6-Gingerol suppresses tumor cell metastasis by increasing YAP<sub>ser127</sub> phosphorylation in renal cell carcinoma

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81602244; Natural Science Basic Research Plan in Shaanxi Province of China, Grant/Award Number: 2017JM8018

Abstract
According to the World Health Organization, the incidence and mortality rates of renal cell carcinoma (RCC) are rapidly increasing worldwide. Serious side effects caused by immune therapy and resistance to targeted drug therapy are urgent clinical problems facing kidney treatment. There is increasing global interest in developing natural products with a reduced number of side effects as adjunctive therapeutic options for RCC. Ginger is a spice and herbal remedy used worldwide, and 6-gingerol is a major pharmacologically active ingredient in ginger. In our study, we found that 6-gingerol suppressed RCC cell migration and metastasis in vitro and in vivo. Moreover, reduction in MMP2, Slug, and Vimentin protein levels was observed following 6-gingerol treatment of 786-O and ACHN cells. Furthermore, we revealed the mechanisms underlying the ability of 6-gingerol to inhibit RCC cell migration and metastasis. 6-Gingerol increased yes-associated protein (YAP)<sub>ser127</sub> phosphorylation and reduced YAP levels in cell nuclei. We also used a series of loss-of-function and gain-of-function experiments to support our results. Western blot results showed that MMP2, Slug, and Vimentin protein expression was downregulated in YAP-silenced cells and upregulated in YAP-overexpressing cells. Transwell data demonstrated that YAP suppressed RCC migration ability. Immunofluorescence images showed that 6-gingerol decreased YAP levels, leading to disordered F-actin and a reduction in cell lamellipodia. Overall, our results indicated that 6-gingerol is a potential antimetastatic compound for use in kidney therapy.

KEYWORDS
6-gingerol, migration, RCC, YAP

1 | INTRODUCTION

According to the World Health Organization, 403,262 cases of renal cell carcinoma (RCC) and 175,098 deaths due to RCC occurred worldwide in 2018. Meanwhile, the global incidence and mortality rates are rapidly increasing (https://www.who.int). Mutation or absence of VHL results in activation of the VHL-HIF1α/2α-mTOR-VEGF/VEGFR pathways, leading to tumor cell growth and metastasis<sup>1,2</sup>

In the last decade, mTOR inhibitors (such as everolimus and temsirolimus) and VEGF/VEGFR inhibitors (such as bevacizumab and
sunitinib) have been used alone or in combination for the treatment of advanced or metastatic renal cancer.\textsuperscript{[3–5]} The combination of nivolumab plus ipilimumab (anti-PD-1 plus anti-CTLA-4), avelumab plus axitinib (anti-PD-L1 plus VEGFR inhibitor), or atezolizumab plus bevacizumab (anti-PD-L1 plus VEGF inhibitor) improves the clinical outcome compared with sunitinib treatment in advanced or metastatic renal cancer.\textsuperscript{[6–8]} However, serious side effects are still an urgent problem in clinical immunotherapy. Chemotherapy is still often used for kidney cancer after targeted drugs and/or immunotherapy have been attempted (http://www.cancer.org/cancer/kidney-cancer/treating.html). Natural products are of increasing interest for investigating adjunctive therapeutic options in clinical therapy due to their nontoxic and wide antitumor effects.

