

## Original Article

# Allicin inhibits human renal clear cell carcinoma progression via suppressing HIF pathway

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Received September 2, 2015; Accepted November 1, 2015; Epub November 15, 2015; Published November 30, 2015

**Abstract:** Hypoxia-inducible factor 1-alpha (HIF-1 $\alpha$ ) protects hypoxic cells from apoptosis or necrosis under ischemic and anoxic conditions. Allicin is characterized by the anti-cancer characteristics. This study aims to explore whether allicin is involved in renal clear cell carcinoma progression through HIF-1 $\alpha$ . A total of 40 RCC tissues and 39 normal renal tissues were collected H&E and immunohistochemistry were applied to study morphology changes. MTT assay and flow cytometry (FCM) were used to analyze cell viability and apoptosis. In vitro colony formation assay and wound healing assay were conducted to explore cell migration. The protein levels of Bcl-2, VEGF and HIF-1 $\alpha$  were increased in RCC tissues. More importantly, treatment with allicin significantly decreased HIF-1 $\alpha$  protein level, thereby reducing Bcl-2 and VEGF expression. In addition, allicin also obviously enhanced apoptotic cells. And colony formation capacity and cell migration rate were reduced in RCC-9863 cells treated with allicin. Further study revealed that overexpression of HIF-1 $\alpha$  could partially repress allicin-induced downstream effects. To conclude, allicin inhibits human renal clear cell carcinoma progression partially by suppressing HIF pathway.

**Keywords:** Allicin, RCC, HIF1 $\alpha$ , apoptosis, migration

## Introduction

Hypoxia pathway plays a key role in the pathologies of cancers [1, 2]. It is reported to mainly maintain the appropriate level of oxygen concentration, thereby regulating the normal cellular metabolism [3]. In normal status, the level of the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is regulated by the VHL tumor suppressor for ubiquitination and proteasomal degradation [4]. To respond to hypoxia, HIF-1 $\alpha$  and HIF-2 $\alpha$  can bind HIF-1 $\beta$  and initiate the expression of downstream genes thereby alleviating hypoxic stress [5]. HIF-1 $\alpha$  induces the expression of VEGF, which regulate the expression of vascular development [6]. And HIF-1 $\alpha$  can also interact with Bcl-2 and Bax, thereby regulating cell apoptosis.

Sporadic clear cell renal cell carcinoma (ccRCC) is the most common adult kidney cancer [7]. The underlying mechanism includes both environmental and genetic risks, such as smoking, obesity and acquired kidney disease [8-10]. Besides, renal cell carcinoma is also suggested

to be characterized by oxidative stress [11, 12]. However, the specific pathologic mechanism has far from been elucidated.

