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Rap2B promotes migration and invasion of human suprarenal epithelioma

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Conflicts of interest
None

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Abstract

The aim of our study was to elucidate the role of Rap2B in the development of human suprarenal epithelioma and to investigate the effect of Rap2B on suprarenal epithelioma cells migration and invasion. We use tissue microarray and immunohistochemistry to evaluate Rap2B staining in 75 suprarenal epithelioma tissues and 75 tumor-adjacent normal renal tissues. And the expression of Rap2B protein in human suprarenal epithelioma cells and tissues was detected by western blot simultaneously. The role of Rap2B in suprarenal epithelioma cells migration and invasion was detected by using wound healing assay, cell migration assay, and matrigel invasion assay. After that, we performed western blot analysis and gelatin zymography to detect MMP-2 protein expression and enzyme activity. Our research showed that Rap2B expression was increased in tumor tissues compared with tumor-adjacent normal renal tissues. But no correlation was found between Rap2B expression and clinicopathological parameters. In addition, we found that Rap2B promoted the cell migration and invasion abilities, and Rap2B increased MMP-2 expression and enzyme activity in suprarenal epithelioma cells. Our data indicated that Rap2B expression is significantly increased in human suprarenal epithelioma and Rap2B can promote the cell migration and invasion abilities, which may provide a new target for the treatment of suprarenal epithelioma.

Keywords

Rap2B; Suprarenal epithelioma; Tissue microarray; Migration; Invasion

Introduction

Renal cell carcinoma (RCC) represents approximately 90 % of all renal neoplasms and accounts for close to 3~5 % of adult overall malignancies [1]; 85 % of RCC is suprarenal epithelioma. At the time of initial diagnosis, about 20~25 % of patients have a local extension or metastasis, and almost 33 % of patients still develop disease recurrence even though they were given curative treatment [2]. Unfortunately, metastatic renal cell carcinoma, with widely chemo- and radio-resistance, is a poor response to immunotherapy, so current treatments are not effective for this tumor [3]. Improvements in screening and advances in treatments have decreased renal cell carcinoma mortality in recent years, but renal cell carcinoma, often behaving aggressively, is associated with a poor prognosis [4]. Thus, there is an urgent need to develop novel treatments for renal cell carcinoma, especially advanced renal cell carcinoma patients.

Rap2B, involved in the Rap2 subfamily, was first discovered when a platelet cDNA library was screened by researchers in 1990 [5, 6]. Rap2B belonged to Ras family members whose expression can be found increasedly in a variety of human tumors. Furthermore, increasing amounts of evidence strongly illustrated the association of the function of Rap2B with

tumor. For example, Rap2B participate the pro-survival program conducted by p53 as a new player and raise the possibility that targeting Rap2B could sensitize tumor cells to apoptosis in response to DNA damage [7]. Rap2B can interact with and activate PLC-ε which was a key enzyme located in the plasma membrane and extremely broad distribution, and played an important role in promoting bladder transitional cancer cell proliferation and the activity could be controlled by small GTPases of the Ras and Rho families [8–13]. These studies suggest a meaningful role for Rap2B in the tumorigenesis of human cancers. However, less is known about the expression and function of Rap2B in RCC.

In the present study, our data demonstrated that expression of Rap2B was increased in suprarenal epithelioma compared with tumor-adjacent normal renal tissue, while was also increased in suprarenal epithelioma Caki-1 and 786O cell lines. Based on these reports, we speculate that Rap2B also plays an important role in renal cell carcinoma. Soon afterwards, we used a tissue microarray (TMA) of human suprarenal epithelioma patients and immunohistochemistry to evaluate the expression of Rap2B in relation to clinicopathologic features. In addition, we found that Rap2B dramatically increased cell migration and invasion abilities by upregulating MMP-2 expression and enzyme activity, which may provide a new target for human renal cancer treatment.

