SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 13: 4872-4878, 2016

Silencing of RTKN2 by siRNA suppresses proliferation, and induces G1 arrest and apoptosis in human bladder cancer cells

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Received March 10, 2015; Accepted December 21, 2015

DOI: 10.3892/mmr.2016.5127

Abstract. Human bladder cancer is the most common urological malignancy in China. One of the causes of carcinogenesis in the cancer may be gene mutation. Therefore, the present study investigated the expression levels of Rhotekin 2 (RTKN2), a Rho effector protein, in human bladder cancer tissues and cell lines, and examined the effect of RTKN2 on the proliferation, cell cycle, apoptosis and invasion of human bladder cancer cell lines. The mRNA expression levels of RTKN2 in 30 human bladder cancer tissue samples were significantly higher, compared with those in 30 normal human bladder tissue samples. The protein expression levels of RTKN2 was markedly higher in T24 and 5637 cells, compared with those in four other human bladder cancer cell lines. The silencing of RTKN2 by small interfering (si)RNA inhibited cell proliferation and arrested cell cycle at the G1 phase, via reducing the expression levels of the MCM10, CDK2, CDC24A and CDC6 cell cycle-associated proteins in the T24 and 5637 cells. Furthermore, RTKN2 knockdown in the cells led to cell apoptosis and the suppression of invasion. These results suggested that RTKN2 is involved in the carcinogenesis and progression of human bladder cancer, indicating that RTKN2 may be a molecular target in cancer therapy.

Introduction

Human bladder cancer has been ranked the seventh most common type of cancer worldwide, the fourth most common type of cancer in men, and the eighth most common type of cancer in women, in economically developed countries (1). However, human bladder cancer is the most common type of urological tumor in China (2), and its frequency has been rapidly increasing in previous years. Therefore, elucidating the carcinogenesis of cancer is important, not only for prevention and prognosis, but also for treatment.

Rhotekin 2 (RTKN2) is a novel Rho effector, which is expressed at high levels in lymphocytes, particularly freshly isolated $CD4^+$ T-cells; and is switched off in activated T-cells (3). The two RTKN proteins, RTKN1 and RTKN2, have homologues in the majority of mammals, including humans, chimpanzee, horses, dogs and rats; and each of the proteins has an N-terminal Rho-GTPase binding domain (4). Although the amino acids are only 65% homologous, the similar protein architecture indicates that they likely share functional characteristics. Previous studies have shown that RTKN2 is expressed at high levels in organs containing sites of lymphopoiesis, including the thymus, spleen, lung, colon and bone marrow (5,6). In addition, stable and low endogenous expression of RTKN2 in HEK cells enhances the survival and suppression of RTKN2 by small interfering (si)RNA in primary human $CD4^+$ T-cells, with high expression levels of RTKN2 reducing viability and increasing sensitivity to 25-OHC (7), which directly associates with apoptosis in several cell types, including hematopoietic and leukemic cells (8-10). These findings suggest an involvement of RTKN2 in cancer progression. However, the expression pattern and biological functions of RTKN2 in human bladder cancer remain to be fully elucidated.

As its gene expression suggests that RTKN2 is important in the development and progression of human bladder cancer, the present study investigated the effects of RTKN2 knockdown on the proliferation, cell cycle, apoptosis and invasion of human bladder cancer cells, and the potential underlying mechanism was examined. The results of these investigations may provide evidence for the upregulation of RTKN2 in human bladder cancer, and may offer potential as an effective therapeutic target for the disease.

Materials and methods

Patients and tissue samples. Tumor and normal human bladder cancer specimens were obtained from 30 patients with bladder cancer (gender, 5 women and 25 men; age range, 44-85 years; median age, 71 years), who underwent surgery at Jingzhou Central Hospital (Jingzhou, China). Among them, 63.3% was

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Key words: human bladder cancer, RTKN2, cell cycle, G1 arrest, apoptosis, invasion

found to have stage II bladder cancer, and 36.7% stage III. The study protocol was approved by the ethics committee of Jingzhou Central Hospital. Written informed consent was obtained from all the individuals involved in the present study, and all investigations were performed in accordance with the Helsinki Declaration of 1975 (11). No patients had received radiotherapy or chemotherapy. Thirty paired normal and bladder cancer tissue samples (0.5 cm^3) were collected from October 2010 to February 2013. The normal bladder tissues were resected within ≥5 cm of the tumor margin during surgery. For histological analysis, resected normal and bladder cancer tissues were fixed in formalin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), embedded in paraffin and Sinopharm Chemical Reagent Co., Ltd.) cut into 5-μm thick sections. The percentage of tumor cellularity in the bladder tissue sections obtained from the patients was at least 70%, determined via pathological examination of histology slides in the patient cohort. The human bladder cancer and normal tissues were immediately snap-frozen in liquid nitrogen (Air Products and Chemicals, Inc., Shanghai, China) and stored at -80˚C until total RNA was extracted. The tumor samples comprised at least 80% viable-appearing tumor cells on histological assessment.

