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SGK2 promotes renal cancer progression *via* enhancing ERK 1/2 and AKT phosphorylation

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Abstract. – OBJECTIVE: Increasing studies reported that the serum- and glucocorticoid-inducible kinases (SGKs) contributed to the tumorigenesis of various cancer. In this article, we are aiming to explore the function of SGK2 in renal cell cancer (RCC).

PATIENTS AND METHODS: In this study, the SGK2 expression was quantified by Western blot (WB) in multiple RCC cell lines. And *in vitro* SGK2 knockdown and overexpression experiments were also performed. In addition, molecular function analysis was performed using FunRich software V3. The Cancer Genome Atlas (TCGA) database was retrieved to verify the association between the SGK2 expression and the prognosis of RCC patients.

RESULTS: We found that SGK2 was up-regulated in RCC tissues compared with adjacent normal tissues, and the SGK2 expression also increased in various RCC cell lines compared to that in the normal epithelial cell line HK-2. Meanwhile, the SGK2 expression was significantly associated with the survival rate of RCC patients. Functional experiments showed that silencing SGK2 expression inhibited RCC cells proliferation, migration, colony formation and invasion abilities *in vitro*, whereas opposite results were uncovered after overexpressing SGK2 in RCC cells. Furthermore, functional analyses showed that SGK2 related genes were associated with protein serine/threonine kinase activity, guanosine triphosphatase (GTPase) activity, guanyl-nucleotide exchange factor activity, and motor activity. Protein interaction analysis identified that growth factor receptor-bound protein 2 (GRB2), one of the most important upstream components in the growth factor signaling pathway, was significantly enriched in SGK2 related genes. In addition, the WB assay validated that SGK2 could promote the phosphorylation of ERK 1/2 and AKT.

CONCLUSIONS: Our results suggested that SGK2 promoted RCC progression by mediating the phosphorylation of extracellular regulated protein kinases (ERK) 1/2 and Protein kinase B (AKT/PKB), indicating that SGK2 might serve as a potential prognostic marker and therapeutic target for renal cancer patients.

Key Words
Serum- and glucocorticoid-inducible kinases (SGKs), Renal cell cancer (RCC), Extracellular regulated protein kinases (ERK), Protein kinase B (AKT/PKB).

Abbreviations

Serum- and glucocorticoid-inducible kinase (SGK), Renal cell cancer (RCC), Western blot (WB), Guanosine triphosphatase (GTPase), Growth factor receptor-bound protein 2 (GRB2), Extracellular regulated protein kinases (ERK), Protein kinase B (AKT/PKB), Copy number variation (CNV), Dulbecco's modification of Eagle medium (DMEM), Fetal bovine serum (FBS), Sodium dodecyl sulfate-polyacrylamide (SDS), Polyvinylidene difluoride (PVDF), Bovine Serum Albumin (BSA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Serine (Ser), Threonine (Thr), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), Mitogen-activated protein kinase (MAPK), Phosphoinositide 3-kinase (PI3K), The Cancer Genome Atlas (TCGA).

Introduction

Renal cell carcinoma (RCC) is one of the most frequent and malignant neoplasms which accounts for 5% in men and 3% in woman of all oncological diagnoses[1]. Although most detected tumors are small in size, metastatic RCC patients continue to

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be diagnosed at a notable proportion, with up to 17% at the time of diagnosis[2]. Furthermore, for these patients with distant metastasis, the 5-year survival rate is less than 10%[3,4]. Currently, surgery or targeted therapies such as immunological therapy[5-7] are preferred as the best treatment option for these patients, while many of them suffered recurrence eventually. Therefore, it is of great significance to identify specific molecular biomarkers for early diagnosis and therapeutics in RCC patients.

The serum- and glucocorticoid-inducible kinases (SGKs) participate in regulating diverse cellular processes, including osmoregulation, cell survival, sodium homeostasis, cell proliferation, and invasion[8-13]. The SGK family comprises three members, SGK1, SGK2, and SGK3. A previous work[14] suggests that the role of SGK2 within the human kidney is different from that of SGK1 and SGK3. SGK1 and SGK3 are commonly expressed in a wide variety of tissues, while SGK2 is more likely expressed in liver, kidney, and brain[15]. Furthermore, aldosterone has been shown to regulate SGK1 and SGK3, rather than SGK2 in the kidney. Although the biological roles of the SGK family are not well characterized, studies have indicated that the SGK family shares approximately 50% amino acid sequence homology with many kinase domains that can promote the phosphorylation of AKT, a key kinase involved in the regulation of the progression of diverse tumors progression[16].

To date, few studies have been conducted concerning the role of SGK2 and tumor progression, and no study was identified investigating the role of SGK2 in renal cancer. We first extracted mRNA data from the TCGA database and found that SGK2 was elevated in renal cancer tissues compared with para-tumor tissues. The SGK2 protein level was also increased in several renal cancer cell lines compared with that in normal epithelial HK-2 cells. In addition, a copy number variation (CNV) of SGK2 referenced in the Oncomine database suggested that a high level of SGK2 CNV was related to a late tumor stage and a high rate of tumor recurrence. Overall, based on previously published work and our preliminary data, we hypothesized that SGK2 might promote renal cancer progression.

Materials and Methods

Cell Culture

ACHN, A498, 786-0, OSRC-2 and Caki-1, as well as an immortalized proximal tubule epithelial cell line, HK-2 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and were cultured in DMEM medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, South-Logan, UT, USA), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) in an incubator at 37°C with 5% CO_2.

