

GRK5 promotes tumor progression in renal cell carcinoma

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Received June 21, 2018 / Accepted November 14, 2018

GRK5 is a multifunctional protein that is able to move within the cell in response to various stimuli to regulate key intracellular signaling from receptor activation on plasma membrane to gene transcription in the nucleus. Thus, GRK5 is involved in the development and progression of several pathological conditions including cancer. Here, we report an important tumor-promoting role for GRK5 in renal cell carcinoma (RCC). We investigated the expression pattern, clinical significance, and function of GRK5 in RCC. By using quantitative real-time polymerase chain reaction (qRT-PCR) and tissue microarray (TMA) immunohistochemistry (IHC), we first demonstrated that compared to the paired adjacent non-tumor (NT) tissues, RCC tissues presented with higher GRK5 expression. Moreover, we found that GRK5 upregulation was associated with poor clinical outcomes in RCC patients. *In vitro*, we found that GRK5 knockdown reduced viability, invasive ability, migratory ability, and decreased proportion of cells in S phase with concomitant increase in G1 phase in RCC cell lines, while GRK5 overexpression promoted tumor cell proliferation, cell invasion, migration and increased proportion of cells in S phase with concomitant decrease in G1 phase. Collectively, our findings describe a tumor-promoting role of GRK5 in RCC and thus provide molecular evidence for new therapeutic options in RCC.

Key words: GRK5, renal cell carcinoma, tumor progression, invasion, proliferation

Renal cell carcinoma (RCC) is the most prevalent adult kidney malignancy and its incidence has been increasing in recent decades [1]. It is associated with high rates of mortality and resistance to chemotherapy and radiotherapy [2–4]. Patients with early-stage disease can be treated with surgical resection, but approximately 20% of patients present with metastatic disease at the initial diagnosis [5]. Moreover, up to 20% of RCC patients suffer from metastatic lesions even if nephrectomy is performed [6]. Therefore, the identification of new sensitive, reliable biomarkers that predict RCC progression and prognosis and the development of new targeted therapies that improve RCC patient prognosis are necessary.

GRKs (G-protein-coupled-receptor kinases) are a family of serine/threonine kinases traditionally known for their ability to recognize and phosphorylate agonist-activated G-protein-coupled receptors (GPCRs), leading to their desensitization [7]. There have been seven subtypes of GRKs (GRK1–7) recognized up to date, while mammalian GRKs are classified into three subgroups based on sequence similarity: the rhodopsin kinases (GRK1 and GRK7), the β -ARK subgroup (GRK2 and GRK3), and the GRK4 subgroup (GRK4, GRK5,

and GRK6) [8, 9]. All mammalian GRKs possess a similar protein structure with a catalytic domain (~270 amino acids), an N-terminal domain (~185 amino acids), and a variable-length C-terminal domain (~105–230 amino acids). The N-terminal domain contains two conserved motifs for ligand binding and plasma membrane insertion [10, 11]. In the existing literature reports, GRKs are associated with thyroid carcinoma [12], hepatocellular carcinoma [13–15], pancreatic cancer [16], breast cancer [17, 18], Kaposi's sarcoma [19], gastric carcinoma, melanoma, prostate cancer, retinoblastoma, oral squamouscarcinoma, ovarian malignant granulosa cell tumor, osteosarcoma, hypopharyngeal squamous cell carcinoma, medulloblastoma and lung cancer [20–33]. However, the role of GRKs in RCC remains less explored. Therefore we want to study whether the GRKs play a role in the development and progression of RCC. By using qRT-PCR we examined the seven subtypes of GRKs in 10 RCC tissues and paired adjacent NT tissues. Except for GRK5, there was no significant difference in the expression of other subtypes, therefore we focused on GRK5 (Figure S1).

GRK5 can promote tumor development by interacting with some intracellular molecules and affecting their stability and

activity [34]. Studies have confirmed that GRK5 can promote tumorigenesis by inhibiting p53-mediated apoptosis both *in vitro*, in cultured human osteosarcoma cells, and *in vivo* [21, 35]. Studies have also shown that GRK5 can interact with moesin on the plasma membrane, catalyzing its phosphorylation at T66 residue, regulating the distribution of moesin in cells, and thereby promoting invasion and metastasis of PC3 cells [20]. Moesin, on the other hand, is part of ERM complex (Ezrin-Radixin-Moesin) that links membrane components to actin cytoskeleton, regulating cytoskeleton remodeling and cell adhesions [36], while altered expression or intracellular distribution of ERMs has been linked to tumor metastasis. Moreover, in a xenograft model of human prostate cancer, knocking down GRK5 could reduce tumor growth, invasion and metastasis [20]. Taken together, these results propose GRK5 as a key contributor to the growth and metastasis of prostate cancer.

