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Effects and mechanisms of silibinin on human hepatocellular carcinoma xenografts in nude mice

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Abstract

AIM: To investigate the *in vivo* effects and mechanisms of silibinin on the growth of hepatocellular carcinoma (HCC) xenografts in nude mice.

METHODS: Nude mice bearing HuH7 xenografts were used to assess the anti-HCC effects and mechanisms of silibinin.

RESULTS: Silibinin resulted in a potent dose-dependent reduction of HuH7 xenografts in association with a significant decrease in Ki-67 and α-fetoprotein production, nuclear NF-κB content, polo-like kinase 1, Rb phosphorylation, and E2F1/DP1 complex, but increased p27/CDK4 complex and checkpoint kinase 1 expression, suggesting that the *in vivo* effects of silibinin are mediated by inhibiting G1-S transition of the cell cycle. Silibinin-induced apoptosis of HuH7 xenografts was associated with inhibited survivin phosphorylation. Silibinin-reduced growth of HuH7 xenografts was associated with decreased p-ERK, increased PTEN expression and the activity of silibinin was correlated with decreased p-Akt production, indicating involvement of PTEN/PI$_3$K/Akt and ERK pathways in its *in vivo* anti-HCC effects. Silibinin-reduced growth of HuH7 xenografts was also associated with a significant increase in AC-H3 and AC-H4 expression and the production of superoxide dismutase (SOD)-1.

CONCLUSION: Silibinin reduces HCC xenograft growth through the inhibition of cell proliferation, cell cycle progression and PTEN/P-Akt and ERK signaling, inducing cell apoptosis, and increasing histone acetylation and SOD-1 expression.

Key words: Apoptosis; Cell cycle; Chemoprevention; Hepatocellular carcinoma; Histone acetylation; Silibinin

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents approximately 6% of all human cancers[1,2]. The global incidence of HCC has risen significantly in the past 2 decades[2], and prognosis of HCC is usually poor[3]. Limited treatment options and the poor prognosis of HCC emphasize the importance of developing an effective chemoprevention for this disease.

Milk thistle (*Silybum marianum*) is a popular dietary supplement that has been reported to be safe, well-tolerated, and protects the liver from drug or alcohol-related injury[4]. Silibinin, the major biologically active compound of milk thistle is a polyphenolic flavonoid, and a strong antioxidant and radical scavenger[5-7]. Studies have demonstrated the inhibitory effects of silibinin on multiple cancer cell lines, including prostate, lung, colon, skin, and bladder cancers[8-16]. Recently, we and Varghese *et al*[17] reported the *in vitro* anti-HCC effects of silibinin[18], however, additional studies are needed to further determine its *in vivo* inhibitory effects and mechanisms on the growth of human HCC. Clearly, nude mice bearing human hepatoma xenografts represent a suitable model for such a study[19,20].

Plasma α-fetoprotein (AFP) has been used as a

clinical marker in the diagnosis and monitoring of HCC[21-23]. We demonstrated that silibinin reduces AFP production and secretion from human hepatoma cells, but the AFP value in monitoring the *in vivo* anti-HCC effects of silibinin has not yet been tested.

Hepatocarcinogenesis is a complicated process that alters cell cycle progression and apoptosis. This may be mediated by altering signal transduction through cell cycle modulators, phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphatidylinositol 3'-kinase (PI_3K) and Akt (PTEN/PI_3K/Akt) pathways[24-30], and histone acetylation[31-33]. p-Rb, p21, and p27 are molecules that are involved in cell cycle regulation[17]. Nuclear factor (NF)-κB activation stimulates G1 to S phase progression and transcription of a wide variety of genes that are involved in cell proliferation[34,35]. Checkpoint kinase 1 (Chk1) and polo-like kinase 1 (Plk1) are the up-stream molecules. Chk1 controls cell cycle progression and inhibits mitosis[36]. Plk1 has long been recognized as a potential target for cancer therapy. Inhibition of Plk1 function may increase anti-tumor activity *in vivo*[37]. It is unclear whether these pathways are involved in silibinin-mediated anti-HCC effects.

