Master of Science

Effect of cytotoxicity in hepatocarcinoma cells by berberine

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Effect of cytotoxicity in hepatocarcinoma cells by berberine

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Abstract

Background: Hepatocellular carcinoma (HCC) is the 7th most common malignancy and has the highest incidence rate of 26.9% in men of Eastern Asia and is the second leading cause of cancer- relative deaths. Most chemotherapeutic agents used to treat HCC patients are mainly cytotoxic medications. However, these drugs are very toxic and have serious side effects. Therefore, I wanted to find a drug that increases the efficacy of anticancer drugs and reduces side effects that target tumor cells without causing cytotoxicity in healthy hepatocytes. Berberine, a plant-derived compound, has very little to no cytotoxic impact on normal liver cells and exerted anti-tumor effects against a variety of carcinoma cells. In previous studies. Berberine has been found to suppress the proliferation and migration of human HCC cells, while the underlying mechanisms are not fully understood.

Methods: It has been analyzed the effect of berberine on HCC growth using *in vivo* mice model. The HCC cells viabilities were determined by using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The HCC cell proliferation was determined by colony forming assay. The effects of berberine on migration were determined by wound healing assay. Various mRNA expressions were detected by qRT-PCR.

Results: Berberine inhibited the Hep3B Xenograft model in does dependent manner and it also inhibited proliferation of HepG2 (wt p53), Hep3B (deficient p53), and (mut p53) cells.

Moreover, berberine inhibited HCC colony formation and migration in HCC Cells.

The treatment of berberine reduced mRNA expression of autophagy-related genes, including *Beclin-1* and *LC3-II* (microtubule-associated protein light chain 3), and decreased the mRNA expression of mitochondria-related apoptosis, including *Caspase-3* and *Caspase-9*, respectively. Besides, berberine regulated various mRNA expressions of p53 and *SHP1*(Src homology region 2 domain-containing phosphatase 1).

Conclusions: These data suggested that berberine has anticancer effects in HCC cells and the Hep3B Xenograft model. Especially, Hep3B and Huh7 cells are less responsive to berberine treatment than HepG2 cell.

Berberine regulated the expression of autophagy-related genes, mitochondria apoptosis signaling pathway-related genes at the mRNA level in HCC cells. In addition, it upregulated the mRNA expression of p53 and *SHP1* in HCC.

From these results, I suggest that berberine promises the additional chemotherapeutic method in HCC.

Key words: Hepatocellular carcinoma (HCC), Berberine, *Becelin-1*, *LC3-II*, *Caspase-3*, *Caspase-9*, p53, *SHP-1*

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Introduction

Hepatocellular carcinoma (HCC) occurs from hepatocytes and is advanced by liver cirrhosis in 80% of the cases [1]. HCC is chronic exposure of the liver to damage from HCV (hepatitis C virus), HBV (hepatitis B virus), NAFLD (nonalcoholic fatty liver disease), NASH (nonalcoholic steatohepatitis). Alcohol cause reiterated hepatocyte damage and results in cirrhosis is induced and persisted leading to HCC [2][Fig.1]. Globally, HCC is the 7th most common malignancy and has the highest incidence rate of 26.9% in men of Eastern Asia in 2020 [3].

HCC is the second leading cause of cancer- relative deaths due to its high recurrence rate [3]. Because HCC is resistant to cytotoxic chemotherapy and more likely to metastasis due to the abundant blood flow to the liver, patients with HCC have a low long-term survival rate. General methods for HCC cancer therapy have conducted surgical elimination of tumors, liver transplantation, radiotherapy and chemotherapy [4]. Though a variety of treatments is available for HCC, chemotherapy is used to treat patients who are thought as being unsuited nominees for surgical elimination of tumors, liver transplantation, that is, patients who have large tumors, have extrahepatic metastasis, show evidence of vascular invasion [5]. Among chemotherapy agents, Sorafenib (Fig.2B) has been widely used as a chemotherapy agent in the treatment of HCC.

Sorafenib, a multiple-target tyrosine kinase inhibitor, is able of targeting *VEGFR2* (vascular

endothelial growth factor receptor2), *PDGFR-β* (platelet-derived growth factor receptor), *c-KIT* (hepatocyte factor receptor), and other proteins to prevent tumor angiogenesis [6]. It also controls tumor cell proliferation by preventing *Raf-1*(proto-oncogene serine/ threonine-protein kinase) , *B-Raf* (v-Raf murine sarcoma viral oncogene homolog B), and kinase activity in the *Ras* (Rat sarcoma virus) / *Raf* /*MEK*(mitogen-activated protein kinase) / *ERK*(extracellular signal-regulated kinases) signaling pathways [6]. Moreover, it has been used in clinical settings to prolong survival, but it is expensive and has serious side effects such as Hypertension [7], Cardiovascular events [8], ATEs (Arterial thromboembolic events) [9], Bleeding [10], HFSR (Hand–Foot Skin Reaction) [11], Diarrhea [12], Renal toxicity [13]. Therefore, I wanted to find a drug that increases the efficacy of anticancer drugs and reduces side effects that target tumor cells without causing cytotoxicity in healthy hepatocytes.

Natural compounds include a large and diverse group of substances from many natural sources such as plants, bacteria, fungi, insects, arachnids, marine organisms, and higher-order animals. Among them, plant-derived compounds have received a lot of attention, and their use has expanded over the last few decades.

Berberine (Fig.2A) has very little to no cytotoxic impact on normal liver cells [14]. Berberine, a small molecule isoquinoline alkaloid extracted from the rhizomes of *Coptis Chinensis and Hydrastis Canadensis* [15].

It was reported that berberine exerted anti-tumor effects against neuroblastoma, lung cancer [16], cervical cancer [17], liver cancer [18], leukemia [19]. Berberine showed that regulated

the cell cycle and inhibited cell proliferation in lung [22], human chondrosarcoma [21], colorectal cancer [22], breast cancer [23], liver cancer [24]. Berberine inhibits cancer cell migration and as well as induces apoptosis [25] or autophagy [26]. Berberine inhibits cancer cell invasion and metastasis in breast cancer [27], neuroblastoma [28] *in vitro*. When tumor cells release cytokines to change the nearby tumor microenvironment [29], berberine prevents osteosarcoma cell [30], peripheral blood mononuclear cells [31] invasion and metastasis by influencing the expression of tumor-related signaling pathways and proteins. Berberine hinders inflammation by regulating *AMPK/mTOR* [32], signaling pathways, and cytokines of TNFα, IL-1β, and IL-6 [33]. Berberine regulates tumor suppressor p53. p53 is known as a tumor suppressor protein because it regulates the cell cycle, DNA replication, and uncontrolled cell division during tumor development [34]. When the p53 protein is mutated, its function is lost, resulting in tumor proliferation and growth [35]. A study revealed that berberine may up-regulate the p53 expression by suppressing its inner inhibitor *MDM2*(Mouse double minute 2 homolog) also known as E3 ubiquitin-protein ligase at the post-transcriptional level in acute lymphoblastic leukemia cells [36]. Berberine-induced miR-23a could suppress the *Nek6* (Never in mitosis A-related kinase 6) , a negative regulator of p53, and induced p53 up-regulation in HCC [37]. Berberine increased wt (wild type) p53 phosphorylation while decreasing mut p53 levels in human glioma cells [38]. In addition, the p53 expressing cells were more sensible against berberine than the p53 null cells upon berberine treatment in neuroblastoma cells [39].

Previous studies of HCC were have revealed that berberine induce growth arrest and cell death in HepG2 [47], MHCC97 L [20], SMMC7721 [47]. Berberine treatment in HepG2, Hep3B, and SNU-182, up-regulated expression of tumor suppressor genes, including *KLF6* (Kruppel-like factor 6), *ATF3* (activating transcription factor 3), and *p21*, On the other hand, down-regulating the expression of *E2F1* (E2F transcription factor 1) and *PTTG1*(pituitary tumor transforming gene 1) [41].

Berberine regulates in Hep3B Cell the Na+ dependent transporter SLC1A5 (Solute Carrier Family 1 Member 5). SLC1A5 is a neutral amino acid transporter included in the SLC1 family and localized in the plasma membrane of several body regions [42]. This SLC1A5 transports glutamate is essential for cancer cells in a Na+-dependent manner[43]. Berberine can inhibit the proliferation of Hep3B by decreasing SLC1A5 expression, and suppression of glutamine uptake [44].

Berberine triggers apoptosis in HCC in a number of different ways. It induces apoptosis via the mitochondrial route in Huh7 [45], HepG2, H22 and Bel-7404 [46]. It induces apoptosis through *AMPK*-dependent in HepG2, SMMC 7721 and Bel 7402 [47]. It also induces apoptosis in HepG2 cells by suppressing the *iPLA2/LOX-5/LTB4* pathway [48].