Lentivirus Packaging and Infection

The shVector, shSGK2, oeVector, oeSGK2, psAX2 packaging plasmid, and pMD2G envelope plasmid, were mixed and then transfected into 293T cells using the protocol of calcium chloride mediated transfection. The virus was collected after 48 h of culture, then immediately frozen at -80°C for future use. Cells were seeded in a 6- well plate, and the virus supernatant was directly added to the cells in the 6- well plate and maintained for 24 h. After 48 h of culture, cell protein was collected to test infection efficiency. The shSGK2 sequences were as follows: Sense: GCACCTGAAGTGCTTCGGAAA, anti-sense: TTTCCGAAGCACTTCAGGTGCT.

Western Blotting Analysis

Total protein was collected from the cells using cell lysis buffer. After quantification, equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The PVDF membrane was blocked in 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 h and then incubated with primary antibody overnight at 4°C, followed by the secondary antibodies for 1 h at room temperature. Antibodies used in the current work included mouse anti-human SGK2, GRB2, ERK1/2, p-ERK1/2 and GAPDH monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and AKT/p-AKT (Thr308/Ser473) monoclonal antibody (CST, Denver, MA, USA). Protein bands were checked using the Bio-Rad Imaging System (Hercules, CA, USA).

Transwell Mediated Migration and Invasion Assays

For the transwell migration assay, approximately 1×10^4 cells/well were seeded. Cells were re-suspended in 180 μL of serum-free DMEM and placed on the upper chamber of the 24-well

Transwell plate with an 8.0 μm pore polycarbonate membrane insert (Corning, Corning, NY, USA). A total of 700 μL of cell culture medium containing 10% FBS was added to the lower chamber. After 24 h in culture, the cells on the upper chamber were scraped off carefully by a moist cotton swab, and then, the cells transferred to the other side of the upper chamber were fixed with 100% methanol for 15 min followed by 0.1% crystal violet solution staining for 15 min. We captured five random fields to calculate the number of migrated cells under a microscope. For the transwell invasion assay, approximately 5×10^4 cells were seeded in each well, and all the procedures were similar to the transwell migration assay, except for the use of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to coat the polycarbonate membrane.

Wound-Healing Assay

We seeded the cancer cells in a 6-well plate, and when the cell density reached approximately 100%, the wound healing assay was conducted. We used a new, 200 μL sterile pipette tip to create a scratch on the confluent cell monolayer. Then, the medium was removed and replaced with fresh, serum-free cell culture medium. Three marked fields were captured under the microscope at the 0 and 12 h time points to evaluate the fusion rate.

Colony Formation Assay

After cell counting, approximately 500 cells were seeded in a six cm plate. Two weeks later, PBS was used to wash the cell plate, and then, the cells were fixed with 100% methanol (15 min) at room temperature. The plates were stained with 0.1% crystal violet for 15 min. After that, the colony numbers on each plate were counted under a microscope.

Molecular Function of SGK2 in RCC Based on the TCGA Database

The expression profiles of RCC were generated from The Cancer Genome Atlas (TCGA) database [https://xenabrowser.net/datapages/?cohort=TCGA%20Kidney%20Clear%20Cell%20Carcinoma%20(KIRC)]. Using R package version 2.10.0 software, we extracted 1000 genes that positively or negatively related to SGK2 at the gene expression level. In addition, gene enrichment and protein interaction tests were conducted using FunRich V3 software.

Statistical Analysis

All the statistical analyses were conducted using GraphPad Prism 6.0 software (San Diego, CA, USA). Data are shown as the mean ± standard deviation (SD). The Student's *t*-test was used to calculate the raw data, and a value of p less than 0.05 was treated as statistically significant.

Results

SGK2 is Significantly Upregulated in RCC Tissues & Cell Lines and Related to RCC Patient Prognosis

Until now, there has been no study documenting the expression and functional role of SGK2 in renal cancer. Here we extracted mRNA data from the TCGA database and found that the SGK2 mRNA level was upregulated in RCC samples compared with that in para-tumor tissues (Figure S1A). We also determined the SGK2 protein level in several RCC cell lines (A498, 786-O, Caki-1, and OSRC-2) compared with proximal tubule epithelial cell line HK-2 and, therefore, we found that SGK2 was overexpressed in the 786-O and A498 cell lines (Figure S1B). In addition, we used the Oncomine database to explore the association between copy number variation (CNV) of SGK2 and renal cancer stage. The results suggested that those patients who have metastases (including lymph node and distant metastases) or a late tumor stage commonly have a high level of SGK2 CNV (Figure S1C, D, and E). Based on the OncoLnc database, we found that a high expression level of SGK2 was also associated with poor survival of RCC patients (Figure S1F). Based on the combined findings, we speculated that SGK2 might be involved in the tumor progression of renal cancer by acting as an oncoprotein.

Knockdown of SGK2 Represses Renal Cancer Cell Growth in Vitro, whereas Overexpression of SGK2 Facilitates its Growth

As shown in Figure S1B, SGK2 was highly expressed in the A498 and 786-O cell lines and showed low expression in Caki-1. Therefore, these three cell lines were employed for subsequent loss/gain-of-function tests. The lentivirus-mediated silencing or overexpression of SGK2 in A498, 786-O cells, and Caki-1 was evaluated by Western blotting. To determine whether silencing SGK2 expression can decrease renal cancer cell growth, MTT and colony formation assays were conducted.

