RBM47 downregulation promotes renal cancer cell malignancy and predicts poor patient survival

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Abstract

RNA-binding proteins (RBPs) is of utmost importance for regulating signaling pathways in cells. RBM47 has been shown to function as a tumor suppressor in various cancers. In this study, we demonstrate the probable mechanism of RBM47 and prognostic significance role in The
Cancer Genome Atlas Kidney Clear Cell Carcinoma database (TCGA - KIRC). RBM47 high expression has a positive correlation with extended overall survival (OS) and disease-free survival (DFS) in TCGA - KIRC. RBM47 could sufficiently distinguish ccRCC and normal tissues. Gene set enrichment analysis (GSEA) shows that RBM47 is associated with epithelial-mesenchymal transition (EMT), and TGF-β signaling pathway. RBM47 is downregulated when compared with their respective control groups with western blot and immunohistochemistry in ccRCC tissues and ccRCC cell lines. The overexpression of RBM47 inhibits the malignant development of renal cancer cells, and suppression of RBM47 by shRNA can promote the proliferation, migration, and invasion activity of cancer cells through EMT signaling. Our results indicates that RBM47 inhibit ccRCC malignancy by reducing EMT signaling. Thus, RBM47 represents as a novel treatment and molecular biomarker for ccRCC.

Introduction

Renal cell carcinoma (RCC) deaths account for 1.8% of all cancer-related deaths worldwide, is one of the third most common cancers in the urology, and ranks 16th in global malignancies [1]. There are approximately 14,830 deaths and 73,750 new cases estimated in USA for 2020 [2]. Clear cell renal cell carcinoma (ccRCC) is the most common pathological type of renal cancer[3]. Locally advanced tumor
constitutes a significant portion of all patients and with about 17% of patients presenting distant metastases at the initial time of diagnosis although most detected lesions are small tumors [4]. Metastasis or local recurrence in postoperative patients is 20-40% which accounts for 90% of patient deaths [5, 6]. Patients of ccRCC still develop poor clinical outcome after a period of tyrosine kinase inhibitors (TKI)-treated because of drug resistance [7]. Therefore, it is necessary to explore more effective prognostic biological markers and the potential mechanisms, which may help to find new strategies for treating ccRCC[8, 9].

RNA binding motif protein 47 (RBM47), also known as NET18, is an RNA binding protein whose main biological function is to bind introns or 3'-UTRs of the target gene mRNA and regulate the stability of these genes [10]. RBM47 consists of three classic RNA recognition motifs (RRM domains). Apobec1 complementation factor (A1CF) and hnRNP-Q are the closest homologs of RBM47 which regulate RNA editing [11], splicing and transcript stability [12]. Recently, RBM47 was demonstrated as a tumor suppressor in lung cancer and colorectal cancer (CRC) [10]. In breast cancer, RBM47 can suppress cancer progression and inactivate widely targeted RNA partners, allowing for statistics on metastasis-promoting [13]. Epithelial-mesenchymal transition (EMT) phenotype of tumor cells had an important role for cancer progress
Low RBM47 was correlated with poor progression and it might inhibit EMT in CRC [18]. RBM47 was discovered as a novel regulator of the p53 / p21 signaling [19]. However, the expression of RBM47 in ccRCC and its role remain unknown.

Here we explored the clinicopathological features and patient survival of ccRCC with RBM47 in an integrate investigation system including public database, patients cancer tissues, gene set enrichment analysis (GSEA) and cellular models. Our study showed that low RBM47 expression predicts poor progression and RBM47 could act as tumor suppressor by affecting EMT signal pathway in ccRCC.

Materials and Methods

Patient samples

All 42 paired human adjacent healthy tissues and ccRCC tissues were from the Department of Urology, Union Hospital, Tongji Medical College (Wuhan, China) in 2014-2017. All patients had informed consent, and the study was approved by the Institutional Review Board of Huazhong University of Science and Technology, in line with the Helsinki Declaration as previously described [20].

