Rab11 Functions as an Oncoprotein via Nuclear Factor kappa B (NF-κB) Signaling Pathway in Human Bladder Carcinoma

Authors' Contribution:
Study Design **A**
Data Collection **B**
Statistical Analysis **C**
Data Interpretation **D**
Manuscript Preparation **E**
Literature Search **F**
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Background: Elevated expression of Rab11 has been reported in different human cancers, including human bladder carcinoma. This study, we investigated the biological effects and mechanism of Rab11 overexpression in human bladder carcinoma for the first time.

Material/Methods: Rab11 expression in bladder cancer tissues was detected using immunohistochemistry and Western blot analysis. Then, Rab11 expression was inhibited in T24 cells and it was overexpressed in BIU-87 cells. The effects of Rab11 perturbations on cell growth rate and invasion were analyzed by CCK8, cell cycle assay, and matrix gel invasion assay. MMP-9, cyclin E, and cyclin D1 levels were studied using Western blot and qPCR. NF-κB activity was studied by luciferase assay.

Results: High expression of Rab11 was detected in 41.5% (66/159) of tumor specimens. We found a significant correlation between high Rab11 expression and depth of tumor invasion (P=0.004). Rab11 overexpression was observed to promote the growth rate and invasiveness of cancer cells through upregulation of MMP9, cyclin E, and cyclin D1 levels. Rab11 overexpression further elevated NF-κB reporter activity and enhanced p-IκB expression. Use of BAY 11-7082, a noted NF-κB inhibitor, partially abolished overexpression of MMP9 and cyclin D1 by Rab11.

Conclusions: Our research proved that high Rab11 expression enhances cellular multiplication and invasiveness of bladder cancer, possibly by regulating the NF-κB signaling pathway.

MeSH Keywords: **Cell Proliferation • Neoplasm Invasiveness • NF-kappa B • rab GTP-Binding Proteins • Urinary Bladder Neoplasms**

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Background

Bladder urothelial carcinoma (BLCA) is the most common form of bladder cancer and its incidence is increasing [1]. It ranks as the ninth most common malignant tumor in women and the fourth most common malignant tumor in men in the United States [2]. BLCA is also the most lethal malignant tumor of the genitourinary track in China. Approximately 70% of BLCA is non-muscle invasive bladder cancer (NMIBC) and about 30% of BLCA is muscle invasive bladder cancer (MIBC), which presents a poor prognosis [3]. Compared with their NMIBC counterparts, MIBC patients showed a higher risk of tumor progression and cancer-related death. The 5-year survival rate of MIBC patients is about 50% [4]. The prognosis is poorest for patients with distant metastasis. Patients with metastatic tumors have a 5-year overall survival rate of only about 20% [5]. Thus, the search for novel molecular targets holds a very important clinical significance.

Growing evidence indicates Rab protein is an essential factor in the process of endocytosis. Rab is a family of small-molecule GTPases that function as a regulator of protein trafficking, vesicular transport, membrane targeting, and fusion. Vesicle trafficking and dynamics are essential for regulation of crucial cell behaviors linked with cell migration and tumorigenesis [6–8]. Rab11 is a member of the Rab family. Recent literature underlines the possibility of regulating the formation and transport of recirculating endosomes [9–12]. Abnormal Rab11 expression is found in diverse cancer types, including esophageal adenocarcinoma, skin cancer, breast cancer, and colorectal cancer. High expression of Rab11 is associated with multiple biological processes of malignant tumors, such as tumor formation, tumor progression, and poor prognosis [13–17]. However, the biological significance of Rab11 levels in BLCA has remained unexplored.

In the present study, we assessed Rab11 levels in BLCA and analyzed the correlation between altered Rab11 expression and clinicopathologic factors. We overexpressed Rab11 in BIU-87, abrogated Rab11 expression in T24, and examined the biological effect of Rab11 on rates of cell growth and invasion. Finally, we investigated the Rab11-regulated molecular signaling pathways.

Material and Methods

Patients and specimens

We collected 159 normal matched BLCA specimens from patients who underwent transurethral resections of bladder tumors or radical cystectomy in China Medical University (CMU) from 2011 to 2014. The study protocol was approved by the CMU Ethics Committee.

Immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) tumor samples from patients were cut into sections 4 micrometers thick. FFPE specimens were immunostained using a staining kit from MaiXin, following the manufacturer's protocol. Deparaffinized sections were first heated in a 95 water bath for 30 min. Hydrogen peroxide was used to block nonspecific immunostaining for 15 min. The sections were treated with Rab11 primary antibody (1: 300, Proteintech, USA) at 4 overnight, followed by incubation with secondary antibody conjugated with HRP for 2 h.

