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ORIGINAL RESEARCH

ITPKA1 Promotes Growth, Migration and Invasion of Renal Cell Carcinoma via Activation of mTOR Signaling Pathway

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Background: Renal cell cancer (RCC) is one of the most lethal malignancies of the kidney in adults. mTOR (mammalian target of rapamycin) signaling pathway plays a pivotal role in RCC tumorigenesis and progression and inhibitors targeting the mTOR pathway have been widely used in advanced RCC treatment. Therefore, it is of great significance to explore the potential regulators of the mTOR pathway as RCC therapeutic targets.

Materials and Methods: Bioinformatics analysis was used to screen out the most significant differentially expressed genes in the RCC dataset of The Cancer Genome Atlas (TCGA). Real-time PCR and Western-blot analysis were utilized to examine the expression of inositol-1,4,5-trisphosphate-3-kinase-A (ITPKA) in four RCC cell lines and one human embryonic kidney cell line. Cell counting Kit-8 and colony formation assay were performed to estimate the effect of ITPKA on the proliferation ability of RCC cells. Wound healing and Transwell assays were used to test the effect of ITPKA on RCC cell migration and invasion. Xenograft formation assay was performed in nude mice to investigate the effect of ITPKA in vivo. mTORC1 pathway inhibitor was added to explore the mechanisms by which ITPKA regulates RCC cell growth and progression.

Results: Based on bioinformatics analysis, ITPKA is screened out as one of the most significant differentially expressed genes in RCC. ITPKA is upregulated and positively correlated with RCC malignancy and poorer prognosis. ITPKA promotes RCC growth, migration and invasion in cultured cells, and accelerates tumor growth in nude mice. Mechanistically, ITPKA stimulates the mTORC1 signaling pathway which is a requirement for ITPKA modulation of RCC cell proliferation, migration and invasion.

Conclusion: Our data demonstrate a critical regulatory role of the ITPKA in RCC and suggest that ITPKA/mTORC1 axis may be a promising target for diagnosis and treatment of RCC.

Keywords: ITPKA1, RCC, growth, migration and invasion, mTORC1 pathway

Introduction

Renal cancer is one of the most lethal malignancies of the kidney in adults, responsible for approximately 90–95% of kidney malignancies.[1] Despite significant improvements in treatment by surgery in many early renal cell cancer (RCC) patients over the past decade, once it reaches the advanced stage, the efficacy of treatment remains poor with a 5-year survival rate of less than 10%, and even 20–30% post-surgery treatment cases eventually develop recurrence.[2] As RCC is resistant to traditional chemotherapy, radiation therapy or hormonal therapy, further investigation of the molecular mechanisms underlying RCC tumorigenesis and progression is crucial for individual treatment of RCC.[3]

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Since the discovery of the mTOR signaling pathway, there have been many studies focusing on the regulatory role of the mTOR signaling pathway on tumors.[4,5] Inhibitors targeting the mTOR signaling pathway have been widely used in advanced RCC treatment. For example, approved mTOR inhibitors everolimus and temsirolimus have served as important therapeutic options within the current RCC treatment paradigm.[6–9] Therefore, it is very important to identify upstream and downstream factors related to the mTOR signaling pathway and design novel markers of RCC diagnosis, treatment and prognosis.

Inositol-1,4,5-trisphosphate-3-kinase-A (ITPKA) are enzymes belonging to the family of transferases, which facilitate phospho-group transfer from adenosine triphosphate to 1D-myo-inositol-1,4,5-trisphosphate 1.[10,11] ITPKA has been found to be overexpressed and implicated in the tumor progression of various cancers, such as of the pancreas, testis, thyroid, breast, lung, colon, liver and prostate breast cancer, etc.[12,13] However, the role and the prognostic value of ITPKA in RCC remain unexplored.

In the current study, we performed bioinformatic analysis based on the RCC database in The Cancer Genome Atlas (TCGA), and screened out ITPKA gene significantly upregulated in RCC and positively correlated with RCC malignancy and poorer prognosis. Further investigations confirmed the role of ITPKA as an oncogene in RCC. Moreover, we found that ITPKA regulated tumorigenesis and invasion via controlling the mTORC1 signaling pathway, thus linking the oncogenic ability of ITPKA in RCC to its activation of the mTORC1 signaling pathway.

