Long Noncoding RNA LINC00460 Facilitates the Proliferation and Metastasis of Renal Cell Carcinoma via PI3K/AKT Signaling Pathway

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Research Article

Keywords: RCC, LINC00460, proliferation, metastasis, PI3K/AKT pathway

Posted Date: May 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-483043/v1

Abstract

Renal cell carcinoma (RCC) is one of the most prevalent cancers. Long noncoding RNAs (LncRNAs) have been indicated as a mediator acted in tumorigenesis of RCC. However, the mechanism of LINC00460 on RCC is yet to be investigated. This study aimed to investigate the potential function of LINC00460 and underlying mechanism of RCC. We detected LINC00460 expression in RCC tissues and the prognosis in RCC patients using Gene Expression Profiling Interactive Analysis (GEPIA) website and The Cancer Genome Atlas (TCGA) database. LINC00460 level in normal renal cell line and RCC cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). We study the effects of LINC00460 on proliferation, migration, invasion, apoptosis in RCC cells lines using a series of in vivo and in vitro experiments. RNA sequencing (RNA-seq) analysis for the whole transcriptome was applied to searching potential LINC00460 related signal pathway in RCC. We identified the significant up-regulated expression level of LINC00460 in RCC tissues and cell lines. Elevated LINC00460 was correlated with shorter survival of RCC patients. Overexpression of LINC00460 promoted cell viability, proliferation, invasion and migration, while down-regulation of LINC00460 exerted inhibitory effect on these activities. We crucially identified that LNC00460 promotes development of RCC by influencing the PI3K/AKT pathway. Knockdown of LNC00460 decreased the phosphorylation of AKT and mTOR. The key finding of our study provided a new evidence suggesting that LINC00460 functions as an oncogene in RCC pathogenesis by mediating the PI3K/AKT pathway, which may provide a new target for the treatment of RCC.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors originating from the renal parenchymal urinary epithelial system, the incidence of which has increased annually (approximately 7% per year) over the past years, only next to that of prostate cancer and bladder cancer, accounting for 2.2% of adult malignant tumors[1]. However, due to the asymptomatic or covert symptoms of kidney cancer in the early stage and the lack of awareness of cancer screening, patients often missed the early diagnosis and treatment, and at the time of diagnosis, local progression had occurred, or the condition was already in the advanced clinical stage[2]. At present, radical surgery remains the mainstay of treatment for patients with early-stage RCC[3]. Although there has been progress in the clinical treatment for RCC, the prognosis of RCC at an advanced stage is still poor, and the expected efficacy of targeted therapy is unsatisfactory due to drug resistance and severe adverse reactions[4, 5]. Thus, it is urgent to clarify the molecular mechanisms and to screen novel biomarkers for RCC.

Long noncoding RNAs (LncRNAs), with more than 200 nucleotides in length and no protein-coding capacity, have been considered as transcriptional, post-transcriptional or post-translational levels regulators of gene expression[6]. LncRNAs participate in various physiological and pathological processes[7, 8], complicating the gene regulation networks, such as gene transcription regulation, RNA processing, chromatin modification, especially in tumorigenic biological activities[9]. Accumulating evidences show that LncRNAs can participate in the occurrence and development of tumor functioning

as oncogenes or tumor suppressor[10–12]. For instance, LncRNA MALAT1, LncRNA HOTAIR, LncRNA TUG1 and LncRNA XIST, promote cell proliferation, invasion and/or migration, including multiple myeloma, triple-negative breast cancer, cervical cancer, pancreatic cancer, bladder cancer, gastric cancer and thyroid cancer [13–17].

Long intergenic noncoding RNA 460 (LINC00460), is a new cancer associated LncRNA whose expression is involved in the development of a variety of human malignancies, including nasopharyngeal carcinoma, lung cancer, thyroid cancer[10, 18, 19]. Unfortunately, the specific mechanism of LINC00460 in RCC is still not clear.

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway is hyperactivated or altered in many cancer types and regulates a broad range of cellular processes including survival, proliferation, growth, metabolism, apoptosis and metastasis[20–22]. Activation of PI3K/AKT/mTOR signaling pathway was reported to promote the occurrence and metastasis of human esophageal cancer and induce the apoptosis of esophageal squamous cell carcinoma cells[23]. The PI3K/AKT/mTOR pathway is regulated by a wide-range of upstream signaling proteins and it regulates many downstream effectors by collaborating with various compensatory signaling pathways[24].

In this work, we investigated the potential involvement of LINC00460 in RCC. We used bioinformatic online tools to detected the increased LINC00460 expression level in RCC tissues and predicted poor survival of RCC patients. We first examined the expression level of LINC00460 in RCC cells and evaluated its effects on cell growth, migration invasion, and apoptosis in vitro and tumorigenesis in vivo. In addition, we explored the underlying mechanism of LINC00460 function in RCC. RNA sequencing (RNA-seq) analysis showed that LINC00460 gene knockout could primarily affect the genes related to proliferation and apoptosis. From the perspective of mechanism, our results indicated that LINC00460 mediates PI3K/AKT signaling to promote the progression of RCC cells. Thus, this research provides a better understanding of RCC pathogenesis.

Materials And Methods

Bioinformatical analyses

Comparison of LINC00460 expression levels in RCC tissue and normal tissue were analyzed with the Gene Expression Profiling Interactive Analysis website (GEPIA, https://gepia.cancer-pku.cn/) and the Cancer Genome Atlas (TCGA, https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The overall survival (OS), disease-free survival (DFS) and clinicopathological features was evaluated in RCC patients based on LINC00460 expression was also analyzed using GEPIA and TCGA.

Cell Culture

The renal cell carcinoma (RCC) cell lines and Human embryo kidney epithelial cell line HK-2 were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). ACHN, 786-O, OSRC-2, Ketr-3, and HK-2 were correspondingly cultured in RMPI-1640 and DMEM Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin, and incubated at 37 °C humidified incubator with 5% CO2.

Cell Transfection

For the overexpression of LINC0046, sequences were constructed into pCDH-CMV-MCS-EF1-GreenPuro lentivirus vector (GenePharma Suzhou, China). For the knockdown of LINC00460, shRNAs (#1: 5'-GCTAAGACCTAATAGCCAATA-3' and #2: 5'-ACCTTGGTCAAACGTTTAACC-3'), as well as the negative control (shCtrl, 5'-GTTCTCCGAACGTGTACGT-3'), were constructed into pLKO.1 (GenePharma Suzhou, China). HEK-293T cells were co-transfected with psPAX2 and pMD2.G with pLKO-shLINC00460#1 / #2 (sh1LINC00460) or pLKO- shCtrl. Forty-eight hours later, lentiviruses with the released lentiviral vectors were harvested. ACHN and 786-O cells were infected with the lentiviruses using 8 mg/mL polybrene. Stable ACHN and 786-O cell lines with shLINC00460#1 / #2 or shCtrl were obtained with a treatment of 5 μg/mL puromycin for 1 week.