Studies have also indicated that signals related to reactive oxygen species (ROS) may play important roles in the development of HCC[38]. The cellular levels of ROS are regulated by the antioxidant defense systems, that is, the enzymatic activities of superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase[39]. Altered expression of SOD has been associated with the development and differentiation of HCC[40,41]. Silymarin significantly increased suppressed SOD activity in patients with chronic alcoholic liver disease[42]. However, it is unclear whether silibinin-reduced growth of HCC cells is mediated by enhanced expression of SOD.

In the present study, we demonstrated that silibinin can effectively inhibit growth of HuH7 xenografts, a human HCC cell line, in nude mice and examined the related mechanisms.

MATERIALS AND METHODS

Reagents

The cell culture media were the same, as previously reported[19,20]. Anti-activated caspase-3 antibody was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibodies against human Ki-67, AFP, p-Rb, E2F1, DP1, CD1, CDK4, p21 and p27, active caspase-9, phosphorylated-AktThr308, PTEN, AC-histone3 and AC-histone4, survivin phosphorylation (p-survivin), Plk1, Chk1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The NF-κB assay kit was from Panomics, Inc. (Redwood City, CA, USA).

Cell culture

HuH7 cells, a human HCC cell line, were cultured in DMEM with 10% FBS, as previously reported[17,19,20], and used to establish HCC xenografts in nude mice as described below.

Development and treatment of nude mice bearing HuH7 xenografts

After subcutaneous inoculation of 5×10^6/0.25 mL of HuH7 cells[19,20], the mice were randomized to 3 groups, 10 mice in each group, respectively. The control group received daily gavage of a vehicle solution. The other 2 groups received silibinin at a dose of 80 mg/kg per day and 160 mg/kg per day, respectively, started 24 h after inoculation. The silibinin dose was adjusted weekly based on changes in body weight. Tumor volumes were recorded weekly and the experiment lasted for 5 wk. At the end of the experiments, xenograft tumors were measured, isolated, and weighted after euthanasia. Blood specimens were collected from the tail vein and plasma was used to quantify AFP. Three HCC xenograft specimens which were closest to the mean volume were taken from each group. Three hundred milligram of tumor tissue from each xenograft was homogenized with lysing buffer. After centrifuging, the clarified supernatants were stored in -80℃ and used for the experiments described below.

Quantification of plasma and tissue AFP levels

The plasma AFP level was quantified using an enzyme immunoassay (EIA) kit as previously reported[20]. A standard curve was obtained using the manufacturer's internal control and was used to calculate plasma AFP levels.

Analysis of apoptosis

Apoptosis was quantified using an EIA kit, as previously reported[19,20]. The degree of apoptosis was expressed based on the ratios of absorbance of the treated *vs* control xenograft tissue specimens.

Immunoprecipitation and Western blotting analysis

The supernatants of xenograft lysates were used to detect Ki-67, p21, p27, E2F1, CDK4, p-Rb, activated caspase-3 and caspase-9, PTEN, AC-H3, AC-H4, p-Akt, p-survivin and p-ERK, Plk1, Chk1, and SOD1. To determine whether silibinin could affect binding of p21 and p27 with CDK4, and binding of DP1with E2F1, an immunoprecipitation technique was used. β-actin was used as an internal control. The relative amount of each protein was quantified by digitally scanning its hybridizing bands, and the optical density of the scanned Western blotting results, as previously reported[19,20].

PTEN activity assay

PTEN protein was immunoprecipitated with 10 μL of rabbit anti-human antibodies at 4℃ overnight, followed by the addition of 25 μL of anti-rabbit IgG-conjugated agarose beads at 4℃ for 2 h. The phosphatase reaction was performed using the PTEN activity assay kit in accordance with the manufacturer's instructions[19,20].