Materials and Methods

Plasmids, Cell Lines and Reagents

786-O, Caki-1, Caki-2, and A498 cell lines were kind gifts from Prof. Xu Zhang in the General Hospital of Chinese People's Liberation Army, tested for mycoplasma contamination and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The use of the cell lines was approved by the ethics committee of the Beijing Institute of Biotechnology. In addition, Caki-2 cells were authenticated by STR profile (Supplementary Figure S1). Stable ITPKA knockdown cell line was established by cloning ITPKA short hairpin RNA (shRNA) fragment into the lentiviral vector pSIH-H1 (System Biosciences). The sequence of ITPKA shRNA was 5′-CTTTCCACCTCGTCGGTCTC-3′. Lentivirus was generated by transfection of the 293T producer cell line with the lentiviral vector and packing vector mix (System Biosciences). Pooled clones were screened by immunoblot with anti-ITPKA. Similar results were obtained with individual clones. Anti-ITPKA was from Abcam (ab251867). Anti-mTOR (#2972), anti-p-mTOR (S2448) (#2971), anti-S6K1 (#9202), anti-p-S6K1 (T389) (#9205), anti-p-4EBP1 (#9456) and anti-4EBP1 (#9452) were from Cell Signaling Technology. Anti-c-myc (sc-40), anti-cyclin D1 (sc8396) and anti-β-actin (sc-47778) were from Santa Cruz Biotechnology.

Transient Transfections

Cell lines were routinely cultured in recommended medium (DMEM for 293T cells, and RPMI 1640 for 786-O, Caki-1, Caki-2 and A498 cells) supplemented with 10% fetal bovine serum at 37 °C in humidified atmosphere of 5% CO_2 in air. Lipofectamine 2000 reagent and Lipofectamine RNAiMAX were used for transfections of plasmids and siRNAs, respectively, according to the manufacturer's guidelines (Invitrogen). siRNAs for ITPKA were chemically synthesized (Genepharma, Shanghai). The sequences of ITPKA siRNAs were 5′-CTTTCCACCTCGTCGGTCTC-3′, 5′-CC TTGTGTGCTCGACTGCATT-3′, and 5′-GGCAGAAGAT CCGGACCATTT-3′.

RNA Isolation and Quantitative Reverse Transcription PCR

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Total RNA was reverse transcribed into cDNA with oligo (dT) primers using Moloney murine leukemia virus reverse transcriptase (Promega). qPCR was performed in triplicate in a reaction mixture containing SYBR Premix Ex Taq Master Mix (2×) (Takara), for each of the primers and cDNA template. The relative expression was calculated by the comparative Ct method. Results were normalized to the expression of β-actin. Specific primer sequences were listed as follows: ITPKA forward, 5′-TGCAAAATGGGCGTCAGGACT-3′, reverse, 5′-CGAGGGTGGTGCTGGAGCT-3′; β-actin forward, 5′-ATCAC CATTGGCAATGAGCG-3′, reverse, 5′-T TGA AGGTAGTTTCGTGGAT-3′.

Cell Growth and Colony Formation Assays

Anchorage-dependent cell growth was determined by the CCK-8 Kit (Dojindo Laboratories) according to the manufacturer's instructions. For colony formation assays, transfected cells were seeded in 6-well plates at 3,000

cells per well. Three weeks later, the colonies were fixed with 4% paraformaldehyde and stained with a crystal violet solution for 30 min. The number of colonies containing at least 50 cells was counted. Colonies with diameters greater than 100 μm were counted after 3 weeks of growth.

Cell Migration and Invasion Assays

Wound healing assays were performed to estimate cell migration. Briefly, transfected cells cultured in 6-well plates as confluent monolayers were mechanically scratched using a 1-mL pipette tip to create the wound. Cells were washed with PBS to remove the debris and were cultured for 24 h to allow wound healing. Cell invasion was determined with Matrigel (BD Biosciences) coated on the upper surface of the transwell chamber (Corning). Twenty-four hours later, cells invaded through the Matrigel membrane were fixed with 4% paraformaldehyde and stained with crystal violet. The number of invaded cells was counted in 5 randomly selected microscopic fields and photographed.

In vivo Tumor Growth

All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Beijing Institute of Biotechnology. 1×10^7 786-O cells were injected into the hind limb of 6-week-old male nude mice (cells suspended in 200 μL of phosphate buffered saline [PBS], n = 8), which were divided into three groups using random number method with no blinding. Tumor size was measured at indicated times using calipers. Tumor volume was calculated according to the following formula: volume = (longest diameter × shortest diameter^2)/2.

Statistical Analysis

All in vitro experiments were performed in triplicate and repeated 3 times. The difference of ITPKA expression between RCC and normal tissues, stage 1 and stage 4, no metastasis and metastasis were assessed by Mann–Whitney *U*-test. Estimation of disease-free survival and overall survival was conducted using the Kaplan-Meier method, and differences between survival curves were examined with the Log rank test. Statistical significance in cell proliferation and invasion assays among constructs was determined by two-tailed Student's *t*-test. The SPSS 17.0 statistical software package was used to perform the statistical analyses. $p<0.05$ was considered statistically significant.

Results

ITPKA is screened out upregulated in RCC and positively correlated with RCC malignancy and poorer prognosis.