RNA extract, reverse transcription-PCR and qRT-PCR

RNA was extracted using TRIzol (Invitrogen) and cDNA was synthesized using the HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech, Nanjing, China). Realtime PCR was carried out on ABI-7500 using UltraSYBR One Step RT-qPCR Kit (CWBIO, Beijing, China). The primers using for quantitative RT-PCR analysis were listed as followed: LINC00460 Forward: ACGCAGTGGATGAGAACGAA, LINC00460 Reverse: GGGGTGACTTCAGAATGCGT, 18S rRNA Forward: GTAACCCGTTGAACCCCATT, 18S rRNA Reverse: CCATCCAATCGGTAGTAGCG.

Cell Counting Kit-8 (CCK-8)

Cell proliferative ability was assessed by Cell Counting Kit-8 reagent (Beyotime, China). In brief, transfected cells (5×10^3 cells/well) were added into a 96-well plate and incubated at 37°C with 5% CO2 for 24-72h. Then, 10 μl of CCK-8 solution was added to each cell well and the plates continued to incubate for 2h. The absorbance (OD) at 450 nm was determined with the microplate reader.

Transwell Assays

ACHN or 786-O cells were first seeded into the upper wells of chambers (BD Biosciences, Bedford, MA, USA) with or without a Matrigel-coated membrane (BD Biosciences) in a 200-μL FBS-free DMEM or RMPI-1640 medium. The lower wells of the chambers were filled with 400-μL DMEM or RMPI-1640 medium containing 10% FBS. 8 hours later, the medium of the upper wells and the filters were removed. 24 hours later, the invasive cells were fixed with 100% methanol and then stained with 0.1% crystal violet for 1 hour. The stained cells were imaged under a microscope (Olympus, Japan).

Wound Healing Assays

Gaps in seeded ACHN or 786-O cells were generated via a plastic pipette tip. After removing the debris or the detached cells, the cells were cultured in DMEM and RMPI-1640 for another 24 hours before calculation of wound width by Wound Healing via ImageJ Analysis Software.

Flow cytometry

Cell apoptosis was determined by flow cytometry (BD, UA). Firstly, ACHN and 786-O cells were digested using ethylenediaminetetraacetic acid (EDTA)-free trypsin and collected in a centrifugal tube. Then, the cells were suspended in 1× binding buffer at 3×10^6/mL. Subsequently, 100 μL of cells were moderately mixed with 5 μL of Annexin V-APC and 5 μL of Propidium Iodide (PI), followed by incubation at room temperature in the dark for 5 min. After 400 μL of 1× binding buffer was added into the sample, cell apoptosis was analyzed using the flow cytometer. Cell apoptosis rate was calculated based on the number of Annexin-APC positive cells and were analyzed by FlowJo v10.6.2 software according to the manufacturer's protocols.

Western Blot

Total protein from harvested RCC cells were extracted by using RIPA lysis buffer (Keygen, Nanjing, China) and qualified using a BCA kit (Keygen, Nanjing, China). Thirty-microgram proteins were separated by SDS-PAGE and then electro-transferred onto PVDF membranes. After blocking with 5% BSA, the membranes were incubated with primary antibodies overnight at 4°C. Following incubation with HRP-labeled secondary antibody (1:5,000; ABclonal), the immunoreactivities were detected by ECL reagent (KeyGen, Nanjin, China) on Tanon 5200 automatic chemiluminescence imaging analysis system (Tanon, Shanghai, China). Anti-GAPDH was used as control (sc-32233, Santa Cruz, USA). Anti-E-cadherin (610181, BD Biosciences, USA), Anti-N-cadherin (610920, BD Biosciences, USA), Anti-Vimentin (10366-1-AP Proteintech, China), Anti-PI3k (67121-1-Ig Proteintech, China), Anti-AKT (101762-2-AP Proteintech, China), Anti-p-AKT (66444-1-Ig Proteintech, China), Anti-p-mTOR (5536S Cell signaling Technology, China), Anti-Bcl-2 (155071S Cell signaling Technology, China), Anti-Cleved-Caspaes 9 (20750S Cell signaling Technology, China), Anti-p53 (10442-1-AP, Proteintech, China) were used for Western blot assays.

Animal Works

The female BALB/c nude mice (6-8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Groups of shCtrl and shLINC00460 786-O cells (5×10^6) were injected subcutaneously into the flanks of mice correspondingly. Tumors volume (V) was monitored every 3 days by measuring the long axis (L) and the short axis (W) of xenograft tumor and calculated with the following formula: $V = (L \times W2)/2$. The tumor tissues were weighed on day 27 and fixed for subsequent analysis.

Meanwhile, groups of shCtrl and shLINC00460 786-O-Luc cells (3×10^6) were injected intravenously via the mice tail vein (two groups of nine mice each). Bioluminescence images were filmed after 6 weeks following the manufacturer's protocol (Night OWL II LB983; Berthold Technologies, Bad Wildbad, Germany). Animal experiments were performed in accordance with National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Care and Use Committee and Ethics Committee of Xuzhou Medical University.

Statistical Analysis

All data were analyzed using Statistical Product and Service Solutions (SPSS) 23.0 (IBM Corp., Armonk, NY, USA) and expressed as mean ± standard deviation. The differences between the two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test. $p<0.05$ suggested statistically significant differences.

Results

LINC00460 was increased and closely correlated with poor prognosis in RCC

GEPIA database and TCGA datasets were used to obtain LINC00460 expression in RCC tumor tissues compared with that in normal tissues and the relationship between LINC00460 expression and the survival of RCC patients. The data in GEPIA database and TCGA (GSE33113, GSE41328) revealed that LINC00460 was highly expressed both in Kidney renal clear cell carcinoma (KIRP) (Fig. 1a) and Kidney renal papillary cell carcinoma (KIRP) (Fig. 1b). In addition, clinicopathological staging are important prognostic factors for RCC patients, we detected the LINC00460 expression with different clinical pathological status of RCC tissues in GEPIA database, we found that LINC00460 was gradually increased with the advanced staging of RCC (Fig. 1c). Furthermore, RCC patients with low expression of LINC00460 exhibited a higher overall survival (OS) rate and disease free survival (DFS) than those with high expression of LINC00460 (Fig. 1d, 1e). The results indicated that aberrant expression of LINC00460 might be strongly associated with poor prognosis in RCC.

