Suppressive effects of RASAL2 on renal cell carcinoma via SOX2/ERK/p38 MAPK pathway

SEN WANG[1], XIAOMIN HAO[2], SAI HE[3], CHANGLI LIU[2] and QILONG WANG[4]

[1]Department of Urinary Surgery, Shaanxi Friendship Hospital, Xi'an, Shaanxi 710068;
[2]Department of Internal Medicine, Shaanxi Province Tuberculosis Hospital, Xi'an, Shaanxi 710100;
[3]Department of Breast Cancer, Shaanxi Province Oncology Hospital, Xi'an, Shaanxi 710061;
[4]Department of General Surgery, Shaanxi Friendship Hospital, Xi'an, Shaanxi 710068, P.R. China

Received September 16, 2019; Accepted March 24, 2020

DOI: 10.3892/etm.2020.9280

Abstract. Metastatic renal cell carcinoma (RCC) is associated with poor prognosis. Ras protein activator like 2 (RASAL2) protein has been previously demonstrated to serves as a tumor suppressor in a variety of malignancies. Therefore, the aim of the present study was to investigate the role of RASAL2 in RCC. Reverse transcription-quantitative PCR, western blot analysis and immunohistochemistry were performed to measure mRNA and protein expression in RCC tissues, whilst immunofluorescence and western blotting were performed to evaluate protein expression in RCC cells. A Cell Counting Kit-8 and 5-bromo-2'-deoxyuridine staining were applied to determine cell viability, and Transwell assays were conducted to measure RCC cell invasion and migration. RASAL2 expression was identified to be downregulated in RCC tissues, which associate negatively with RCC pathological grade. Sox2 expression, in addition to ERK1/2 and p38 MAPK phosphorylation, were demonstrated to be increased in RCC tissues. In RCC cells, RASAL2 overexpression decreased the expression of Sox2 and the activation of ERK1/2 and p38 MAPK. Physiologically, RASAL2 overexpression decreased RCC cell viability, invasion and migration. The expression of metalloproteinase-2/9 and tissue inhibitor of metalloproteinase 1 were also identified to be decreased and increased by RASAL2 overexpression, respectively. By contrast, RASAL2 knockdown exerted opposite effects on RCC cells compared with those observed following RASAL2 overexpression. RASAL2 expression decreased RCC cell viability, migration and invasion, which was demonstrated to be associated with the inactivation of SOX2/ERK1/2/p38 MAPK signaling. These results suggest that RASAL2 may potentially serve as a potential target for the development of novel therapeutic intervention strategies against RCC.

Correspondence to: Mr. Qilong Wang, Department of General Surgery, Shaanxi Friendship Hospital, 227 Friendship West Road, Xi'an, Shaanxi 710068, P.R. China
E-mail: wangqilongmr@163.com

Key words: ras protein activator like 2, invasion, migration, renal cell carcinoma, extracellular signal-regulated kinase, p38 mitogen-activated protein kinase

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney malignancy (1,2). About 190,000 new cases are diagnosed, and over 91,000 RCC patients were related with death in 2003 worldwide, with the incidence of RCC increasing by ~2% every 10 years (3). The morbidity and mortality associated with RCC are also typically increased in males compared with females (4,5). The high rates of morbidity and mortality as a result of RCC are mainly due to RCC metastasis (6). Although improvements have been made in cancer therapy, RCC remains relatively insensitive to conventional therapeutic interventions (7), as metastasis is a common feature of late-stage RCC. Therefore, metastatic tumor cells further aggravate the condition of patients with RCC. Effective treatment for metastatic RCC that are available remain insufficient, leading to poor prognoses (8). Molecular targeted therapy against cancer has garnered significant attention worldwide, where research effort has been focused on the exploration and development of targeted therapy against RCC. Therefore, the identification of a potential target that can interfere with RCC progression is of utmost importance.

Ras protein activator like 2 (RASAL2) is a Ras GTPase-activating protein. RASAL2 catalyzes GTP to GDP, which inactivates Ras. Dysregulation of the Ras signaling pathway is frequently observed in cancer cells (5). RASAL2 expression has also been previously revealed to be aberrantly altered in a number of different tumors (9-14), where it appears to serve as a tumor suppressor gene (5,15,16). Although RASAL2 has been reported to inhibit epithelial-mesenchymal transition (EMT), a process which serves important roles in cancer cell metastasis (17,18), the functional profile of RASAL2 in RCC remains poorly understood. Therefore, in the present study, RASAL2 expression and function was investigated in RCC.

Ras-MAPK signaling pathway dysfunction, particularly those of ERK1/2 and p38 MAPK, is frequently observed in human malignancies (19-21). In particular, a number of

important cellular processes, including cell growth and apoptosis, are associated with the ERK1/2 and p38 MAPK signaling pathways (22,23). The MAPK signaling pathway has been reported to regulate EMT by modulating the expression of Sox2 (24-27), a transcription factor that is involved in the regulation of cancer cell physiology.

In the present study, the hypothesis that RASAL2 may regulate RCC progression by modulating the Sox2/MAPK signaling pathway was explored. The results from the present study may provide novel insights into the mechanism underlying RASAL2-mediated regulation of RCC progression, which can be a potential therapeutic target in RCC therapy.

Materials and methods

Tissue collection. The experiment protocol on human tissues was approved by the Ethics Committee of the Shaanxi Friendship Hospital. Tumor tissues and the matched para-carcinoma tissues from the same patient were collected from 21 patients with RCC (Average age: 64.5±11.3 years; male/female: 13/8) who were admitted to the Shaanxi Friendship Hospital between August 2016 and November 2017. Inclusion criteria included the pathological diagnosis was clearly RCC; successful surgical treatment; and recurrence or metastasis of RCC was the immediate cause of death. Exclusion criteria included unclear pathological diagnosis; multiple basic diseases; no surgical treatment; and patients with fatal cardiovascular or cerebrovascular diseases or accidental death.

The patients enrolled were pathologically diagnosed with RCC and signed informed consent forms. According to the World Health Organization grading system (28), RCC tissues were divided into the following three groups in the present study: i) High-grade, ii) middle-grade; and iii) low-grade.

Cell culture. ACHN cells, a human renal carcinoma cell line, was purchased from the American Type Culture Collection (ATCC). Cells were cultured routinely in Eagle's Minimum Essential Medium (EMEM; ATCC) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 10% penicillin/streptomycin, at 37˚C with 5% CO_2.

Cell transfection. Cells were seeded into 6-well plates at a density of $1.0x10^4$ cells/well. Following starving overnight without serum, cells were transfected with the 1 μg control vector (pcDNA3.1), 1 μg RASAL2 overexpression plasmid (pcDNA3.1-RASAL2), 1 μg short hairpin (sh) RASAL2 plasmid (pGPU6-RASAL2; targeting RASAL2 sequence, 5'-CCCTCGTGTTCTTGCTGATAT-3') or 1 μg shRASAL2 or 1 μg scramble plasmid (negative control shRNA plasmid). All plasmids were purchased from Shanghai GenePharma Co., Ltd.. Transfection reactions were performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. All cells were collected and subjected to further experiments 24 h following transfection.