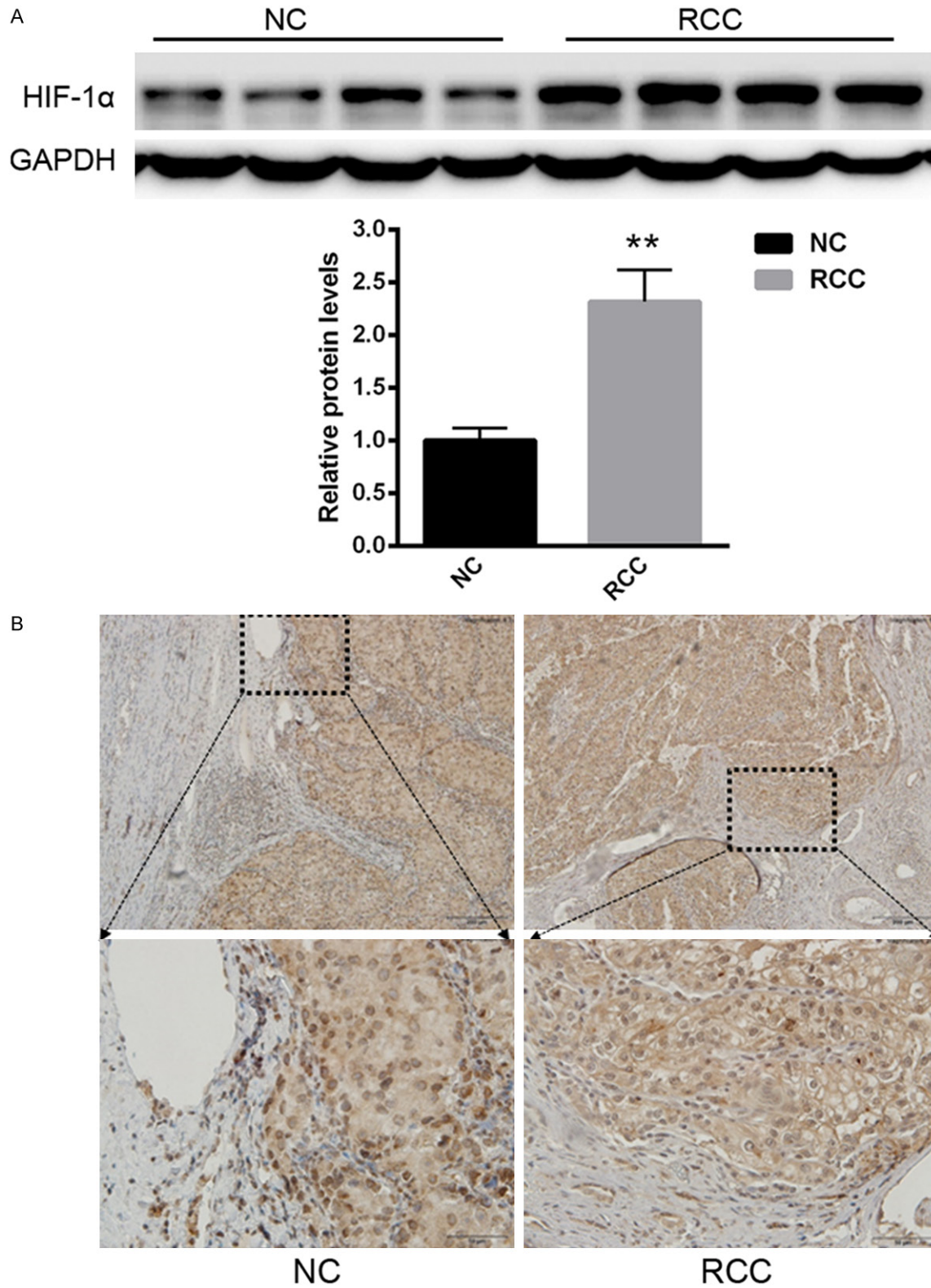
Garlic is widely used as food and remedy in China. It has been found that garlic is characterized by a variety of biological functions, such as anti-atherosclerotic, anti-hypertensive, anti-microbial, and anti-cancer roles [13]. Allicin is the major component of garlic, which is proved to inhibit cancer progression partially through relieving oxidative stress in gastric cancer, colon cancer, liver cancer and so on [14]. However, the mechanism by which allicin exerts its anti-oxidative function has not been fully elucidated. The present study offers new evidence showing that allicin suppresses RCC partially through inhibition of HIF-1 $\alpha$ .

## Materials and methods

### Patients

A sample of 40 patients receiving curative radical or partial nephrectomy with a histological