In our study, we demonstrated that compared to the paired adjacent non-tumor (NT) tissues, RCC tissues present with higher GRK5 expression. Moreover, we found that GRK5 upregulation was associated with poor clinical outcomes in RCC patients. *In vitro*, we found that GRK5 knockdown reduced viability, invasive ability, migratory ability, and led to decreased proportion of cells in S phase with concomitant increase in G1 phase in RCC cell lines, while GRK5 overexpression promoted tumor cell proliferation, cell invasion, migration and led to increased proportion of cells in S phase with concomitant decrease in G1 phase. Overall, our findings describe a tumor-promoting role of GRK5 in RCC and thus provide molecular evidence for new therapeutic options in RCC.

Patients and methods

Patients and clinical samples. All cases included in the study were clinically and pathologically identified as RCC. This study included 40 RCC tissues and paired adjacent NT tissues obtained from patients who underwent surgery at Changzheng Hospital, Second Military Medical University (Shanghai, China). No patients received anticancer treatments prior to the surgery. The median follow-up time of these 40 RCC patients was 50 months. Written informed consent was obtained from all patients. The Ethics Committee of Changzheng Hospital, Second Military Medical University, approved the use of these tissues in the study.

Cell lines and culture conditions. Human RCC cell lines (A498, ACHN, CAKI-1, CAKI-2, OS-RC-2, 769P and 786-O) and the normal kidney cell line HK-2 were obtained from the Shanghai Institute of Life Sciences Cell Resource Center (Shanghai, China). OS-RC-2, 769P and 786-O cell lines were cultured in RPMI modified medium (HyClone, USA), and A498 and ACHN cell lines were cultured in minimum essential medium (Eagle) (Corning). The CAKI-1 and CAKI-2 cell line were cultured in McCoy's 5A medium (Gibco, USA), and the HK-2 cell line was cultured in DMEM (HyClone,

USA). All media were supplemented with 10 % fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, USA), according to the American Type Culture Collection. All cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO₂. According to the expression of GRK5 in the cell line of renal cell carcinoma, OS-RC-2 cells were selected to generate GRK5-knockdown cells and ACHN cells to generate stable GRK5-overexpression cells.

Tissue microarray (TMA) construction and immunohistochemical (IHC) detection. IHC was performed with the TMA using a two-step immunoperoxidase technique. After heating the sections in 10 mmol/l citrate buffer for antigen retrieval, the sections were incubated with primary antibody against GRK5 (ab64943, Abcam, CA, USA; dilution 1:60) at 4 °C overnight and then with appropriate secondary antibody for 1 h at room temperature.

RNA extraction, cDNA preparation and qRT-PCR. Total RNA was extracted from cells and tissues using TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. Total RNA quality was assessed using a Nanodrop 2000 and agarose gel electrophoresis. First-strand cDNA was generated from 2 µg of total RNA using M-MLV reverse transcriptase (Invitrogen, CA) with random primers. qRT-PCR was performed according to the SYBR Green protocol in a Step One Plus System (Applied Biosystems, Foster City, CA, USA), and β-actin served as the endogenous control. Primer sequences were as follows: GRK5 5'-CGTCAATCTGGACCACACAG-3' (forward) and 5'-CCAAACACGTTTCAGCTCCTT-3' (reverse); and β-actin 5'-CTGGTGCCTGGGGCG-3' (forward) and 5'-AGCCTCGCCTTTGCCGA-3' (reverse). Other primer sequences used in qRT-PCR are listed in supplementary Table S1. Relative mRNA expression levels were calculated based on the corresponding relative quantity (RQ) values and were normalized to β-actin expression.

