indicated that expression of miR-335 was lower in MIBC compared with that in NMIBC, suggesting that miR-335 expression was significantly associated with an aggressive tumor phenotype (Fig. 1B). These results indicated that miR-335 may represent a potential tumor suppressor in bladder cancer.

miR-335 is downregulated in bladder cancer cell lines. When compared with the TCHu169 normal bladder cell line, the expression of miR-335 was significantly downregulated in T24 and 5637 bladder cancer cells, indicating that low levels of miR-335 may be relevant to the development of bladder cancer (Fig. 2A). After transfection of T24 cells with miR-335 mimics for 48 h, upregulation of miR-335 expression levels compared to those in miR-NC-transfected cells were verified by RT-qPCR (Fig. 2B).

miR-335 suppresses bladder cancer cell proliferation and migration, and induces apoptosis. In order to assess whether miR-335 is able to suppress bladder cancer progression *in vitro*, the present study performed a gain-of-function study via trans-fection of miRNA-335 into T24 cells. The results of the MTT assay revealed that overexpression of miR-335 significantly inhibited the proliferation of miR-335-transfected T24-cells (Fig. 3A). Flow cytometric analysis revealed a significant increase in the number of apoptotic cells in miR-335-trans-fected cells, when compared with that in the control group (Fig. 3B). Furthermore, the Transwell assay indicated a signifi-cant inhibition of T24 cell migration following transfection with miR-335 (Fig. 3C).

miR-335 exerts is tumor-suppressor effects in bladder cancer through targeting MAPK1. The present study aimed to eluci-date the underlying mechanism of the tumor-suppressive role of miR-335 in bladder cancer. MAPK1 has been verified as a functional target of miR-335, as miR-335 efficiently controls MAPK1 expression by directly targeting a sequence motif in the coding region of MAPK1 in pediatric acute lympho-blastic leukemia (17). MAPK signaling is associated with proliferation and drug resistance in a variety of cancers (19). However, the possible involvement of MAPK1 in the tumor suppressor role of miR-334 in bladder cancer has remained elusive.
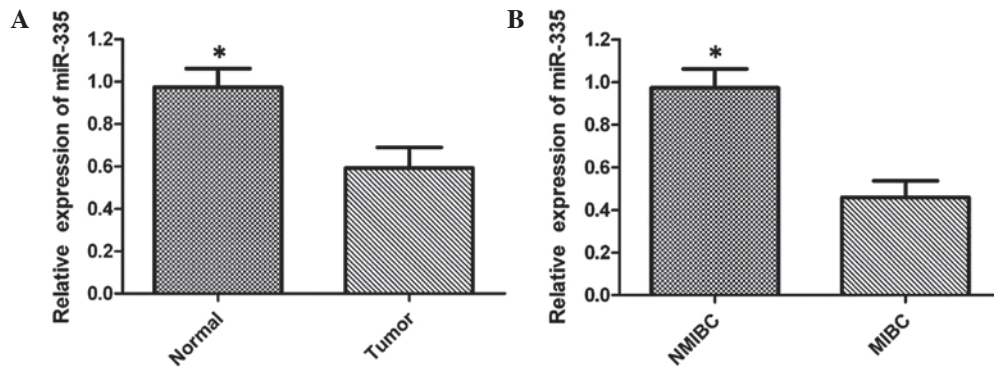
Figure 1. miR-335 is downregulated in bladder cancer tissues compared with the corresponding adjacent non-neoplastic tissues. (A) Relative expression levels of miR-335 in 50 pairs of bladder cancer tissues and the adjacent non-neoplastic tissues were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (B) The relative expression levels of miR-335 in MIBC and NMIBC were determined by RT-qPCR. Values are expressed as the mean ± standard deviation; n=3. *P<0.05 vs. tumor/MIBC group. MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; miR, microRNA.
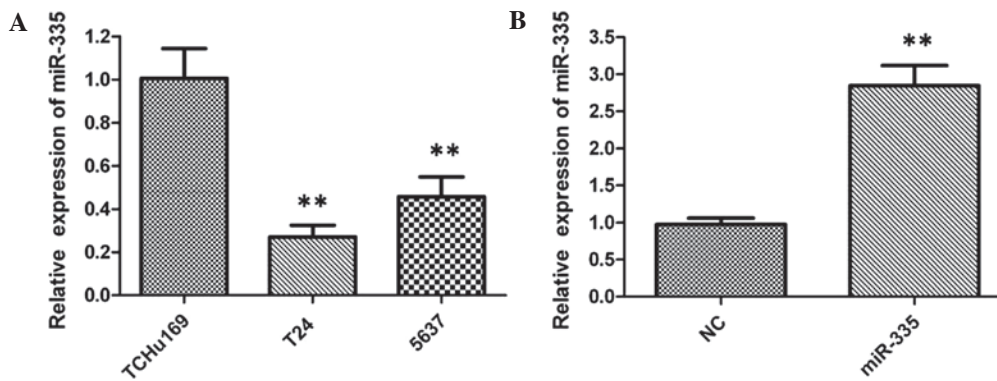