Experimental procedures

Patients and samples

The suprarenal epithelioma TMA was purchased from Shanghai Xinchao Biotechnology (Shanghai, China). The array dot diameter was 1.5 mm, and each dot represented a tissue spot from one individual specimen. Fifty-seven WHO-I, 57 WHO-II, 18 WHO-III, and 18 WHO-IV were selected and pathologically confirmed. At the same time, it includes 75 cases of tumor-adjacent normal renal tissue. In addition, four RCC tissues and paired non-cancerous tissues were obtained from the affiliated hospital of Xuzhou Medical College.

Cell line and culture conditions

All the cell lines were purchased from the Chinese Academy of Science (Shanghai, China). H-K_2 and HEK293 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15 % fetal calf serum (FCS) at 37 °C in 5 % CO_2 using a humidified incubator. While human 786O and Caki-1 renal carcinoma cells supplemented with 15 % superior placental bovine serum at 37 °C in a humidified atmosphere with 5 % CO_2 and maintained in RPMI-1640 medium (Thermo) and McCoy's 5A Medium, respectively.

Immunohistochemistry of TMA

We performed immunohistochemistry according to the avidin-biotinylated HRP complex (ABC) method by using a standard ABC kit (Zhongshan biotech, Beijing, China). TMA slides was incubated with monoclonal Rabbit anti-Rap2B antibody (1:500) (Abcam, Cambridge, USA) overnight at 4 °C, while diaminobenzidine (DAB; Zhongshan Biotech, Beijing, China) was used to produce a brown precipitate. Then, a biotinylated secondary antibody (Zhongshan Biotech, Beijing, China) was incubated in the sections at room temperature for 30 min. The sections were followed by the incubation with streptavidin-

peroxidase (Zhongshan Biotech, Beijing, China) for an additional 30 min. After rinsing with PBS three times for 5 min, the sections were stained using DAB (Zhongshan Biotech, Beijing, China) for 15 min, rinsed in distilled water, and counterstained with hematoxylin. The immunoreactivity was assessed blindly by two qualified pathologists using light microscopy (Olympus BX-51 light microscope), and the image was collected by Camedia Master C-3040 digital camera. The expression of Rap2B was graded as positive when >5 % of tumor cells showed immunopositivity. Biopsies with <5 % tumor cells showing immunostaining were considered negative.

Transfection

The pDNA3-control and pDNA3-Rap2B expression plasmids were gifts from Dr. Yan-Ping Zhang (The University of North Carolina at Chapel Hill, NC, USA). Transfection of the plasmids into the 786O cells was carried out using Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China) following the manufacturer's protocol. Twenty-four hours after transfection, cells were harvested to do wound healing assay, cell migration and invasion assay. Transfection of small-interfering RNA (siRNAs) was carried out using siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Then, cells were subjected and assayed 48 h after transfection.

Wound-healing assay and transwell assay

The supernatant of cell was collected after transfection with plasmid or siRNA, in which 786O cells were cultured. Meanwhile, a scratch was drawn in the center of the well. The distance between the cells bordering the scratch was measured every 6 h within 12 h. Transwell, pre-coating at 37 °C for 3 h, was incubated in serum-free MEM/EBSS for 30 min. 786O cells were harvested, and then seeded in serum-free medium at a density of 4×10^4 cells/ml. Altogether, 100 μl of the cell suspension was put onto the upper layer of transwell. After coating 12 or 24 h, the cells were fixed by formalin and stained by trypan blue and counted.

Western blot analysis

To determine Rap2B protein levels by western blot, cells were transiently transfected with plasmids for 24 h. Then, cells were harvested and washed twice with PBS. After cell lysis, the protein concentration was determined by using the bicinchoninic acid (BCA) assay. Proteins were separated by SDS-PAGE and then electrotransfer onto nitrocellulose membranes. The primary antibodies were utilized for western blot: rabbit anti-Rap2B (Abcam, Cambridge, MA, USA), rabbit anti-MMP-2 (Cell Signaling Technology, Beverly, MA, USA) and mouse anti-β-actin (Boster Biotechnology, Wuhan, China). The membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) for 2 h at room temperature. Detection was performed by an enhanced chemiluminescence method.

