Original Article
Silibinin induces apoptosis by caspase activation, down-regulating Bcl-2 and activating YAP/p53 in renal cell carcinoma

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Abstract: Silibinin, a *flavanone* in milk thistle, has been shown effective anticancer effects in various kinds of human tumors. In the present study, we investigated whether and how silibinin induces cell apoptosis in human renal cell carcinoma (RCC) cells. We found that silibinin could markedly inhibit the growth of 786-O and ACHN RCC cells by MTT assay and cell apoptosis by flow cytometric analysis. Moreover, using laser scanning confocal microscope, we found 786-O and ACHN showed typical pyknosis and nuclei splitting after treatment with 150 μM silibinin for 24 h. Meanwhile, it was found that silibinin activated cleaved caspase 3 and cleaved PARP and decreased expression of Bcl-2 in 786-O and ACHN cells by western blotting analysis. Furthermore, we found that silibinin could increase YAP and p53 expression in a time-dependent manner, but the induction of p53 was about 2 h behind the induction of YAP. Additionally, knockdown of YAP could not cause the p53 protein overexpression by silibinin treatment. Taken together, our study provided the first evidence that silibinin is imparted strong inhibitory, and apoptotic effects on RCC cells through increasing YAP/p53 index. Silibinin merits further investigation as an apoptosis inducer as well as a novel RCC chemo therapeutic agent in the clinical setting.

Keywords: RCC, silibinin, YAP, p53, apoptosis

Introduction

Renal cell carcinoma (RCC) was the sixteenth most common cause of death from cancer worldwide in 2012 [1]. Approximately 33.3% of patients were metastases at diagnosis, and 40% of patients will have disease recurrence after surgical resection, furthermore the 5-year survival rate is less than 5% in metastatic patients [2]. Although mTOR inhibitor and VEGF inhibitor had plays a principal role in the targeted treatment of RCC, but the response rates of targeted drug for metastatic RCC (mRCC) is still poor [3-5], and the resistance to chemotherapy remains to be a major obstacle for RCC to cure.

Silibinin, a natural flavonolignan isolated from *milk thistle* seeds (*Silybum marianum*), has been found pleiotropic anticancer capabilities in different human malignant tumors, including anti-proliferative, anti-apoptosis and anti-invasion/metastasis properties [6-9]. In particular,

silibinin has been reported to induce cell apoptosis in other cancer cells, and the molecular mechanism including by nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) in ALK-positive anaplastic large cell lymphoma [10], by modulating p53, p21, Bak and Bcl-xl pathways in breast cancer cells [6], by inhibiting NF-κB activation in estrogen receptor (ER)-negative breast carcinoma cells [11], et al. However, to the best of our knowledge, the apoptosis mechanism of silibinin on RCC remains unknown.

In the present study, we firstly investigated the apoptotic effect of silibinin on RCC cells, and then focused on whether silibinin induced apoptosis through increasing YAP/p53.

Materials and methods

Cell culture and silibinin treatment

Human Renal cancer cells 786-O, ACHN purchased from American Type Culture Collection

(Manassas, VA) were maintained in RPMI 1640 (Gibco) containing 10% (v/v) fetal bovine serum (Hyclone) at 37°C in a humidified 5% CO_2 incubator. For silibinin treatment, appropriate volumes of stock solution of were added to the medium to achieve the indicated final concentrations and were then incubated with cells for the indicated periods of time (24, 48, and 72 h).

MTT assay

To compare the sensitivities of different kidney cancer cells to silibinin treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Amresco) proliferation assays were performed to determine cell viability. Two kidney cancer cells were seeded at the density of 4×10^3 cells/well in 96-well plates, and then fed fresh medium and treated with various doses of silibinin (0, 1, 50, 100, 150, and 250 μM) for 24, 48 and 72 h. After the exposure period, 20 μL MTT (5 mg/mL in PBS) was added to each well for 4 h incubation at 37°C in 5% CO_2. Thereafter the medium containing MTT was removed and 150 μL DMSO was added to solubilize the formazan crystals. The absorbance (OD) was then measured at a wavelength of 490 nm by a Microplate Autoreader (Bio-Tek Instruments). The growth inhibitory rate was calculated by the following formula: growth inhibitory rate = (average OD value in the control group-average OD value in the treatment group)/average OD value in the control group ×100%.