Cell counting Kit-8 (CCK-8) assay. Cells were seeded into 96-well plates at a density of $1x10^2$ cells/well prior to transfection using the protocol aforementioned. After 24 h, 10 μl CCK-8 reagent (Beyotime Institute of Biotechnology) were added into each well. The cells were then incubated at 37˚C for a further 4 h. Absorbance values were measured in each well at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.), which were then correlated to cell proliferation activity. Cell proliferation activity (%)=(OD value in the experimental group-OD value in the blank group)/(OD value in the control group-OD value in the blank group) x100%.

5-bromo-2'-deoxyuridine (BrdU) staining. Cells ($2x10^4$ cells) from each group were inoculated on cell culture slides and incubated at 37˚C for 72 h, followed by being labeled with 10 μmol/l BrdU solution (Sigma-Aldrich; Merck KGaA; cat. no. B5002) at 37˚C for 48 h. After washing with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min. After washing with PBS, the cells were treated with 2 mol/l HCl at 37˚C for 5 min, neutralized with 0.1 mol/l sodium tetraborate at room temperature for 10 min and then washed with 0.2% triton X-100 at room temperature for 5 min. After sealing with 3% BSA (Thermo Fisher Scientific, Inc.; cat. no. BP9704-100) at room temperature for 1 h, Brdu antibodies (1:200; Abbiotec, Inc.; cat. no. 251163) was applied at 4˚C overnight. After washing with PBS, cells were incubated with sheep anti-mouse IgG/Alexa Fluor594 (1:100, ProteinTech Group, Inc.; cat. no. SA00006-3) at room temperature in darkness for 1 h. After washing with PBS, 1 mg/ml DAPI (Invitrogen, Thermo Fisher Scientific, Inc.; cat. no. D1306) was added to the cells at room temperature for 10 min. The BrdU positive cells were observed under a fluorescence microscope (Leica FW 4500 B microscope; Leica Microsystems GmbH), magnification x100.

Transwell assays. Cell invasion and migration were evaluated using Transwell assays. the upper chamber (BD Biosciences) were precoated with or without Matrigel (cat. no. M8370; Beijing Solarbio Science and Technology Co., Ltd.) for 30 mins at 37˚C. Chambers that were coated with Matrigel were used for invasion assays whilst those without were used for migration assays. Briefly, the transfected RCC cells with serum-free medium were seeded into the upper chamber (BD Biosciences) at a density of $1.0x10^5$ cells/ml. The lower chamber was added with medium supplemented with 10% FBS. Cells that invaded or migrated through the membrane were subsequently stained with 500 μl 0.1% crystal violet at 37˚C for 30 min after incubation for 36 h. The cells were observed using a light microscope (magnification, x100). Five different fields were observed and photographed. The relative cell migration and invasion rates were counted through the number of the migrated or invaded cells/the number of the inoculated cells in the same field.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. cDNA was obtained from 1 μg total RNA using GoScript™ Reverse Transcription kit (Promega Corporation) according to manufacturer's protocol, using the following temperature setting: 25˚C for 5 min, at 42˚C for 60 min and 70˚C for 15 min. Subsequent qPCR amplification was performed using Fast SYBR™ Green Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.)

according to manufacturer's protocol. The following thermocycling conditions were used: Initial denaturation at 95˚C for 20 sec, followed by 40 cycles at denature at 95˚C for 3 sec, and annealing/extension at 60˚C for 30 sec. The sequences of primers used were as follows: *RASAL2* forward, 5'-TCCCTC GTGTTCTTGCTGAT-3' and reverse: 5'-GTCTGTGTTGTC CTGGCTTG-3'; GAPDH forward, 5'-TGCACCACCAAC TGCTTAGC-3' and reverse, 5'-GGCATGGACTGTGGTCAT GAG-3'. Relative *RASAL2* expression was normalized to that of GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (12).

Western blotting. Total proteins were extracted from tissues and cells using Total Protein Extraction kit (Merck Millipore; cat. no. XY-AP200A). Protein concentration was measured using the Bicinchoninic Acid protein assay kit (Shanghai Qcbio Science & Technologies Co., Ltd.). A total of 1 μg total protein were loaded per well and separated by 10% SDS-PAGE, proteins were transferred onto PVDF membranes (EMD Millipore). The membranes were then blocked using 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. Following incubation with primary antibodies at 4˚C overnight, the membranes were then treated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos., ab205719 and ab205719; Abcam) at room temperature for 1 h. ECL Plus reagent (Beyotime Institute of Biotechnology) was used to visualize the membranes, following which Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.) was used for densitometric analysis according to the manufacturer's protocols. The following primary antibodies were used: Anti-RASAL2 antibody (cat. no. ab121578; 1:100; Abcam), anti-SOX2 (cat. no. ab97959; 1:200; Abcam), anti-phosphorylated (p)-ERK1/2 (cat. no. ab201015; 1:400; Abcam), anti-ERK1/2 (cat. no. ab54230; 1:1,000; Abcam), anti-p38 (cat. no. ab27986; 1:1,000; Abcam), anti-p-p38 (cat. no. ab47363; 1:2,000; Abcam), anti-MMP-2 (cat. no. ab97779; 1:2,000; Abcam), anti-MMP-9 (cat. no. ab38898; 1:2,000; Abcam), anti-TIMP1 (cat. no. ab61224; 1:1,000; Abcam) and anti-GAPDH (cat. no. ab9485; 1:2,500; Abcam).

Immunohistochemical (IHC) staining. The tumor and para-carcinoma normal tissues were fixed using 4% paraformaldehyde overnight at 4˚C, embedded in paraffin and sliced into sections of 3-4 μm thickness. RCC was divided into low-grade and high-grade according to previously published criteria (29). Slides were subsequently dewaxed with dimethylbenzene, hydrated with dimethylbenzene and treated with an alcohol gradient (100, 95, 80 and 75%). After washing, the antigen retrieval was performed by heating the sections at 95˚C in sodium citrate buffer (pH 6.0; 10 mM; cat. no. 25229-1; Wuhan Sanying Biotechnology). The cooled sections were then incubated in 3% hydrogen peroxide (H_2O_2) solution for 10 min at room temperature. Following blocking with 10% normal goat serum (Thermo Fisher Scientific, Inc.) at 37˚C for 30 min, slides were incubated with anti-RASAL2 antibody (1:50; cat. no. ab216127; Abcam) at 4˚C overnight. The slides were then incubated with biotin-labeled secondary antibody (cat. no. A0279; 1:200; Beyotime Institute of Biotechnology) at room temperature for 1 h. Slides were first treated with horseradish peroxidase-linked streptavidin (1:500, Vector Laboratories; cat. no. SA-5014) at room temperature for 30 min, following which they were incubated with 3'3'-diaminobenzidine (DAB) for 5 min at room temperature. The sections were then stained with hematoxylin at room temperature for 2 min. Sections were observed using a light microscope (magnification x100). The results were assessed by two senior pathologists. RASAL2 was positive with brown-yellow staining of nucleus/cytoplasm. According to the percentage of positive cells: 1 point: 1-10%; 2 points: 10-50%; 3 points: 50-80%; 4 points: >80%.