To explore more genes associated with RCC growth and development, we analyzed the RNA-Seq data of RCC from TCGA database according to the strategy shown in Figure 1A (Figure 1A). Briefly, we screened the differentially expressed genes (DEGs) based on the malignancy, stage, and metastatic status of RCC respectively. We identified 2203 upregulated and 2652 downregulated DEGs between paired RCC and non-tumor group, 133 upregulated genes and 192 downregulated genes between Stage 1 and Stage 4, and 195 upregulated and 176 downregulated DEGs between the metastatic and non-metastatic groups (Figure 1B–D). Collectively, there were 14 overlapped DEGs based on the above-mentioned screening methods (Figure 1E and Supplementary Table 1). Combined with the survival analysis (http://gepia.cancer-pku.cn/index.html), 11 genes exhibited significant clinical outcome and accorded with the malignancy of the genes. Among the 11 significant DEGs, 4 genes, such as AIM2,[14] IGF2BP3,[15] CXCL13[16] and IL20RB[17] had been demonstrated to play critical roles related to the development of RCC. The other 7 genes had not been reported to be associated with RCC growth and progression. Among them, ITPKA was the most differentially expressed one. Therefore, we chose ITPKA for further study. The database analysis showed that ITPKA expression was upregulated in RCC tumor tissue, higher stage and metastatic groups compared with those of non-tumor, lower stage and non-metastatic counterparts (Figure 1F). Based on the clinical information of TCGA data, the survival analysis showed that a higher expression of ITPKA was significantly correlated with poorer overall survival (OS) and disease-free survival (DFS) in RCC patients (Figure 1G). Taken together, these data indicate that ITPKA may be a promoter in RCC growth and progression.

ITPKA promotes RCC Proliferation, Migration and Invasion both in vitro and in vivo.

Since ITPKA has been shown to correlate with RCC malignancy and clinical outcome, we tested if ITPKA mediates proliferation, migration and invasion in RCC cells. To select appropriate cell lines, we used qRT-PCR and Western-blot analysis to examine the expression of ITPKA in four different RCC cell lines (786-O, Caki-1, Caki-2 and A498) and one human embryonic kidney cell line (293T). All four RCC cell lines and one human embryonic kidney cell line expressed endogenous ITPKA protein. Among them, 786-O and Caki-1 cell lines expressed ITPKA at the highest mRNA