Western blot. Total cell and tissue lysates were prepared in 1× sodium dodecyl sulfate buffer. Identical quantities of protein were separated by SDS gel electrophoresis and transferred onto nitrocellulose filter membranes. After incubating with antibodies specific for GRK5 (ab64943, Abcam, CA, USA) and GAPDH (sc-25778; Santa Cruz, CA, USA), the blots were incubated with IRDye 800-conjugated goat anti-rabbit IgG, and bands were detected using an Odyssey infrared scanner (Li-Cor). GAPDH was used as the loading control.

siRNA transfection. GRK5 siRNAs was synthesized by GenePharma (Shanghai, China) with a sequence of si-GRK5-1: 5'-CCUGAAGUCCCCUCACAUUTT-3' (forward) and 5'-AAUGUGAGGGAACUUCAGGTT-3' (reverse) and si-GRK5-2: 5'-CCCUCAAUGAGAAGCAGAUTT-3' (forward) and 5'-AUCUGCUUCUCAUUGAGGGTT-3' (reverse). A non-silencing siRNA oligonucleotide that does not recognize any known mammalian gene homolog (GenePharma, Shanghai, China) was used as a negative control. We selected OS-RC-2 cells to generate GRK5-knockdown cells, which

were named si-GRK5 cells; control cells were named si-NC cells. OS-RC-2 cells were transfected with GRK5 siRNAs (50 nmol/l) or control siRNA (50 nmol/l) via Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Lentiviral vectors and infection. The lentivirus encoding GRK5 plasmids was packaged and purified at HanBio Biotechnology (Shanghai, China) and cells were infected following the manufacturer's instructions. We selected ACHN cells to generate stable GRK5-overexpression cells, which we named ACHN-GRK5 cells; control cells were named ACHN-Ctrl cells.

Flow cytometry analysis. Cell cycle progression was quantified by flow cytometry analysis (BD Biosciences, San Jose, CA). For cell cycle progression, RCC cells were collected, washed with PBS and fixed with 75% ethanol. Cells were stained with PI and RNase overnight at 4°C. Samples were analyzed by flow cytometry.

Plate colony formation assay. RCC cells (500 cells) were seeded into 6-wells plates and cultured in the 37°C incubator for 10 days until most single colonies were composed of more than 50 cells. The plates were washed by PBS, fixed with 4% paraformaldehyde, and stained with crystal violet. The number of colonies containing more than 50 cells was counted in each well.

Wound-healing migration assay. RCC cells were seeded at 5×10^5 cells/well in 6-well plates and cultured until the plates were confluent. The cell monolayer was scraped in a straight line using a 10- μ l pipette tip and was washed with PBS twice and the medium was replaced with serum-free medium. To evaluate cell migration, images were captured at 0 and 48 h following the initial scratch.

Transwell assays. Polycarbonate membranes with a pore size of 8 μ m and 24-well culture insert plates (Millipore, USA) were used for transwell assays. First, the insert plates were equilibrated with 0.5 ml of serum-free culture medium for 1 h at 37°C in 5% CO₂. Then, the medium in the lower chambers was replaced with 0.5 ml of culture medium supplemented with 10% FBS. Serum pre-starved RCC cells (5×10^4) in 400 μ l of serum-free medium were seeded into the upper chambers. After a 48-h incubation period, the inserts were rinsed with PBS and cells on the upper surface of the membrane were scraped off. Cells on the bottom side of the membrane were stained with crystal violet stain and counted by a microscope. Cells were counted from 8 randomly chosen fields (200 \times magnification).

Cell counting kit 8 (CCK8) assay. RCC cells were cultured for 12, 24, 36, 48 and 60 h. Wells with only culture medium added served as blanks. At different time points, the supernatant was removed, and 100 μ l of culture medium containing 10 μ l of CCK8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well for another 2 h incubation at 37°C. Absorbance was recorded at 450 nm using a microplate reader (Varioskan Flash; Thermo Scientific, Waltham, MA, USA). Viability (%) was calculated

based on optical density (OD) values as follows: (OD of time sample – blank)/(OD of control sample – blank) \times 100. All experiments were independently repeated in triplicate on separate occasions.

Statistical analysis. Data are expressed as means \pm s.d. of three independent experiments. All statistical analyses were performed using SPSS version 17.0 software (Abbott Laboratories, Chicago, IL, USA). For comparisons, Student's t-test (two-tailed), Fisher's exact test, Pearson correlation analysis, Log-rank test and non-parametric Mann-Whitney U-test were performed as appropriate. The data met the assumptions of the tests and the variance was similar between the groups being compared. A p-value <0.05 was considered significant.

Results

GRK5 was highly expressed in RCC. We examined GRK5 mRNA and protein expression levels in 40 RCC tissues and paired adjacent NT tissues. GRK5 expression was significantly upregulated in RCC tissues compared to paired adjacent NT tissues (Figures 1A and B). Using IHC analysis of RCC tissues and paired adjacent NT tissues, we confirmed GRK5 upregulation at the protein level in RCC patients (Figure 1C). To explore the biological functions of GRK5 in RCC *in vitro*, we detected GRK5 expression levels

Table 1. Correlation between GRK5 protein level and clinical characteristic in RCC patients.

