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Rab25 upregulation correlates with the proliferation, migration, and invasion of renal cell carcinoma

Yuanyuan Li [a, b], Qingzhu Jia [a, b], Qian Zhang [c], Ying Wan [a, b, *]

[a] Biomedical Analysis Center, Third Military Medical University, Chongqing, China
[b] Chongqing Key Laboratory of Cytomics, Chongqing, China
[c] Department of Urology, Xinqiao Hospital, Third Military Medical University, Chongqing, China

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ABSTRACT

Renal cell carcinoma (RCC) is a common urological cancer with a poor prognosis. A recent cohort study revealed that the median survival of RCC patients was only 1.5 years and that <10% of the patients in the study survived up to 5 years. In tumor development, Rab GTPase are known to play potential roles such as regulation of cell proliferation, migration, invasion, communication, and drug resistance in multiple tumors. However, the correlation between Rabs expression and the occurrence, development, and metastasis of RCC remains unclear. In this study, we analyzed the transcriptional levels of 52 Rab GTPases in RCC patients. Our results showed that high levels of Rab25 expression were significantly correlated with RCC invasion classification ($P < 0.01$), lymph-node metastasis ($P < 0.001$), and pathological stage ($P < 0.01$). Conversely, in 786-O and A-498 cells, knocking down Rab25 protein expression inhibited cell proliferation, migration, and invasion. Our results also demonstrated that Rab25 is a target gene of let-7d, and further suggested that Rab25 upregulation in RCC is due to diminished expression of let-7d. These findings indicate that Rab25 might be a novel candidate molecule involved in RCC development, thus identifying a potential biological therapeutic target for RCC.

1. Introduction

Renal cell carcinoma (RCC) is a common urological tumor and its morbidity rate ranks tenth in China [1]. RCC accounts for about 3% of adult malignancies and for approximately 90%–95% of neoplasms arising from the kidney [2]. Although RCC diagnosis and treatment have greatly improved over the years, in a recent cohort study, the median survival of RCC patients was only 1.5 years, and <10% of the patients survived up to 5 years [3]. Moreover, evidence of metastases is obtained in nearly 25%–30% of RCC patients at initial presentation [4]. Because RCC development is a complex biological process, it is often accompanied by changes in certain cytokines and signaling pathways. Therefore, comprehensively understanding the molecular biology of RCC is critical for developing improved therapeutic strategies and identifying prognostic markers for RCC.

The interplay between cancer cells and vesicle transport is recognized to play a crucial role in tumorigenesis and cancer progression [5]. Rab GTPases are key regulators of vesicle transport and they are considered to serve as a “molecular switch” in vesicle transport [6,7]. Rab GTPases are deregulated in cancers, and several of these proteins have been reported to play a central role in tumor cell migration, invasion of the ECM, proliferation, signaling to and from stromal cells, and drug resistance [8–11]. Rab25 is a member of the Rab11 subfamily (Rab11a, Rab11b, and Rab25), and it is involved in selectively regulating apical recycling and/or transcytotic pathways [12,13]. Recent studies has demonstrated that Rab25 is abnormally expressed in cancer: Rab25 was shown to be overexpressed in human ovarian cancers [14,15] and gastric cancers [16], where its expression correlated with poor prognosis and increased cell invasion and tumorigenesis. Interestingly, although Rab25 has been reported to clearly function as an oncogene in certain types of cancer, it has also been regarded to act as a tumor suppressor in other cancers such as colorectal cancer [17,18], triple-negative breast cancer [19], and esophageal squamous cell carcinoma [20]. Thus, the exact functions of Rab25 in tumor progression are likely decided by multiple factors.

The involvement of Rab25 in tumorigenesis has been widely examined, but the correlation between Rab25 expression and the occurrence, development, and metastasis of RCC remains unclear. In this study, we examined the altered expression patterns of 52 Rabs in RCC patients, and our results showed that Rab25

* Corresponding author. Biomedical Analysis Center, Third Military Medical University, #30 Gaotanyan Street, Shapingba District, Chongqing, 40038, China.
E-mail address: wanying_cn@163.com (Y. Wan).

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upregulation was positively associated with the clinical phenotype. Conversely, siRNA-mediated knockdown of Rab25 protein expression in the human RCC cell line 786-O or A-498 inhibited cell proliferation, migration, and invasion. Moreover, our results demonstrated that Rab25 is a target gene of let-7d, and further suggested that Rab25 might be upregulated in RCC because of a reduction in let-7d expression in RCC.