Cell apoptosis analysis

Human renal cancer cells, 786-O and ACHN were grown in RPMI-1640 medium supplemented with 10% FBS in 6 cm-dishes, and then fed fresh medium and treated with various doses of silibinin (0, 50, 100, and 150 μM) for 48 h. Subsequently, cells were harvested, washed with PBS, subjected to annexin V and propidium iodide (PI) staining using an Annexin V-FITC Apoptosis Detection Kit. Then cells were incubated at room temperature for 15 min and were analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Calibur). These data were analyzed by using GraphPad Prism software.

Nuclear condensation and fragmentation analysis

Human renal cancer cells, 786-O and ACHN cells were grown in RPMI-1640 medium supple-

mented with 10% FBS in Millicell EZSLIDE 8-well Glass Slide, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After the fixation, the cells were washed with PBS and a 300 nM DAPI solution was added to the fixed cells for 5 min. After the nuclei were stained, the cells were examined by laser scanning confocal microscope (Nikon A1R/A1).

Lentivirus transduction of RCC cell lines and generation of stable cell lines 786-O cells were grown in RPMI-1640 medium supplemented with 10% FBS (Gibco, USA) in Coning 24-well-dishes, the cells were transfect with the lentiviral plasmids (Shanghai GenePharma Co.,Ltd), which has the shRNA sequences directed against human YAP1. After 24 h, the 786-O/YAP KD and 786-O/NC stable cell lines were selected by puromycin (Sigma, China). The efficiency of lentivirus was detected by Western blotting and Real-time Quantitative PCR.

Real-time quantitative PCR (QPCR)

Total RNA was isolated by RNA fast200 (Feijie, China) and reverse-transcribed to cDNA by using PrimeScript™ RT reagent kit (Takara, China). Relative gene expression was studied using real-time PCR system (Bio-Rad, CA) with SYBR Green PCR Master Mix (Takara, China) to determine the transcriptional expression of specific genes and calculated by the 2-ΔΔCt method. GAPDH was used for normalization. GAPDH primer: F-ATGGGGAAGGTGAAGGTCGG, R-GACGGTGCCATGGAATTTGC. YAP1 primer: F-AATTTGCCCAGTTATACCTCAGTG, R-CA-CATCAA-GGCTATGATTCAAACTC.

Western blotting analysis

Total cellular protein was extracted from cells with radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, and 0.5% sodium deoxycholate] containing protease inhibitors, 1% Cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma, St Louis, MO). 30 μg of protein was separated on SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (pH7.6, TBST) at room temperature for 1 h and then incubated at 4°C overnight with primary antibody, including primary antibodies against
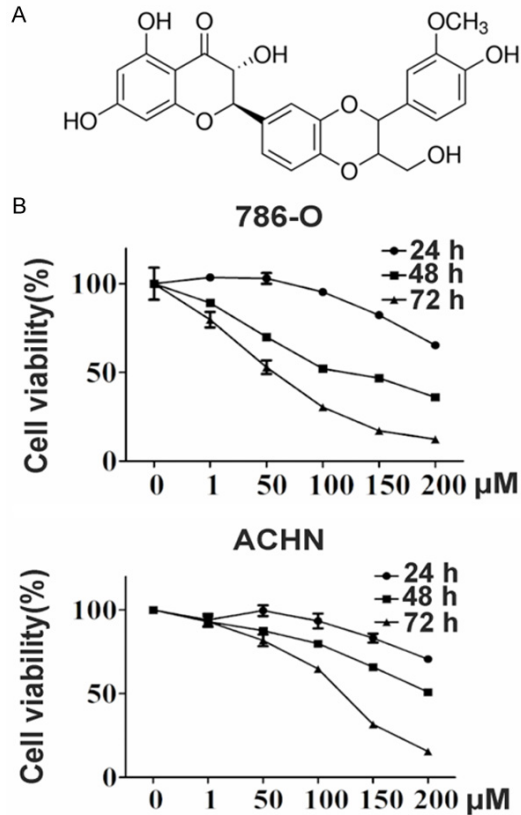
Figure 1. Silibinin inhibit the growth of RCC cells. A: Chemical structure of silibinin. B: Cells were treated with various concentration of silibinin, and the cell viabilities were detected at different time points by MTT assay.