Gelatin zymography analysis

Gelatinase activity was determined by gelatin zymography according to the method previously described. Cells were incubated in RPMI-1640 without serum overnight after 24

h of transfection in a 60-mm culture dish. After overnight, culture conditioned medium was obtained with Ultracel 30-k centrifugal filters (Millipore, Billerica, MA) at 5,000×*g* for 20 min at 4 °C to measure the gelatinase activity. After concentrating, 25-μg proteins was loaded in non-redenaturing conditions on a 10 % polyacrylamide gel containing 0.1 % gelatin in order to electrophoresis. Then, the gel following rinsing with 2.5 % Triton X-100 for 1 h at room temperature was followed by incubation buffer (20-mM Tris–HCl, pH 8.0, 150-mM NaCl, 5-mM $CaCl_2$, and 0.01 % NaN_3) overnight at 37 °C, stained with 0.5 % Coomassie blue R250 (Sigma) for 1 h, and destained with 30 % methanol and 10 % glacial acetic acid until depicting MMPs as clear white bands appeared on the blue background.

Calculations and statistical analysis

Data expressed in figures or given in the text are the means± SD from >3 independent experiments. The differences significance of TMA was assessed using the SPSS 16.0 software (SPSS). X^2 test was used to evaluate the association between staining of Rap2B and the clinicopathologic parameters of the suprarenal epithelioma patients, including age, gender, WHO grade, and histologic type. Statistical analysis in treatment groups were assessed by two-factor analysis of variance procedures and the Dunnett's *t* test. A value was regarded statistically significant when $P<0.05$.

Results

The expression of Rap2B is increased in suprarenal epithelioma

To determine whether there is a different expression of Rap2B between normal renal cells and carcinoma cells, we performed western blot to measure the Rap2B expression in normal renal cells and different carcinoma cells. Our results showed that the level of Rap2B expression is drastically increased in human 786O and Caki-1 renal carcinoma cells, compared with H-K_2 and HEK293 normal renal cells (Fig. 1a).

To further study the expression of Rap2B in suprarenal epithelioma, we performed to investigate the expression of Rap2B protein between tumor-adjacent normal renal tissue and four suprarenal epithelioma patients' tissue. These results are consistent with the previous cell study, a significant difference in Rap2B staining was observed between tumor-adjacent normal renal tissue and malignant tumor (Fig. 1b). And Fig. 2 presented that positive Rap2B staining was recorded in 84.5 and 4.9 % of the biopsies in tumor-adjacent normal renal tissue and malignant tumor in TMA, respectively. Figure 2 also displayed that Rap2B mainly located at the membrane suprarenal epithelioma patients.

Association between the expression of Rap2B and clinicopathological parameters

There is the association between the suprarenal epithelioma prognosis and clinicopathological parameters. We assessed the correlation between the expression of Rap2B and the clinicopathological data, including WHO grade and histologic type. We did not find significant correlations between the expression level of Rap2B and biological factors such as patients' age ($P=0.591$), gender ($P=0.315$), and WHO grade ($P=0.575$). As we all know, WHO grade and histologic type are important prognostic markers for the

patients with suprarenal epithelioma. But significant correlations were not found between Rap2B expression and these markers (Table 1).

Over-expression of Rap2B could promote cell motility in human 786-O renal carcinoma cell line in vitro

Since the expression of Rap2B is notably increased in suprarenal epithelioma tissue, we detected the further involvement of Rap2B in the migration and invasion of human 786O renal carcinoma cell. We transiently transfected 786O cells with pDNA3-control and pDNA3-Rap2B plasmids, Rap2B protein was significantly over-expressed in cancer cell (Fig. 3a). And Rap2B protein expression decreased in cancer cells after knockdown Rap2B (Fig. 4a). In cell wound-healing assay, we found that the healing speed of the scratch in 786O cells cultured with supernatant from 786O/Rap2B was higher than that in 786O cells cultured with supernatant from 786O/pDNA3 ($P<0.05$) (Fig. 3b). Similarly, the migration and invasion results corroborated the wound-healing assay. Our data revealed that 786O cell cultured in supernatant from 786O/Rap2B migrated and invaded faster than 786O cells cultured in supernatant from 786O/pDNA3 ($P<0.05$) (Fig. 3c, d), while knockdown Rap2B inhibited the suprarenal epithelioma cell growth and motility (Fig. 4b–d). Those findings illustrated that Rap2B could promote the migration and invasion of 786O cell in vitro.

