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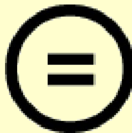

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**Master of Science**

# Anticancer effects of berberine on human cholangiocarcinoma cells and mouse model

**The Graduate School**

**of the University of Ulsan**

**Department of Medical science**

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# Anticancer effects of berberine on human cholangiocarcinoma cells and mouse model

Supervisor by Professor Shin Hwang

Master's thesis

Submitted to
the Graduated School of the University of Ulsan
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science

by

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Ulsan, Korea
August 2022

# Anticancer effects of berberine on human cholangiocarcinoma cells and mouse model

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# Abstract

**Background and Aim:** Cholangiocarcinoma (CCA) is an uncommon epithelial cell malignancy arising from the bile duct that is resistant to chemotherapy. So, there are no highly effective therapeutic agents for CCA. Recently, many of natural compounds including of berberine have attracted the attention of researchers and their use has increased during the past decades. Berberine is isolated from the stems, rhizomes, and roots of several plants such as *Coptis chinensis* which has been used as a therapeutic agent in the treatment of bacterial infections, diabetes, cardiovascular, and inflammatory diseases. Previous other studies have consistently reported the anticancer effect of berberine on various tumors such as breast, cervical, colon, hepatoma, lung, and ovarian cancer cells through inhibition of proliferation, migration, and invasion. However, little is known about the anticancer activities of berberine in CCA. The purpose of this study was to explore the effects and underlying mechanism of berberine on CCA.

**Methods**: Human CCA cell lines (SNU-308, -478, -1079, and -1196) were used in this study. Cell viability was determined by MTT assay and the long-term proliferation of cells was determined by colony formation assay. Moreover, the cancer cell migration was examined by wound healing assay. MMP-9 and -2 activities were examined by zymography. The mRNA expression of MMP-9, MMP-2, Bax, Bcl-2, Capsae-9, Caspase-3, Beclin-1, LC3-II, and Snail1 was measured by qRT-PCR.

**Results**: Berberine inhibited cell proliferation, migration, and colony formation in various CCA cell lines and also increased Bax, Caspase-9, -3 in SNU-478 cells. Berberine blocked MMP-9 activity in SNU-478 cells. In the xenograft model, berberine inhibited tumor growth and it increased Bax, Bcl-2, Caspase-9, Caspase-3, and decreased Snail1 in SNU-478 xenograft tumor model.

**Conclusion**: This study suggested that berberine inhibits cell proliferation, migration, colony formation through mechanisms of apoptosis and autophagy *in vitro* and *in vivo* model. Therefore, berberine might be a potential therapeutic agent to treat patients with this devastating malignancy.

**Keywords:** Cholangiocarcinoma, Berberine, Apoptosis, Migration

# Contents

Abstract ------------------------------------------------------------------------------------ i

List of Table and figures ------------------------------------------------------------------ iii

Introduction ------------------------------------------------------------------------------- 1

Materials and methods -------------------------------------------------------------------- 3

Results ------------------------------------------------------------------------------------- 7

Discussion ----------------------------------------------------------------------------------- 26

References ---------------------------------------------------------------------------------- 30

국문요약 ------------------------------------------------------------------------------------- 35

# List of Table and figures

**Table 1** -------------------------------------------------------------------------------- 12

**Figure 1** -------------------------------------------------------------------------------- 13

**Figure 2** -------------------------------------------------------------------------------- 14

**Figure 3** -------------------------------------------------------------------------------- 15

**Figure 4** -------------------------------------------------------------------------------- 17

**Figure 5** -------------------------------------------------------------------------------- 19

**Figure 6** -------------------------------------------------------------------------------- 20

**Figure 7** -------------------------------------------------------------------------------- 21

**Figure 8** -------------------------------------------------------------------------------- 22

**Figure 9** -------------------------------------------------------------------------------- 23

**Figure 10** ------------------------------------------------------------------------------ 24

**Figure 11** ------------------------------------------------------------------------------ 25

# Introduction

Cholangiocarcinoma (CCA) is an epithelial cell malignancy tumor arising from the bile ducts that connect the liver and gallbladder to the small intestine. CCAs are divided into three subtypes depending on their location in relation to the liver. Intrahepatic CCA (iCCA) is in small bile ducts within the liver. Extrahepatic CCA (eCCA) is subdivided into perihilar CCA (pCCA) which is in an area of the hilum, and distal CCA (dCCA) is in the cystic duct and the ampulla of Vater [1,2].

The incidence and mortality of CCA increasing globally. iCCAs account for 10% to 12% of liver cancers, whereas eCCAs account for one-third of biliary track cancers [3]. In the USA, pCCA is accounting for 50–60% of all CCAs, followed by dCCA (20–30%) and iCCA (10–20%) [1,4-6]. Unfortunately, most CCA patients in the early stage generally remain asymptomatic until tumor progression to the advanced stage, when the primary tumor grows enough to produce a large liver mass or when jaundice has developed because of biliary tree obstruction [7], therefore patients are diagnosed at a late stage of disease [7,8]. Types of treatment with advanced patients may be given chemotherapy with or without surgery to kill cancer cells that are left. The CCA can easily induce metastasis and recurrence [9-11] via lymph node metastasis [12,13], surgical margin [14,15], liver remnant [16], and vascular invasion [12].

It has been used to conventional chemotherapeutic agents including gemcitabine [17,18], cisplatin [17], and oxaliplatin [18] have limited efficiency and also could induce resistance in CCA patients [19,20]. However, these chemotherapeutic agents have numerous undesirable side effects. Gemcitabine has vomiting [21], nausea [21] and fever [22]. Cisplatin has nephrotoxicity [23,24]. In case of treatment with cisplatin, it was reported that kidney has a tendency of accumulating cisplatin to higher compared to other organ in the body [25,26]. In contrast to cisplatin, oxaliplatin is not nephrotoxic, but it cause various adverse effect such as neurotoxicity [27, 28], fever [29], anemia [30], nausea [31], and gastrointestinal disorders [32]. Therefore, it is necessary to research new agents that are more effective and with fewer side effects.

Traditionally, natural compounds have been used for treatment of various disease including cancer. Especially, plant-derived nature compounds have gained much attention and its use has increased over the past decades. Among of them, berberine is a bioactive compound extracted from the stems, rhizomes and roots of traditional Chinese medicine *Coptis chinensis* [33]. Berberine has extensive biological activities, thus applied to anti-inflammation, anti-oxidative, anti-diabetic, anti-viral infections [34-36]. Berberine has anti-cancer activity by means of interference in tumorigenesis and tumor development such as breast cancer, cervical cancer, colon cancer, hepatoma, lung cancer, and ovarian cancer [37-42]. Berberine inhibits cancer cells through various mechanisms thus inducing apoptotic cell death including inhibition of proliferation [41], suppression of tumor invasion [43,44] and metastasis [45,46] thus inducing apoptotic cell death.

It was reported that berberine has anti-cancer effects on KKU-213, KKU-214 and QBC939 cells (human CCA cell lines) through the induction of cell cycle arrest [47,48], but the autophagy mechanism of berberine in CCA has not been investigated. Therefore, this study aimed to investigate the anti-cancer activities using human CCA cell lines from Korean patients which are established from pathologically proven primary biliary tract and ampulla of Vater cancer samples [49], and xenograft mouse model.