6-Gingerol (1-[4'-hydroxy-3'-methoxyphenyl]-5-hydroxy-3-decanone) is extracted from ginger, and ginger is a major spice and herbal remedy used by people globally.\textsuperscript{[9,10]} 6-Gingerol, 6-shogaol, 8-gingerol, and 10-gingerol are all phytochemicals in ginger. 6-Gingerol was confirmed to have wide antitumor activity in multiple cancer types.\textsuperscript{[12–18]} In oral and cervical tumor cells, 6-gingerol arrests the cell cycle in the G2 phase and promotes tumor cell apoptosis through activating caspases 3 and 7.\textsuperscript{[19]} In HeLa cells, 6-gingerol showed antitumor activity by stimulating autophagy via drug-DNA interactions.\textsuperscript{[20]} In colon cancer cells, 6-gingerol was able to inhibit tumor cell proliferation by regulating mitogen-activated protein kinase-activator protein 1 signaling.\textsuperscript{[21]} In breast cancer cells, 6-gingerol suppressed tumor cell adhesion, invasion, and motility by decreasing MMP2 and MMP9 levels in MDA-MB-231 cells.\textsuperscript{[21]} In RCC, 6-gingerol was proven to inhibit cell proliferation via the AKT-GSK 3β-cyclin D1 pathway in vitro and in vivo.\textsuperscript{[22]}

Up to approximately 30% RCC is known to be metastasis on the first diagnosis, and the underlying mechanisms remain elusive.\textsuperscript{[23]} Yes-associated protein (YAP) was found to be a key effective drug-resistant responsive protein in a screen that used 275,000 short hairpin RNAs targeting 5046 signaling components.\textsuperscript{[24]} YAP was reported to interact with the actin remodeling, Notch pathway, HIF pathway, and AMPK pathway to influence tumor cell metastasis.\textsuperscript{[25–30]} In RCC, YAP expression was confirmed to correlate with high grade and stage, migration, invasion, and angiogenesis.\textsuperscript{[31–34]} Furthermore, YAP regulated chemosensitivity by upregulating p53 levels in RCC.\textsuperscript{[35]} In our study, we observed that 6-gingerol inhibited RCC cell migration by decreasing YAP levels. We further identified that 6-gingerol phosphorylated YAP at YAP Ser127 and reduced YAP nuclear localization. These results indicate that 6-gingerol can re sensitize RCC to drug therapy.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human clear cell RCC cell lines 786-O and ACHN were purchased from American Type Culture Collection (Manassas, VA). The two cell lines were authenticated by STR analysis by the committee of type culture collection at the Chinese Academy of Sciences (Room 301, NW-10, Suzhou Nanopolis, 99 Jinji Lake Avenue, Suzhou Industrial Park, Jiangsu, China). Cell lines were maintained in RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc, Waltham, MA) with 10% (vol/vol) fetal bovine serum (FBS; HyClone) at 37°C in a 5% CO\textsubscript{2} incubator.

2.2 | Chemicals

6-Gingerol (#S3836; Selleckchem) was dissolved in dimethyl sulfoxide (DMSO) or corn oil. Phalloidin-iFluor 594 reagent (#ab176757; Abcam) was dissolved in DMSO. α-Luciferin potassium salt (ab1436655; Abcam) was purchased from Abcam Co Ltd. and was dissolved in water and stored in the dark. 4′,6-Diamidino-2-phenylindole (DAPI; #ST007) was purchased from HAT heart biological technology Co Ltd.

2.3 | Wound healing assay

A total of 40 × 10\textsuperscript{4} 786-O and ACHN cells/well were plated in six-well plates. After 6 hours, cells were starved overnight in medium containing 0.5% FBS. A wound was made by using a 200 μL pipette tip to scratch wells in vertical and horizontal directions, which was followed by washing the cells with phosphate buffer saline (PBS) twice. Cell migration images were captured by a microscope every 12 hours during the wound healing of the samples.

2.4 | Transwell migration assay

786-O and ACHN cells were treated with 30 μM 6-gingerol for 48 hours, and then the cells were harvested and resuspended in FBS-Free RPMI-1640 medium. A total of 200 μL of cell suspension, which contained 4 × 10\textsuperscript{4} cells, was seeded in the upper chamber, and 600 μL of 10% FBS RPMI-1640 medium served as the chemotactant. After 24 hours, the insert was removed, and the cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature and then were washed with PBS twice. Cells at the bottom of the insert were stained with 0.05% crystal violet for 20 minutes and washed with PBS twice. The number of migrated cells were visualized by a microscope, and three randomly chosen fields per membrane were photographed and quantitated using Adobe Photoshop CS6.