Variables	Total (n=85)	GRK5		p-value
		Low expression (n=44)	High expression (n=41)	
Gender				0.0914
Male	60	31	29	
Female	25	13	12	
Age				0.0637
<60y	58	34	24	
\geq 60y	27	10	17	
Tumor size				0.0067
\leq 4 cm	52	33	19	
>6 cm	33	11	22	
Fuhrman Grade				0.0027
I-II	57	36	21	
III-IV	28	8	20	
Tumor thrombus				0.3487
No	79	42	37	
Yes	6	2	4	
Metastasis				0.0169
No	74	42	32	
Yes	11	2	9	
Pathology				0.5790
ccRCC	75	38	37	
non-ccRCC	10	6	4	

in several RCC cell lines, the HK-2 cell line was used as control (Figure 1D).

GRK5 upregulation served as a prognostic factor for patients with RCC. To determine the prognostic value of GRK5 in RCC, we measured GRK5 levels in 85 RCC samples from patients after surgery. According to the median expres-

sion of GRK5 in immunohistochemistry, the 85 samples were divided into high GRK5 expression group (n=41) and low GRK5 expression group (n=44). High GRK5 expression was associated with larger tumor size (p=0.0067), advanced pathological stage (p=0.0027) and distant metastasis (p=0.0169) (Table 1). Then, we generated Kaplan-Meier survival curves