LINC00460 was elevated in RCC cell Lines and promoted cell proliferation

We firstly detect the expression level of lINC00460 in RCC cell lines to explore the effect of LINC00460 on RCC progression. qRT-PCR analysis demonstrated that LINC00460 was significantly upregulated in RCC cell lines (ACHN, 786-O, OSRC-2, and Ketr-3) compared with normal renal cell line HK-2. (Fig. 2a). Subsequently, qRT-PCR was conducted to determine the transfection efficiency by examining the expression patterns of LINC00460 in cells after different transfection. The results showed that the expression of LINC00460 was significantly up-regulated in cells transfected with LINC00460 overexpression vector compared with the control vector (Fig. 2b), while the expression of LINC00460 was remarkably down-regulated in cells transfected with the pLKO.1-shRNA compared with that in cells transfected with pLKO.1-shCtrl (Fig. 2c). Next, we tested cell proliferation by CCK-8 assay. The data of

CCK-8 revealed that upregulated LINC00460 expression increased cell proliferation as relative to the control groups (Fig. 2d), while downregulated LINC00460 expression suppressed cell proliferation (Fig. 2e).

LINC00460 facilitated RCC cell migration, invasionand induced EMT phenotype *in vitro*

The results of Transwell assays demonstrated that LINC00460 overexpression enhanced cell migration and invasion in ACHN and 786-O cells (Fig. 3a, 3c), while LINC00460 knockdown suppressed cell migration and invasion in ACHN and 786-O cells (Fig. 3b, 3d). In Wound healing assays, we conformed the same results that LINC00460 overexpression enhanced cell migration (Fig. 3e, 3g), while LINC00460 knockdown suppressed cell migration (Fig. 3f, 3h) respectively.

Western blot analysis were employed to detect the expression patterns of EMT-related proteins, as the results obtained from Western blot analysis (Fig. 4c, 4d), LINC00460 knockdown significantly increased the levels of E-cadherin but decreased those of N-cadherin and Vimentin in ACHN cells. Consistent with the results in RCC cells, it can be concluded that LINC00460 induced the migration, invasion and EMT of RCC cells.

LINC00460 affected the apoptosis of RCC cells

Flow cytometry after Annexin V-APC/Propidium Iodide (PI) staining and Western blot assay were used to verify whether promotion of cell malignant progress by LINC00460 was associated with cell apoptosis. Then, we found that the percentage of apoptotic cells was remarkably decreased in the LINC00460 overexpression transfected ACHN and 786-O cells group compared to the empty vector group, (Fig. 4a, 4b). Compared with shCtrl group, the expression level of anti-apoptotic protein Bcl-2 was decreased after LINC00460 knockdown, conversely, pro-apoptotic protein Cleaved Caspase-9 and p53 expression were increased in ACHN cells (Fig. 4c, 4d). All of these results proved that LINC00460 affects the malignant progression of RCC cells by affecting apoptosis.

LINC00460 promoted RCC cell growth and metastasis *in vivo*

To evaluate the effects of LINC00460 on tumor growth in nude mice, we subcutaneously injected 786-O cells transfected with shLINC00460 or shCtrl into nude mice. Knockdown of LINC00460 significantly decreased tumor growth (Fig. 5a), as shown by the significantly reduced tumor volumes and weights in the knockdown group compared with the control group (Fig. 5b, 5c). Furthermore, the expression of LINC00460 in xenograft tumor tissues was confirmed by qRT-PCR, which showed that LINC00460 expression was significantly decreased in shLINC00460-treated groups compared to shCtrl groups (Fig. 5d).

To assess whether LINC00460 knockdown could inhibit metastasis *in vivo*, we administered tail vein injections with 786-O-Luc cells stably transduced shLINC00460 or shCtrl vector into two groups of mice each respectively. Six weeks after tail vein injections, the mice were sacrificed, and the metastatic nodules formed on the lung surfaces were examined by bioluminescence imaging system. LINC00460 knockdown

cells formed fewer metastatic foci in lungs than the control group (Fig. 5e). Overall, the functional data demonstrated that silencing of LINC00460 blocked tumor growth and metastasis of RCC cells *in vivo*.

Dysregulation of LINC00460 was related to PI3K/AKT pathway

To unbiasedly explore the LINC00460-related pathway in RCC, RNA-seq analysis for 786-O cells was conducted after LINC00460 knockdown (Fig. 6a). To get further insights into the mechanism of LINC00460 in RCC tumorigenesis, we performed Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis for the target genes of LINC00460 (>1.5-fold change). KEGG pathway clustering demonstrated that the most significantly overexpressed biological pathways included cell growth and death, cell motility, repair and replication. Markedly, the dysregulated key genes that were related to the PI3K/AKT pathways (Fig. 6b).

PI3K/AKT pathway was involved in different cancer cell behaviors, especially cell proliferation and apoptosis[25]. Thus, we investigated whether LINC00460 control cancer cell progress by the PI3K/AKT pathway. The Western blot assay revealed a reduction of PI3K, phosphorylated AKT (p-AKT) and phosphorylated mTOR (p-mTOR) expression in ANCH and 786-O cells transfection with shLINC00460 as relative to the control groups, Meanwhile, an increased expression of PI3K, p-AKT and p-mTOR were observed in cells transfection with overexpressed LINC00460 as relative to the control groups (Fig. 6c, 6d, 6e, 6f). These results suggested that dysregulation of LINC00460 could regulate the PI3K/AKT pathway.

Discussion

Renal cell carcinoma (RCC) accounts for approximately 90% of renal malignancies and is a heterogenous group of various subtypes of cancer[26], and was regarded as a multistage process involving genetic and epigenetic alterations [27]. The most critical biological features of RCC are uncontrolled cell proliferation, apoptosis and metastasis which are the major reasons for death. Surgery is currently the treatment of choice for organ-confined tumor, while locally advanced or metastatic disease often requires pharmacological or targeted therapy[28]. Unfortunately, many RCC patients were diagnosed at the advanced stage due to the lack of early-detection techniques, due to no effective diagnosis biomarker and a poor understanding of the mechanism involving cancer progression have limited the effectiveness of therapy for RCC patients.

