

Downregulation of Keap1 contributes to poor prognosis and Axitinib resistance of renal cell carcinoma via upregulation of Nrf2 expression

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Abstract. Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) signaling has a protective effect on normal cells. A number of previous studies demonstrated that Keap1/Nrf2 signaling is associated with drug resistance in numerous tumors. The aim of the present study was to investigate the roles of Keap1 in renal cell carcinoma (RCC) and its effect on sensitivity to chemotherapy. Reverse transcription-quantitative polymerase chain reaction was used to detect the mRNA expression of Keap1 in 45 cases of RCC tumors and adjacent normal tissues. A total of five randomly selected patients with RCC, five RCC cell lines and normal renal tubular cells were examined to detect the protein and mRNA expressions of Keap1. The 5-year survival rate was analyzed by Kaplan-Meier analysis. The cell viability was assessed by a Cell Counting kit-8 assay. The cell apoptosis and reactive oxygen species (ROS) were determined by flow cytometry. The expressions of associated proteins were determined by western blot analysis. It was identified that in RCC tissues and RCC cell lines, the expression of Keap1 was downregulated, which was considered to be associated with poor prognosis. In total, 1 μ M Axitinib significantly decreased cell viability, promoted ROS release and induced cell apoptosis in ACHN cells. Silencing Keap1 was able to reverse the inhibitory effect of Axitinib and enhance the protein expressions of Nrf2, NAD(P)H dehydrogenase [quinone] 1 and heme oxygenase 1. However, silencing Nrf2 increased the cell sensitivity to Axitinib. Under Axitinib condition, overexpressing Nrf2 was able to increase cell

viability; however, overexpressing Keap1 resulted in an opposite effect. Keap1 serves as a tumor suppressor; its low expression was associated with poor prognosis and a decreased sensitivity of RCC cells to Axitinib. A possible mechanism underlying Axitinib resistance may involve Nrf2 overexpression.

Introduction

Renal cell carcinoma (RCC) is a common malignant tumor in the urinary system. Among all the malignant tumors of the urinary system, the incidence rate of RCC is third following prostate cancer and bladder cancer, accounting for 2.7% of male malignant tumors worldwide in 2018 (1). Annually, there are >200,000 novel diagnosed cases of renal cancer and >100,000 mortalities occur worldwide. According to the latest statistics of the American Cancer Society, the morbidity and mortality of RCC in the last two years were among the leading 10 types of malignant tumors with highest morbidity and mortality (2,3). The morbidity and mortality of male patients with RCC are significantly higher compared with women (2,3). In recent years, with the emergence of molecular targeted drugs, the prognosis of patients with RCC has significantly improved; the clinical effect of molecular targeted drugs has been fully tested (4,5). Axitinib, a second generation vascular endothelial growth factor receptor inhibitor approved by the US Food and Drug Administration in 2012, is a small molecular tyrosine kinase inhibitor and a multi-target drug used for patients with advanced RCC that are not responding to other systemic therapies (6,7). Axitinib selectively acts on multiple targets, blocking the proliferation of tumor cells by blocking specific targets (8,9).

As a cytoplasmic protein, Kelch-like ECH-associated protein 1 (Keap1) is an important regulatory factor of the oxidative stress signaling pathway. It has five protein domains, which are in the N-terminal region (Broad complex, Tramtrack, Bric-a-Brac, linker intervening region and Kelch domain) and a C-terminal region (10). Numerous previous studies demonstrated that the expression of Keap1 in tumor tissues was significantly different compared with adjacent tissues. In particular, the Keap1 gene was inactivated or mutated at

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different structural sites in cancer tissues of patients with lung, ovarian and liver cancer (11-13). In a previous clinical analysis, it was identified that low expression of Keap1 was associated with a high mortality in patients with ovarian cancer (14).

The specific mechanism of Keap1 regulating tumorigenesis and development remains unclear. However, it was hypothesized that the progression of tumor is affected by three different ways; Keap1/nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, Keap1/inhibitor of nuclear factor κ -B kinase subunit b/nuclear factor- κ B signaling and Keap1/B cell lymphoma-2 (15-21). Keap1 forms a complex with cullin-3 (a ubiquitin ligase E3), which subsequently binds to a key transcription factor of cellular oxidative stress, Nrf2. It ubiquitinates and degrades Nrf2, thus regulating the Nrf2-antioxidant responsive element (ARE) signaling pathway and affecting anti-oxidation protein and phase II detoxification enzyme expression, which in turn affects tumor cell proliferation, apoptosis and sensitivity to chemotherapy drugs and radiation therapy (22-26). The aim of the present study was to investigate the effect of Keap1 on Axitinib resistance of RCC.

Patients and methods

Patients and tissue samples. All tissue specimens were collected from patients with RCC admitted to The Second Affiliated Hospital of Guangxi Medical University (Nanning, China) between May 2015 and April 2016. It was confirmed that all patients were diagnosed with RCC by pathology. All patients that were treated for the first time for renal tumor, clear cell RCC, papillary RCC, chromophobe RCC, multilocular cystic RCC and renal medullary carcinoma were included in the present study. All patients who had received preoperative radiotherapy, chemotherapy and cancer drug therapy were excluded from the present study. A portion of the RCC tissues and adjacent normal tissues were stored in 4% formaldehyde solution for pathological diagnosis routinely, and the rest were stored in a liquid nitrogen tank to perform reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. In total, 45 cases of RCC and corresponding adjacent tissues were analyzed. The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Guangxi Medical University. All tissue samples were obtained with written informed consent from the patients.

Cell culture. Normal human renal tubular epithelial cells (HK-2 cells) and five RCC cell lines (ACHN, 769-P, A-498, 786-0 and Caki-1) were obtained from China Union Medical University Basic Medical Cell Center (China). The cells were cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 100 μ /ml penicillin and 100 μ g/ml streptomycin, and placed in an incubator with 5% CO₂ at 37°C. When confluence reached 70-80%, the cells were digested and subcultured.