Berberine induces autophagic cell death in HepG2 and MHCC97-L cells through *Beclin-1* up-regulation and activation of *mTOR* down-regulation by suppressing the activation of *Akt* and up-regulating P38 MAPK signaling [18]. Berberine also induced *Beclin-1* and *LC3-II*(microtubule-associated protein light chain 3) upregulation, by inhibiting *mTORC1* via

AMPK activation in HepG2 cells [49].

Previous studies have demonstrated that berberine induce cell death by apoptosis, autophagy and other in HCC. However, the role of berberine signaling pathways which are related with downstream of wt p53 and mut p53 in HepG2 (wt p53), Huh7(mut p53), and Hep3B (p53 null) cells is not fully understood. Therefore, the aim of this study is investigating the anticancer mechanism of berberine in HCC using *in vitro* and *in vivo* models.

Material and Method

Animals

Specific pathogen-free NOD-SCID (Nonobese diabetic/severe combined immunodeficiency) male mice (weight 20-22g) were purchased from Central Laboratory Animal Inc. (Seoul, Republic of Korea). The animals were housed and maintained under controlled specific pathogen-free conditions at 21–24°C and 40–60% relative humidity under a 12-h light/dark cycle with free access to food and water. The mice were provided with veterinary/supportive care when they began to show signs of illness. All animal experiments were performed in accordance with the Korean Ministry of Food and Drug Safety guidelines.

In vivo tumor model

NOD-SCID (Nonobese diabetic/severe combined immunodeficiency) mouse was implanted with Hep3B cells at a density of $2x10^6$ cells/ each. After making a CDX (Cell Line-Derived Xenograft) model, the cell was transferred to another NOD-SCID mouse and transplanted. When the tumor volume reached 100 mm^3, mice were randomly divided into three groups. The experimental group was intraperitoneally injected with berberine (5 mg/kg and 10 mg/kg) (Sigma-Aldrich) daily for 11day. The control group received daily intraperitoneal injections with an equal volume of PBS (Phosphate-buffered saline, Capricorn). The long axis (D) and the short axis (d) of the tumor were measured daily. Tumor volume (Tv) was calculated by the formula: $Tv = 0.5 \times D \times d^2$. On day 11 post-administration, all mice were sacrificed, tumor

tissues were weighed.

Cell culture

Human hepatoma cancer cell line HepG2, Hep3B, and Huh7 cells were obtained from Korean Cell Line Bank. HepG2 cells were cultured in RPMI (Roswell Park Memorial Institute) medium with 5% FBS (fetal bovine serum). Huh7 and Hep3B cells were cultured in DMEM (Dulbecco's Minimum Essential Medium) supplemented with 5% FBS. All cells were cultured at 37°C in a humidified chamber with 95% air and 5% CO_2.

MTT assay

Cell proliferation rates were assessed using the MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Briefly, cells were seeded into 48-well plates at a density of $0.7x10^4$ cells/well (HepG2) and $1.4x10^4$ cells/well (Hep3B and Huh7). The experimental groups were treated with different concentrations of berberine ranging from 5, 10, 15 μM. After 24 h ,48 h, and 72 h, MTT was added into each well at a concentration of 333μg/ml per well, incubated for 2 h and then replaced with 100 μl of DMSO (Dimethyl sulfoxide). The number of viable cells was evaluated by measuring the absorbance at an O.D. (optical density) of 595 nm using ELISA (enzyme-linked immunosorbent assay).

Wound-healing assay

Wound-healing assay was used to analyze cell migration. After seeding the cells at $8x10^5$ cells/well in a 12-well plate, overnight at 37°C, 5% CO_2 incubation. Then, after making a scratch line on the cells using a 200 µl sterile pipette tip, the plates with FBS 2% media were incubated at 37°C in 5% CO_2. Wound healing was observed at 0 and 24 h using an inverted microscope system (Olympus).

Colony formation assay

Human HCCs were made into single-cell suspensions with trypsin and then incubated in six-well plates at a density of $5x10^2$ cells per well. Cells were treated with berberine for 10 days and then cells were washed with PBS (phosphate buffered saline, Capricorn) twice, fixed in 4% paraformaldehyde and stained with 0.5% crystal violet for 15 min. After washed by PBS, images were captured.

RT-PCR experiments

Total RNA was isolated from cultured cells using Trizol (Cellconic) reagent and reverse transcribed using the Maxime™ RT PreMix Kit (Oligo dT15 Primer) (iNtRON) according to the manufacturer's protocol. Real-time PCR was carried out using Luna ® Universal qPCR Master Mix Kit (NEB) under standard reaction conditions: 45 cycles at 95 °C for 5 mins, 95 °C for 10 s and 60 °C for 30 s with the 7500 Real-Time PCR Detection System (Bio molecular systems). Lists of primers summarized in Table 1.

Statistics analyses

Values are expressed as the mean ± standard error of the mean (SEM). Data were statistically analyzed using the independent samples *t*-test and an analysis of variance.

Results

1. Berberine inhibits Hep3B growth *in vivo*.

First, I investigated that whether berberine could inhibit the Hep3B growth *in vivo* animal model. After 11 days, the animals were sacrificed, the 10 mg/kg berberine caused a 67.37% decrease in tumor volume as compared with the control group, whereas 5mg/kg berberine caused a 39.72% decrease respectively (Fig.3A). Similarly, the 10mg/kg berberine showed significantly decreased tumor weight (Fig. 3C and 3D). During the experiments, no weight loss was observed in mice treated with berberine (Fig. 3E). As the results, berberine inhibited the Hep3B growth in NOD-SCID mouse in does dependent manner.

2. Berberine suppressed HCCs proliferation.

To test the effect of cell viability, berberine treated to HepG2, Hep3B and Huh7, respectively. Figures 4A, 4B and 4C showed the MTT assay of cell viability after berberine treated with different concentrations and indicated times. At the concentration of 15uM of berberine, cell viability was decreased 55.4 % at 24 h, 27.7 % at 48 h and 15 % at 72 h in HepG2, at 24 h, 78.09 % at 48 h and 66.53 % at 72h in Huh7, and by 88.8 % at 24 h, 78.09 % at 48 h, and 66.05 % at 72 h in Hep3B which are comparing with control groups. Notability, the HepG2 cell viability was effectively inhibited compared with Huh7 and Hep3B.

3. Berberine suppressed migration in HCCs.

To investigate the effect of berberine on cell migration in HepG2 and Hep3B cell, cell migration was performed using wound-healing assay. Figure 5A showed that the region of the wounded area, between cell layers after generating a scratch was at 76.07% occupied by migrating cells after 24 h in the control group in HepG2 cells. The HepG2 cells treated with 10, 20, 30μM of berberine, on the other hand, did not occupy at 44.78%, 25.92%, 24.62% of the vacant area of the cells. The region of the wounded area by 200 μl tip, between cell layers after generating a scratch was at 52.41% occupied by migrating cells after 48 h in the control group in Hep3B cells (Fig. 5B). While, Hep3B cells treated with 10, 20, 30μM of berberine did not occupy at 20.49%, 20.51%, 14.79% of the vacant area of the cells. These findings indicated berberine's ability to suppress cell migration in HCCs.

4. Berberine suppressed colony formation in HCCs.

To further examined the cell proliferation, the colony formation assay performed in HepG2 and Hep3B. Berberine treatment showed a clear reduction of colony formation in a dose dependent manner. In comparison to the control group, colony formation rates were decreased in berberine-treated HepG2 cells at 1 μM (72.15 %), 2 μM (31.81%), 3 μM (23.29 %), 4 μM (22.15%), 5 μM (13.63 %), respectively. Notability, the colony rate was decreased at 2uM of berberine treated cells (Fig. 6A).

Additionally, the colony formation rate in Hep3B decreased in 1 μM (76.17 %), 2 μM (58.82%), 3 μM (54.11 %), 4 μM (35.29 %), and 5 μM (32.64 %) (Fig. 6B), respectively.

Compared with HepG2, Hep3B showed that the colony formation rate was considerably reduced from 4 μM. Collectively, berberine inhibited colony formation more effectively in HepG2 cells than in Hep3B cells.

5. Berberine regulated *Beclin-1, LC3-II* associated to autophagy in HCC cells at the mRNA level.

It was reported that berberine upregulates the autophagy-related factor *Beclin-1* and *LC3-II*, thus induces the autophagy in HCC [49]. To investigate the effect of berberine in autophagy formation, the mRNA expression of *Beclin-1* and *LC3-II* were examined in HepG2, Hep3B and Huh7 cell by using qRT-PCR assay.