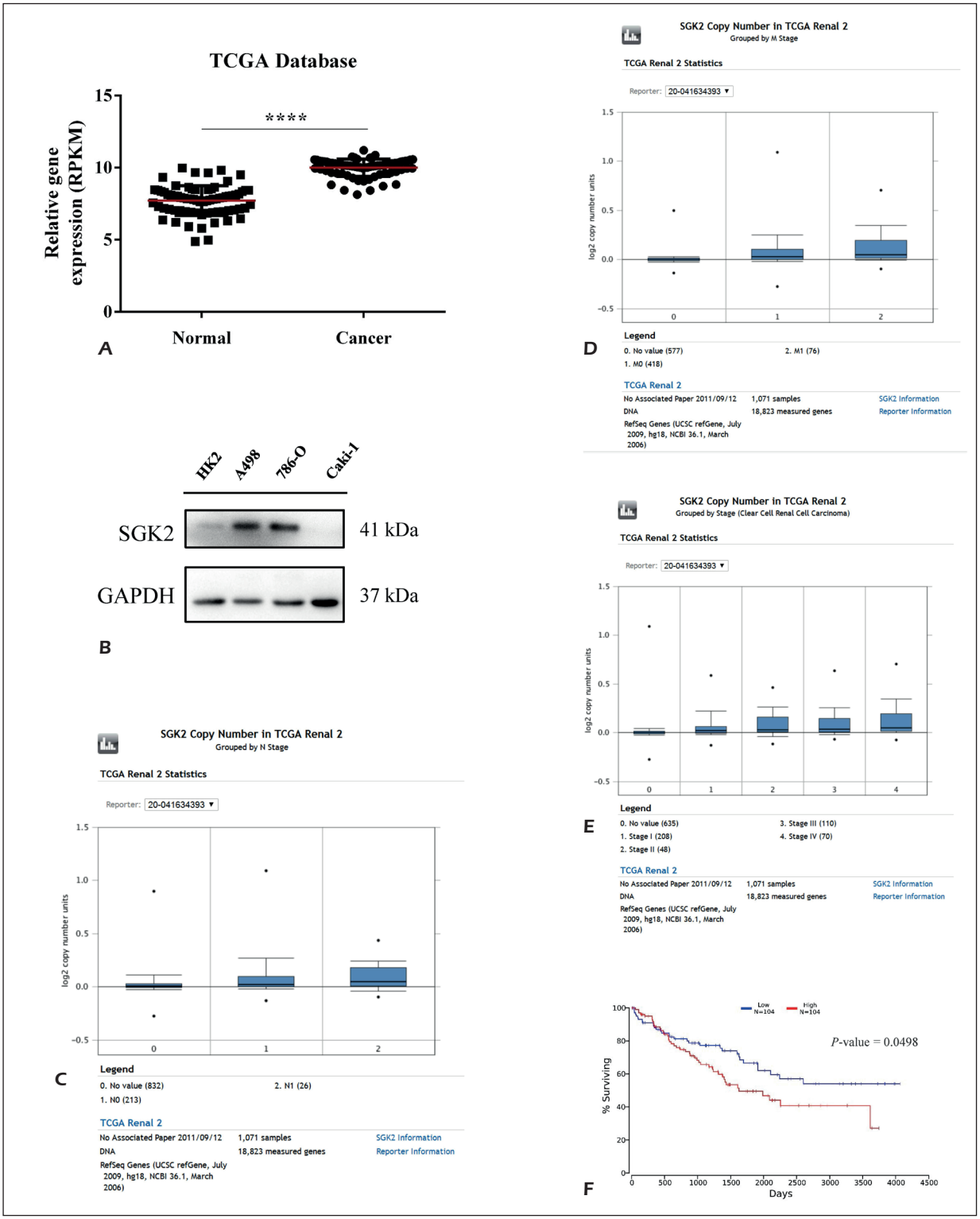

Figure S1. SGK2 expression in renal cancer patients and cell lines. **A**, The SGK2 mRNA expression between renal tumor vs. para-tumor tissues. Patients' data was retrieved from TCGA database; **B**, Western blot (WB) showed SGK2 protein expression in several renal cancer cell lines (A498, 786-O, and Caki-1) compared with normal epithelial cell line HK-2; C-E, Copy number variation comparison among different lymph node metastatic status **C**, distant metastatic status **D**, and clinical stage status **E-F**, Survival analysis of SGK2 expression and renal cancer patients. * indicated $p<0.05$. ** indicated $p<0.01$. *** indicated $p<0.001$. **** indicated $p<0.0001$.