2.5 | Real-time quantitative polymerase chain reaction

An RNA Fast 200 kit (Feijie Biotech, Shanghai, China) was used to isolate total RNA from 786-O and ACHN cell lines, and complementary DNA synthesis was performed using a Prime Script RT reagent kit (Takara Biotechnology Co, Ltd, Dalian, China). Relative gene expression was detected with an SYBR Green PCR Master Mix.
(Takara Biotechnology Co, Ltd, Dalian, China) and was calculated by the 2^(-ΔΔCt) method, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. GAPDH and YAP primer sequences were as follows:

GAPDH forward, 5'-ATGGGGAAGGTAAGGTCCGG-3', GAPDH reverse, 5'-GACGCTGCGGATTTTGGC-3', YAP forward, 5'-AATTGCCAATTATACCTCAGTG-3', YAP reverse, 5'-CACATCAAGGGCTATGTTCAACTC-3'.

2.6 | Western blot assay

The protocol was performed as described previously. Antibodies against YAP (1:1000; #14074; Cell Signaling Technology), p-YAP (1:1000; #13008T; Cell Signaling Technology), Slug (1:1000; #9585; Cell Signaling Technology), Vimentin (1:1000; #5741; Cell Signaling Technology), MMP2 (1:1000; #40994S; Cell Signaling Technology), Histone 3 (1:1000; #94817; Abcam), AMPKα (1:1000; #2532S; Cell Signaling Technology), p-AMPKα (1:1000; #2535S; Cell Signaling Technology), Lats1 (1:1000; #3477S; Cell Signaling Technology), and β-actin (1:1000; #JB09; Absin) were all applied to detect protein expression levels in cells, and β-actin was used as an internal control. After incubation with primary antibodies for 24 hours, membranes were washed with TBST three times and then incubated with a secondary antibody (goat anti-mouse immunoglobulin G [IgG; 1:2000; #ZB-2305, Beijing Zhongshan Golden Bridge Biotechnology] or goat anti-rabbit IgG [1:2000; #ZB-2301; Beijing Zhongshan Golden Bridge Biotechnology]) for 1 hour at room temperature. A Western Bright Quantum HRP substrate kit (Advansta, Inc, Menlo Park, CA) was applied for detecting immunoreactive signals, and they were visualized by a Molecular Imager ChemiDoc XRS system (Bio-Rad Laboratories, Inc, Hercules, CA).

2.7 | Gene set enrichment analysis

cCRCC transcriptome data (count and FPKM value), clinical data, and mutation data were downloaded from The Cancer Genome Atlas database (TCGA; https://www.cancer.gov/tcga). edgeR and clusterProfiler packages were used in gene set enrichment analysis (GSEA) to assess the significantly changed pathways between the YAP high-expression group (top 25% of cCRCC patients in TCGA FPKM cohort) and the YAP low-expression group (top 25% of cCRCC patients in TCGA FPKM cohort).

2.8 | F-actin staining assay

786-O and ACHN cells were cultured in Millicell EZ SLIDE 8-well glass slides (Millipore, Germany) and then were treated with 30 μM 6-gingerol for 48 hours. Cells were fixed in 4% paraformaldehyde for 30 minutes, washed with PBS twice, treated with a permeabilization solution (1% Triton X-100 in PBS) for 10 minutes, washed with PBS twice, and blocked with 1% bovine serum albumin in PBS for 1 hour. Then, cells were incubated with Phalloidin-iFluor 594 reagent for 30 minutes, washed with PBS twice, stained with DAPI (to label nuclei) for 10 minutes, washed with PBS twice, and examined by laser scanning confocal microscopy (Nikon A1R/A1).

