RASA1 inhibits the progression of renal cell carcinoma by decreasing the expression of miR-223-3p and promoting the expression of FBXW7.

Running title: RASA1 is involved in the progression of RCC.

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Abstract

RAS p21 protein activator 1 (RASA1), also known as p120-RasGAP, is a RasGAP protein that functions as a signaling scaffold protein, regulating pivotal signal cascades. However, its biological mechanism in renal cell carcinoma (RCC) remains unknown. In this study, RASA1, F-box/WD repeat-containing protein 7 (FBXW7) and miR-223-3p expression was assessed via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot. Then, the targeted correlations of miR-223-3p with FBXW7 and RASA1 were verified via a dual-luciferase reporter gene assay. CCK-8, flow cytometry, and Transwell assays were implemented independently to explore the impacts of RASA1 on cell proliferation, apoptosis, migration, and cell cycle progression. Finally, the influence of RASA1 on tumor formation in RCC was assessed in vivo through the analysis of tumor growth in nude mice. Results showed that FBXW7 and RASA1 expression was decreased in RCC tissues and cell lines, while miR-223-3p was expressed at a higher level. Additionally, FBXW7 and RASA1 inhibited cell proliferation but facilitated the population of RCC cells in the G0/G1 phase. Altogether, RASA1 may play a key role in the progression of RCC by decreasing miR-223-3p and subsequently increasing FBXW7 expression.

Keywords: Renal cell carcinoma, RASA1, miR-223-3p, FBXW7
Introduction

Renal cell carcinoma (RCC) is a common cancer with approximately 65,000 new cases each year [1]. RCC makes up 2-3% of all adult cancers and ranks eighth among the most common causes of death associated with cancers [2]. Thus, RCC greatly affects quality of life and patient life span while burdening health and economic systems owing to increased risks of metabolic syndrome and cardiovascular and kidney diseases in late stages [3]. Additionally, the incidence and death rate of RCC is increasing worldwide [4]. Thus, studying the molecular basis of RCC is essential for the development of new treatments for RCC patients.

*Ras* small GTPases exert regulatory effects in various signaling cascades as well as in different cellular processes [5, 6]. Roughly 30% of human cancers have mutations in *Ras* genes (K-*Ras*, H-*Ras* and N-*Ras*) making them constantly active in their GTP-bound form [7], giving rise to constitutively active *Ras* signal transduction even without extracellular stimuli. RAS p21 protein activator 1 (RASA1), also known as p120-RasGAP, is a RasGAP protein. In addition to its RasGAP domain, RASA1 has two Src homology 2 (SH2) domains, an SH3 domain, a Pleckstrin homology (PH) domain, and a Calcium-dependent phospholipid-binding (C2) domain. It functions as a signaling scaffold protein regulating pivotal signal cascades [8, 9]. RASA1 has also been implicated in many biological processes including actin filament polymerization, blood vessel development, and cell apoptosis and movement [10]. Mice deficient in *RASA1* have aberrantly growing blood vessels and exhibit large-scale neuronal apoptosis and embryonic death at E10.5 [11]. In mouse endothelial cells, loss of...
RASA1 increases endothelial proliferation and tube formation [10].

Human RASA1 germline mutations are related to an autosomal dominant disorder, capillary malformation-arteriovenous malformation (CM-AVM), featuring malformed atypical capillaries [12]. Despite its physiological functions, the role of RASA1 in tumor formation, and specifically in RCC, has not yet been elucidated.

The purpose of this study was to inquire into the functions of RASA1 in the occurrence and progression of RCC and to explore its potential mechanisms, in order to provide novel protocols for the diagnosis and therapy of RCC.
Materials and methods

Clinical specimens

Renal cancer tissues and corresponding non-cancerous tissues were collected from renal cancer patients who underwent surgical resection at the First Affiliated Hospital of Xinjiang Medical University from 2016 to 2018. All clinical specimens were preserved at -80°C until use. No routine treatments were performed before surgery.

All research subjects provided written informed consent in advance, and this project was approved by the Institutional Review Board from the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University in accordance with the Code of Ethics in the Declaration of Helsinki.

Cell culture

ACHN, SN12C, 786-0, SKRC-39, A-498 and HTB-46 (RCC cells lines), HK-2 (a normal proximal tubule epithelial cell line) and HEK293T cell lines were purchased from BeNa Culture Collection. The culture medium used contained 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in an environment with 5% CO2.