NF-κB assay

NF-κB was quantified using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Briefly, after incubation

Table 1 Silibinin reduced the frequency and volume of HuH7 xenografts in nude mice

Groups	Treatment	Tumor frequency (%)	Tumor volume (cm^3)
Group 1	Placebo	100	4.0 ± 0.9
Group 2	80 mg/kg per day	50[a]	2.1 ± 0.3[a]
Group 3	160 mg/kg per day	30[a]	0.6 ± 0.2[a]

[a]$P < 0.05$ *vs* Group 1, $n = 10$/group.

for 1 h with 10 μL of the sample solution at room temperature the sample was washed 3 times, NF-κB p50 antibody (1:1000) was added and incubated for another hour at room temperature, followed by anti-rabbit HRP antibody (1:1000) and substrate reaction. The *A* absorbance at 450 nm was recorded.

Statistical analysis

The descriptive statistics are provided with mean ± SD. *t*-test was used to assess the effect (i.e. mean differences) of silibinin treatment on AFP production, apoptosis, as well as the scanning data of Western blots. $P < 0.05$ was considered statistically significant.

RESULTS

Silibinin reduced the frequency and volume of HuH7 xenograft growth

As shown in Table 1, silibinin treatment significantly reduced the frequency and volume of HuH7 xenografts in a dose-dependent fashion. The frequency of HuH7 xenografts was reduced by 50% in the group treated with silibinin 80 mg/kg per day and by 70% in the group treated with 160 mg/kg per day. The mean reduction in HuH7 xenograft volume was 48% in the group treated with silibinin 80 mg/kg per day and was 85% in the group treated with 160 mg/kg per day. The silibinin-reduced frequency and volume of HuH7 xenografts was associated with a significant decrease in Ki-67 expression (Figure 1A). These findings demonstrated that silibinin produced a significant *in vivo* inhibition of HCC growth through a reduction in HCC cell proliferation.

Silibinin-reduced growth of HCC xenografts was associated with decreased AFP production and secretion

Consistent with our previous *in vitro* report[17], we found that silibinin treatment significantly reduced AFP levels in both xenograft tissue and plasma obtained from the mice (Figure 2A and B). This indicated that silibinin-reduced HuH7 xenograft growth was associated with decreased production of AFP in xenograft tissue and secretion of AFP into blood circulation.

Effects of silibinin on cell cycle progression

Uncontrolled progression of the cell cycle promotes multiplication of cancer cells. We have reported the inhibitory effects of silibinin on p-Rb formation *in vitro*[17]. In the present study, we demonstrated that silibinin