Pathologists examined all slides (5 views per slide, 100 cells per view) in a random manner. The evaluated slides were assigned a histological score (using a semiquantitative method) based on intensity of cytoplasmic staining, and the number of cells staining positively were counted. The intensity of staining was assigned scores as follows: 0 (no staining), 1 (for moderate), and 2 (for intense). Cells that stained positively were also marked (depending on the count of positive cells) as 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The values of staining intensity and percentage of positively stained cells were multiplied to get total histological score on a scale of 0 to 8 and the total scores ascribed to different grades of protein expression. Net scores less than 4 were considered as low expression of Rab11 while scores greater than 4 were considered overexpression of the protein.

Cell culture and transfection

T24, RT4, 5637, and BIU-87 cells were procured from ATCC (Manassas, VA, USA) and cultured using 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen). Passaging was done every 2 days by trypsinization using 0.25% trypsin solution (Invitrogen).

siRNA Cells were transfected using DharmaFECT1 as transfection reagent (GE Healthcare, USA). ONTARGETplus siRNA against Rab11 and nontargeting siRNAa (Dharmacon, GE Healthcare, USA) were used for knockdown studies.

Attractene transfection reagent (Qiagen, Hilden, Germany) was used to transfect pCMV6-Rab11 plasmid (Origene, USA), and pCMV6 empty vector was used as control.

BAY 11-7082 (Sigma, USA) was used to inhibit NF-κB pathway in BIU-87 at a final concentration of 5 μM for 5 h.

Western blot analysis

Lysis buffer (Pierce, Rockford, IL) was used to lyse fresh tissues and total protein content was estimated by Bradford assay.

About 20 µg of lysate was resolved in 10% SDS-PAGE, blotted onto a PVDF membrane, and incubated with antibodies. The following antibodies against target proteins were used: Rab11 (1: 800, Proteintech, USA), MMP9, cyclin E, cyclin D1, IκB, p-IκB (1: 1000, Cell signaling, USA), β-actin (1: 2000, Santa Cruz). Membranes were incubated with primary antibodies overnight at 4°C followed by treatment with HRP-conjugated secondary antibody (1: 2000, Cell Signaling Technology, USA) and incubated for 2 h at 37°C. Specific proteins were detected using chemiluminescent substrate ECL (Pierce, USA) and signal-documented in the Chemiluminescent BioImaging System (DNR, Israel).

CCK-8 assay

Proliferation of cells was studied using the CCK-8 kit (Dojindo, MD) following the manufacturer's instructions. At 48 h after transfection, cells were seeded at a density of 6×10^3 cells/well in 96-well plates. The cells in every well were treated with 10 µL CCK-8 reagent every day and cultured for 4 h at 37°C in a humidified incubator in 5% CO_2. The absorbance values were measured by a microplate reader at 490 nm.

Cell cycle analysis

At 48 h after transfection, cells were treated using 1% paraformaldehyde for fixation and then washed with PBS. We used 5 mg/ml of propidium iodide to stain the fixed cells by incubation for 30 min at room temperature. Cell cycle analysis was determined using a FACS Calibur flow cytometer (Becton Dickinson, USA).

Transwell invasion assay

Matrigel invasion assay was performed using a 24-well Transwell chamber (Corning, USA). The chamber was coated with 20 µL of matrigel at a dilution of 1: 4 (BD Bioscience, USA). At 48 h after transfection, cells were resuspended in 1640 medium without serum and 200 µL (1×10^5/ml) suspension cells were seeded in the top chamber. The lower compartment contained 800 µL 1640 medium supplemented with 20% fetal bovine serum. After incubation for 24 h, the cells that had invaded the Matrigel layer were fixed and stained with hematoxylin.

Quantitative real-time PCR

Total cellular RNA was extracted using Trizol reagent as per standard protocol. PCR was performed in the 7500 Real-Time PCR System (Applied Biosystems, USA). Quantitative RT-PCR was performed from extracted RNA using a SYBR Green master mix kit (Applied Biosystems, USA). The expression values were normalized against the reference gene β-actin. The normalized expression value was calculated as ΔCt=Ct gene–Ct reference. The relative quantification was obtained by the formula: $2^{-\Delta\Delta Ct}$.