2. Materials and methods

2.1. Patients

We obtained 31 paired RCC tissues and adjacent normal tissues together with informed consent from RCC patients who underwent surgical resection at Xinqiao Hospital of Third Military Medical University (Chongqing, China) between 2013 and 2014 (Table S1). The clinical staging of tumors was performed according to the 7th edition of American Joint Committee on Cancer Staging Manual. The study protocol was approved by the institutional review board of our institution, and written informed consent was obtained from each study participant.

2.2. Cell culture and transfection

We obtained 786-O cells, A-498 cells, and 293FT cells from ATCC and grew them in RPMI 1640 medium or DMEM medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin (100 U/mL) and streptomycin (100 μg/mL; Invitrogen). For transfection, 786-O and A-498 cells were seeded in 6-well plates (2×10^5 cells/well). After 24 h, the cells were

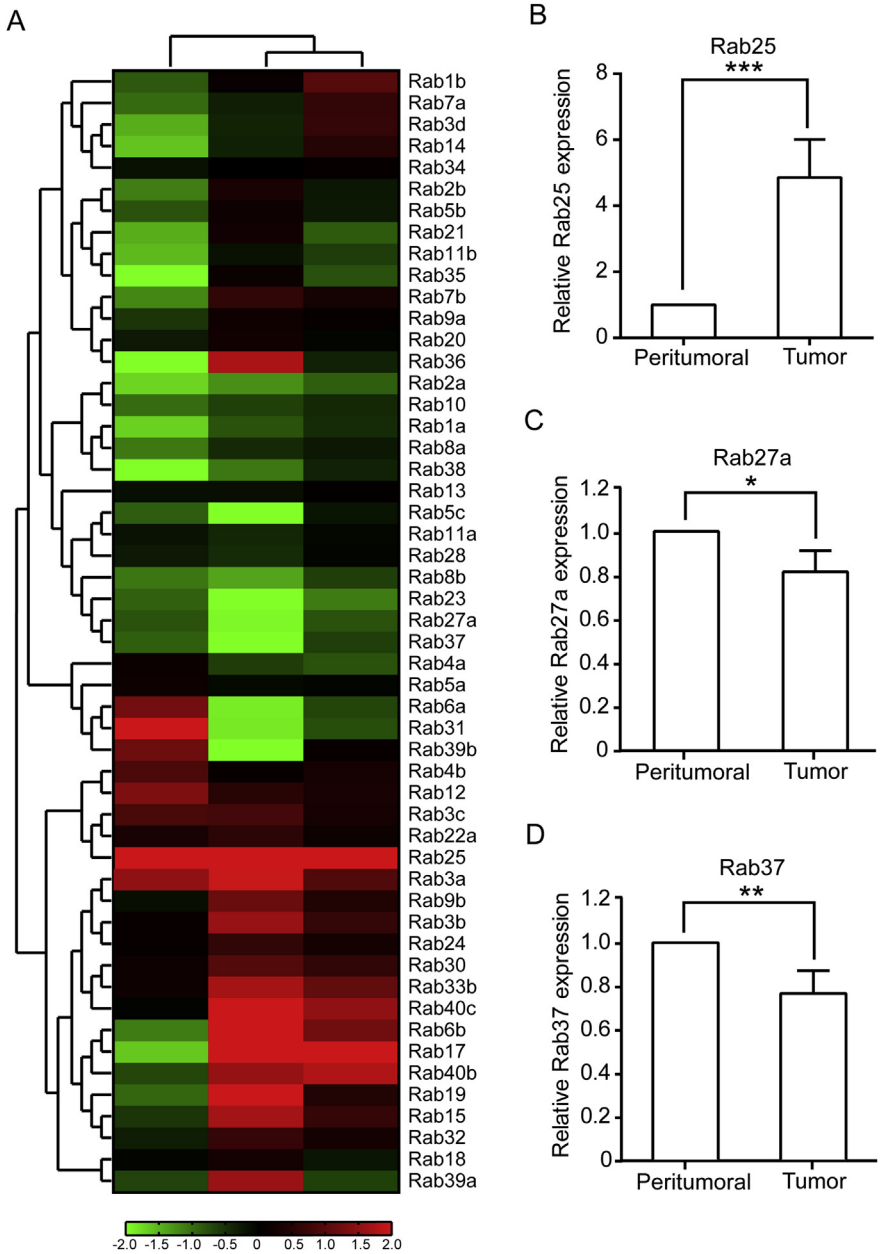

Fig. 1. The transcriptional levels of Rabs in RCC. (A) Hierarchical clustering analysis of the transcriptional levels of 52 Rabs in three renal cell carcinoma (RCC) patients. Upregulated (red) and downregulated (green) genes in compared to corresponding peritumoral tissues. (B–D) The relative transcriptional levels of 3 dysregulated Rabs (Rab25, Rab27a and Rab37) were determined by performing qRT-PCR analyses in 31 RCC patients. GAPDH was used as an internal control. Each qRT-PCR experiment was performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transfected with 3 targeting Rab25 siRNA oligonucleotides or non-silencing siRNA oligonucleotides (RiboBio, Chongqing, China) by using the X-tremeGENE siRNA Transfection Reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. Cultures were incubated for 72 h before collecting samples for western blotting and qRT-PCR analysis.

2.3. RNA extraction and real-time RT-PCR

Total RNA was isolated from tissues or cultured cells by using TRIzol reagent (Invitrogen) as per the manufacturer's protocol. For mRNA analyses, 1.0 μg of total RNA was reverse-transcribed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada). For microRNA (miRNA) quantification, 1.0 μg of total RNA was polyadenylated using polyA polymerase (QuantoBio, Beijing, China) and then reverse-transcribed with an oligo-dT adapter primer into cDNAs for use in quantitative real-time PCR (QuantoBio). We used the SsoAdvanced™ SYBR® Green Supermix (BioRad, CA, USA) to perform real-time quantitative PCR and the CFX96™ Real Time System (BioRad) to perform real-time quantitative RT-PCR (qRT-PCR) analyses. The mRNA or miRNA expression levels were normalized relative to GAPDH or U6, respectively. Table S2 list all the primers used.

2.4. Western blotting