Cell Counting kit-8 (CCK-8). Cells were plated into 96-well plates at a density of 1x10⁴ cells/well for 24 h. Subsequent to the cells being treated with different concentrations (0.1, 1, 10, 50 and 100 μ M) of Axitinib (cat. no. PZ0193; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or transfected at 37°C,

10 μ l CCK-8 solution (Invitrogen; Thermo Fisher Scientific, Inc.) was added in each well and incubated for another 3 h at 37°C. Cell viability was determined by recording the optical density at a test wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Cell transfection. ACHN cells were seeded in a 6-well plate (1x10⁵) for 24 h prior to transfection. Small interfering (si)Keap1, siNrf2, overexpressing Keap1, overexpressing Nrf2 and empty control plasmids were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The sequence of siKeap1 was 5'-GAATGATCACAGCAATGAA-3'; the sequence of siNrf2 was 5'-GGTTGCCACATTCCCAAATC-3'; the sequence for overexpressing Keap1 was 5'-ATACTCGAGATGCAGCCAGATCCCAGGCC-3'; the sequence for overexpressing Nrf2 was 5'-GTACTAGTATGATGGACTTGGAGTT-3'; and the NC sequence was 5'-GTTCTCCGAACGTGTCACGT-3'. Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to perform the transient transfection, according to the manufacturer's protocol. In total, 2 μ g si/overexpressing RNA, negative control (NC) and Lipofectamine[®] 2000 were added to Opti-Minimum Essential Medium (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 25°C for 20 min. Lipofectamine[®] 2000 was subsequently mixed into each well, which was cultured in Opti-MEM RPMI 1640. After 6 h of culturing, the fluid was replaced with RPMI 1640 medium containing 10% FBS.

Flow cytometry. The collected cells were stained with Annexin V-fluorescein isothiocyanate and propidium iodide (cat. no. CA1020; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 4°C for 15 min. Within 1 h, a flow cytometer and analysis software (BD CellQuest[™] Pro Software; version 1.2; BD Biosciences, San Jose, CA, USA) was used to assess cell apoptosis.

The reactive oxygen species (ROS) level was detected by 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were treated with Axitinib or siKeap1 as previously described and 2x10⁶ cells/ml were seeded into a 6-well plate. In total, 10 μ M DCFH-DA with 1 ml PBS were added to cells at 37°C for 20 min. DCF fluorescence was determined using a flow cytometer and the data were analyzed using Summit Software v4.3 (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA).

RT-qPCR. Total RNA from tissues or cultured cells were obtained using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using GoScript[™] RT kit (Promega Corporation, Madison, WI, USA). The RT temperature protocol consisted of 37°C for 15 min and at 85°C for 5 sec. RT-qPCR was conducted using SYBR Fast qPCR Mix (Invitrogen; Thermo Fisher Scientific, Inc.) for Keap1 and GAPDH. The primer sequences used were as follows: Keap1: Sense, 5'-CTGGAGGATCATACC AAGCAGG-3' and antisense, 5'-GGATACCTCAATGG ACACCAC-3'; Nrf2 sense, 5'-CAACTCAGCACCTTGT ATC-3' and antisense, 5'-TTCTTAGTATCTGGCTT CTT-3'; GAPDH sense, 5'-GAAGGTGAAGGTCGGAGTC-3'

and antisense, 5'-GAAGATGGTGATGGGATTTTC-3'. The cycling parameters were set as: 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 30 sec. Subsequent to completing PCR amplification, comparative quantification was determined using the $2^{-\Delta\Delta C_q}$ method (27). Primers were purchased from BioSune Biotechnology (Shanghai, China).

Western blot analysis. Total protein was extracted from tissues or cultured cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Protein concentrations were quantified using a bicinchoninic acid protein kit (Beyotime Institute of Biotechnology). Aliquots of protein (30 μ g) were separated by 12% SDS-PAGE, and resolved proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% milk PBS with 0.1% Triton X-100 at 4°C for 2 h, and incubated with different primary antibodies at 4°C overnight as follows: Anti-Keap1 antibody (cat. no. ab119403; 1:1,000; Abcam, Cambridge, UK), anti-Nrf2 antibody (cat. no. ab156883; 1:1,000), anti-Lamin B1 antibody (cat. no. ab133741; 1:1,000), anti-NAD(P)H dehydrogenase [quinone] 1 (NQO1) antibody (cat. no. ab28947; 1:1,000), anti-heme oxygenase 1 (HO1) antibody (cat. no. ab13243; 1:2,000), anti-phosphorylated (p)-extracellular signal-regulated kinase (ERK) antibody (cat. no. ab223500; 1:400), anti-ERK antibody (cat. no. ab17942; 1:1,000) and anti-GAPDH antibody (cat. no. ab9485; 1:2,500; all Abcam). The membranes were subsequently incubated with appropriate horse-radish peroxidase-conjugated secondary antibodies (anti-rabbit; cat. no. ab205718; 1:2,000 and anti-mouse; cat. no. ab205719; 1:5,000) for 2 h at room temperature. Protein bands were detected with enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and visualized using Quantity One software, version 4.6.9 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Statistical analysis was detected by Prism GraphPad version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The correlation between Keap1 expression and the survival was analyzed using the Kaplan-Meier method. All data are presented as the mean \pm standard deviation from three independent experiments. χ^2 test was used to analyze the discontinuous data. Differences were performed using one-way analysis of variance and Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Keap1 is downregulated in RCC tissues and is associated with a poor survival rate. A total of 45 patients with RCC were recruited in the present study and the mRNA expressions of Keap1 in cancer and adjacent normal tissues were detected. The result demonstrated that Keap1 mRNA expressions were lower in cancer tissues compared with normal paired tissues ($P = 0.001$; Fig. 1A). The association between Keap1 expression in RCC and patient survival was analyzed, and it was identified that low Keap1 expression was associated with a poor 5-year survival rate ($P = 0.140$; Fig. 1B). A total of five patients with RCC were subsequently randomly selected to assess the expressions of Keap1 at the protein and mRNA expression level. The western blot analysis and RT-qPCR demonstrated that the expressions of Keap1 significantly decreased in three

Table I. Analysis between the clinical features and expression of Keap1 in patients with renal cell carcinoma.

Characteristics	n	Keap1 expression		P-value
		Low group	High group	
Age, years		60 \pm 5.2	59 \pm 6.2	0.560
Sex				0.556
Male	22	10	12	
Female	23	13	10	
Location				0.554
Left	24	11	13	
Right	21	12	9	
T stage				0.514
T1	32	17	15	
T2	12	5	7	
T3	1	1	0	
T4	0	0	0	
Recurrence				0.003
No	22	6	16	
Yes	23	17	6	

Keap1, Kelch-like ECH-associated protein 1.

patients with cancer ($P < 0.01$; Fig. 1C and D); however, an increased expression of Keap1 was detected in specific patients with cancer suggesting that the phenomenon may be affected by individual differences or different subtypes of RCC. The patients were divided into high and low Keap1 expression group based on the median value (0.36) of the relative Keap1 mRNA expression. The association between Keap1 expression and pathological classification data is presented in Table I. The low expression of Keap1 was identified to be associated with the recurrence of RCC.