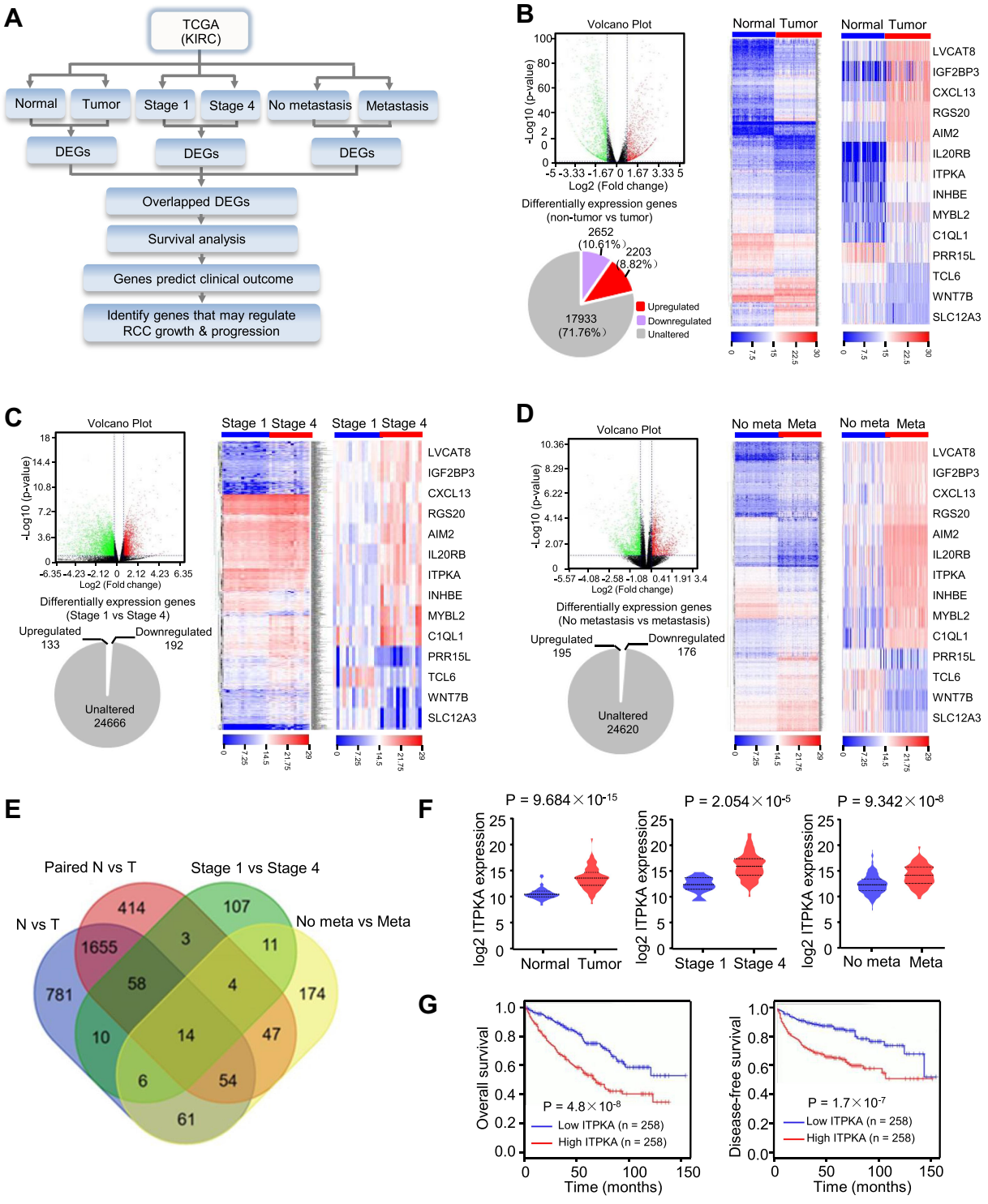

Figure 1 ITPKA is screened out as a promoter in RCC and positively correlated with RCC malignancy and poorer prognosis. (**A**) Schematic diagram of screening strategy. (**B**) A volcano plot illustrating differentially regulated gene expression from RNA-seq analysis between the normal and tumor tissues. Genes upregulated and downregulated are shown in red and green, respectively. Values are presented as the log2 of tag counts. Pie chart revealed a total of 17,933 genes expressed, of which 2203 genes were upregulated and 2652 genes were downregulated. The hierarchical clustering of the RNA-seq analysis results shows all genes that were significantly differently expressed (left panel) and the overlapped 14 genes that were differentially expressed (right panel). (**C**) Comparision between stage 1 and stage 4 analyzed as in (**B**). (**D**) Comparision between non-metastasis and metastasis analyzed as in (B). (**E**) Venn analysis of the DEGs based on the strategy. The four circles in the figure represent the DEGs in each screening methods, and the middle part represented the intersection of the results. (**F**) ITPKA expression scores between normal and tumor, stage 1 and stage 4, non-metastasis and metastasis group were displayed in violin plot and compared using Mann–Whitney *U*-test. (**G**) Kaplan–Meier estimates of disease-free survival and overall survival of RCC patients from The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/). Marks on graph lines represent censored samples.

or protein level, while 293T cell line expressed ITPKA at the lowest level (Figure 2A). Therefore, we chose two relatively high ITPKA-expressed cell line Caki-1 and 786-O cells to knockdown endogenous ITPKA (Figure 2B). 786-O and Caki-1 cells transfected with ITPKA siRNAs grew more slowly than those transfected with Control siRNA. These effects were reversed by ITPKA re-expression in the ITPKA knockdown cells transiently transfected with the ITPKA siRNA-resistant expression vector. Colony formation assays revealed similar trends to those of growth curves mentioned above (Figure 2C). These data indicate that ITPKA accelerates RCC cell proliferation. As expected, wound-healing and transwell assays demonstrated that ITPKA knockdown decreased migration and invasion ability of 786-O and Caki-1 cells while ITPKA re-expression in the knockdown cells rescued these effects in those cells (Figure 2D and E).

To investigate the in vivo phenotype of the ITPKA in RCC, 786-O cells harboring Control shRNA or ITPKA shRNA or ITPKA shRNA plus ITPKA were subcutaneously injected into the right flanks of male nude mice. Compared with the Control shRNA, tumors with ITPKA shRNA construct grew more slowly, and again, ITPKA re-expression reversed the ITPKA knockdown effect to regulate the growth of tumor xenografts (Figure 2F). Collectively, these data suggest that ITPKA may play an important role in RCC cell proliferation, migration and invasion both in vitro and in vivo.