PARP (CST 1:1000), Caspase 3 (CST 1:1000), YAP (CST 1:1000), p53 (Santa Cruz 1:300), and Bcl-2 (Santa Cruz 1:200), then incubated horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive signals were detected using the ECL detection system, followed by exposure to X-ray film. Immunoblotting against glyceraldehyde-3-phosphate dehydrogenase (GAPDH Shanghai Kangchen 1:10000) was performed as an internal control.

Statistical analysis

Data are presented as the mean ± SEM from at least three independent experiments, and the differences between two groups were compared by the student's t test. All data analyses were done by GraphPad Prism software. $P <$ 0.05 was regarded as the threshold value for statistical significance.

Results

Silibinin inhibit the growth of renal cancer cells

To investigate the potential inhibitory role of silibinin (structure shown in **Figure 1A**) on cell growth of RCC cells, the 786-O and ACHN renal cancer cell lines were treated with silibinin (0, 1, 50, 100, 150, and 200 μM). As shown in **Figure 1B**, the cell growth of 786-O and ACHN were obviously inhibited by silibinin, comparing to vehicle treated control groups. In concentration-dependent experiments, 1 μM of silibinin could not effectively reduce the cell viabilities in 786-O and ACHN cells, whereas only 20.3% growth inhibition was observed in 786-O cells after treatment for 72 h. When treated with silibinin at 100 μM concentration for 48 h, the cell growth was inhibited 53.1% and 34.0% in 786-O and ACHN cells, respectively. On the other hand, different treatment time of silibinin (24 h, 48 h and 72 h) showed a time-dependent cell growth inhibition in the two cell lines. These results indicate that silibinin has an inhibitory effect on the growth of renal cancer cells in both a dose- and time-dependent manner.

Silibinin induces cell apoptosis in renal cancer cells

To investigate why silibinin could inhibit the viability of RCC cells, we detected the effect of silibinin on cell apoptosis by flow cytometry array and nuclear staining analysis. 786-O and ACHN cells were exposed to various concentrations of silibinin for 48 h, the cells were subjected to annexin V and PI staining and determined by flow cytometry. Compared to the control cells, silibinin treatment at 50, 100, and 150 μM resulted in 2.85%, 8.54%, and 41.43% apoptosis cells in the 786-O cells, respectively, while silibinin treatment at 50, 100, and 150 μM resulted in 3.67%, 6.85%, and 19.80% apoptosis cells in the ACHN cells, respectively (**Figure 2A**). Next, we used nuclear staining to detect the pyknosis and nuclei splitting, and we found 786-O and ACHN showed typical pyknosis and nuclei splitting after treatment with 150 μM silibinin for 24 h (**Figure 2B**).

