Silibinin induces apoptosis through inhibition of the mTOR-GLI1-BCL2 pathway in renal cell carcinoma

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Abstract. The downstream transcriptional factor of the hedgehog (Hh) pathway, GLI family zinc finger 1 (GLI1), plays a crucial role in regulating tumor progression. In the present study, we demonstrated that silibinin, a natural flavonoid antioxidant isolated from extracts of the milk thistle herb, exerts its anticancer capabilities by restraining GLI1 function in renal cell carcinoma (RCC) cells in vitro and in vivo. In the present study, we confirmed that silibinin induced growth inhibition of RCC through caspase-dependent apoptosis and downregulation of GLI1 and BCL2, which could be partially reversed by GLI1 overexpression. Moreover, we determined that the decreased GLI1 expression by silibinin was mediated by the mammalian target of rapamycin (mTOR) pathway. The in vivo mouse xenograft study also showed that silibinin significantly reduced RCC tumor growth and specifically targeted the mTOR-GLI1-BCL2 signaling pathway. In conclusion, our findings demonstrated for the first time that silibinin induces apoptosis of RCC cells through inhibition of the mTOR-GLI1-BCL2 pathway. These findings also indicate that GLI1 is a novel regulator for the potential therapeutic application of silibinin against RCC.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors of the urinary system, accounting for 80-90% of kidney neoplasms. The American Cancer Society estimated that the number of new cancer cases and deaths caused by cancer of the kidney and renal pelvis were 63,920 and 13,860 in 2014 in the US (1). Surgical resection is considered to be the first choice for the curative treatment of localized RCC and some locally advanced RCC. However, ~25-30% patients initially present with metastases, and a substantial part of patients have subclinical metastases at that time (2,3). Some patients undergoing surgical treatment relapse to metastatic renal cell carcinoma (mRCC). mRCC is usually resistant to radiotherapy and chemotherapy, while immunotherapy shows limited response rates of 15-20% (4-6). Therefore, understanding the mechanisms involved in the progression of RCC especially mRCC and exploring more effective targeted therapies are urgently needed.

The hedgehog (Hh) pathway was firstly emphasized in embryonic developmental studies (7,8). Recent research has demonstrated that the Hh pathway is constitutively activated in cancer compared with normal tissue and plays a crucial role in cancer progression (9-13). Inhibition of Hh signaling was found to significantly induce apoptotic cell death in pancreatic cancer cell lines both in vitro and in vivo (9). Hh signaling-mediated survival of gastric cancer cells is known to be associated with the regulation of apoptosis inhibition protein BCL2 (12). Selective downregulation of GLI family zinc finger 1 (GLI1) by antisense oligodeoxynucleotides results in decreased BCL2 expression and cancer cell survival (13). Clinical trials suggest benefits for patients administered specific Hh pathway inhibitors. GDC-0449, a small-molecule inhibitor of SMO, has shown encouraging antitumor activity in locally advanced or metastatic solid tumors in two separated phase I trials (14,15). Another Hh inhibitor irtraconazole decreased cancer cell proliferation by 45%, Hh pathway activity by 65%, and reduced the tumor area by 24% in phase II trials (16). Dormoy et al also reported that the sonic Hh signaling pathway is reactivated in RCC compared with normal tissue, and inhibition of this pathway dramatically decreases tumor cell proliferation and induces apoptosis in vitro and in vivo (17). Thus, the Hh pathway may be a promising target for RCC especially mRCC therapy.

Silibinin is a natural flavonoid which is widely used for the treatment of drug- or alcohol-induced liver injury and cirrhosis. Recent studies have shown the significant anticancer activity of silibinin. For many tumors such as prostate (18,19), bladder (20,21), lung (22) and colon (23,24) cancers, silibinin exhibits growth inhibitory and anti-inflammatory effects, cell cycle arrest, apoptosis induction, chemosensitization, inhibition of angiogenesis, reversal of multi-drug resistance and inhibition of invasion and metastasis, which involves regulation of receptor tyrosine kinases, the androgen receptor, and

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cell cycle regulatory and apoptotic signaling pathways (25). Cheung et al showed that oral administration of silibinin suppressed local and metastatic tumor growth in an orthotopic xenograft model of RCC. This anti-neoplastic action of silibinin might involve insulin-like growth factor-binding protein 3 (IGFBP-3) (26). Our previous studies demonstrated the apoptosis induction and metastatic inhibitory effects of silibinin against RCC by targeting the epidermal growth factor receptor (EGFR) pathway (27,28). Recently, we found that autophagy induction by silibinin positively contributes to its anti-metastatic capacity via the adenosine 5'-monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway in RCC cells (29). However, whether the Hh pathway is regulated by silibinin followed by induction of apoptosis remains to be explored.