Cell culture and transfection with RTKN2 siRNA. The T24, 5637, BIU-87, J28, ScaBER and UM-UC-3 bladder cancer cell lines were cultured in RPMI 1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All culture media were supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 mg/ml penicillin G and 50 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were maintained at 37˚C in 5% CO_2. RTKN2 siRNA was used to target RTKN2. The siRNA target position was 1144-1166 (5'-TGGTAGAAGGTCTGATTAG-3') in human RTKN2 mRNA. The cells were transfected with siRNA (40 nM) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Nonspecific siRNA was used as a negative control (NC), and the selective silencing of RTKN2 was confirmed using polymerase chain reaction (PCR) analysis. The cells were analyzed 48 h following transfection. The siRNAs were obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

Cell proliferation assay. Cell proliferation was measured using a Cell Counting Kit 8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, the control, NC and RTKN2 siRNA-treated cells were seeded onto 96-well plates at an initial density of $5x10^3$ cells/well. At specified time points (at 0, 12, 24, 48 and 72 h), 10 μl of CCK-8 solution was added to each well of the plate. Then the plate was incubated for 1 h at 37˚C. Cell proliferation was determined by scanning with a microplate reader (SM600 Labsystem; Shanghai Utrao Medical Instrument Co., Ltd., Shanghai, China) at 450 nm.

Cell cycle assay. Cell proliferation was measured by propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) and flow cytometery (BD Accuri™ C6 version 1.0.264.21 software; BD Biosciences, Franklin Lakes, NJ, USA) BD Biosciences, San Diego, CA, USA). Approximately $1x10^6$ cells were removed at specified time points, washed twice with phosphate-buffered saline (PBS), fixed in cold ethanol for 30 min, and then incubated with PI for 30 min at 37˚C.. The cells were then analyzed by flow cytometry.

Cell apoptosis assay. Apoptosis was determined by flow cytometric analysis. The cells were collected following treatment with RTKN2 siRNA. Annexin-V fluorescein isothiocyanate (FITC)/PI double staining assays (Biovision, Inc, Mountain View, CA, USA) were performed, according to the manufacturer's protocol. The floating and trypsinized (trypsin; JRDUN Biotech Co., Ltd., Shanghai, China) adherent cells were collected and resuspended in 500 μl binding buffer, containing 2.5 μl annexin-V FITC and 5 μl PI, following which the cells were incubated for 10 min in the dark at room temperature prior to flow cytometric analysis.

In vitro invasion assay. The upper well of a Transwell (Corning, Corning, NY, USA) was coated with Matrigel (BD Biosciences) at 37˚C in a 5% CO_2 incubator for 1 h. The T24 and 5637 cells were serum-starved for 24 h, following which $5x10^4$ cells in 500 μl serum-free RPMI 1640 were seeded into the upper well of the Transwell chamber. Culture medium supplemented with 10% FBS (750 μl) was added to the lower well of the chamber. After 48 h, the cells in the upper well were removed with a cotton swab. The cells that had migrated into the lower well were washed with PBS (JRDUN Biotech Co., Ltd.), fixed in 3.7% paraformaldehyde (paraformaldehyde (Beinuo Biotech Co., Ltd., Shanghai, China) and stained by 0.2% crystal violet (JRDUN Biotech Co., Ltd.). Images of the cells were captured and the number of cells were counted under a microscope (CX41RF; Olympus Corporation, Tokyo, Japan).