ITPKA Activates mTORC1 Signaling Pathway in RCC

To further explore the molecular mechanisms of ITPKA regulating RCC cell growth and progression, we performed gene set enrichment analysis (GSEA) based on RNA-seq data of RCC downloaded from public databases (TCGA) and identified signaling pathways that were closely correlated with ITPKA expression. Interestingly, we found that activation of mTORC1 signaling, which is the critical signaling pathway for cell proliferation and invasion, was positively related to ITPKA expression level in the TCGA RCC cohort (Figure 3A). Therefore, we investigated whether ITPKA activates mTORC1 signaling pathway in RCC cells. Notably, Western blot analysis showed that, consistent with the GSEA analysis of ITPKA activation of mTORC1 signaling, knockdown of ITPKA in 786-O and Caki-1 cells decreased the levels of phosphorylation of mTOR, phosphorylation of S6K1 and 4E-BP1, two mTOR kinase targets, as well as the mTOR downstream effectors c-myc and cyclin D1, whereas reexpression of ITPKA reversed the effects (Figure 3B). In addition, ITPKA had little effect on AKT S473 phosphorylation activated by mTORC2 (Supplementary Figure 2). These data suggest that ITPKA is an upstream regulatory factor of mTORC1 and stimulates the mTORC1 signaling pathway in RCC.

ITPKA Regulates RCC Cell Proliferation, Migration and Invasion via Activation of mTORC1 Signaling Pathway

To further determine the mechanisms by which ITPKA regulates RCC proliferation and invasion, we tested whether activation of the mTORC1 signaling pathway is responsible for ITPKA modulation of RCC cell proliferation and invasion. As expected, ITPKA overexpression in 786-O cells promoted cell proliferation and migration, accompanied by elevated levels of phosphorylation of mTOR, S6K1 and 4E-BP1, and increased expression of c-myc and cyclin D1. As expected, the mTORC1 pathway inhibitor, Rapamycin reduced 786-O cell proliferation and invasion. More importantly, Rapamycin almost abolished the ability of ITPKA to increase RCC cell proliferation, migration and invasion as well as the mTOR pathway molecules (Figure 4A–D), indicating that ITPKA promotes RCC cell proliferation, migration and invasion through activating the mTORC1 signaling pathway.

Discussion

Recently, accumulating evidence has revealed that ITPKA is overexpressed and functions as a promoter of varieties of cancers, including breast,[18] lung,[19,20] and liver cancer,[21] etc. However, the role of ITPKA in RCC remains unclear. Our work suggests a critical role of ITPKA regulating RCC growth and progression. First, ITPKA is overexpressed in RCC patients, positively correlates with pathological stage and predicts poor prognosis. Second, ITPKA promotes RCC cell growth, migration and invasion both in vitro and in vivo. Third, mechanistically, the oncogenic ability of ITPKA in RCC can be linked to its activation of the mTORC1 signaling pathway. Collectively, our data suggest that ITPKA may be a promising target for RCC therapy.

The PI3K family and the Inositoltrisphosphate 3-kinase (ITPK) family are closely related because