Keap1 is downregulated in RCC cell lines. The expression of Keap1 in normal human renal tubular epithelial cells (HK-2 cells) and five RCC cell lines (ACHN, 769-P, A-498, 786-0 and Caki-1) was detected. All RCC cells demonstrated a downregulation of Keap1 protein ($P < 0.01$; Fig. 1E) and mRNA expression ($P < 0.01$; Fig. 1F), compared with normal HK-2 cells.

Inhibitory effect of Axitinib on RCC cell lines. In order to test the effect of Axitinib on RCC cell lines, a CCK-8 assay was performed and different concentrations (0.1, 1, 10, 50 and 100 μ M) of Axitinib were used to treat two of the five RCC cell lines, ACHN and 769-P cells, which were randomly selected, at 37°C for 24 and 48 h. In ACHN and 769-P cells, it was observed that Axitinib decreased the cell viability in a dose-dependent manner (Fig. 2). In ACHN cells, 1 μ M Axitinib was able to significantly attenuate cell viability for 24 h of treatment ($P < 0.05$; Fig. 2A) and 0.1 μ M Axitinib treatment for 48 h was able to significantly decrease cell viability ($P < 0.05$). In 769-P cells, treatment with 0.1 μ M Axitinib for 24 and 48 h significantly decreased cell viability ($P < 0.05$; Fig. 2B). The

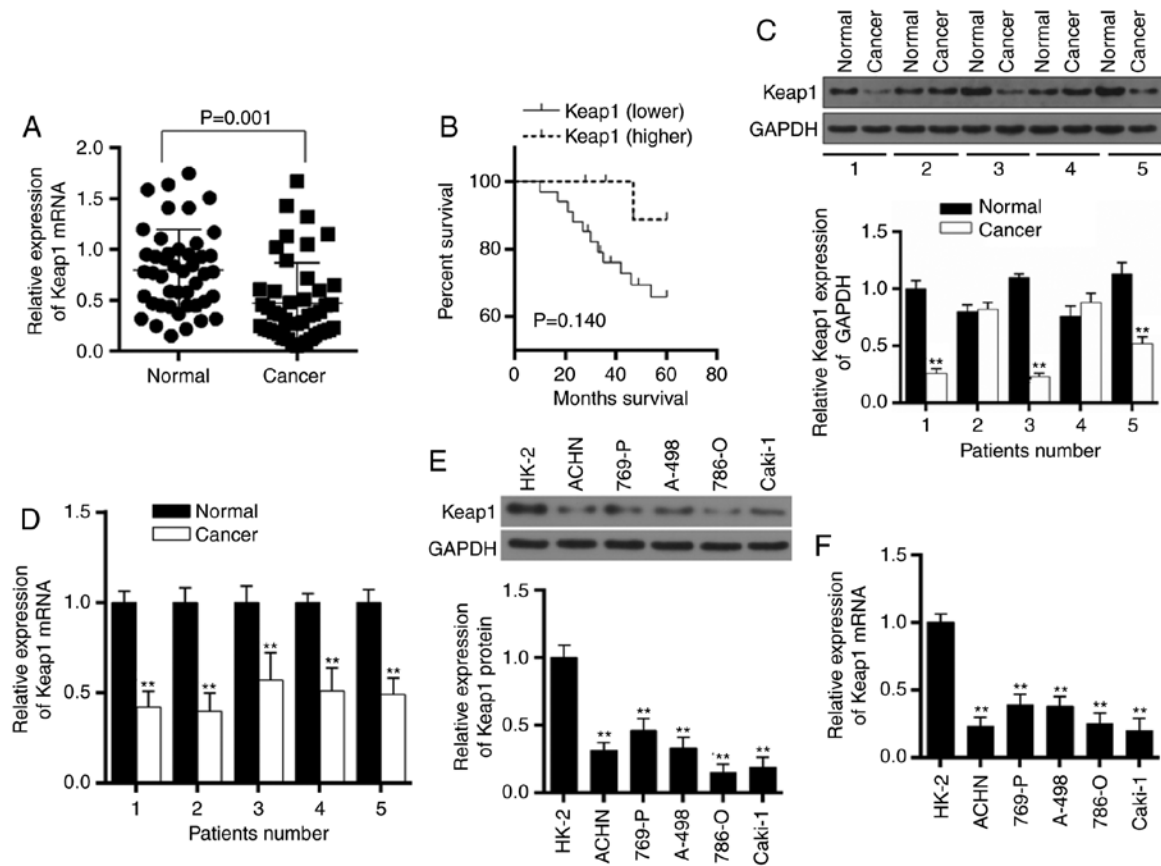


Figure 1. Low expression of Keap1 in RCC is associated with a poor survival rate. (A) In total, 45 cases of patients with RCC were analyzed to determine the mRNA expression of Keap1 in normal and cancer tissues ($P=0.001$). (B) Kaplan-Meier survival curve was determined to assess the association between the expression of Keap1 and survival rate ($P=0.140$). A total of five patients with RCC were randomly selected to assess the expression of Keap1 at the (C) protein and (D) mRNA level. GAPDH served as an internal control. Data are presented as the mean \pm standard deviation from three independent experiments. $^{**}P<0.01$ vs. respective normal RCC tissue. (E) Western blotting was used to detect the protein expression in normal human renal tubular epithelial cells (HK-2 cells) and five RCC cell lines (ACHN, 769-P, A-498, 786-O and Caki-1). (F) Reverse transcription-quantitative polymerase chain reaction was used to detect the mRNA expression in normal human renal tubular epithelial cells and five RCC cell lines. GAPDH served as an internal control. Data are presented as the mean \pm standard deviation from three independent experiments. $^{**}P<0.01$ vs. HK-2 cells. Keap1, Kelch-like ECH-associated protein 1; RCC, renal cell carcinoma.