Allicin inhibits RCC through HIF signaling

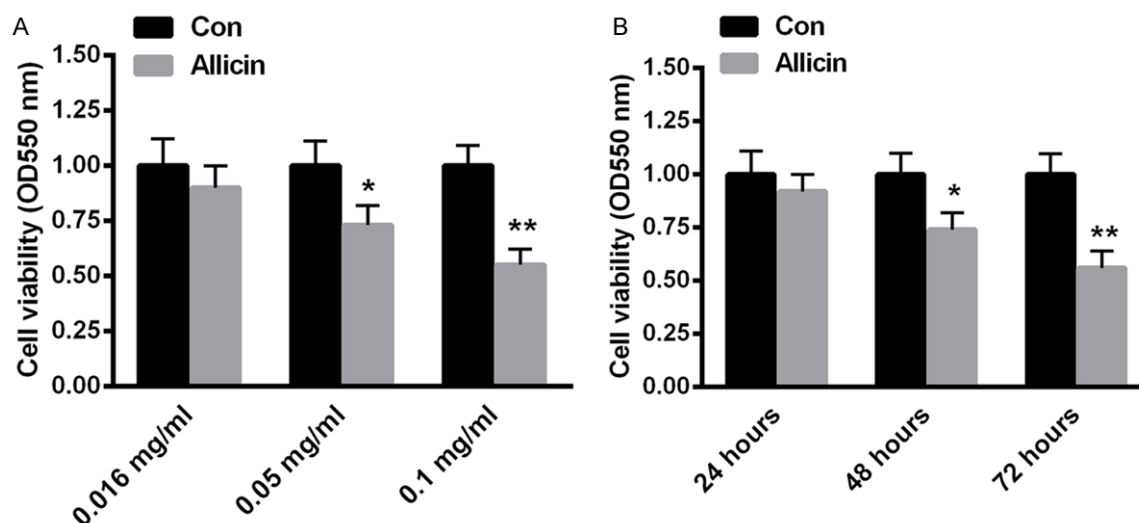


**Figure 1.** The protein level of HIF-1 $\alpha$  was increased in RCC tissues compared with normal tissues. A. Western blot analysis of HIF-1 $\alpha$  expression. B. IHC staining of HIF-1 $\alpha$  expression. \*\* $P < 0.01$  versus control.

diagnose of RCC at West China Hospital of Sichuan University were collected between

2012 and 2014. None of these patients received adjuvant therapy. And the normal tis-

## Allicin inhibits RCC through HIF signaling



**Figure 2.** RCC-9863 cell viability was affected by allicin in a dose- and time-dependent manner. RCC-9863 cells were exposed to allicin at the concentration of 0.016 mg/mL, 0.05 mg/mL, and 0.1 mg/mL for 48 h (A). (B) RCC-9863 cells were treated with 0.05 mg/mL allicin for 24 hours, 48 hours and 72 hours. Cell viability was determined by MTT assay using the spectrophotometer at 550 nm. Data represent the means  $\pm$  SEM, n=6 independent experiments. \*P<0.05, \*\*P<0.01 versus control.

sues were collected due to biopsy. Experiments involving human subjects are in accord with the Helsinki Declaration. The consent form was signed by all patients.

### Histological examination

Samples obtained from patients were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections of 4  $\mu$ m were stained with hematoxylin and eosin. Two senior pathologists performed the evaluations of the specimens.

### Cell culture

RCC-9863 Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 IU/ml) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Effect of allicin on cell proliferation

RCC-9863 cells were cultured in six-cell chamber at 60-70% confluent. And different concentrations (0.016 mg/ml, 0.05 mg/ml, 0.1 mg/ml) of allicin were added to the cells for 48 hours. To determine the time-dependent effect, RCC-9863 cells were treated with 0.05 mg/ml allicin for 24 h, 48 h and 72 h, respectively. MTT assay was performed as previously described [15].