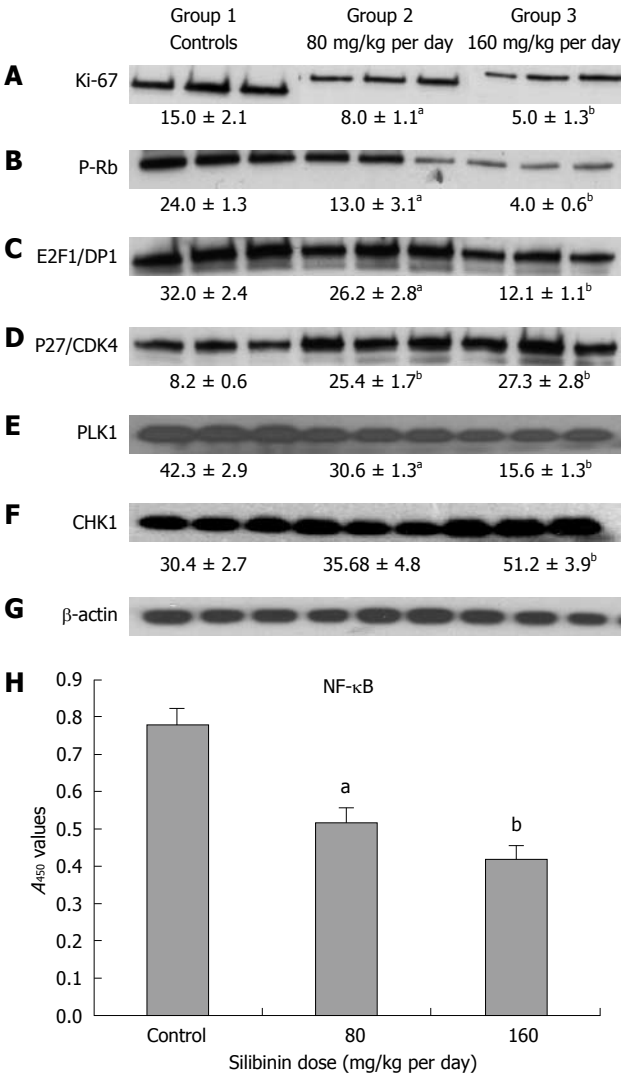

Figure 1 Effects of silibinin on proliferation and cell cycle progression in HuH7 xenograft tissue specimens. A: Silibinin inhibited Ki-67 expression; B: Silibinin inhibited Rb phosphorylation; C: Silibinin inhibited E2F1/DP1 complex formation; D: Silibinin increased p27/CDK4 complex formation; E: Silibinin inhibited Plk1 expression; F: Silibinin increased Chk1 expression; G: β-actin as internal control; H: Silibinin inhibited nuclear NF-κB content. [a]$P < 0.05$; [b]$P < 0.01$ *vs* control.

resulted in a significant and dose-dependent inhibition of p-Rb production (Figure 1B), which was associated with decreased E2F1/DP1 complex formation in HuH7 xenograft tissue (Figure 1C).

By binding to the cyclin/CDK complexes, cyclin dependent kinase inhibitors, such as p21 and p27, halt uncontrolled cell proliferation. P21/CDK4 and p27/CDK4 complexes are involved in the transition from G1 into S phase. Consistent with our *in vitro* report[17], silibinin treatment significantly and dose-dependently increased p27/CDK4 complex (Figure 1D), but did not affect p21/CDK4 complex formation (data not shown) in HuH7 xenograft tissue. To further determine whether silibinin could also alter the levels of up-stream molecules that control cell cycle progression, the changes in Plk1, Chk1 and nuclear NF-κB were determined. As shown in Figure 1E-H, silibinin increased Chk1 expression, but inhibited Plk1 expression and nuclear NF-κB level.

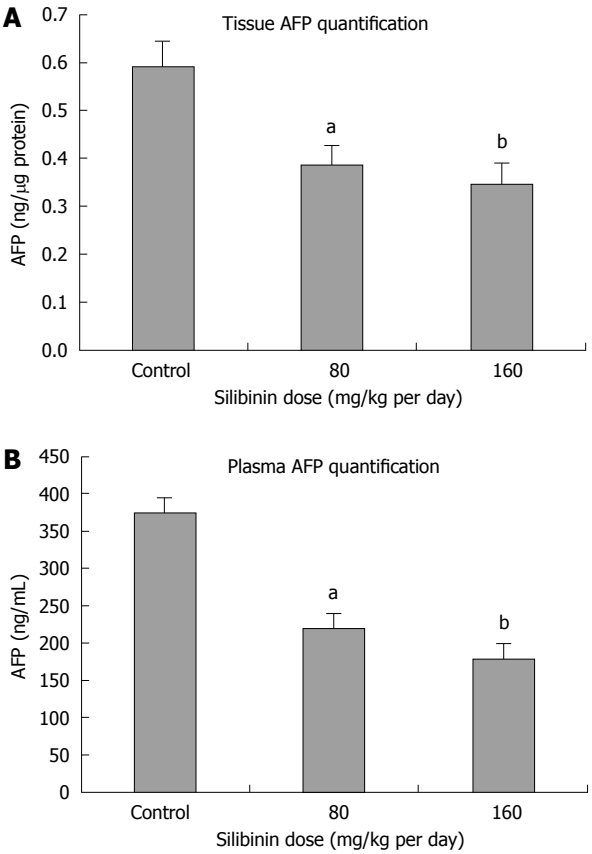

Figure 2 Effects of silibinin on AFP production and secretion. A: Silibinin reduced AFP production; B: Silibinin reduced AFP secretion. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* control.