Silibinin activated the caspase activity

To investigate the mechanism of silibinin targeting RCC cell apoptosis, we performed western blot analysis to detect the expression of
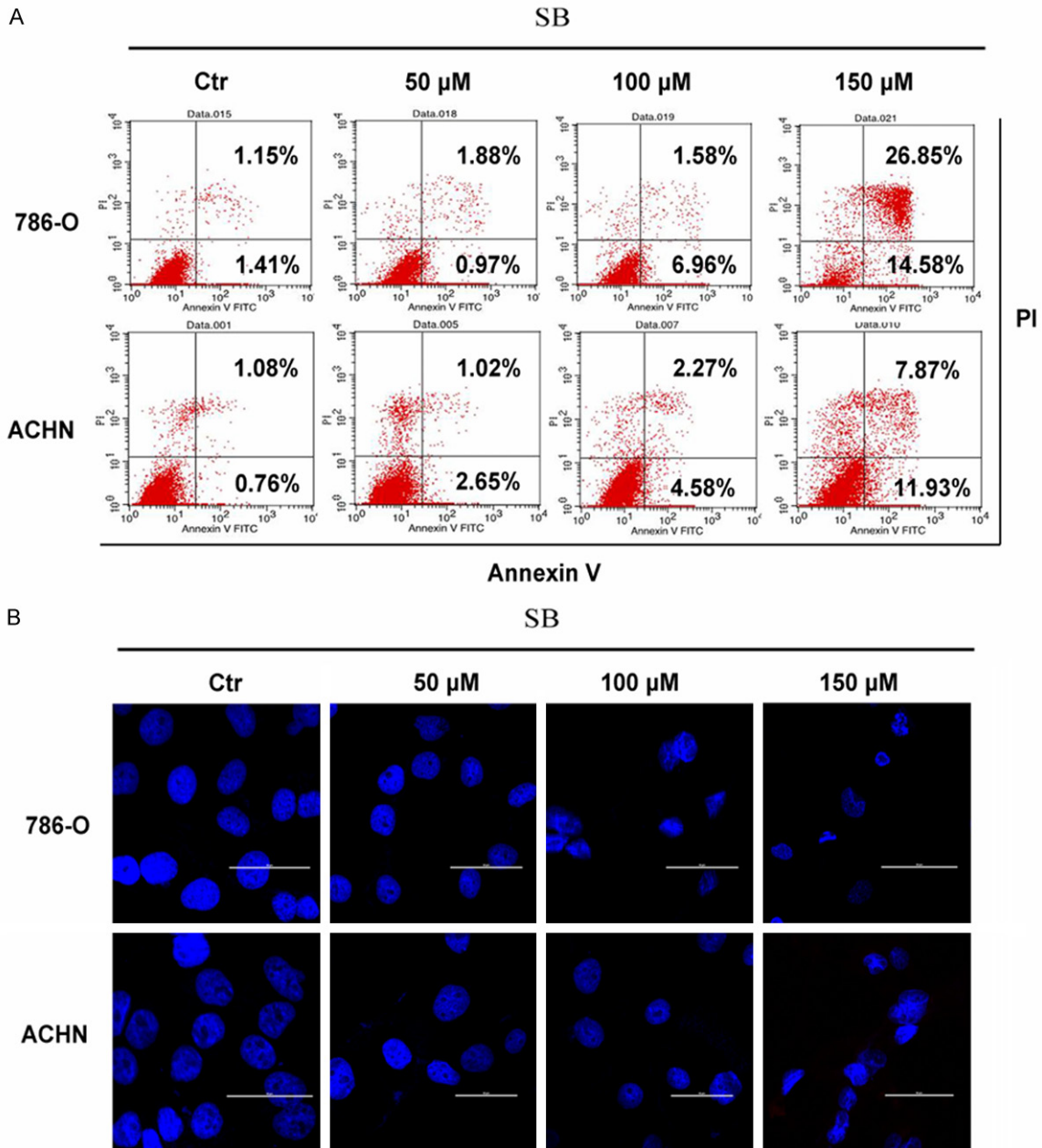
Figure 2. Silibinin induced apoptosis in RCC cells. A: RCC cells were treated with different concentration of silibinin for 48 h, then the apoptotic index was calculated by flow cytometry. B: Nuclear condensation and fragmentation were detected after RCC cells were treated with various concentration of silibinin. Cell nuclear were stained with DAPI and analyzed by laser scanning confocal microscope.

