MicroRNA-335 is downregulated in bladder cancer and inhibits cell growth, migration and invasion via targeting ROCK1

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Abstract. The expression of microRNA-335 (miR-335) has been demonstrated to be downregulated in numerous types of cancer. Thus far, no previous studies have investigated the miR-335 expression in bladder cancer. In the present study, the expression and effects of miR-335 were assessed in bladder cancer. The results of the present study provided, to the best of our knowledge, the first evidence that miR-335 is downregulated in the tumor tissue of patients with bladder cancer. Following transfection of miR-335, MTT, cell migration and invasion, luciferase and western blot assays were conducted in bladder cancer cell lines. The results demonstrated that miR-335 inhibited cell proliferation, migration and invasion in T24 and EJ cells. In addition, the results suggested that miR-335 directly targets Rho-associated protein kinase 1 (ROCK1) in bladder cancer. The present study provided a novel therapeutic target, the miR-335/ROCK1 axis in bladder cancer. The suggested approach will be beneficial in developing an effective treatment against bladder cancer.

Introduction

Bladder cancer is the second most common malignant tumor in the urogenital tract in the USA, and the seventh most common cancer worldwide (1). In the USA, there was an estimated 72,500 newly diagnosed cancer cases and 17,960 cases of cancer-associated mortality in 2013 (2). The most common type of bladder cancer is comprised of transitional cell carcinoma that arises from transitional epithelium (3). Bladder cancer is classified as having either non-muscle-invasive or muscle-invasive tumors. Upon initial diagnosis, ~75% cases are classified as non-muscle-invasive and ~25% of cases as muscle-invasive (4). Patients with non-muscle-invasive bladder cancer have a high rate of recurrence, and in certain cases become muscle-invasive (5). Despite the development of various surgical and chemotherapeutic methods for the treatment of bladder cancer, it remains a highly prevalent and lethal malignancy (1). Therefore, there is a requirement for sensitive and reliable biomarkers, therapeutic targets and approaches for the treatment of bladder cancer.

Previous studies have demonstrated that microRNAs (miRNAs) are expressed in numerous types of human cancer (6-8). miRNAs are a class of small non-coding RNAs whose mature products are 17-27 nucleotides long (9). miRNAs negatively regulate protein expression by inducing degradation or impairing the translation of target mRNAs, by specifically binding to the 3 prime untranslated region (3'-UTR) of target mRNAs (10,11). Although the biological functions of the miRNAs are unknown, previous studies demonstrated that they serve a role as regulators, involved in all hallmarks of cancer (12-14). miRNAs may regulate diverse biological processes, including cell proliferation, development, differentiation, apoptosis and tumorigenesis of cancer (9). Increasing evidence has indicated that deregulation of miRNAs may function as either a tumor suppressor or an oncogene in the tumorigenesis of numerous types of human cancer (7). Thus, identifying the targets of the miRNAs is important to understand their function in cancer development and progression, as miRNAs may be a target for cancer therapy.

Previous studies demonstrated that the expression of miR-335 is downregulated in multiple tumor types (15-18). However, miR-335 expression remains to be investigated in bladder cancer. The current study demonstrated that miR-335 was downregulated in tumor tissues from human bladder cancer compared with their normal adjacent tissues (NATs). Furthermore, results demonstrated that miR-335 inhibited cell proliferation, migration and invasion by directly targeting Rho-associated protein kinase 1 (ROCK1). The data of the present study have diagnostic and therapeutic implications, and may be exploited for the development of further treatment strategies in bladder cancer.

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Materials and methods

Clinical specimens. Bladder cancer and adjacent normal tissues (27 samples) were obtained from patients diagnosed histopathologically with bladder cancer and who had received radical cystectomy at The Fourth Affiliated Hospital of Nantong Medical College (Yancheng, China), between 2008 and 2012. None of the patients had received other therapies, such as chemotherapy and radiotherapy, prior to radical cystectomy. All samples were immediately placed in liquid nitrogen following excision from patients, and were subsequently frozen at -80°C for RNA extraction. The present study was approved by the Hospital's Protection of Human Subjects Committee, and informed consent was obtained from all patients. The clinicopathological features of the patients are summarized in Table I.