2.9 | Immunofluorescence assay

786-O and ACHN cells were cultured in Millicell EZ SLIDE 8-well glass slides (Millipore, Germany), and treated, fixed, permeabilized, and blocked for immunofluorescence assays as they were for F-actin staining assays. Then, cells were incubated with a primary antibody against YAP (1:100) in antibody dilution buffer overnight at 4°C, which was followed by cells being washed with PBS twice and incubated with secondary antibody for 1 hour. Cell nuclei were stained with DAPI (1:5000) for 10 minutes in the dark. Cells were examined by laser scanning confocal microscopy (Nikon A1R/A1).

2.10 | Lentiviral, plasmid transfection

786-O and ACHN cells were seeded in 6-cm dishes and then were transfected with a lentivirus (Shanghai Gene Pharma Co, Ltd, Shanghai, China). The sequence for YAP silencing was 5'-CGTTTCCCA GACTACCTT-3', and the NC sequence was 5'-TTCTCCGAACG TGTCAGT-3'. YAP overexpression plasmids were purchased from Shanghai Genechem Co, Ltd (Shanghai, China). 769-P cells were seeded in six-well plates, were grown overnight and then were transfected with plasmids (GV230 and GV230-YAP). After 24 hours, stable cells were selected by puromycin treatment (Sigma-Aldrich, Shanghai, China), and the transfection efficacy was determined by Western blotting and real-time quantitative PCR assay.

2.11 | Nuclear and cytoplasmic extraction

786-O cells were seeded in 10 cm dishes and then were treated with 30 μM 6-gingerol for 48 hours. Then, 786-O cells were harvested. NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833; Thermo Fisher Scientific) were used to isolate nuclear and cytoplasmic fractions of 786-O cells according to the manufacturer’s instructions.

2.12 | Kidney cancer metastasis animal model

Animal experiments were approved by the institutional review board of Xi’an Jiaotong University. Ten BALB/c 4-week-old nude mice (male) were randomly separated into two groups, and then 2 × 10^6 ACHN cells were injected into the tail veins of 4-week-old nude mice.
After 3 days, one group of nude mice (n = 5) was treated with corn oil (control), and the other group of nude mice (n = 5) was treated with 6-gingerol (diluted in corn oil, 5 mg/[kg body weight]) every 3 days by gavage. Moreover, 10 nude mice were weighed after every treatment. α-Luciferin potassium salt 300 mg/kg was injected intraperitoneally into nude mice 10 min before imaging, and images were captured by weekly bioluminescence imaging using IVIS Lumina II (Xenogen, Inc, Waltham, MA). For 30 days, 10 nude mice were injected with α-luciferin potassium salt to enable imaging of tumor growth and metastasis. At the end of the experiment, all the animals were euthanized according to the guidelines of the institutional review board of Xi’an Jiaotong University.

2.13 | Statistics

The data in this study, including standard deviations, medians, and ranges, were analyzed by GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The significant differences between two groups were analyzed with unpaired the Student t tests. *P < .05 is considered significant.

3 | RESULTS

3.1 | 6-Gingerol inhibits renal cancer cell migration in vitro

To confirm the antitumor effect of 6-gingerol in renal cell tumorigenesis, a series of in vitro experiments were conducted to assess the biological role of 6-gingerol in 786-O and ACHN cells. Our results showed that 6-gingerol repressed 786-O and ACHN cell migration in vitro (Figure 1). First, we performed transwell migration assays to assess the effect of 6-gingerol on RCC cell migration. As shown in Figure 1A, the migration of 786-O and ACHN cells was abolished by treating the cells with 6-gingerol for 48 hours. Meanwhile, we found that 6-gingerol inhibited 786-O and ACHN cell migration in a dose-dependent manner (P = .002 in 30 μM, P = .004 in 60 μM, P = .003 in 30 μM, P = .002 in 60 μM, respectively). The IC50 of 6-gingerol was 30 μM in our previous data,[21] so 30 μM 6-gingerol was chosen for the following experiments. Furthermore, the inhibitory effect of 6-gingerol on RCC cell migration was confirmed by wound healing assays. As shown in Figure 1B, unlike the results of the 786-O and ACHN cells treated with 30 μM 6-gingerol, the wound area was almost closed in control cells at 24 hours. Vimentin, Slug, and MMP2 are considered to be critical prerequisites for metastasis in many human cancers, so we tested Vimentin, Slug, and MMP2 protein levels in our study by Western blotting. As shown in Figure 1C, Vimentin, Slug, and MMP2 were decreased in 786-O and ACHN cells that were treated with 30 μM 6-gingerol. Taken together, the results shown in Figure 1 confirmed that 6-gingerol restrained RCC cell migration in vitro.