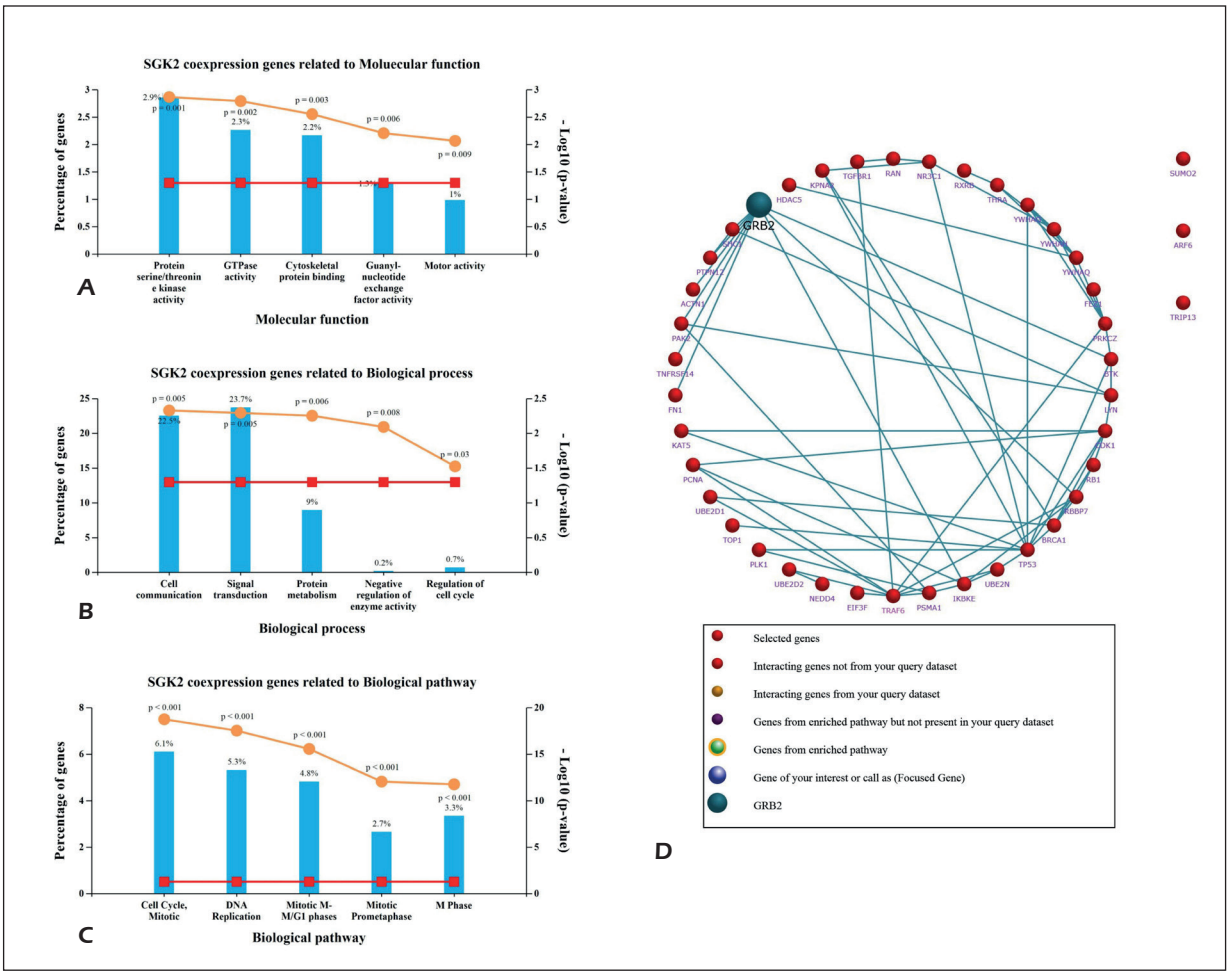

Figure S2. Molecular function analysis of SGK2. Gene expression results were obtained from TCGA database. **A**, Molecular function analysis suggested that SGK2 related genes were mainly enriched in protein serine/threonine kinase activity, GTPase activity, cytoskeletal protein binding, Guanyl-nucleotide exchange factor and motor activity; **B**, Biological process analysis indicated that SGK2 related genes were enriched in cell communication, signaling transduction, protein metabolism, negative regulation of enzyme activity and regulation of cell cycle processes; **C**, Biological pathway analysis indicated that SGK2 related genes were enriched in cell cycle mitotic, DNA replication, mitotic M-M/G1 phases, mitotic prometaphase and M phase; **D**, The protein network using FunRich 3 software identified that GRB2 was the key enriched interactive protein in SGK2-related gene network.

We found that after knocking down the SGK2 expression, both cell proliferation and colony formation ability were significantly decreased in A498 and 786-O cells (Figure 1A and 1B). In addition, cell proliferation and colony formation ability were significantly increased after overexpressing SGK2 in Caki-1 cells (Figure 1C). These data suggested that SGK2 promotes renal cancer cell growth.

Knockdown of SGK2 Suppresses Renal Cell Migration while Overexpression of SGK2 Promotes this Process

To evaluate the association of SGK2 and renal cancer cell migration, wound healing and transwell chamber migration assays were conducted to determine the rate of wound closure and migration in SGK2-silenced A498 and 786-O, and SGK2 overexpressing Caki-1 renal cancer cells. The wound-healing rate was dramatically lower in the knockdown groups compared with the vector control (Figure 2A). In contrast, SGK2 overexpression facilitated wound closure in Caki-1 cells compared with the vector group (Figure 2C). Furthermore, the SGK2-silencing groups displayed a reduction in migration ability as determined by the transwell chamber migration assay (Figure 2B), whereas SGK2 overexpressing Caki-1 cells exhibited a remarkable enhancement (Figure 2D). These data suggested that SGK2 promotes renal cancer cell migration.