Over-expression of Rap2B promoted MMP-2 expression and activity in 786-O cells in vitro

We next investigated the mechanism by which Rap2B promoted the migration and invasion of 786O cells. In cancer cells, the invasive ability can be regulated by MMPs [14]. Western blot and gelatin zymography was performed to measure the MMPs expressions and ability, respectively, in 786O cells. Figure 5a illustrated that the MMP-2 protein level were dramatically increased in 786O-Rap2B cells. And the MMP-2 protein level dramatically decreased in 786O-siRap2B cells compared with the control cells in Fig. 5b. In addition, further result showed the MMP-2 activity was significantly increased after transfecting an expression plasmid for Rap2B in 786O cells when compared with control cells (Fig. 5c, d).

Discussion

Rap2B gene was initially cloned from the complementary DNA (cDNA) library of human platelet by Ohmstede in 1990 [5]. Rap2B gene is located at 3q25.2 of human chromosome which is the hot area of cancer research, and its cDNA contains an open reading frame of 552 bp. Though Rap2B contains conserved domain and belongs to the Ras superfamily, and previous studies pointed out that Rap2B have a relationship with the occurrence and development of some tumors, including lung cancer, but little is known about the expression and role of Rap2B in renal carcinoma. In this work, our findings demonstrated that Rap2B expression is significantly increased in renal carcinoma compared with tumor-adjacent normal renal tissue by TMA technique (Fig. 2). And consistent with the above findings, the expression also highly increased in suprarenal epithelioma cell and tissue with relatively lower levels seen in the normal renal cell and tissue by Western blot (Fig. 1a, b). This observation implied that Rap2B might play some potential role in the development and progression of renal carcinoma. We further carried out experimental studies on the function

of Rap2B. In the function study, we first test the effects of Rap2B on the motility of 786O cells by wound-healing and migration analysis.

In wound-healing assay, Rap2B could accelerate growth rate of 786O cells (Fig. 3b). But what will take occurrence if Rap2B was knocked down? RNA interference (RNAi) and small-interfering RNA molecule (siRNA) corresponding to any endogenous gene of interest, can be used as a mechanism of post-transcriptional gene silencing to result in the mRNA degradation of the targeted gene [15, 16]. Therefore, we referred siRNA as an extremely powerful method for assessing and eliminating Rap2b gene function. After inhibiting the expression of Rap2B in vitro assay (Fig. 4a), Fig. 4b exhibited that knockdown of Rap2B in human suprarenal epithelioma cell lines reduced the movement. And the results are in agreement with that Rap2B staining also played important role in migration of human suprarenal epithelioma cell lines (Figs. 3c and 4c).

Previous reports have suggested that Rap2B, encoding 183 amino acids, is a Ras-related low molecular weight GTP-binding protein. Rap proteins are widely expressed, but the expression and location of isoforms still have the tissue-specific. The function of Rap2B in cancers may also have tissue/cell-type specificity. We reviewed that Rap2B located at the membrane in TMA (Fig. 2) similar to Torti and Lapetina's research [17]. Simultaneously, the above findings implied that some secreted proteins did participate in Rap2B inhibiting effect on the migration of 786O cells.