3.2 | 6-Gingerol suppresses renal cancer cell migration by decreasing YAP protein levels

To explore the molecular mechanisms underlying the inhibition of cell migration by 6-gingerol in RCC, we first tested the effect of 6-gingerol on the levels of the Hippo pathway effector YAP; YAP plays a key role in tumor migration. The results showed that treatment with 10 to 50 μM 6-gingerol did not affect YAP messenger RNA levels (Figure S1A). However, 6-gingerol increased YAP phosphorylation at Ser127, resulting in a dose-responsive decrease in the YAP amount in 786-O and ACHN cells from 10 μM 6-gingerol to 50 μM 6-gingerol (Figure 2A). To find the mechanism of 6-gingerol phosphorylates YAP, Lats1, the upstream of YAP, was detected in our study. As shown in Figure 2A, Lats1 was activated in a dose-responsive increase in 786-O and ACHN cells from 10 μM 6-gingerol to 50 μM 6-gingerol. Furthermore, we found 6-gingerol enhanced phosphorylated AMPKα amount in a dose-dependent manner (Figure 2A). In GSEA analysis results, 32 of 50 gene sets were up-regulated in the YAP high-expression group, including the gene sets of “hallmark_epithelial_mesenchymal_transition” (Figure 2B).

Furthermore, we tested the effect of YAP on Vimentin, Slug, and MMP2 expression. As expected, our data showed that knockdown of YAP in 786-O and ACHN cells downregulated the protein expression of Vimentin, Slug, and MMP2 relative to that of the control (Figure 2C). We also conducted a loss-of-function experiment to test the biological role of YAP in cell migration. Upon YAP deletion, we observed significantly decreased numbers of migrating 786-O and ACHN cells (P < .001, and P < .001, respectively; Figure 2D). In sum, our results revealed that 6-gingerol inhibits cell migration by decreasing YAP protein levels in RCC.

3.3 | The inhibition ability of 6-gingerol is not reversed by ectopic expression of YAP in RCC cells

To further biochemically validate the effect of YAP on kidney cancer cell migration, ectopic expression of YAP was introduced in 769-P cells. As shown in Figure 3A, overexpression of YAP in 769-P cells resulted in a significantly higher number of migrating cells than what was observed in the vector-transfected cells (P = .013). In addition, 6-gingerol treatment was assessed in migration assays, and we found that 6-gingerol significantly decreased the number of cells that passed through the transwell membrane compared to that of the control (P = .002). However, following the ectopic expression of YAP cells, there was no significant change in migrating cells passing through the transwell membrane than there were in the group of cells treated with 30 μM 6-gingerol (P = .471). Similarly, we tested Vimentin, Slug, MMP2, and p-YAP protein levels in 769-P cells ectopically expressing YAP. Overexpression of YAP unexpectedly increased Vimentin, Slug, and MMP2 protein levels (Figure 3B), and Vimentin, Slug, and MMP2 protein levels were decreased in ectopic expression of YAP 769-P cells that were also treated with 30 μM 6-gingerol. However, the p-YAP amount was
increased in the ectopic expression of YAP 769-P cells that were also treated with 30 μM 6-gingerol.

These findings show that overexpression of YAP can increase the migration cells, however, YAP could not restore the ability of 6-gingerol to inhibit RCC cell migration.