Silibinin-reduced HuH7 xenograft growth was associated with increased apoptosis and reduced survivin phosphorylation

Apoptosis is another important mechanism that controls cancer cell growth. We previously reported that silibinin promotes HuH7 cell apoptosis *in vitro*[17]. In the present study, we examined apoptosis in HuH7 xenograft tissue specimens. As showed in Figure 3A, we observed that silibinin significantly increased apoptosis in HuH7 xenograft tissue. To further define the mechanisms involved in the apoptosis pathway, activated caspase-3 and 9, Bcl-2, and p-survivin expression were assessed. We demonstrated that silibinin treatment significantly inhibited p-survivin (Figure 3B), as previously reported in the *in vitro* system[17]. However, inconsistent with our previous *in vitro* findings, silibinin did not affect production of activated caspase 3 and 9, or Bcl-2 (data not shown).

In vivo effects of silibinin on p-Akt and P-ERK pathways

P-Akt and p-ERK pathways are involved in modulating cancer development and growth[24-30]. Our previous study indicated that silibinin increased PTEN activity and reduced p-Akt expression *in vitro*[17]. In the present study, we found that significantly reduced p-Akt production was only seen in HuH7 xenograft tissue treated with silibinin at a dose of 160 mg/kg per day, but not 80 mg/kg per day (Figure 4A). However, silibinin-reduced HuH7 xenograft growth was associated with a silibinin dose-dependent increase in PTEN production (Figure 4B) and its activity (Figure 4E). In addition, silibinin-reduced HuH7 xenograft growth was also dose-dependently associated with a decrease in p-ERK (Figure 4C).

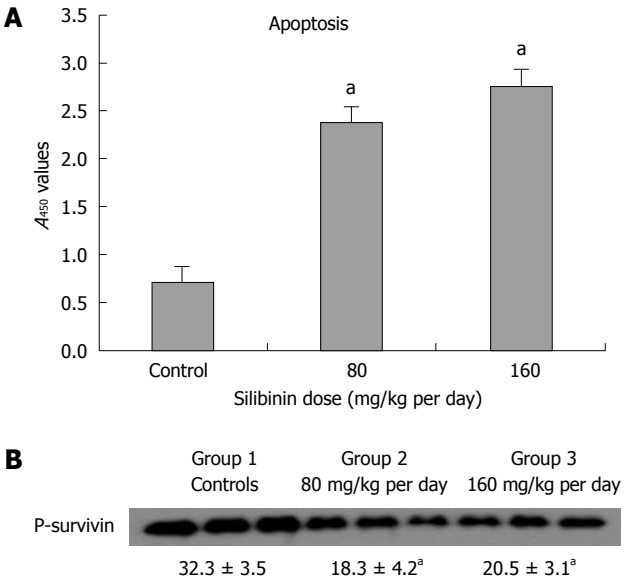

Figure 3 Effects of silibinin on apoptosis in HuH7 xenograft tissue. A: Silibinin induced apoptosis; B: Silibinin inhibited survivin phosphorylation. $^{a}P < 0.05$ *vs* control.