Currently, LncRNAs have been considered as active biological molecules rather than transcriptional noise[29]. They were proved to drive carcinogenesis via regulating various cellular processes, including proliferation, apoptosis, angiogenesis, invasion and metastasis[30]. In cervical cancer, upregulation of LncRNA ZEB1-AS1 enhances cell invasion and epithelial to mesenchymal transition by elevating ZEB1 expression[31]. LncRNA UCA1 is increased in thyroid cancer and represses cell proliferation and cell invasion by interacting with miR-204/IGFBP5[32]. LncRNA CA3-AS1 inhibits colorectal cancer cell proliferation and invasion, and it induces cell apoptosis by miR-93/PTEN axis[33]. The downregulation of LINC00152 suppresses the progression of gastric cancer through controlling miR-193b-3p/ETS1 axis[34].

In the present study, LINC00460 have been proved overexpressed in RCC according to the TCGA analysis of clinical specimens, higher LINC00460 expression level was correlated with poor OS and DFS and advanced clinicopathological staging in RCC patients, which is key finding of prognostic significance of LINC00460 for RCC patients. Our study is the first to clarify the biologic function of LINC00460 correlated with malignant progress of RCC cells. *In vitro* and *in vivo* assays revealed that LINC00460 down-regulation suppressed cell proliferation and tumor growth and reduced cell migration and invasion, whereas its overexpression promoted cell proliferation, migration, and invasion. Flow cytometry assays showed that overexpressed LINC00460 inhibited apoptosis in RCC cells. To unbiasedly explore the pathways that were related to the effects of LINC00460 in tumorigenesis of RCC, RNA-seq analysis was conducted, and PI3K/AKT pathway was found to be regulated by LINC00460. Then we confirmed this finding via Western blot assays. These findings indicate that LINC00460 has an oncogenic role in RCC tumorigenesis and could be a potential prognostic indicator for RCC patients.

Emerging literature has identified that the PI3K/AKT signaling pathway is crucial for normal cell growth, and its deregulation influences various cellular responses that are associated with cancer phenotypes, such as cell apoptosis and cell proliferation[35–40]. PI3K activation phosphorylates AKT and active AKT can lead to a number of downstream effects including the activation of mTOR, also in the form of phosphorylates mTOR, which in turn directly impacts cell growth and survival[41–43]. The main influence of the activation of AKT is the increasing survival in cell that normally undergoes death by apoptosis[44], and also involved in many other progressions, such as cell proliferation, angiogenesis, invasiveness and migration, modulating the initiation and progression of cancer[45, 46]. Therefore, in accordance with our RNA-seq data analysis, we found that the knockdown of LINC00460 downregulated the protein expression levels of PI3K, p-AKT, and p-mTOR, confirmed our hypothesis that LINC00460 might act as one upstream of PI3K/AKT pathway to control RCC progression. Furthermore, our findings displayed in Western blot assay indicating that LINC00460 could promote tumor migration and invasion via EMT.

In conclusion, our study illustrated that LINC00460 expression was upregulated in RCC tissues and cells, and its high level could be associated with poor prognosis in RCC patients, which made it a potential prognostic factor for RCC. LINC00460 influenced the proliferation, migration, invasion and apoptosis of RCC cells *in vitro* and restrains tumor growth and metastasis *in vivo*. Functionally, LINC00460 inactivating the PI3K/AKT signaling pathway. Our findings might provide novel insights into the mechanism of LINC00460 in RCC and the molecular targets for the treatment of RCC. Further clinical experiments are needed to illustrate and verify the role of LINC00460 in regulating RCC.

Abbreviations

RCC
Renal cell carcinoma; KIRC:Kidney renal clear cell carcinoma; KIRP:Kidney renal papillary cell carcinoma; KICH:Kidney Chromophobe; LincRNA:Long intergenic noncoding RNA; GEPIA:Gene Expression Profiling Interactive Analysis; TCGA:The Cancer Genome Atlas; qRT-PCR:Quantitative real-time polymerase chain reaction; RNA-seq:RNA sequencing; OS:Overall survival; DFS:Disease-free survival; LncRNAs:Long

noncoding RNAs; LINC00460:Long intergenic noncoding RNA 460; PI3K:The phosphatidylinositol 3-kinase; mTOR:Mammalian target of rapamycin; p-AKT:Phosphorylated AKT; p-mTOR:Phosphorylated mTOR; EMT:Epithelial-mesenchymal transition; PI:Propidium Iodide.

Declarations

Funding This work was supported by grants from the National Natural Science Foundation of China (No. 82072649, 81872304), the Outstanding Youth Foundation of Jiangsu Province (BK20200046), the Education Department of Jiangsu Province (No. 19KJA130001).

Conflict of interest The authors have declared that no competing interests exist.

Availability of data and material Publicly available datasets were analyzed in this study. This data can be found here: http://gepia.cancer-pku.cn/detail.php?gene=LINC00460.

Code availability Not applicable.

Author' contributions PF Hou provided study concept and design. J Bai provided study concept. ML Li and HM Yong collected the patients' samples and performed the experiments. SF Chu, S Meng and FJ Zhou collected, analyzed and interpreted the data. FJ Zhou and S Meng wrote the manuscript.

Ethics approval This study was performed under a protocol approved by the Institutional Review Boards of the Affiliated Hospital of Xuzhou Medical University. Animal experiments were performed in accordance with National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Care and Use Committee and Ethics Committee of Xuzhou Medical University.

Consent to participate Not applicable.

Consent for publication Not applicable.