### 3.4 6-Gingerol decreases YAP nuclear localization

YAP is the major mediator of the Hippo-YAP pathway, and YAP translocates to the cell nucleus to activate downstream gene transcription. Our results show that 6-gingerol phosphorylated YAP (Figure 2A). We thus speculate that 6-gingerol inhibits RCC cell migration by decreasing YAP nuclear localization. We first tested cytoplasmic and nuclear protein fractions from 786-O cells after treatment with 30 μM 6-gingerol for 48 hours. As expected, dephosphorylated YAP levels were decreased in 786-O cell nuclei; in contrast, phosphorylated YAP levels were increased in the cytoplasm of 786-O cells (Figure 4A).

To further validate that 6-gingerol regulates YAP nuclear localization, we conducted a YAP immunofluorescence staining assay using 786-O and ACHN cells. As shown in Figure 4B, YAP levels in the nucleus of 786-O and ACHN cells were decreased...
FIGURE 2  6-Gingerol decreases cell migration by increasing the phosphorylation of YAPser127. A, 6-gingerol induced YAP phosphorylation in a dose-dependent manner. 786-O and ACHN cells were seeded in 10 cm dishes overnight and then were treated with 0, 5, 10, 20, and 40 μM 6-gingerol for 48 hours. Then, the cells were lysed, and the protein levels of YAP, YAPser127, and actin were analyzed by Western blot assay. B, GSEA results showed that “hallmark_epithelial_mesenchymal_transition” was a response to the high YAP expression. We downloaded the TCGA KIRC database (11 September 2019) and separated it into the YAP low-expression group (25% of 611 patients) and the YAP high-expression group (25% of 611 patients). Then, the data were submitted to GSEA, and the 50 hallmark gene sets were analyzed. C, Immunoblot analysis of AMPKα, p-AMPKα, Lats1, Vimentin, Slug, and MMP2 was performed in 786-O and ACHN cells with YAP silenced. 786-O and ACHN cells were seeded in 6 cm dishes overnight, and then cells were transfected with lentiviruses containing sequences to knockdown YAP and an NC for 48 hours. Then, the cells were lysed, and the protein levels of AMPKα, p-AMPKα, Lats1, Vimentin, Slug, and MMP2 were analyzed by Western blot assay. D, Transwell assays were performed to test the effect of YAP on cell migration. 786-O and ACHN cells with silenced YAP were harvested and seeded in the upper chamber (4 × 10^4 cells), and they were incubated for 24 hours. Then, the cells were stained with crystal violet and visualized by a microscope. The cell numbers were determined using ImageJ. Statistical analysis of the data between two groups was performed using the Student t tests, and error bars indicate ±SD. GSEA, gene set enrichment analysis; SD, standard deviation; TCGA, The Cancer Genome Atlas database; YAP, yes-associated protein.
following 48 hours of treatment with 30 μM 6-gingerol compared with that of the control cells. In contrast, the location of YAP in the cytoplasm was increased in 786-O and ACHN cells compared that of the 6-gingerol-treated cells. In addition, we also found that F-actin was decreased in 6-gingerol-treated 786-O and ACHN cells. At the same time, we observed that cell lamellipodia were also reduced in 6-gingerol-treated 786-O and ACHN cells. Taken together, the molecular mechanism underlying 6-gingerol inhibition of RCC cell migration was 6-gingerol-mediated phosphorylation of YAP, resulting in a decrease in the nuclear localization of YAP.