Cellular protein lysates were boiled for 5 min and then the denatured samples were separated in 4%–12%-gradient NuPAGE® Novex Bis-Tris gels (Invitrogen). The separated proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) and probed with rabbit anti-Rab25 (1:1000; Abcam, Cambridge, MA, USA) and anti-β-actin (1:1000; CST, Boston, MA, USA) primary antibodies; the immunoreactive bands were detected by staining with HRP-conjugated goat anti-rabbit secondary antibodies (1:1000; CST).

2.5. Cell-proliferation assays

To perform cell-proliferation assays, we seeded 2×10^3 Rab25-knockdown cells or control cells in 96-well plates and then quantified the proliferation by using the Cell Counting Kit-8 (CCK-8) (Beyotime, Chongqing, China) at 0, 24, 48, and 72 h. The CCK-8 solution was added to each well at various time points and incubated at 37 °C for 2 h, after which the optical density was measured at 450 nm.

2.6. Scratch assay

Cells in complete media were plated in poly-D-lysine-coated 24-well plates (2×10^4 cells/well). After overnight incubation, one artificial wound was scratched into the monolayers by using the tip of a 10-μL micropipette; after wounding, the cells were washed at least twice in PBS to eliminate floating cells. We monitored wound closure and photographed the cultures at 48 h post-wounding.

2.7. Transwell-migration assay

We examined the invasive behavior of cells by performing transwell-migration assays. Briefly, 8-μm-pore-size Transwell inserts (Corning, New York, NY, USA) were coated with 1.0 mg/mL Matrigel (BD) in cold serum-free medium. Cells (1×10^5) were plated on the top side of the transwell insert (upper chamber), and serum-free medium was added to the bottom chamber. After 24 h, stationary cells were removed from the top side of the membrane, and the cells that had migrated to the bottom side of the inserts were stained with 0.1% crystal violet and counted under a light microscope. In each well, we counted cells in 5 fields and then calculated the mean number of cells per field. Each experiment was performed in triplicate and repeated at least twice.

2.8. Dual luciferase activity assay

The human Rab25-3′-UTR wild-type (wt) sequence and mutant (mut) sequence were cloned into the pGL3 luciferase reporter vector (Promega, Madison, WI, USA). We seeded 293FT cells in 24-well plates (1.5×10^5/well), and 24 h later, cotransfected them with 0.5 μg each of FUGW-let-7d plasmid and pGL3 plasmid expressing Rab25-3′ UTR-wt, Rab25-3′ UTR-mut, or control sequence by using Lipofectamine 2000 (Invitrogen). After 48 h, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). For each well of transfected cells, firefly luciferase activity was normalized relative to Renilla luciferase activity. The results were obtained from 3 independent experiments, each performed in triplicate.