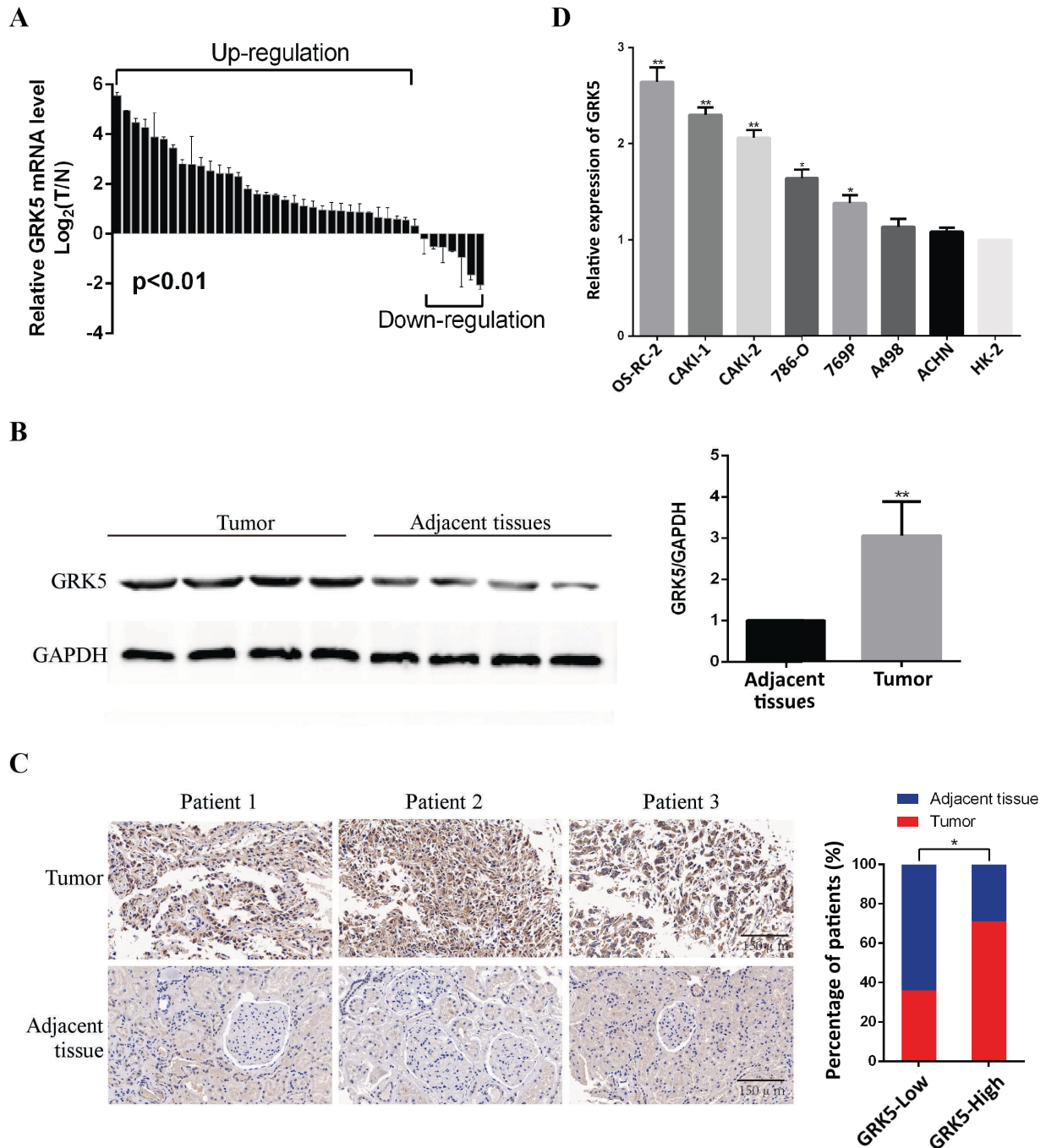


Figure 1. GRK5 is upregulated in RCC. A) qRT-PCR analysis of GRK5 in RCC tissues and adjacent non-tumor tissues from 40 ccRCC patients. B) Western blot analysis of GRK5 in RCC tissues and paired adjacent non-tumor tissues. GAPDH was used as an internal standard. C) Immunohistochemical analysis of GRK5 in RCC tissues and adjacent non-tumor tissues. D) qRT-PCR analysis of GRK5 in RCC cell lines. * $p < 0.05$ and ** $p < 0.01$

and performed log-rank tests in this cohorts and we found that progression free survival (PFS) and overall survival (OS) were significantly shorter in patients with increased GRK5 protein levels than in those with reduced GRK5 protein levels (Figures 2A and B). Collectively, our findings indicate that GRK5 can be used as a prognosis predictor in RCC patients.

GRK5 knockdown reduced tumor cell proliferation, invasion, migration and led to decreased proportion of cells in S phase with concomitant increase in G1 phase *in vitro*. GRK5 knockdown efficiency was confirmed by qRT-PCR (Figure 3A) and western blotting (Figure 3B). CCK-8 assays revealed that si-GRK5 cells presented with a significant reduction in cell proliferation compared with si-NC cells (Figure 3C). Plate colony formation assay indicated that the cloning ability of si-GRK5 cells was reduced (Figure 3D). In wound-healing migration assays, microscopic examination at 0 and 48 h revealed that compared with si-NC cell migration, si-GRK5 cell migration was significantly reduced (Figure 3E). Transwell assays revealed that compared to si-NC cells, si-GRK5 cells displayed decreased invasion (Figure 3F). Afterwards, we evaluated the cell cycle distribution by flow cytometry, which indicated that knockdown of GRK5 decreased the proportion of cells in S phase with concomitant increase in G1 phase (Figure 3G).

GRK5 overexpression led to increased viability, invasive ability, migratory ability and increased proportion of cells in S phase with concomitant decrease in G1 phase *in vitro*. GRK5 overexpression efficiency was confirmed by qRT-PCR (Figure 4A) and western blotting (Figure 4B). CCK-8 assays revealed that ACHN-GRK5 cells exhibited increased viability (Figure 4C). Plate colony formation assay indicated that the cloning ability of ACHN-GRK5 cells increased (Figure 4D). We also found that GRK5 overexpression increased ACHN-GRK5 cell migration (Figure 4E) and invasion (Figure 4F). The flow cytometry indicated that overexpression of GRK5 increased proportion of cells in S phase with concomitant decrease in G1 phase (Figure 4G).

Discussion

Partial nephrectomy is the recommended standard treatment for localized RCC [37]; however, cancer metastasis is a serious problem in clinical treatment, warranting a significant change in therapeutic strategies and predicting poor outcomes in RCC [3, 38]. Once a tumor has metastasized, the mortality burden faced by RCC patients is significant [39]. Thus, the ability to determine which patients at high risk for developing metastasis may benefit from radical nephrectomy and adjuvant treatment is urgently needed. Targeted therapy is the main treatment for metastatic RCC patients. Unfortunately, because of acquired resistance and other drawbacks, both therapies for metastatic RCC have limited efficacy and remain unsatisfactory in respect to patient outcomes [40–42]. Therefore, gaining a better understanding of the molecular mechanisms that underlie RCC metastasis may