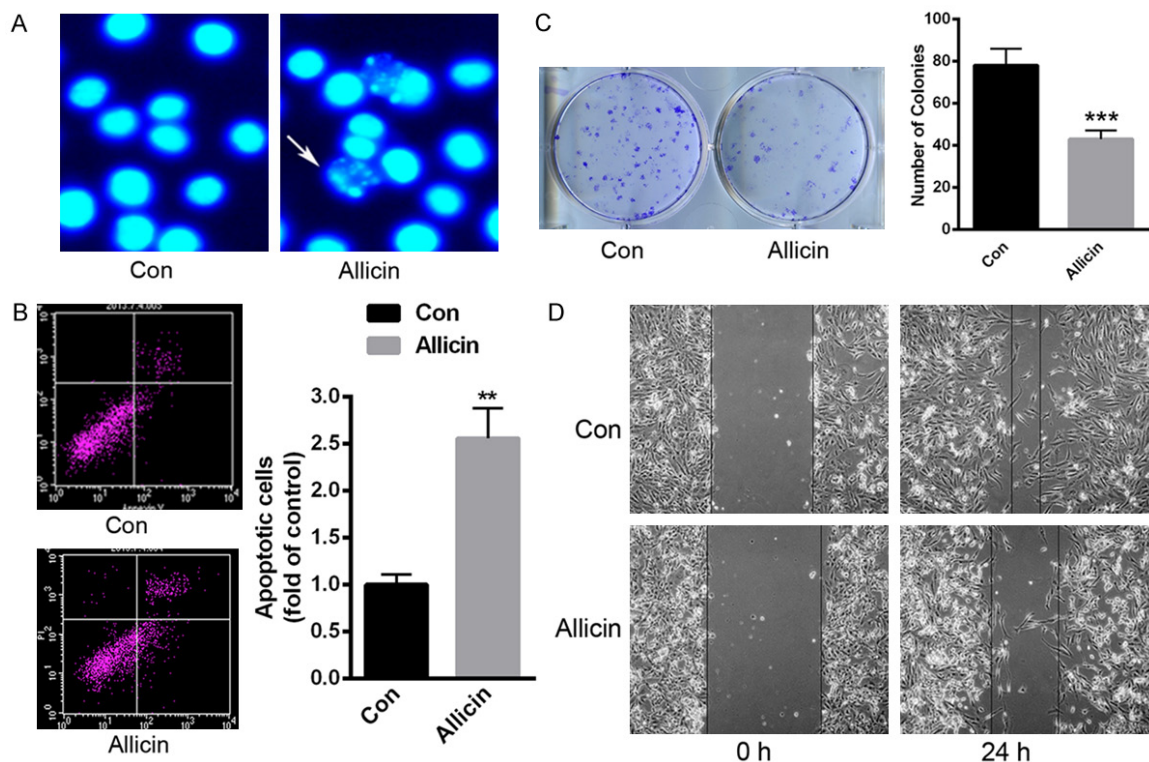
### Western blotting analyses

15 mg of RCC tissues was collected in RIPA buffer (SolarBio, Beijing, China) containing 1% (v/v) PMSF (SolarBio), 0.3% (v/v) protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation, the supernatant was collected and the protein concentration was determined using a BCA protein assay kit (Millipore, Billerica, MA, USA). Then, proteins were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was incubated with primary antibodies against HIF-1 $\alpha$ , Bcl-2, Bax, VEGF and GAPDH (Cell signaling, Danvers, MA, USA; 1:1000) overnight at 4°C. GAPDH was used as the internal control.

### Hoechst 33258 staining

RCC-9863 cells ( $1 \times 10^5$  cells per well) were cultured in six-well tissue culture plates. At 70-80% confluence, the cells were incubated for 16 h in serum-free RPMI-1640 medium. Then, 0.05 mg/ml allicin was added to the fresh medium and pre-incubated with the cells for 48 h. After drug treatment, the medium was removed, and the cells were fixed with 4% formaldehyde (Zhongshan Technology) in PBS for 20 min at room temperature. Then, the cells were washed three times with cold PBS and stained with Hoechst 33258 (10  $\mu$ g/mL) (Si-

## Allicin inhibits RCC through HIF signaling



**Figure 3.** RCC-9863 cell apoptosis was enhanced, while colony formation and cell migration was decreased when these cells were incubated with 0.05 mg/ml allicin for 48 h. A. Hoechst staining demonstrated the enhanced cell apoptosis. B. An annexin V and PI kit was applied to detect cell apoptosis. C. In vitro colony formation assay was performed to determine the number of colonies in RCC-9863 cells with 0.05 mg/ml allicin. D. The in vitro wound healing assay was conducted to explore cell migration in RCC-9863 cells with 0.05 mg/ml allicin. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

gma-Aldrich, St. Louis, MO, USA) for 5 min. After staining, the cells were further washed with cold PBS and examined under fluorescence microscope (Leica CM3000; Leica Microsystems GmbH).