3.5 | 6-Gingerol inhibits tumor migration in nude mice

The migration inhibition capacity of 6-gingerol was validated in an ACHN xenograft model. A total of 2 × 10^6 cultured ACHN cells were injected into nude mice by tail vein injection (n = 10), and the mice were randomly separated into two groups (five mice in one group). Control group mice (n = 5) were treated with 100 μM corn oil, and the 6-gingerol treatment group (n = 5) was treated with 2.5 mg/kg 6-gingerol every 3 days (Figure 5A). Meanwhile, mice were weighed every 3 days, and the physical state of the mice was monitored throughout all experiments. As previously reported, there was no significant body weight loss between the 6-gingerol treatment group and the control group (Figure 5B). We observed that tumor metastasis was strongly inhibited in the 6-gingerol group compared with that of the control group by measuring the value of maximum and minimum luminescence signal (Figure 5C). As shown in Figure 5C, there were multiple tumor metastases in the control group (n = 5), including leg metastases, spinal metastases, and lung metastases. However, in the 6-gingerol treatment group, tumor cell numbers at metastatic sites were reduced compared with those in the control group. Our results suggested that 6-gingerol inhibits RCC cell migration in vivo.

4 | DISCUSSION

Because RCC is considered to be an incurable disease that commonly develops drug resistance, prior drugs targeting VEGF and mTOR signaling caused by VHL mutations and immunotherapeutic treatments have shown limited clinical benefit in RCC clinical treatment. 6-Gingerol is one of the most consumed dietary components worldwide. Our study conducted a series of experiments to expand the understanding of 6-gingerol antitumor activity in RCC. In our study, we found that 6-gingerol suppresses kidney cancer metastasis in vivo and in vitro. Furthermore, we demonstrated that 6-gingerol decreased cell migration primarily by decreasing Vimentin, Slug, and MMP2 protein levels. Meanwhile, F-actin staining assays showed that cell pseudopods and F-actin were reduced in 6-gingerol-treated cells. The mechanism underlying the 6-gingerol inhibition of RCC migration was that the amount of YAP in the nucleus was decreased by 6-gingerol.

Ginger is a food product and has been used in medicine and daily life. In our study, a significant decrease in organ metastasis...
and tumor cell numbers in metastatic sites were observed in the 6-gingerol treatment group (Figure 5). In addition, our results confirmed that 6-gingerol had no effect on mouse body weight and lifespan when we compared the 6-gingerol treatment group with the control group (Figure 5B). These findings were verified and are consistent with previous reports in other cancers. 6-Gingerol has been previously shown to suppress tumor cell growth by inhibiting the AKT-GSK 3β pathway and cyclins in RCC, pancreatic cancer, cervical adenocarcinoma cells, and colorectal cancer cells. Previous research results showed that 6-gingerol induced tumor cell apoptosis by activating caspases 3 and 9 and by modulating mitochondrial functions in colorectal cancer cells, oral tumor cells, and cervical tumor cells. 6-Gingerol has also been shown to suppress breast cancer cell invasion by decreasing MMP2 and

**FIGURE 4**  6-Gingerol decreases YAP localization in the nucleus. A, YAP in nuclear and cytoplasmic protein fractions was measured after 6-gingerol treatment. 786-O cells were seeded in 10 cm dishes and treated with 30 μM 6-Gingerol for 48 hours. Cells were lysed, and the amount of YAP protein in the nucleus and cytoplasm was analyzed by Western blot assay. B, YAP and F-actin were detected using an immunofluorescence assay. 786-O and ACHN cells were treated with 6-gingerol for 48 hours. YAP expression and distribution were detected using immunofluorescence, F-actin was stained by Phalloidin-iFluor 594, yellow arrow represents lamellipodia, and nuclei were labeled with DAPI. DAPI, 4′,6-diamidino-2-phenylindole; YAP, yes-associated protein.
However, the mechanism behind these decreases is unclear. In our experiments, we confirmed that the activities of YAP were decreased by 6-gingerol treatment and found that the levels of phosphorylated YAP were increased in 786-O and ACHN cells that were treated with 6-gingerol (Figure 2B). Meanwhile, 6-gingerol enhanced phosphorylated AMPKα amount in a dose-dependent manner (Figure 2A), and AMPKα was reported to activate Lats1.