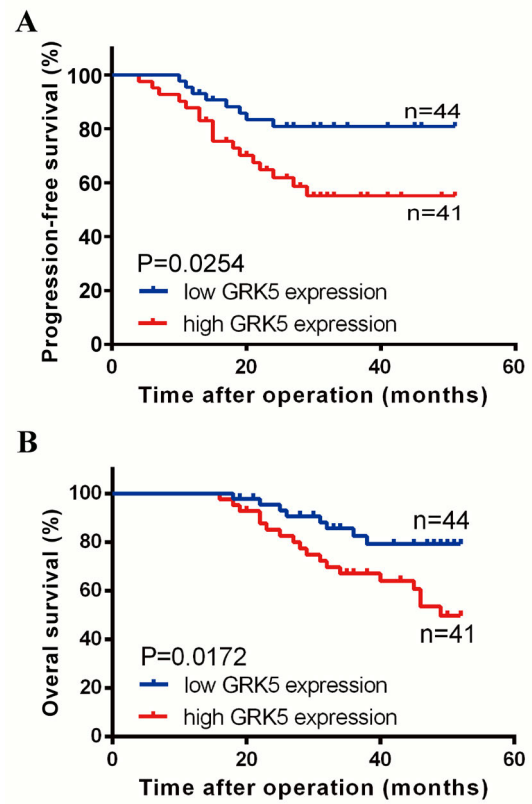


Figure 2. GRK5 upregulation serves as a prognostic factor in patients with RCC. A) Progression free survival and B) overall survival was significantly shorter in patients with increased GRK5 protein levels than in those with reduced GRK5 protein levels.

enable researchers to identify reliable biomarkers to clinically diagnose affected patients, predict the prognosis of these patients and target therapies to treat these patients.

The significant correlation between GRK5 expression levels in tumors and poor RCC patient prognosis prompted us to investigate whether GRK5 plays a promoting role in RCC invasion and metastasis. We subsequently found that silencing GRK5 using siRNA decreased RCC cell invasion and metastatic abilities, whereas GRK5 overexpression significantly increased RCC invasion and metastatic abilities *in vitro*. These results also suggest that GRK5 may be a potential therapeutic target in RCC metastasis.

Next, we will explore the molecular mechanisms affected by GRK5, potentially those that promote RCC progression and metastasis. Among the regulators of cell cycle progression in cancer, GRK5 could represent a candidate molecule, given its nuclear localization and the identification of new nuclear substrates of this kinase [34]. In HeLa cells, breast cancer cells and non-transformed cells, GRK5 localizes to the centrosome during the interphase and promotes G2/M transition, which affects cell cycle progression [43]. It has also been reported that GRK5 can interact with and phosphorylate