proteins related to apoptosis. 786-O and ACHN cells were exposed to various concentrations of silibinin for 48 h, and total lysates of 786-O and ACHN cells were collected for caspase 3 and PARP detection. Total caspase-3 protein decreased and cleaved caspase-3 protein increased accordingly after silibinin treatment (**Figure 3**). Consistent with this result, PARP, a chromatin-associated enzyme that plays an important role in DNA repair and the recovery
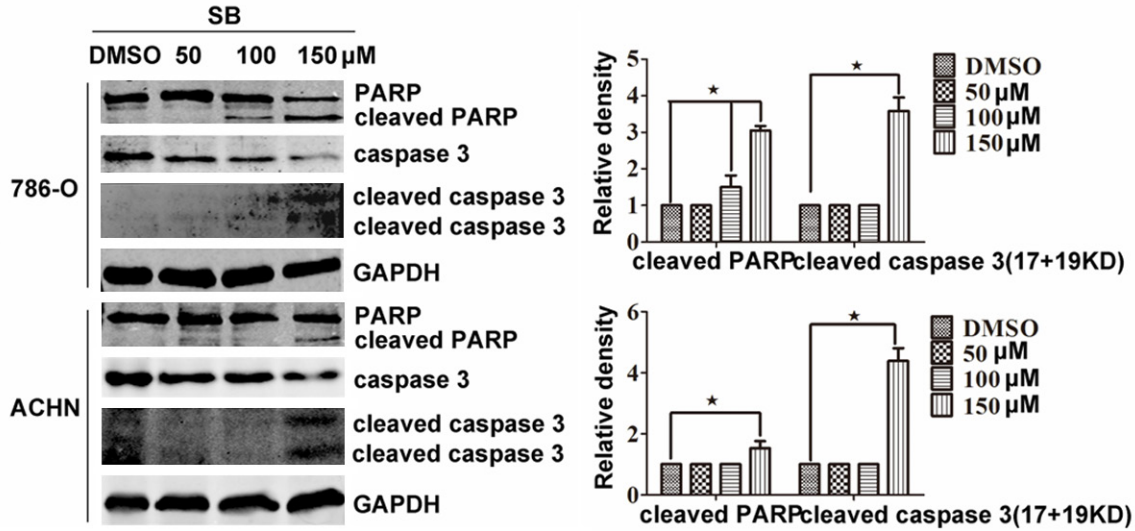
of cells from DNA damage, were decreased in both 786-O and ACHN cells, but cleaved PARP were increased in both 786-O and ACHN cell lines (**Figure 3**).

Silibinin induces cell apoptosis through the YAP/p53 index

To elucidate the apoptosis mechanisms of silibinin in RCC cells. We examined the expression

Figure 3. Silibinin treatment activated caspases in RCC cells. after treatment with various concentration of silibinin for 48 h, total lysates of 786-O and ACHN cells were analyzed for caspase 3, cleaved caspase 3 (17 KD and 19 KD), and PARP cleavage were detected by western blot.

of Bcl-2 protein which is an anti-apoptotic protein for the induction of apoptosis by anti-cancer drugs [12]. As shown in **Figure 4A**, expression level of Bcl-2 protein was both significantly down-regulated in 786-O and ACHN cell lines. The tumor suppressor protein p53, which is a major defense against cancer and dictates the fate of cell [13], was also measured by western blotting analysis. In this study, it was amazed to find that p53 proteins increased at ACHN cell line but decreased in 786-O cell line.

Next, the expression levels of YAP, which was reported to improve cell apoptosis by binding on the p53 promoter and enhances p53 expression in other systems [14], were measured by western blotting analysis. To verify the above mechanisms in RCC cells, the specific YAP-shRNA lentiviral vectors were constructed and transfected into 786-O cells to knockdown YAP (**Figure 4B**, **4C**), and then the cells were treated with 150 μM silibinin for 24 h, and the expression of PARP, YAP, and p53 were detected by western blotting analysis. As shown in **Figure 4D**, YAP protein gradually increased at 2 h and then decreased at 8 h in a time-dependent manner, which is similar with the expression pattern of p53 when cells was treated with silibinin, but the induction of p53 was about 4 h behind the induction of YAP. Meanwhile, cleaved PARP was detected at 12 h in 786-O/NC cells. Nevertheless, the p53 expression in

786-O/YAP KD was not activated comparing with the control group (**Figure 4E**), and the cleaved PARP was not detected at 12 h in 786-O/NC cells. These results revealed that YAP is essential for p53 activation and cell apoptosis induction by silibinin RCC cell line.

Discussion

Deregulated cell proliferation is a hallmark of cancer. p53 was reported to be a tumor suppressor protein and it is induced to activate growth-inhibitory genes or triggers the activation of apoptotic associated genes in the condition of damage [15, 16]. In our study, ACHN and 786-O RCC cell lines were used as cell model. According to International Agency for Research on Cancer, ACHN cell line contains wild type p53, while the gene of p53 in 786-O cell line contains nonsense mutation (according to International Agency for Research on Cancer). Our results showed that the expression of p53 is increased by treatment with silibinin in both ACHN and 786-O RCC cell lines (**Figure 4B**). It is worth to mention that p53 expression is increased at 24 h, and then decreased when cells are treated with silibinin for 48 h (**Figure 4D**), but the expression of p53 was also increased when ACHN are treated with silibinin for 48 h (**Figure 4A**).