In order to verify the migration and invasion ability after transfecting Rap2B plasmid, proliferation-associated proteins are commonly used. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidase that are capable of degrading components of the basement membrane and the extracellular matrix (ECM), allowing cancer cells' ability of migration and invasion [18, 19]. Moreover, cell migration was associated with high level of MMP-2 [20] and increasing expression of MMP-2 is associated with enhanced tumor invasion and metastasis [21]. In this study, we also found that Rap2B promoted suprarenal epithelioma cells motility by regulating the MMP-2 protein expression (Fig. 5a) and knockdown of Rap2B in suprarenal epithelioma cells resulted in significantly inhibiting cell abilities of migration and invasion (Fig. 5b). Our further data showed that the MMP-2 enzyme activity could be up-regulated after Rap2B over-expression in suprarenal epithelioma cell (Fig. 5c, d). However, it remains to be elucidated how Rap2B regulates MMP-2 expression and activity. Previous reports have shown that PI3K/Akt and MAPK signaling pathway, NF-κB activation and transcription factor Sp1 is involved in the regulation of MMP-2 expression and activation [22–24]. Our further study in the future will try to find the exact mechanism underlying the MMP-2 regulation by Rap2B.

In conclusion, our study shows that the expression of Rap2B is significantly increased in human suprarenal epithelioma. Strikingly, our data indicate that over-expression of Rap2B may promote suprarenal epithelioma cell migration through increasing MMP-2 protein expression and ability. The clear molecular mechanisms of how Rap2B regulates suprarenal epithelioma cell migration and invasion still need further investigation, but the findings regarding Rap2B provide evidences for the contribution of developments to human

suprarenal epithelioma. Taken together, our data strongly suggest that Rap2B might be considered as a novel therapeutic target for suprarenal epithelioma.

Acknowledgments

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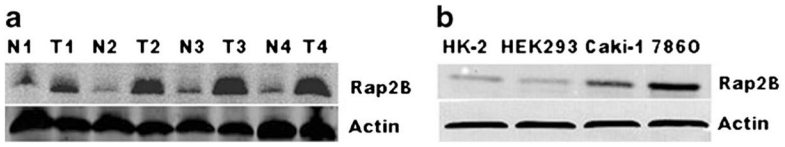

Fig. 1.

Rap2B protein expression in suprarenal epithelioma cells and tissues. **a** In contrast to normal renal cells, the Rap2B expression is increased in 786-O cell lines. **b** Whole-cell protein extracts were further prepared from four paired tumor-adjacent normal renal tissues (*N*) and RCC tissues (*T*). The Rap2B protein level was determined by Western blot analysis