Luciferase promoter activity study

Bladder cancer cells were transfected with NF-κB reporter vector (Beyotime Biotechnology, China) and Renilla luciferase reporter plasmid (Promega, USA) using Attractene (Qiagen, Germany). Promoter activity was measured using a dual luciferase reporter kit (Promega, USA) as per the manufacturer's protocol in cellular lysates 30 h after transfection.

Statistical analysis

All data were analyzed with SPSS 19 statistical software. The χ^2 test was used to analyze categorical variables and the t test was used to compare the rest of the data. $p<0.05$ was considered to be statistically significant.

Results

Rab11 expression in BLCA samples and cell lines

Rab11 level was examined in 159 BLCA samples and respective adjacent normal tissue using immunohistochemistry. The expression of Rab11 remained undetected in normal bladder urothelium (Figure 1A). Of the 159 patients studied, high expression of Rab11 was detected in 66 specimens (41.5%) and was mainly localized in the tumor cytoplasm (Figure 1B, 1C). Rab11 protein levels were examined in 10 cases of fresh normal matched tumor specimens through immunoblotting. The results showed higher Rab11 expression in bladder cancer samples compared to adjacent normal counterparts (Figure 1D).

We further investigated the correlation between elevated expression of Rab11 and clinicopathological characteristics (Table 1). We found no significant differences between high expression of Rab11 and the factors of age, sex, and tumor grade. Interestingly, high expression of Rab11 was correlated with local invading depth (Ta–T1 *vs*. T2–T4, p=0.004).

Subsequently, we studied Rab11 expression levels in BIU-87, T24, 5637, and RT4 cells through Western blot analysis. Rab11 expression was observed to be high in T24, 5637, and RT4 cell lines but BIU-87 exhibited relatively low levels of the protein (Figure 2A). The molecular role of Rab11 was investigated by increasing Rab11 expression in BIU-87 and silencing Rab11 expression in the T24 cell lines. Finally, we tested the efficiency of transfection by immunoblotting (Figure 2B).

Regulation of cell proliferation and cell cycle progression by Rab11

We used CCK8 assay to study Rab11-regulated tumor cell proliferation. Rab11 overexpression was observed to promote cell
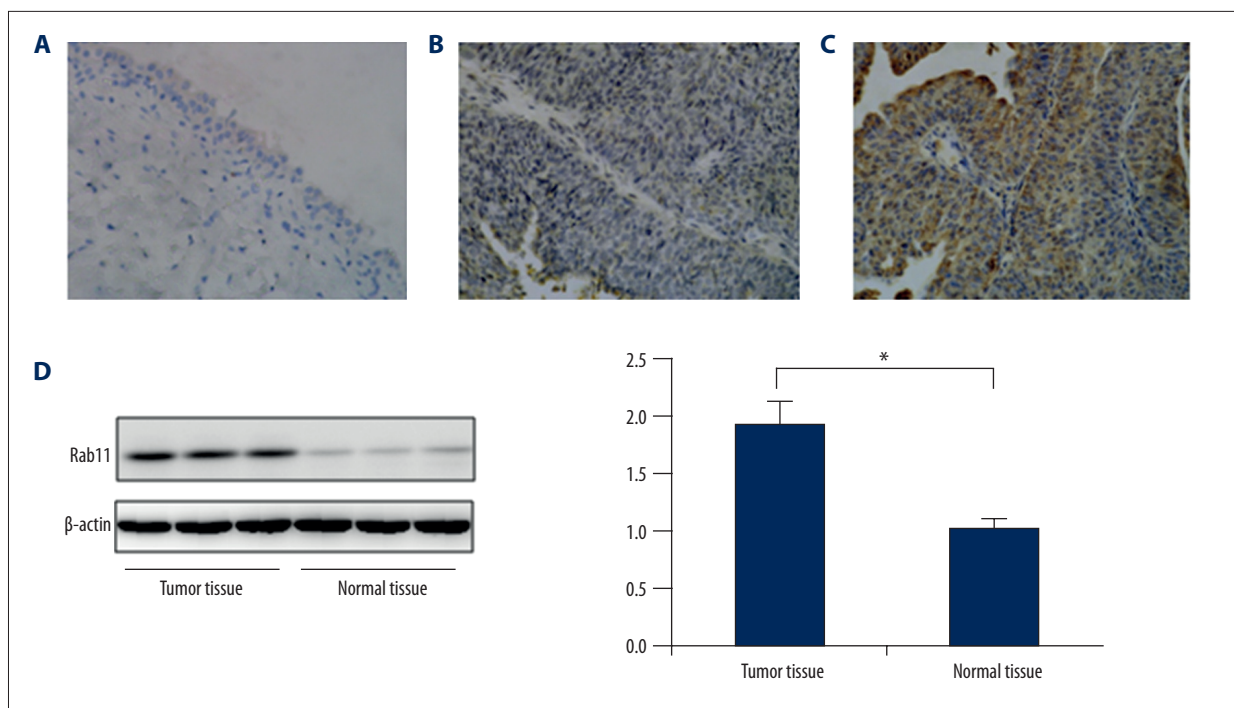
Figure 1. Expression pattern of Rab11 in bladder cancer tissues. (**A**) Negative Rab11 expression in normal bladder epithelial tissue. (**B**) Negative Rab11 expression in bladder cancer tissue. (**C**) Positive Rab11 expression in bladder cancer tissue. (**D**) The average Western blot intensity of Rab11 expression was higher in bladder cancer tissues compared with adjacent normal tissues, * p<0.05.