Immunofluorescence (IF) staining. Cells ($2x10^5$ cells/ml) were fixed with 4% paraformaldehyde at room temperature for 15 min and treated with 0.5% Triton X-100 for 20 min at room temperature. Following blocking with 10% normal goat serum (Thermo Fisher Scientific, Inc.) at 37˚C for 30 min, slides were incubated with the anti-RASAL2 primary antibody (1:100; cat. no. ab121578; Abcam) at 4˚C overnight. The slides were then incubated with Alexa Fluor® 594-conjugated secondary antibody (1:200; cat. no. ab150120; Abcam) for 1 h at room temperature, following which nuclei were stained using DAPI (1:10; cat. no. D1306; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 5 mins and the slides were observed using a fluorescence microscope (Leica FW 4500 B microscope; Leica Microsystems GmbH).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 6.0 (GraphPad Software, Inc.). Data are presented the mean ± SD from at least three replicates of the experiment, where comparisons were calculated using one-way ANOVA followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RASAL2 expression is decreased in RCC tissues. RT-qPCR and western blotting were performed to measure RASAL2 mRNA and protein expression levels in RCC tissues. RASAL2 expression was revealed to be significantly decreased in RCC tissues at mRNA and protein levels compared with adjacent normal tissues (Fig. 1A and B). Specifically, RASAL2 expression was significantly decreased in high-grade RCC tissues compared with the middle-grade samples, whilst RASAL2 expression was in turn significantly decreased in middle-grade RCC tissues compared those in low-grade RCC tissues ($P<0.05$; Fig. 1A and B). In IHC staining, the brown staining represented RASAL2 staining. And it was discovered that RASAL2 expression was significantly decreased in RCC tissues compared with in normal tissues. RASAL2 expression was significantly decreased in high-grade RCC tissues compared with middle-grade RCC tissues, whilst RASAL2 expression in middle-grade RCC tissues was significantly decreased compared with low-grade RCC tissues ($P<0.05$; Fig. 1C). Therefore, these results suggest that RASAL2 was downregulated in RCC tissues compared with corresponding normal tissues, where its expression level is negatively associated with histological tumor grade.

SOX2 expression, and ERK and p38 MAPK activation levels are increased in RCC tissues. The expression levels of SOX2, p-ERK/ERK and p-p38/p38 were identified to be

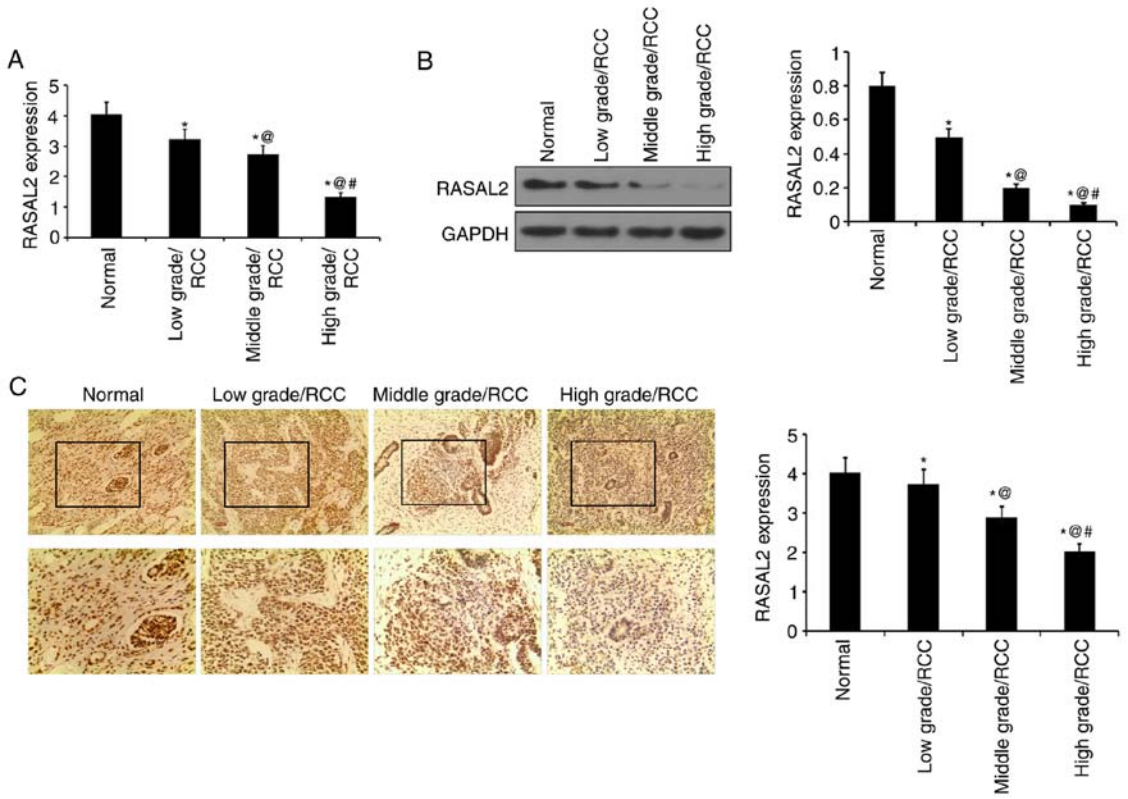

Figure 1. RASAL2 expression was decreased in RCC tissues. (A) Comparison of RASAL2 mRNA expression in normal and RCC tissues, measured using reverse transcription-quantitative PCR. (B) Comparison of RASAL2 protein expression in normal tissue and RCC tissues, measured using western blotting. (C) Comparison of RASAL2 protein localization in normal and RCC tissues, as measured using immunohistochemical staining. Scale bars=100 μm (upper panel); Scale bars=50 μm (lower panel). *P<0.05 vs. normal RCC tissue. $^{@}$P<0.05 vs. low-grade RCC. $^{\#}$P<0.05 vs. middle-grade RCC. RCC, renal cell carcinoma; RASAL1, ras protein activator like 2.

significantly increased in RCC tissues compared with those in normal tissues; a trend that was observed to be positively associated with tumor grade, suggesting that SOX2 and the phosphorylated ERK and P38 were significantly enhanced in RCC tissues (P<0.05; Fig. 2).

Overexpression and knockdown of RASAL2 expression in ACHN cells. To investigate the potential underlying mechanism and function of RASAL2 in RCC, ACHN cells were transfected with plasmids encoding either the RASAL2 protein or RASAL2 shRNA to overexpress or silence RASAL2 expression, respectively. Data from RT-qPCR, western blotting and IF staining demonstrated that RASAL2 expression was significantly increased in cells transfected with RASAL2 plasmids compared with those transfected with corresponding control, whilst RASAL2 expression was significantly lower in cells transfected with shRASAL2 compared with those transfected with the scramble shRNA (P<0.05; Fig. 3). Therefore, these observations suggest that the manipulation of *RASAL2* expression ACHN cells was successful.

Effect of RASAL2 expression on RCC cell viability, invasion and migration. The effects of RASAL2 overexpression and knockdown on RCC cell viability, invasion and migration were subsequently evaluated on ACHN cells. As shown in Fig. 4A, compared with the control group, overexpression of RASAL2 significantly decreased cell viability (P<0.05), and the cell viability was significantly increased in RASAL2 knockdown group relative to scramble group (P<0.05). In addition, the migration and invasion abilities of ACHN cells were significantly decreased in RASAL2 overexpression group with respect to the control group; by contrast, the migration and invasive capacities of ACHN cells were significantly increased in the RASAL2 knockdown group versus that in the scramble group (P<0.05; Fig. 4B-D). Moreover, the results of BrdU staining also disclosed that overexpression of RASAL2 prominently suppressed the proliferation of RCC cells, and knockdown of RASAL2 had a significant promoting effect on the RCC cell proliferation (P<0.05; Fig. 4E).