The *Beclin-1* and *LC3-II* mRNA levels were higher in HepG2 cells treated with berberine for 12 h than in control cells (Fig. 7A). The *Beclin-1* levels were higher in Hep3B cells treated with berberine for 12 h than in control cells. The *LC3-II* mRNA levels were higher in Hep3B cells treated with berberine for 24 h than controls (Fig. 7B). The *Beclin-1* mRNA levels were higher in Huh7 cells treated with berberine for 24 h than in control cells. The *LC3-II* mRNA levels were higher in Huh7 cells treated with berberine for 12 h than controls (Fig. 7C).

6. Berberine regulated *Caspase-3, Caspase-9* associated to mitochondrial cell apoptosis signaling pathway in HCC cells at the mRNA level.

To investigate the effect of apoptosis-related signaling molecules in berberine treated HCC, *Capase-3* and *Caspase-9* mRNA expression examined in HepG2, Hep3B and Huh7.

The *Caspase-9* mRNA levels were higher in HepG2 cells treated with berberine for 6h than control. In addition, the *Caspase-3* mRNA levels were higher in HepG2 cells treated with berberine for 12 h than in control, but were lower in HepG2 cells treated with berberine for 24 h than controls (Fig. 8A).

The *Caspase-9* mRNA levels were higher in Hep3B cells treated with berberine for 6 h than control, but were decreased in Hep3B cells treated with berberine for 12 h than control. The *Caspase-3* mRNA levels were lower in Hep3B cells treated with berberine for 24 h than controls (Fig. 8B).

The mRNA levels of *Caspase-9* were no difference in Huh7 cells treated with berberine for 12 h and 24 h in control and treated, but the mRNA levels of *Caspase-3* were higher in Huh7 cells treated with berberine for 24 h than controls (Fig. 8A).

7. Berberine regulated p53 in HCC cells at the mRNA level.

It was reported that berberine regulates tumor suppressor p53 [34]. p53 is known as a tumor suppressor protein because it regulates the cell cycle, DNA replication, and uncontrolled cell division during tumor development mRNA of P53 was identified in HepG2.

The RNA level of p53 was higher in HepG2 cells treated with berberine for 6 h,12 h and 24 h than controls (Fig. 9).

8. Berberine regulated *SHP-1* in HCC cells at the mRNA level.

mRNA of *SHP-1* (Src homology region 2 domain-containing phosphatase 1) was identified in HepG2 and Huh7. *SHP-1* acts as a tumor suppressor in Hepatocarcinogenesis and HCC Progression. The mRNA level of *SHP-1* was higher in HepG2 cells treated with berberine for 12 h than controls (Fig. 10A). The *SHP-1* mRNA levels were higher in Huh7 cells treated with berberine for 12 h, 24 h than control (Fig. 10B).

Discussion

Hepatocellular carcinoma (HCC) is the common malignancy and has the highest incidence rate in men of Eastern Asia [3]. The treatment of HCC patients is still a major problem. Most chemotherapeutic agents used to treat HCC patients of unsuited nominees for surgical removal of tumors, liver transplantation, are mainly cytotoxic medications. However, these drugs are very toxic and has serious side effects [7-13].

Previous research found berberine has been reported to exert anti-tumor effects against liver cancer [18], and it is has very little to no cytotoxic impact on normal liver cells [14].

This study confirmed that berberine had therapeutic effects on HCC in the Hep3B-xenografts model by inhibition of tumor cell growth.

In order to check the mechanism related to the anticancer effect, various of apoptosis mechanisms were studied in cell lines. Previous research found that the p53 WT cells were more sensible against berberine than the p53 null cells upon berberine treatment in Neuroblastoma cells [39] and also found that the p53 WT cells were more sensible against berberine than the p53 Mut cells upon berberine treatment in human glioma cells [38].

I confirmed that berberine inhibited HepG2, Hep3B and Huh7 cell proliferation and also demonstrated that the p53 wild type (wt) HepG2 cell lines, emerged to be the most responsive, whereas p53 deficient Hep3B cell lines and p53 Mutation type (mut) Huh7 cell lines, were the less responsive to berberine treatment. These data suggests that berberine induced p53-dependent HCC growth inhibition.

Moreover, berberine significantly inhibited both HepG2 and Hep3B cell migration. This result is correspond with previously reported in HepG2 [51], but little is known regarding the effects of berberine on migratory in Hep3B cells. Collectively, I confirmed found that berberine induced p53 independent cells migration inhibition.

Berberine suppressed colony formation in both HepG2 and Hep3B cells, although in HepG2 cells more effectively than in Hep3B cells. This result is in line with what has been previously reported [41]. Next, I investigated that berberine regulate various mechanism in HepG2, Hep3B, and Huh7 cells. I checked that berberine regulate autophagy mechanism in HepG2, Hep3B, and Huh7 cells. Previous research found that berberine induces HepG2 [49] and Huh7 [50] cells autophagy in protein level, but little is known regarding the effects of berberine on autophagy of mRNA level in HepG2 and Huh7 cells. I found that berberine increases the expression of autophagy-related genes *Beclin-1* and *LC3-II* at the mRNA level in HepG2 and Huh7 cells. Furthermore, little is known regarding the effects of berberine on autophagy of mRNA and Protein level in Hep3B. I found that Berberine increases the expression of autophagy-related genes *Beclin-1* and *LC3-II* at the mRNA level in Hep3B cells. We suggest that Berberine induces the autophagy at the mRNA level in HCC Cells.

I confirmed that berberine regulate mitochondrial cell apoptosis signaling pathway in HepG2, Hep3B, and Huh7 cells.

Previous research found that berberine decreases HepG2 cells the expression of pro-*caspase-3* and pro-*caspase-9* and increases HepG2 cells the expression of cleaved-*caspase-3* and

cleaved-*caspase-9* in protein level [47], but little is known regarding the effects of berberine on mitochondrial cell apoptosis signaling pathway of mRNA level in HepG2. I found that Berberine increases the expression of *caspase-9* and *caspase-3* at the mRNA level in HepG2 . Previous research found that berberine decreases Huh7 cells the expression of pro *caspase-3* and pro *caspase-9* in protein level [45], but little is known regarding the effects of berberine on mitochondrial cell apoptosis signaling pathway of mRNA level in Huh7. I found that Berberine increases the expression of *caspase-3* at the mRNA level in Huh7. Furthermore, little is known regarding the effects of berberine on mitochondrial cell apoptosis signaling pathway of mRNA and Protein level in Hep3B. I found that Berberine decreases the expression of *caspase-3* and *caspase-9* at the mRNA level in Hep3B treated for 12 h. I suggest that Berberine regulated the mitochondrial cell apoptosis signaling pathway at the mRNA level in HCC Cells.

I also confirmed that berberine regulate p53 in HepG2. Previous research found that berberine increases p53 at protein level in SK-N-SH(p53 wt) neuroblastoma cell[39]. In addition, previous research found that berberine increases p53 at protein level in HepG2(p53 wt) [37]. However, little is known regarding the effects of berberine on p53 of mRNA level in HepG2. our result suggests that berberine upregulated p53 at m RNA level in HepG2 (p53 wt). Finally, I investigated berberine influence on *SHP 1* mRNA expression in HCC.

SHP-1 was discovered to be a tumor suppressor in hepatocarcinogenesis and HCC progression in previous studies [52]. I found that berberine regulate the mRNA expression of *SHP1* in

HepG2 and Huh7. This result suggests berberine upregulate the m RNA expression of *SHP1* in HCC.

I investigated that berberine inhibits HCC growth by regulating various mechanisms. I suggest that berberine might be effective as an anticancer drug for HCC.

Table

Genes	Forward (5'-3')	Reverse (5'-3')	Annealing temperature (ºC)
S18	TTT GCG AGT ACT CAA CAC CAA CA	CCT CTT GGT GAG GTC AAT GTC TG	60
SHP-1	ACC ATC ATC CAC CTC AAG TAC C	GTG TCC GTT CAC CAA CAG GAA G	58
p53	ATG TTT TGC CAA CTG GCC AAG	TGA GCA GCG CTC ATG GTG	60
LC3-II	GAG AAG CAG CTT CCT GTT CTG G	GTG TCC GTT CAC CAA CAG GAA G	68
Beclin-1	GGC TGA GAG ACT GGA TCA GG	CTG CGT CTG GGC ATA ACG	60
Caspase-3	AGG ACT CTA GAC GGC ATC CA	CAG TGA GAC TTG GTG CAG TG	60
Caspase-9	AAC CCT AGA AAA CCT TAC CCC	CAT CAC CAA ATC CTC CAG AAC	60

Table 1. List of primer sequences used for RT-PCR analysis.

Figures

Fig. 1 Pathogenesis of Hepatocellular Carcinoma.