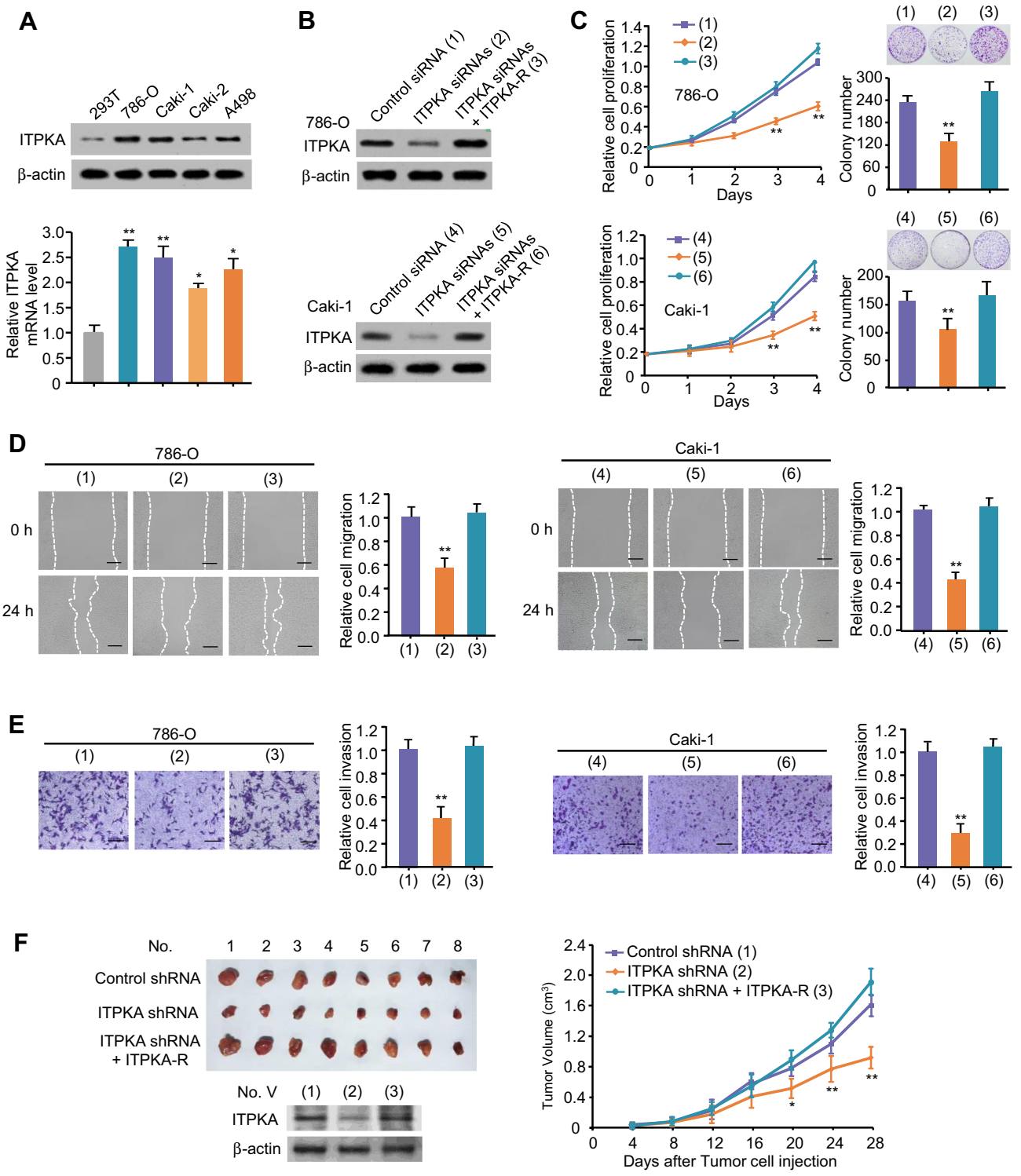

Figure 2 ITPKA promotes RCC proliferation, migration and invasion both in vitro and in vivo. (**A**) Total proteins extracted from the indicated RCC cell lines were analyzed by Western-blot with anti-ITPKA. β-actin was used as a loading control. (**B**) 786-O and Caki-1 cells were transfected with Control siRNA or ITPKA siRNAs or ITPKA siRNAs plus ITPKA. The immunoblot shows ITPKA expression. (**C**) Cell proliferation and colony formation, wound healing (**D**) and transwell (**E**) assays of 786-O and Caki-1 cells transfected as in (**B**). (1), (2), (3) represent 786-O cells transfected with Control siRNA, ITPKA siRNAs, ITPKA siRNAs plus ITPKA, respectively. (4), (5), (6) represent Caki-1 cells transfected with Control siRNA, ITPKA siRNAs, ITPKA siRNAs plus ITPKA, respectively (**B**–**D**). Illustrative images show colonies in plates, cell migration and invasion. Histograms show colony number, comparative cell migration and invasion. All values displayed are mean ± SD and have been duplicated 3 times with similar results (**A**–**D**). *p < 0.05 and **p < 0.01 versus corresponding Control siRNA. (**F**) 786-O cells stably infected with lentivirus carrying the indicated constructs were injected into nude mice as indicated. After 28 days, mice were sacrificed to harvest tumors. At the indicated times, the tumors were measured (mean ± SD, n = 8), and the growth curve was plotted. **p < 0.01 vs. Control shRNA group.