Quantitative real-time PCR (qRT-PCR)

Using manufacturer’s instructions, TRIzol reagent (Invitrogen) was used for total RNA extraction from RCC tissues and cells. Through determination via a NanoDrop 2000 (Thermo Fisher Scientific), 200 ng of RNA from each sample was used for reverse transcription with the ReverTra Ace qPCR RT Kit. THUNDERBIRD SYBR® qPCR Mix (Toyobo) was utilized for the calibration of mRNAs in the three groups through qRT-PCR. Reaction conditions were as follows: 94 °C for 2 min, 94 °C for 10
s, 56 °C for 30 s, 72°C for 1 min and 72 °C for 10 min. The reaction was conducted three times. Finally, with GAPDH and U6 as endogenous controls, mRNA expression levels were normalized to GAPDH expression and quantified via the $2^{\Delta\Delta Ct}$ method. In addition, miRNA expression was normalized to U6 expression and also quantified via the $2^{\Delta\Delta Ct}$ method. The qRT-PCR primers used in this study are listed in Table 1.

**Cell transfection and culture**

siRNAs targeting RASA1 or FBXW7 (F-box and WD repeat domain containing 7), scrambled siRNAs, miR-223-3p inhibitor, miR-223-3p NC inhibitor, miR-223-3p mimic and miR-223-3p NC mimic were obtained from GenePharma. The pcDNA3.1 plasmid (Thermo Fisher Scientific) was utilized for RASA1 or FBXW7 overexpression. Prior to transfection, 786-O and ACHN cells were trypsinized (0.25%) and inoculated in six-well plates with $1\times10^5$ cells per well. In the case of 80-90% cell fusion, original medium was replaced with fresh medium lacking serum and antibiotics. Transfection was conducted using Lipofectamine 2000 (Life Technologies Corporation (Gaithersburg, MD, USA)), followed by cultivation of the transfected cells at 37 °C in an environment with 5% CO₂ for 48 h.

**Dual-luciferase reporter gene assay**

Through bioinformatical analysis (miRDB, http://www.mirdb.org/), the targeted association between miR-223-3p and FBXW7 was predicted. Enzymes were used to digest the FBXW7 3'UTR using Xbal. Subsequently, with the use of Lipofectamine 2000 reagent (Invitrogen), HEK293T cells were treated with a pGL3-control luciferase reporter gene vector provided by Promega (Madison, WI, USA) containing
FBXW7-wt/FBXW7-mut and miR-223-3p mimics/miR-223-3p control. Finally, after 48 h of treatment, a Dual-Glo Luciferase Assay System (Promega) was utilized to examine the luciferase activity in cell lysates following manufacturer’s protocols. Each assay was implemented in triplicate at the minimum.

CCK-8 assay

In a 6-well plate, 10 μL of CCK-8 solution (Dojindo) was added to each cell following cell transfection for 0, 24, 48 and 72 h. Next, the RCC cells underwent 2 h of incubation in a humid chamber with 5% CO₂ at 37 °C. Then, a plate luminometer from Bio-Rad was employed to determine the optical density of the cells at 450 nm. The assay was implemented in triplicate at the minimum.

Flow cytometry analysis

To analyze the cell cycle, cells were collected from different groups at 24-48h after transfection, and a cell suspension was obtained by digestion of the cells. The supernatant was removed following centrifugation, the cells were washed in PBS twice to discard any residue, and the cells were immobilized in ethanol (75%) for 4 h at 4 °C. Later, the immobilized cells underwent centrifugation and PBS washes, followed by mixing with 40 μg propidium iodide (PI) and 1 mL of a 100 μg RNase staining solution from BD Biosciences, and incubation at room temperature for 15 min. Cell cycle analysis was conducted using a FACSCalibur flow cytometer subsequent to staining, and FACSDiva was adopted for statistical data analysis. In apoptosis assays, transfected cells were obtained from each group and underwent trypsinization (0.25%) 24-48h later. Following inoculation into 96-well plates (2×10⁴
cells per well), 5 μL PI, 200 μL HEPES and 5 μL Annexin V/FITC was added and the mixtures were incubated for 25 min at room temperature. A FACSCalibur FCM from BD Biosciences was used for observing apoptosis. The above three assays were independently implemented to reduce errors, and data were analyzed via FACSDiva software.