MMP2, Vimentin, and Slug significantly contribute to the migration and metastasis of tumor cells. In our study, we confirmed that 6-gingerol suppressed MMP2, Vimentin, and Slug protein levels by decreasing the nuclear levels of YAP. YAP has been reported to act as a promoter in tumor metastasis in many cancers. Bin You et al. demonstrated that ERK1/2 inhibitors inhibited the migratory and invasive activity of NSCLC by promoting YAP degradation. Haskins et al. reported that members of the epidermal growth factor

YAP is the key downstream target of the Hippo signaling pathway, it has been reported to be highly expressed in human kidney cancer and other types of cancers. Previous studies confirmed that YAP is an important regulator of organ growth, stem cell self-renewal, differentiation, and tumor cell growth/migration. MMP2, Vimentin, and Slug significantly contribute to the migration and metastasis of tumor cells. In our study, we confirmed that 6-gingerol suppressed MMP2, Vimentin, and Slug protein levels by decreasing the nuclear levels of YAP. YAP has been reported to act as a promoter in tumor metastasis in many cancers. Bin You et al. demonstrated that ERK1/2 inhibitors inhibited the migratory and invasive activity of NSCLC by promoting YAP degradation. Haskins et al. reported that members of the epidermal growth factor
receptor family ERBB4 promote breast cancer cell migration by activating YAP to regulate its target genes. Feng et al.\(^\text{[42]}\) showed that thromboxane A2 receptors stimulate vascular smooth muscle cell migration by upregulating YAP/TAZ. Furthermore, F-actin homeostasis and tumor cell lamellipodia were all disordered in 6-gingerol-treated 786-O and ACHN cells (Figure 2B). In addition, we demonstrated that F-actin was regulated by YAP in our study. These results were consistent with those reported by Kim et al.\(^\text{[27]}\) in SKMel28 and WM3248 cells and by Feng et al.\(^\text{[42]}\) in vascular smooth muscle cells. Therefore, we concluded that 6-gingerol induces RCC cell migration inhibition though the Hippo-YAP signaling pathway in vitro.

In conclusion, our results confirmed that 6-gingerol inhibits metastasis of the 786-O and ACHN kidney cancer cell lines in vivo and in vitro. The mechanism behind the anticancer effect of 6-gingerol was the phosphorylation of YAP, which led to a decrease in YAP nuclear localization. Considering that 6-gingerol is one chemical component of ginger, which is commonly ingested in the human diet (250 mg-1 g/day), our results suggested that 6-gingerol would be safe and useful for preventing or treating kidney cancer.

**ACKNOWLEDGEMENTS**

This study was supported by the National Natural Science Foundation of China (NSFC No. 81602244 to Shan Xu) and Natural Science Basic Research Plan in Shaanxi Province of China (Program No. 2017JM8018 to Shan Xu).

**CONFLICT OF INTERESTS**

The authors declare that there are no conflict of interests.

**AUTHOR CONTRIBUTIONS**

Pengsheng Zheng and Dalin He conceived and supervised the study; Haibao Zhang, Tianjie Liu, and Shan Xu conducted the Western blot, transfection, wound healing, and transwell assay; Haibao Zhang and Shan Xu conducted the animal experiment; Tao Hou and Wenjie Yang performed the immunofluorescence staining; Wei Lv performed statistical analysis; Zixi Wang performed the visualization of mechanism; Shan Xu wrote the manuscript; Dalin He, Pengsheng Zheng, and Xinyang Wang were all major contributor to the editing of the manuscript. All the authors read and approved the final manuscript.

**ETHICS STATEMENT**

The protocol for animal care was followed under the guidelines of the Institutional Animal Care and Committee of Xi’an Jiaotong University.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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**How to cite this article:** Xu S, Zhang H, Liu T, et al. 6-Gingerol suppresses tumor cell metastasis by increasing YAPser127 phosphorylation in renal cell carcinoma. *J Biochem Mol Toxicol*. 2021;35:e22609. https://doi.org/10.1002/jbt.22609