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Figures

Fig. 1

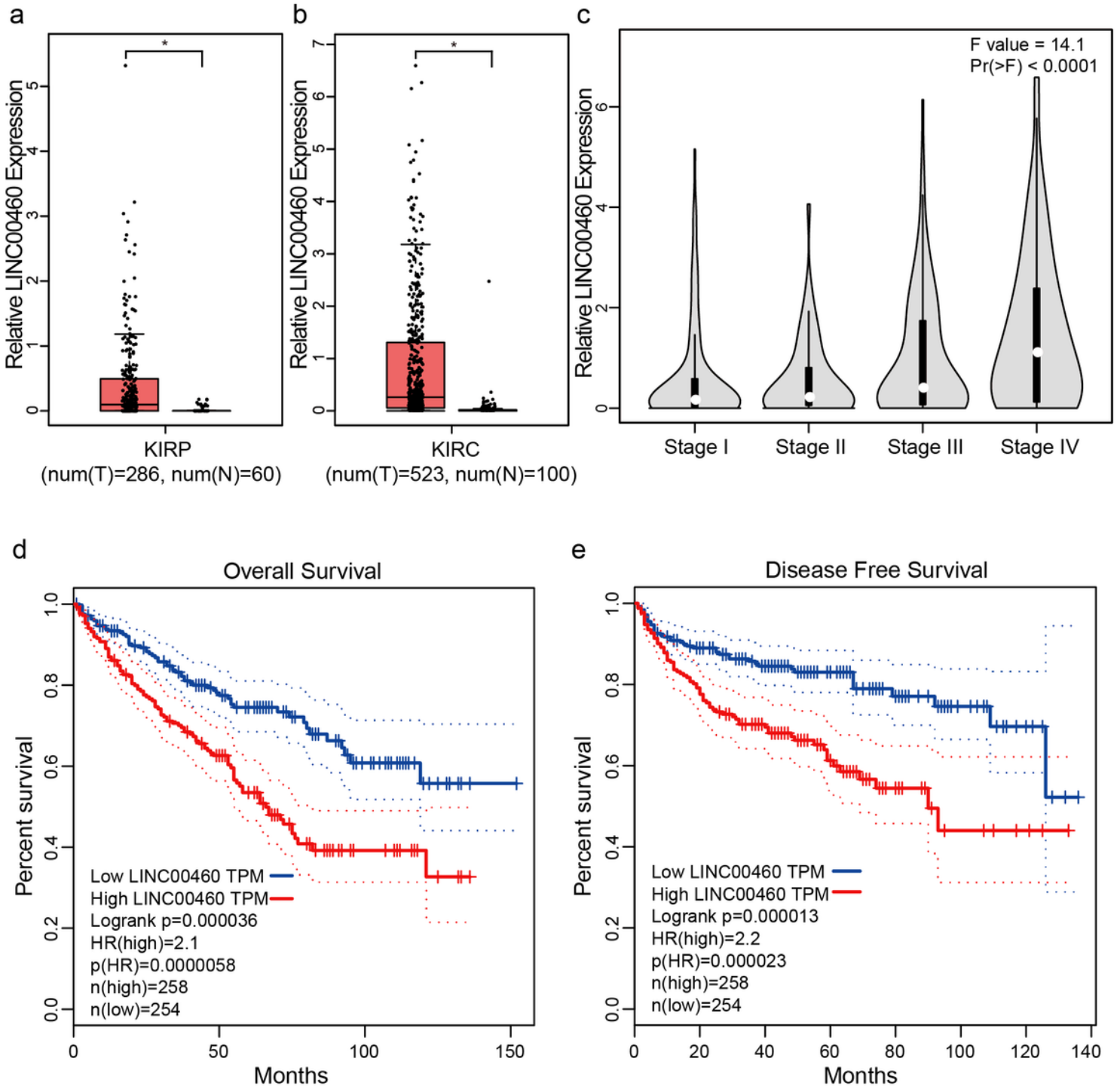

Figure 1

Expression of LINC00460 in RCC tissues based on the data from the GEPIA and TCGA. a, b Expression level of LINC00460 in KIRP tissues (n=286) and normal tissues (n=60), KIRC tissues (n=523) and normal tissues (n=100), analyzed in TGCA database (fold change>2.0, * p<0.05). c LINC00460 was gradually elevated with advanced staging in RCC. d, e OS and DFS rate of RCC patients with lowly or highly LINC00460 analyzed using the Kaplan-Meier analyses and log-rank test.