Chronic exposure of the liver to damage from HBV, HCV, alcohol abuse and NASH, NAFLD causes reiterated hepatocyte damage and results in cirrhosis is induced and persisted leading to HCC.

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis: NAFLD, nonalcoholic fatty liver disease.

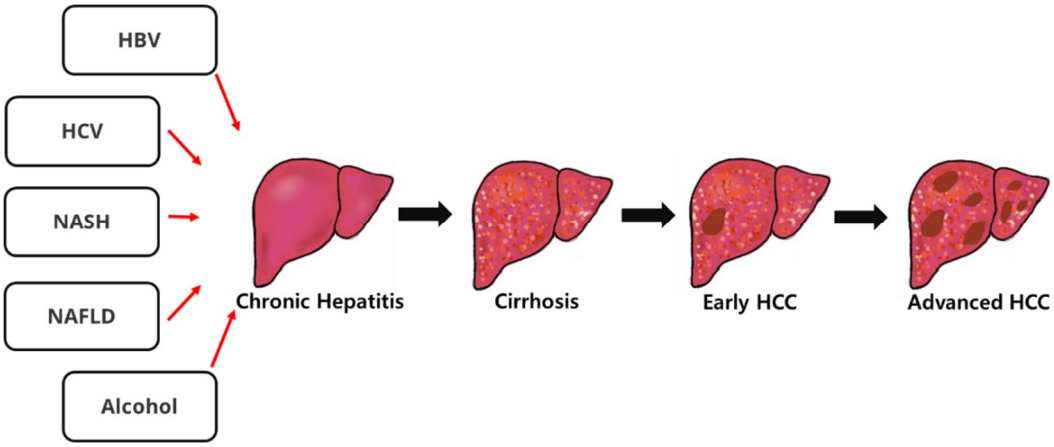


Fig. 2 Structural formula for berberine and sorafenib.

(A) The chemical structure of berberine. (B) The chemical structure of Sorafenib.

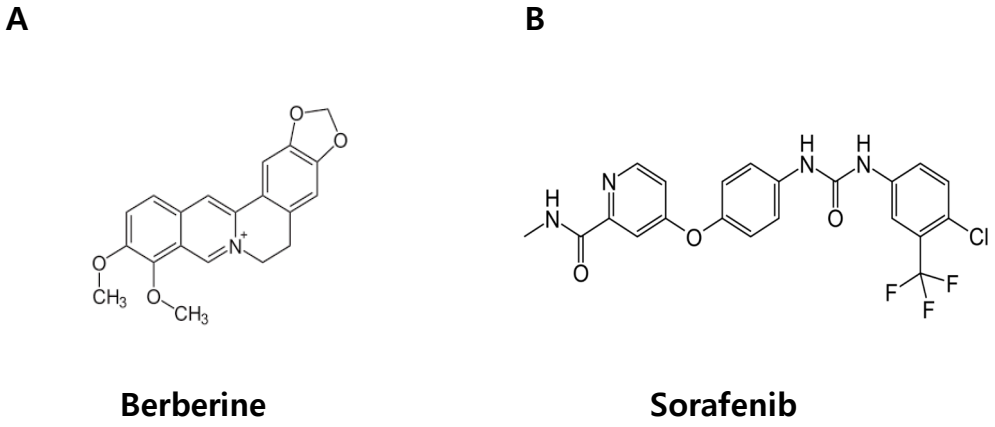


Fig.3 Berberine inhibits HCC growth *in vivo*.

After NOD-SCID mice were inoculated with Hep3B cells, The tumor volume was measured in CDX Hep3B for 11 days with or without berberine treatment (A). Berberine inhibits HCC growth in vivo (B) Mice were sacrificed 11 days of Berberine treatment, and the tumor weight, volume and mouse body weight were measured, Berberine treated CDX Hep3B suppressed the tumor weight and volume compared with control group, $p<0.01$ (C-E).

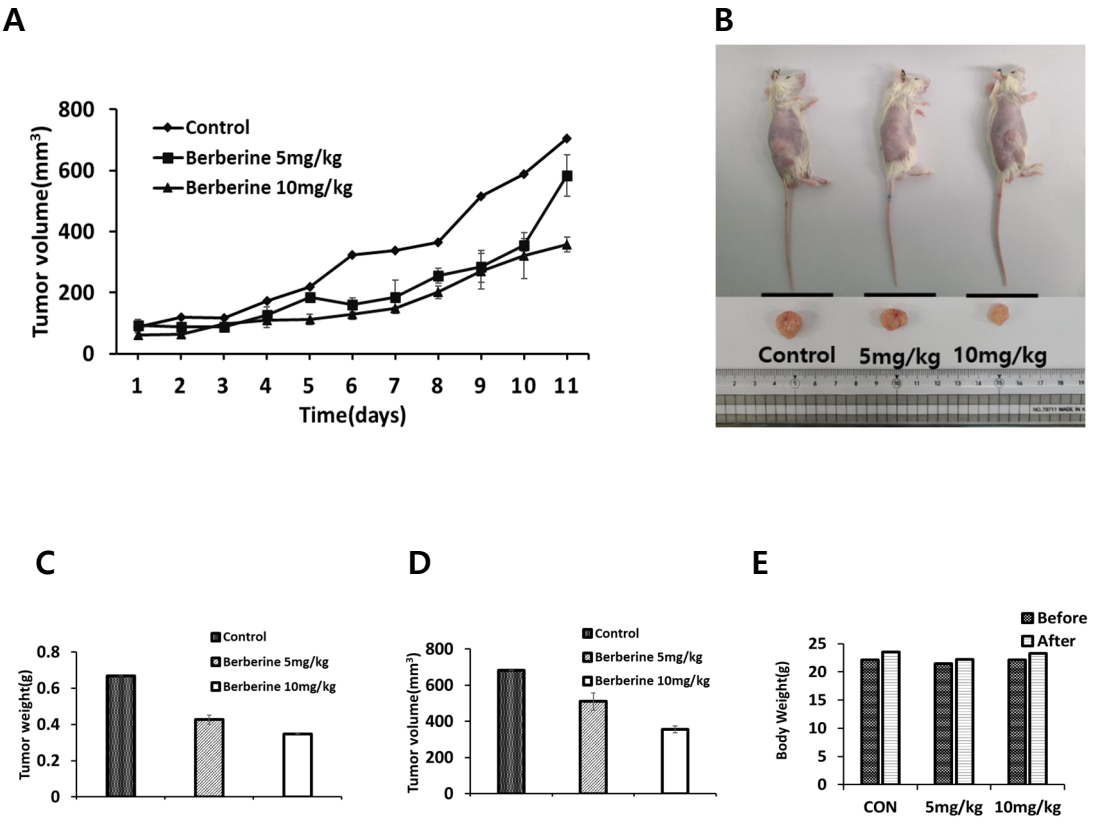


Fig. 4 Berberine suppressed HCCs proliferation.

HepG2 (A), Hep3B (B), and Huh7 (C) cells were cultured in the 48-well plates 3hour and then were treated with different concentrations of berberine (5 μM, 10 μM, 15μM) for 24, 48 and 72 h. The cell viability was analyzed by MTT assay. The percentage was calculated by comparing the O.D.