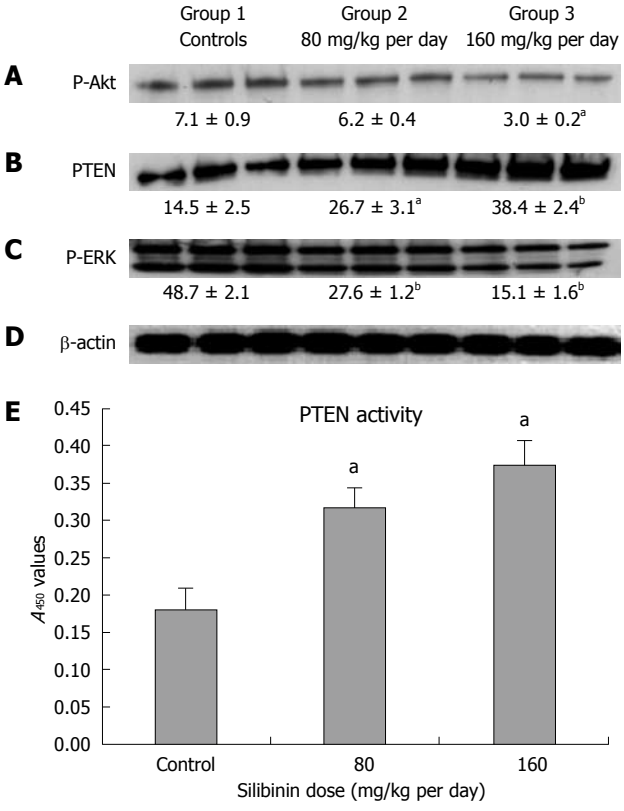

Figure 4 Effects of silibinin on P-Akt and P-ERK pathways in HuH7 xenograft tissue. A: Silibinin inhibited p-Akt expression; B: Silibinin increased PTEN expression; C: Silibinin inhibited P-ERK expression; D: β-actin as internal control; E: Silibinin increased PTEN activity. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* control.

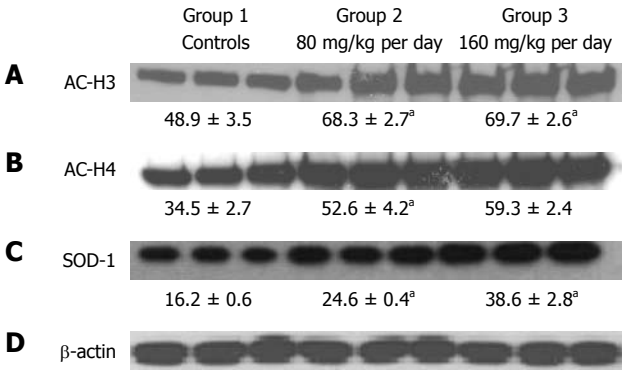

Figure 5 Effects of silibinin on AC-H3, AC-H4, and SOD1 in HuH7 xenograft tissue. A: Silibinin increased AC-H3 expression; B: Silibinin increased AC-H4 expression; C: Silibinin increased SOD1 expression in HuH7 xenograft tissue; D: β-actin as internal control. $^aP < 0.05$ *vs* control.

In vivo effects of silibinin on histone acetylation

Histone acetylation plays an important role in controlling cell proliferation and cell cycle progression[31-33]. Our previous *in vitro* results indicated silibinin increases AC-H3 and AC-H4[17]. In the present study, we found that silibinin-reduced HuH7 xenograft growth was associated with significantly increased AC-H3 and AC-H4 production (Figure 5A and B). These results further confirmed the *in vivo* effects of silibinin on AC-H3 and AC-H4 production, indicating their potential role in HCC growth.

In vivo effects of silibinin on SOD1 expression

SOD1 is one of the most important enzymes in reducing ROS levels. It was reported that SOD1 may play a role in the effects of silymarin on alcoholic-induced liver injury[42]. We demonstrated that silibinin-reduced growth of HuH7 xenografts was associated with a significant and dose-dependent increase in SOD1 production in the xenograft tissue (Figure 5C). Our results indicate a possible mechanistic role of SOD1 in silibinin-reduced growth of HuH7 xenografts.

DISCUSSION

HCC is one of the most common malignancies globally. A rise in incidence, limited treatment options, and poor prognosis of this disease emphasize the importance of developing effective chemoprevention for this disease. Silibinin is the major biologically active compound of milk thistle which has been reported to be safe and well-tolerated, and protects the liver from drug or alcohol-related injury[5-7]. Recently, the potent *in vitro* anti-HCC effects of silibinin have been demonstrated[5,17], which have provided us with a rationale to further define the *in vivo* effects and mechanisms of silibinin on HCC growth.