RNA isolation, reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions, and RT reactions were performed using the M-MLV Reverse Transcriptase system (Promega Corporation, Madison, WI, USA). RT-qPCR was performed using a standard protocol from the SYBR Green PCR kit (Toyobo Co., Ltd., Osaka, Japan). Each sample was analyzed in triplicate. The primer sequences used were as follows: miR-335, F 5'-TCAAGAGCAATAACGAAAAATGT-3' and R 5'-GCTGTCAACGATACGCTACGT-3'; and U6, F 5'-CGC TTCGGCAGCACATATAC-3' and R 5'-TTCACGAATTTG CGTGTCAT-3'. Data were normalized using the endogenous U6 snRNA and fold changes were calculated using the $2^{-\Delta\Delta C_q}$ normalization method with the following formula: $\Delta\Delta C_q = C_q miR\text{-}335 - C_q U6$ (19).

Cell culture. The bladder cancer cell lines, T24 and EJ, were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were maintained in Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell transfection. Mature miR-335 mimics and miRNA negative control mimics (NC) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). For functional analysis, T24 and EJ cells were seeded into 6-well plates 24 h prior to transfection to ensure 60-70% confluency at the time of transfection. Cells were transfected with miR-335 mimics or NC using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Cell proliferation assay. Human bladder cancer cells were seeded into 96-well plates in triplicate at a density of $3x10^3$ cells/well, 24 h subsequent to transfection with miR-335 or NC mimics. Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at various time points subsequent to transfection. Briefly, MTT solution (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated at 37°C for 4 h. The MTT solution was then discarded and 200 μl dimethyl sulfoxide was added to the cells to dissolve the formazan crystals precipitated. Absorbance was measured at a wavelength of 490 nm using an enzyme-linked immunosorbent assay reader (Elx800; Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were performed in triplicate. The suppression rate was calculated using the following formula: Suppression rate = [1-optical density (OD) miR-335/ODmiR-NC] x 100%.

Cell migration and invasion assay. Corning Costar transwell chambers (Thermo Fisher Scientific, Inc.) with 8 μm pore size polycarbonate membrane were used to assess cell migration and invasion. For the invasion assay, the membrane was pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). Transfected cells ($5x10^4$) were seeded on the upper chamber with serum-free medium. A volume of 0.5 ml of 20% FBS-containing medium was added to the lower chamber as a chemoattractant. Cells were then incubated for another 12 h for the migration assay and 24 h for the invasion assay. The membranes were stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China), and five visual fields of x200 magnification of each membrane were randomly selected for cell counting under an inverted CKX41 microscope (Olympus Corporation, Tokyo, Japan).

Western blot analysis. Subsequent to a 72-h transfection with miR-335 or NC mimics, human bladder cancer cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). Lysates were centrifuged at 12,000 x g for 40 min at 4°C, and the protein sample was diluted, heated for denaturation, and then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 20 min at 70 V and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) for 70 min at 110 V. Membranes were then incubated with rabbit anti-human polyclonal anti-ROCK1 (cat no. 4035S; 1:1,000) or anti-β-actin (cat no. 4970L 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) primary antibodies overnight. Membranes were rinsed in Tris-buffered saline containing 0.05% Tween 20 (Beyotime Institute of Biotechnology) three times, and then incubated for 2 h with the corresponding goat anti-rabbit horseradish peroxidase conjugated secondary antibody (sc-2054; 1:10,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Proteins were visualized with an ECL kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and analyzed using Quantity One 1-D analysis software (version 4.62; Bio-Rad Laboratories, Inc.).

Luciferase assay. Human bladder cancer cells were seeded into a 12-well plate at ~90% confluency and transfected with the reporter plasmid, miR-335 or NC mimics. The Renilla and firefly luciferase activity were measured 48 h subsequent to transfection with the Dual-Luciferase Reporter Assay System (Promega Corporation) and a luminometer (Infinite 200 PRO NanoQuant; Tecan Group Ltd., Männedorf, Switzerland). The firefly luciferase activity was normalized to the renilla luciferase activity for each transfected well. All experiments were performed in triplicate.

Table I. Summary of clinicopathological features of bladder cancer.