Effect of RASAL2 expression on MMP-2, MMP-9 and TIMP-1 in RCC cells. Subsequently, the possible regulatory effects of RASAL2 overexpression or knockdown on the MMP-2, MMP-9 and TIMP-1 expressions in RCC were further investigated. The results of western blot assay exhibited that relative to the control group, RASAL2 overexpression significantly downregulated MMP-2 and MMP-9 expressions, and significantly upregulated TIMP-1 expression in ACHN cells. By contrast, compared with the scramble group, RASAL2 knockdown significantly increased MMP-2 and MMP-9 expressions, and markedly decreased TIMP-1 expression in ACHN cells (P<0.05; Fig. 5).

Effect of RASAL2 expression on SOX2 expression and the activation of ERK and p38 MAPK in RCC cells. Subsequently, western blot assays were used to evaluate the effects of RASAL2 overexpression and silencing on SOX2

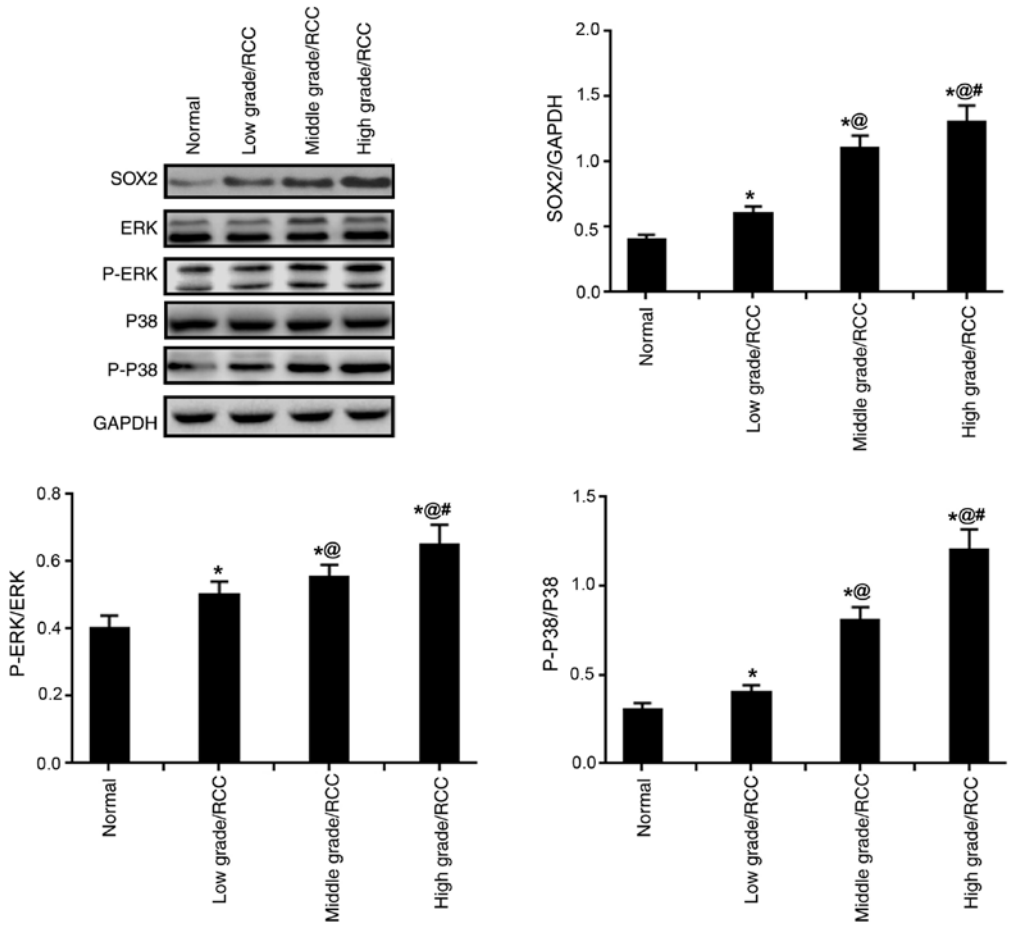

Figure 2. SOX2, p-ERK and p-p38 MAPK expressions were notably elevated in RCC tissues. Protein expression levels of SOX2, ERK1/2, p-ERK1/2, p38 and p-p3 were measured in RCC tissues by western blotting analysis. And the relative expressions were calculated in accordance with the gray value. *P<0.05 vs. normal RCC tissue. @P<0.05 vs. low-grade RCC. #P<0.05 vs. middle-grade RCC. RCC, renal cell carcinoma; p-, phosphorylated.

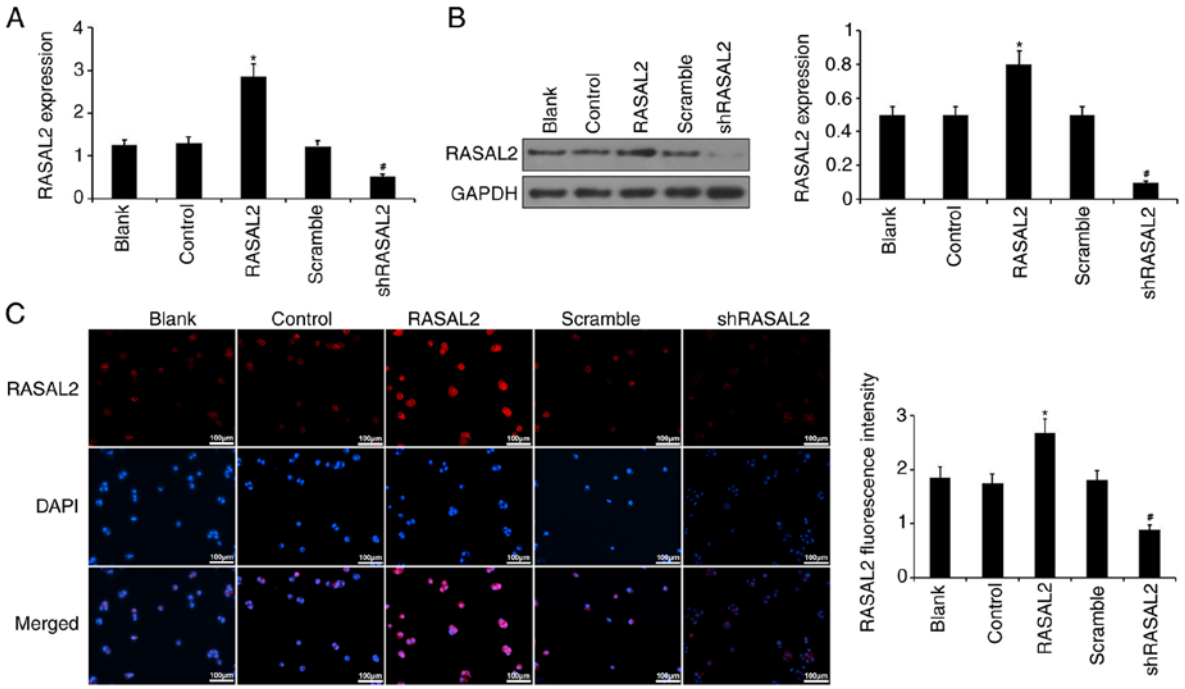

Figure 3. Overexpression and knockdown of RASAL2 expression in ACHN cells. (A) RASAL2 expression was evaluated in ACHN cells using reverse transcription-quantitative PCR, (B) western blotting and (C) immunofluorescence staining following transfection with plasmids expressing either the RASAL2 protein or shRASAL2. Magnification, x200. *P<0.05 vs. Control group. #P<0.05 vs. scramble group. Blank, un-transfected control; Control, control pcDNA.31 plasmid; sh, short hairpin RNA; scramble, scrambled shRNA; RASAL2, ras protein activator like 2; shRASAL2, RASAL2 shRNA.