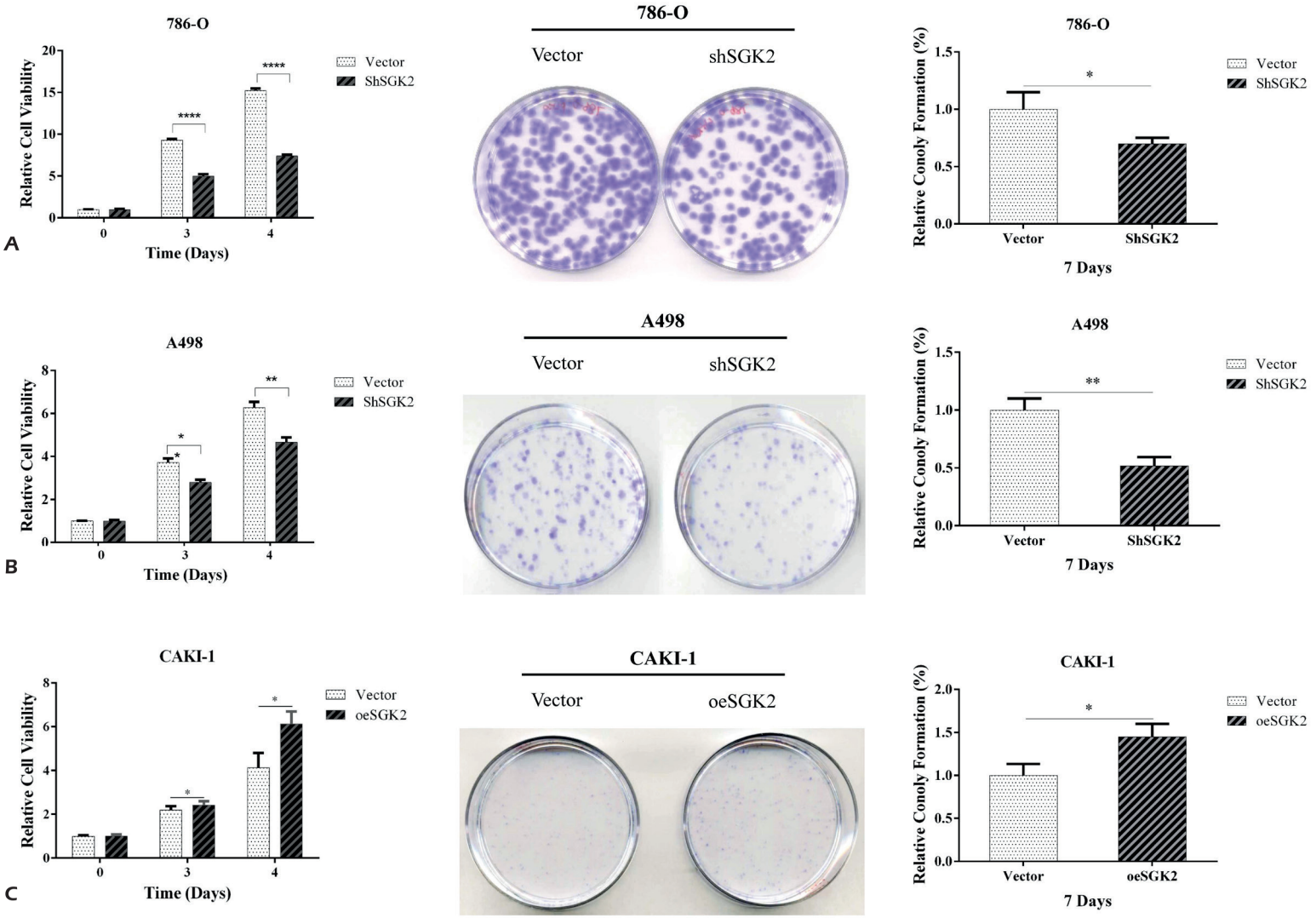

Figure 1. SGK2 promotes renal cancer cell growth and colony formation. MTT and colony formation assays indicated that cell growth and colony formation were inhibited after knocking down SGK2 in 786-O and A498 cell lines ($p<0.05$) (**A** and **B**). And cell growth and colony formation were significantly increased after overexpressing SGK2 in Caki-1 cell line using MTT and colony formation assays ($p<0.05$) (**C**). Data are representative of three independent experiments. * indicated $p<0.05$. ** indicated $p<0.01$. *** indicated $p<0.001$. ****indicated $p<0.0001$.