2.9. Statistical analysis

All data are presented as the mean ± SEM. GraphPad Prism 5 Software was used for statistical analysis. The two-tailed unpaired *t*-test was used for statistical analysis to evaluate the differences between 2 groups of samples, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Rab transcription levels are aberrant in RCC patients

To compare the transcription levels of Rabs between RCC tissues and paired adjacent noncancerous tissues, we performed an

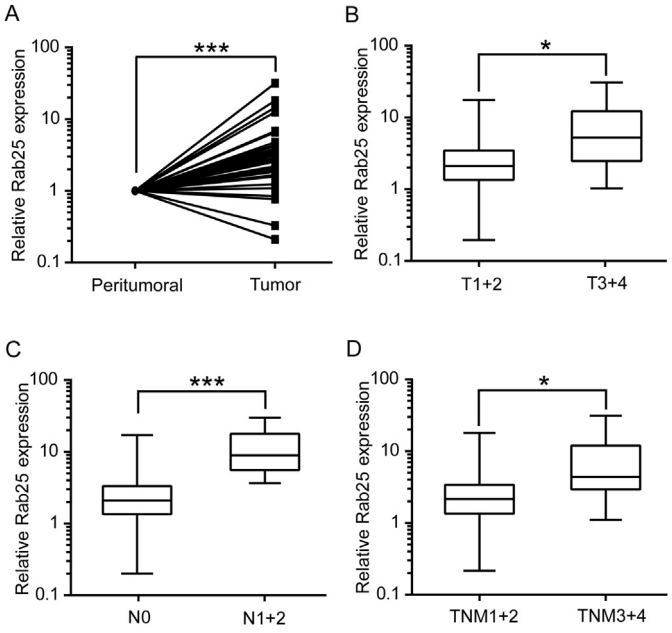

Fig. 2. Rab25 expression level in RCC patients was associated with tumor invasion classification, the status of lymph-node metastasis, and pathological stage. (A) Rab25 expression in RCC tissues in comparison with the corresponding peritumoral tissues (n = 31). (B) Rab25 expression was significantly higher in patients with high invasion classification (T3+4) than in those with low invasion classification (T1+2; $^*P < 0.05$). (C) Rab25 expression was significantly higher in patients with lymph-node metastasis (N1+2) than in those without lymph-node metastasis (N0; $^{***}P < 0.001$). (D) Rab25 expression was significantly higher in patients with a high pathological stage (TNM3+4) than in those with low pathological stage (TNM1+2; $^*P < 0.05$).

unbiased screen on 52 Rabs by using 3 clinical specimens. We performed clustering analysis and assembled the clustered data to obtain a visual network of Rab transcription levels (Fig. 1A, Table S3). Our results showed that 2 Rabs (Rab25 and Rab3a) were upregulated and 4 Rabs (Rab8b, Rab23, Rab27a, and Rab37) were downregulated in RCC tissues as compared with their expression in the paired adjacent noncancerous tissues. To validate the data on the transcription levels of the Rabs, we analyzed the expression levels of the 6 Rabs in 31 RCC patients (Table S3). As compared with their expression in adjacent noncancerous tissues, Rab27a and Rab37 were downregulated and Rab25 was upregulated in RCC tissues in a statistically significant manner (Fig. 1B–D).

3.2. Rab25 expression levels are correlated with high invasion classification, lymph-node metastasis, pathological stage in RCC

Examination of 31 matched normal and RCC tumor tissues revealed that Rab25 was significantly upregulated in 27 cancer tissues as compared with its expression in the corresponding non-tumor tissues (Fig. 2A). Rab25 levels were previously shown to be markedly elevated in several tumors, and this upregulation was correlated with a diminished survival rate of patients and poor prognosis [21]. Therefore, we examined the correlation between Rab25 expression and the clinicopathological factors of RCC (Fig. 2B–D). The results showed that Rab25 upregulation was correlated with high invasion classification ($P < 0.01$), lymph-node metastasis ($P < 0.001$), and pathological stage ($P < 0.001$) of RCC. Thus, we concluded that Rab25 upregulation might play key roles in RCC progression and development.