Figure 2. (A) Relative expression levels of miR-335 in the T24 and 5637 human bladder cancer cell lines and the TCHu169 normal bladder epithelial cell line; (B) Transfection of miR-335 mimics into T24 increased the expression of miR-335 as indicated by reverse-transcription quantitative polymerase chain reaction analysis. Values are expressed as the mean ± standard deviation; n=3. **P<0.01 vs. TCHu169/NC. miR, microRNA; NC, negative control.
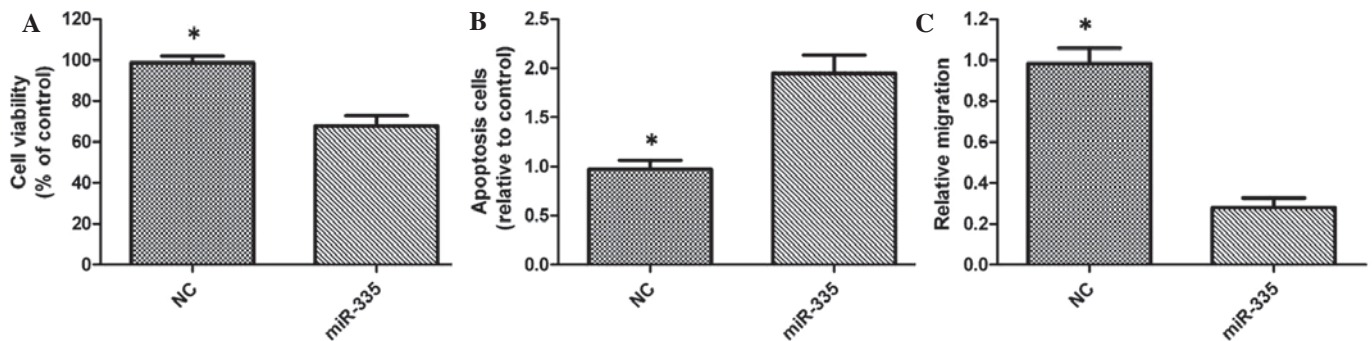

Figure 3. (A) An MTT assay revealed significant inhibition of cell proliferation in T24 cells following miR-335 transfection. (B) Analysis of apoptosis revealed a significant increase in the apoptotic rate of miR-335-transfected cells compared with the NC group. (C) A Transwell assay displayed significant inhibition of cell migration following miR-335 transfection. Values are expressed as the mean ± standard deviation; n=3. *P<0.05 vs. miR-335. NC, negative control; miR, microRNA.

To further confirm that MAPK1 is a target gene of miR-335, RT-qPCR and western blot analysis were used to detect effects of miR-335 mimics on the expression of MAPK1 in T24 cells. The expression of MAPK1 was evidently decreased after overexpression of miR-335 at the mRNA (Fig. 4A) and protein level (Fig. 4B) compared with that in the negative control-transfected cells. These results indicated that miR-335 downregulated the expression of MAPK1 in bladder cancer cells.