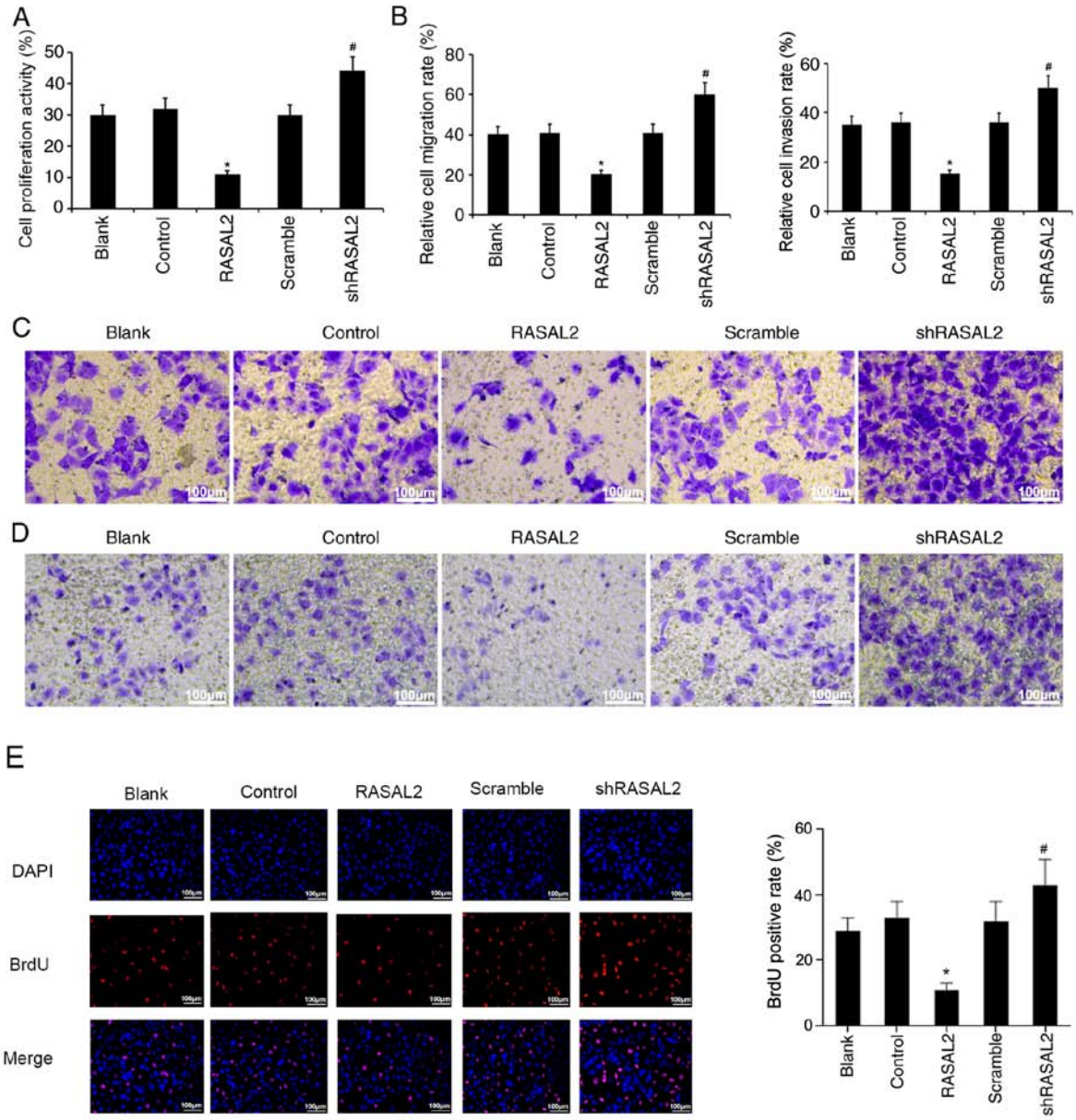

Figure 4. Effect of manipulating RASAL2 expression on RCC cell viability, migration and invasion. (A) Following RASAL2 overexpression or knockdown in ACHN cells, cell viability was measured using Cell Counting Kit-8 assay. (B-D) ACHN cell migration and invasion ability was evaluated using Transwell assays following RASAL2 overexpression or knockdown. Magnification, x200; scale bars=50 μm. Relative cell migration and invasion rates were calculated depending on the number of cells in different fields. (E) BrdU staining was conducted to evaluate the proliferation of RCC cells after RASAL2 overexpression or knockdown. Magnification, x100; scale bars=100 μm. *P<0.05 vs. Control group. $^{\#}$P<0.05 vs. scramble group. OD, optical density; Blank, un-transfected control; Control, control pcDNA.31 plasmid; sh, short hairpin RNA; scramble, scrambled shRNA; RASAL2, ras protein activator like 2; shRASAL2, RASAL2 shRNA.

expression and the activations of ERK and p38 MAPK in ACHN cells. It was discovered that the levels of SOX2, p-ERK/ERK and p-p38/p38 were significantly downregulated in ACHN cells transfected with RASAL2 plasmid compared with that cells transfected with corresponding control plasmid. Meanwhile, compared with ACHN cells transfected with the scramble, silence of RASAL2 exhibited significant promoting effects on the expressions of SOX2, p-ERK/ERK and p-p38/p38 in ACHN cells (P<0.05; Fig. 6). Taken together, these results suggest that RASAL2 overexpression suppressed the activation of SOX2/ERK/p38 MAPK signaling pathway, and RASAL2 knockdown potentiated the SOX2/ERK/p38 MAPK signaling pathway in RCC cells.

Discussion

The clinical prognosis of patients with metastatic RCC is poor, where the numbers of therapeutic methods that are effective in prolonging the survival time of patients with RCC remain insufficient (30,31). Therefore, it is of importance to elucidate the specific mechanism and function underlying the pathophysiology of RCC. The present study demonstrated that RASAL2 exerted inhibitory effects on RCC by inhibiting cell migration and invasion through regulation of the SOX2/MAPK signaling pathway.