RT-qPCR. Total RNAs were extracted from the normal and human bladder cancer tissues or cell lines using 1 μl TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), as described previously (12). Briefly, they were centrifuged at 400 x g at 25˚C for 20 sec for homogenization and immediately placed in ice, and then centrifuged at 400 x g at 4˚C for 10 min and stored at -80˚C prior to RT-qPCR. Complementary (c)DNA (2 μl) was synthesized using RevertAid First Strand cDNA Synthesis kit (K1622; Thermo Fisher Scientific, Inc.). Maxima SYBR Green/ROX qPCR Master Mix (K0223; Finnzymes Oy, Espoo, Finland) was used, according to the manufacturer's protocol. qPCR was performed to detect the mRNA levels of the indicated genes. An MxPro™ qPCR system (version 4.10; Agilent Technologies, Santa Clara, CA, USA) was used. The primer sequences used were as follows: RTKN2, forward 5'-ACAGTTCGCGTTGGAGATGGAG-3' and reverse 5'-GTC GAGCATTGCACACCATGAG-3'; and GAPDH, forward 5'-CACCCACTCCTCCACCTTTG-3' and reverse 5'-CCA CCACCCTGTTGCTGTAG-3'. The PCR cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 45 sec, and a final extension step of 95˚C for 15 sec, 60˚C for 1 min, 95˚C for 15 sec and 60˚C for 15 sec. The thermal cycler used was an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) Relative quantification of the signals was performed by normalizing the signals of different genes with that of GAPDH. The gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (13).

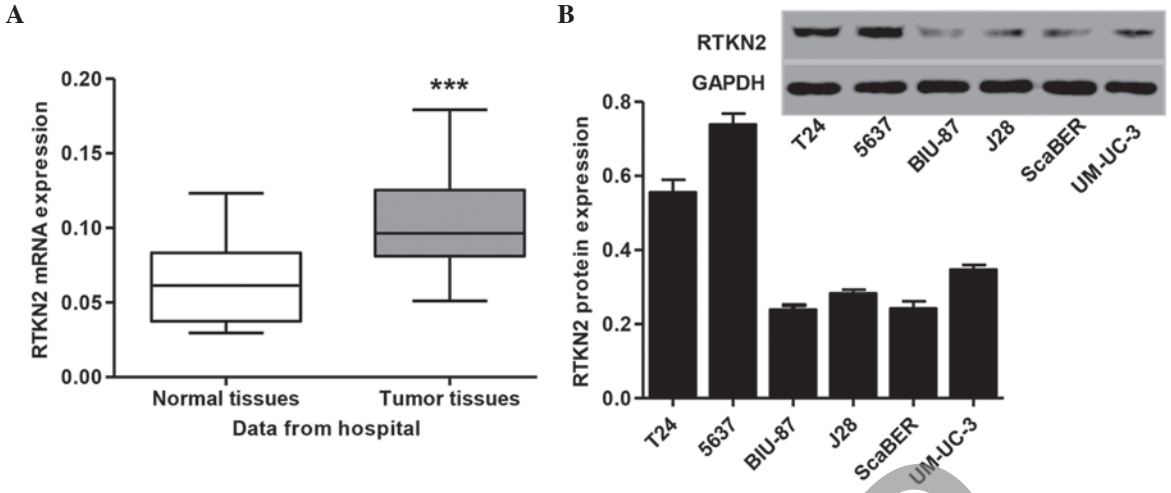

Figure 1. Expression of RTKN2 in human bladder cancer tissues and cell lines. (A) mRNA expression of RTKN2 was significantly higher in human bladder cancer tissues, compared with normal human bladder cancer tissues. (B) Protein expression levels of RTKN2 in the T24 and 5637 cells were higher, compared with four other human bladder cancer cell lines, and were selected for further analysis. ***P<0.0001. Data are expressed as the mean ± standard deviation. RTKN2, Rhotekin 2.