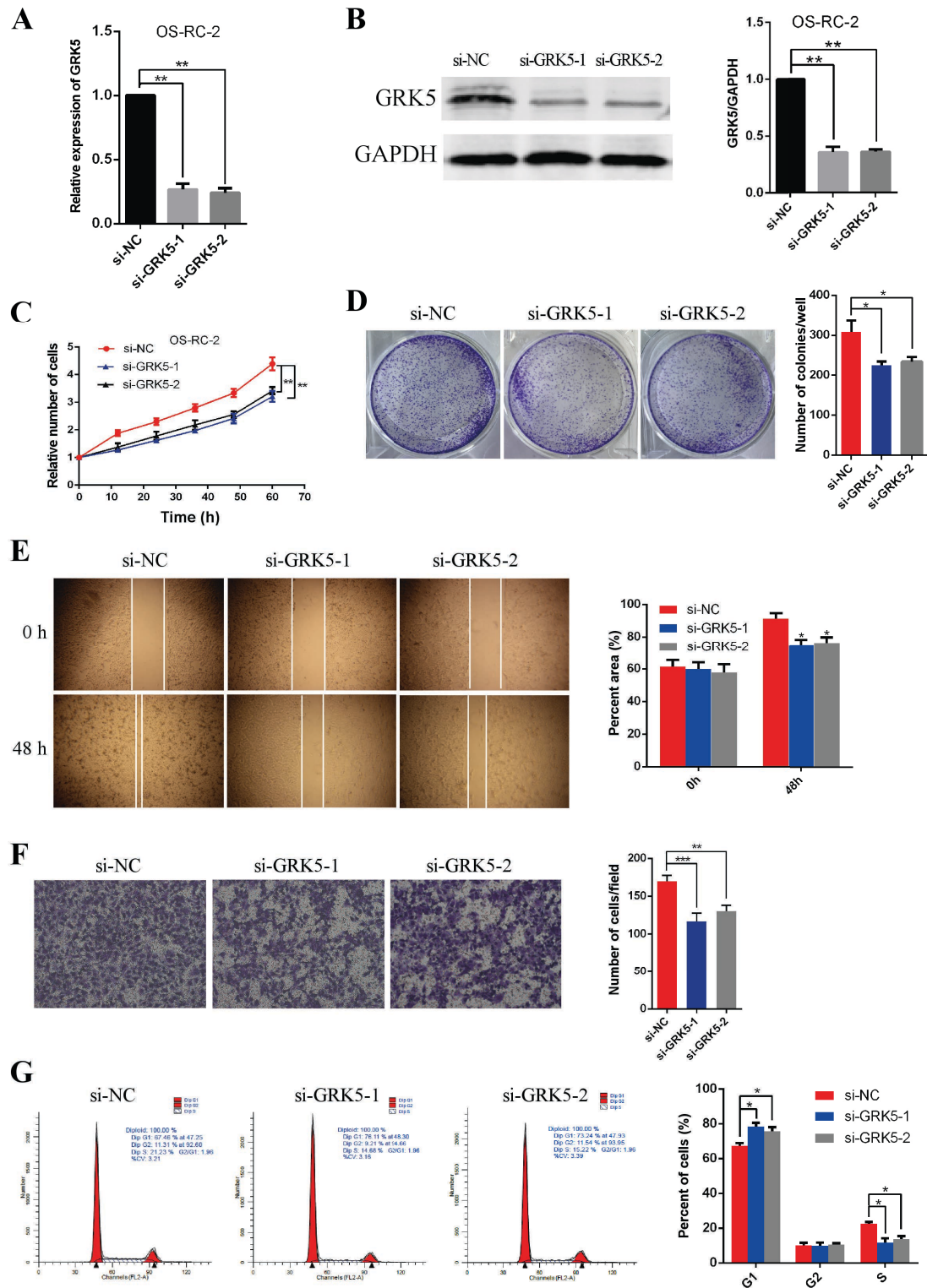


Figure 3. GRK5 knockdown in OS-RC-2 inhibits viability, invasive ability, migratory ability and decreases the proportion of cells in S phase with concomitant increase in G1 phase *in vitro*. A) qRT-PCR analysis and B) western blotting were performed to test the siRNA efficiency. C) CCK8 assays of si-GRK5 and si-NC cells at the indicated times. D) Plate colony formation assay of si-GRK5 and si-NC cells. E) Representative images of the wound-healing assay with si-GRK5 and si-NC cells photographed at 0 h and 48 h after the scratch. F) Transwell assays were performed to evaluate cell invasive ability in si-GRK5 and si-NC cells. G) Flow cytometric analysis of cell cycle in si-GRK5 and si-NC cells. * $p < 0.05$ and ** $p < 0.01$