Inhibition of MAPK1 suppresses the proliferation and migration of T24 cells. To investigate whether miR-335 exerts its tumor-suppressive function through the MAPK1 pathway, MAPK1 was silenced in T24 cells using siRNA. RT-qPCR and western blot analyses confirmed that the siRNAs siMAPK1_1 and siMAPK1_2 significantly decreased the mRNA and protein expression of MAPK1 (Fig. 5A and B). Following MAPK1 silencing, the growth rate of T24 cells was significantly reduced compared with that in the negative control-transfected group
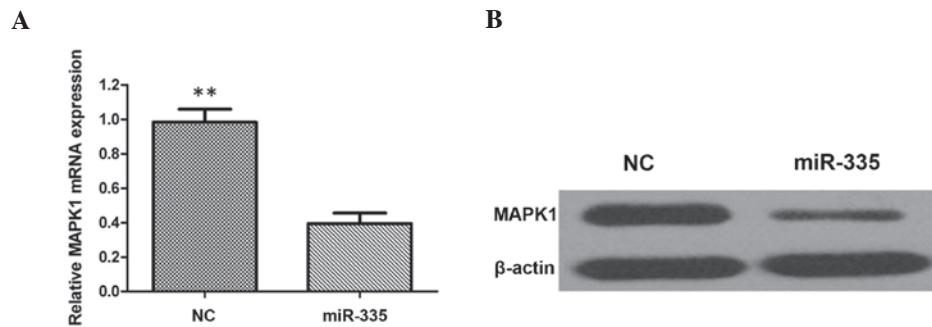
Figure 4. miR-335 negatively regulates MAPK1 gene expression. miR-335 inhibited the expression of MAPK1 in bladder cancer cells at (A) the mRNA level and (B) the protein level. Values are expressed as the mean ± standard deviation; n=3. **P<0.01 vs. miR-335. NC, negative control; miR, microRNA; MAPK1, mitogen-activated protein kinase 1.
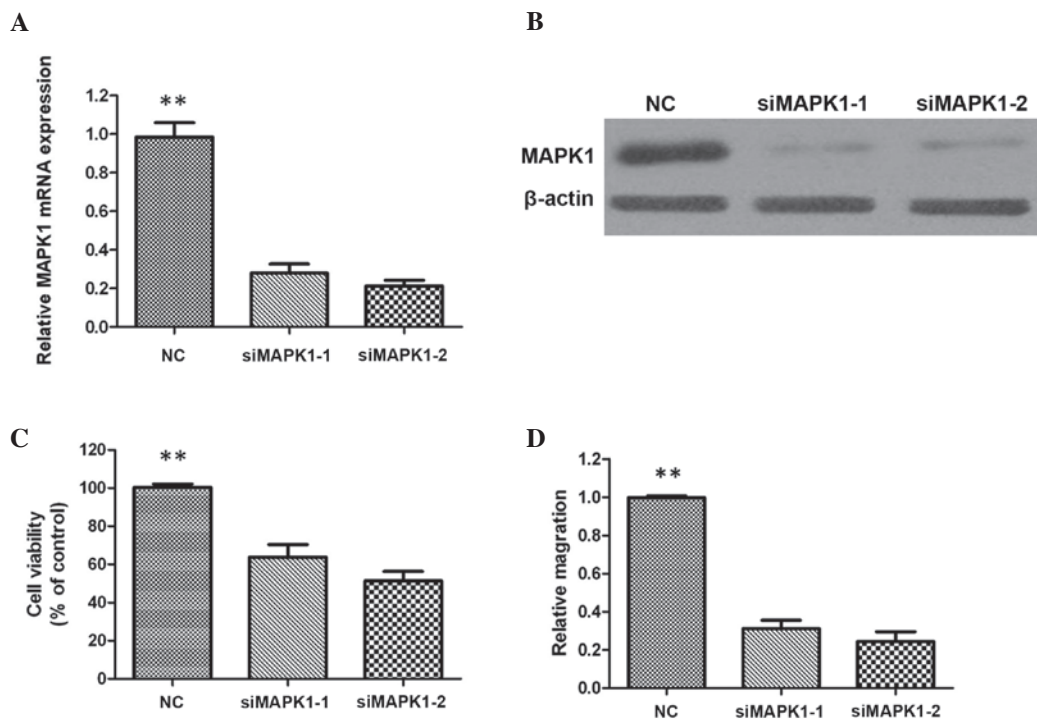