Fig. 2

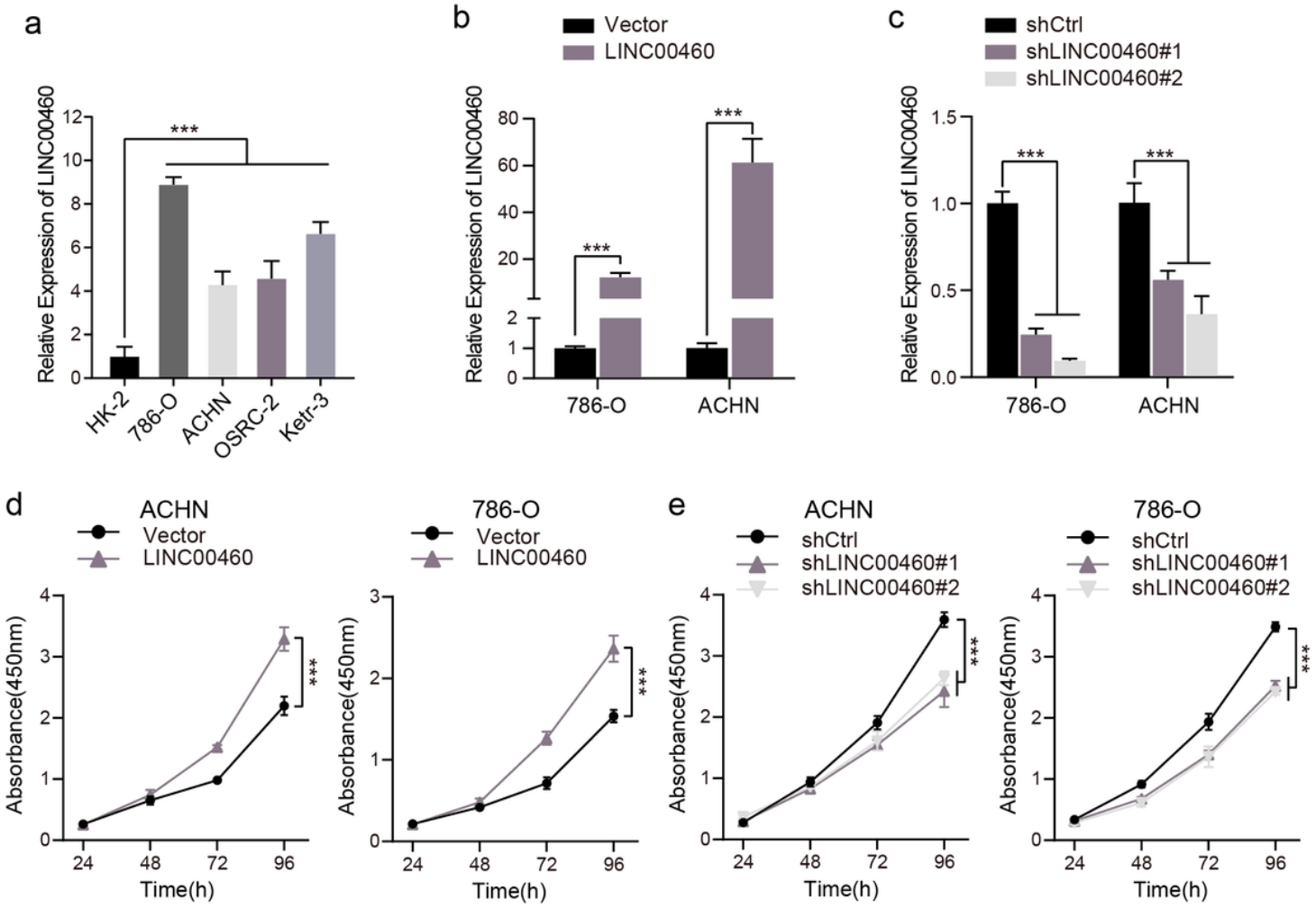

Figure 2

LINC00460 expression level in RCC cell lines and effects on cell proliferation. a Expression patterns of LINC00460 in RCC cell lines and normal renal cell lines detected by qRT-PCR (*** p<0.001). b, c Expression patterns of LINC00460 in RCC cells treated with over-expressed or silenced LINC00460 detected by qRT-PCR (*** p<0.001). d, e CCK-8 assay of ACHN and 786-O cells transfected with over-expressed or silenced LINC00460 (*** p<0.001).

fig. 3

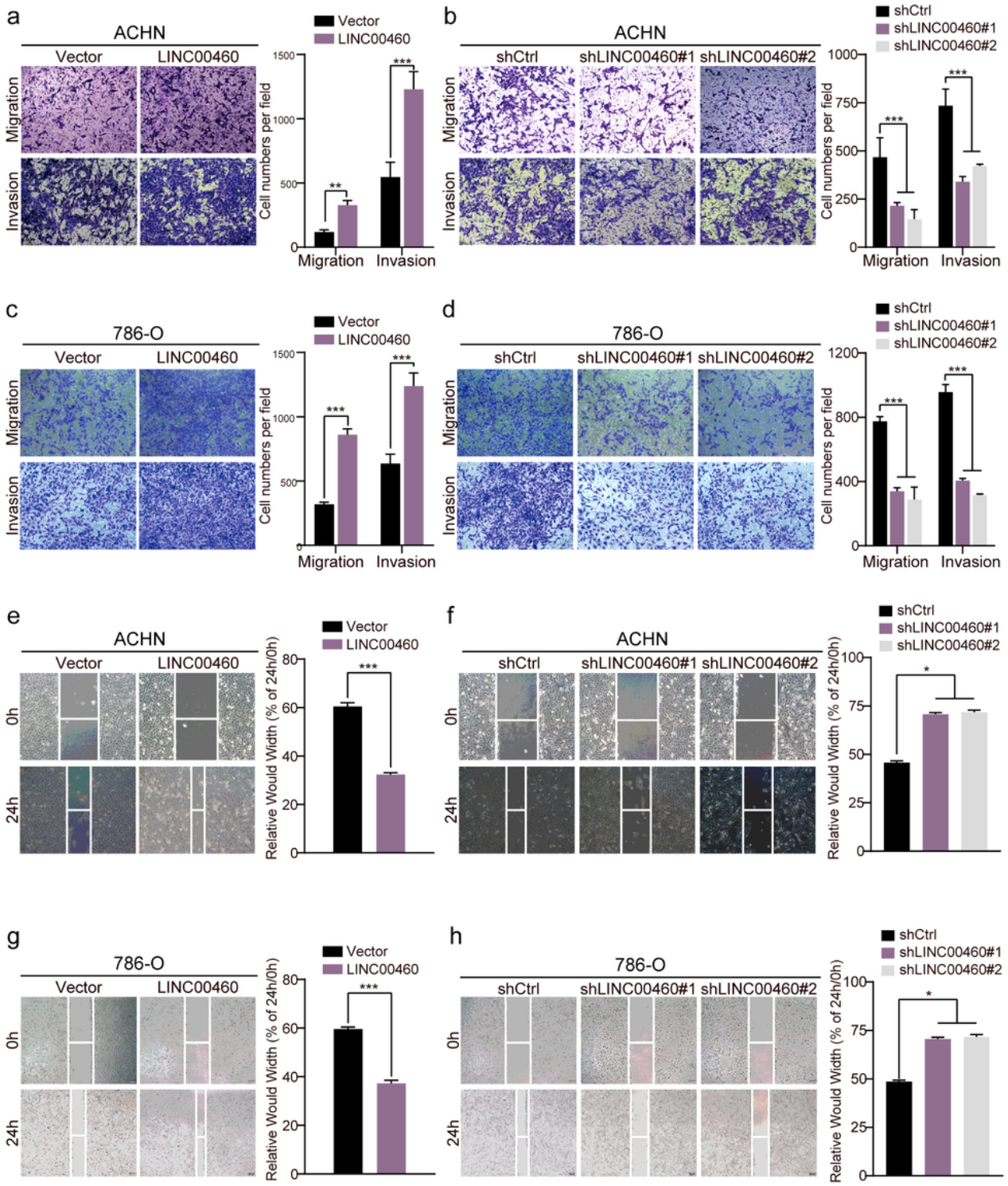

Figure 3

LINC00460 facilitated RCC cells migration, invasion in vitro. a, b, c, d The cell migration and invasion abilities of ACHN and 786-O cells transfected with overexpressed or silenced LINC00460 were determined by Transwell assays (*** p<0.001). e, f, g, h Wound healing assay was performed to examine the effect of LINC00460 overexpression or knockdown on ACHN and 786-O cells migration (* p<0.05, *** p<0.001).