In the present study, 769-P, 786-O, OS-RC-2 (cell lines derived from primary clear cell adenocarcinoma, which have the similar genetic and molecular features of RCC) and ACHN (a cell line derived from the malignant pleural effusion) were applied as the model system. We first examined whether the Hh pathway is regulated by silibinin followed by induction of apoptosis remains to be explored.

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Materials and methods

Cell culture and reagents. Human RCC cell lines 769-P, 786-O, ACHN and OS-RC-2 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, New York, NY, USA) at 37°C, in humidified air containing 5% CO2. Silibinin, cyclopamine, LY294002, rapamycin and 3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for western blot analysis against poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, p-mTOR, mTOR, p-ERK, ERK, p-AKT (Thr308), p-AKT (Ser473) and AKT were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-GLI1 was from Bios, Inc. (Beijing, China). Anti-BCL2 was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from KangChen Bio-tech, Inc. (Shanghai, China). Antibodies for immunohistochemistry against p-mTOR and BCL2 were from Abcam (Cambridge, MA, USA), and the antibody against GLI1 was from Santa Cruz Biotechnology, Inc.

Cell viability assay. Cell viability was measured by a tetrazolium-based assay. Briefly, cells were seeded in a 96-well plate and treated with doses of silibinin from 0 to 200 µM for the indicated time. The medium was then removed and cells were incubated with 0.5 mg/ml MTT for 4 h and the formazan products were resolved with dimethyl sulfoxide (DMSO) (150 µl/well). The OD value of each well was measured by a microplate reader at a wavelength of 490 nm. The experiments were performed in triplicate.

 Colony formation assay. Cells were seeded in a 6-well plate (1,000 cells/well) and incubated for 48-72 h, followed by treatment with different doses of silibinin. The mediums and drugs were replaced every 3 days. Ten days later when a single cell formed a colony (≥50 cells), the plates were washed with phosphate-buffered saline (PBS), fixed in 10% formalin, and then stained with 0.1% crystal violet solution. Cell colony numbers were counted under a microscope. The experiments were performed in triplicate.

Quantitative detection of apoptosis. To evaluate the apoptosis induced by the reagents, flow cytometry was performed. Cells were treated with the indicated doses of silibinin for 48 h, and collected for Annexin V and propidium iodide (PI) staining (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions, and the apoptotic cells were detected and analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Cell lysates were prepared in lysis buffer [10 mM of Tris-HCl (pH 7.4), 150 mM of NaCl, 0.1% of SDS, 1 mM of EDTA, 1 mM of EGTA, 0.3 mM of PMSF, 0.2 mM of sodium orthovanadate, 1% of NP-40, 10 mg/ml of leupeptin and 10 mg/ml of aprotinin]. Proteins were separated by sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) on 10 or 12% Tris-glycine gels, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBS for 1 h at room temperature, and probed the with primary antibody overnight at 4°C followed by secondary antibody incubation for 1 h at room temperature. The protein expression was visualized with the ECL detection system or Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Reverse transcription and real-time PCR. Total RNA was isolated with RNAfast 200 reagents (Fastagen Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's instructions and was quantitated by absorbance at 260 nm. The RNA (0.5 µg) sample was used for reverse transcription with PrimeScript™ RT Master Mix according to the manufacturer's instructions, and quantitative PCR was performed with SYBR-Green PCR Master Mix (both from Takara Bio, Inc., Dalian, China) and GAPDH mRNA was used as the internal control. The primer sequences were: GLI1 forward, 5'-GAT ATCCCCACATCCTCAGTC-3' and reverse, 5'-ACTTGGCA ACCAGCATGTC-3'; GAPDH forward, 5'-ATGGGGAAGG TGAAGGTCGG-3' and reverse, 5'-GACGGTGCCATGGAA TTTGC-3'.