# Materials and methods

### Chemicals and materials

The reagents used in this study were purchased from the following sources: berberine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide], PMA [Phorbol 12-myristate 13-acetate] from Sigma-Aldrich (USA); RPMI, fetal bovine serum, 1% antibiotic-antimycotic (anti-anti), SYBR Green Supermix, and Trizol were supplied by Gibco BRL (USA). All chemicals and reagents were of analytical grade.

### Cell culture

SNU-308, SNU-478, SNU-1079, and SNU-1196 cell lines were obtained from Korean Cell Line Bank (KCLB) and cells were cultured in RPMI supplemented with 5% fetal bovine serum (FBS), 1% anti-anti and cultured in an incubator at 37°C and atmosphere of 5% $CO_2$.

### Cell treatment and Cell viability assay

Berberine was dissolved in Dimethyl sulfoxide (DMSO) and directly treatment to the culture media. Cell viability was determined using the MTT assay. Cells were seeding at $3x10^4$ cells/well in 48-well plates with overnight incubation. After 24 hours, the cells were treated with Berberine at 0 to 15 μM. After 24 to 72 hours incubation, each well was given 100 μl MTT (1mg/ml) and incubated for 2 h at 37°C in a humidified atmosphere of 5% $CO_2$. The culture medium was removed and 100 μl DMSO was added to each well for 1 min at room temperature. The absorbance was determined with an ELISA reader (PerkinElmer, USA) at 595 nm. This wavelength was found not to interfere with berberine.

**Wound healing assay**

The cells (SNU-308, SNU-478, SNU-1079, SNU-1196) were seeding at $3x10^5$ cells/well in 12well plates and incubated for 24 h in RPMI with 5% FBS, 1% anti-anti. The cellular monolayer was wounded with a sterile 200 µl pipette tip and washed with phosphate buffered saline to remove floating cells. Then cells were treated with 0–15 µM berberine for 24 h and 48 h. The effect of Berberine on wound closure observed by inverted microscope (Olympus, Japan).

**Colony formation assay**

Cells (SNU-308, -478, -1079, -1196) were seeding at $1x10^3$ cells/well in 6 well plates. adherent cells were treated with different concentrations of berberine. The cells were maintained in a 37°C, 5% $CO_2$ incubator for 7 days. Colonies were washed with PBS, then fixed with 10% Neutral Buffered Formalin for 30 min and stained with 1% crystal violet for 30 min.

**Reverse transcription-polymerase chain reaction analysis (RT-PCR)**

Total RNA was isolated from the cells and tissues using TRIzol® (Gibco BRL, USA), and 20µg volume of total RNA was used for cDNA synthesis. Synthesized cDNA was performed in Bio molecular system using SYBR Green Master Mix. The expression levels Bax, Bcl-2, Caspase-9, Caspase-3, LC3-II, Beclin-1, Snail, MMP-9 and MMP-2 in the exposed cells were compared to those in control cells at each time point using the comparative cycle threshold (Ct) method. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of S18, which is a housekeeping gene. The primer sequences were presented in Table 1.

**Zymography**

Matrix Metalloproteinase-2 (MMP-2) and Matrix Metalloproteinase-9 (MMP-9) enzymatic activities were assayed by gelatin zymography with some modifications. SNU-308 was treated berberine of 5 µM, 10 µM and 15µM with 100nM PMA for 12 h and 24 h. Cell supernatants were diluted 3:1 with sample buffer. 15µl of sample was added to the wells. After electrophoresis, gels were soaked in buffer containing 2.5% Triton X-100 to remove SDS from gel and incubated in developing buffer at 37℃ for 22 h. After developing, the gel was stained with 0.5% Coomassie Blue R-250 for 30 min and then destained appropriately.

**Animal xenograft model**

Six to 8-week-old female and male non-obese diabetic/severe-combined immunodeficiency (NOD/SCID) mice were housed under the pathogen-free condition at room temperature for the xenograft model. SNU-478 cells ($1x10^6$) in Matrigel (Corning, USA) were subcutaneously injected into the right flanks of mice. The treatment initiated when xenografts reached volumes of about 50 $mm^3$ and injected intraperitoneally daily with 100 µl of PBS control vehicle, berberine 10mg/kg or 20mg/kg. Tumor volume was recorded daily. Tumor length (L) and width (W) were measured with a Vernier caliper, and tumor volume was calculated by the following formula: Tumor volume ($mm^3$) = ½ x (tumor length) x (tumor width)$^2$. At the end point of the experiment, the tumors were removed and weighed. All sacrifices were performed under $CO_2$, and every effort was made to minimize animal suffering. Some parts of the xenograft tissues were stocked in 10% Neutral Buffered Formalin and embedded in paraffin for pathologic assessment, the rest were processed for RNA extraction and preserved at -20˚C.

## Hematoxylin and Eosin (H&E) staining

The mice were sacrificed and the tumors were fixed with 10% neutral buffered formalin, then paraffin embedded and cut into 4 μm thick sections using a microtome. The sections were heated to 60°C for 2 h in a drying oven. Paraffin sections were deparaffinized with xylene, dehydrated in a series of different ethanol concentrations and stained with H&E. The sections were mounted in Canada Balsam. The slides were observed under a Digital microscope Coolscope II (Nikon, Japan)

## Statistical analysis

All the experiments were repeated at least three times. The results are expressed as a mean ± S.D., and the data were analyzed using one-way ANOVA followed by Student's t-test for significant difference. A p value $< 0.05$ was considered significant.

# Results

**Berberine reduces cholangiocarcinoma cancer (CCA) cell viability**

The anti-proliferation of berberine examined in CCA cells. The cells were treated with 0, 5, 10 and 15 μM of berberine for 24 h to 72 h by using MTT assay. The cell viability of berberine 15 μM at 24 h in SNU-308, SNU-478, SNU-1079 and SNU-1196 was each of 66.9%, 75.5%, 71.5% and 67.6%, respectively. The cell viability of berberine 15 μM at 48 h in SNU- 308, SNU-478, SNU-1079 and SNU-1196 was each of 40.6%, 49.6%, 42.5% and 40.2%, respectively. The cell viability of berberine 15 μM at 48 h in SNU-308, SNU-478, SNU-1079 and SNU-1196 was each of 40.6%, 49.6%, 42.5% and 40.2%, respectively. The cell viability of berberine 15 μM at 72 h in SNU-308, SNU-478, SNU-1079 and SNU-1196 was 31.74%, 30.87%, 30.82% and 30.11%, respectively (Fig. 1). These data indicated that the CCA cells were decreased with time- and dose-dependent manners, although no significant differences among CCA cells.

**Berberine suppresses colony formation in CCA cells**

We assessed the anti-proliferation ability of CCA cell lines after treatment with different concentrations (0 μM, 1 μM, 5 μM, and 10 μM) of berberine for 7 days using colony formation assay. As shown in the Fig. 2A, berberine markedly suppressed the colony formation of CCA Cells in a dose-dependent manner. The percentage of colony number of berberine 1 μM, 5 μM and 10 μM in SNU-308 was each of 86.9%, 72.1% and 48.4%, respectively. The percentage of colony number of berberine 1 μM, 5 μM and 10 μM in SNU-478 was each of 79.4%, 53.6% and 32.4%, respectively. The percentage of colony number of berberine 1 μM, 5 μM and 10 μM in SNU-1079 was each of 88.4%, 75.7% and 56.4%, respectively. The percentage of colony number of berberine 1 μM, 5 μM and 10 μM in SNU-1196 was each of 95.4%, 68.7% and 34.5%, respectively. SNU-478 and SNU-1196 are more sensitive to berberine compared with SNU-308 and SNU-1079 (Fig. 2B). In colony formation

assay, the results also demonstrated that berberine treatment could significantly inhibit the proliferation of CCA cells.