**Transwell assay**

Matrigel (BD Bioscience) and serum-free DMEM were thoroughly mixed and placed in a Transwell chamber (Corning Incorporated). Cells in serum-free medium at a concentration of 2.5×10^4 cells/mL were placed into the upper chamber (500 μL for each chamber) of 24-well invasion chambers, while culture medium containing 20% FBS was added to the lower chamber. A 4% paraformaldehyde solution was utilized to immobilize the cells, and 0.1% crystal violet was used to stain RCC cells after 24-48 hours of incubation. Pictures were taken in each chamber, and the cells in 5-10 independent fields were counted. The assay was repeated in triplicate at the minimum.

**Western Blot assessment**

A BCA Protein Assay Kit from Pierce was utilized to measure the protein concentration of each sample. Following isolation via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane provided by Millipore (Billerica) for 120 minutes. Next, TBST with 5% skim milk was used to seal the membrane, followed by one hour of incubation with primary antibodies (anti-FBXW7 and anti-GAPDH) from Abcam and secondary antibody (anti-rabbit IgG H&L) from Abcam at
37°C. An ECL system from Life Technology was used to visualize the antibody signals. Differences in the relative protein levels among samples were detected by GAPDH density as an endogenous control.

**Nude mouse tumorigenesis experiment**

BALB/c nude mice were obtained from Xinjiang Medical University. Animal experiments took place in SPF Animal Laboratory at Xinjiang Medical University. All animal experiments were approved by the Changji Branch Hospital of the First Affiliated Hospital of Xinjiang Medical University. Female athymic BALB/c nude mice were injected with control, NC or RASA1-transfected ACHN cells in the subcutaneous tissue of the right flank. Six nude mice were used per group. Tumor growth was monitored by two-dimensional measurements using electronic calipers starting from the seventh day after tumor transplantation, once every 7 days for a total of 28 days. All surgeries were performed under sodium pentobarbital anesthesia via intraperitoneal injection (40 mg/kg) and all efforts were made to minimize suffering. At 4 weeks post-injection, the mice were anesthetized with 40 mg/kg sodium pentobarbital and then sacrificed by 10% formalin perfusion fixation of central nervous system; death was confirmed by complete cessation of heartbeat and breathing. The tumor tissues were isolated and weighed.

**Statistical analysis**

Data from each assay implemented thrice were presented as mean ± standard deviation (SD). The comparison between two different groups with parametric variables was conducted via Student's t-test, while the comparison among multiple
groups was performed via one-way ANOVA. GraphPad Prism 6.0 was adopted for statistical processing. $P<0.05$ indicated statistically significant differences.

**Results**

**RASA1 was down-regulated in RCC tissues and cells**

$RASA1$ mRNA was expressed at lower levels in RCC tissue samples than in adjacent (paracancerous) tissue (Figure 1A). In RCC cell lines, we also found that $RASA1$ expression was decreased compared to the HK2 cell line (Figure 1B). Additionally, the $RASA1$ protein expression, as detected by western blot, was lower in RCC tumor tissue samples compared to adjacent tissue (Figure 1C).

**Effects of $RASA1$ on RCC cell apoptosis**

The transfection efficiency of the $RASA1$ over-expression plasmid was verified in 786-0 cells (Figure 2A). Then, a western blot was utilized to detect the overexpression of $RASA1$ and the expression of Caspase 3. Previous studies have reported that Caspase-3 is involved in cell apoptosis. Here, no differences were found in $RASA1$ and Caspase-3 protein expression between the control cells and NC cells, after $RASA1$ transfection. However, the expression of $RASA1$ and Caspase-3 proteins in cells with $RASA1$ overexpression was strikingly increased relative to the NC cells, indicating that $RASA1$ may regulate Caspase-3 expression and promote cell apoptosis (Figure 2B). To confirm the effect of $RASA1$ on cell apoptosis, a flow cytometry assay was used to detect apoptosis following $RASA1$ overexpression. The results showed that the apoptosis rate increased after $RASA1$ overexpression (Figure 2C). These experiments were also performed in ACHN cells and the same results were observed (Figure 2D-F).
Influence of RASA1 on the proliferation, migration and cell cycle progression of RCC cells