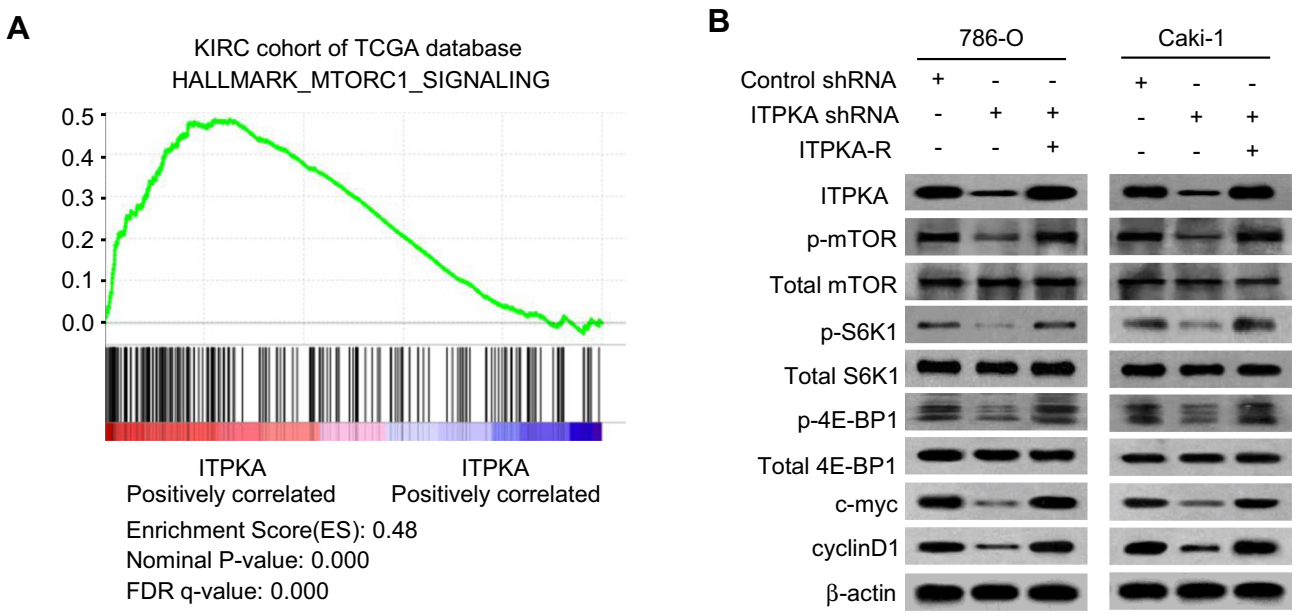

Figure 3 ITPKA activates mTORC1 signaling pathway in RCC. (**A**) GSEA plot showing that ITPKA expression is positively correlated with mTORC1 signaling in the TCGA KIRC dataset. (**B**) 786-O and Caki-1 cells were transfected with Control shRNA or ITPKA shRNA or ITPKA shRNA plus ITPKA-R. Western-blot assay was performed to detect the protein expression changes of mTORC1 signaling pathway related-molecules (including t-mTOR, p-mTOR, t-S6K1, p-S6K1, t-4E-BP1, p-4E-BP1, c-myc, cyclin D1) between indicated groups. The protein expression levels of β-actin were detected as the internal control.

both are phosphorylate inositol phosphates. However, compared to the PI3K family, much less is known about the exact process and mechanisms of ITPKA in regulating cancer growth and progression. Ashour et al reported that ITPKA was a bi-functional protein which phosphorylated Ins (1,4,5) P3 by its kinase activity and cross-linked F-actin by its F-actin bundling activity, which accounts for the ITPKA-promoting effects on cancer development.[22] However, the mechanism of how ITPKA executes its function in tumors still needs further investigation. Our current work first demonstrates that ITPKA promotes RCC proliferation, migration and invasion via activation of the mTORC1 pathway, which provides novel mechanisms for regulations of RCC growth and development by ITPKA.

The mTOR signaling pathway is frequently activated in many cancers, including lung cancer,[23] colorectal cancer,[24] breast cancer[25] and renal cell carcinoma,[6] which play critical roles in carcinogenesis and progression. Activated mTOR pathway predicts poor clinical outcome for patients with tumors. In line with their driver roles in tumor development and progression, tumor-therapeutic drugs that block the mTOR signaling pathway have become one of the most promising targets, more than any other hubs of signal transduction.[26] mTOR inhibitors, such as everolimus and temsirolimus, have been put into use in the treatment of advanced renal cancer.[27] However, drug resistance of everolimus and temsirolimus, limits their applications in advanced RCC treatment.[28] Therefore, elucidating the mechanisms of mTOR signaling may provide new therapeutic targets for overcoming mTOR inhibitor drug resistance and improving clinical outcomes.

mTOR exists in two distinct complexes: mTORC1 and mTORC2.[29] mTORC1 is highly sensitive to rapamycin, whereas mTORC2 is relatively insensitive to rapamycin. The role of the mTORC1 complex, which is based on the interaction between mTOR and Raptor (regulatory associated protein of mTOR), is responsible for activating the downstream substrates, S6K1 and 4E-BP1. The role of the mTORC2 complex, which is based on the interaction between mTOR and Rictor (rapamycin-insensitive companion of mTOR), is mainly related to the regulation of AKT S473 phosphorylation. Our results demonstrate that ITPKA alters the levels of phosphorylation of S6K1 and 4E-BP1, two mTORC1 kinase targets, whereas it has little effect on AKT S473 phosphorylation activated by mTORC2, collectively indicating that ITPKA controls the mTORC1 signaling pathway.

In summary, our findings display the pivotal role of ITPKA in promoting RCC cell proliferation, migration and