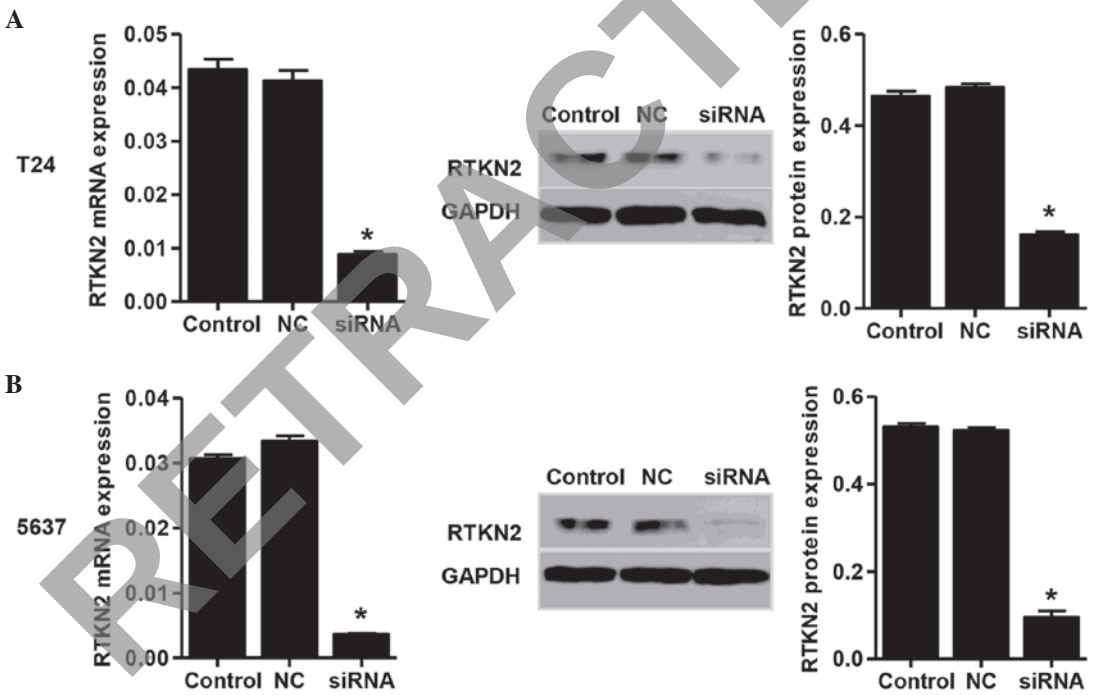

Figure 2. Silencing of RTKN2 by siRNA in T24 and 5637 cells. Reverse transcription-quantitative polymerase chain reaction and Western blot analyses revealed that the expression levels of RTKN2 were significantly inhibited by RTKN2 siRNA in the (A) T24 and (B) 5637 cells. *P<0.01. Data are expressed as the mean ± standard deviation. RTKN2, Rhotekin 2; NC, negative control; siRNA, small interfering RNA.

Western blot analysis. Western blot analysis was performed according to standard procedures. The total proteins were isolated from the tumor samples and corresponding normal tissues, and the human bladder cancer cell lines using radio-immunoprecipitation buffer (Amyjet Scientific, Inc. Wuhan, China) at 10 min at 95˚C and then centrifuged at 400 x g at 25˚C for 10 min. Total protein (50 μg) was separated using 10 (for higher molecular weight proteins) or 15% (for lower molecular weight proteins) sodium dodecyl sulfate polyacrylamide gel electrophoresis (Amyjet Scientific Inc.). Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins were then transferred to polyvinylidene difluoride membranes (Sigma-Aldrich), which were blocked with fat-free milk for 1 h at room temperature (25˚C) and subsequently incubated with primary antibodies against RTKN2 (mouse monoclonal; cat. no., ab118069; dilution, 1:1,000; Abcam, Cambridge, MA, USA), MCM10 (rabbit polyclonal antibody; cat. no., ab3733 dilution, 1:1,000; Abcam), CDK2 (rabbit monoclonal antibody; cat. no., ab32147 dilution, 1:1,000; Abcam), CDC24A (mouse polyclonal; cat. no., 3652 dilution, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and CDC6 (rabbit