Table 1. The association of Rab11 expression with clinicopathological characteristics in BLCA patients.

	Total	Rab11 low expression	Rab11 high expression	P
Cases	159	93	66	
Age				
≥65	88	49	39	0.424
<65	71	44	27	
Gender				
Male	132	77	55	0.929
Female	27	16	11	
T staging				
pTa–T1	84	58	26	0.004
pT2–T4	75	35	40	
Pathologic Grading				
G1	63	42	21	0.090
G2 and G3	96	51	45	

growth and its depletion inhibited cell proliferation (Figure 3A). Cell cycle profiling showed that G1 phase was shortened upon Rab11 overexpression in BIU-87 cells but Rab11 depletion in T24 cells prolonged the G1 phase in the cells. BIU-87 cells showed increased S phase population upon ectopic expression of Rab11. T24 cells, on the contrary, reflected shortened S phase upon Rab11 silencing (Figure 3B).

In addition, we found that Rab11 depletion decreased the protein and mRNA levels of cyclin E and cyclin D1 in T24 cells.
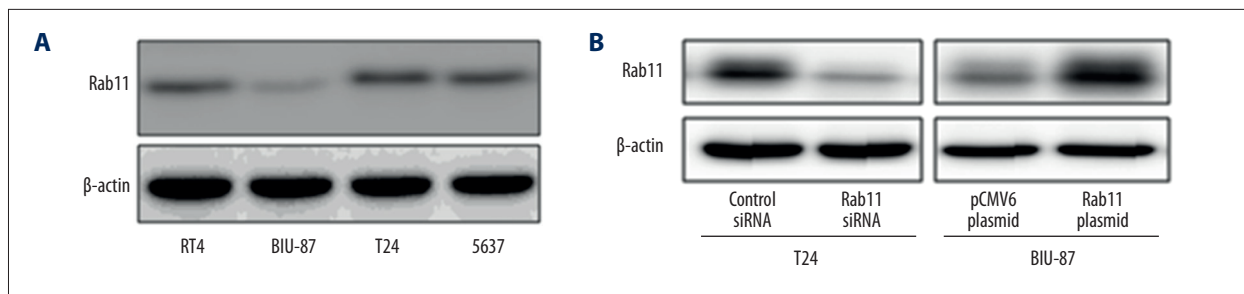
Figure 2. Expression of Rab11 in bladder cancer cell lines. (**A**) Western blot analysis of Rab11 expression in 4 bladder cancer cell lines (BIU-87, T24, RT4, 5637). (**B**) Western blot analysis demonstrated that Rab11 siRNA markedly decreases protein levels in T24 cells, while transfection of Rab11 plasmid upregulated its protein expression in BIU-87 cell line, * p<0.05.

BIU-87 exhibited increased levels of the cyclin targets upon Rab11 overexpression (Figure 3C). Thus, these results indicate the regulatory role of Rab11 in cell cycle transition.

Rab11 promotes invasion and upregulates MMP9 expression

The correlation of Rab11 expression with the extent of invasion in immunohistochemistry results suggests that Rab11 plays an important part in cancer cell intrusion. Matrigel invasion assay was performed to determine if Rab11 mediated the invasiveness of T24 and BIU-87. A direct correlation between Rab11 levels and invasiveness of cancer cells was observed in both Rab11-depleted and Rab11-overexpressed conditions (Figure 4A).

We further studied the protein and mRNA expression of MMP9, a protein closely related to invasion control. As shown in Figure 4B, Rab11 knockdown also decreased MMP9 expression in the T24 cell line but BIU-87 cells with elevated Rab11 showed upregulated MMP9.