RASAL2 has been previously demonstrated to be a Ras GAP tumor suppressor in gastric cancer (32). In the present study, RASAL2 expression was identified to be

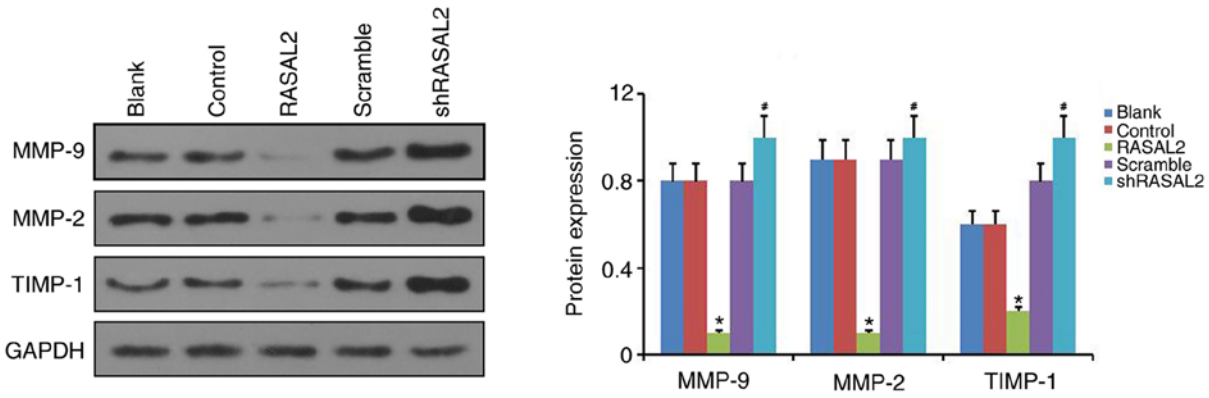

Figure 5. Effect of manipulating RASAL2 expression on MMP-2, MMP-9 and TIMP-1 expression in RCC cells. Protein expression of MMP-2, MMP-9 and TIMP-1 was assessed by western blotting in ACHN cells following RASAL2 overexpression or knockdown. GAPDH was used as the loading control. *P<0.05 vs. Control group. #P<0.05 vs. scramble group. Blank, un-transfected control; Control, control pcDNA.31 plasmid; sh, short hairpin RNA; scramble, scrambled shRNA; RASAL2, ras protein activator like 2; shRASAL2, RASAL2 shRNA; MMP, matrix metalloproteinase; TIMP-1, tissue inhibitor of metalloproteinases 1.

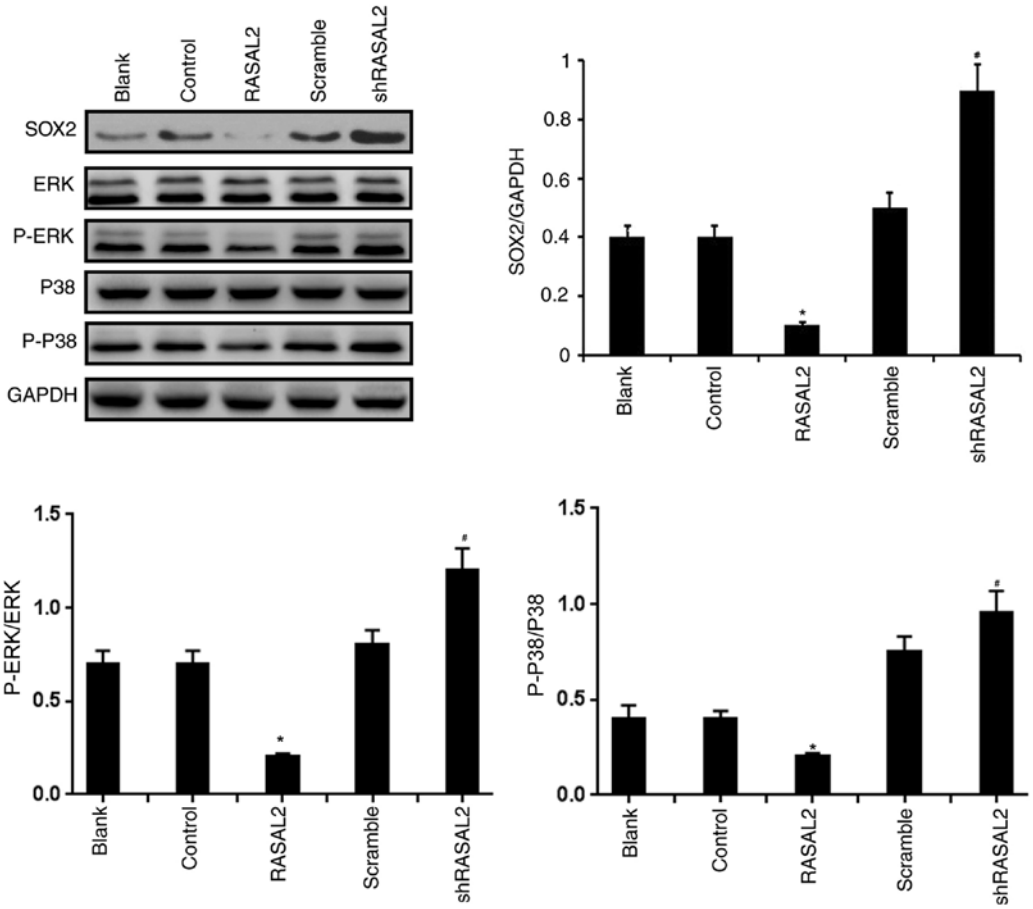

Figure 6. Effect of manipulating RASAL2 expression on SOX2 expression, ERK phosphorylation and p38 MAPK phosphorylation in RCC cells. SOX2, ERK, p38 MAPK expression, in addition to ERK and p38 MAPK phosphorylation, were assessed in ACHN cells by western blotting following RASAL2 overexpression or knockdown. GAPDH was used as loading control. *P<0.05 vs. Control group. #P<0.05 vs. scramble group. Blank, un-transfected control; Control, control pcDNA.31 plasmid; sh, short hairpin RNA; scramble, scrambled shRNA; RASAL2, ras protein activator like 2; shRASAL2, RASAL2 shRNA; p-, phosphorylated.

decreased in RCC tissues, particularly in high-grade RCC. This suggests that the inhibition of RASAL2 may contribute to the progression of RCC. Consistent with this notion, RASAL2 suppression promoted tumorigenesis in breast cancer in a previous study (5) In addition, the expression levels of RASAL2 were identified to be inversely associated with pathological grade in triple-negative or estrogen receptor-negative breast tumors (11). In the present study, the role of RASAL2 in RCC was investigated *in vitro*, where the overexpression and suppression of RASAL2 expression

were successfully performed in ACHN cells, a model RCC cell line. Subsequently, the effect of RASAL2 on RCC cell viability was investigated, as excessive cell proliferation is one of the primary hallmarks of cancer physiology (33). ACHN cell viability was revealed to be inhibited by RASAL2 overexpression but was increased by RASAL2 knockdown. Additionally, invasion and migration levels were decreased by RASAL2 overexpression but enhanced by RASAL2 knockdown in ACHN cells, suggesting that decreased RASAL2 expression promotes the invasive capabilities of RCC. Supporting this, a previous study has reported that RASAL2 downregulation in ovarian cancer cells increased EMT and metastasis (34). During the EMT process, epithelial cells lose their polarity and transform into mesenchymal cells, where they acquire migratory and invasive phenotypes (35). During this process, matrix metalloproteinases (MMPs) such as MMP-2/9 and tissue inhibitor of metalloproteinases 1 (TIMPs) such as TIMP-1, their corresponding endogenous inhibitors, exert pivotal roles (36,37). Therefore, the effects of RASAL2 which support the observations from the invasion assay were subsequently investigated. MMP-2/9 expression was demonstrated to be decreased by RASAL2 overexpression, whilst it was increased by RASAL2 knockdown. By contrast, the opposite trend was observed in TIMP-1 expression compared with that of MMP2/9 following the manipulation of RASAL2 expression. Altogether, these results suggest that RASAL2 served as an inhibitory factor in RCC by suppressing invasion, consistent with results identified in previous studies on RASAL2 (14). However, as previous reports have also previously demonstrated that RASAL2 serves as an oncogene in other types of cancer (16,38), the role of RASAL2 is most likely to be dependent on the type of malignancy, where its physiological role remains controversial.