**Berberine inhibits the migration of CCA cells**

Wound healing assay was conducted to investigate the effect of berberine on migration of CCA cells. After 24 h and 48 h treatment, as compared with the control group, inhibition of cell migration at all time points. The percentage of wound width of berberine 10 μM and 20 μM at 48h in SNU-308 was each of 23.8% and 33.1%, respectively. The percentage of wound width of berberine 10 μM and 20 μM at 48 h in SNU-478 was each of 45.6% and 58.1%, respectively. The percentage of wound width of berberine 10 μM and 20 μM at 48 h in SNU-1079 was each of 32.0% and 40.2%, respectively. The percentage of wound width of berberine 10 μM and 20 μM at 48 h in SNU-1196 was each of 63.5% and 68.8%, respectively. Compared to the control, berberine obviously disrupted the migration of CCA cells after treating 24 h and 48 h (Fig. 3). The experimental results revealed that berberine inhibited the migration of CCA cells.

**Berberine suppresses MMP-2 and MMP-9 activity**

The expression of MMP-2 and MMP-9 has been implicated in the invasion and metastasis of various types of cancer cells. Therefore, in this study the activities of MMP-2 and MMP-9 were investigated by gelatin zymography and qRT-PCR to examine the possible anti-invasive ability of berberine. There were no significant MMP-9 and MMP-2 activities in SNU-478, SNU-1079 and SNU-1196 in the pilot study. On the other hand, MMP-9 activity was observed only in SNU-308 cells. We tested gelatinase zymography, in which SNU-308 cell showed one band corresponding to MMP-2 and induction of MMP-9 with 100 nM PMA treatment. SNU-308 was stimulated with 100 nM PMA for 24 h and gelatinolytic activity in the supernatant was examined by gelatin zymography. As shown in Fig. 4A, gelatinolytic bands were detected. The mRNA levels of MMP-9 were also reduced following different concentrations of berberine treatment (Fig. 4B). This result

showed that berberine significantly blocked PMA-induced MMP-9 expression. However, there were no significant changes in the activity and expression of MMP-2.

**Berberine reduces cell viability of SNU-478 cells.**

We treated berberine high concentrations at 20 μM, 40 μM and 60 μM for 6 h to 24 h in SNU-478 cells to examine whether berberine induces apoptosis or autophagy and decreases the epithelial-to-mesenchymal transition (EMT) in short term. As the result, berberine decreased cell viability of CCA cells with time- and dose-dependent manners (Fig. 5A). The growth inhibitory effect of berberine confirmed in microscopic images showing fewer cells present in berberine-treated cultures after 24 h incubation (Fig. 5B).

**Berberine upregulated of apoptosis related genes in SNU-478 cell**

The mRNA expression levels of Bax, Bcl-2, caspase-9 and caspase-3 were measured using real-time RT-PCR to assess whether berberine is involved in apoptosis. Level of pro-apoptotic gene Bax was upregulated at 40 μM, anti-apoptotic gene Bcl-2 was downregulated at 24 h in SNU-478 (Figs. 6A-B). As shown in Figs. 6C-D, the level of caspase-3 was decreased at 6 h, 12 h and 24 h. The level of Caspase-9 was increased at 6 h and 12 h but deceased at 24 h. These results showed that berberine may be involved in apoptosis in SNU-478.

**Berberine upregulated of autophagy related genes in SNU-478 cell**

Autophagy, as a route of programmed cell death, is contribute to autophagic cell death through lysosome-related cell degradation. Beclin-1 is a central player in autophagy and serves a pivotal function in autophagosome formation. We investigated autophagy induction in SNU-478 cells treated with berberine of 20 μM, 40 μM and 60 μM. The mRNA expression of Beclin-1 is higher than control at 12 h (Fig. 7A). The mRNA expression of LC3-II, autophagosome marker, is higher than control (Fig. 7B).

**Effects of berberine on mRNA expression of EMT associated gene in SNU-478 cells.**

To determine whether berberine is associated with Snail1, the EMT Marker, expression was examined by using RT-PCR. The result indicated that berberine reduced mRNA expression of Snail1 at 24 h in SNU-478 cell (Fig. 8).

**Berberine treatment inhibited xenograft growth.**

To further determine the antitumor effect of berberine in vivo, SUN-478 xenograft model was established by using NOD SCID mice. Tumors reached a size of about 50 $mm^3$, mice were randomly divided into two groups: control (PBS) vs. berberine (10mg/kg). After treatment with 10mg/kg of berberine, there was a significant decrease in both tumor volume and weight compared to control (Figs. 9A-D). H&E staining showed that berberine treatment did not induce marked histological changes compared with the control (Fig. 9E). These results suggested that berberine inhibited SNU-478 xenograft tumor growth.

### Berberine induces of apoptotic signaling in SNU-478 xenograft tissue

Apoptosis, known as a form of programmed cell death, is triggered by activation of caspases sequentially. The mitochondrial pathway (known as intrinsic pathway) is activated by pro-apoptotic and anti-apoptotic from bcl-2 family at the mitochondria and cytosol, resulting in the release of cytochrome c from the mitochondria, which in turn activates caspase-9. Caspase-9 activate capase-3 resulting in apoptosis. We tested the expression of Bcl-2 family and whether they were associated with the damage to the mitochondria. As a result, berberine increased the expression of Bax and decreased the expression of Bcl-2 (Figs. 10A and B). It also increased the expression on caspase-9 and caspase-3 (Figs. 10C and D).

### Berberine induced LC3-II and Beclin-1 in SNU-478 xenograft mouse tissue

Expressions of Beclin-1 and light chain 3 (LC3) B-II, the two essential autophagy indicators, in berberine-treated SNU-478 xenograft mouse tissue was examined. Beclin-1, LC3-II mRNA levels were assessed by qRT-PCR. Related results showed increased expressions of Beclin-1 and LC3-II in berberine treated SNU-478 xenograft mouse tissue compared with control (Fig. 10A-B).

### Berberine downregulated Snail1 in SNU-478 xenograft mouse tissue

EMT produces cancer cells that are invasive, migratory, and exhibit stem cell characteristics, hallmarks of cells that have the potential to generate metastases. To evaluate whether berberine block the Snail1 in the SNU-478 xenograft mouse tissue changes in the mRNA expression level of Snail1 was evaluated using real-time RT-PCR. Berberine downregulated Snail1 expression, which is considered to be a regulator of the EMT (Fig. 11).