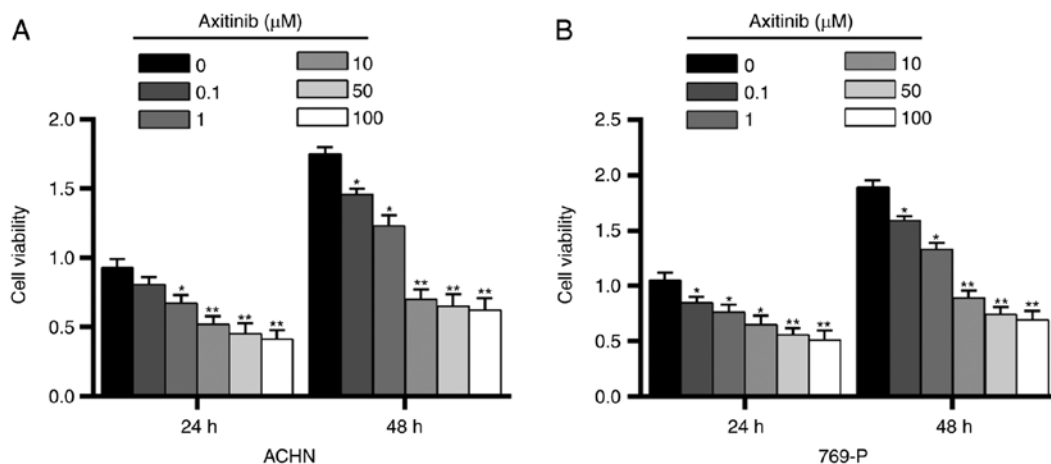


Figure 2. Different concentrations of Axitinib are administered to renal cell carcinoma cell lines. Cell Counting kit-8 was used to detect the effect of different concentrations (0.1, 1, 10, 50 and 100 μ M) of Axitinib on (A) ACHN and (B) 769-P cells for 24 and 48 h. Data are presented as the mean \pm standard deviation from three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. respective 0 μ M Axitinib.

results suggested that Axitinib had a similar inhibitory effect in ACHN and 769-P cells. Therefore, one cell line, ACHN, was randomly selected and 1 μ M Axitinib was selected for subsequent experimentation.

Transfection efficiencies of Keap1 and Nrf2 on ACHN cells. The transfection efficiencies of Keap1 and Nrf2 were assessed by RT-qPCR. It was demonstrated that the mRNA expressions of Keap1 ($P<0.01$; Fig. 3A) were downregulated by transfecting

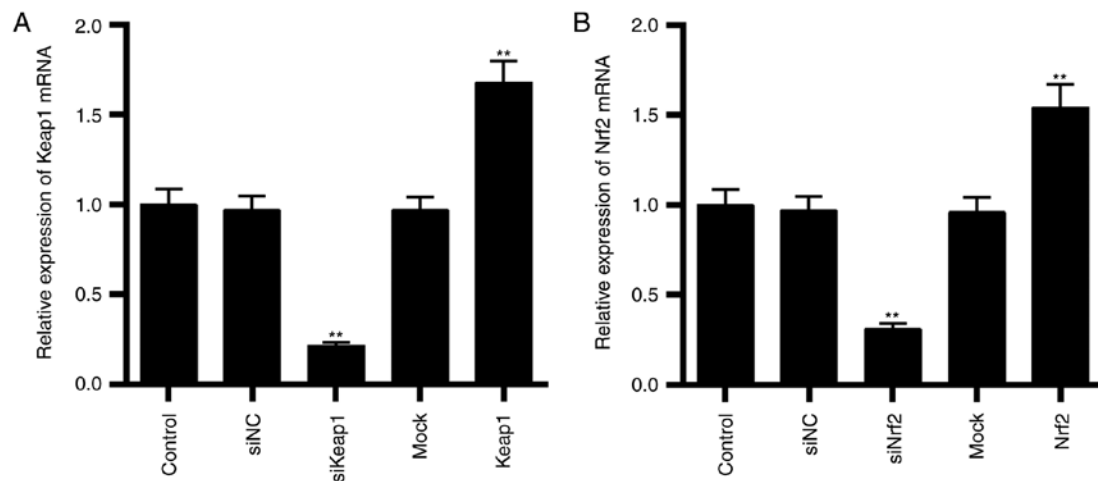


Figure 3. Transfection efficiencies of Keap1 and Nrf2 in ACHN cells. (A) Keap1 was silenced and overexpressed in cells and the mRNA expression of Keap1 was determined by RT-qPCR. (B) Nrf2 was silenced and overexpressed in cells and the mRNA expression of Nrf2 was determined by RT-qPCR. Data are presented as the mean \pm standard deviation from three independent experiments. ** $P < 0.01$ vs. control. Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; si, small interfering; NC, negative control.

cells with siKeap1 and were upregulated by overexpressing Keap1 ($P < 0.01$; Fig. 3A). As presented in Fig. 3B, the mRNA expressions of Nrf2 ($P < 0.01$; Fig. 3B) were downregulated by transfecting cells with siNrf2 and were upregulated by overexpressing Nrf2 ($P < 0.01$; Fig. 3B).

Silencing Keap1 attenuates the inhibitory effect of Axitinib on ACHN cells. As presented in Fig. 4A, treatment with 1 μ M Axitinib was able to significantly decrease cell viability, compared with the control ($P < 0.01$; Fig. 4A); however, silencing Keap1 demonstrated an opposite result that cell viability was significantly increased compared with the Axitinib group ($P < 0.01$). Cell viability was increased by siKeap1 alone compared with treatment with Axitinib alone ($P < 0.05$). The result suggested that silencing Keap1 attenuated the inhibitory effect of Axitinib on ACHN cells.

Silencing Keap1 decreases the ROS level induced by Axitinib in ACHN cells. Keap1 is known to be associated with oxidative stress (28). Therefore, the ROS level was detected by flow cytometry. The ROS level was significantly increased with treatment with Axitinib in ACHN cells compared with the control cells ($P < 0.01$; Fig. 4B and C), suggesting that Axitinib had a damaging effect on RCC cells. However, Axitinib in combination with silencing Keap1 significantly decreased the ROS level compared with Axitinib treatment alone ($P < 0.01$; Fig. 4B).