Tissue and cell RNA extraction and qRT-PCR

The total RNA of adjacent healthy, ccRCC tissues or cells was extracted
with TRizol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA concentration and purity were measured with NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). A total of 1 μg of enriched tissue or cell RNAs was utilized for cDNA synthesis (Advantage RT-for-PCR Ki; Clontech, Japan). All qPCR analysis was performed (Stepone plus; ABI, Massachusetts, USA) using the SYBR Green mix (Thermo Fisher, Massachusetts, USA). Normalized to GAPDH: \( 2^{-\Delta Ct} (\Delta Ct = C_{RBM47} - C_{GAPDH}) \) was used to calculate the relative expression of RBM47. All operations are performed according to the manufacturer’s instructions as previously described [21, 22]. Gene primers were purchased from Qinke (Qinke, Wuhan, China):

GAPDH Forward 5’-GAGTCAACGGATTTGGTCGT-3’
Reverse 5’-GACAAGCTTCCCCGTTCAG-3’

RBM47 Forward 5’-TGTCATTCCCACTGTGTCGA-3’
Reverse 5’-GTAGCCTGCGTATCCTCCAT-3’

Vim Forward 5’-GACGCCATCAACACCGAGTT-3’
Reverse 5’-CTTTGTCGTTGGTATGCTGGT-3’

Snail Forward 5’-TCGGAAGCCTAACTACGAGGTG-3’
Reverse 5’-TTGGCATTGGGCAGCGAG-3’

CDH1 (E-cad) Forward 5’-CGAGAGCTACGCTCCACGG-3’
Reverse 5’-GGGTGTCGAGGGAAAAATAGG-3’

TGFβ Forward 5’-GGCCAGATCCTGTCCAAGC-3’
Reverse 5’-GTGGGTTTCCACCATTAGCAC-3’

**Immunohistochemistry (IHC)**

Human adjacent healthy tissue and ccRCC tissue of the human body are removed, and then placed in liquid nitrogen. Fixed tissues with formalin and dehydrated, and then embedded, and the sections are incubated with rabbit RBM47 primary antibody (1:100; A17350; ABelonal Biotech Co., Ltd., Wuhan, China) at 4°C overnight. The sections were washed three times with PBS, then incubated with goat anti-rabbit secondary antibody (1:200; GB23303; Servicebio, Inc., Woburn, MA, USA) as previously described[23]. IHC of human normal renal tissue and ccRCC tissue were download from The Human Protein Atlas (https://www.proteinatlas.org).

Cell culture and transient transfection

The human normal control renal cells HK2 and renal cancer cell lines (786-O, A498, OSRC-2, ACHN and CAKI) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultivated in high-glucose DMEM (HyClone; GE Healthcare, Logan, UT, USA), which contained 10% FBS (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 5% CO2 incubator at 37°C. The plasmid vectors specifically targeting RBM47 (sh-RBM47) and negative control shRNA (CON) or expressing RBM47 (RBM47) or negative control (NC) were constructed by GeneChem (Shanghai, China).
A total of 3μg of sh-RBM47 CON, RBM47 or NC plasmid were transfected to cancer cells with the Lipofectamine® 2000 reagent (Thermo Fisher Scientific). All operations are performed according to the manufacturer’s instructions.

Cell proliferation analysis
Firstly, transfected 786-O and A498 cells with RBM47, NC, sh-RBM47 or CON, and then seeded the 2×10³/cells/well to a 96-well plate. OD value (the cell proliferation rate) was detected with cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc, Rockville, MD, USA). 1 hour later, measured the OD value at 450 nm. All operations are according to the manufacturer protocol. Three independent trials were conducted for each experiment.

Migratory and invasion assays
Following 24 hours FBS plasma withdrawal, 2×10⁴ cells/well were seeded into the upper chamber in serum-free medium, whereas the bottom chamber (Corning Incorporated, Corning, NY, USA) was filled with complete medium containing 10% FBS. In the invasion assays, the membrane was first covered with Matrigel (Thermo Fisher Scientific) and cultured with 4 x 10⁴ cells / well. After 24 hours of incubation, the cells were fixed with 100% methanol and then stained with 0.05% crystal
violet. Five randomly selected fields were used to count cells numbers under a microscope (Olympus CX41-32C02; Olympus Corporation, Tokyo, Japan). Three independent experiments were conducted in each experiment as previous study.