Plasmid, siRNA transfection. GLI1 cDNA was cloned into the pcDNA3.1 vector. Small interfering RNAs were designed and synthesized by Gene Pharma (Shanghai, China). Cells achieved 70-80% confluence for plasmid transfection or 30-50% confluence for siRNA transfection, and were transfected with X-tremeGENE HP DNA or X-tremeGENE siRNA transfection reagents (Roche Diagnostics GmbH) for 2-3 days, and harvested for subsequent experiments.

Xenograft model. Cells (786-O) were mixed with Matrigel (5:1, v/v) and injected subcutaneously into the right hind flank...
of male BALB/c (nu/nu) mice. Twelve days after injection, the mice were randomly divided into two groups (5 mice/group), and were fed by oral gavage with saline or silibinin (200 mg/kg). The volume of tumors were measured with a vernier caliper every three days. Thirty days after implantation, the mice were sacrificed and the tumors were weighed with electronic scales and fixed in 10% formalin and embedded in paraffin. Animal experiments were approved by the Institutional Animal Care and Use Committee of Xi’an Jiaotong University (permit no. SCXK2014-0155, 5 March 2014).

Immunohistochemistry. Immunohistochemical staining was performed with the EnVision™ system (Dako, Carpinteria, CA, USA), and the slices were de-paraffinized, rehydrated, followed by 5 min antigen retrieval, 10 min of endogenous enzyme block and incubated with the primary antibody overnight at 4°C. Then the slices were incubated with EnVision-HRP secondary antibody for 1 h, and the signals were detected by diaminobenzidine (DAB) followed by hematoxylin counterstaining. The results were observed by a microscope. Stained cells were quantified as the number of positive cells x 100/total number of cells in 10 random microscopic (magnification, x200) fields in each slice.

Statistical analysis. All the statistical analyses were performed by GraphPad Prism version 5.0 software, and the Student’s t-test was used for two-group comparisons. P<0.05 was considered statistically significant.

Results

Silibinin inhibits the proliferation and colony formation of RCC cells. To evaluate the effects of silibinin on RCC cells, four cell lines 769-P, 786-O, ACHN and OS-RC-2 were treated with different doses of silibinin. The results showed that silibinin significantly inhibited the proliferation of RCC cells in a dose- and time-dependent manner as detected by MTT assay (Fig. 1A). Similarly, silibinin treatment also decreased the colony numbers in the 769-P, 786-O and ACHN RCC cells as determined by the colony formation assay (Fig. 1B and C).
Figure 2. Silibinin induces the apoptosis of RCC cells involving activation of caspase-3 and PARP. (A) Cells were treated with the indicated doses of silibinin (SB) or DMSO (control) for 48 h, and stained with Annexin V and PI. Flow cytometry was performed to detect the percentages of apoptotic cells. (B) Cell lysates were collected for western blot analysis to detect the expression of PARP and caspase-3. GAPDH was used as an internal control. The values represent the mean ± SD of three independent experiments (*P<0.05). RCC, renal cell carcinoma.

Figure 3. Silibinin induces apoptosis through downregulation of GLI1 and BCL2. (A) The expression levels of GLI1 and downstream BCL2 were detected by western blot analysis after 48 h of treatment with silibinin (SB). (B) Cells were treated with hedgehog signaling inhibitor cyclopamine (15 µM) for 48 h, and apoptosis was detected by flow cytometry. (C) The expression levels of GLI1 and BCL2 were analyzed by western blot analysis after cyclopamine treatment. (D) Cells (786-O and ACHN) were transfected with the pcDNA3.1-GLI1 or pcDNA3.1 (vector control) plasmids for 72 h, and the mRNA expression of GLI1 was analyzed by real-time PCR. (E) After 48 h of transfection, 786-O and ACHN cells were treated with 100 µM silibinin for an additional 48 h, and apoptosis analysis was performed by flow cytometry. (F) The expression levels of GLI1 and BCL2 were detected by western blot analysis. The values represent the mean ± SD of three independent experiments (*P<0.05).
Silibinin induces the apoptosis of RCC cells involving activation of caspase-3 and PARP. Since we observed the anti-proliferative effects of silibinin on RCC, we next examined the apoptotic effects of silibinin. RCC 769-P, 786-O and ACHN cells were treated with silibinin (50, 100 and 200 µM) or DMSO (control) for 48 h. Cells were then collected for flow cytometric assay and western blot analysis. The percentages of apoptotic cells after treatment with different dosages of silibinin (50, 100 and 200 µM) vs. the control cells were 5.77/22.72/51.10 vs. 4.91% (769-P), 7.79/61.9/77.69 vs. 5.05% (786-O), 6.23/9.71/15.77 vs. 5.24% (ACHN), respectively (P<0.05) (Fig. 2A). Western blot analysis data showed that cleaved subunits of caspase-3 and PARP were increased upon silibinin treatment in a dose-dependent manner (Fig. 2B), indicating the activation of the caspase cascade by silibinin. Taken together, our results demonstrated that silibinin induces RCC cell apoptosis via activation of caspase cascade signaling.