Silencing Keap1 enhances the expression of Nrf2 and promotes ERK signaling. In order to understand how siKeap1 affects ACHN cell sensitivity to Axitinib, the protein expression of Nrf2, and downstream target genes of Nrf2 and ERK signaling were detected. It was identified that treatment with Axitinib was able to promote the protein expression of Nrf2 ($P < 0.01$; Fig. 4D) and downregulate the expression of Keap1 ($P < 0.01$; Fig. 4E) compared with the control cells. Silencing Keap1 significantly promoted the effect of Axitinib by further upregulating the expression of Nrf2 protein ($P < 0.01$; Fig. 4D). When silencing Keap1 was combined with treatment with Axitinib, the expression of Keap1 protein was further

downregulated and was significantly decreased compared with treatment with Axitinib alone ($P < 0.05$; Fig. 4E). The downstream target proteins of Nrf2 demonstrated a similar result to that of Nrf2 protein. As presented in Fig. 4F, treatment with Axitinib significantly increased the protein expressions of NQO1 and HO1 compared with the control ($P < 0.01$). Furthermore, silencing Keap1 enhanced the effect of Axitinib in increasing NQO1 and HO1 protein ($P < 0.05$; Fig. 4F). The protein expressions of p-ERK and ERK were detected. The result demonstrated that the expression of ERK had no marked differences among the control, Axitinib, NC+ Axitinib and siKeap1+ Axitinib groups, and groups that were treated with Axitinib demonstrated significant downregulated expressions of p-ERK/ERK, compared with the control ($P < 0.05$; Fig. 4F). However, in the siKeap1 group, the protein expression of p-ERK/ERK demonstrated a significant increase compared with the treatment with Axitinib alone group ($P < 0.05$).

Co-transfection of siKeap1 and siNrf2 affects the protein expression of Nrf2 and Keap1. It was identified that transfection with siKeap1 and siNrf2 alone resulted in the downregulation of the respective mRNA and protein expressions ($P < 0.01$; Fig. 3 and Fig. 5A). To further investigate the mechanism underlying the effect by Keap1, Keap1 and Nrf2 were silenced. Silencing Nrf2 in combination with Axitinib significantly decreased the protein expression of Nrf2, compared with co-transfection of siKeap1 and siNrf2, and treated with Axitinib ($P < 0.05$). It was observed that co-transfection of siKeap1 and siNrf2 resulted in a similar effect on the Keap1 protein expression compared with siKeap1 alone ($P > 0.05$; Fig. 5B). It was demonstrated that silencing Nrf2 only significantly increased the protein expression of Keap1 compared with the siNrf2+Axitinib group ($P < 0.01$).

Co-transfection of siKeap1 and siNrf2 affects the cell viability and cell apoptosis in ACHN cells. Co-transfection of siKeap1 and siNrf2 was able to decrease the increased cell viability that was induced by silencing Keap1+Axitinib ($P < 0.05$; Fig. 5C). Furthermore, silencing Nrf2 enhanced the ACHN cell injury

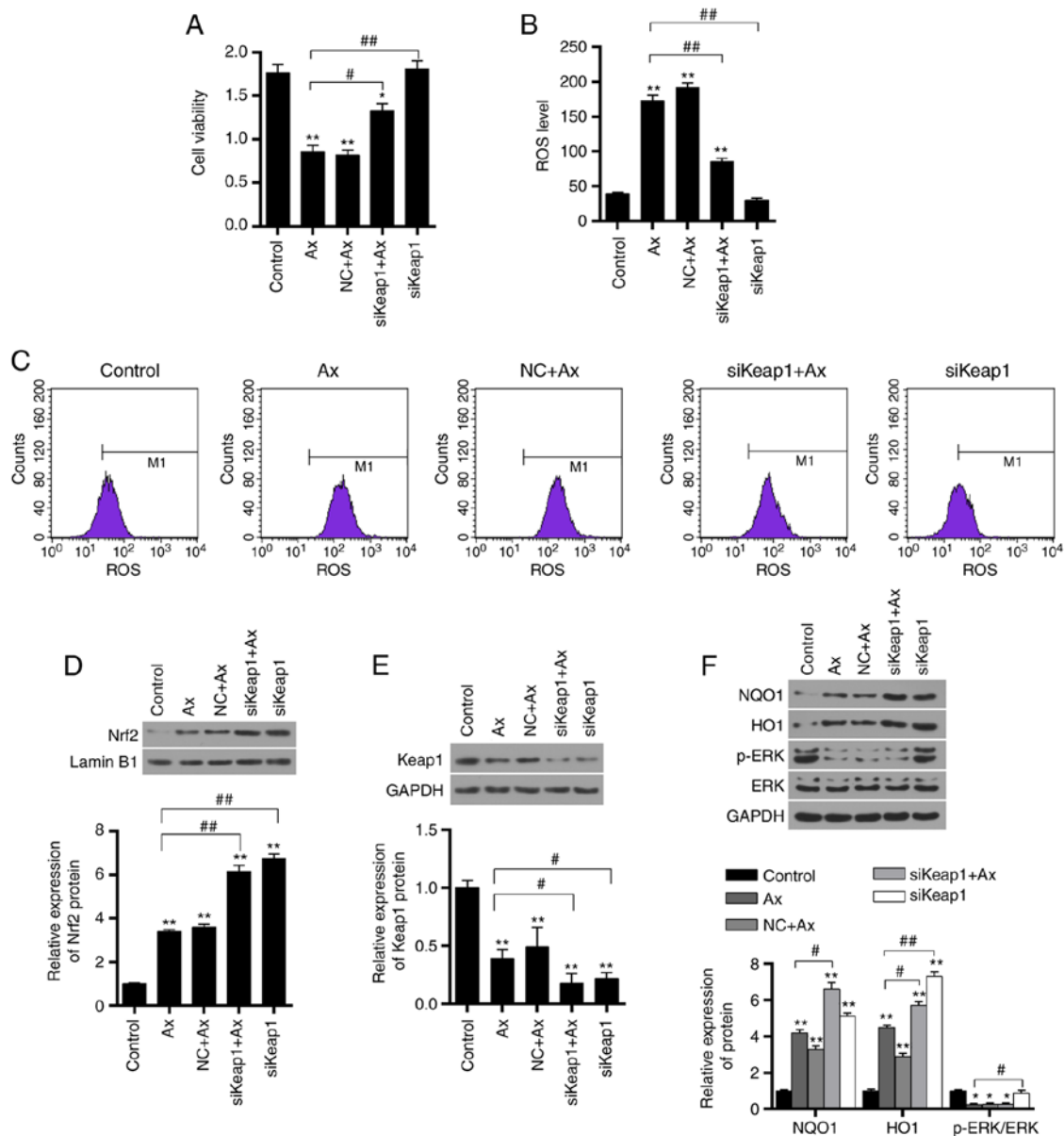


Figure 4. Effect of silencing Keap1 on the cell viability, ROS level and protein expression of Nrf2 and ERK signaling. ACHN cells were transfected with siKeap1 and divided into five groups, which were the control, Ax (1 μ M Ax), NC+Ax, siKeap1+Ax and siKeap1 groups. (A) Cell viability was determined by a Cell Counting kit-8 assay. (B) ROS levels are presented in a bar graph. (C) Flow cytometry was used to detect the ROS level. (D) Nrf2 protein expression was detected by western blotting. Lamin B1 served as an internal control. (E) Keap1 protein expression was detected by western blotting. GAPDH served as an internal control. (F) NQO1, HO1, p-ERK and ERK protein expressions were detected by western blotting. GAPDH served as an internal control. Data are presented as the mean \pm standard deviation from three independent experiments. * P <0.05, ** P <0.01 vs. control; # P <0.05, ## P <0.01. Keap1, Kelch-like ECH-associated protein 1; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; ERK, extracellular signal-regulated kinase; Ax, Axitinib; NC, negative control; si, small interfering; NQO1, anti- NAD(P)H dehydrogenase [quinone] 1; HO1, heme oxygenase 1; p-, phosphorylated.