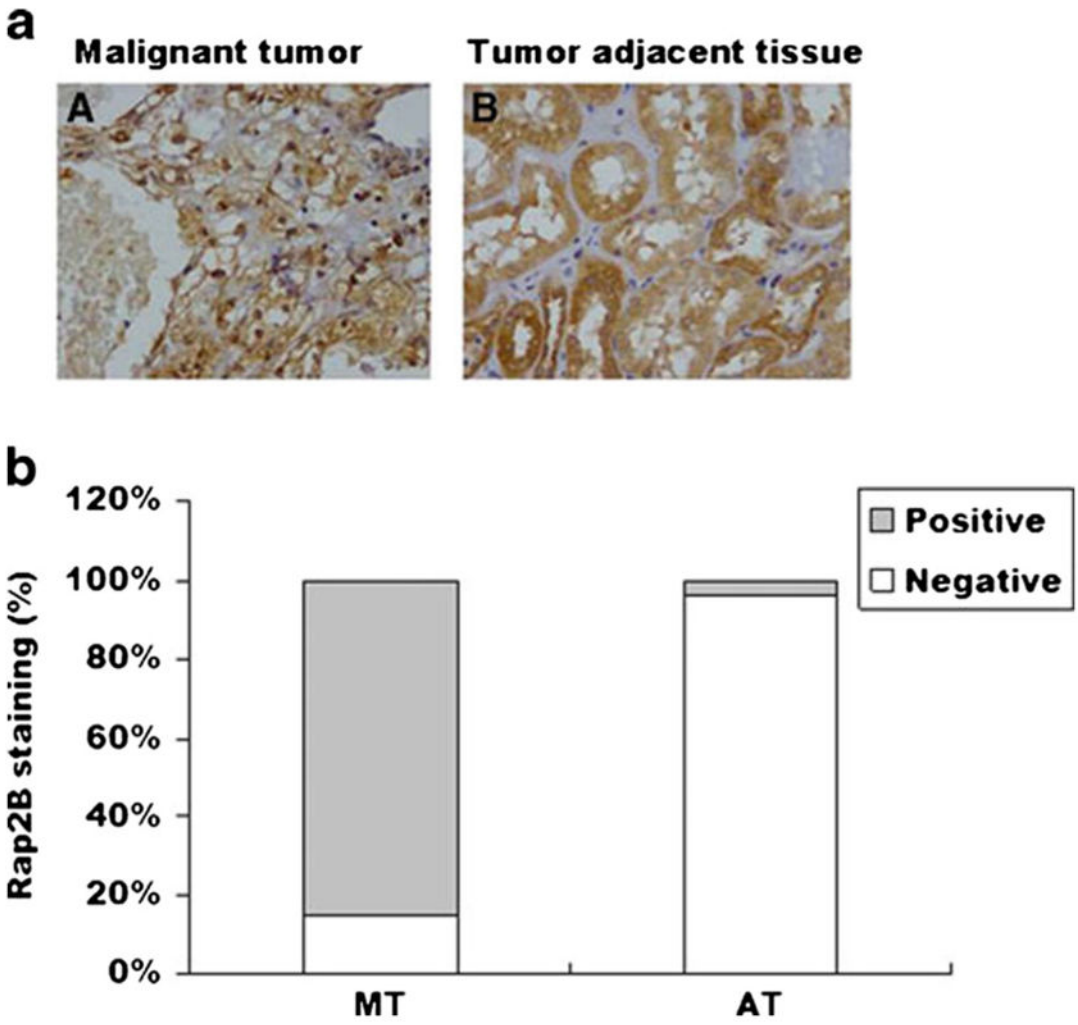

Fig. 2.

Correlation between Rap2B expression and suprarenal epithelioma progression is shown. **a** Significant difference in Rap2B staining was observed between malignant tumor (MT) and tumor-adjacent normal renal tissue (AT). **b** The distribution of the difference in Rap2B staining ($P<0.05$, X^2 test). Magnification ×400