Silibinin induces apoptosis through downregulation of GLI1 and BCL2. Since we demonstrated that silibinin treatment inhibits cell proliferation, the underlying mechanism was investigated. As shown in Fig. 3A, silibinin markedly downregulated GLI1 expression in all three RCC cell lines in a dose-dependent manner, and BCL2, the downstream molecule of GLI1, was also decreased. As known, GLI1 plays an important role in cancer development and progression. In order to detect the function of GLI1 in RCC, we used cyclopamine, a potent Hh signaling inhibitor. The results showed that cyclopamine induced the apoptosis of 769-P cells (Fig. 3B), while the levels of GLI1 and BCL2 were reduced (Fig. 3C). When the GLI1-overexpressing plasmid was transfected into 786-O and ACHN cells, the apoptosis and reduced BCL2 expression induced by silibinin was partially reversed (Fig. 3D-F), suggesting that the GLI1-BCL2 pathway participates in the silibinin-induced apoptosis of RCC cells.

Downregulation of p-mTOR is involved in the silibinin-induced GLI1 decrease. To further investigate the mechanisms of the silibinin-induced decrease in GLI1, we detected the GLI1-associated pathway which has been previously reported to regulate GLI1 expression. Western blot results showed that p-AKT, p-mTOR but not p-ERK were downregulated after the treatment with silibinin (Fig. 4A). Additionally, only the p-mTOR inhibitor (rapamycin, 100 nM) but not the p-AKT inhibitor (LY294002, 20 µM) could inhibit GLI1 and BCL2 expression in the 786-O cells (Fig. 4B). Furthermore, knockdown of mTOR by siRNA...
in the 786-O and ACHN cells reduced GLI1 and BCL2 expression (Fig. 4C), indicating that the mTOR pathway regulated GLI1 expression in the RCC cells. When knockdown of mTOR by siRNA was combined with silibinin treatment or DMSO (control) in ACHN cells, we found this combination did not increase the percentage of apoptotic cells compared with silibinin alone or mTOR knockdown alone (Fig. 4D), which implied that the silibinin-induced apoptosis of RCC cells was mediated by mTOR. Taken together, our results indicate that silibinin inhibits GLI1 expression via reduction of mTOR activity.

Silibinin inhibits the growth of RCC xenografts in vivo. To further evaluate the anticancer effects of silibinin in vivo, we chose 786-O cells, which have a high potential for tumor formation, as a model system in vivo. Silibinin (200 mg/kg) inhibited tumor growth compared with the control group in vivo (Fig. 5A and B). After 30 days, the average tumor weight of the silibinin-treated group was 98.8 ± 77.6 mg while the control group was 281.2 ± 118.87 mg (P<0.05) (Fig. 5C). Immunohistochemistry assay showed that the expression levels of p-mTOR, GLI1 and BCL2 were decreased in the silibinin (200 mg/kg) group compared with the saline group (control) in the xenograft samples, which was consistent with the results observed in vitro (Fig. 5D).

Discussion
There have been only a limited number of studies on the apoptotic effects of silibinin on RCC cells. Our previous studies showed the apoptotic and anti-metastatic effects of silibinin on RCC cells, in which downregulated p-ERK was observed after the treatment of silibinin (27,28). However, in our present study, no significant change was detected in p-ERK expression. Such disparate findings from our results could be due to the difference in cell type used (we used RCC cell line Caki-1 in our previous study, while 769-P, 786-O and OS-RC-2 cell lines were used in our present study), implying that there may be fundamental differences in the individual gene expression pattern among various cell lines. Therefore, precautions should be taken when extrapolating results from one to another study. More recently, we reported the role of autophagy and the AMPK/mTOR pathway in the silibinin-induced anti-metastatic effects on RCC cells (29). As known, when cells are subjected to adverse stress, such as nutrient starvation or chemical agents, cancer cells may trigger an autophagic response followed by degradation of unnecessary molecules or organelles to prevent cells from death. On the other hand, treatment with many anticancer reagents or ionizing radiation, has been shown to induce autophagic cell death, directly leading to the inhibition of...
cancer cell growth (30-32). Silibinin has been reported to induce autophagic death in cervical and breast cancer (33,34). Another study indicated that inhibition of autophagy with a specific inhibitor enhanced cellular death, suggesting a cytoprotective function of autophagy in silibinin-treated cells (35). Thus, there is considerable interest in elucidating the mechanisms of interplay between autophagy and apoptosis induced by silibinin, which clearly requires further research.