induced by Axitinib (P <0.05). However, silencing Nrf2 alone significantly increased the cell viability compared with the siNrf2+Axitinib group (P <0.01). It was additionally observed that treatment with Axitinib alone was able to significantly increase cell apoptosis, compared with the control (P <0.01; Fig. 5D and E) and that silencing Keap1 was able to significantly decrease the apoptosis induction by Axitinib (P <0.01). However, apoptosis was significantly increased by siNrf2 in cells transfected with siKeap1 (P <0.01).

Overexpressing Nrf2 reverses the decrease of cell viability induced by Axitinib. The transfection efficiency of the overexpression plasmid of Nrf2 was assessed and it was demonstrated

that the mRNA expression of Nrf2 was upregulated by overexpressing Nrf2 mRNA transfected in cells (P <0.01; Fig. 4B). Furthermore, the effect of overexpressing Nrf2 on ACHN cells was detected. Nrf2 overexpression was observed to significantly increase the Nrf2 protein expression induced by Axitinib (P <0.05; Fig. 6A). Under treatment with Axitinib, overexpressing Nrf2 did not influence the expression of Keap1 protein (P >0.05; Fig. 6B). Overexpressing Nrf2 in combination with Axitinib significantly increased cell viability, compared with the treatment with Axitinib alone (P <0.05; Fig. 6C).

Overexpressing Keap1 enhances cell sensitivity to Axitinib. The transfection efficiency of the overexpression of Keap1

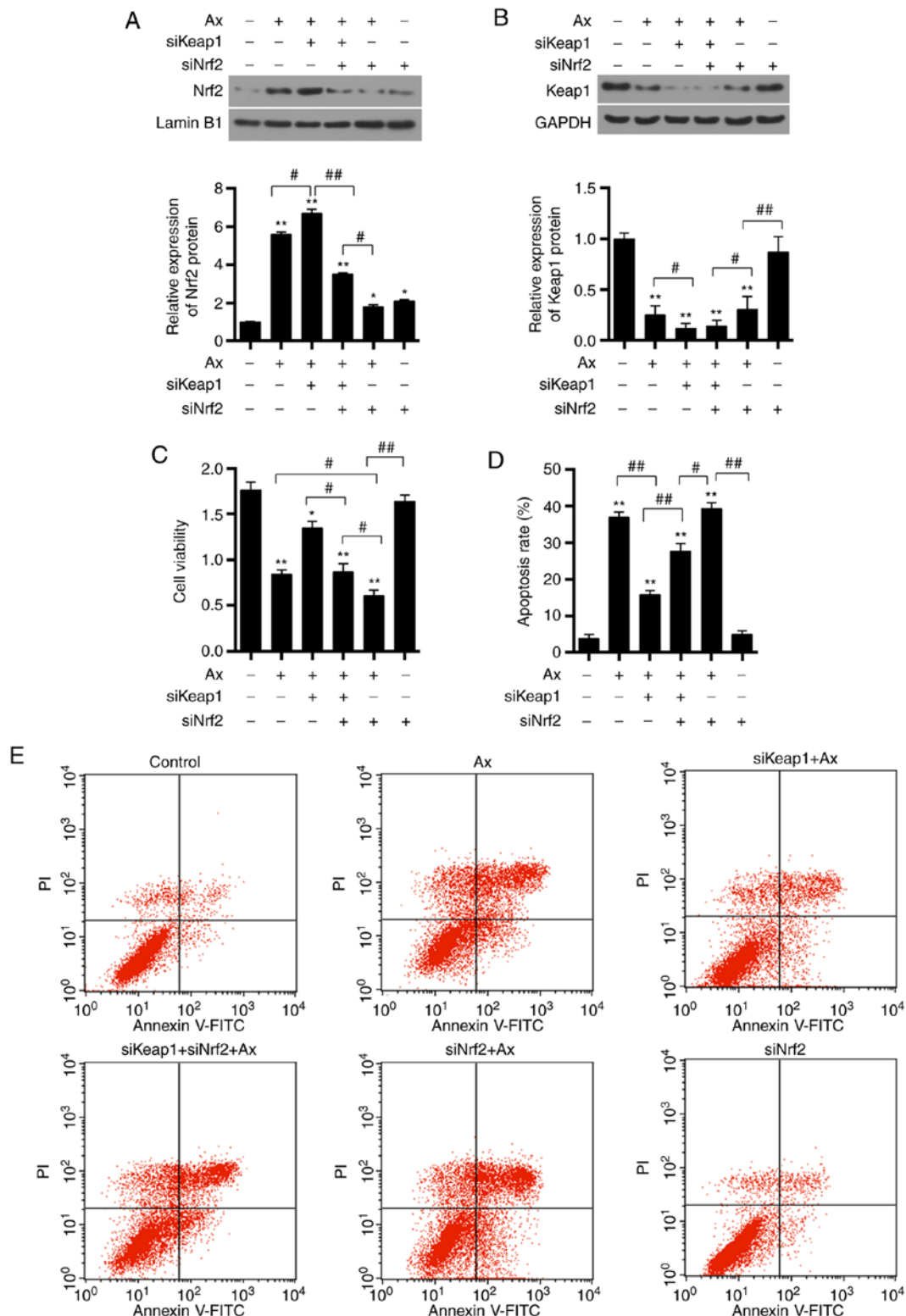


Figure 5. Effect of co-transfection of siKeap1 and siNrf2 on protein expression of Nrf2 and Keap1, cell viability and apoptosis. ACHN were transfected with siKeap1 and siNrf2 and were divided into six groups, which were the control, Ax (1 μ M Ax), siKeap1+Ax, siKeap1+siNrf2+Ax, siNrf2+Ax and siNrf2 groups. (A) Nrf2 protein expression was detected by western blotting. Lamin B1 served as an internal control. (B) Keap1 protein expression was detected by western blotting. GAPDH served as an internal control. (C) Cell viability was assessed by a Cell Counting kit-8 assay. (D) Apoptosis rate is presented as a bar graph. (E) Flow cytometry was used to detect the apoptosis level. Data are presented as the mean \pm standard deviation from three independent experiments. * P <0.05, ** P <0.01 vs. control; # P <0.05, ## P <0.01. Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; Ax, Axitinib; si, small interfering; PI, propidium iodide; FITC, fluorescein isothiocyanate.