In order to explain the molecular mechanisms of the above phenomenon, the expression of
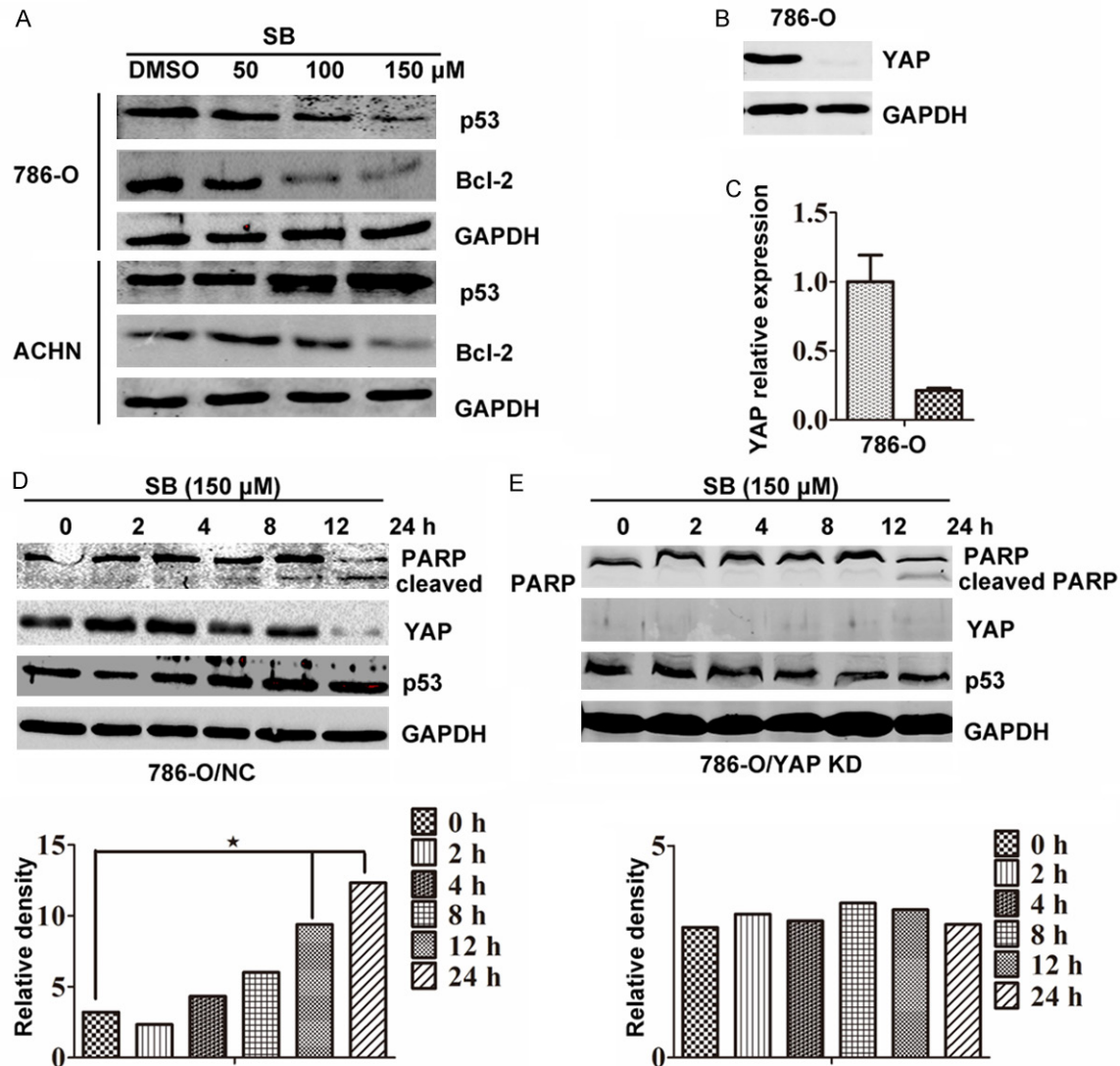
Figure 4. Silibinin induced RCC cells apoptosis through regulating YAP/p53 index. A: After treatment with silibinin for 48 h, 786-O and ACHN cell lysates were analyzed for p53 and Bcl-2 expression by western blot. GAPDH was detected as an internal control. B, C: 786-O cells were knockdown of YAP1 by Lentivirus, 24 h after transfection, cells lysed and the expression of YAP was analyzed by western blot and QPCR. D, E: 786-O/NC and 786-O/YAP KD cells were treated with 150 μm/ml silibinin for 24 h and then analyzed for PARP, YAP and p53 protein level at different time points by western blots. ★P < 0.05.