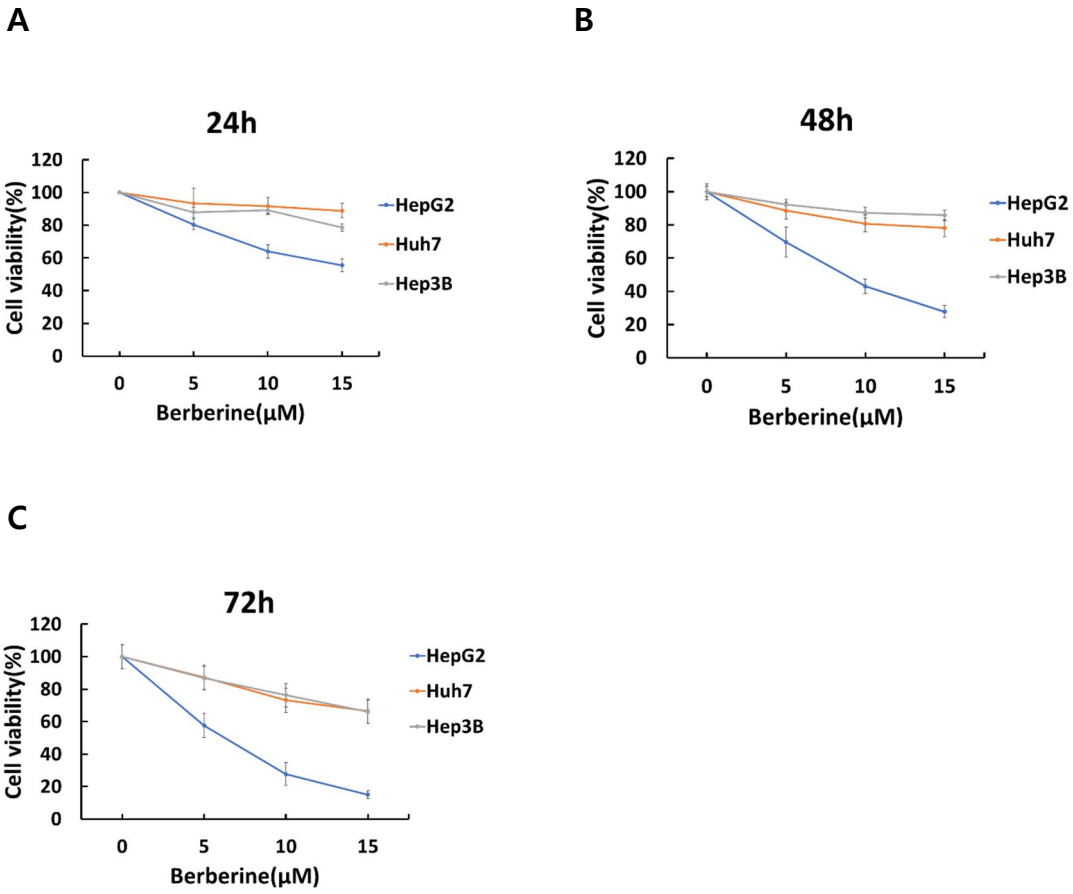


Fig. 5 Inhibition of migration after berberine treatment.

Berberine inhibited HCC migration. Wound healing assay shows Berberine inhibit the migration of HepG2 (A) and Hep3B (B) cells. HepG2 and Hep3B cells were cultured in the 12-well plates overnight and then were scratched and treated with different concentrations of berberine (10 μM, 20 μM, 30μM) for 24 h. Left: wounded areas (marked by white lines), Right: By measuring the width of the wounds, migration was compared with untreated control cells. HCCs with berberine showed significantly lower migration capacity than the control cells.

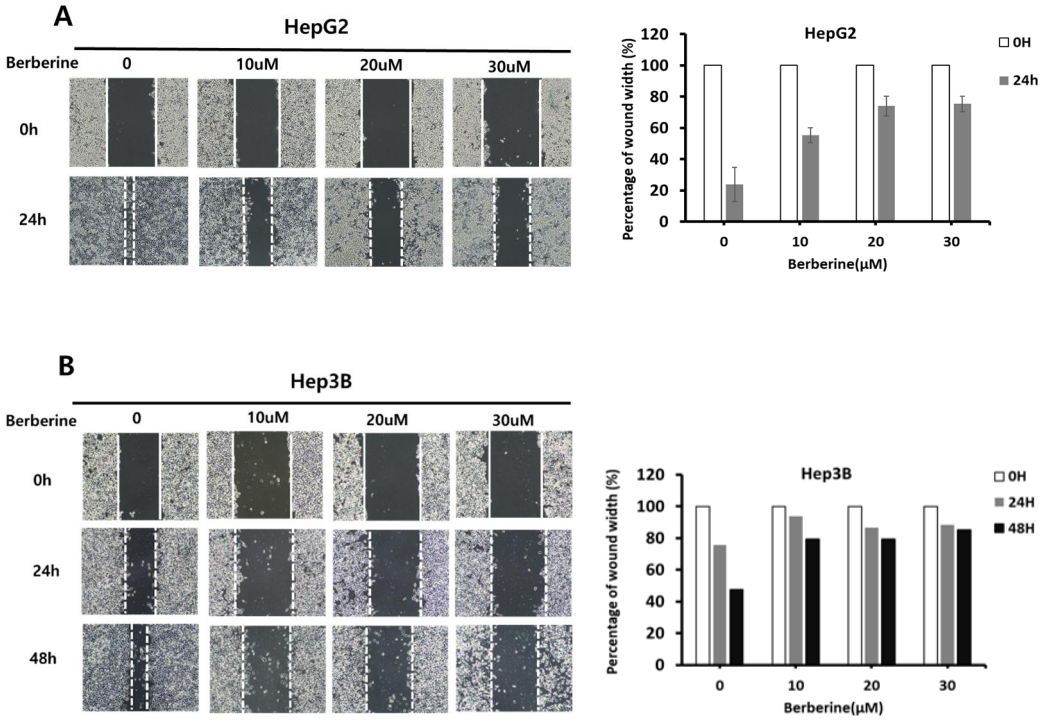


Fig. 6 The inhibition effects of berberine on proliferation of HCC cells including HepG2 and Hep3B. Berberine inhibited HCC colony formation. colony formation assay shows Berberine inhibit the colony formation of HepG2 (A), Hep3B (B) cells. HepG2 and Hep3B cells were cultured in the 6-well plates at a density of 5x102 cells per well overnight and then were treated with different concentrations of berberine (1μM- 5μM) for 10day. Cells fixed in 4% paraformaldehyde and stained with 0.5% crystal violet for 15 min.