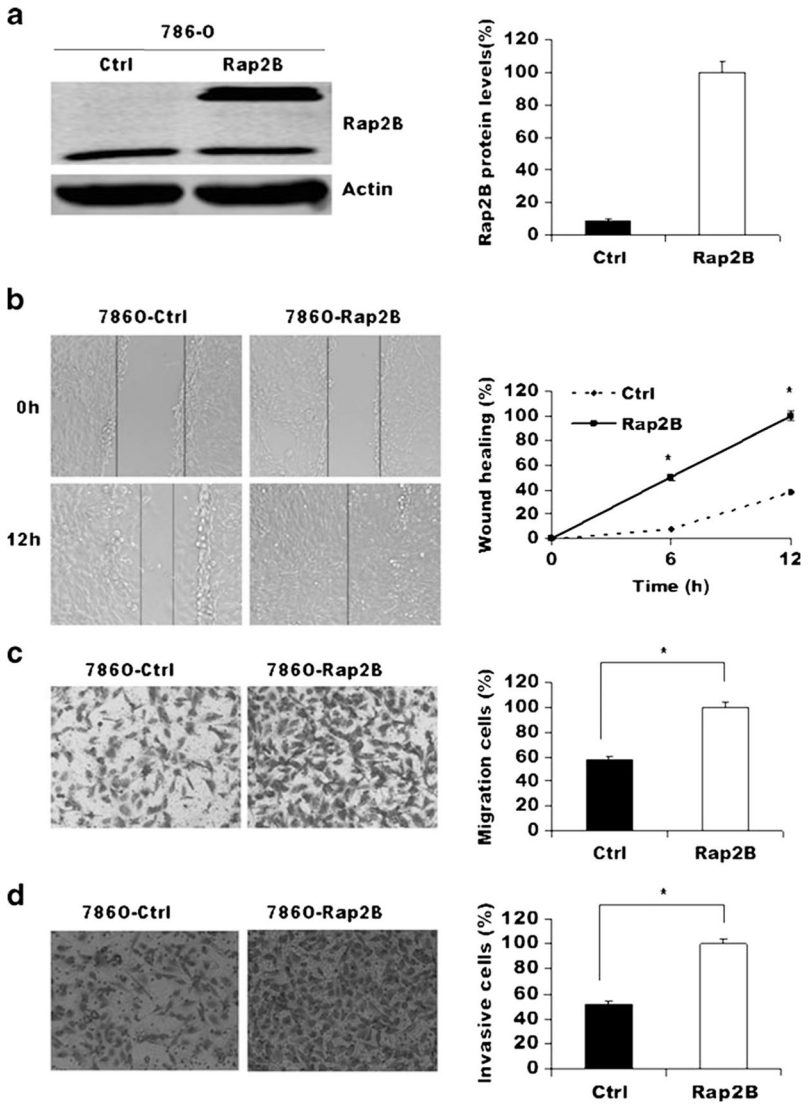

Fig. 3.

Overexpression of Rap2B promotes the cell motility in suprarenal epithelioma cells. **a** Twenty-four hours after transfection, the expression of Rap2B was evaluated by Western blot in 786O cell and quantitative analysis shows that Rap2B protein was over-expressed after transfection. Actin was used as an internal control. **b** Wound-healing assay was executed that over-expression Rap2B promoted proliferation ability in wound closure compared with pcDNA3-control transfected group; **c**, **d** Cell migration assay and Matrigel cell invasion assay were performed after Rap2B over-expression in 786O cells. Rap2B over-expression promoted the ability to migrate and invade through Boy-den chamber. All experiments were carried out in triplicate. Data are shown as mean±SE. (EB, endogenous band) *P<0.05, **P<0.01, ***P<0.001