was assessed and it was demonstrated that the mRNA expression of Keap1 was upregulated by overexpressing Keap1 mRNA transfected in cells (P <0.01; Fig. 4A). As presented

in Fig. 7A, treatment with Axitinib significantly increased the protein expression of Nrf2, compared with the control (P <0.01; Fig. 7A). Overexpressing Keap1 significantly

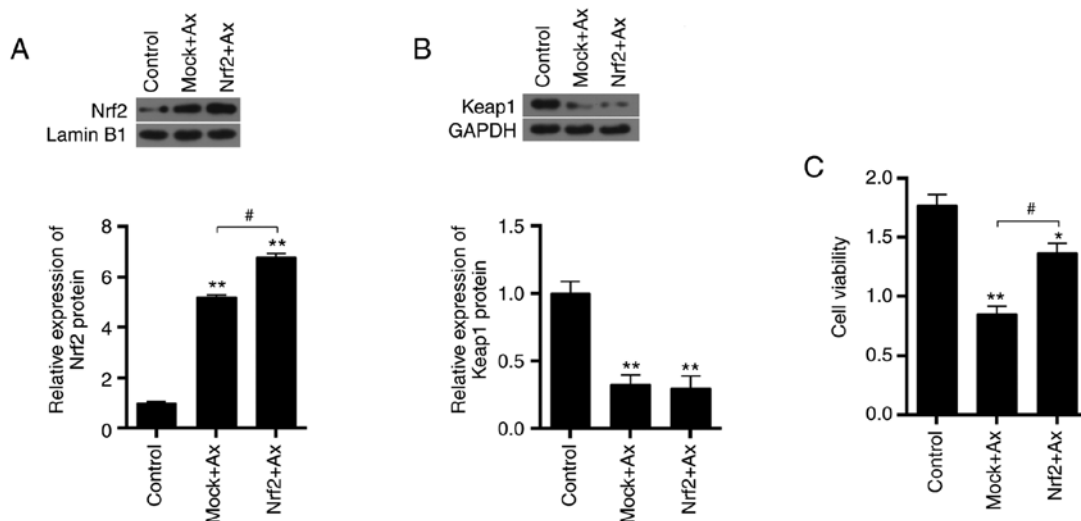


Figure 6. Effect of overexpressing Nrf2 on cell viability. Nrf2 was overexpressed in ACHN cells and were divided into three groups, which were the control, Mock+Ax and Nrf2+Ax groups. (A) Nrf2 protein expression was detected by western blotting. Lamin B1 served as an internal control. (B) Keap1 protein expression was detected by western blotting. GAPDH served as an internal control. (C) Cell viability was assessed by a Cell Counting kit-8 assay. Data are presented as the mean \pm standard deviation from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$. Keap1, Kelch-like ECH-associated protein 1; Ax, Axitinib; Nrf2, nuclear factor erythroid 2-related factor 2.

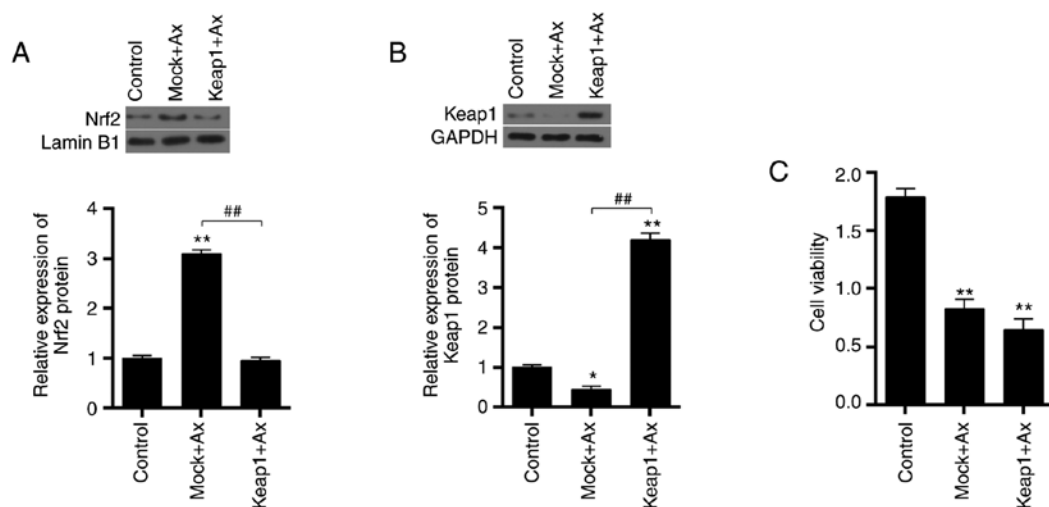


Figure 7. Effect of overexpressing Keap1 on cell viability. Keap1 was overexpressed in ACHN cells and were divided into three groups, which were the control, Mock+Ax and Keap1+Ax groups. (A) Nrf2 protein expression was detected by western blotting. Lamin B1 served as an internal control. (B) Keap1 protein expression was detected by western blotting. GAPDH served as an internal control. (C) Cell viability was detected by a Cell Counting kit-8 assay. Data are presented as the mean \pm standard deviation from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control; ## $P < 0.01$. Keap1, Kelch-like ECH-associated protein 1; Ax, Axitinib; Nrf2, nuclear factor erythroid 2-related factor 2.

decreased the expression of Nrf2 caused by Axitinib ($P < 0.01$). Keap1 protein expression was significantly upregulated by overexpressing Keap1 ($P < 0.01$; Fig. 7B). Furthermore, overexpressing Keap1 decreased the cell viability ($P < 0.01$ vs. control; $P > 0.05$ vs. Mock+Ax; Fig. 7C).