Involvement of NF-κB pathway in Rab11-mediated cell cycle progression and invasion

Luciferase reporter assay revealed that Rab11 overexpression enhanced functionality of NF-κB promoter but Rab11 knockdown decreased NF-κB promoter function. Moreover, immunoblotting showed that Rab11 transfection increased p-IκB expression but the reverse was found for Rab11 depletion (Figure 5A). These results indicate Rab11-NF-κB axis can regulate cell cycle progression and cancer tissue invasion. To verify the above hypothesis, NF-κB inhibitor Bay 11-7082 was used. The drug inhibits p-IκB levels in BIU-87 cells upon Rab11 overexpression. As shown in Figure 5B, Bay 11-7082 blocked Rab11-induced expression of cyclin D1 and MMP9.

Discussion

Our study demonstrated elevated expression of Rab11 in BLCA tissues and cell lines, especially in MIBC. We found that Rab11 induces BLCA growth and invasion, and regulates the expression of cyclin D1, cyclin E, and MMP9 via the NF-κB pathway. These results indicated Rab11 might act as an oncoprotein in bladder cancer.

Rab11 is a small GTPase involved in the vesicular trafficking, endosomal recycling, and regulation of cell motility. Rab11 also monitors mitotic spindle organization and orientation [18]. The role of Rab11 has not been well studied in human cancers. It has been reported that Rab11 upregulated E-cadherin to induce the transformation of colorectal cancer cells [19]. Epidermal growth factor (EGF) is known to induce proliferation and motility in an immortal breast cell line. Rab11 was observed to differentially affect EGF function towards this end [17]. These reports indicate Rab11 is a candidate oncoprotein in different human cancers. Some studies reported that Rab11-family interacting proteins (Rab11-FIPs) are associated with progression and poor survival in various tumors. Overexpression of Rab11-FIP4 was detected in colorectal cancer and pancreatic cancer tissues, it may predict poor survival of patients, and is also correlated with tumor progression [20,21]. Xu et al. reported that Rab11-FIP2 upregulation enhanced invasion and migration in colorectal cells via regulation of matrix metalloproteinase 7 (MMP7) [22]. Dong et al. reported that a significant increase of Rab11-FIP2 expression is associated with nodal metastasis in gastric cancer tissue [13]. It has also been demonstrated that Rab11-FIP3 is associated with recycling endosomes and regulates breast cancer cell motility via regulating the actin cytoskeleton [15]. However, the biological role and mechanism of Rab11 expression in BLCA have not been previously defined.