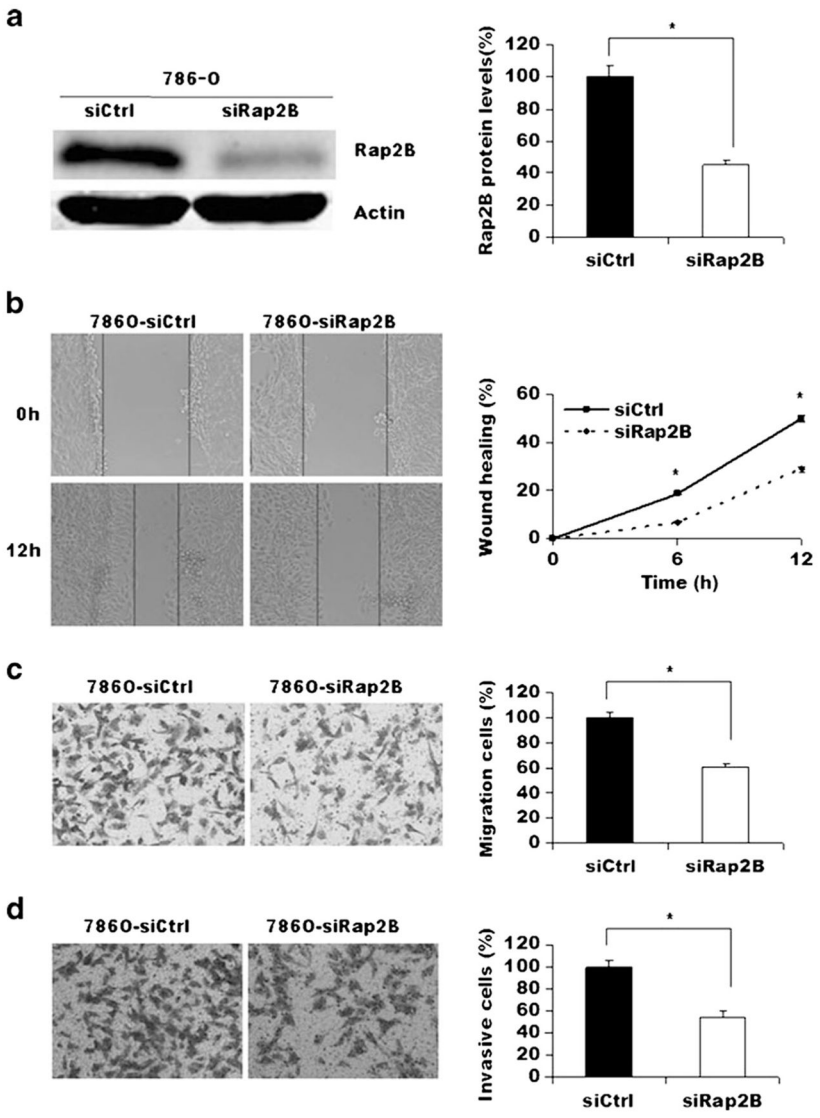

Fig. 4.

Knockdown Rap2B inhibits the suprarenal epithelioma cells motility. **a** Forty-eight hours after transfection, the expression of Rap2B was evaluated by western blot in 786O cell and quantitative analysis shows that Rap2B protein was decreased after Rap2B knockdown. Actin was used as an internal control. **b** Wound-healing assay was executed after Rap2B knockdown in 786O cells. There was a significant delay in wound closure compared with pDNA3-control transfected group. **c**, **d** Cell migration assay and matrigel cell invasion assay were performed after Rap2B knockdown in 786O cells. The knockdown of Rap2B decreased the ability to migrate and invade through Boy-den chamber. All experiments were carried out in triplicate. Data are shown as mean±SE. **P*<0.05, ****P*<0.001

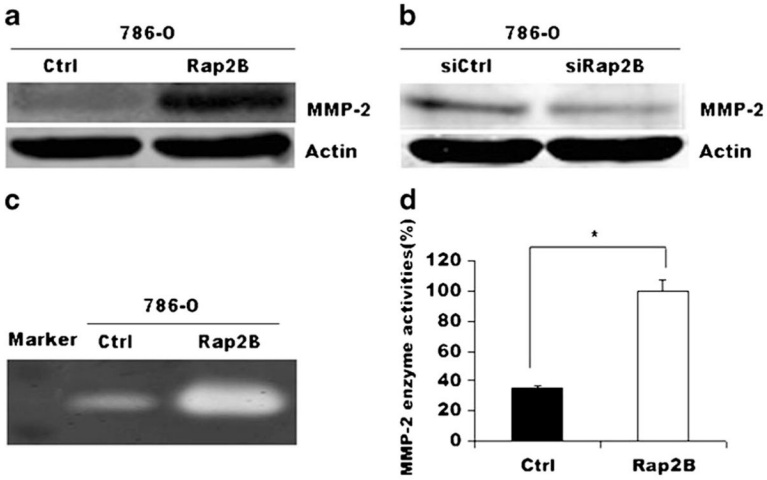

Fig. 5.

Rap2B increases MMP-2 expression and ability in 786O cell. **a** Western blot analysis showed that MMP-2 expression was increased in suprarenal epithelioma cells after transfection of Rap2B. **b** In siRap2B and control group for 786O cell lines, the MMP-2 protein level was dramatically decreased in 786O-siRap2B cells. **c**, **d** Gelatin zymography confirmed that Rap2B over-expression can increase the MMP-2 ability compared to control. All experiments were carried out in triplicate. ***P<0.01

Table 1

Rap2B staining and clinicopathological characteristics of 75 suprarenal epithelioma patients

Variables	Rap2B staining			
	Negative, no. (%)	Positive, no. (%)	Total	*P**
Age (years)				
<57	9 (21.4)	33 (78.6)	42	0.591
≥57	7 (21.9)	25 (78.1)	32	
Gender				
Male	12 (24.0)	38 (76.0)	50	0.315
Female	4 (16.0)	21 (84.0)	25	
WHO grade				
Benign (I–II)	12 (21.4)	45 (78.9)	57	0.575
Malignant (III–IV)	4 (22.2)	14 (77.8)	18	

* χ^2 test