Discussion

In the present study, Keap1 mRNA expression was lower in RCC tissues compared with adjacent normal tissues collected from 45 patients with RCC. This was additionally confirmed by the expressions of mRNA in five randomly selected patients with RCC. With regards to the protein expression level, it was identified that Keap1 protein expression increased in cancer tissues

of two of the selected patients and this variation may be due to individual differences or different subtypes of RCC. A lower expression of Keap1 was associated with a poor 5-year survival analyzed by a Kaplan-Meier survival curve. In agreement with this result, a low expression of Keap1 is associated with various malignancies, including non-small cell lung cancer, colorectal cancer, liver cancer, gallbladder cancer, breast cancer, and head and neck squamous cell carcinoma (16,29-34). Solis *et al* (35) additionally observed that downregulated expression of Keap1 and high expression of Nrf2 were common abnormal phenomena in non-small cell lung carcinoma, and they were associated with a poor prognosis. The expression of Keap1 in normal human renal tubular epithelial cells and five RCC cell lines was further detected; as hypothesized, Keap1 expression

was significantly decreased in RCC cell lines. As the protein expression of Keap1 was detected in five patients, the results may be limited as the Keap1 expression was not detected in the remaining patients. Furthermore, there were other limitations of the present study, including that the other two pathways involving NF- κ B and Bcl-2 were not investigated.

Keap1 is not only associated with the poor prognosis of RCC; however, additionally serves an important role in chemotherapeutic resistance. It was previously demonstrated that Axitinib is effective in breast cancer, non-small-cell lung, pancreatic cancer and thyroid cancer (36-39). The present results additionally demonstrated that Axitinib had a similar inhibitory effect on RCC. In particular, it was able to inhibit RCC cell viability in a dose-dependent manner. In addition, treatment with Axitinib decreased cell viability, promoted ROS release and induced cell apoptosis. When Keap1 was silenced, the sensitivity of ACHN cells to Axitinib was decreased, specifically, cell viability was increased, the release of ROS was decreased and tumor cell apoptosis was suppressed by siKeap1. A previous study additionally observed that Keap1 mutations increased radio-resistance and was able to predict local tumor recurrence in patients with laryngeal squamous cell carcinoma subjected to radiotherapy (40). The present results demonstrated that siKeap1 decreased the ROS level and increased the cell viability.

The Keap1-Nrf2 signaling pathway has a protective effect on normal cells in addition to tumor cells (39,31). Numerous previous studies demonstrated that the signaling was able to induce drug resistance by reducing the sensitivity of tumor cells to chemotherapeutic drugs (41-44). Therefore, the effect of silencing Keap1 on the expression of Nrf2 and its effect on ERK signaling was investigated. The result demonstrated that treatment with Axitinib was able to decrease Keap1 expression and stimulate Nrf2 expression. Furthermore, the downstream proteins of Nrf2, NQO1 and HO1 were significantly enhanced under treatment with Axitinib. Silencing Keap1 increased the expression of Nrf2, NQO1 and HO1.

Nrf2 is a basic leucine zipper structural transcription factor and cap 'n' collar family transcription factor (45). Human Nrf2 has 605 amino acid residues and forms conserved domains from Neh1 to Neh7 (46,47). Nrf2 has the function of activating the transcription and expression of the ARE gene, binding to Keap1, and regulating transcriptional activation and degradation (46,48). Nrf2 has been identified as one of the most important antioxidative regulators (49). Although a number of previous studies demonstrated that Nrf2 served an important role in tumor prevention (50,51), other previous studies observed that a high expression level of Nrf2 in tumor cells was additionally able to reduce its sensitivity to chemotherapeutic drugs and promote tumor growth (52-54). Stacy *et al* (55) identified that Nrf2 was highly expressed in head and neck squamous cell carcinoma, and that the high expression of Nrf2 was considered as one of the markers of tumor drug resistance. The Keap1-Nrf2 signaling pathway is activated in mammary cancer cells tolerant to tamoxifen, and the tolerance of tumor cells to tamoxifen may be altered subsequent to silencing Nrf2 with siRNA (42). Nrf2 may be a prognostic indicator of gastric cancer, and it may predict the efficacy of 5-Fu in patients with gastric cancer (56). Previous studies demonstrated that the target genes of Nrf2, including HO1, glutathione S-transferases, multidrug resistance associated protein and NQO1, were the core components

of the drug resistance mechanism of tumors (57-59). HO1 has anti-oxidation and anti-apoptotic effects, whereas, Nrf2 may inhibit apoptosis of tumor cells and produce drug resistance by regulating the expression of HO1 (60). NQO1 is a flavozyme that catalyzes the redox reaction of cytotoxic substances to produce non-toxic or low-toxic substances (61). The present results suggest that decreased sensitivity to Axitinib in renal carcinoma cells induced by the downregulation of Keap1 is associated with the increase of Nrf2 expression.

Numerous previous studies demonstrated that activation of the mitogen-activated protein kinase/ERK signaling pathway contributed to resistance to receptor tyrosine kinase inhibitors (62-64). The protein expression of ERK signaling was assessed, and it was identified that the phosphorylation of ERK expression was decreased in Axitinib-treated cells. The activity of ERK signaling was not affected by siKeap1, suggesting that Axitinib resistance induced by silencing Keap1 was independent of the ERK pathway.

Silencing Keap1 and Nrf2, and overexpressing Nrf2 and Keap1 were performed to assess ACHN cell survival. The present results demonstrated that Axitinib decreased the cell viability and the knockdown of Keap1 increased the viability of Axitinib-treated cells, whereas, overexpression of Keap1 enhanced the effects of Axitinib. Taken together, it was concluded that the downregulation of Keap1 may contribute to the resistance of RCC cells to Axitinib.

In conclusion, the present study suggested that Keap1 serves as a tumor suppressor, and that its decreased expression is associated with a poor prognosis and a reduced Axitinib sensitivity of RCC cells. A possible mechanism involved Nrf2 overexpression. The present study suggested that regulation of Keap1 may be an attractive strategy to increase the therapeutic effect of cancer-drug resistance.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

HH wrote the manuscript. HH, YuW, WF, XW, LZ, XX, FH and YiW performed the experiments. HH, YuW and YiW designed the study. WF, XW, LZ, XX, FH and YiW performed data analysis. HH, YuW and YiW contributed to manuscript revisions and all the authors reviewed the manuscript.

Ethics approval and consent to participate

The project protocol was approved by the Ethics Committee of The Second Affiliated Hospital of Guangxi Medical

University. All patients recruited in the present study provided written informed consent for the use of their tissue samples for clinical research.

Patient consent for publication

Written informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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