Western blotting
Tissues or cells were pyrolyzed in a protein lysis system containing RIPA (Beyotime Institute of Biotechnology, Shanghai, China), protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), and phenylmethylsulfonyl fluoride (PMSF) (Wuhan Boster Biological Technology, Ltd.). The protein concentrations were measured with bicinchoninic acid kit (Beyotime Institute of Biotechnology, Shanghai, China) at 562 nm. A total of 30 μg of protein was subjected to 10% SDS-PAGE, then, separated and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA) at 250 mA for 90 minutes. The transferred PVDF membranes were blocked in PBS containing 5% nonfat milk for 1 h at room temperature, and were then incubated with primary antibodies against RBM47 (1:1,000; ABclonal Biotech Co., Ltd.), snail (1:1,000; ABclonal Biotech Co., Ltd.), E-cadherin (1:1,000; ABclonal Biotech Co., Ltd.), Vim (1:1,000; ABclonal Biotech Co., Ltd.), TGF-β (1:1,000; ABclonal Biotech Co., Ltd.), and β-actin (1:1,000; Proteintech, USA) at 4°C overnight. The
membranes were washed and incubated with secondary antibodies (1:10,000; BA1020; Wuhan Boster Biological Technology, Ltd.) on the next day at room temperature for 2 hours. Finally, the membranes were washed and proteins were detected by ChemiDoc-XRS+ (Bio-Rad Laboratories Inc., Hercules, CA, USA) as previously described [24].

Bioinformatics analysis

The RNA-seq data and the clinical information of ccRCC patients in TCGA (TCGA-KIRC) were obtained from the Xena Functional Genomics Explorer (https://xenabrowser.net/heatmap/) [25-27]. Gene set enrichment analysis (GSEA) elucidated the potential pathway that RBM47 may be participated in TCGA-KIRC (http://www.broadinstitute.org/gsea) [28]. A false discovery rate of < 25% and a nominal P<0.05 following 1,000 permutations was considered to be significantly enriched for enriched gene sets analysis as previous study [29]. The Z-values of protein levels in ccRCC samples from CPTAC (https://proteomics.cancer.gov/programs/cptac) as previously study described[30]

**Statistical analyses**

Paired sample t-test were used to analyze paired samples and one-way ANOVA or t-test were used to analyze unpaired samples. The area under
the curve (AUC) and receiver operator characteristic (ROC) curve were used to distinguish clinical subtype feature classifications as previously described [31]. The survival rate of all 532 patients from TCGA-KIRC with RBM47 expression level were evaluated by Kaplan–Meier (KM) curve with a log-rank test [32]. Due to the mission part of grade, N, M, TNM stage, 518 samples contained all clinical information, and were used for analysis of the correlation between RBM47 mRNA expression and clinicopathological parameters and Cox proportional hazard regression of overall survival. The prognostic significance of RBM47 on survival prognosis of patients with ccRCC by univariate and multivariate Cox proportional hazard regression. All statistical analyses were performed using SPSS Statistics 23.0 (IBM Corporation, Armonk NY, USA). Values of P <0.05 were considered statistically significant [20].

Results

RBM47 is significantly down-regulated in TCGA-KIRC and correlated with various clinicopathological parameters

The expression of RBM47 mRNA in cancer tissues and corresponding normal tissues in the TCGA-KIRC database was investigated firstly. Detailed clinical data of TCGA-KIRC was showed in Table 1. RBM47 expression was significantly declined in ccRCC cancer tissues than in
parallel healthy noncancer tissues (Figure 1A). Similar results were detected in 72 paired ccRCC tissues and matching healthy noncancerous tissues (Figure 1B). Interestingly, the expression of RBM47 was higher in women than in men (Figure 1D). And we found that the estrogen receptor (ESR) was downregulated (Supplementary Figure 1A), ESR had a positive correction with RBM47 in ccRCC tumor tissues (Supplementary Figure 1B), high ESR had higher RBM47 in ccRCC cancer (Supplementary Figure 1C), which may be the reason for the low incidence of ccRCC in women. Meanwhile, lower RBM47 expression level was correlated with higher T grade, M grade, Fuhrman grade, and TNM grade (Figure 1E, 1G-1I). However, RBM47 was not significantly associated with lymph node metastasis or age. These data indicate that the expression of RBM47 was reduced in ccRCC tumor tissues, and its expression was significantly correlated with different clinical clinicopathological parameters.