3.3. Rab25 promotes RCC cell proliferation, migration and invasiveness in vitro

To study the biological functions of Rab25 in RCC cells, we used 3 siRNAs to deplete Rab25 in 786-O and A-498 cells. The effectiveness of the siRNAs was evaluated by performing qRT-PCR analysis and western blotting (Fig. 3A–C). The results showed that siRab25-3 most strongly interfered with Rab25 expression, and thus it was used in subsequent studies. First, to determine whether Rab25 affects the proliferation of RCC cells, we used the CCK-8 assay to measure cell viability. The results showed that Rab25-depleted 786-O and A-498 cells grew significantly more slowly than control cells did (Fig. 3D). This indicated that Rab25 expression likely promotes the proliferation of RCC cells in vitro. Next, to assess

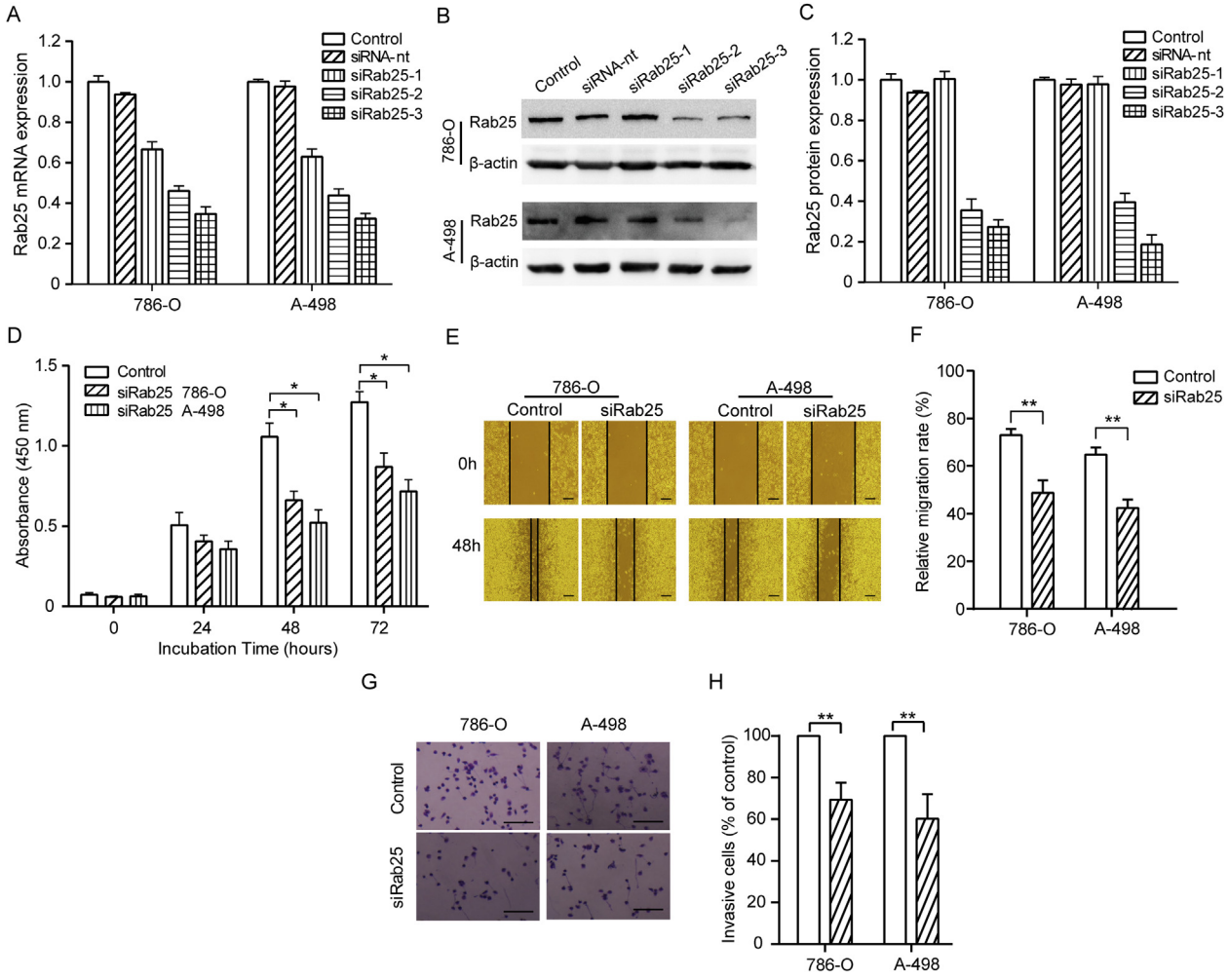

Fig. 3. Rab25 promoted proliferation, migration, and invasion in the RCC cell lines 786-O and A-498. (A–C) 786-O and A-498 cells were transfected with a non-targeting siRNA (siRNA-nt) and 3 Rab25-targeting siRNA (siRab25-1, -2, -3). Three days after transfection, cells were collected for use in qRT-PCR (A) and western-blot analyses (B–C). The results showed that siRab25-3 most potently interfered with Rab25 expression, and it was used in subsequent studies. (D) The proliferation of Rab25-knockdown cells and control cells were evaluated using the CCK-8 assay (at 0, 24, 48, and 72 h). (E–F) The migration of Rab25-knockdown cells compared with the control cells by using a scratch-healing assay. (G–H) The invasiveness of Rab25-knockdown cells compared with the control cells by using a Matrigel-transwell assay system. Five fields of cells were counted for each well, and the mean number of cells per field was calculated. All data shown are from 2 independent experiments conducted in triplicate; Bar = 200 μm; $^{*}P < 0.05$; $^{**}P < 0.01$.