In the present study, we examined the *in vivo* effects and mechanisms of silibinin on HCC growth using the nude mouse model bearing human HCC xenografts following inoculation of HuH7 cells[19,20]. We demonstrated that silibinin treatment resulted in a significant dose dependent decrease in both frequency and mean volume of HuH7 xenograft growth. Our previous data recently reported the *in vivo* anti-HCC effects of silymarin[43]. The silibinin dose used in our study was lower than that of silymarin[43]. The fact that silibinin is a purified bioactive component from silymarin may explain why silibinin at the lower dose can achieve a potent anti-HCC effect. The anti-HCC effects of silibinin were associated with a significant reduction in Ki-67 expression, a biomarker of cell proliferation. These findings were consistent with our previous *in vitro* results and those of Varghese *et al*[17], and were further supported by the recent reported effects of silibinin on colorectal cancer[14,17]. Thus, our data suggest that silibinin-reduced *in vivo* growth of human HCC xenografts is associated with down regulation of cell proliferation.

Plasma AFP has been widely used as a noninvasive biomarker for HCC[21-23]. As we previously reported in the cell culture system, it was demonstrated that silibinin treatment resulted in a significant decrease in xenograft production and plasma levels of AFP which was correlated with growth inhibition of HCC xenografts. Since AFP overexpression has been associated with uncontrolled growth of HCC, our data provided additional *in vivo* evidence that silibinin-reduced growth of human HCC is associated with down regulation of cell proliferation. These findings also indicate the potential value of using plasma AFP as a non-invasive biomarker to determine the *in vivo* anti-HCC effects of silibinin.

Uncontrolled G1-S progression results in continued proliferation with potential malignant transformation and carcinogenesis. Increased E2F1/DP1 complex promotes cell cycle progression. Our results indicated that silibinin could significantly inhibit E2F1/DP1 complex formation in association with inhibition of HCC xenograft growth. Consistent with these findings, we also demonstrated that silibinin significantly decreased p-Rb expression, an important modulator that induces E2F1/DP1 formation.

P21 and p27 inhibit cell cycle progression by forming p21/CDK4 or p27/CDK4 complexes. Consistent with our previous *in vitro* report[17], we demonstrated that silibinin significantly increased p27/CDK4 complexes in HuH7 xenograft tissue. Similar effects of silibinin were previously reported in a skin carcinogenesis model. In contrast to the *in vitro* data[17], silibinin did not enhance p21/CDK4 complex formation in HuH7 xenograft tissue.

Chk1 is a critical enzyme in DNA damage-induced G2/M arrest, and blocks mitosis by phosphorylating Cdc25C and has been proposed as a novel tumor suppressor[36]. Both Plk1 and NF-κB promote cell cycle progression. NF-κB mediates activation of cyclin D1 gene transcription, induces cell cycle progression and inhibits cell apoptosis[34]. Inhibition of NF-κB activation induced an early G1 cell cycle arrest in primary rat hepatocytes[35]. In human cells, Plk1 has

been implicated in the regulation of different processes, including mitotic entry, spindle formation, and plays a role at multiple points during the restart of the cell cycle following DNA damage[37]. Our results demonstrated that silibinin at 80 mg/kg and 160 mg/kg significantly reduced Plk1 expression and the level of nuclear NF-κB. The higher dose of silibinin (160 mg/kg) also increased Chk1 expression. Taken together, our data indicate that silibinin reduced *in vivo* HCC xenograft growth by decreasing HCC cell proliferation and cell cycle progression which was mediated by inhibiting translocation of NF-κB to the nucleus, Plk1, p-Rb expression, E2F1/DP1, and increasing Chk1 expression and formation of the p27/CDK4 complex.

Increasing cell apoptosis is another important step that inhibits tumor growth[44]. We demonstrated that silibinin promotes *in vivo* apoptosis in HuH7 xenografts, which reconfirmed the previous *in vitro* findings[17,18]. Survivin is an apoptosis inhibitor that is overexpressed in most cancers in a cell cycle-dependent manner. P-survivin is necessary for cancer cell viability[45]. Our results demonstrated that silibinin inhibited p-survivin in association with increased apoptosis in HuH7 xenograft tissue. These results reconfirmed our previous *in vitro* findings[17] and indicated the important role of the survivin-mediated decrease in apoptosis in HCC growth.