We observed high Rab11 expression among BLCA samples. To confirm the results, we examined the expression of Rab11 with Western blot analysis, and the results showed the Rab11 level was higher in tumor specimens than in the adjacent non-cancerous counterparts. We retrospectively analyzed the correlation
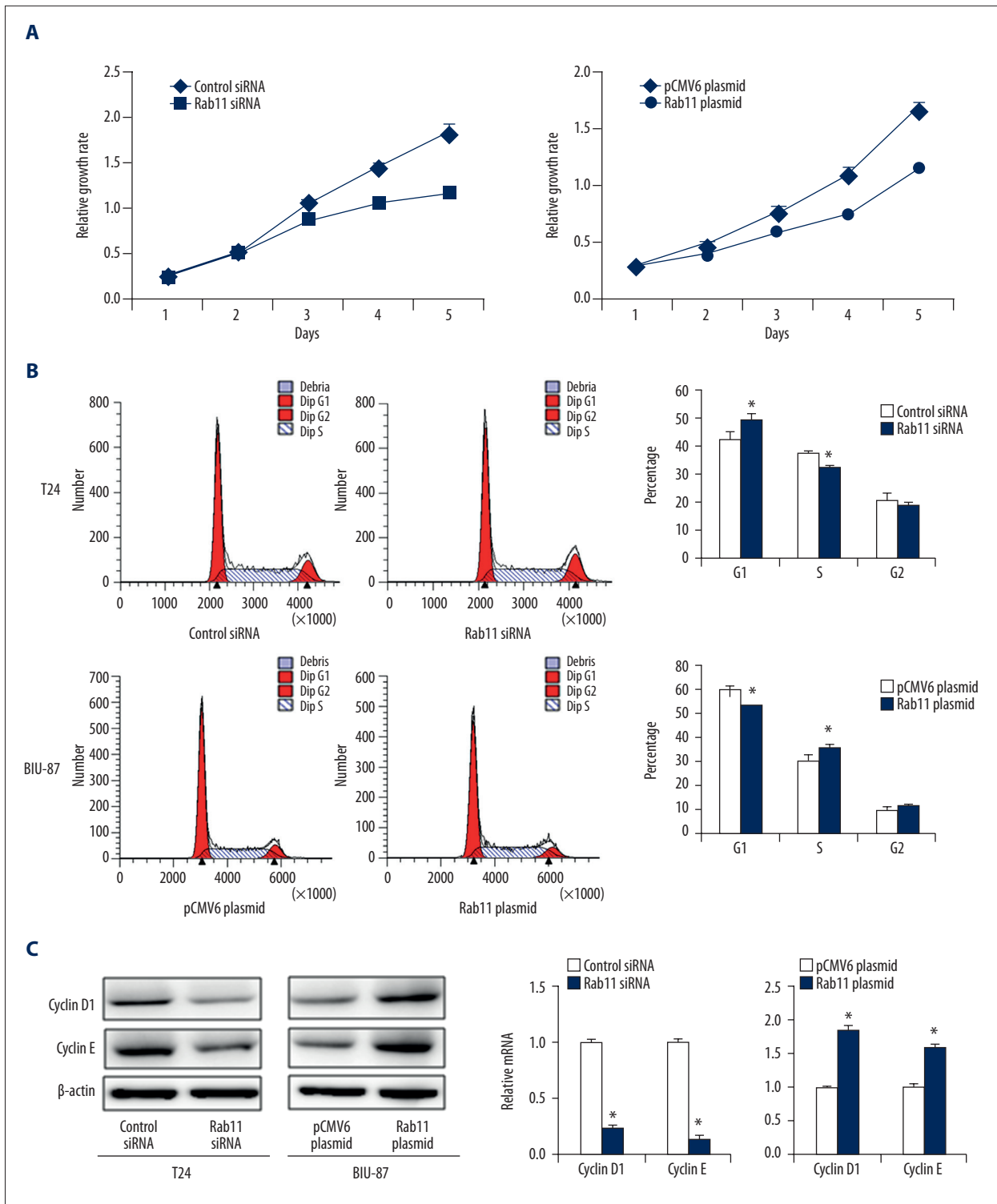
Figure 3. Rab11 promotes proliferation and cell cycle transition of bladder cancer cells. (**A**) CCK8 assay showed that Rab11 overexpression promoted cell growth rate in BIU-87 cells while Rab11 knockdown inhibited cell growth rate in T24 cells. (**B**) Cell cycle analysis showed that Rab11 overexpression increased S phase percentage and decreased G1 phase cells percentage in BIU-87 cells, while Rab11 knockdown increased G1 phase percentage and decreased S phase percentage in T24 cells. (**C**) Rab11 