Next, we aimed to verify the influence of RASA1 on cell proliferation, migration and cell cycle progression. A CCK-8 assay was conducted which demonstrated that RASA1 overexpression significantly decreased the viability of 786-0 cells (Figure 3A). Furthermore, a cell colony formation assay disclosed that the rate of colony formation was lower in 786-0 cells with RASA1 overexpression (Figure 3B). In addition, CCK-8 results showed that overexpression of RASA1 in 786-0 cells lowered their proliferation ability with a time gradient compared to the NC cells (Figure 3C). A Transwell assay uncovered that RASA1 overexpression drastically reduced cell migration (Figure 3D). Likewise, RASA1 overexpression greatly decreased cell proliferation, colony formation and migration in ACHN cells (Figure 3E-H). Finally, flow cytometry results revealed that RASA1 overexpression increased the RCC cell proportion in G0/G1 phase in both 786-0 and ACHN cells (Figure 4A, 4B).

RASA1 regulates miR-223-3p

To explore the underlying mechanism of RASA1 in cells, the effect of RASA1 on the expression of miRNAs closely related to kidney cancer was examined. Here, we assessed miR-132, miR-630, miR-223-3p, miR-508-3p, miR-486 and miR-296-3p [13-19]. RASA1 overexpression reduced the expression of miR-223-3p (Figure 5A), but had no notable impacts on the expression of other miRNAs tested (Supplement Figure 1). Next, 786-0 cells were transfected with a miR-223-3p mimic or inhibitor to alter miR-223-3p expression (Figure 5B). A CCK-8 assay showed that miR-223-3p
The effect of miR-223-3p on cell proliferation was also seen in ACHN cells (Figure 5D-F). Further, based on a Transwell assay, miR-223-3p could partially attenuate the inhibition of cell migration caused by RASA1 overexpression (Figure 5G). The above results indicate that RASA1 might inhibit cell proliferation and migration by regulating miR-223-3p.

**FBXW7 is a target of miR-223-3p**

Through bioinformatic analysis, FBXW7 was identified as a potential target gene of miR-223-3p (Figure 6A). There’re some other potential target genes of miR-223-3p, which have high binding scores, but we found they are not dysregulated in RCC tissues. Therefore, FBXW7 was chosen for the further study, A dual luciferase reporter gene assay confirmed a binding association between FBXW7 and miR-223-3p (Figure 6B, 6C). Further, we detected the expression level of FBXW7 mRNA and protein level in RCC tissues. FBXW7 mRNA and protein were down-regulated in RCC tumor tissues compared to adjacent tissues (Figure 6D, 6E). Interestingly, the mRNA and protein levels of FBXW7 were pronouncedly decreased after up-regulation of miR-223-3p expression, but increased when miR-223-3p was down-regulated (Figure 6F-I), confirming that miR-223-3p could modulate the expression of FBXW7. In RCC cell lines, the expression of FBXW7 was generally reduced compared to HK2 cells (Figure 6J). By Pearson correlation analysis, the expression of FBXW7 was found to be negatively correlated with miR-223-3p expression, but remarkably positively correlated to the expression of RASA1 in renal cancer tissues, indicating that RASA1
might up-regulate *FBXW7* expression by down-regulating miR-223-3p expression (Figure 6K-L).