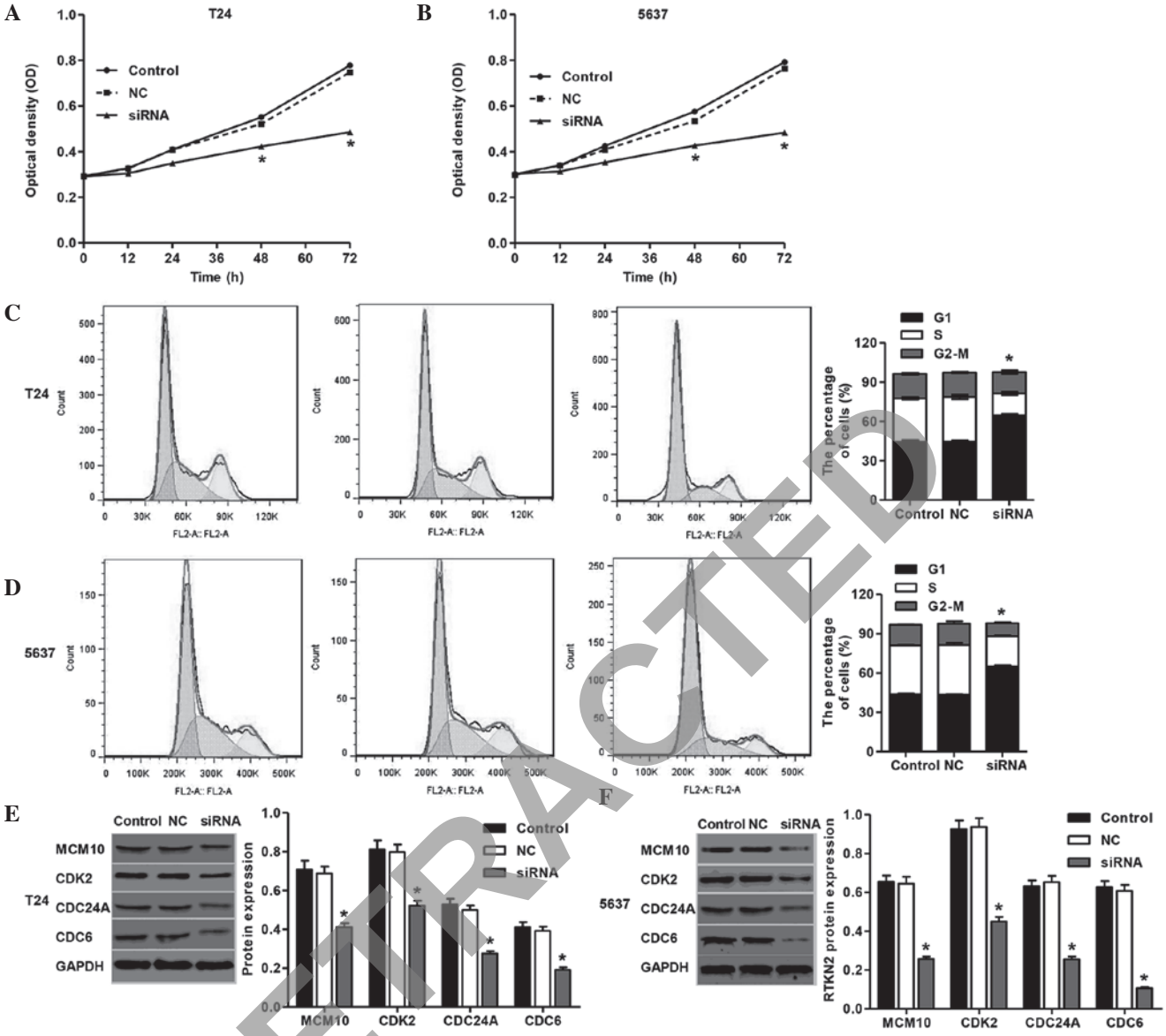

Figure 3. Knockdown of RTKN2 inhibits cell proliferation and arrests cell cycle at the G1 phase. Analysis using a Cell Counting Kit-8 identified significant inhibition in the proliferation of (A) T24 and the (B) 5637 cells. Flow cytometric analysis identified significant arrest at the G1 phase of the cell cycle in the (C) T24 and (D) 5637 cells. Western blot analysis showed that the expression levels of cell cycle-associated proteins were significantly decreased in the (E) T24 and (F) 5637 cells. P<0.01 compared with the control. Data are expressed as the mean ± standard deviation. RTKN2, Rhotekin 2; NC, negative control; siRNA, small interfering RNA.

monoclonal cat. no., 3387 dilution, 1:1,000; Cell Signaling Technology, Inc.,) for 2 h at 25˚C, or anti-GAPDH antibody (cat. no. 5174S; dilution, 1:1,000; Cell Signaling Technology, Inc.,) for 2 h at 25˚C, which was used as a loading control. The membranes were subsequently washed three times with Tris-buffered saline with Tween 20 (Amresco, Solon, OH, USA). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. A0208; 1,000; Beyotime Institute of Biotechnology, Haimen, China) and goat anti-mouse IgG (cat. no. A0216; 1:1,000; Beyotime Institute of Biotechnology) secondary antibodies for 1 h at 37˚C, and washed three times with Tris-buffered saline with Tween 20 (Amresco). Signals were detected by incubation with secondary antibodies labeled with horseradish peroxidase, and signal intensity was determined using ImageJ 1.46 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. The significant differences between groups were analyzed using unpaired two-tailed Student's t-test. Statistical analysis were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