**Low RBM47 expression predicts poor survival of ccRCC**

All 532 patients from TCGA-KIRC were divided into two groups based on the median RBM47 expression value. Low RBM47 expression (n=266) patients had a shorter overall survival (OS) (Figure 2A). Furthermore, various subgroups of ccRCC patients with different OS analyses were performed according to the RBM47 mRNA expression. These results
indicated that the RBM47 might be a possible diagnosis marker for ccRCC patients, including gender (male, female; Figure 2B, 2C), age (≤60 years, > 60 years; Figure 2D, 2E), T stage (T1+T2, T3+T4; Figure 2F, 2G), Nx+N0 stage (Figure 2H), Mx+M0 stage (Figure 2I), TNM stage (I+II, III+IV; Figure 2J, 2K), Fuhrman G3+G4 grade (Figure 2L). However, no significant differences were observed in N1, M1, or G1+G2 subgroups in ccRCC patients. The prognostic values based on RBM47 expression in various clinicopathological subclasses of OS with univariate and multivariate Cox proportion hazard ratio (HR) analysis are shown in Table 2.

Kaplan-Meier analysis (KM) was used to verify the relationship between RBM47 expression and disease-free survival (DFS). The results demonstrated that patients with high RBM47 expression exhibited better DFS (Figure. 3A; P<0.0001). Furthermore, subgroups in patients with ccRCC of DFS analysis demonstrated that high RBM47 expression was a potential prognostic indicator with the following characteristics for patients with ccRCC: male (Figure 3B), age (≤60 years, > 60 years; Figure 3C, 3D), T stage (T1+T2, T3+T4; Figure 3E, 3F), Nx+N0 stage (Figure 3G), M stage (Mx+M0, M1; Figure 3H,3I), TNM stage (I+II, III+IV; Figure 3J, 3K), and G3 + G4 grade (Figure 3L). These results suggest that RBM47 may be an independent survival prognostic factor for OS and DFS.
Diagnostic value of RBM47 in ccRCC

Higher RBM47 expression in ccRCC predicts a good prognosis for patients, the diagnostic value of RBM47 was investigated with ROC curves to analyze the clinicopathological parameters. It was demonstrated that RBM47 could sufficiently distinguish ccRCC from healthy tissues with the AUC of 0.9527 (95% CI: 0.9333–0.9723; P<0.0001) as shown in Figure 4A. Additionally, ccRCC subgroups had a significant difference of RBM47 expression, including T stage, M stage, G stage, TNM stage, recurrent status ((T1 + T2)/(T3 + T4) stage (Figure 4B, AUC=0.6045, P<0.0001), ((Mx + M0)/(M1) stage (Figure 4C, AUC =0.5776, P = 0.0280), (G1 + G2)/(G3 + G4) stage (Figure 4D, AUC=5676, P = 0.0079),TNM (I + II)/(III + IV) stage (Figure 4E, AUC = 0.5961, P = 0.0002), nonrecurrent/recurrent (Figure 4F, AUC = 0.6087, P=0.0004). These results elucidated that RBM47 expression could be a potentially useful diagnostic indicator for ccRCC.

Validation of down regulation of RBM47 in ccRCC tissues and cells

To explore how RBM47 was involved in ccRCC pathogenesis, gene set enrichment analysis (GSEA) was performed to gain insight into the biological pathways in the TCGA-KIRC database. These GSEA results illustrated that epithelial-mesenchymal transition (EMT), and the TGF-β
signaling pathway might be interrelated with patients with higher RBM47 expression compared with lower RBM47 expression in TCGA-KIRC (Figure 5A; P<0.05). The correlation of e-cadherin, TGF-β, snail, vim, and RBM47 was shown by the TCGA-KIRC and qRT-PCR (Figure 5B and Supplementary Figure 2A). Furthermore, protein level of RBM47, e-cadherin, TGF-β, snail, vim was showed in ccRCC patient tumor tissues by western blot (WB) (Figure 5C). The results indicated that the TGF-β, snail and vim genes were negatively correlated with RBM47 and had a positive relationship with e-cadherin. To further validate the results of ccRCC patients in the TCGA-KIRC data, qRT-PCR analysis revealed that RBM47 mRNA expression levels were down-regulated in samples from ccRCC patients (Figure 5D). Furthermore, protein level of RBM47 was also downregulated in ccRCC patient tumor tissues by immunohistochemistry and a public database (Figure 5E, Supplementary Figure 2B and 2C). These results indicated that RBM47 mRNA and protein levels from cancerous tissues were significantly lower than corresponding adjacent non-cancerous kidney tissues.