**RASA1 inhibits RCC tumorigenesis in vivo**

As shown in Figure 7A, overexpression of *RASA1* inhibited tumor growth, while downregulation of *RASA1* promoted tumor growth *in vivo*, which was confirmed by the detection of tumor volume and weight (Figure 7B, 7C). Additionally, over-expression of *RASA1* greatly upregulated the RASA1 protein expression, which was downregulated with an siRNA targeting *RASA1* in tumor tissues (Figure 7D). Furthermore, western blot and IHC results revealed that the upregulation of *RASA1* greatly reduced the expression of Ki67, which was upregulated when *RASA1* was silenced (Figure 7E, 7F).
Currently, 14 RasGAPs have been identified in mammals [20], and several of them are epigenetically modified in various cancers [21], including *NF1* [22], *DAB2IP* [23], *RASAL1* [24], and *RASAL2* [25]. Within the RasGAP family, the RASA subfamily is composed of five RasGAPs [21]: RASA1/p120GAP, three GAP1 members (RASA2/GAP1m, RASA3/GAP1IP4BP and RASA4/CAPRI), and RASA5/SynGAP. The RASA group inversely modulates Ras signaling and could therefore curb tumor formation in cancer pathogenesis. The *RASA1* inactive mutation, called a driver mutation, together with the NF1 mutation defines a genetically new subclass of non-small cell lung cancer [26, 27]. Allelic deletion of *RASA1* is common in triple-negative breast cancer (BC) with TP53 mutations, and its decrement gives rise to the malignant phenotype of breast cells [28]. However, the role of *RASA1* in RCC remains unclear. This study disclosed that *RASA1* was down-regulated in RCC tissues and cells. Furthermore, we found that the up-regulation of *RASA1* could inhibit cell proliferation and migration while promoting cell apoptosis. Caspases are cysteine-containing aspartate-specific hydrolases and are the primary executor of cell apoptosis [29]. Caspase-3 is a key protease in mammalian cell apoptosis and is one of the most important proteins in caspase family [30, 31]. Here, we demonstrated that RASA1 could promote cell apoptosis by regulating Caspase-3 expression.

MicroRNAs, non-coding RNAs with 19-22 nucleotides, inversely modulate translation and are implicated in a number of cellular processes. Augmentation of certain miRNAs has been intimately correlated with diverse diseases, including
diabetes, obesity, cancers and cardiovascular diseases [32, 33]. Aberrant modulation
of miRNAs is an important component in cancers due to both the oncogenic and
tumor-suppressive functions of miRNAs [34]. miR-223 is found in the q12 segment
of the human X chromosome, whose expression is modulated by transcription factors
such as C/EBPa, PU.1 and NFI-A [35]. miR-223 is implicated in modulating
functions of the cardiovascular system, human immune system, muscle and bone
[36]. Additionally, miR-223 also functions as a tumor suppressor gene in different
tumors. A study by Fabris et al. ascertained that overexpression of miR-223 could
inhibit BC relapse through the mediation on the EGF signaling pathway, implying that
miR-223 overexpression also has crucial impacts on BC progression [37].
Additionally, it has been ascertained that miR-223-3p promotes cell proliferation and
metastasis by downregulating SLC4A4 in RCC [15]. Here, the expressions of
RCC-correlated miRNAs were examined upon RASA1 overexpression and only
miR-223-3p had altered expression. In order to confirm whether RASA1 regulates
miR-223-3p in RCC cells, a rescue experiment was conducted. We found that RASA1
inhibits cell migration and proliferation but boosts cell apoptosis by inhibiting miR-
223-3p expression.
Reports show that miRNAs participate in cell differentiation, apoptosis and
proliferation by targeting downstream mRNAs [38]. It was found in this work that
F-box/WD repeat-containing protein 7 (FBXW7) is a target gene of miR-223-3p, and
is expressed at lower levels in RCC tissues and cell lines. FBXW7 is pivotal for
modulating cell growth and differentiation as well as cell cycle progression in human
cells, and its decrement is able to drive cancer cells to proliferate [39, 40]. Deletion of
FBXW7 results in poor prognosis in multiple cancers [41, 42]. Several recent studies
have shown that FBXW7 is the target of miRNAs and functions in the regulation of
cancers. For instance, miR-223 regulates acute lymphoblastic leukemia by
suppressing FBXW7 expression [43]. Additionally, miR-27a promotes lung cancer
 cell growth by repressing FBXW7. These results imply that FBXW7 acts as a tumor
suppressor in human cancers [44]. In this study, we confirmed an association
between miR-223-3p and FBXW7 by dual-luciferase reporter and found that
miR-223-3p regulates the expression of FBXW7 in RCC cell lines. Moreover, we also
found FBXW7 was overexpressed in RCC tumor tissues and that miR-223-3p and
FBXW7 mRNA expression is negatively correlated, while RASA1 and FBXW7 mRNA
levels are positively correlated. Hence miR-223-3p may directly regulate FBXW7.
Lastly, we demonstrated that RASA1 functions in RCC by regulating miR-223-3p.
Taken together, we hypothesize that RASA1 has a role in the development of RCC by
decreasing miR-223-3p expression, thus increasing FBXW7 expression.
In conclusion, RASA1 is lowly expressed in RCC tissues and cell lines and has a role
in RCC occurrence and progression by inhibiting the expression of miR-223-3p and
enhancing the expression of FBXW7. However, this study has several limitations.
Firstly, the sample size was small. Further studies using a large sample size will
validate the results shown here. Secondly, this study found that RASA1 can regulate
miR-223-3p, but its specific mechanism has not yet been elucidated. Future work will
aim to elucidate the mechanism behind RASA1 regulation.
Abbreviations