knockdown inhibited while its overexpression upregulated cyclin D1 and cyclin E expression at protein and mRNA levels.
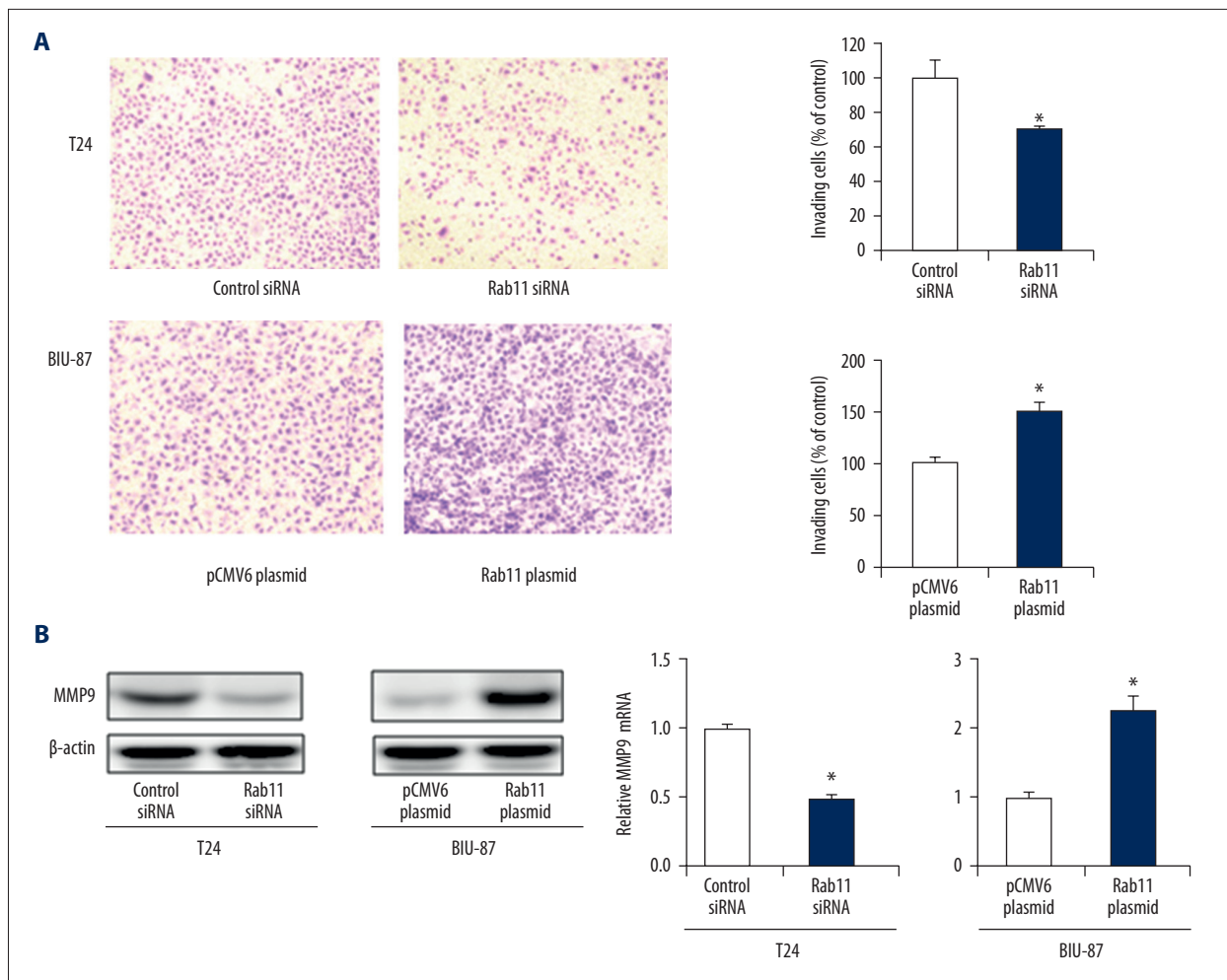
Figure 4. Rab11 induced bladder cancer cell invasion. (**A**) Matrigel invasion assay demonstrated that Rab11 overexpression upregulated invading ability of BIU-87 cells, while Rab11 knockdown inhibited invading ability of T24 cells. (**B**) Rab11 knockdown inhibited while its overexpression upregulated MMP9 expression at protein and mRNA levels.
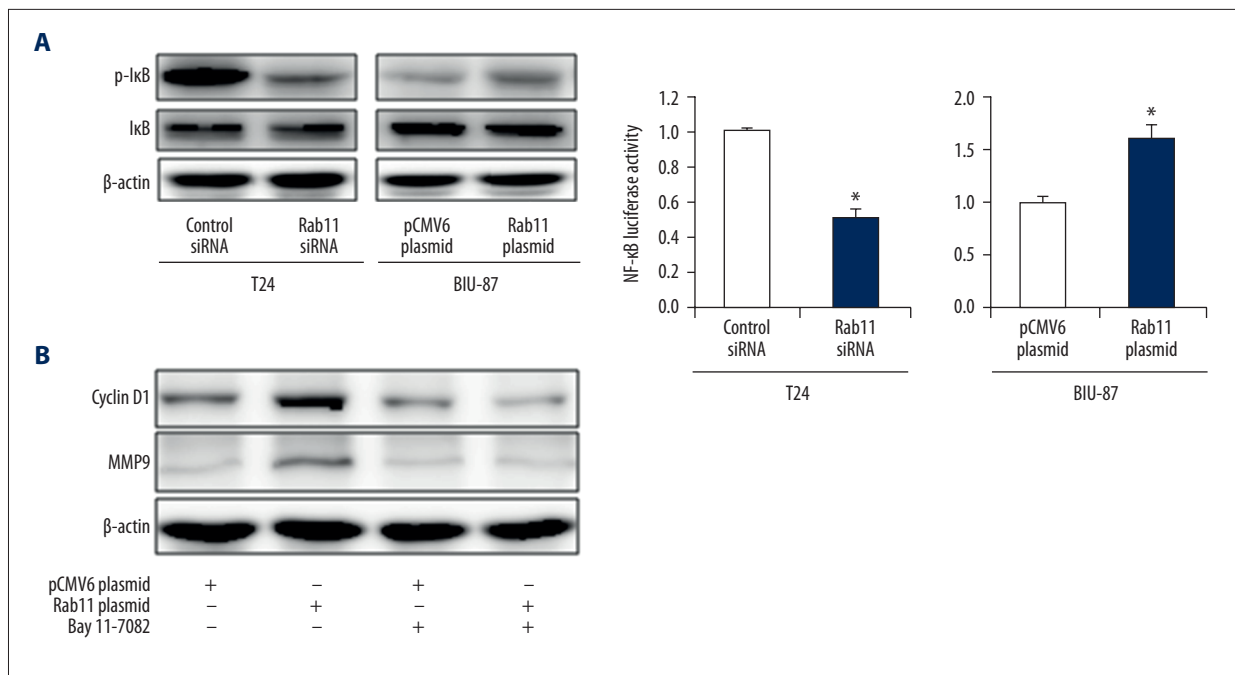
between high Rab11 expression and clinicopathological attributes in 159 BLCA patients. High Rab11 expression was significantly correlated with extent of tumor invasion. MIBC also showed higher Rab11 levels than in NMIBC, suggesting Rab11 regulates invasion and progression of MIBC.