Fig. 4

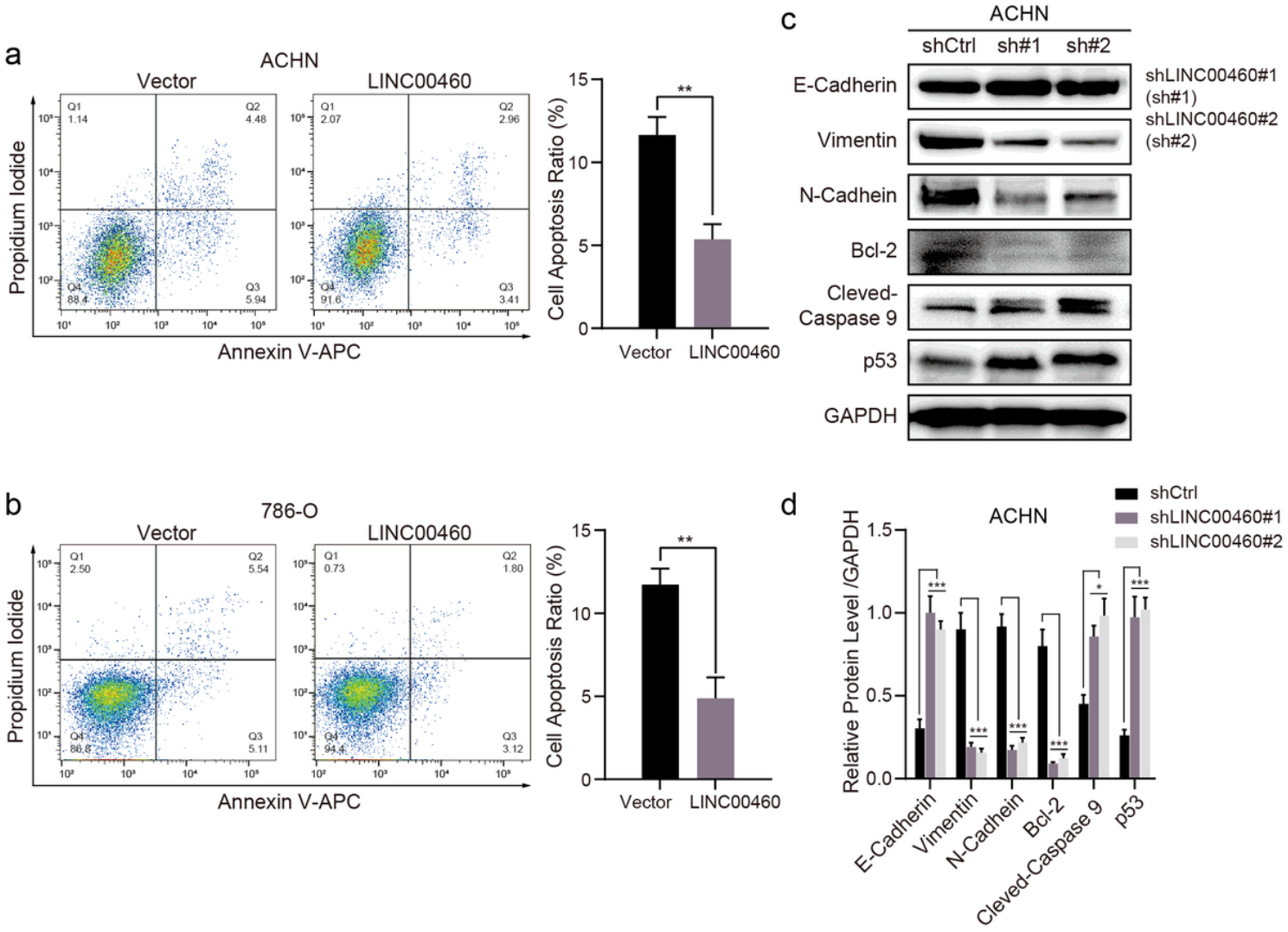

Figure 4

Influence of LINC00460 on RCC cells apoptosis and EMT phenotype. a, b Cell apoptosis was determined using flow cytometry analysis after LINC00460 overexpression in RCC cell lines (** $p<0.01$). c, d Cell apoptosis and EMT markers was detected by Western blot analysis when silenced LINC00460 was transfected in RCC cells. Data statistics was also shown (GAPDH as negative control, *$p < 0.05$, *** $p<0.001$).