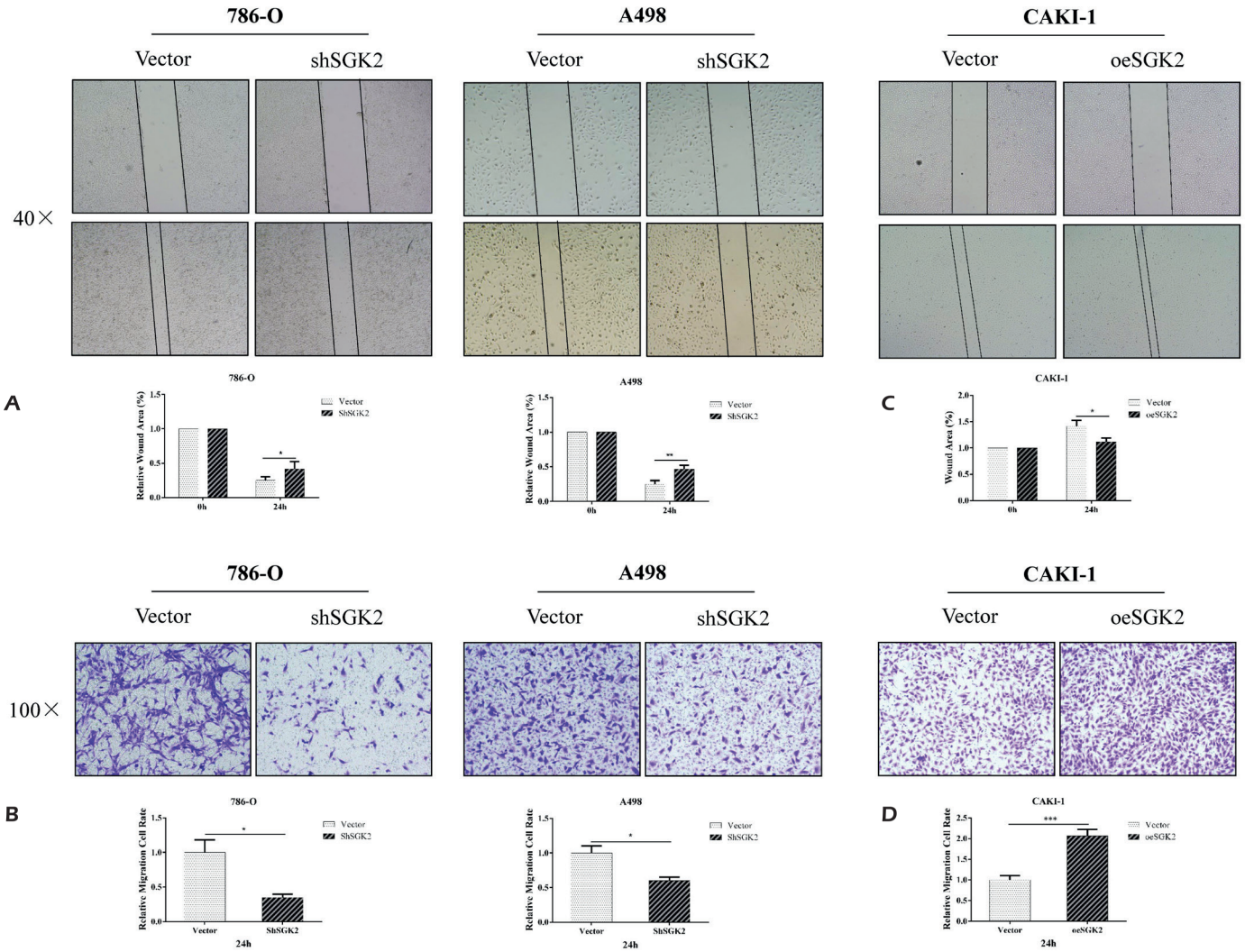

Figure 2. SGK2 promotes renal cancer cell wound healing and migration abilities *in vitro*. Wound healing and Transwell migration assays which indicated cell wound healing and migration abilities were inhibited after knocking down SGK2 in 786-O and A498 cell lines (**A** and **B**) ($p<0.05$). Inversely, cell wound healing and migration abilities were significantly increased after overexpressing SGK2 in Caki-1 cell line (**C**) ($p<0.01$). Data are representative of three independent experiments. * indicated $p<0.05$. ** indicated $p<0.01$. *** indicated $p<0.001$.

Knockdown of SGK2 Suppresses Renal Cell Invasion whereas Overexpression of SGK2 Promotes this Process

To evaluate the association of SGK2 and renal cancer cell invasion, the transwell chamber invasion assay was applied to examine the invasion capacity in of SGK2-silenced A498 and 786-O and SGK2 overexpressing Caki-1 renal cancer cells. We found that the SGK2-silencing groups showed a substantial reduction in invasion capacity assayed by the transwell chamber invasion assay, while oeSGK2-Caki-1 exhibited a significant enhancement (Figure 3). These data suggested that SGK2 promotes renal cancer cell invasion.

Molecular Function Analysis of SGK2

To predict the functions of SGK2 in renal cancer, we constructed a gene co-expression network according to Pearson-correlation coefficients based on the TCGA database. Next, the top 1000 SGK2-associated genes (500 positively and 500 negatively associated genes) were classified according to GO term using FunRich software V3 (Table S I) https://www.europeanreview.org/wp/wp-content/uploads/Table_S_I.xlsx

Based on a molecular function analysis, we found that the SGK2-related genes were significantly enriched in protein serine/threonine kinase activity, GTPase activity, cytoskeletal protein binding, guanyl-nucleotide exchange factor activity, and motor activity pathways, etc. (Figure S2A). For the biological process analysis, we found that SGK2 related genes were associated with cell communication, signal transduction, protein metabolism, negative regulation of enzyme activity, regulation of cell cycle, etc. (Figure S2B). Moreover, functional pathway enrichment was also conducted, and the most significantly enriched pathway was the mitotic cell cycle pathway, suggesting that SGK2 related genes potentially control the tumorigenesis process by regulating cell cycle processes (Figure S2C). The details of these analyses are listed in Table S II https://www.europeanreview.org/wp/wp-content/uploads/Table_S_II.xlsx

To further evaluate the interactive relationships among proteins that are related to SGK2, we mapped these proteins to protein networks using FunRich software and identified GRB2 as a key enriched interactive protein in the SGK2-related protein network (Figure S2D). In reviewing the literature, we found that GRB2 functions as upstream of growth factor signaling pathways and plays a critical role during tumorigenesis by regulating the AKT, ERK (MAPK) or growth factor signaling pathways.