IncRNA: Long non-coding RNA; RASA1: RAS p21 protein activator 1; RCC: Renal cell carcinoma; FBXW7: F-box/WD repeat-containing protein 7.

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None.

Authors’ contributions

All authors have participated in study design and manuscript preparation. Rui-Li Zhang performed all experiments, Ainiwaer Aimudula and Jiang-Hong Dai performed the statistical analysis and drafted the manuscript. Yong-Xing Bao designed the work and revised the manuscript. All authors have approved the final article.

Ethic statement

This study protocol was approved by the First Affiliated Hospital of Xinjiang Medical University. All patients provided written informed consent.

Conflicts of interests

None

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Figure legends:

Figure 1: RASA1 is expressed at lower levels in RCC tissues and cell lines than in control ones. A: qRT-PCR manifests that RASA1 is expressed at lower levels in RCC tissues than in paracancerous tissues. B: Through examination, the expression levels of RASA1 in six RCC cell lines are found to be reduced relative to those in HK-2 cell line. C: RASA1 was down-regulated in RCC tissues at protein level. Data are presented as mean ± SD in three independent assays. * P<0.05, ***P<0.001.

Figure 2: RASA1 promotes cell apoptosis. A: In 786-0 cells, the expression of RASA1 is tested by qRT-PCR; B: The expressions of RASA1 and apoptosis index Caspase-3 are detected by Western blot; C: Flow cytometry is employed to examine the apoptosis rate after overexpression of RASA1. D: In ACHN cells, qRT-PCR assay verifies the overexpression efficiency of RASA1; E: Western blot is applied to examine the expressions of RASA1 and Caspase-3; F: Flow cytometry is adopted for examining the apoptosis rate after overexpression of RASA1. * P<0.05.

Figure 3: Effects of RASA1 on the proliferation, migration and cell cycle of RCC cells. A: Detection of cell viability after transfection of RASA1 in 786-0 cells via CCK-8 assay. B: cell colony formation assay verifies the colony formation rate of 786-0 cells after transfection of RASA1. C: Detection of the time gradient effects of RASA1 on 786-0 cell proliferation via CCK-8 assay. D: Examination of cell migration after transfection of RASA1 via Transwell assay. E: Examination of cell viability after
transfection of RASA1 in ACHN cells via Transwell assay. F: Cell colony formation assay validates the colony formation rate of ACHN cells after transfection of RASA1. G: CCK-8 assay is used to detect the time gradient effects of RASA1 on the proliferation of ACHN cells. H: Detection of the migration of ACHN cells after transfection of RASA1 via Transwell assay. *P<0.05.

**Figure 4: Overexpression of RASA1 greatly increases RCC cell proportion in G0/G1 phase.** A: Cell cycle is detected using flow cytometry after overexpression of RASA1 in 786-0 cells. B: Cell cycle changes are examined by flow cytometry after overexpression of RASA1 in ACHN cells. *P<0.05.