Then, we silenced Rab11 in the T24 cells (high endogenous Rab11) and transfected Rab11 plasmid to overexpress Rab11 in BIU-87 cells. CCK-8 assay proved proliferation to be significantly inhibited after Rab11 was knocked down and strongly increased upon Rab11 overexpression, which is consistent with previous studies. Our study also investigated the role of Rab11 on cell cycle progression. The result indicated that Rab11 knockdown inhibited G1 to S phase transition, thereby inhibiting cell cycle progression, while Rab11 overexpression promoted cell cycle progression. To further analyze how Rab11 plays a role in cell cycle progression, we examined the expression of cell cycle-related proteins and found that Rab11 monitors

levels of cyclinsD1 and E. The cyclins are well known in terms of their regulatory role in the G1-S transition, and Rab11 overexpression is associated with the invasiveness of bladder cancer cells [23–25]. The research results have strongly verified that Rab11 upregulates the expression of the cyclin family of proteins to facilitate proliferation and cell cycle progression of bladder cancer cells.

Regulation of cellular invasion by Rab11 was well documented through Matrigel invasion assay. We observed that MMP9, a key player in cellular invasion, was targeted by Rab11. MMP9 plays a crucial role in metastasis of cancer cells by degrading collagen of basement membranes [26]. Thus, our study showed that Rab11 modulates MMP9 to regulate the invasion ability of bladder cancer cells.

In general, NF-κB binds to a series of inhibitory proteins and exists in the cytoplasm in an inactive state. When a molecular

Figure 5. Rab11 regulates NF-κB signaling activity. (**A**) Luciferase reporter and Western blot analysis revealed that knockdown of Rab11 decreased the level of NF-κB luciferase activity and p-IκB in T24 cell line, while Rab11 overexpression increased NF-κB luciferase activity and p-IκB in BIU-87 cells. (**B**) NF-κB inhibitor BAY 11-7082 blocked the roles of Rab11 on upregulation of cyclin D1 and MMP9 in BIU-87 cells.

signal is received, the bound inhibitory protein IκB undergoes phosphorylation-mediated degradation, and NF-κB is activated. Activated NF-κB enters the nucleus from the cytoplasm and exerts its function of promoting the transcription of the target genes in the nucleus. As reported, NF-κB signaling pathways have been implicated in many malignant tumors, including bladder cancer. This signaling pathway can promote tumor cell proliferation, angiogenesis, invasion, and metastasis [27,28]. This also suggests that NF-κB and its upstream and downstream networks may be a very reasonable and effective therapeutic target in the treatment of bladder cancer.

Our results demonstrated that Rab11 plays a biological role in bladder cancer cells and is associated with the NF-κB signaling pathway. As reported, cyclin D1 and MMP9 proteins are transcriptional downstream targets of the NF-κB signaling pathway [23, 29, 30]. To further verify the relationship, the BIU-87 cell line transfected with Rab11 plasmid was treated with BAY 11-7082. It

was found that the upregulation of Rab11 on cyclin D1, MMP9, and cell proliferation was partially abrogated, suggesting Rab11 regulates proliferation and invasion of bladder cancer cells, at least in part through activation of the NF-κB signaling axis.

Conclusions

Our study found elevated expression of Rab11 in bladder cancer. Rab11 promotes cancer progression by facilitating cell cycle transition and invasion. Rab11 also regulates expressions of cyclin proteins and MMP9 through the NF-κB pathway. Therefore, Rab11 may be a potential therapeutic target in bladder cancer.

Conflict of interest

None.

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