**Table 1. List of primer sequences used for RT-PCR analysis in this study.**

| Gene | Sequence | Annealing temperature (°C) |
|---|---|---|
| S18 | F : AAG TTT CAG CAC ATC CTG CGA GTA<br>R : TTG GTG AGG TCA ATG TCT GCT TTC | 68 |
| Bax | F : TCA GGA TGC GTC CAC CAA GAA G<br>R : TGT GTC CAC GGC GGC AAT CAT C | 60 |
| Bcl-2 | F : ATC GCC CTG TGG ATG ACT GAG T<br>R : GCC AGG AGA AAT CAA ACA GAG GC | 60 |
| Caspase-9 | F : GTT TGA GGA CCT TCG ACC AGC T<br>R : CAA CGT ACC AGG AGC CAC TCT | 60 |
| Caspase-3 | F : GCG GTT GTA GAA GAG TTT CGT G<br>R : CTC ACG GCC TGG GAT TTC AA | 60 |
| Beclin-1 | F : GGC TGA GAG ACT GGA TCA GG<br>R : CTG CGT CTG GGC ATA ACG | 60 |
| LC3-II | F : GAG AAG CAG CTT CCT GTT CTG G<br>R : GTG TCC GTT CAC CAA CAG GAA G | 60 |
| Snail1 | F : CTG GGT GCC CTC AAG ATG CA<br>R : CCG GAC ATG GCC TTG TAG CA | 60 |
| MMP-9 | F : TTG ACA GCG ACA AGA AGT GG<br>R : GCC ATT CAC GTC GTC CTT AT | 60 |
| MMP-2 | F : CGC TCA GAT CCG TGG TGA<br>R : CGC CAA ATG AAC CGG TCC TT | 65 |

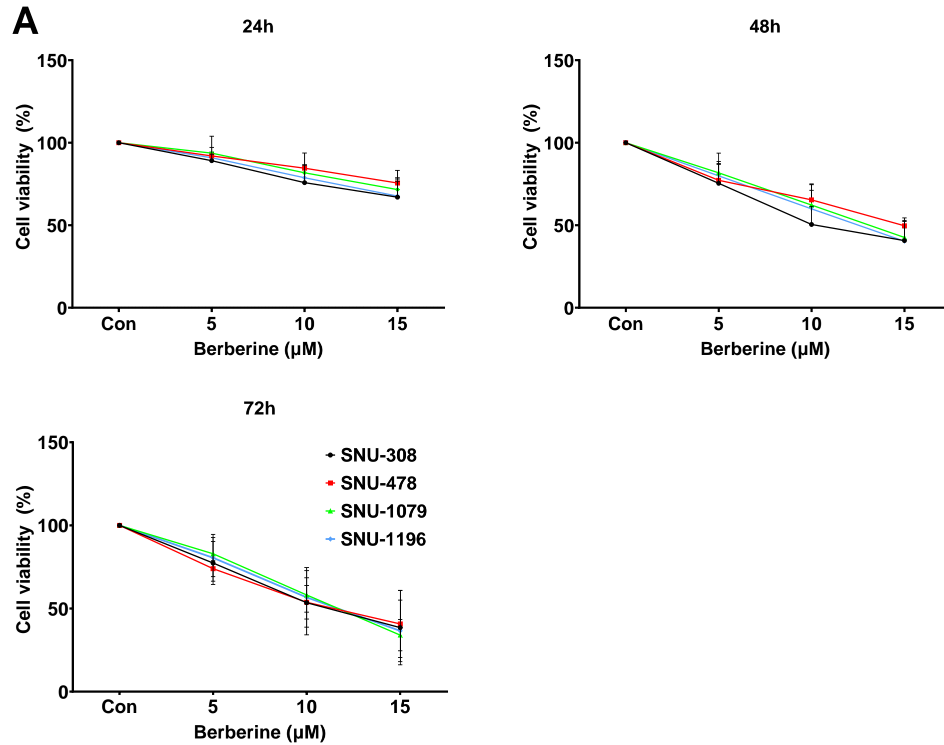



**Figure 1. Effect of berberine on the proliferation of CCA Cells.**

Berberine suppresses CCA cells proliferation in vitro. Cell viability was determined by MTT assay after treatment with indicated concentrations of berberine for 24 h, 48 h, and 72 h in SNU-308, SNU-478, SNU-1079 and SNU-1196 cells.

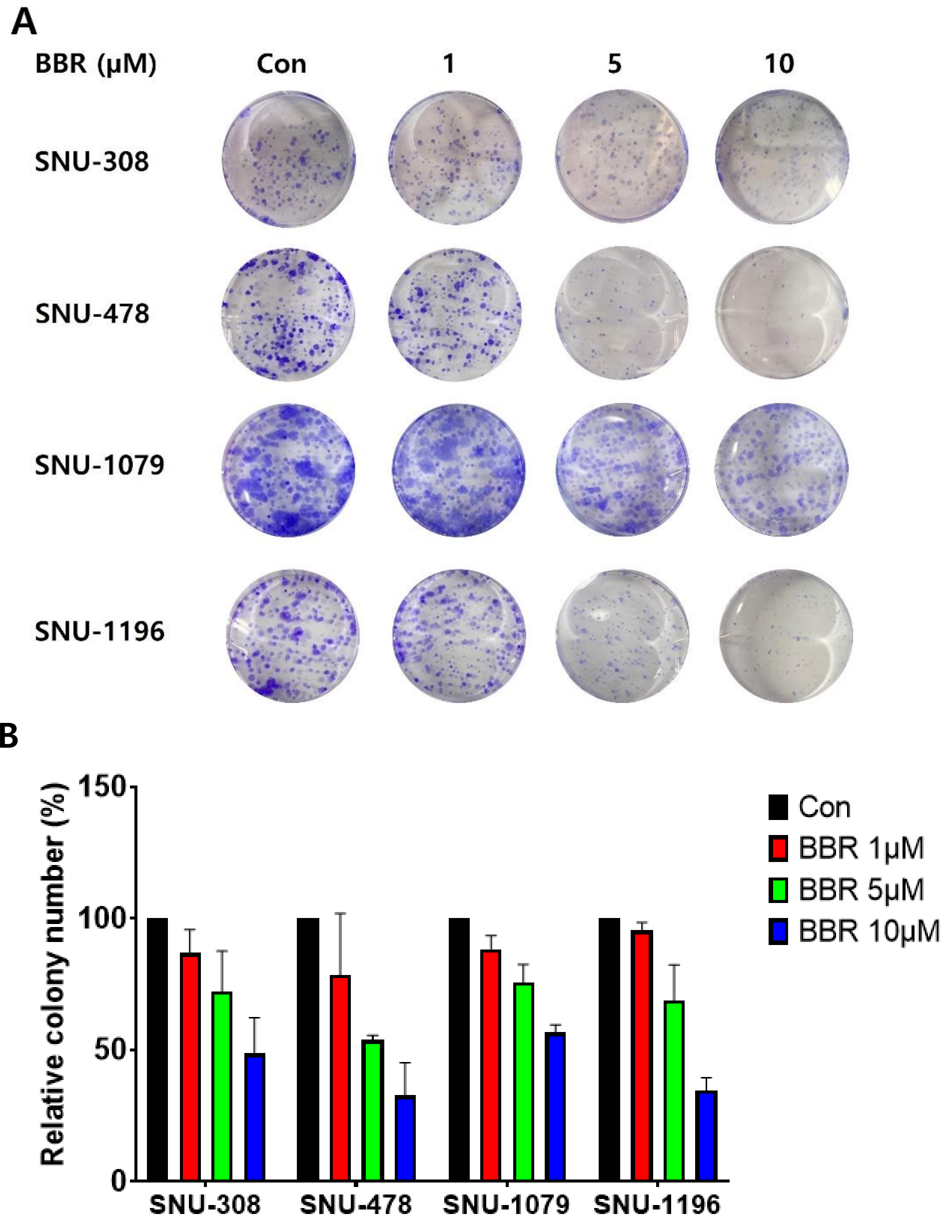
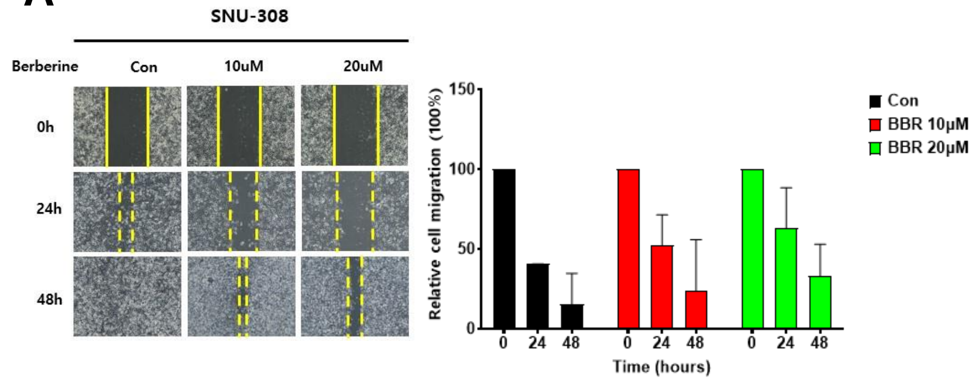