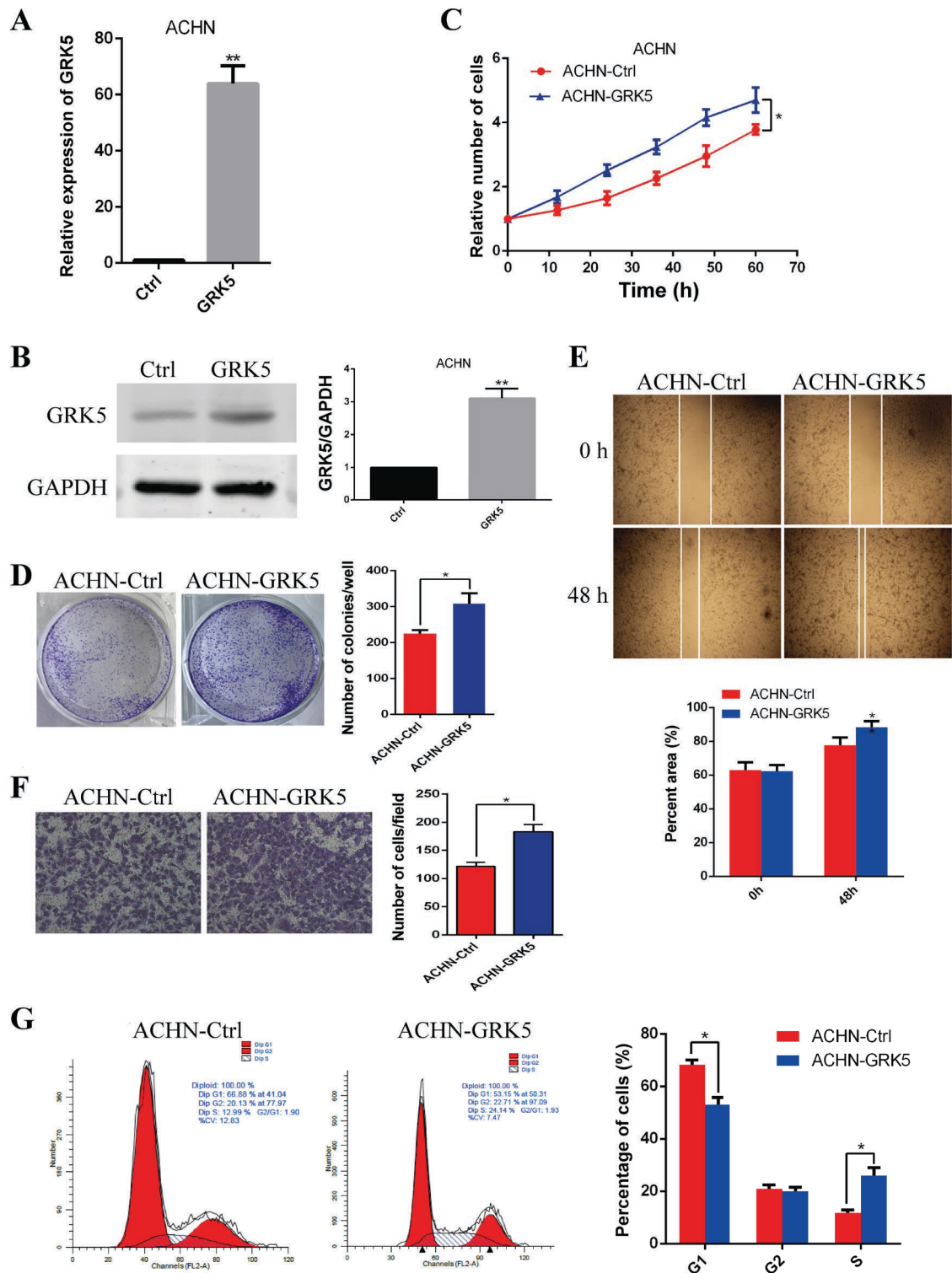


Figure 4. GRK5 overexpression in ACHN cells promotes tumor growth, cell invasion and migration and increased proportion of cells in S phase with concomitant decrease in G1 phase. A) qRT-PCR analysis and B) western blotting were performed to test GRK5 overexpression efficiency. C) CCK8 assays of ACHN-GRK5 and ACHN-Ctrl cells at the indicated times. D) Plate colony formation assay of ACHN -GRK5 and ACHN-Ctrl cells. E) Representative images of wound-healing assays with ACHN-GRK5 and ACHN-Ctrl cells photographed at 0 and 48 h after the scratch. F) Transwell assays were performed to evaluate cell invasive ability in ACHN-GRK5 and ACHN-Ctrl cells. G) Flow cytometry analysis of cell cycle in ACHN-GRK5 and ACHN-Ctrl cells. * $p < 0.05$ and ** $p < 0.01$

nucleophosmin (NPM1), which is a multifunctional protein that is closely related to cell cycle, centrosomal duplication and apoptosis, and NPM1 is highly expressed in several tumors [44]. NPM1 function is regulated primarily through phosphorylation by PLK1 that leads to the protection from cell death [45]; Indeed, the inhibitor of PLK1, which induces apoptosis, is used in the treatment of several cancers including esophageal cancer [46], neuroblastomas [47], and others [48]. GRK5 phosphorylates NPM1 at Ser-4, a site shared with PLK1, suggesting the possibility of an interplay between GRK5 and PLK1 in the regulation of NPM1. In particular, GRK5-depleted cells were more sensitive to apoptosis induced by PLK1 inhibition, while cells with high GRK5 levels exhibited reduced sensitivity to PLK1 inhibition [44].

In summary, GRK5 plays an important role in promoting cell proliferation and metastasis in RCC and may serve as an independent predictor of RCC patient clinical outcomes. Based on these findings, targeting GRK5 may represent a potential therapeutic strategy by which to curb RCC progression.

Supplementary information is available in the online version of the paper.

Acknowledgments: The present study was supported by the National Nature Science Foundation of China (no. 81572521).

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