### Quantification of apoptotic cells

An Annexin-V FITC-PI Apoptosis Kit (Invitrogen, Carlsbad, CA) was applied to determine cell apoptosis. Briefly, cells were washed with PBS (5 min/time, 3 times) and suspended at  $2-3 \times 10^6$  cells/ml in  $1 \times$  Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, respectively. Then, they were incubated at room temperature for 15 min in darkness. After incubation, the cells were filtered by a filter screen and the cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining.

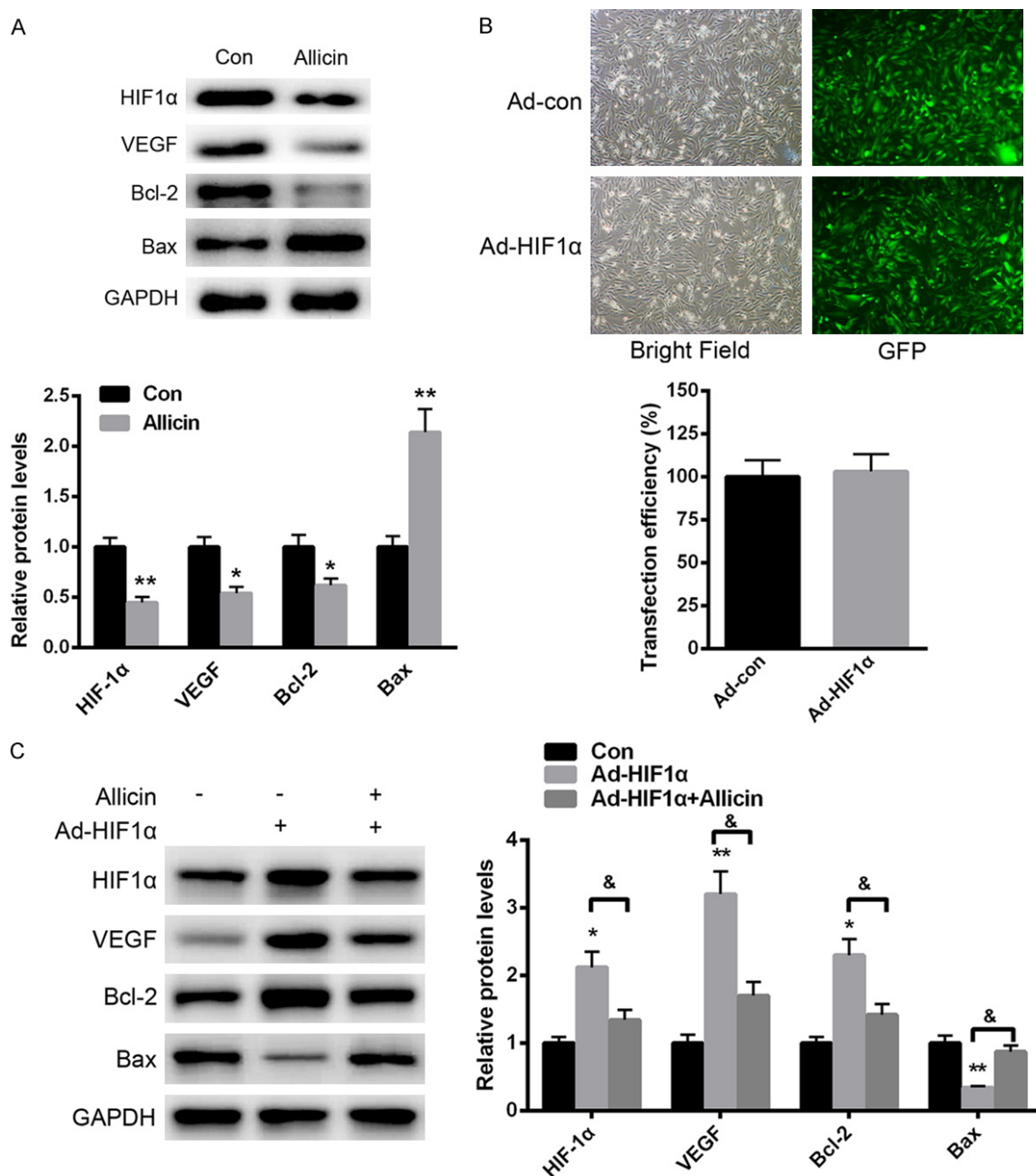
### In vitro colony formation assay

RCC-9863 cells that were treated with 0.05 mg/ml were seeded into 6-well plates at 60-70% confluent. The cells were cultured at  $37^\circ\text{C}$  for two weeks and then stained with 0.1% crystal violet for 10 minutes. After washing 3 times with PBS, the clones were counted, and the data represent the mean  $\pm$  standard deviation (SD) from experiments repeated three times.

### Cell migration assay

The in vitro wound healing assay was performed according to a published protocol [16]. Briefly, RCC-9863 cells were seeded in 6-well plates to form a confluent monolayer. The monolayer was scratched with a sterile 10  $\mu\text{l}$  pipette tip, and the floating cells were carefully removed by PBS. Then, the cells were cultured in RPMI-1640 medium without FBS at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. The wound scratches we-

## Allicin inhibits RCC through HIF signaling



**Figure 4.** Allicin suppressed RCC progression partially through HIF-1 $\alpha$ . **A.** Western analysis of HIF-1 $\alpha$  expression after allicin treatment. **B.** Transfection efficiency was determined in RCC-9863 cells transfected with ad-con or ad-HIF-1 $\alpha$ . **C.** Allicin treatment could significantly reverse HIF-1 $\alpha$  overexpression-induced VEGF and Bcl-2 upregulation. Data represent the means  $\pm$  SEM, n=3 independent experiments. \*P<0.05, \*\*P<0.01 versus control.

re photographed at 0 hour and 12 hours after scraping.

### Adenovirus vector construction

The adenovirus vector (Ad)-HIF-1 $\alpha$  and Ad-con were purchased from the Chinese National Human Genome Center (Beijing, China).