RTKN2 is upregulated in human bladder cancer tissues and cell lines. The microarray expression profile of human bladder cancer in the present study indicated that RTKN2 was expressed at high levels in human bladder cancer (data not shown). To further verify this finding, RT-qPCR analysis was performed on 30 pairs of human bladder cancer tissues and normal tissues samples, obtained from patients at

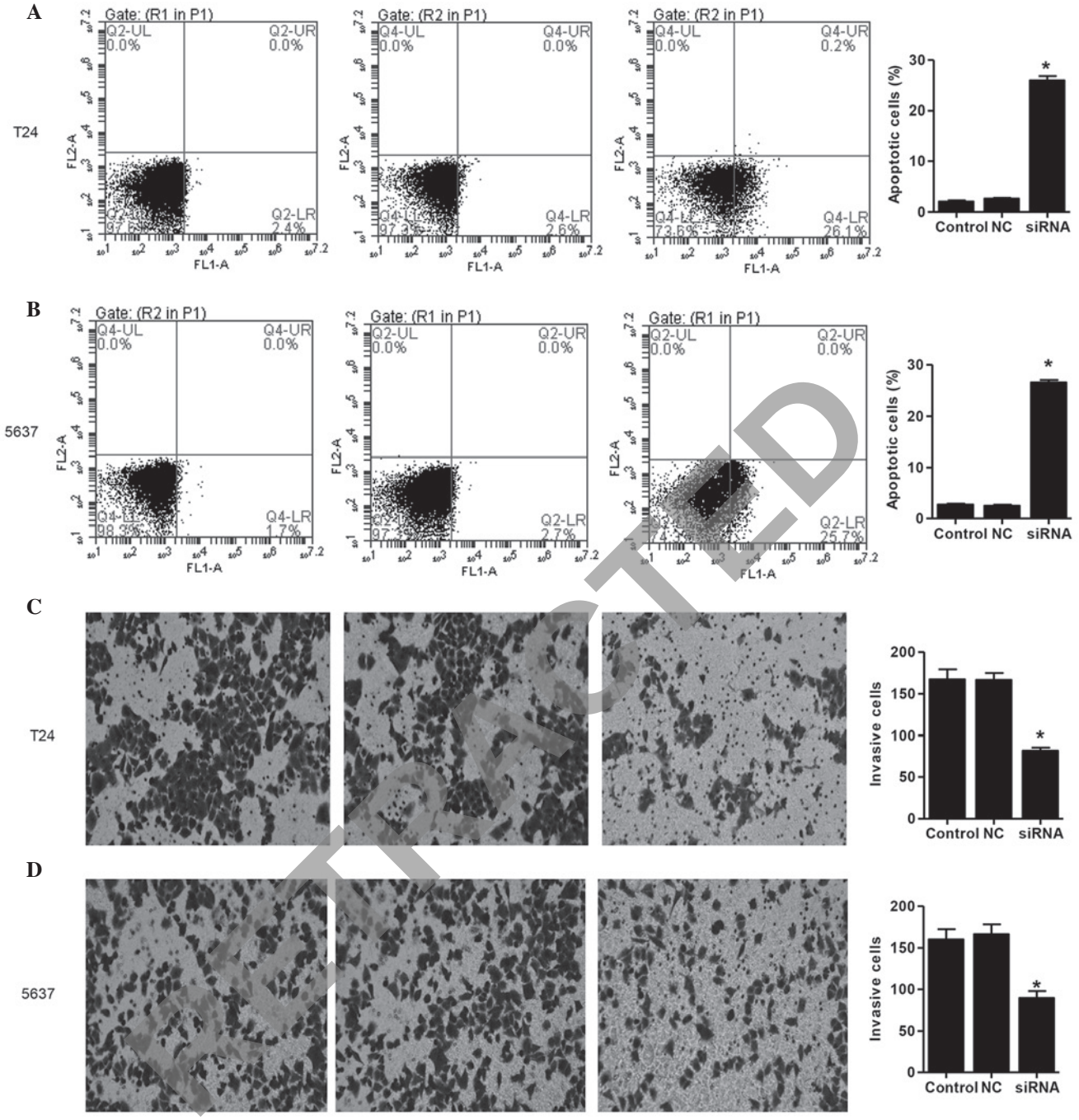

Figure 4. Knockdown of RTKN2 induces cell apoptosis and inhibits cell invasion. (A) T24 and (B) 5637 cells were stained with annexin V-fluorescein, and apoptotic cells were analyzed using flow cytometry. Lower left quadrant, normal cells; lower right quadrant, early apoptotic cells; upper right quadrant, late apoptotic cells; upper left quadrant, necrotic cells. Invasion assays of the (C) T24 and (D) 5637 cells were performed using a Transwell chamber coated with Matrigel. The cells, which migrated from the upper well of a Transwell chamber into the lower well were stained, images were captured and the numbers of cells were counted. Magnification, x200. *P<0.01, compared with the control. Data are expressed as the mean ± standard deviation. RTKN2, Rhotekin 2; NC, negative control; siRNA, small interfering RNA.

Jingzhou Central Hospital. The mRNA levels of RTKN2 was significantly increased in the human bladder cancer tissues, compared with those in the normal tissue samples (Fig. 1A). The present study also examined the protein levels of RTKN2 in human bladder cancer cell lines. The results showed that the protein expression levels of RTKN2 were markedly higher in the T24 and 5637 human bladder cancer cell lines, compared with the BIU-87, J28, ScaBER and UM-UC-3 cell lines (Fig. 1B). Therefore, T24 and 5637 cells were selected for the subsequent assays.

RTKN2 is knocked down by siRNA in T24 and 5637 cells. To investigate the functions of RTKN2 in human bladder cancer, an siRNA was designed and transfected into the T24 and 5637 cells. The mRNA and protein expression levels of RTKN2 in response to the specific siRNA were then assessed. The mRNA and protein expression levels of RTKN2 were reduced by 78±3.9 and 67±2.1% in the T24 cells transfected with siRNA, respectively (Fig. 2A). In the 5637 cells transfected with siRNA, the reductions in the mRNA and protein expression levels of RTKN2 were 89±4.2 and 81±3.1%, respectively.

(Fig. 2B). No significant changes were identified in the NC group.