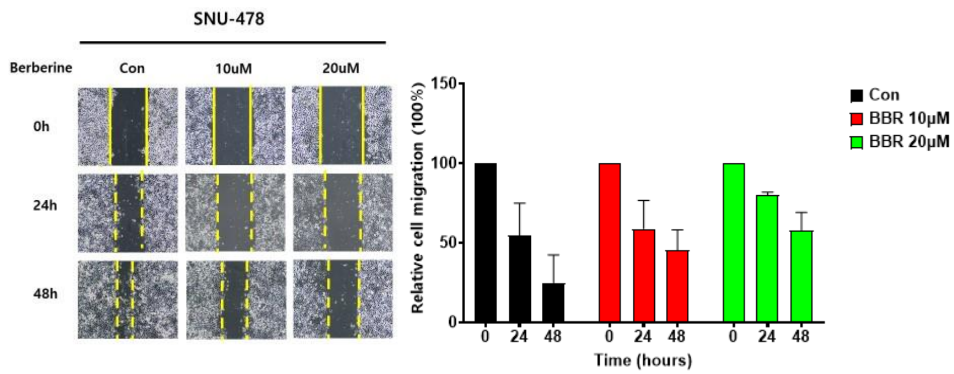

**Figure 2. Effect of berberine on colony formation potential of CCA Cells.**

The inhibition effects of BBR on proliferation of CCA cells including SNU-308, SNU-478, SNU-1079 and SNU-1196. The crystal violet staining of CCA cell lines after cultured in the concentration of 1 μM, 5 μM, and 10 μM for 7 days. The colony formation rate (%) of each well in the panel A.

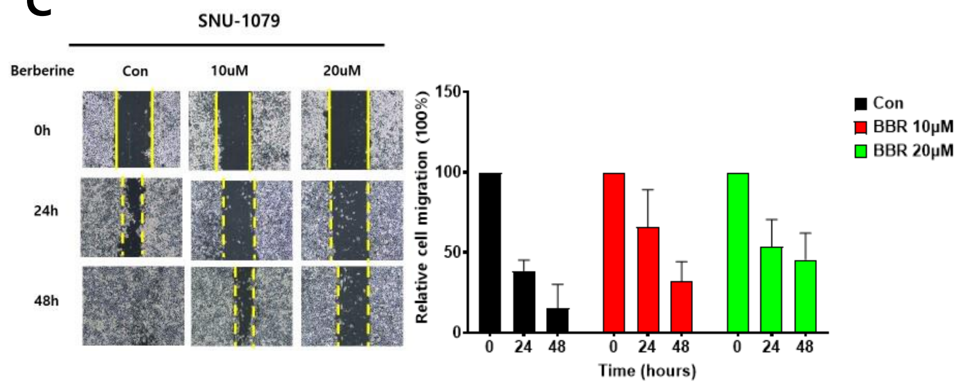
**A**




**B**




**C**



**D**

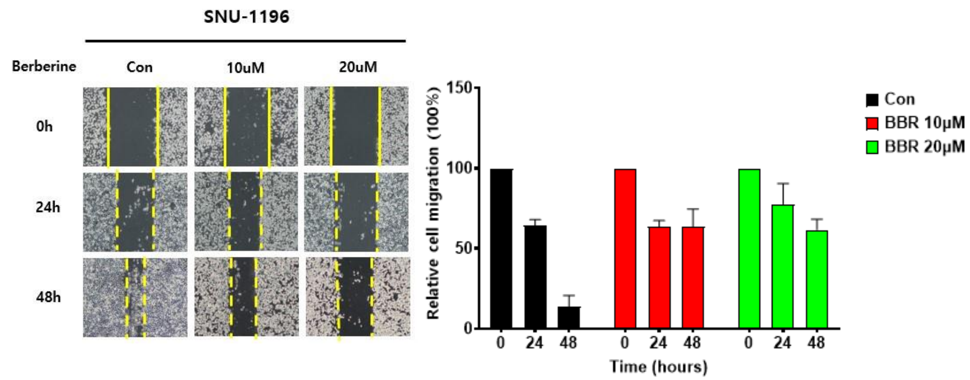
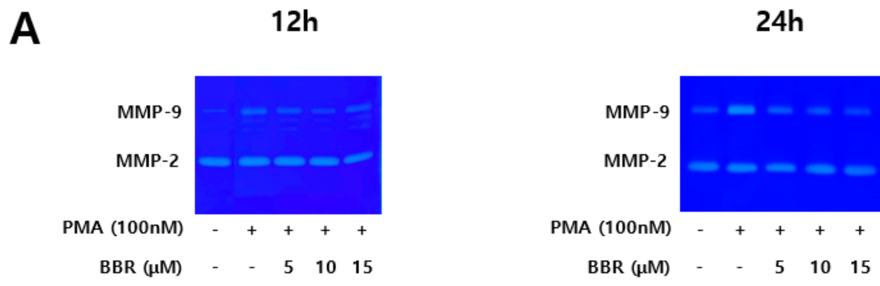
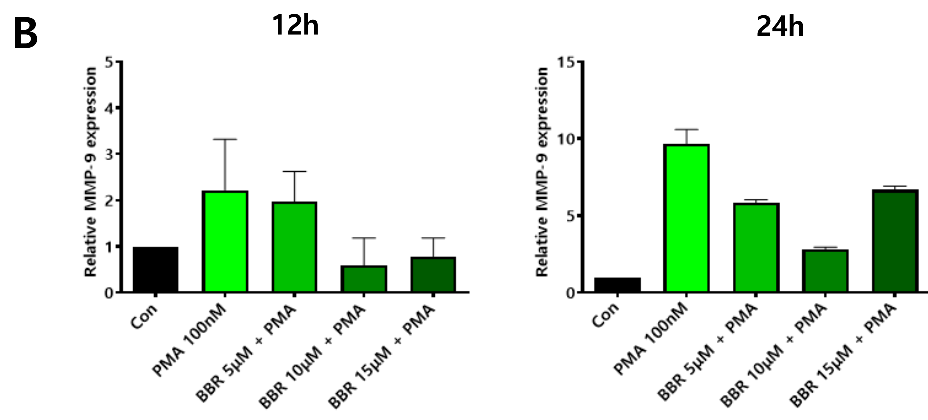



**Figure 3. Effect of berberine on the migration of CCA cells.**

(A) SNU-308, (B) SNU-478 (C) SNU-1079 (D) SNU-1196 with 10 μM and 20 μM berberine at 0 h, 24 h and 48 h after wound healing. The yellow lines show the gap distance of cell wound.