Patient No.	Sex	Age	Grade	Stage
1	M	61	2	T2N0M0
2	F	59	1	T2N0M0
3	M	55	1	T2N0M0
4	M	55	3	T1N0M0
5	M	65	2	T1N0M0
6	M	45	3	T4N0M0
7	M	58	1	T4N0M0
8	F	62	2	T2bN0M0
9	M	62	3	T2N0M0
10	M	77	2	T2N0M0
11	M	82	3	T1N0M0
12	F	69	2	T2bN0M0
13	M	82	1	T4N0M0
14	M	76	3	T1N0M0
15	M	77	2	T1N0M0
16	M	68	2	T4N2M0
17	M	81	1	T2N0M0
18	M	73	1	T1N0M0
19	M	66	2	T1N0M0
20	M	68	3	T3bN0M0
21	M	77	1	TaN0M0
22	M	52	3	T1N0M0
23	M	43	2	T1N0M0
24	M	67	2	T1N0M0
25	M	61	1	T1N0M0
26	M	53	3	T3aN0M0
27	F	64	2	TaN0M0

M, male; F, female.

Figure 1. (A) Expression patterns of miR-335 in urinary bladder cancer tissues. The miR-335 levels were determined in 27 surgical specimens of human bladder cancer tissues and NATs. (B) Reverse transcription-quantitative polymerase chain reaction analysis indicated a statistically significant suppression of miR-335 expression in bladder cancer tumor tissues compared with NATs. **P<0.01 vs. negative control. miR-335, microRNA-335; NAT, normal adjacent tissue.

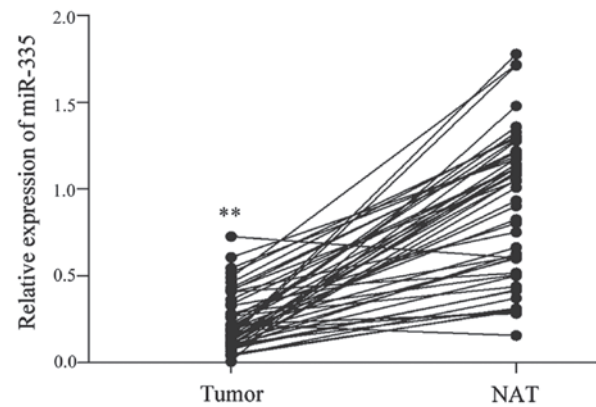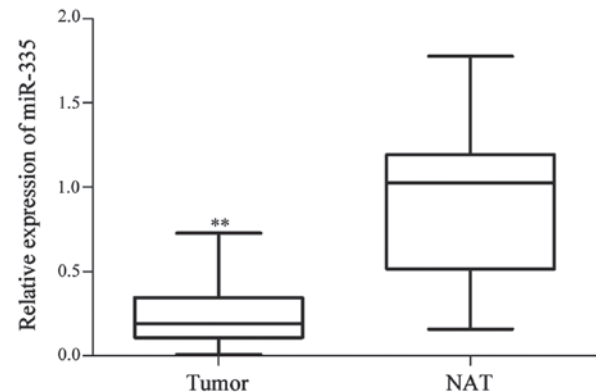*Statistical analysis.* Data are presented as the mean ± standard deviation, and were analyzed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed using the Chi-square test. Kaplan-Meier curves were constructed, and the log-rank test was performed for analysis of survival data. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-335 expression in bladder cancer tissues and association with clinicopathological factors. A total of 27 bladder cancer samples were included in the present study. As demonstrated in Fig. 1, miR-335 was significantly downregulated in bladder cancer tissues compared with the NAT (P<0.01). These results suggested that miR-335 may serve an important role in human bladder cancer.

miR-335 suppressed cell proliferation in the T24 and EJ cells. the MTT assay was conducted to investigate whether miR-335 has a biological function in cell proliferation. As demonstrated

in Fig. 2, upregulation of miR-335 markedly inhibited cell proliferation compared with the NC. The results indicated that after a 120-h treatment, the suppression rate of miR-335 reached 38.98±4.5% in T24 cells and 24.97±4.9% in EJ cells (P<0.05; Fig. 2).

miR-335 suppressed cell migration and invasion in the T24 and EJ cells. Taking the fact that T24 and EJ cells are metastatic cells into consideration, it was investigated whether a reduction in miR-335 had an effect on the capacities of cells to migrate and invade. As demonstrated in Fig. 3, the migratory and invasive capacities of T24 and EJ cells transfected with miR-335 were markedly reduced compared with the NC. These results indicated that miR-335 reduced cell migration and invasion in the bladder cancer cells.