Knockdown of RTKN2 inhibits cell proliferation and induces G1 cell cycle arrest in T24 and 5637 cells. To substantiate the role of RTKN2 on the proliferation of human bladder cancer cells, the present study detected the proliferation of T24 and 5637 cells transfected with RTKN2 siRNA using a CCK-8 assay. As shown in Fig. 3A and B, cell proliferation was reduced by 36±1.8 and 39±1.9% 72 h following siRNA transfection in the T24 and 5637 cells, respectively.

The present study then determined the possible inhibitory effect of RTKN2 knockdown on cell cycle progression. In the absence of RTKN2 siRNA, the populations of cells in the G1, S and G2 phases were determined. Transfection of the cells with siRNA was accompanied by a concomitant increase in the G1 phase population in the T24 cells (44±1.9%) and 5637 cells (50±2.1%), as shown in Fig. 3C and D. These results suggested that RTKN2 knockdown induced G1 cell cycle arrest in the T24 and 5637 cells, which may be associated with the inhibition of cell proliferation. Western blot analysis was performed to detect cell cycle-correlated proteins in the T24 and 5637 cells. RTKN2 knockdown resulted in significant reductions in the levels of MCM10, CDK2, CDC24A and CDC6, in the T24 and 5637 cells, compared with the NC group of cells (Fig. 3E and F). These data obtained in the present study suggested that the silencing of RTKN2 inhibited the expression of cell cycle-associated proteins, which may have contributed to the induction of G1 cell cycle arrest.

Knockdown of RTKN2 induces cell apoptosis and inhibits invasion of T24 and 5637 cells. To examine the effects of RTKN2 on cell apoptosis, Annexin V/PI staining was performed. The ratio of cells undergoing apoptosis was significantly increased, by 26±0.88%, in the RTKN siRNA-treated T24 cells, and by 27±1.1% in the RTKN siRNA-treated 5637 cells, compared with the NC group (2.5%; Fig. 4A and B). These data suggested that RTKN2 may have an important anti-apoptotic role in human bladder cancer cells.

To investigate whether RTKN2 affected the invasive ability of the human bladder cancer cells, a Matrigel-coated membrane chamber invasion assay was performed. As shown in Fig. 4C and D, marked reductions in invasive ability were observed in the RTKN2-knockdown T24 and 5637 cells, respectively, compared with the NC group. The numvers of invasive RTKN2 siRNA-treated T24 and 5637 cells were 51±2.1 and 46±1.7% of that in the NC group, respectively.