A
12h
24h
MMP-9
MMP-2
PMA (100nM) - + + + +
BBR (μM) - - 5 10 15


B
12h
24h
Relative MMP-9 expression
Con
PMA 100nM
BBR 5μM + PMA
BBR 10μM + PMA
BBR 15μM + PMA

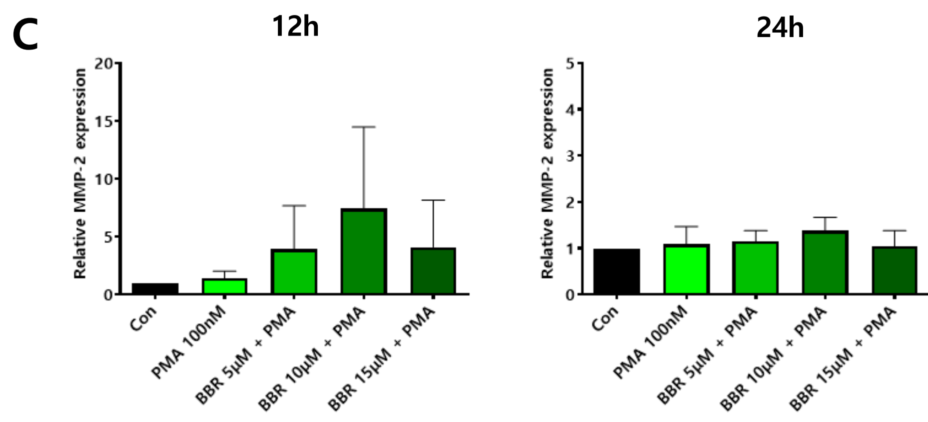

C
12h
24h
Relative MMP-2 expression
Con
PMA 100nM
BBR 5μM + PMA
BBR 10μM + PMA
BBR 15μM + PMA

**Figure 4. Effects of MMP-9 and MMP-2 activities and mRNA expression in SNU-308 cells.**

(A) Effects of berberine on MMP-9 and MMP-2 activities. SNU-308 cells were incubated with varying concentrations of berberine in the presence of PMA (100 μM) for 12 h and 24h. (B) MMP-9 expression was analyzed by qRT-PCR. (C) MMP-2 expression was analyzed by qRT-PCR.

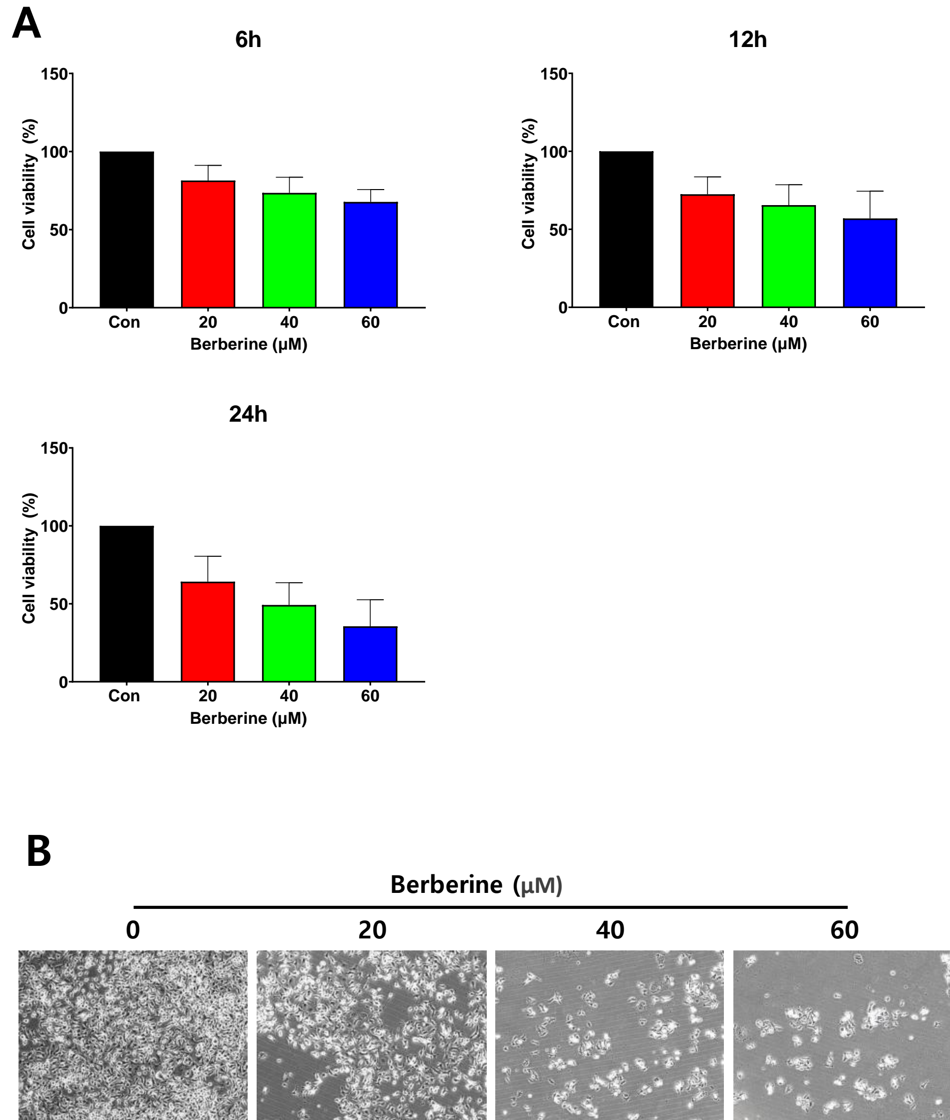



**Figure 5. Effect of berberine on the proliferation of SNU-478.**

Berberine suppresses CCA cells proliferation *in vitro*. (A) Cell viability was determined by MTT assay after treatment with indicated high concentrations of berberine for 6 h, 12 h and 24 h in SNU-478. (B) SNU-478 cells were treated with berberine 0 μM, 20 μM, 40 μM and 60μM for 24 h.