ROCK1 is a direct target gene of miR-335 in the T24 and EJ cells. To identify the target gene of miR-335, a public database (TargetScan; http://www.targetscan.org) was used, and ROCK1 was predicted to be a target of miR-335 (Fig. 4A). To verify whether miR-335 directly targeted ROCK1, luciferase reporter assays were performed. As
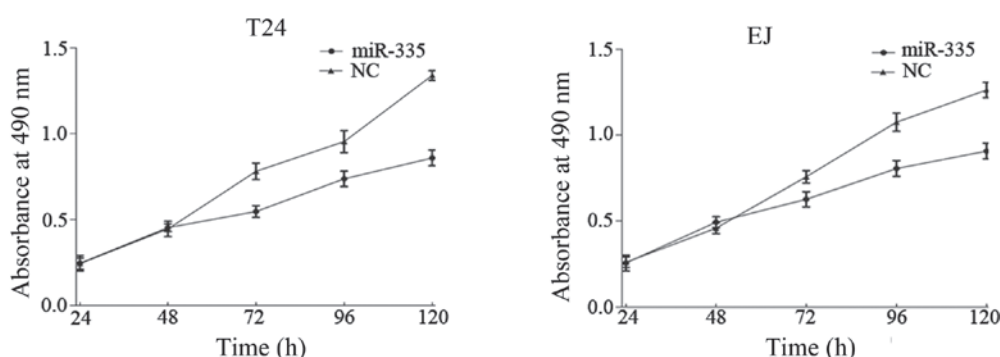
Figure 2. Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Upregulation of miR-335 significantly suppressed cell proliferation in T24 and EJ cells. miR-335, microRNA-335; NC, negative control.
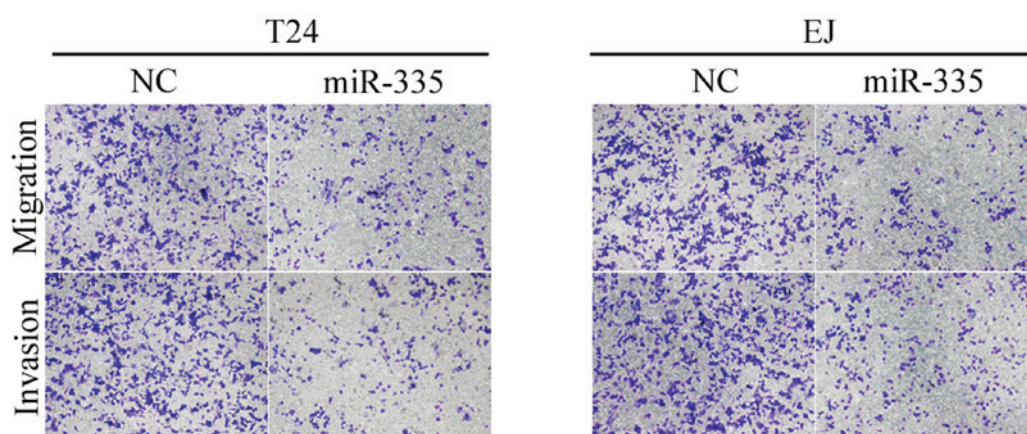

Figure 3. Forced expression of miR-335 suppressed cell migration and invasion in the Transwell assay. T24 and EJ cells were transfected with NC or miR-335 mimics. Cells were then incubated for 12 h for the migration assay and 24 h for the invasion assay. NC, negative control; miR-335, microRNA-335.

demonstrated in Fig. 4B, miR-335 significantly inhibited the wild type, and not the mutated luciferase activity of ROCK1 in the T24 and EJ cells (P<0.05). Furthermore, western blot analysis was conducted to investigate whether ROCK1 protein expression levels were reduced following transfection of miR-335 in the T24 and EJ cells. As demonstrated in Fig. 4C, ROCK1 protein expression levels were significantly downregulated following transfection of miR-335 (P<0.05). The results indicated that ROCK1 is a direct target gene of miR-335 in the bladder cancer T24 and EJ cells.

Discussion

The aberrant expression of miRNAs in bladder cancer has been investigated previously (20). Certain miRNAs were indicated to be upregulated in bladder cancer tissues and may function as tumor oncogenes by negatively regulating tumor suppressors (21). miRNAs are small, stable, easy to deliver and be detected, thus, their abnormal expression may certify them as potential biomarkers and novel targets for bladder cancer therapy (22). However, further studies are required to address the potential diagnostic and therapeutic roles of these miRNAs in bladder cancer, and whether this may be beneficial for the treatment of bladder cancer.