Discussion

RTKN belongs to the group of proteins containing a Rho-binding domain, which are target peptides (effectors) for Rho-GTPases (14). A previous study identified a novel cDNA exhibiting homology with human RTKN, designated RTKN2, and was observed in the cytosol and nucleus of CHO cells (4). The involvement of RTKN2 in different types of cancer has been an area of interest, and different expression levels of RTKN2 have been reported in several types of cancer and cells (15-17). The molecular mechanisms underlying the development and progression of human bladder cancer remain to be fully elucidated. In the present study, RT-qPCR analyses of data from patients at Jingzhou Central Hospital indicated that RTKN2 was expressed at high levels in human bladder cancer tissues (Fig. 1A). Western blot analysis showed that the expression levels of RTKN2 were significantly higher in T24 and 5637 human bladder cancer cells, compared with the four other cell lines (Fig. 1B).

In the present study, the expression of RTKN2 was knocked down in the T24 and 5637 cells by siRNA (Fig. 2). Suppression of the expression of RTKN2 notably inhibited the proliferation (Fig. 3A and B) and invasion (Fig. 4C and D) of the T24 and 5637 cells, which was consistent with a previous study on human $CD4^+$ T-cells (5). The data further indicated the roles of RTKN2 in human bladder cancer carcinogenesis.

Cell cycle regulation is frequently abnormal in the majority of types of common malignancy, resulting in aberrant cell proliferation (18,19). In the present study, the knockdown of RTKN2 by siRNA significantly induced G1 cell cycle arrest in the T24 and 5637 cells (Fig. 3C and D), which indicated that the inhibition of cell proliferation in human bladder cancer cells was due to the arrest of cell cycle progression. The cell cycle is mediated, directly or indirectly, by misregulation of cyclin-dependent kinases (CDKs) (20). However, only certain CDK-cyclin complexes are considered to control cell cycle progression (21). Experimental evidence suggests that certain human cancer cell lines exhibit a selective dependence on interphase CDKs. For example, whereas colon cancer cell lines efficiently proliferate in the absence of CDK2, the downregulation or inhibition of this kinase in cell lines derived from glioblastomas and osteosarcomas prevents their proliferation (22,23). Cell division cycle 6 (CDC6) is an essential regulator of DNA replication in eukaryotic cells. The expression of CDC6 at the end of mitosis suggests the protein is involved during the G1 phase (24,25). It was subsequently found that the downregulation of CDC6 by RNA interference prevents cell proliferation and promotes apoptosis (26,27). CDC24A is another regulator of DNA replication, the phosphorylation of which by checkpoint kinase (CHK)1 and CHK2 induces the intra-S-phase checkpoint, allowing repair of the DNA in the phase of the cell cycle (28). Human minichromosome maintenance 10 (MCM10) is an essential protein in chromosomal DNA replication (29), which is decreased in the late M and G1 phases, and accumulates in the S phase (30). In the present study, the mRNA expression levels of the MCM10, CDK2, CDC24A and CDC6 proteins were notably decreased in the T24 and 5637 cells treated with RTKN2 siRNA (Fig. 3E and F), which was consistent with the results showing the induction of G1 cell cycle arrest in the T24 and 5637 cells treated with RTKN2 siRNA. This indicated the presence of an association between the function of RTKN2, and the regulation of DNA replication and cell cycle progression in human bladder cancer cells.

G1 phase arrest in cell cycle progression provides an opportunity for cells to either undergo repair or enter the apoptosis process (31). In the present study, the effects of RTKN2 knockdown on the induction of apoptosis were determined in the T24 and 5637 cells. The flow cytometry data indicated that the silencing of RTKN2 resulted in significant induction of apoptosis (Fig. 4A and B), which was consistent with previous studies on lymphocytes (3,5), human leukemic cells (6) and a

human keratinocyte line (16). Due to its anti-apoptotic role in human bladder cancer, RTKN2 may be a potential therapeutic target, which merits further investigation.

In conclusion, the results of the present study indicated that RTKN2 was upregulated in human bladder cancer tissues and cell lines. Silencing of RTKN2 inhibited proliferation and invasion, and induced cell apoptosis. In addition, knockdown of RTKN2 arrested the cell cycle at the G1 phase via inhibition of the expression of cell cycle-associated proteins. These data suggested that RTKN2 may be a tumor promoter gene and may provide an effective therapeutic target in the treatment of bladder cancer in humans.

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