**Figure 5: RASA1 functioned by modulating miR-223-3p.** A: In 786-0 cells, the expression of miR-223-3p is reduced after up-regulating the expression of RASA1; B: miR-223-3p mimic and inhibitor transfection efficiency are detected; C: Up-regulation of miR-223-3p can partially reverse the inhibition of RASA1 on cell proliferation; D: In ACHN cells, the expression of miR-223-3p is reduced after up-regulating the expression of RASA1; E: miR-223-3p mimic and inhibitor transfection efficiencies are detected; F: Up-regulation of miR-223-3p can partially reverse the inhibition of RASA1 on cell proliferation; G: Up-regulation of miR-223-3p in both cell lines partially reverses the inhibitory effect of RASA1 on cell migration. *P<0.05.
Figure 6: **FBXW7 is the target gene of miR-223-3p.** A: *FBXW7* has a potential binding site with miR-223-3p. B-C: Dual luciferase reporter gene results in two RCC cell lines. D-E: *FBXW7* was down-regulated in RCC tissues at protein level. F: The transfection efficiency of miR-223-3p mimic or inhibitor in 786-0 cells. G: *FBXW7* protein expression is down-regulated after miR-223-3p increment in 786-0 cells, whereas it is elevated after down-regulation of miR-223-3p. H: The transfection efficiency of miR-223-3p mimic or inhibitor in ACHN cells. I: *FBXW7* protein is decreased after up-regulation of miR-223-3p in ACHN cells, while it is increased after down-regulation of miR-223-3p. J: The expression of *FBXW7* is pronouncedly decreased in renal cancer cell lines. K: The expression of *FBXW7* and miR-223-3p are notably negatively correlated in renal cancer tissues. L: *FBXW7* is prominently positively correlated with *RASA1* expression in renal cancer tissues. *P<0.05, **P<0.01.

Figure 7: **RASA1 inhibited RCC tumorigenesis in vivo.** A: Morphology of tumor xenograft. B: The change in tumor volume was determined every 7 d during the tumor growth. C: Tumor weight of nude mice was significantly increased by the transfection with si-*RASA1* and reduced when *RASA1* overexpressed. D: The protein expression of *RASA1* in each group tumor tissues. E: The protein expression of *Ki-67* in each group tumor tissues. F: The expression of Ki67 in each group tumor tissues was determined by IHC. *P<0.05, **P<0.01, ***P<0.001.
| Gene    | Primer sequences                                      |
|---------|-------------------------------------------------------|
| RASA1   | Forward: 5'-ACTTGACAGAACGATAGCAGAAG -3'               |
|         | Reverse: 5'-GCCTCCGATCCTCTCTTATA -3'                 |
|         | Forward: 5'-GTTCCGCTGCTAATCTTCTCT -3'                |
|         | Reverse: 5'-CCCTCCAGGGATTTCTGTCCTCC -3'              |
| FBXW7   | Forward: 5'-CCATGTTCGTCATGGGTGTTGCATTCA -3'          |
|         | Reverse: 5'-GCCAGTAGAGGGGATGTGTTTG -3'               |
| GAPDH   | Forward: 5'-CCATGTTCGTCATGGGTGTTGCATTCA -3'          |
|         | Reverse: 5'-GCCAGTAGAGGGGATGTGTTTG -3'               |
| U6      | Forward: 5'-AAATATGGAAACGCTTACGACGA -3'              |
|         | Reverse: 5'-AAATATGGAAACGCTTACGACGA -3'              |
| miR-223-3p | Forward: 5'-CAGAAAGCCCAATTCCATCT -3'               |
|         | Reverse: 5'-GGGCAAATGGATACCATACC -3'                 |
A. 

FBXW7 WT 5' GTAAGACTGACA 3'  
miR-223-3p 3' GUUUGACUG 5'  
FBXW7 MUT 5' GTUUUGACUGA 3'

B. 

\[ \text{Relative luciferase activity} \]

C. 

\[ \text{Relative luciferase activity} \]

D. 

\[ \text{Relative FBXW7 protein level} \]

E. 

\[ \text{Relative FBXW7 protein level} \]

F. 

\[ \text{Relative mRNA expression levels of FBXW7} \]

G. 

\[ \text{Relative mRNA expression levels of FBXW7} \]

H. 

\[ \text{Relative mRNA expression levels of FBXW7} \]

I. 

\[ \text{Relative mRNA expression levels of FBXW7} \]

J. 

\[ \text{Relative expression of FBXW7} \]

K. 

\[ \text{Relative expression of miR-223-3p} \]

L. 

\[ \text{Relative expression of RASA1} \]
Supplement Figure 1: The effect of RASA1 on the expression of some renal cancer-associated miRNAs is detected in 786-0 cells. Except for miR-223-3p, no obvious differences are found in other indexes. $P<0.05$, **$P<0.01$, ***$P<0.001$. 
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**Title of the Paper**
RASA1 inhibits the progression of renal cell carcinoma by decreasing the expression of miR-223-3p and promoting the expression of FBXW7

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