Aberrant expression of miR-335 has been demonstrated in numerous types of tumor, suggesting a complex role during tumorigenesis. Previous studies have demonstrated that it is downregulated in osteosarcoma (15), gastric cancer (16), small cell lung cancer (17), breast cancer (18), ovarian cancer (23), clear cell renal cell carcinoma (24), hepatocellular carcinoma (25) and prostate cancer (26). Furthermore, upregulation of miR-335 has been demonstrated in colorectal cancer (27), glioma (28) and myeloma (29). However, the expression, function and mechanism of miR-335 in human bladder cancer remains to be elucidated. The present study demonstrated that miR-335 was downregulated in human bladder cancer tissues compared with NATs, suggesting that miR-335 may have a tumor suppressive role in bladder cancer development and progression.

Identification of the miR-335 target genes is important for understanding its role in tumorigenesis, and defining novel therapeutic targets. Thus far, dishevelled associated activator of morphogenesis 1 (30), paired box 6 (31,32), ROCK1 (15), retinoblastoma 1 (33), zinc finger E-box binding homeobox 2 (34), met proto-oncogene (35) and OCT4 (36) have been identified as targets of miR-335. The current study demonstrated that miR-335 transfection resulted in reduced cell proliferation, migration and invasion in bladder cancer T24 and EJ cells by targeting ROCK1. These results are in agreement with previous studies in human osteosarcoma (15). The results of the present study suggested that miR-335 may be used for the development of novel molecular markers and
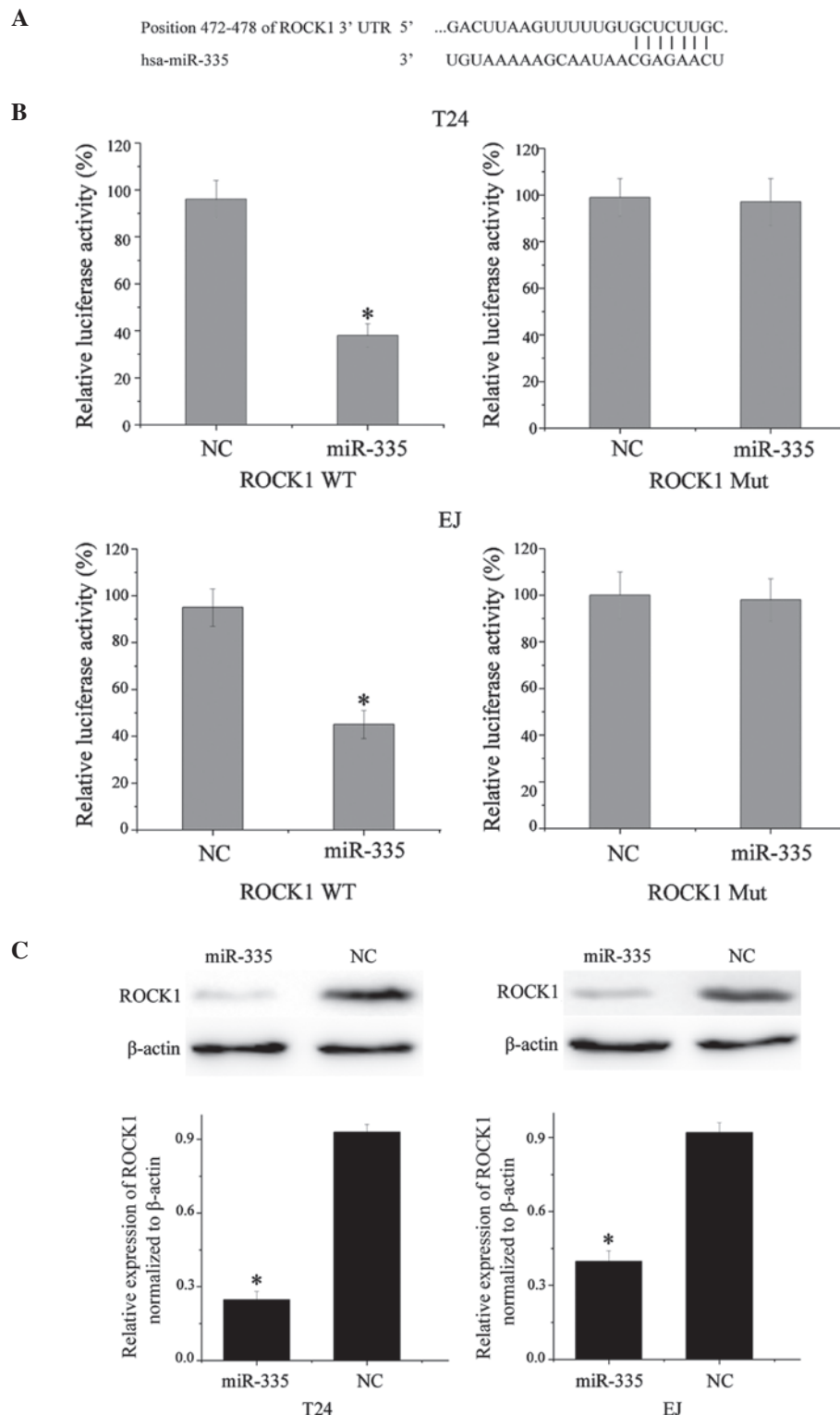
Figure 4. (A) Targetscan assessment indicated that ROCK1 mRNA contained a miR-335 seven-nucleotide seed match at position 472-478 of the ROCK1 3'-UTR. (B) ROCK1 may be a direct target of miR-335 *in vitro*. Overexpression of miR-335 significantly inhibited the WT, however not the Mut, luciferase activity of ROCK1 in T24 and EJ cells. *P<0.05 vs. NC. (C) Downregulation of ROCK1 was confirmed by western blotting, following transfection, β-actin was used as the control. *P<0.05 vs. NC. 3'-UTR, 3 prime untranslated region; hsa-miR-335, human microRNA-335; NC, negative control; ROCK1, rho-associated protein kinase 1; WT, wild type; Mut, mutated.