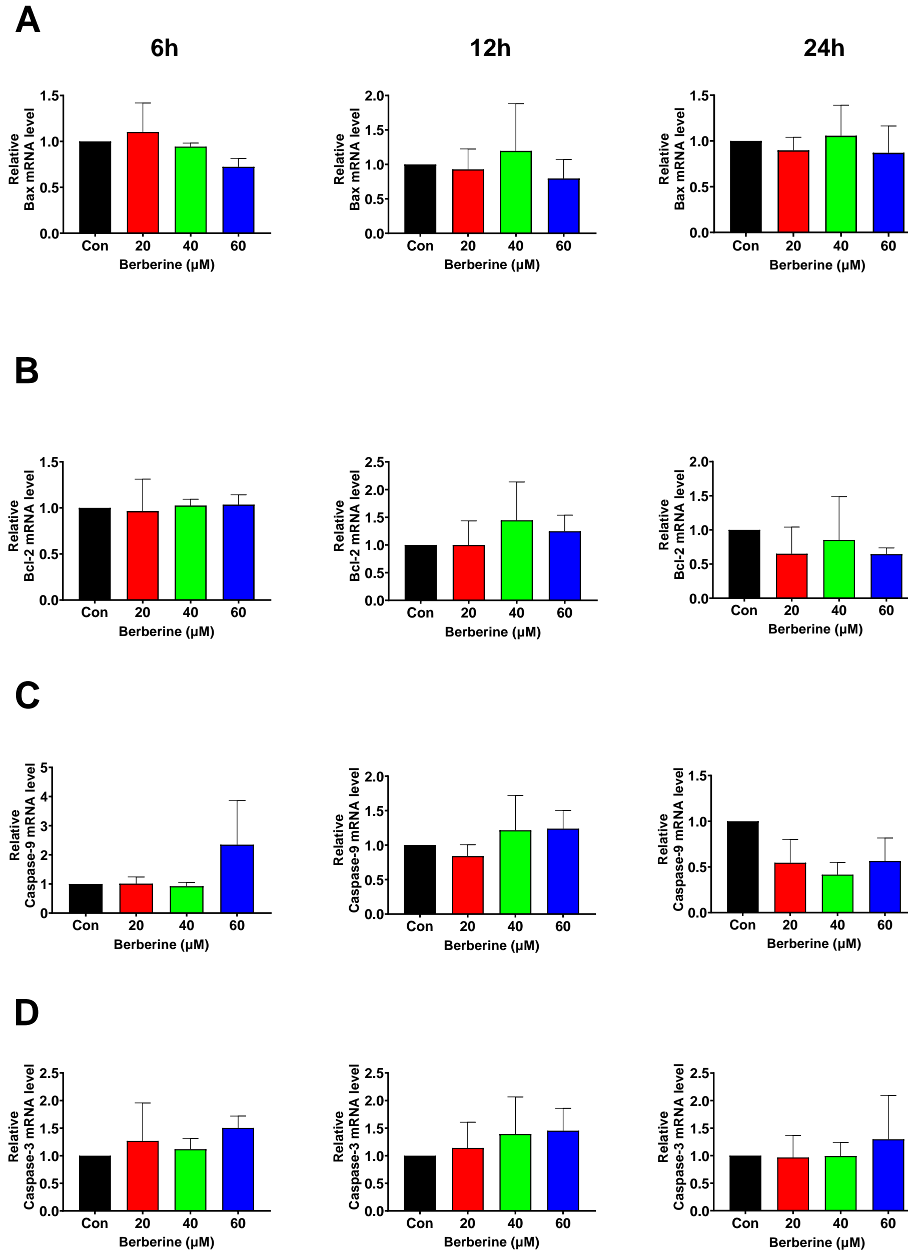



**Figure 6. Effects of berberine on mRNA expression of apoptosis associated genes in SNU-478 cells.**

Effect of berberine on relative mRNA expression in SNU-478 cell. SNU-478 cell was analyzed by reverse transcription-quantitative polymerase chain reaction to measure the levels of Bax, Bcl-2, Caspase-9 and Caspase-3.

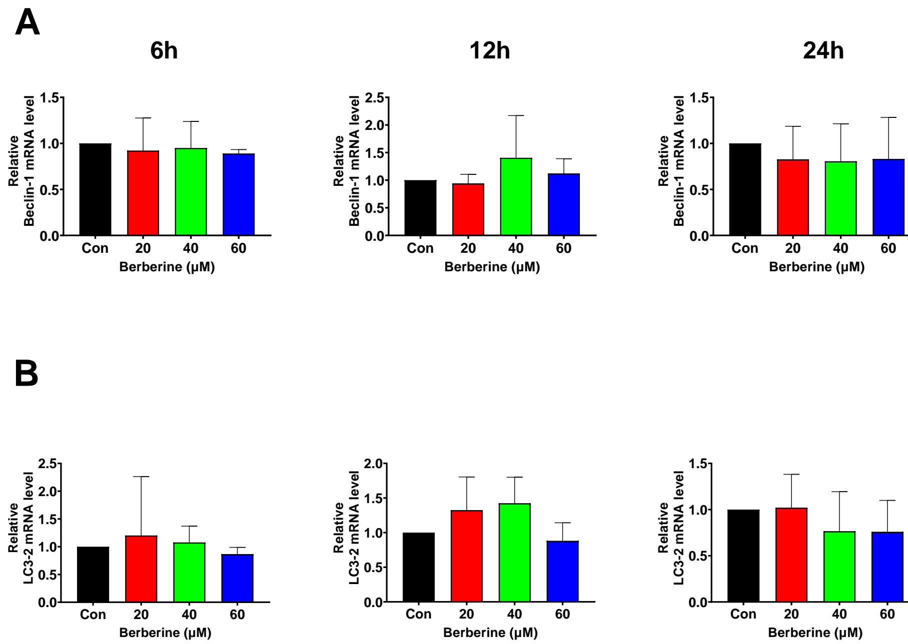



**Figure 7. Effects of berberine on mRNA expression of autophagy associated genes in SNU-478 cells.** Effect of berberine on relative mRNA expression in SNU-478 cell. SNU-478 cell was analyzed by reverse transcription-quantitative polymerase chain reaction to measure the levels of Beclin-1 and LC3-II.

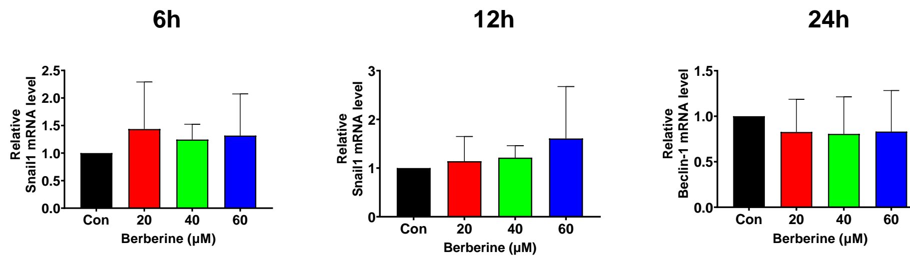



**Figure 8. Effects of berberine on mRNA expression of EMT associated gene in SNU-478 cells.**

Effect of berberine on relative mRNA expression in SNU-478 cell. SNU-478 cell was analyzed by reverse transcription-quantitative polymerase chain reaction to measure the levels of Snail1.