**RBM47 inhibits malignant transformation of renal cancer cells**

Then, we detected RBM47 expression in ccRCC cell lines by qRT-PCR and WB (Figure 6A and 6B). Since the RBM47 gene might play a significant role in ccRCC, we explored the functional role of RBM47 in
RCC cell lines through gene overexpression or knockdown. After transfection with plasmid vectors, the mRNA and protein level of RBM47 expression were tested in 786-O and A498 cells (Figures 6C-6F). The proliferation ability of 786-O and A498 cells was obviously inhibited following transfected with RBM47 compared with the NC group (Figure 6G and 6H). Knockdown RBM47 promoted the proliferation ability of 786-O and A498 cells by CCK-8 assays (Figure 6I and 6J). These results illustrated that RBM47 might act as a tumor suppressor in renal cancer cells.

**RBM47 affects renal cancer cell invasion and migration via EMT signaling**

The snail, e-cad, and vim genes play an essential role in EMT signaling pathway. The relationship between RBM47 and e-cadherin, vim or snail was verified by western blot (WB) and qRT-PCR (Figure 7A and 7B). These results showed that RBM47 was positively correlated with e-cadherin and negatively correlated with snail and vim in 786-O and A498 cells. Overexpression of RBM47 could inhibit the migration ability of 786-O and A498 cells in wound-healing assay (Figures 7C). Overexpression of RBM47 significantly inhibited RCC cell migration and invasion in transwell assays when compared with the NC group (Figure 7D). The knockdown of RBM47 expression promoted cell
migration and invasion in RCC cell lines (Figure 7E). These results demonstrated that RBM47 might affect ccRCC biological characteristics through the EMT signaling pathway.

Discussion

The ccRCC is a common fatal urological malignancy worldwide, which has an identical high rate of metastasis and mortality [4]. This study reports the expression of RBM47, biological function roles and its clinical significance in ccRCC for the first time.

Regulation and processing of RNA is the basis of biological complexity in different states [33, 34]. RNA-binding proteins (RBPs) can regulate the transcriptome through a variety of mechanisms, such as alternative splicing, variable polyadenylation, and transcription stability [35-37]. Increasing evidence shows that RBP plays a significant role in the occurrence and development of cancer[38]. For example, splicing factors in the SR and hnRNP families can act as regulators of cancer cell apoptosis [39]. RBM10 suppresses lung cancer progression by eukaryotic translation initiation factor 4H splicing [40]. RBM47 is a newly discovered RBP containing three classic RRM domains [13]. RBM47 can bind to Nanog mRNA in mouse embryonic stem cells [41]. Experimental results verified exogenous RBM47 could bind to the 3'UTR of mRNA via high-throughput sequencing and cross-linked immunoprecipitation
analysis [42]. In addition, RBM47 has been shown to regulate C to U RNA editing through APOBEC1[43]. But RBM47 has not been studied in ccRCC. This study is the first demonstration of RBM47 expression, prognostic role, and possible mechanisms in ccRCC. Apobec1 complementation factor (A1CF) and hnRNP-Q are the closest homologs of RBM47 which regulate RNA editing [11], but the expression of A1CF and hnRNP-Q had no difference in ccRCC cancer tissues and normal kidney tissues in Supplementary Figure 3A and 3B.