We reported that silibinin-enhanced apoptosis of cultured HuH7 cells was associated with increased production of activated caspase 3 and 9, however, these changes were not reproducible in HuH7 xenograft tissue. Additionally, silibinin seemed not to alter Bcl-2 expression, another modulator of apoptosis, in HuH7 xenograft tissue. These data indicated that a discrepancy of silibinin-mediated apoptosis signaling may occur in these two systems.

Studies have indicated the important roles of PTEN/PI3K/Akt and ERK signaling in carcinogenesis and cancer progression[24-30,46]. Phosphorylation of Akt results in its activation, which promotes cell cycle progression by phosphorylating several other key proteins[47-51]. PTEN is an up-stream molecule that inhibits p-Akt. We found that silibinin significantly increased PTEN expression and activity that was further associated with reduced p-Akt production in HCC xenograft tissue. These results indicate a possible pathogenic role of the PTEN/PI3K/p-Akt pathway in HCC growth that may also serve as an important silibinin target. Increased p-ERK activates transcription of the mitogenic and cell regulatory genes and promotes oncogenesis[46]. P-ERK was reportedly increased in HCC[52], suggesting its involvement in HCC development. A previous *in vitro* study reported that silibinin can inhibit ERK phosphorylation in human osteosarcoma[53]. In the present study, we found silibinin-reduced HuH7 xenograft growth was also associated with a significant inhibition of p-ERK production. These results are also in agreement with an effect on colorectal cancer reported by Singh *et al*[14]. The results revealed that the p-ERK pathway is likely involved in silibinin-reduced HCC growth, another possible novel target of HCC chemoprevention and therapy in future research.

Histone acetylation has been reported to be involved in cell proliferation, differentiation, and cell cycle regulation. A decrease in acetylation status in the cells is associated with carcinogenesis[31-33]. Our results demonstrated that silibinin significantly increased AC-H3 and AC-H4 expression, suggesting that increased histone acetylation may mediate silibinin-reduced HCC growth.

ROS stress has been associated with the development of HCC. SOD is one of the important enzymes in reducing ROS levels. Altered expression of SOD has been associated with the development and differentiation of HCC. Although the effects of silibinin on SOD were reported in patients with alcoholic liver disease[42], it is unknown whether the same mechanism has any role in the anti-HCC effects of silibinin. We demonstrated that silibinin-reduced growth of HuH7 xenografts was associated with a significant increase in the production of SOD1 in the xenograft tissue of nude mice. This was particularly evident when a higher dose of silibinin was used. Thus, our results indicate a possible mechanistic role of SOD1 in silibinin-reduced growth of HuH7 xenografts.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common malignancies. Silibinin has been reported to be safe, well-tolerated, and protects the liver from drug or alcohol-related injury. A recent demonstration of the potent *in vitro* anti-HCC effects of silibinin has provided us with a rationale to further assess the *in vivo* effects of silibinin on HCC growth. The present study examined the *in vivo* effects and mechanisms of silibinin on HCC growth using a nude mouse model bearing HuH7 (a human HCC cell line) xenografts.

Research frontiers

The search for safe and well-tolerated chemopreventive agents is one of the significant research frontiers in HCC chemoprevention. Many studies have demonstrated that silibinin can effectively inhibit the growth of various types of tumor cells, however, little is known about the *in vivo* effects and mechanisms of silibinin on HCC growth.

Innovations and breakthroughs

Previous study demonstrated that silibinin can inhibit HCC cell growth *in vitro*. In the present study, we confirmed that silibinin can effectively inhibit growth of human HCC xenografts in mice by affecting cell cycle progression, apoptosis, and several other pathways.

Applications

These results provide a rationale to further pre-clinical investigations which may result in clinical trials assessing the application of silibinin in HCC chemoprevention.

Terminology

Xenografts: Tissue or organs from an individual of one species inoculated, transplanted into or grafted onto an organism of another species, genus, or family. Chemoprevention: The use of chemical compounds to intervene in the early stage of carcinogenesis and thereby reverse tumor formation.

Peer review

This is a well-designed and very interesting study, methods are appropriated and results are consistent with the conclusions.

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