SGK2 Enhances the Phosphorylation of ERK 1/2 and AKT in Renal Cancer Cells

Based on the results of the molecular function analysis, we examined several key proteins of the PI3K/AKT and ERK/MAPK signaling pathways, including GRB2, AKT, p-AKT-308, p-AKT-473, ERK 1/2 and p-ERK 1/2, which are closely linked to cancer cell proliferation, migration, and invasion. We found that p-AKT-308, p-AKT-473 and p-ERK 1/2, but not GRB2 (data not shown), AKT or ERK 1/2 were decreased in SGK2-silenced A498 and 786-O cells (Figure 4A) and were increased in SGK2 overexpressing Caki-1 cells (Figure 4B), consistent with previously published work, showing that high levels of p-AKT or p-ERK 1/2 indicated poor survivals of renal cancer patients[17,18]. Taken together, these results showed that SGK2 activates ERK 1/2 and AKT signaling by phosphorylation in RCC cells.

Discussion

SGK2 is involved in the regulation of cell growth, proliferation, migration and invasion processes[19-27]. This work found that SGK2 mRNA was up-regulated in renal cancer tissues and its protein expression was moderately increased compared with that of the normal controls, based on the TCGA and Protein Atlas database (data not shown), respectively. In addition, SGK2 protein expression was also increased in several RCC cell lines compared with that in HK-2 cells. Functional assays suggested that downregulation of SGK2 inhibited cell proliferation, migration, invasion and colony formation abilities in renal cancer cells, while overexpression promoted these processes. Previous studies suggested that the SGK family shares about 55% sequence identity with the catalytic domains of the Akt family[28-30]. Akt plays an important role during cell survival and proliferation and is known as the best effector of the PI3K signaling pathway, which is regarded as one of the most pivotal pathways in cancer metabolism and growth[31]. For example, the enhancement of the PI3K/AKT signaling pathway is known to promote cell proliferation and invasion in renal carcinoma[32]. Based on SGK2 sequence identity with the Akt family, and the function of Akt in renal cancer, we predicted that SGK2 regulates renal cancer cell fate potentially mediated by the PI3K/

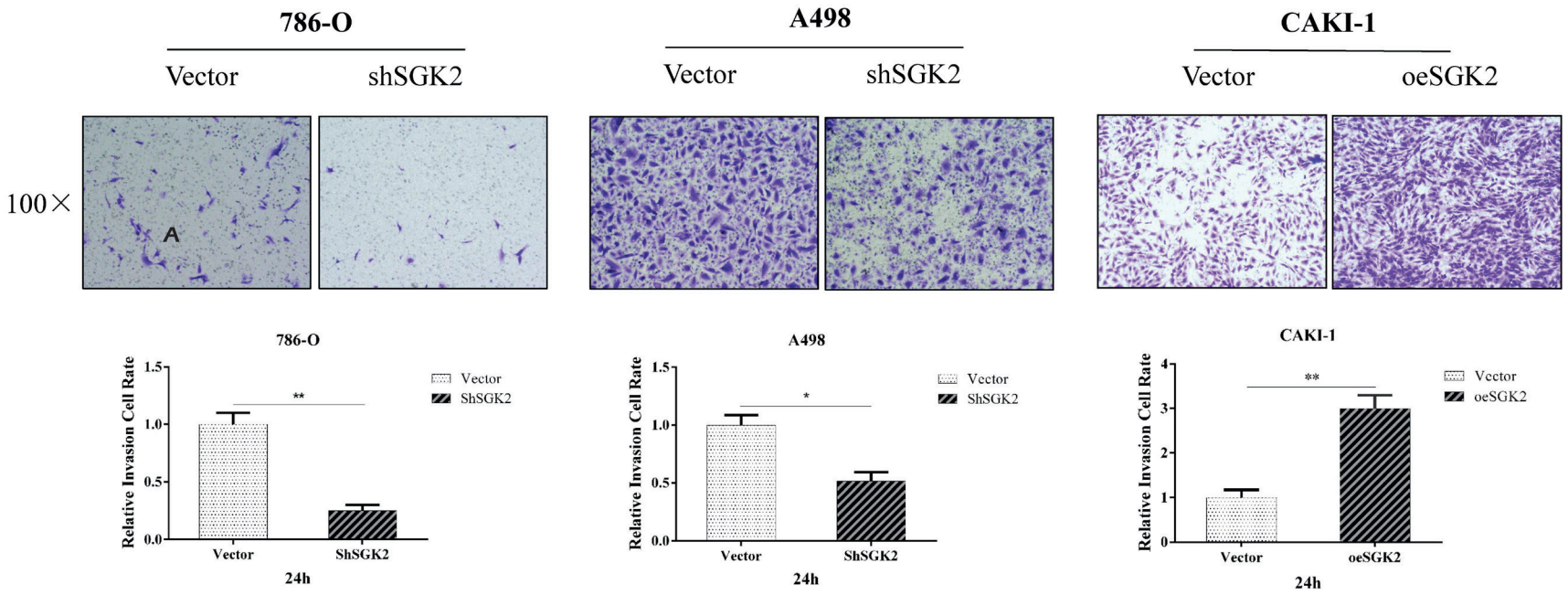

Figure 3. SGK2 promotes renal cancer cell invasion ability in vitro. The Transwell invasion assay suggested that cell invasion ability was significantly decreased after knocking down SGK2 expression in 786-O and A498 cell lines, while it was increased after overexpression SGK2 in Caki-1 cell lines. Data are representative of three independent experiments. * indicated $p<0.05$. ** indicated $p<0.01$.