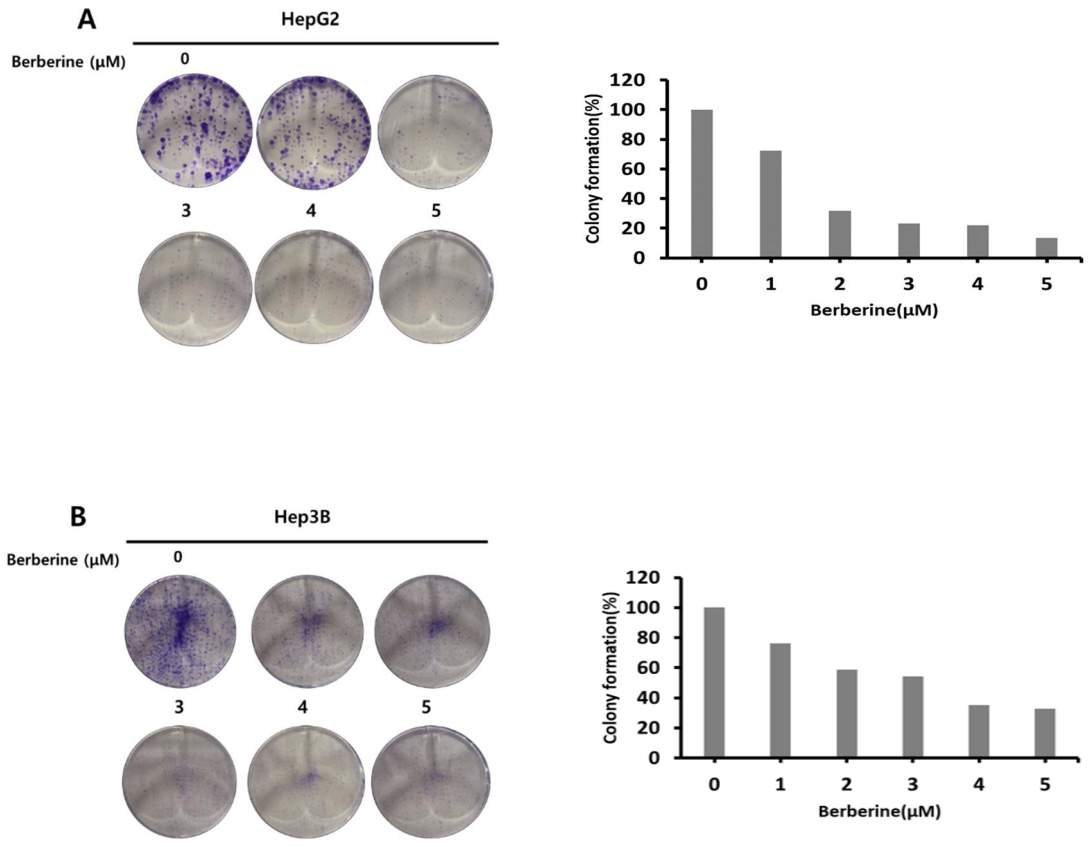
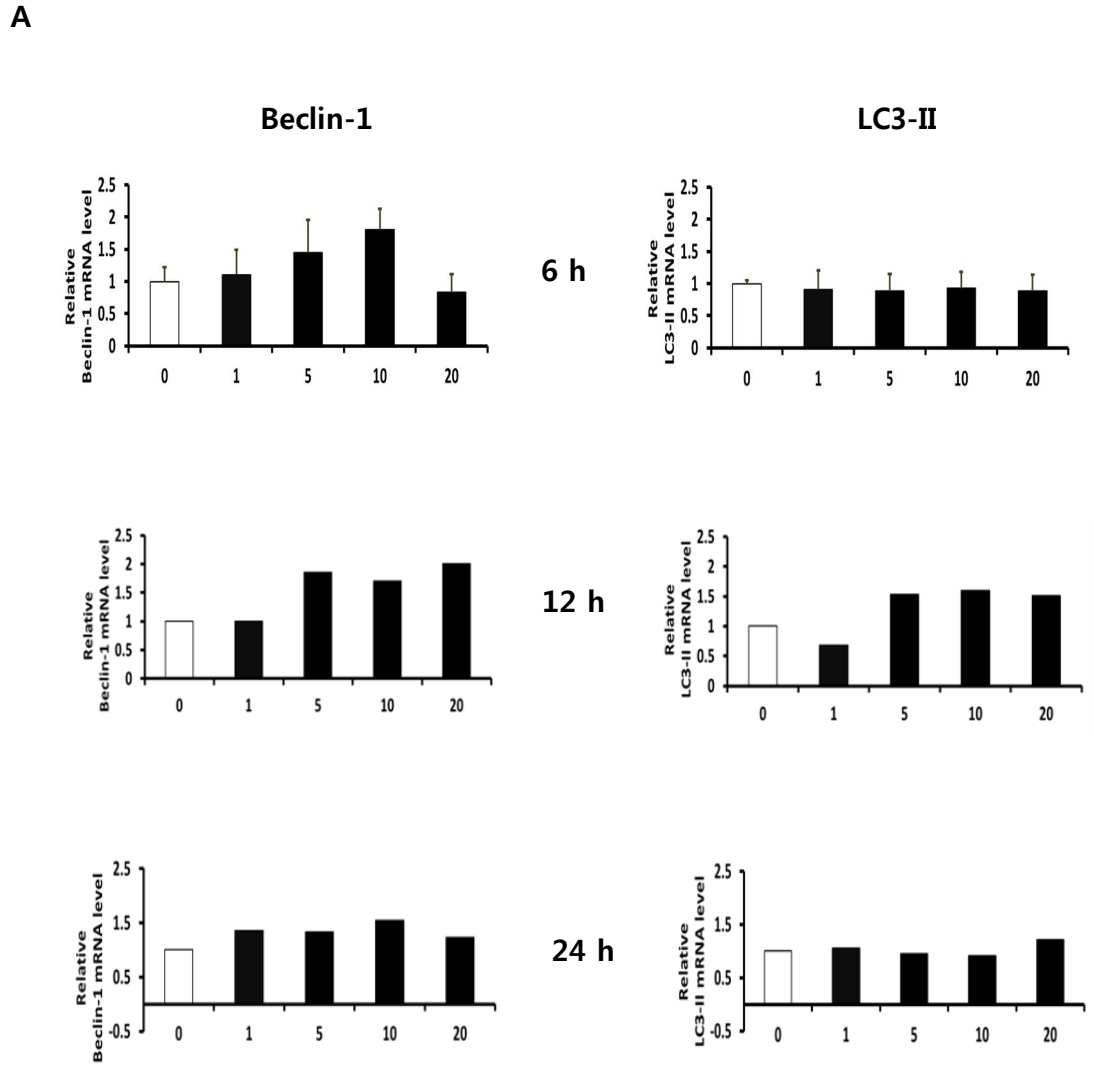
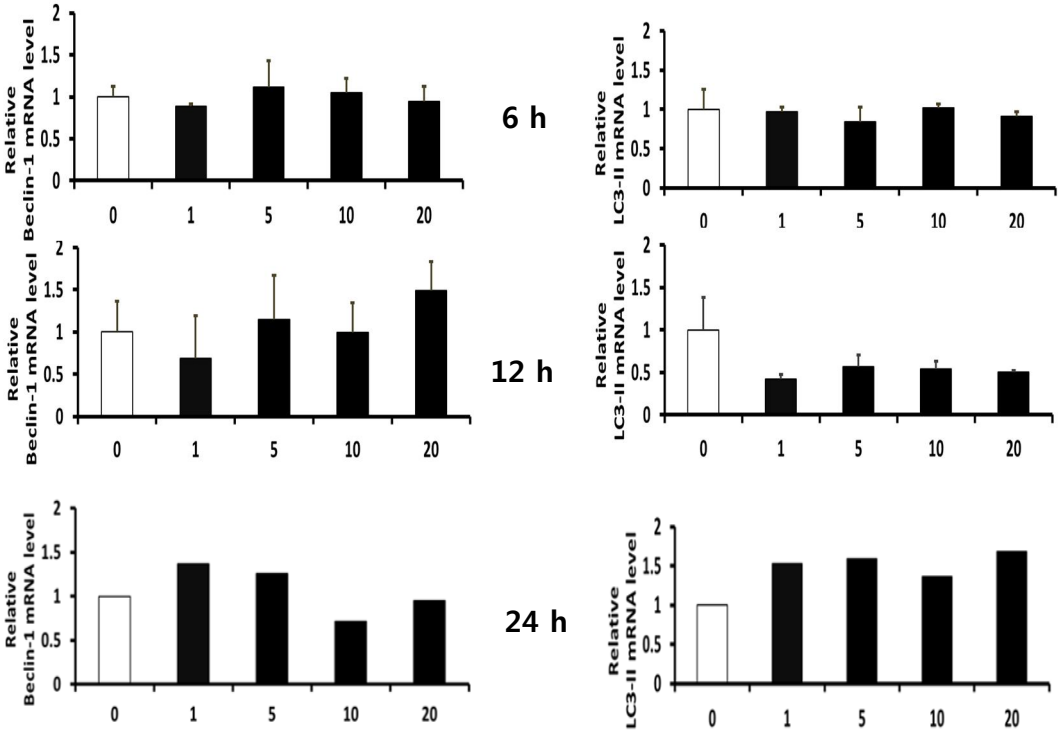


Fig.7 The mRNA levels of Becilin-1 and LC3-II were determined by quantitative RT-PCR. HepG2(A), Hep3B(B) and Huh7(C) are treated with different concentrations of berberine (1 μM, 5 μM, 10 μM, 20 μM) for 6, 12 and 24 h.



B


Relative Beclin-1 mRNA level
Relative LC3-II mRNA level
6 h
12 h
24 h
0
1
5
10
20