therapeutic approaches to inhibit the metastasis of bladder cancer.

The Rho guanosine triphosphate (GTP)ase family consists of small proteins that bind to and hydrolyze GTP, and control a wide variety of cellular processes, such as cell motility, proliferation, adhesion, differentiation and apoptosis (37). ROCK is one of the best characterized Rho effectors (38). The two ROCK isoforms, ROCK1 and ROCK2, share 65% identity in amino-acid sequence and 92% identity in their kinase domains, and display certain similarities in the kinase activity

domain (39). Upregulation of ROCK promotes invasion and metastasis in numerous solid tumors, such as bladder (40), hepatocellular (41), breast (42) and colon cancers (43). In addition, overexpression of ROCK1 has been associated with the progression of bladder cancer (40). This is consistent with the results of the present study which demonstrated that exogenetic overexpression of miR-335 inhibited the migration and invasion of human bladder cancer cells. These observations provide the evidence that miR-335 has an effect on cell migration and invasion via regulating the expression of ROCK1.

Human ROCK1 maps to chromosome 18 (18q11.1) (44). Previous studies demonstrated that ROCK1 functioned as an oncogene and was regulated by a number of miRNAs in human cancer. An *et al* (45) and Xu *et al* (46) demonstrated that miR-124 inhibited cell migration and invasion via directly targeting ROCK1 in bladder cancer and glioma. In gastric cancer, miR-148a suppressed cell metastasis by downregulating the expression of ROCK1 (47). ROCK1 was also targeted in other types of human cancer, including miR-335 in neuroblastoma (48), miR-584 in renal cell carcinoma (49) and miR-146a in prostate cancer (50). The current study demonstrated that miR-335 inhibited bladder cancer cell proliferation and motility by downregulation of ROCK1. This may be further investigated as a predictive value for early detection of tumor recurrence and target therapy drugs to prevent bladder cancer from becoming invasive.

In conclusion, to the best of our knowledge, this is the first study to demonstrate that miR-335 is downregulated in bladder cancer. The current study provided evidence that miR-335 suppressed cell proliferation, migration and invasion by directly targeting ROCK1 in bladder cancer. Identifying the candidate target genes of miR-335 may provide an understanding of potential carcinogenic mechanisms in bladder cancer. These observations indicated that miR-335 may serve an important role as a diagnostic and prognostic marker in bladder cancer, and may be exploited for further treatment of bladder cancer.

Further research is required to identify the full potential of miR-335 in cancer treatment, and its benefits in the treatment of bladder cancer.

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