Interaction between growth factors and their corresponding receptors is essential for the initiation of signaling cascades through activation of downstream signaling pathways, with the MAPK pathway one of the most widely studied (39). MAPK is of great significance in signal transduction (40) and can result in a variety of effects on cellular processes (41). The MAPK signaling family includes ERK1/2, JNK1/2 and p38 MAPK (40). Previous studies have demonstrated that the activation status of ERK1/2 and p38 were associated with metastatic tumors (42-44), including that of RCC (45-47). As SOX2 has also been reported to associate with ERK1/2 and p38 signaling in bladder cancer (48), the involvement of the SOX2/ERK/p38 pathway on RCC was evaluated. SOX2 expression, in addition to the activation of ERK1/2 and p38 MAPK, were identified to be elevated in RCC tissues.

RASAL2 is involved in a number of cancer types through the SOX2/MAPK signaling pathway (17,49). Therefore, in the present study it was hypothesized that a potential association between RASAL2 and SOX2/ERK/p38 signaling may exist during RCC onset. To study the mechanism underlying the effects of RASAL2 on RCC, the activity of the SOX2/ERK/p38 signaling pathway was subsequently determined *in vitro* following manipulation of RASAL2 expression. ERK and p38 MAPK activation were found to be decreased and increased by RASAL2 overexpression and knockdown in ACHN cells, respectively. According to previous studies, the inhibition of ERK1/2 and p38 MAPK signaling may prove to be beneficial to the inhibition of prostate cancer progression (50,51). In addition, it has also been previously reported that SOX2 bridges RASAL2 to the MAPK pathway in the regulation of EMT in bladder cancer cells (7,8). In the present study, RASAL2 overexpression increased the expression of SOX2, whilst its RASAL2 knockdown decreased SOX2 expression, suggesting that SOX2 may function as a bridge between RASAL2 and the ERK/p38 MAPK signaling pathway. Intracellular mechanisms underlying tumor pathophysiology are highly complex. Although the involvement of ERK1/2 and p38 MAPK in cell invasion has been reported in prostate cancer (51), the role of ERK1/2 and p38 MAPK in RCC cells has yet to be fully elucidated. Therefore, the use of ERK1/2 or p38 MAPK inhibitors may be useful to illustrate the role of ERK1/2 and p38 MAPK further in RCC in future studies, which serve as a limitation to the present study. In addition, the effect of RASAL2 on the SOX2/ERK/p38 MAPK signaling pathway should be determined *in vivo* in future studies, to confirm the role of RASAL2 in RCC.

Taken together, the present study demonstrated that RASAL2 overexpression can inhibit RCC cell viability, migration and invasion. In addition, these results support the notion that RASAL2 can serve as a potential therapeutic target for RCC.

Acknowledgements

Not applicable.

Funding

Not funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SW and QW conceptualized and designed the present study. XH and SH performed the experiments and contributed to data collection. SW and CL performed the statistical analysis and drafted the manuscript. QW examined and corrected the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experiment protocol on human tissue was approved by the Ethics Committee of the Shaanxi Friendship Hospital. The patients enrolled signed the informed consent form.

Patient consent for publication

The patients enrolled signed the informed consent form.

Competing interests

The authors declare that they have no competing interests.