Figure 5. MAPK1 was silenced in bladder cancer cells by transfection with specific siRNA, which was confirmed by (A) polymerase chain reaction and (B) western blot analyses. (C) Knockdown of MAPK1 inhibited the growth of bladder cancer cells. (D) Knockdown of MAPK1 by siRNA inhibited the migration of bladder cancer cells. Values are expressed as the mean ± standard deviation; n=3. **P<0.01 vs. NC. NC, negative control; siRNA, small interfering RNA; MAPK1, mitogen-activated protein kinase 1; NC, negative control.

(Fig. 5C). Furthermore, a Transwell assay demonstrated that the migratory capacity of cells transfected with siMAPK1_1 or siMAPK_2 was significantly inhibited compared with that of control-transfected cells (Fig. 5D).

Discussion

Aberrant expression of miRs is implicated in the pathogenesis of most cancer types (20), and miRs have important roles in regulating tumor development. Deregulation of miRs in bladder cancer has been comprehensively reviewed (21), however, the function and mechanisms of miR-335 in bladder tumorigenesis have remained to be elucidated.

miR-335 is transcribed from its genomic region on chromosome 7q32.2 and acts as a tumor suppressor in types of various malignancy (22). Studies have reported that miR-335 is downregulated in various cancer types, including breast, prostate (23) and gastric cancers (15,24); however, the function of miR-335 and its target genes in bladder cancer have remained elusive. To the best of our knowledge, the present study was the first to investigate the expression of miR-335 in bladder cancer tissues and to assess the underlying mechanisms. The expression of miR-335 was detected to be significantly decreased in human bladder cancer tissues compared with that in adjacent normal tissues. Furthermore, low expression of miR-335 in bladder cancer was associated with a more aggressive phenotype. Similar results were obtained by analysis of miR-335 expression in two bladder cancer cell lines and a normal bladder epithelial cell line, which further strengthened the conclusion that miR-335 was downregulated in bladder cancer.

A gain-of-function study was further performed in the T24 bladder cancer cell line. Overexpression of miR-335

through transfection with miR-335 mimics was observed to significantly inhibit the proliferation of T24 cells, which was consistent with the results of previous studies, which reported the tumor-suppressive role of miR-335 in other cancer types (15,24). In addition, a Transwell assay revealed a significant inhibition of T24-cell migration following transfection with miR-335 mimics, demonstrating that miR-335 impaired the migratory ability of bladder cancer cells. Thus, downregulation of miR-335 may have a critical function in bladder cancer development. The role of miR-335 in bladder cancer remains to be confirmed *in vivo*.

To further assess the mechanism by which miR-335 functions as a tumor suppressor in bladder cancer, the present study examined the association between miR-335 and MAPK1 in bladder cancer cells, revealing that miR-335 exerted its regulatory role in bladder cancer cells by targeting MAPK1. MAPK is an intracellular serine/threonine protein kinase which regulates cell proliferation, differentiation, development and apoptosis. The MAPK signal pathway consists of ERK, c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), p38 and ERK5/BMK1 subgroup. Several studies have suggested that JNK/SAPK and p38MAPK pathways are related to cellular stress and apoptosis, and ERK pathway serves an important role in cell proliferation and differentiation (25,26). Furthermore, it was demonstrated that miR-335 inhibited bladder cancer cell proliferation and migration via the MAPK1 pathway. The MTT assay revealed a significant cell-growth inhibition following transfection of T24 cells with MAPK1-targeted siRNA compared with si-control transfection. Furthermore, the Transwell assay demonstrated a significant inhibition of T24-cell migration following transfection with siMAPK1_1 and siMAPK_2. These results suggested that reduced miR-335 levels lead to elevated MAPK1 levels, which drives the progression of bladder cancer.

In conclusion, the present study suggested that miR-335 is a potential tumor suppressor in bladder cancer. By targeting MAPK1, miR-335 inhibits bladder cancer cell proliferation and migratory potential bladder cancer cells. Therefore, restoration of miR-335 may represent a promising therapeutic strategy for bladder cancer.

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