### Statistical analysis

The data were expressed as the mean  $\pm$  SEM. The number of independent experiments was represented by "n". Multiple comparisons were performed using one-way ANOVA followed by Tukey's multiple-comparison test, where P<0.05 was considered significant.

### Results

#### *Increased HIF-1 $\alpha$ protein level in RCC tissues*

First of all, we explored the protein levels of HIF-1 $\alpha$  in RCC tissues. As shown in **Figure 1A**, the protein level of HIF-1 $\alpha$  was increased in RCC tissues compared with normal tissues. Immunohistology assay also showed extensive HIF-1 $\alpha$  expression in RCC tissues.

#### *RCC-9863 cell viability was affected by allicin in a dose- and time-dependent manner*

To investigate the impacts of allicin on cell viability, RCC-9863 cells were treated with allicin at the concentration of 0.016 mg/ml, 0.05 mg/ml, 0.1 mg/ml for 48 h. Then, cell viability was analyzed by MTT assay. As shown in **Figure 2A**, cell viability of RCC-9863 cells was significantly decreased by about 35% when the concentration of allicin was 0.1 mg/ml. Furthermore, RCC-9863 cells were also treated with 0.05 mg/ml allicin for 24 hours, 48 hours and 72 hours, the corresponding cell viability was determined by MTT. According to the statistics, RCC-9863 cell viability was reduced by about 16% and 24% at 48 hours and 72 hours respectively (**Figure 1B**). These data suggested that RCC-9863 cell viability was significantly reduced by allicin in a dose- and time-dependent manner.

#### *Allicin enhanced RCC-9863 cells apoptosis and decreased colony formation, cell migration*

We further treated RCC-9863 cells with 0.05 mg/ml allicin for 48 h. Hoechst staining showed obvious apoptotic cell morphology (**Figure 3A**). Besides, flow cytometry assay was also conducted to determine cell apoptosis rate. As shown in **Figure 3B**, allicin enhanced cell apoptosis by over ~1.5 fold. In vitro colony formation assay demonstrated that the number of colonies was significantly decreased in RCC-9863 cells with 0.05 mg/ml allicin (**Figure 3C**). Moreover, the in vitro wound healing assay indicated that allicin could significantly decrease cell migration (**Figure 3D**). These data suggested the anti-tumor function of allicin in renal cancer cells.