The Hh pathway was proven to play a crucial role in pancreatic cancer tumorigenesis in early and late stages, and the maintenance of Hh signaling is important for aberrant proliferation and tumorigenesis (9). Our results confirmed that GLI1 sustained the proliferation of RCC, and inhibition of GLI1 by cyclopamine induced apoptosis by downregulation of BCL2, which was consistent with previously published results (12). We first documented that silibinin effectively decreased the expression of GLI1 in a dose-dependent manner and induced apoptosis by downregulating the expression of BCL2 in RCC. Overexpression of GLI1 could partially reverse the apoptosis induced by silibinin, which implied that GLI1 is a regulator for the potential therapeutic application of silibinin against mRCC.

As the downstream transcriptional factor of the Hh pathway, GLI1 plays a key role in regulating cancer progression not only in an SMO-dependent manner but also in an SMO-independent manner. The canonical SMO-dependent pathway was found to be activated by the binding of ligand HH to the membrane receptor PTC, which triggered another membrane receptor SMO disassociated from PTC and activated GLI1. However, Desch et al confirmed the autonomous role of GLI1 in B-cell chronic lymphocytic leukemia (CLL) cell apoptosis independent of SMO (36). The well known SMO-independent pathway includes the AKT pathway (37), MAPK/ERK pathway (38) and KRAS pathway (39). Wang et al found that activation of the mTOR/S6K1 pathway could enhance GLI1 transcriptional activity and oncogenic function through S6K1-mediated GLI1 phosphorylation at Ser84, which released GLI1 from its endogenous inhibitor SuFu (40). In order to explore the mechanisms involved in the silibinin-induced GLI1 downregulation, we detected p-ERK, p-AKT and p-mTOR expression which have been reported to regulate GLI1 expression after silibinin treatment in RCC. The results showed that p-AKT and p-mTOR were downregulated but p-ERK had no significant change. Then we treated RCC cells with inhibitors of PI3K (LY294002, 20 μM) and p-mTOR (rapamycin, 100 nM), followed by detection of GLI1 protein expression. However, only the mTOR inhibitor decreased GLI1 expression in RCC. Meanwhile, the results of the knockdown of mTOR by siRNA also supported the conclusion, indicating that GLI1 is regulated by the mTOR pathway in RCC. As known, mTOR has two existing complex forms exerting various effects, mTORC1 (raptor) and mTORC2 (rictor). mTORC1 is regulated by AKT whereas mTORC2 is the upstream of AKT. Our results indicated that GLI1 might be potentially regulated by mTORC2 but not mTORC1. However, the exact evidence remains to be explored in further studies.

The activation of the mTOR pathway has been found in RCC and is correlated with high grade and poor prognostic patient features (41,42). Recently, the mTOR pathway has been considered as one of the most important targets for mRCC therapy, and mTOR antagonists rapamycin and three analogs of rapamycin (temsirolimus, everolimus and deforolimus) have undergone clinical evaluation as cancer therapeutics. It was reported that silibinin could dephosphorylate mTOR and its effectors ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4E-BPI) and suppress HIF-1α accumulation (43). Silibinin inhibited colon cancer stem-like cell self-renewal and sphere formation by suppressing the PP2Ac/AKT Ser473/mTOR pathway (44). Our data also supported that silibinin could reduce the activation of the mTOR pathway in RCC. However, the mechanisms that mediate the silibinin-regulated mTOR pathway still need to be identified.

In conclusion, our in vitro and in vivo results first demonstrated that silibinin-induced apoptosis of RCC cells was mediated by the regulation of the mTOR-GLI1-BCL2 pathway. This finding also implies that GLI1 is a novel regulator mediated by silibinin for potential therapeutic application in mRCC, providing a basis for future clinical trials of silibinin for the treatment of patients with mRCC.

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References