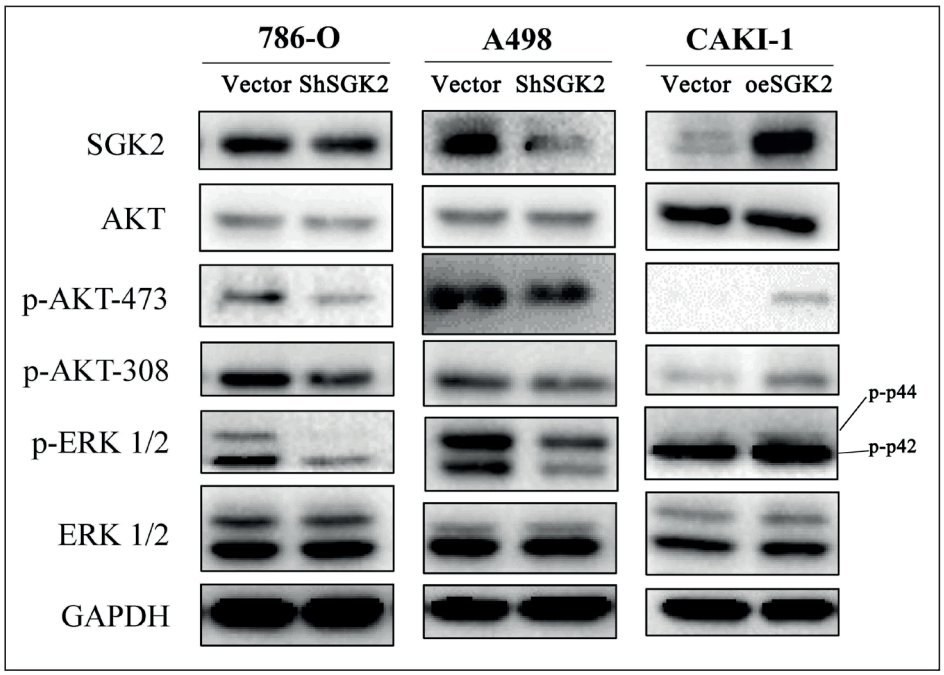

Figure 4. SGK2 promotes the phosphorylation of AKT and MAPK (ERK) families. **A**, WB results showed that the phosphorylation levels of AKT-493, AKT-308 and ERK1/2 were significantly decreased after down-regulating SGK2 expression in 786-O and A498 cells. **B**, WB results showed that the phosphorylation levels of AKT-493, AKT-308, and ERK1/2 were significantly increased after up-regulating SGK2 expression in Caki-1 cells.

Akt and MAPK/ERK signaling pathway. Therefore, we conducted a Western blotting assay to verify whether SGK2 can regulate these pathways. The results demonstrated that knockdown of SGK2 was associated with a decreased protein level of p-AKT (Thr473 and Ser308) and p-ERK 1/2, whereas overexpression of SGK2 increased the levels of p-AKT (Thr473 and Ser308) and p-ERK 1/2, implying that SGK2 contributes to the activation of the PI3K/AKT and ERK pathways. Recently, an increasing number of studies have been published demonstrating extensive cross-talk between the AKT and ERK pathways, and these studies also mentioned that their roles were interactive rather than independent[33,34]. Zitzmann et al[34] found that in human neuroendocrine tumor cell lines, AKT phosphorylation could be enhanced after inhibition of ERK 1/2 phosphorylation, indicating a feedback loop between the MAPK and AKT pathways. Conversely, using epidermal growth factor to induce PI3K signaling pathway could lead to MEK inhibition[35]. Moreover, it was also well demonstrated that inhibition of MEK/ERK substantially enhanced their antitumor effects on cancer progression both *in vitro* and *in vivo*[36]. The major limitation of this study was the lack of animal experiments, due to which we could not fully illustrate our conclusions. We will try to improve this deficiency in future work.

Conclusions

We showed that SGK2 was up-regulated in renal cancer tissues and cells compared with normal tissues and HK-2 cells. SGK2 promoted RCC cell proliferation, migration, invasion and colony formation abilities. Furthermore, the results of the current study indicated that SGK2 may activate AKT and ERK1/2 by promoting phosphorylation at Thr308/Ser473 for AKT and Thr202/204 for ERK1/2. These findings may aid in disease diagnosis and the development of small-molecule inhibitors targeting SGK2 for renal cancer patients.

Acknowledgments

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Author contributions

Y.L., J.C. and M.Z. designed the studies and drafted the manuscript. Y.L., M.Z., J.C., X.Z. J.M and J.Z. performed the cell experiments. Y.L., M.Z., J.C., Z.H. and L.Z. carried out the clinical data analysis. M.Z. and J.C. performed the statistical analysis. C.L. and X.Z. managed the experimental design, reviewed the manuscript. All authors read and approved the final manuscript.

Conflict of Interests

The Authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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