Fig. 5

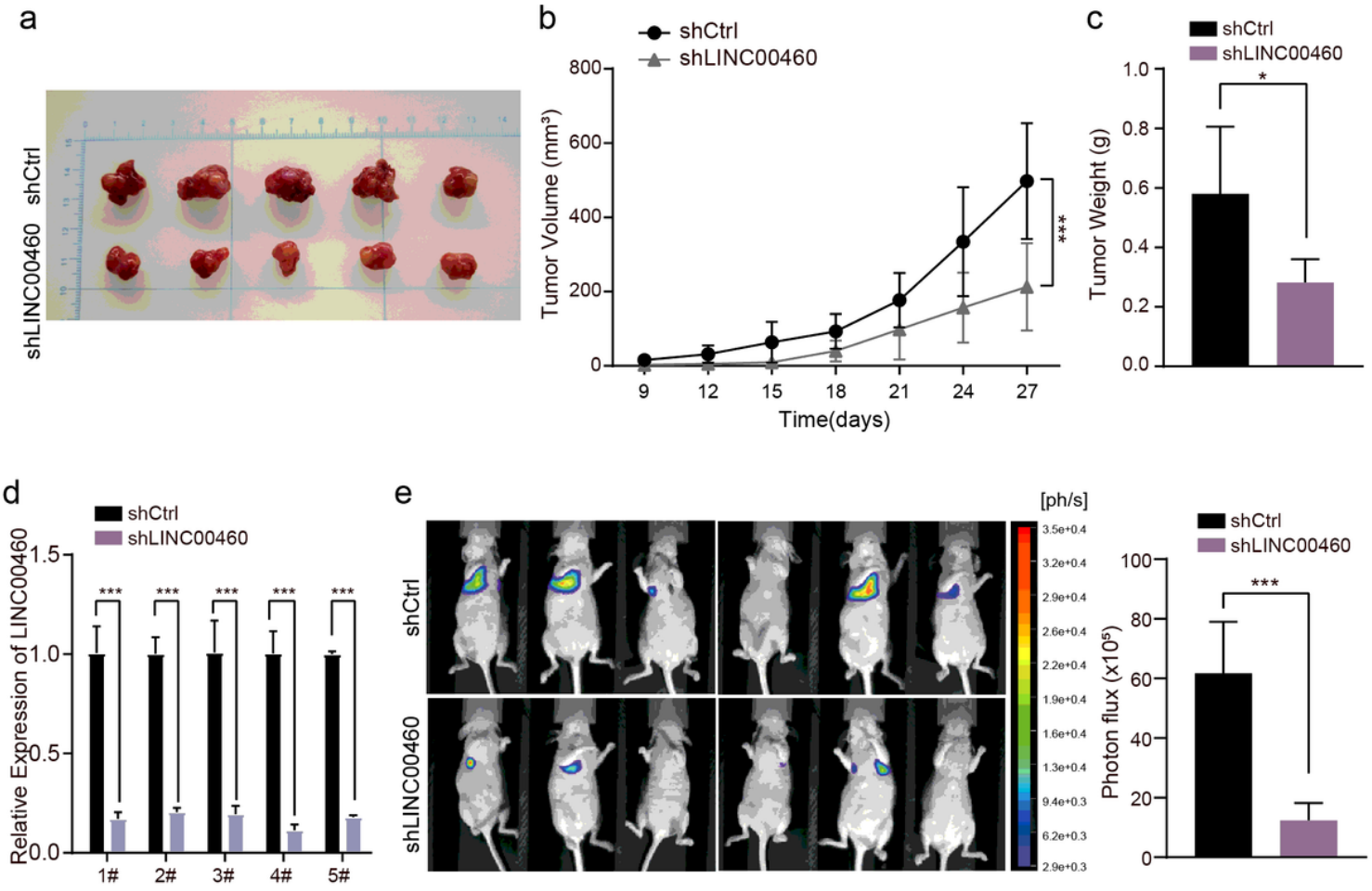

Figure 5

LINC00460 promoted RCC cells growth and metastasis in vivo. a 786-O cells were injected into nude mice after the transfection of shLINC00460 and shCtrl vector. b The growth curves of tumors from subcutaneously injected nude mice treated with shLINC00460 or shCtrl (*** $p<0.001$). c The weights of tumors from subcutaneously injected nude mice treated with shLINC00460 or shCtrl (* $p<0.05$). d The levels of LINC00460 expression in tumor tissues formed from subcutaneously injected nude mice treated with shLINC00460 or shCtrl, determined by qRT-PCR (*** $p<0.001$). e Representative bioluminescence images and statistical analysis of lung metastases in mice via tail vein injection of indicated cells (** $p < 0.01$, *** $p < 0.001$).

Fig. 6

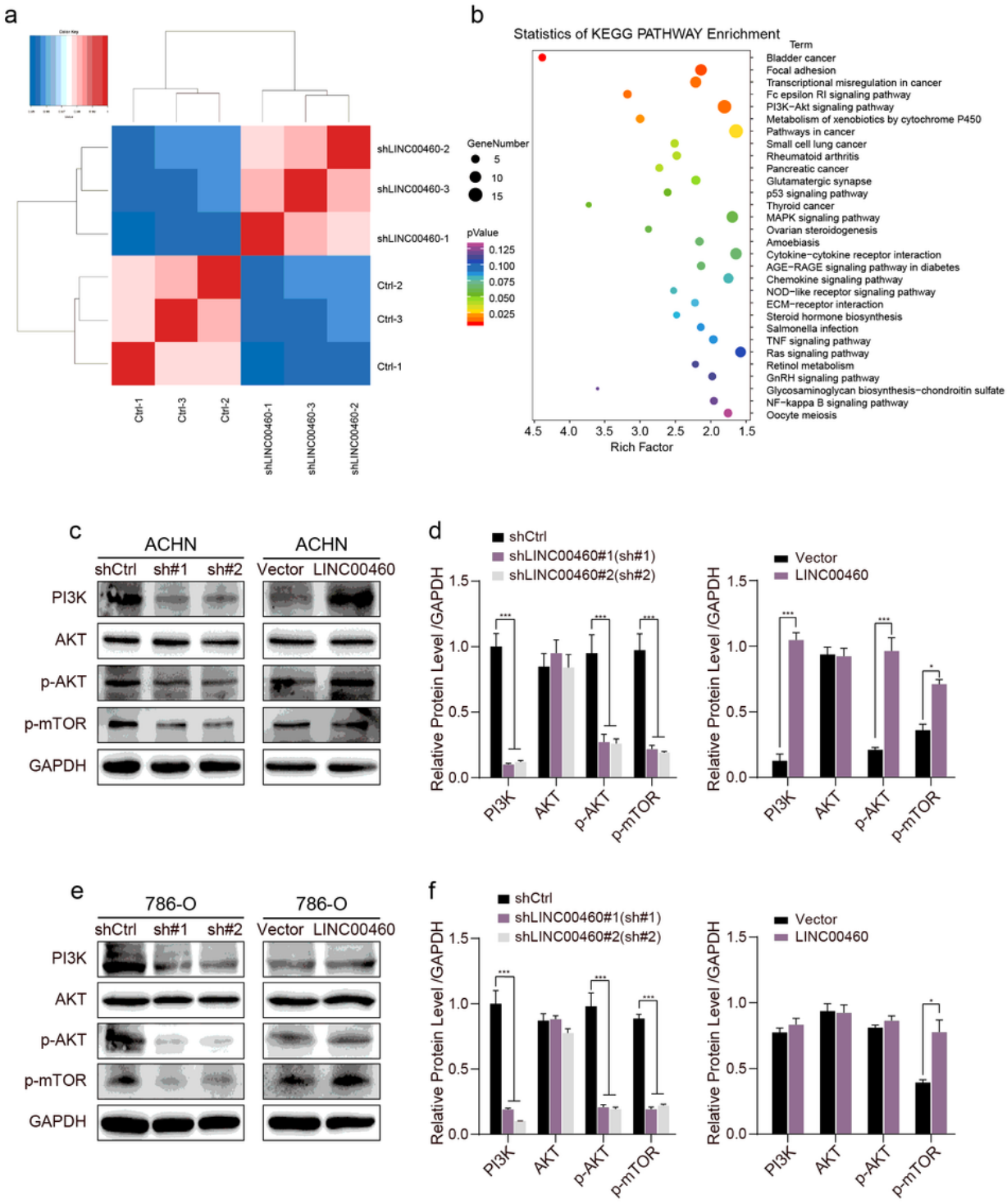

Figure 6

Effect of LINC00460 knockdown on activation of PI3K/AKT pathway in RCC cell lines. a Hierarchically clustered heatmap of the upregulated and downregulated genes in 786-O cells after shLINC00460 and shCtrl transfections. b Pathway classification of differentially expressed genes (DEGs). Bubble plots represented the number of DEGs, x axis represented rich factor, y axis represented the functional classification of KEGG. c, d, e, f The Western blot assay was used to detect PI3K, AKT, p-AKT, and p-mTOR

expression in cells transfection with shLINC00460 or overexpressed LINC00460 as relative to the control groups. (GAPDH as negative control, *p < 0.05, *** p<0.001).