References

1. Matalliotakis M, Velegrakis A, Laliotis A, Niraki E and Trivli A: Exacerbation of neurofibromatosis and adverse pregnancy outcome. A case report and review of the literature. Clin Exp Obstet Gynecol 45: 126-128, 2018.
2. Stühler V and Bedke J: Overview of treatment of localized and metastatic renal cell carcinoma (RCC). MMW Fortschr Med 160: 45-51, 2018 (In German).
3. Osawa T, Takeuchi A, Kojima T, Shinohara N, Eto M and Nishiyama H: Overview of current and future systemic therapy for metastatic renal cell carcinoma. Jpn J Clin Oncol 49: 395-403, 2019.
4. Cohen HT and McGovern FJ: Renal-cell carcinoma. N Engl J Med 353: 2477-2490, 2005.
5. McLaughlin SK, Olsen SN, Dake B, De Raedt T, Lim E, Bronson RT, Beroukhim R, Polyak K, Brown M, Kuperwasser C and Cichowski K: The RasGAP gene, RASAL2, is a tumor and metastasis suppressor. Cancer Cell 24: 365-378, 2013.
6. Curtis SA, Cohen JV and Kluger HM: Evolving immunotherapy approaches for renal cell carcinoma. Curr Oncol Rep 18: 57, 2016.
7. Liu M, Wu H, Shangguan D, Jiang Y, Li X, Liu S, Zhou B, Yin T and Gong Z: Immunomodulatory therapies for renal cell carcinoma. Protein Pept Lett 25: 534-547, 2018.
8. Abel EJ and Wood CG: Cytoreductive nephrectomy for metastatic RCC in the era of targeted therapy. Nat Rev Urol 6: 375-383, 2009.
9. Matheu A, Collado M, Wise C, Manterola L, Cekaite L, Tye AJ, Canamero M, Bujanda L, Schedl A, Cheah KS, *et al*: Oncogenicity of the developmental transcription factor Sox9. Cancer Res 72: 1301-1315, 2012.
10. Malki S, Bibeau F, Notarnicola C, Roques S, Berta P, Poulat F and Boizet-Bonhoure B: Expression and biological role of the prostaglandin D synthase/SOX9 pathway in human ovarian cancer cells. Cancer Lett 255: 182-193, 2007.
11. Lu X, Wan F, Zhang H, Shi G and Ye D: ITGA2B and ITGA8 are predictive of prognosis in clear cell renal cell carcinoma patients. Tumour Biol 37: 253-262, 2016.
12. Büttner F, Winter S, Rausch S, Reustle A, Kruck S, Junker K, Stenzl A, Agaimy A, Hartmann A, Bedke J, *et al*: Survival prediction of clear cell renal cell carcinoma based on gene expression similarity to the proximal tubule of the nephron. Eur Urol 68: 1016-1020, 2015.
13. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
14. Hui K, Yue Y, Wu S, Gu Y, Guan B, Wang X, Hsieh JT, Chang LS, He D and Wu K: The expression and function of RASAL2 in renal cell carcinoma angiogenesis. Cell Death Dis 9: 881, 2018.
15. Wang Z, Wang J, Su Y and Zeng Z: RASAL2 inhibited the proliferation and metastasis capability of nasopharyngeal carcinoma. Int J Clin Exp Med 8: 18765-18771, 2015.
16. Li N and Li S: RASAL2 promotes lung cancer metastasis through epithelial-mesenchymal transition. Biochem Biophys Res Commun 455: 358-362, 2014.
17. Hui K, Gao Y, Huang J, Xu S, Wang B, Zeng J, Fan J, Wang X, Yue Y, Wu S, *et al*: RASAL2, a RAS GTPase-activating protein, inhibits stemness and epithelial-mesenchymal transition via MAPK/SOX2 pathway in bladder cancer. Cell Death Dis 8: e2600, 2017.
18. Micalizzi DS and Ford HL: Epithelial-mesenchymal transition in development and cancer. Future Oncol 5: 1129-1143, 2009.
19. Santarpia L, Lippman SM and El-Naggar AK: Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. Expert Opin Ther Targets 16: 103-119, 2012.
20. Kohno M and Pouyssegur J: Targeting the ERK signaling pathway in cancer therapy. Ann Med 38: 200-211, 2006.
21. Bradham C and McClay DR: p38 MAPK in development and cancer. Cell Cycle 5: 824-828, 2006.
22. Chuang SM, Wang IC and Yang JL: Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. Carcinogenesis 21: 1423-1432, 2000.
23. Wakeman TP, Wyczechowska D and Xu B: Involvement of the p38 MAP kinase in Cr(VI)-induced growth arrest and apoptosis. Mol Cell Biochem 279: 69-73, 2005.
24. Otsubo T, Akiyama Y, Hashimoto Y, Shimada S, Goto K and Yuasa Y: MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. PLoS One 6: e16617, 2011.
25. Chen Y, Huang Y, Huang Y, Chen J, Wang S and Zhou J: The prognostic value of SOX2 expression in non-small cell lung cancer: A meta-analysis. PLoS One 8: e71140, 2013.
26. Thu KL, Becker-Santos DD, Radulovich N, Pikor LA, Lam WL and Tsao MS: SOX15 and other SOX family members are important mediators of tumorigenesis in multiple cancer types. Oncoscience 1: 326-335, 2014.
27. Huang W, Chen Z, Shang X, Tian D, Wang D, Wu K, Fan D and Xia L: Sox12, a direct target of FoxQ1, promotes hepatocellular carcinoma metastasis through up-regulating Twist1 and FGFBP1. Hepatology 61: 1920-1933, 2015.
28. Kanamaru H, Akino H, Suzuki Y, Noriki S and Okada K: Prognostic value of nuclear area index in combination with the world health organization grading system for patients with renal cell carcinoma. Urology 57: 257-261, 2001.
29. Sun M, Abdollah F, Bianchi M, Trinh QD, Jeldres C, Tian Z, Shariat SF, Widmer H, Zorn K, Menon M, *et al*: A stage-for-stage and grade-for-grade analysis of cancer-specific mortality rates in renal cell carcinoma according to age: A competing-risks regression analysis. Eur Urol 60: 1152-1159, 2011.
30. Lalani AA, McGregor BA, Albiges L, Choueiri TK, Motzer R, Powles T, Wood C and Bex A: Systemic treatment of metastatic clear cell renal cell carcinoma in 2018: Current paradigms, use of immunotherapy, and future directions. Eur Urol 75: 100-110, 2019.
31. Zarrabi K, Fang C and Wu S: New treatment options for metastatic renal cell carcinoma with prior anti-angiogenesis therapy. J Hematol Oncol 10: 38, 2017.
32. Wang S, Tie J, Wang R, Hu F, Gao L, Wang W, Wang L, Li Z, Hu S, Tang S, *et al*: SOX2, a predictor of survival in gastric cancer, inhibits cell proliferation and metastasis by regulating PTEN. Cancer Lett 358: 210-219, 2015.
33. Goodlad R: Workshop report: Increased cell proliferation as a cause of human cancer. Food Cancer Prev: 300-301, 2005.
34. Huang Y, Zhao M, Xu H, Wang K, Fu Z, Jiang Y and Yao Z: RASAL2 down-regulation in ovarian cancer promotes epithelial-mesenchymal transition and metastasis. Oncotarget 5: 6734-6745, 2014.
35. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.
36. Orlichenko LS and Radisky DC: Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development. Clin Exp Metastasis 25: 593-600, 2008.
37. Bourboulia D and Stetler-Stevenson WG: Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. Semin Cancer Biol 20: 161-168, 2010.
38. Feng M, Bao Y, Li Z, Li J, Gong M, Lam S, Wang J, Marzese DM, Donovan N, Tan EY, *et al*: RASAL2 activates RAC1 to promote triple-negative breast cancer progression. J Clin Invest 124: 5291-5304, 2014.
39. Sebolt-Leopold JS and Herrera R: Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 4: 937-947, 2004.
40. Johnson GL and Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298: 1911-1912, 2002.
41. Sun Y, Liu WZ, Liu T, Feng X, Yang N and Zhou HF: Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct Res 35: 600-604, 2015.
42. Suthiphongchai T, Promyart P, Virochrut S, Tohtong R and Wilairat P: Involvement of ERK1/2 in invasiveness and metastatic development of rat prostatic adenocarcinoma. Oncol Res 13: 253-259, 2003.
43. Ge X, Fu YM and Meadows GG: U0126, a mitogen-activated protein kinase kinase inhibitor, inhibits the invasion of human A375 melanoma cells. Cancer Lett 179: 133-140, 2002.
44. Graf K, Xi XP, Yang D, Fleck E, Hsueh WA and Law RE: Mitogen-activated protein kinase activation is involved in platelet-derived growth factor-directed migration by vascular smooth muscle cells. Hypertension 29: 334-339, 1997.

45. Serrano-Oviedo L, Ortega-Muelas M, García-Cano J, Valero ML, Cimas FJ, Pascual-Serra R, Fernandez-Aroca DM, Roche O, Ruiz-Hidalgo MJ, Belandia B, *et al*: Autophagic cell death associated to Sorafenib in renal cell carcinoma is mediated through Akt inhibition in an ERK1/2 independent fashion. PLoS One 13: e0200878, 2018.
46. Li JK, Chen C, Liu JY, Shi JZ, Liu SP, Liu B, Wu DS, Fang ZY, Bao Y, Jiang MM, *et al*: Long noncoding RNA MRCCAT1 promotes metastasis of clear cell renal cell carcinoma via inhibiting NPR3 and activating p38-MAPK signaling. Mol Cancer 16: 111, 2017.
47. Wu K, Hu L and Hou J: Selective suppression of Notch1 inhibits proliferation of renal cell carcinoma cells through JNK/p38 pathway. Oncol Rep 35: 2795-2800, 2016.
48. Uchiyama A, Nayak S, Graf R, Cross M, Hasneen K, Gutkind JS, Brooks SR and Morasso MI: SOX2 epidermal overexpression promotes cutaneous wound healing via activation of EGFR/MEK/ERK signaling mediated by EGFR ligands. J Invest Dermatol 139: 1809-1820.e8, 2019.
49. Zhou B, Zhu W, Jiang X and Ren C: RASAL2 plays inconsistent roles in different cancers. Front Oncol 9: 1235, 2019.
50. Uzgare AR, Kaplan PJ and Greenberg NM: Differential expression and/or activation of P38MAPK, erk1/2, and jnk during the initiation and progression of prostate cancer 1 1 Prostate 2003;55:128-39. Urol Oncol 22: 82-83, 2004.
51. Chen L, He HY, Li HM, Zheng J, Heng WJ, You JF and Fang WG: ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion. Cancer Lett 215: 239-247, 2004.