C

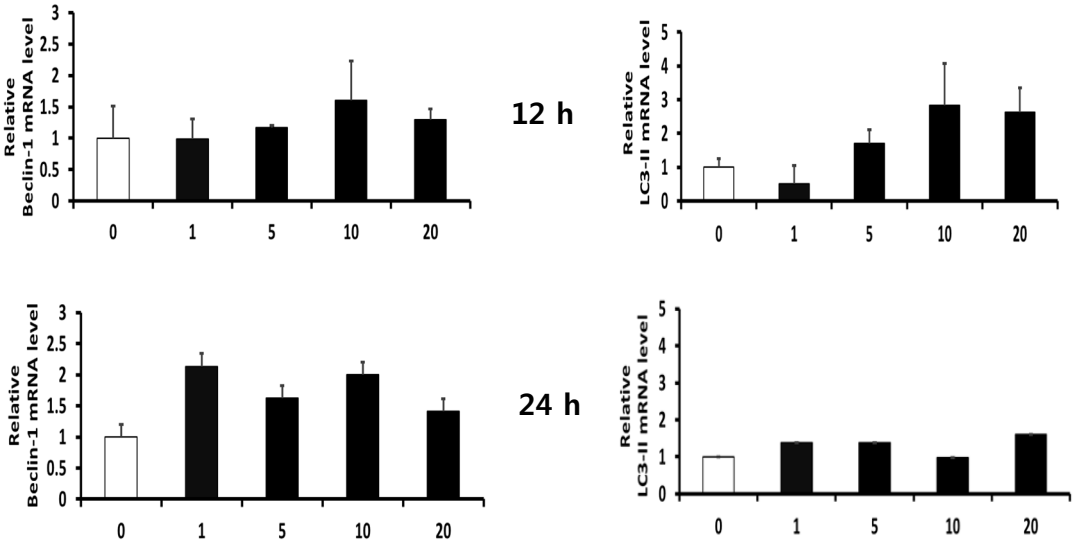
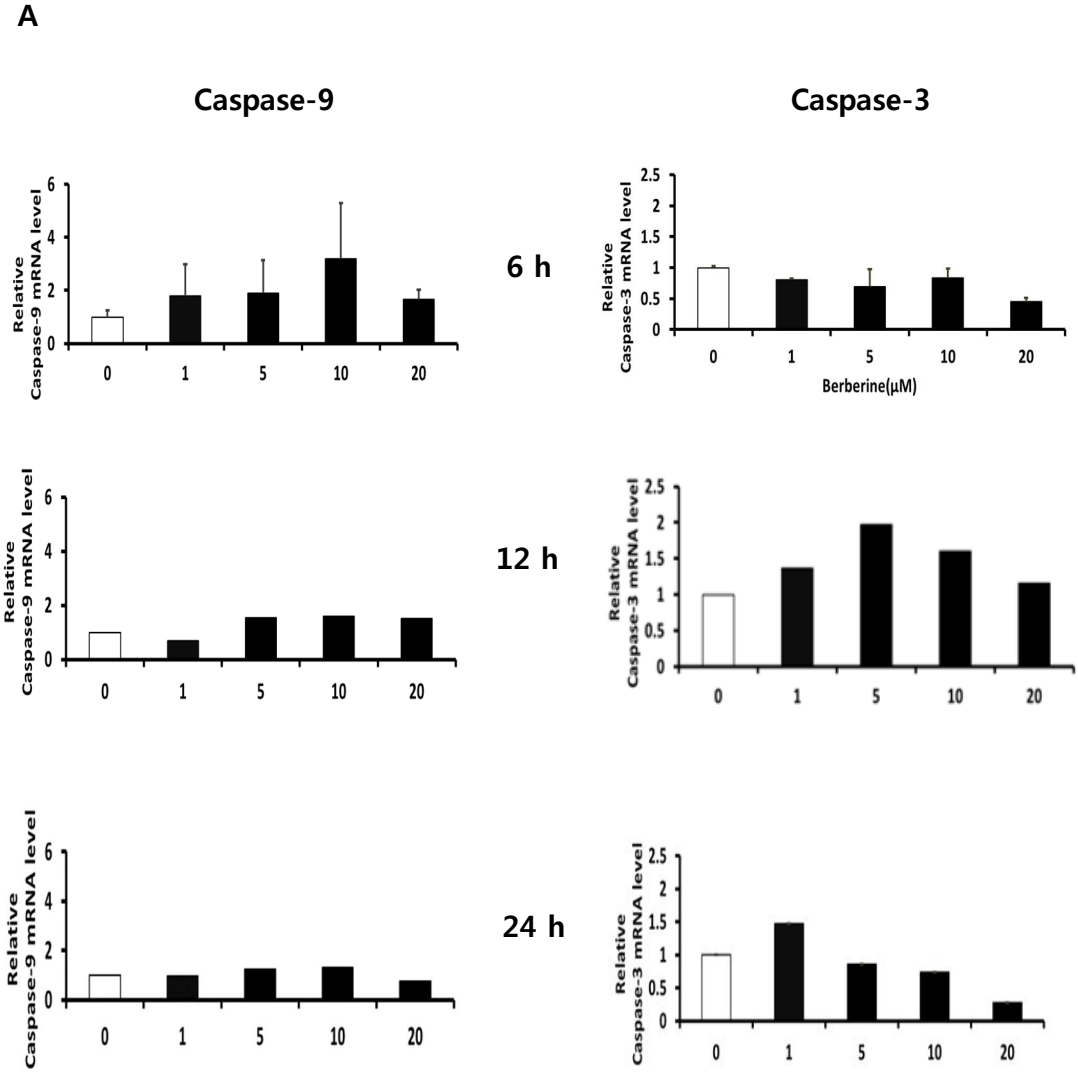

Relative Beclin-1 mRNA level
Relative LC3-II mRNA level
12 h
24 h
0
1
5
10
20

Fig. 8 The mRNA levels of Caspase-9 and Caspase-3 were determined by quantitative RT-PCR. HepG2(A), Hep3B(B) and Huh7(C) are treated with different concentrations of berberine (1 μM, 5 μM, 10 μM, 20 μM) for 6, 12 and 24 h.



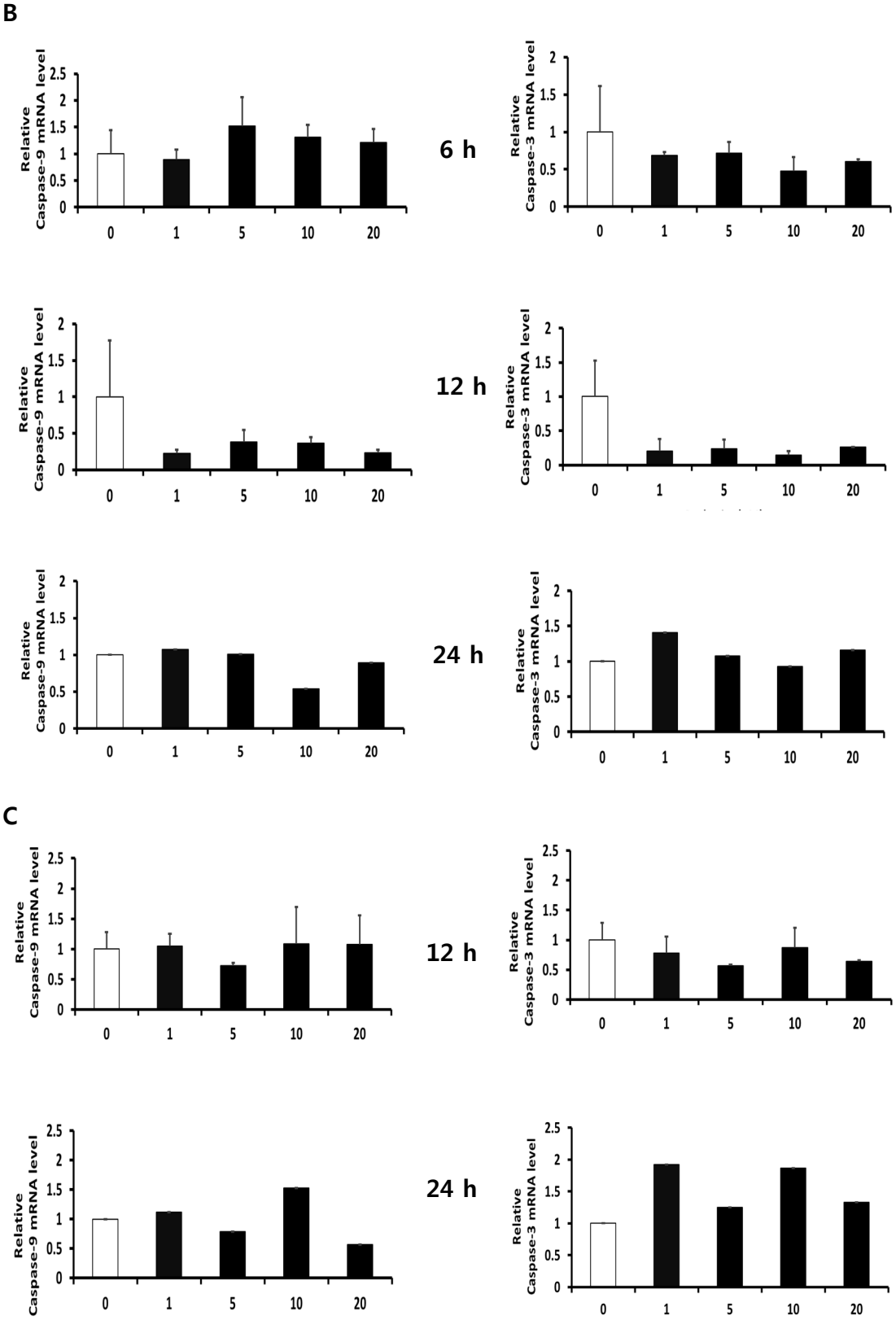

B
Relative Caspase-9 mRNA level
Relative Caspase-3 mRNA level
6 h
12 h
24 h
0
1
5
10
20
C
12 h
24 h

Fig. 9 The mRNA levels of p53 were determined by quantitative RT-PCR.