whether Rab25 affects the migration of RCC cells, we examined the change in the invasiveness of 786-O and A-498 cells after Rab25 knockdown by using scratch assays and transwell-migration assays. The results of the scratch assay showed that at 48 h, the mobility of Rab25-depleted 786-O and A-498 cells was lower than that of control cells (Fig. 3E–F). Furthermore, the results of the transwell-migration assay also showed that knocking down Rab25 inhibited cell migration (Fig. 3G–H). The results demonstrated that the suppression of Rab25 protein expression led to a reduction in the migration and invasion of 786-O and A-498 cells.

3.4. Rab25 is a direct let-7d target gene in RCC cells

Recently, the downregulation of several miRNAs was shown to be associated with high levels of expression of various Rabs in cancer cells [22–25]. To identify the miRNAs that might regulate Rab25, we first performed *in silico* searches for target miRNAs by using 4 algorithms (miRanda, miRWalk, PicTar, and TargetScan) and obtained a list of predicted target miRNAs of Rab25. Next, by means of data mining, we selected the miRNAs potentially involved in the tumor growth, metastasis, and chemotaxis activity of RCC. Based on the findings, we predicted that Rab25 could be a direct target of let-7d (Fig. 4A). In vitro assays, we found that luciferase activities were decreased significantly in 293FT cells transfected with a Rab25-3′-UTR reporter plasmid, but not in cells transfected with the control or Rab25-3′-UTR-mutation plasmid (Fig. 4B). Furthermore, we found that the let-7d expression levels was significantly downregulated in cancer tissues as compared with its expression in the corresponding non-tumor tissues (Fig. 4C). Therefore, our results suggested that Rab25 is a target gene of let-7d, and that Rab25 upregulation in RCC is due to the diminished of let-7d.

4. Discussion

Intracellular vesicle trafficking is responsible for maintaining and regulating the dynamic balance of materials in all cells [7,26].