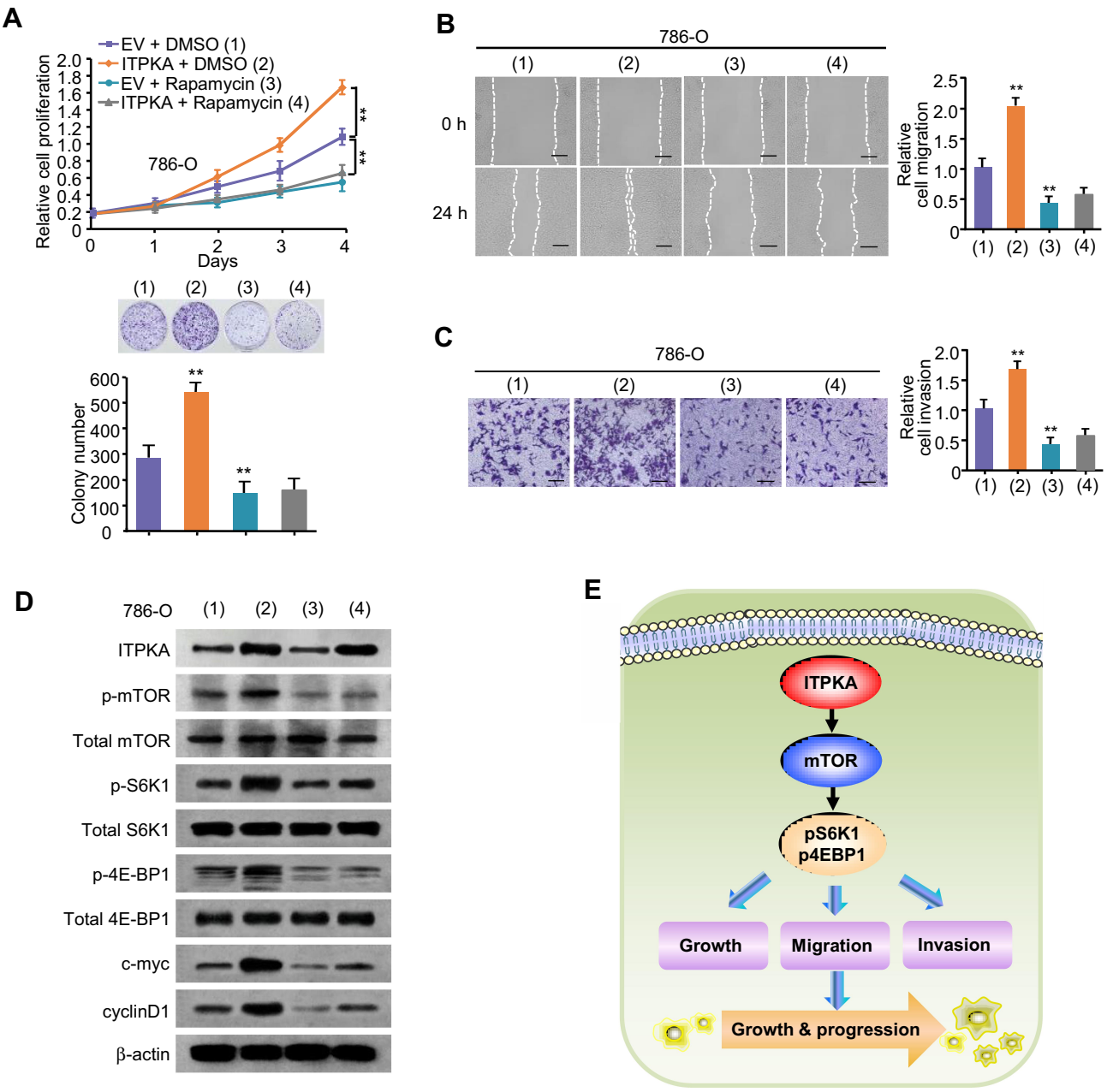

Figure 4 ITPKA regulates RCC cell proliferation, migration and invasion via activation of mTORC1 signaling pathway. (**A**) 786-O cells were transfected with pcDNA3.0-Empty vector (EV) or pcDNA3.0-ITPKA (ITPKA). The cell proliferation and colony formation differences were detected by CCK-8 assay among 4 groups (including EV [DMSO], ITPKA [DMSO], EV [Rapamycin], and ITPKA [Rapamycin]). (**B** and **C**) The cell migration (**B**) and invasion changes (**C**) were detected by wound-healing and transwell assay among the above-mentioned 4 groups. (**D**) The Western-blot assay was performed to detect the proteins involved in the mTORC1 signaling pathway of the 4 groups. (1), (2), (3), (4) represent 786-O cells treated with EV + DMSO, ITPKA + DMSO, EV + Rapamycin, ITPKA + Rapamycin, respectively (**A**–**D**). All experiments were performed three times independently, and the data were presented as the mean ± SD. **p < 0.01 versus corresponding EV + DMSO. (**E**) Proposed model for ITPKA activation of mTORC1 signaling pathway and subsequent enhancement of growth, migration and invasion of RCC cells, thus leading to RCC growth and progression.

invasion and demonstrate the mechanisms of ITPKA activation of mTORC1 signaling pathway (Figure 4E). Therefore, exploring the more exact roles of ITPKA in RCC oncogenesis and progression as well as the underlying mechanisms will provide powerful and direct evidence to prove ITPKA as a therapeutic target for RCC.

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Disclosure

The authors report no conflicts of interest in this work.

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