Numerous experimental evidences indicated that RBM47 expression was significantly reduced in ccRCC RBM47 expression was associated with important clinicopathogenesis factors, and higher RBM47 expression was an independent predictor of good prognosis in OS and DFS. Furthermore, in vitro results showed that overexpression of RBM47 inhibited malignant characteristics of renal cancer cells. Mechanically, RBM47 might affect the biological functions of ccRCC through epithelial-to-mesenchymal transition (EMT) signaling pathway or TGF-β signaling pathway. And TGF-β signaling could induce EMT signaling in cancer or hepatic stellate cells [17, 44-47]. In vitro experiments illustrated that RBM47 can inhibit EMT signaling in ccRCC with mRNA and protein level. Previous studies have shown RBM47 was directly inhibited during EMT as the conservative binding motifs in the RBM47 promoter by these EMT factors [18]. RBM47 could bind and stabilize the AXIN1
mRNA, and enhance suppression of Wnt/beta-catentin signaling in non-small-cell lung cancer [48]. But the role of RBM47 had not been studied in ccRCC.

The results of this study indicate that RBM47 could be a potential new biomarker for predicting the prognosis of ccRCC. The incidence of renal cancer was higher in men [49]. Interestingly, we found that the expression of RBM47 was higher in women than in men. The ESR was downregulated and had a positive correction with RBM47 in ccRCC tumor tissues, high ESR patient had higher RBM47 in ccRCC cancer, which may be a reason for the lower incidence of renal cancer in women. We found that RBM47 expression was downregulated in RCC cell lines (786-O, A498, OSRC-2, ACHN and CAKI). We chose 786-O and A498 as the research object. Our results suggested that 786-O had higher growth, invasion, and migration abilities, which may be related to the low expression of RBM47. RBM47 could inhibit the EMT pathway in ccRCC with mRNA and protein level. However, the detailed mechanism of how RBM47 inhibits EMT expression has not been studied.

**Conclusion**

The results provided by all these studies helps to understand the prognostic role of RBM47 and the possible mechanisms that affect in ccRCC, and suggests that RBM47 may be a new therapeutic target for
ccRCC patients.

Acknowledgements

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Author Contributions

CW, WX, XGM performed the experiments and bioinformatics analysis, ZYX, XGM, YZY, CFY, JS, ZXC, DL collected clinical data. WX and XPZ contributed to study design and manuscript writing.

Competing Interests

No.

References

clinically-significant clear cell renal cell carcinoma subtypes with convergent evolutionary trajectories into an aggressive type. EBioMedicine. 2020; 51: 102526.
Fig. 1. RBMC47 is down-regulated in ccRCC tissues and has significant correlation with various clinicopathological parameters. RBM47 expression of 72 healthy tissues and 533 ccRCC tissues and their clinical data was downloaded from the TCGA-KIRC dataset. Different clinicopathological parameters were compared with expression of RBM47: (A) cancer versus para-cancer, (B) cancer versus paired para-cancer, (C) and (D) Age and gender, (E) and (F) T stage, (G) M stage, (H) grade and (I) TNM stage. Data are presented as the means ± standard. *P<0.05 and **P<0.01.
Fig. 2. Low expression of RBM47 predicts poor overall survival in patients with ccRCC. (A) Patients from the TCGA-KIRC database were divided into two group according to the median expression value of RBM47 mRNA. Kaplan-Meier was used to analyze the correlation between RBM47 expression and overall survival time (B-L) Subgroups of ccRCC patients were compared with RBM47 mRNA to overall survival analysis: (B) female, (C) male, (D) Age ≤ 60 years, (E) Age > 60 years, (F) T1+T2, (G) T3+T4, (H) N0 stage, (I) non-metastasis status, (J) TNM I+II stage, (K) TNM III+IV stage, (L) G3+G4 stage.
Fig. 3. Low expression of RBM47 predicts poor disease-free survival in patients with ccRCC. (A) Patients from the TCGA-KIRC database were divided into two group according to the median expression value of RBM47 mRNA. Kaplan-Meier was used to analyze the correlation between RBM47 expression and disease-free survival time: (B) male, (C) Age ≤ 60 years, (D) Age > 60 years, (E) T1+T2, (F) T3+T4, (G) N0 stage, (H) non-metastasis status, (I) metastasis status, (J) TNM I+II stage, (K) TNM III+IV stage, (L) G3+G4 stage.
Fig. 4. Low RBM47 expression serves as a diagnostic indicator in ccRCC patients. (A) The ROC curve showed that RBM47 could effectively distinguish ccRCC from para-cancer tissues; the AUC was 0.9527 (p<0.0001). (B-F) ROC curve analysis regarding the expression of RBM47 mRNA in subgroups of ccRCC patients against (B) T stage, (C) metastasis status, (D) Grade, (E) TNM stage, (F) recurrence status.
Fig. 5. RBM47 is downregulated in ccRCC and negatively related to EMT signaling. (A) Enrichment curves are shown for activated gene sets related to epithelial-mesenchymal transition (EMT), and the TGF-β signaling pathway. (B) The correlation of snail, e-cad, vim, TGF-β and RBM47 was performed using the TCGA-KIRC data. (C) Protein expression of snail, e-cad, vim, TGF-β and RBM47 in ccRCC tissues and para-cancer tissues. (D) RBM47 gene expression in ccRCC tissues and para-cancer tissues. (E) Immunohistochemistry (IHC) analysis of RBM47 in ccRCC tissues and para-cancer tissues in public database. Data are presented as the means ± standard deviation from three independent experiments. *P<0.05.
Fig. 6. The effects of RBM47 on renal cancer cell proliferation. (A, B) RBM47 expression in renal cancer cell lines. (C, D) RBM47 mRNA and protein expression were successfully overexpressed in 786-O and A498 cells. (E, F) RBM47 mRNA and protein expression were successfully knocked down in 786-O and A498 cells. (G)-(J) Cell counting kit-8 assays detected the effects of RBM47 overexpression and
knockdown on proliferation of 786-O and A498 cells. Data are presented as the means ± standard deviation from three independent experiments. *P<0.05 and **P<0.01.