#### *Allicin suppressed RCC progression partially through HIF-1 $\alpha$*

We further explored the specific molecular mechanism of allicin in RCC progression. As shown in **Figure 4A**, allicin treatment signifi-

cantly decreased the protein level of HIF-1 $\alpha$ , which is an important regulator in tumors. Moreover, the downstream signaling effectors, such as VEGF and Bcl-2, were increased, while the protein level of Bax was enhanced. Besides, an adenovirus vector overexpressing HIF-1 $\alpha$  was introduced into RCC-9863 cells. No significant difference was identified in cells transfected with ad-con or ad- HIF-1 $\alpha$  (**Figure 4B**). More importantly, we found that over expression of HIF-1 $\alpha$  significantly enhanced the expression of VEGF and Bcl-2, thereby prompting cell proliferation. In comparison, allicin treatment could significantly reverse HIF-1 $\alpha$  overexpression-induced VEGF and Bcl-2 upregulation (**Figure 4C**). These data indicated that allicin suppressed RCC progression partially through HIF-1 $\alpha$ .

### Discussion

Due to the high rate of recurrence and distant metastases in RCC, although the clinical treatment strategies and health surveillance have been greatly improved, the RCC prognosis remains unsatisfactory [17]. In the present study, we explored the protein level of HIF-1 $\alpha$  in RCC tissues. Compared with normal tissue, we found HIF-1 $\alpha$  expression was significantly enhanced. As the bulb of *Allium*, allicin is suggested to provide a new therapeutic method due to its potential anti-carcinoma characteristics [18]. Recent study suggests that allicin induces cancer cell apoptosis through the activation of Caspase-3, Caspase-8 and caspase-9 [19]. In addition, allicin is also characterized by anti-inflammatory and anti-oxidant functions [20, 21]. However, the correlation between allicin and HIF-1 $\alpha$  has never been explored.

As an oxygen-sensitive transcriptional factor, HIF mainly acts as a heterodimer including HIF-1 $\alpha$  and HIF-1 $\beta$  [22]. Under hypoxia, HIF-1 $\alpha$  was obviously increased and it was easily decreased under normoxia [23]. HIF-1 $\alpha$  significantly prompts the downstream gene expression, such as VEGF and EPO [24]. These factors play key roles in the maintenance of cell survival. In this study, we found that the expression of HIF-1 $\alpha$  was significantly increased in RCC tissues. More importantly, we found that allicin treatment significantly decreased HIF-1 $\alpha$  expression in RCC-9863 cells. Meanwhile, the downstream effectors, VEGF and Bcl-2 were also decreased. Besides, allicin treatment could partially reverse HIF-1 $\alpha$  overexpression induced effects, indicating the protective role of allicin.

## Allicin inhibits RCC through HIF signaling

MTT assay demonstrated that allicin reduced RCC-9863 cell viability in a time- and dose-dependent manner. Hoechst staining and flow cytometry analysis indicated that allicin induced RCC cell apoptosis, which suggested that allicin acts as an anti-tumor drug. We also demonstrated that allicin reduced the level of HIF-1 $\alpha$ , which is a biomarker for cancers due to the relative hypoxia environment. Further analysis revealed that allicin treatment also reduced the level of Bcl-2, an anti-apoptotic member. By controlling mitochondrial membrane channels, the decrease of Bcl-2 and the enhancement of Bax significantly lead to cancer cell apoptosis. Therefore, the ability of allicin to enhance the ratio of Bax/Bcl-2 suggests a potential pro-apoptotic function in RCC cancer cells.

To conclude, our results suggest that HIF-1 $\alpha$  expression was significantly increased in RCC tissues, while allicin leads to the reduction of HIF-1 $\alpha$  thereby inhibiting RCC cancer progression.

### Disclosure of conflict of interest

None.

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