YAP was detected. YAP, the key effector of hippo pathway, was reported to be an oncoprotein and important for tumor progression and metastasis [17], including cellular proliferation, migration, and invasion in renal cell carcinoma [18, 19]. Meanwhile, some researcher reported that the expression of p53 is induced by YAP through binding to the p53 promoter [20]. In our study, we used the specific YAP-shRNA lentiviral vectors to knock down YAP in 786-O cells, and then treat cells with 150 μM silibinin for 24 h. We found that p53 is up-regulated in 786-O/

NC cells, but the expression of p53 was not increased in 786-O/YAP KD cells (**Figure 4D**, **4E**). Furthermore, our results showed that p53 expression in 786-O cell line is higher than in ACHN cell line (Supplementary Figure 1). It was worth to mention that YAP was inhibited by p53 through feedback to bind the YAP promoter. Take message home, cell apoptosis were occurred earlier in 786-O cell line than ACHN cell line, part of the reason is that the YAP expression in 786-O cell line is higher than in ACHN cell line. It seems contradictory, but we

believe the chemo-resistance of cancer cells are not only controlled by pro-proliferation and anti-apoptosis signals, they are also regulated by other cell metabolism as well as changes in drug treatment. YAP may be regulated by other cell signaling pathway without the hippo signaling pathway. Taken together, our results indicate that YAP is essential for p53 activation, and silibinin inducing cell apoptosis is through up-regulation of YAP protein expression, and subsequently cause the up-regulation of p53, YAP might function as an apoptotic enhancer with chemotherapeutics in renal cell carcinoma by modulation of p53 family. The molecular mechanisms were also reported in other carcinoma cells.

Silibinin has been investigated as an anti-cancer and chemo-preventive drug in various human cancers [7]. As far as we know, silibinin has been reported through the IGFBP-3 pathway to inhibit RCC cells growth [21], by activating caspase, down-regulating survivin and blocking EGFR-ERK activation to inhibits cell growth and induces apoptosis in RCC cells [9], and by decreasing MMP-2, MMP-9, u-PA, p-p38, and p-Erk1/2 expressions to inhibits the invasion, migration and xenografts growth in RCC cells [22]. The above results indicated that silibinin is an effective drug for RCC cells treatment, but the molecular mechanisms of silibinin inducing RCC cells apoptosis has not been clearly elucidated yet. In our study, we found that silibinin could induce cell apoptosis by regulating p53 expression and decreasing Bcl-2 expression. In the present study, we found that silibinin could activate the expression of YAP and p53 in a time-dependent, and YAP is necessary for p53 activation.

In summary, our results demonstrate that silibinin induces apoptosis of RCC cells through up-regulation of YAP protein expression, and subsequently cause the up-regulation of p53, moreover this is the first report about silibinin regulating YAP/p53 index to inducing cell apoptosis. Taken together, silibinin might be as one of the candidate chemopreventive agents for renal cell carcinoma therapy, and YAP might be a new candidate target for silibinin to activate cell apoptosis singling pathway.

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Disclosure of conflict of interest

None.

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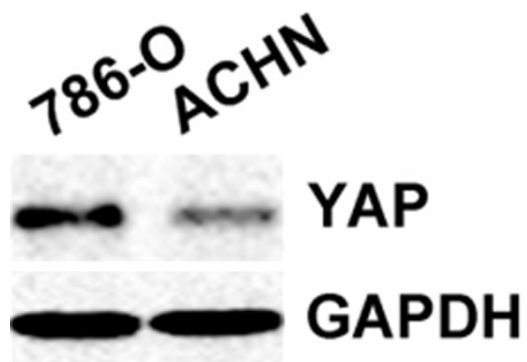
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