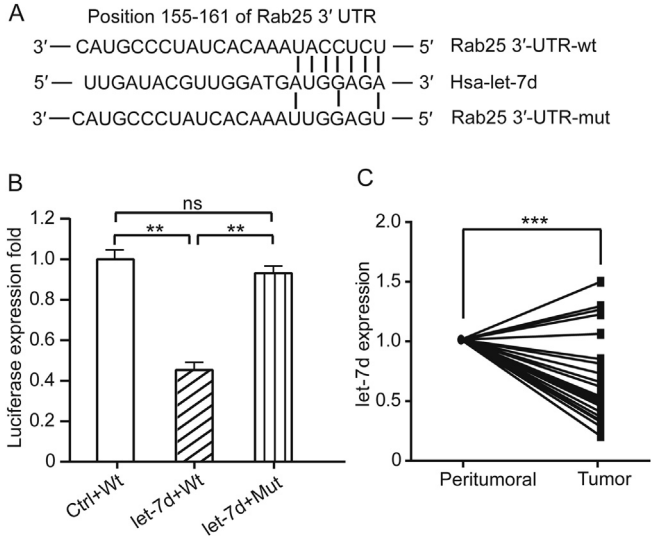

Fig. 4. Rab25 is a direct target of let-7d in RCC cells. (A) Putative let-7d-binding sites in the 3′-UTR of human Rab25. The mutated sequence in the putative let-7d binding sites for Rab25 is shown at the bottom. (B) Results of luciferase-reporter assays performed on 293T cells cotransfected with pGL3-Luc vector containing Rab25 3′-UTR-wt or Rab25 3′-UTR-mut and a plasmid encoding either let-7d or miR-scramble (control). Data represent the mean ± SEM from 3 independent experiments; $^{**}P < 0.01$. (C) Expression of let-7d in RCC tissues in comparison with the corresponding peritumoral tissues (n = 31). Data were normalized using U6, and each qRT-PCR experiment was performed in triplicate; $^{***}P < 0.001$.

Recently, potential functions of Rab GTPases in various tumor-related processes have been identified [9,11], and the importance of the intracellular-trafficking functions of Rab GTPases has been elucidated by demonstrating that these proteins coordinate the transformation between normal and cancerous cells [5]. Furthermore, Rabs have been shown to direct the cross-talk between cancerous cells and stromal cells and to be capable of creating a favorable environment for tumor growth, invasion, and migration [8–10]. Rab25, like other members of the Rab family, has been implicated to function in various cancers, although its mechanism of action has remained controversial: both upregulation and downregulation of Rab25 have been associated with tumor growth, differentiation, and poor prognosis [21]. Moreover, Rab25 is involved in several cellular processes such as proliferation, apoptosis, angiogenesis, cell cycle, trafficking of adhesion molecules, and cell migration [21].

In this study, we determined the dysregulated expression profiles of 52 Rabs at the transcriptional level in RCC patients, and found that Rab25 was expressed at considerably higher levels in RCC tissues than in paired adjacent noncancerous tissues. Our database revealed that the high expression level of Rab25 in RCC patients was associated with tumor differentiation, pathological stage, and the status of lymph-node metastasis. This strongly suggested that Rab25 likely operates in RCC in the same manner as it does in other cancers [14–16]. Moreover, knocking down Rab25 protein expression in the human RCC cell lines 786-O and A-498 inhibited cell proliferation, migration, and invasion. These findings favor a model in which Rab25 acts as a tumor promoter, as suggested in the case of other cancers. However, with regard to cancer development, a loss of Rab25 alone might not be sufficient for initiating tumorigenesis, because Rab25 knockout mice do not develop any spontaneous tumors [18]. Although we did not investigate the potential mechanisms by which Rab25 regulates cell proliferation, invasion, and migration, a previous study suggested that Rab25 might promote the invasive migration of cells by collaborating with CLIC3 (chloride intracellular channel protein 3) to recycle $\alpha5\beta1$ integrin from late endocytic compartments [27,28]. Moreover, Rab25 increases cellular ATP and glycogen stores and thus protects cancer cells from bioenergetics stress [15].

Interestingly, recent analyses of the expression profile of miRNAs in cancer samples have revealed that the downregulation of several miRNAs is associated with high levels of expression of various Rabs [22–25]. Based on bioinformatics predictions and the results of luciferase-activity assays, we identified Rab25 as a target gene of let-7d, which belongs to the let-7 family of molecules that function as tumor suppressors in several types of cancer [29]. In clinical RCC samples, the downregulation of let-7d was found to be associated with advanced tumor grade and T stage and increased vascular invasion [30]. Functional studies indicated that ectopic expression of let-7d potently inhibited RCC cell proliferation and migration and the recruitment of peripheral blood monocytes in vitro, as well as tumor growth, metastasis, and tumor macrophage infiltration in vivo [30]. Our findings agree with these results; we found that let-7d was downregulated in cancer tissues in a statistically significant manner as compared with its expression in normal tissues. Therefore, Rab25 mRNA levels were inversely correlated with let-7d levels in our clinical RCC specimens.

In conclusion, we have reported the altered expression pattern of 52 Rab GTPases in tissues of RCC patients and have shown that high Rab25 expression is positively correlated with the clinical phenotype. Although its mechanism of action remains unclear, Rab25 can alter cell proliferation, migration, and invasion in RCC cell lines. Further evaluating the functions of Rab25 in RCC and conducting studies on an increased number of RCC samples will lay a foundation for exploring the nature of RCC metastasis and

recurrence. Moreover, this it will also provide a scientific basis for the treatment of RCC.

Conflict of interest

There are no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.144.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.144.

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