HepG2 is treated with different concentrations of berberine (1 μM, 5 μM, 10 μM, 20 μM) for 6, 12 and 24 h

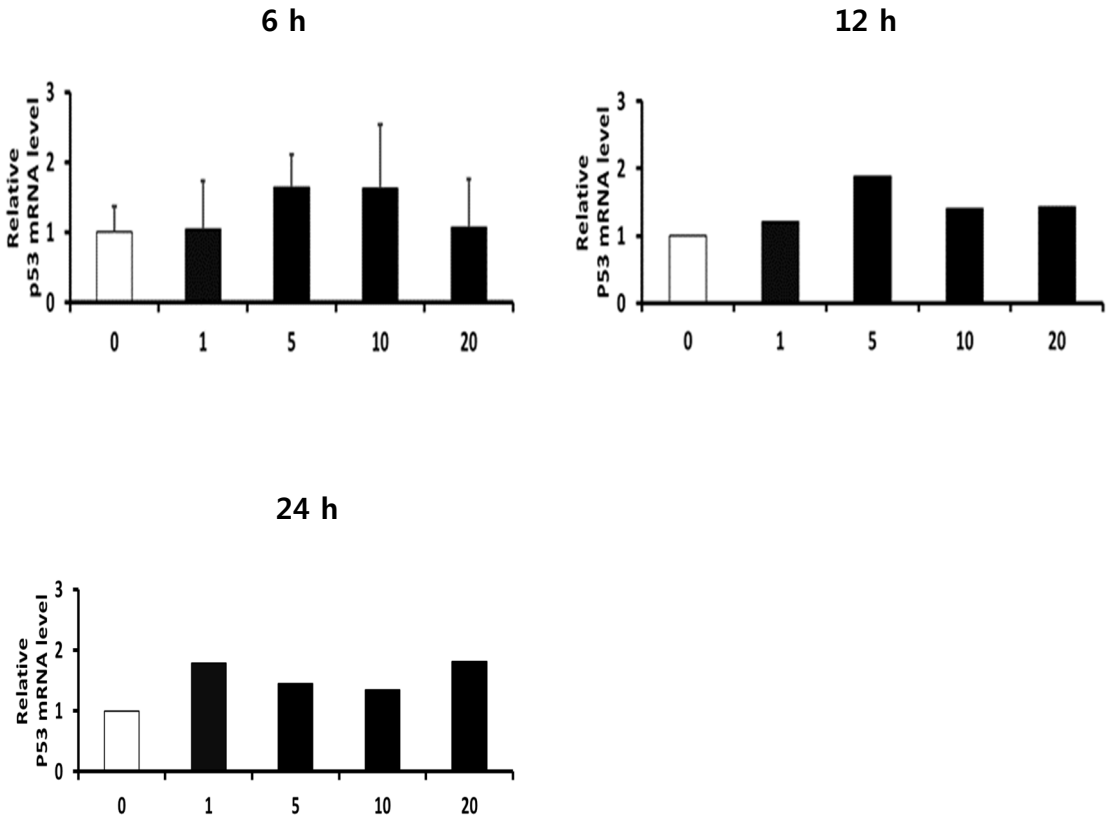
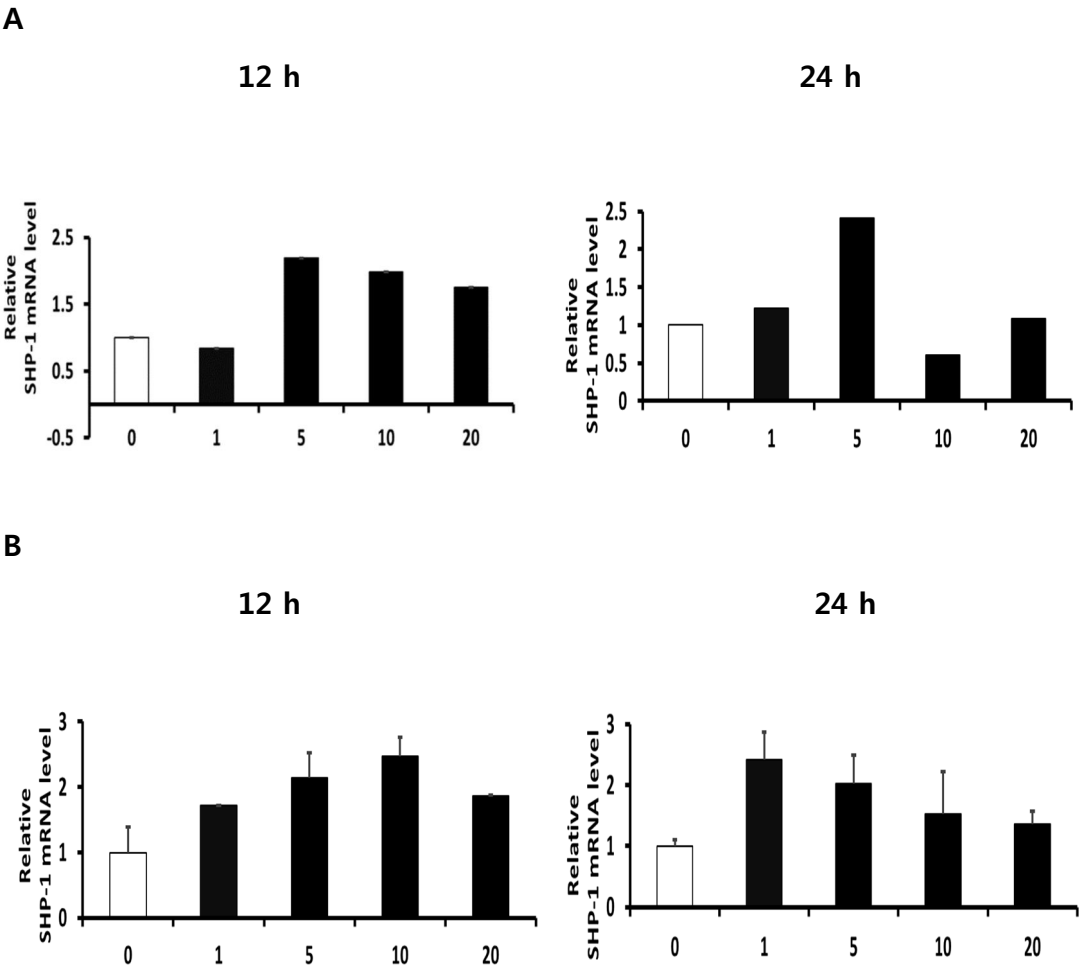


Fig. 10 The mRNA levels of SHP1 were determined by quantitative RT-PCR.

HepG2 (A) and Huh 7 (B) are treated with different concentrations of berberine (1 μM, 5 μM, 10 μM, 20 μM) for 12 and 24 h.



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국문요약

연구 목적: 간세포 암종은 7번째로 흔한 악성종양으로 동아시아 남성에서 26.9%로 가장 높은 발병률을 보인다. HCC는 암 관련 사망의 두 번째 주요 원인이다. 간세포 암종 환자를 치료하는 데 사용되는 대부분의 화학요법제는 주로 세포독성 약물이다. 그러나 이러한 약물은 독성이 매우 강하고 심각한 부작용이 있다. 따라서 나는 정상 간세포에서 세포독성을 일으키지 않으며 종양 세포를 표적하는 항암제의 효능을 높이고 부작용을 줄이는 약물을 찾고 싶었다.

식물유래 화합물인 베르베린은 정상 간 세포에 대한 세포 독성 영향이 거의 또는 전혀 없다. 최근 연구에 따르면 베르베린은 다양한 암종 세포에 대해 항종양 효과를 나타냈다. 베르베린은 인간 간암세포의 증식과 이동을 억제하는 것으로 밝혀졌지만 기본 메커니즘은 완전히 밝혀지지는 않았다.

연구 방법: 생체 내 마우스 모델을 사용하여 간암세포 성장에 대한 베르베린의 효과를 분석하였다. HCC 세포 생존율은 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) 분석을 사용하여 결정되었다. 간암세포 증식은 군집 형성 분석에 의해 결정되었다. 세포 이동에 대한 베르베린의 효과는 상처 치유 분석에 의해 결정되었다. mRNA 발현은 qRT-PCR에 의해 검출되었다.

연구 결과: Berberine은 NOD-SCID 마우스에서 Hep3B 성장을 의존적으로 억제했으며 HepG2(정상군 p53), Hep3B(결핍 p53) 및 (돌연변이 p53) 세포의 증식도 억제했다. 또한, 베르베린은 군집 형성 및 이동을 억제했다. 베르베린 처리는 *Beclin-1* 및 *LC3-II*(microtubule-associated protein light chain 3)를 포함한 자가 포식 관련 유전자의 mRNA 발현을 감소시켰고, *Caspase-3* 및

*Caspase-9*를 포함한 미토콘드리아 관련 세포자멸사의 mRNA 발현을 감소시켰다. 또한, 베르베린은 p53 및 *SHP1*(Src homology region 2 domain-containing phosphatase 1)의 다양한 mRNA 발현을 조절하였다.

결론: 이러한 결과는 베르베린이 간암세포와 Hep3B NOD-SCID 모델에서 항암효과가 있음을 제안한다. 특히, Hep3B 및 Huh7 세포는 HepG2 세포보다 베르베린 처리에 덜 반응한다. 베르베린은 간암세포의 mRNA 수준에서 자가포식 관련 유전자, 미토콘드리아 세포자멸사 관련 신호 전달 유전자의 발현을 조절하였으며 또한, 간암세포에서 p53 및 *SHP1*의 mRNA 발현을 상향 조절하였다. 이러한 결과로부터 나는 베르베린이 간세포 암종에서 추가적인 화학요법적 치료제임을 제안한다.