Fig. 7. RBM47 affects renal cancer cells via EMT signaling. (A) and (B) The effects of RBM47 overexpression and knockdown on mRNA and protein level of snail, e-cad, and vim in renal cancer cells. (C) Representative images of cell scratch assays performed using 786-O and A498 cells. (D) and (E) The effects of RBM47 overexpression and knockdown on cell migration and invasion in renal cancer cells. Data are presented as the means ± standard deviation from three independent experiments. *P<0.05, **P<0.01, and ***P<0.001.
Table 1 Correlation between RBM47 mRNA expression and clinicopathological parameters of ccRCC patients.

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<td>0.015</td>
</tr>
<tr>
<td>Variables</td>
<td>Univariate analysis</td>
<td>Multivariate analysis$^c$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------</td>
<td>--------------------------</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>HR$^a$</td>
<td>95%CI$^b$</td>
<td>P value</td>
<td>HR$^a$</td>
<td>95%CI$^b$</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years) ≤60 vs. &gt;60</td>
<td>1.728</td>
<td>1.260-2.369</td>
<td>0.001</td>
<td>1.644</td>
<td>1.198-2.256</td>
</tr>
<tr>
<td>Sex Female vs. Male</td>
<td>0.965</td>
<td>0.703-1.324</td>
<td>0.825</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3 or T4 vs. T1 or T2</td>
<td>3.346</td>
<td>2.446-4.576</td>
<td>0.000</td>
<td>1.746</td>
<td>1.203-2.534</td>
</tr>
<tr>
<td>N stage</td>
<td>2.954</td>
<td>1.556-5.609</td>
<td>0.001</td>
<td></td>
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</tr>
<tr>
<td>N1 vs. N0 or NX M stage</td>
<td>4.540</td>
<td>3.303-6.241</td>
<td>0.000</td>
<td>2.803</td>
<td>1.945-4.039</td>
</tr>
<tr>
<td>M1 vs. M0 or MX Grade</td>
<td>2.627</td>
<td>1.855-3.721</td>
<td>0.000</td>
<td>1.706</td>
<td>1.175-2.477</td>
</tr>
<tr>
<td>G3 or G4 vs. G1 or G2 RBM47 High vs. Low</td>
<td>0.517</td>
<td>0.377-0.71</td>
<td>0.000</td>
<td>0.571</td>
<td>0.414-0.787</td>
</tr>
</tbody>
</table>

$^a$ Hazard ratio, estimated from Cox proportional hazard regression model.
$^b$ Confidence interval of the estimated HR.
$^c$ Multivariate models were adjusted for T, N, M classification, age and ge.