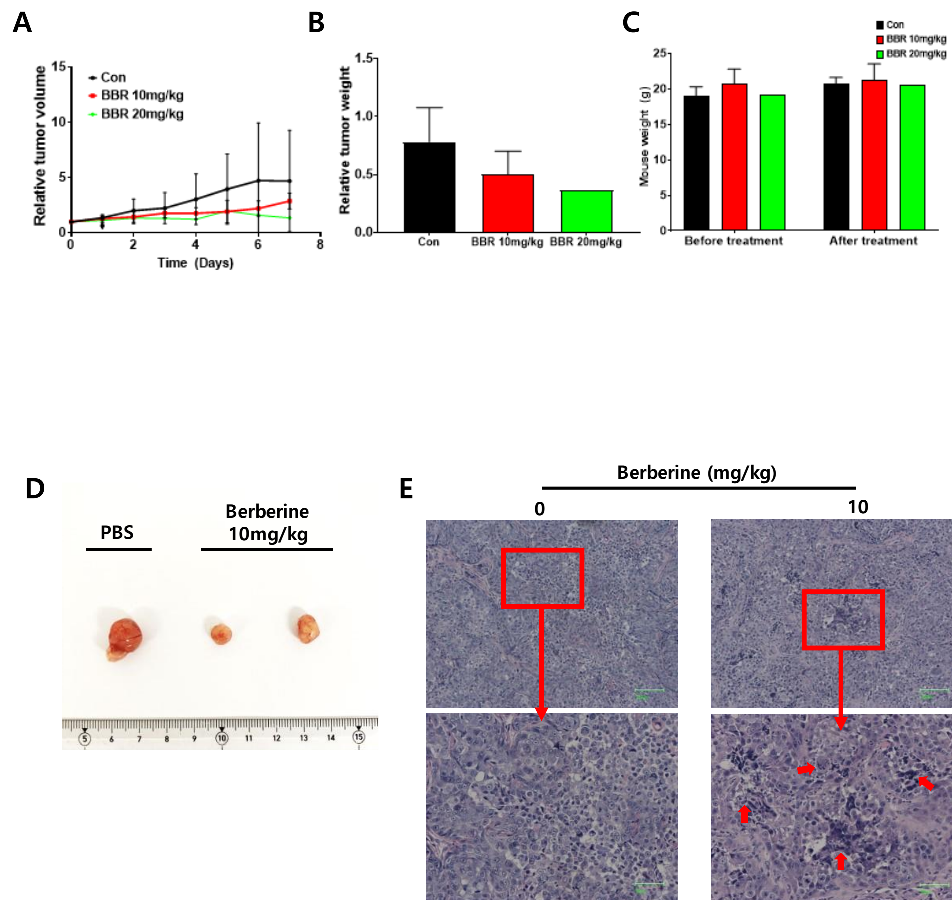



**Figure 9. Effect of berberine on SNU-478 xenograft model**

Berberine suppresses tumor xenograft growth *in vivo*. (A) NOD SCID mouse with subcutaneous SNU-478 cells were intraperitoneally treated with PBS (as Control) or BBR (10 mg/kg and 20 mg/kg) and the tumor growth was monitored. (B) Treatment with berberine suppressed CCA tumor growth. (C) The weight of mouse before and after treatment. (D) Tumor photographs (PBS and Berberine 10mg/kg) (E) H&E staining histological analysis of CCA tumor with PBS, Berberine 10mg/kg treatment. Arrows indicate nuclear condensation and fragmentation.

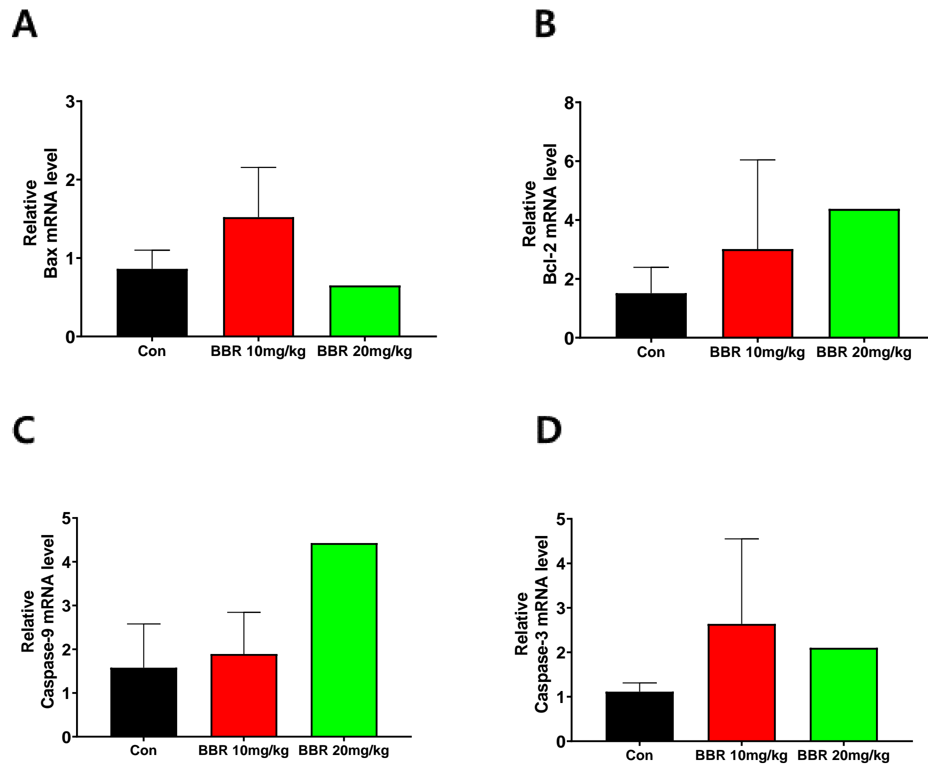



**Figure 10. Effects of berberine on mRNA expression of apoptosis associated genes in SNU-478 xenograft mouse tissue**

Effect of berberine on relative mRNA expression in tumor of xenograft mice. Mice were treated with BBR 10 mg/kg and 20 mg/kg or PBS (as control). CCA tumors were analyzed by reverse transcription-quantitative polymerase chain reaction. mRNA levels of four apoptosis related genes, (A) Bax (B) Bcl-2 (C) Caspase-3 and (D) Caspase-9.

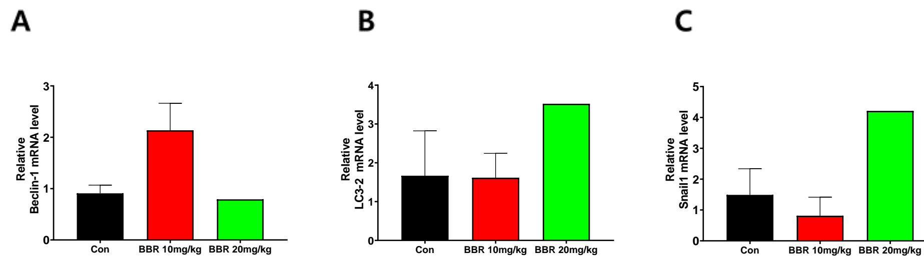



**Figure 11. Effects of berberine on mRNA expression of apoptosis associated genes and EMT related gene in SNU-478 xenograft mouse tissue.**

Effect of berberine on relative mRNA expression in tumor of xenograft mice. Mice were treated with BBR 10mg/kg and 20mg/kg or PBS (as control). CCA tumors were analyzed by reverse transcription-quantitative polymerase chain reaction. mRNA levels of two autophagy related genes, (A) Beclin-1 and (B) LC3-II. mRNA level of EMT related gene, (C) Snail1.

# Discussion

The incidence and mortality of CCA have been increasing worldwide. Chemotherapy is the major treatment for CCA. But long-term chemotherapy not only caused side effects, but also inevitably induced drug resistance [19,20]. Thus, researchers are increasingly turning to natural compound for reducing side effects, resistance and enhancing anticancer effect recently. Among of them, this study investigated the anticancer mechanism using berberine, which has not been studied extensively as a treatment for CCA.

We first examined that CCA cells (Korean patient-derived CCA cell lines) were treated with 0 μM, 5 μM, 10 μM and 15 μM of berberine for 24 h to 72 h. This result showed that the CCA cells were decreased with time- and dose-dependent manner. In the previous studies, similar effects were reported in KKU-213, KKU-214 (Thai patient-derived CCA cell lines) which treated berberine (0–20 μM) [47] and QBC939 (Chinese patient-derived CCA cell lines, 0–80 μM) [48]. In the previous studies, the proliferation of each normal cell and cancer cell was compared. The CCA cell lines are more sensitive compared with normal cell lines to berberine. However, there was no comparison between CCA cells and normal cells in this study. Further study is needed to comparison between cancer cells and normal cells from Korean CCA patients.

Various studies have been reported about berberine's anti-proliferation and anti-migration functions which were measured by colony formation assay [50] and wound healing assay [38] in several types of cancers. However, the effect of anti-proliferation and anti-migration functions of berberine using colony formation and wound healing assay has not yet been studied in CCA cells. This study was the first to show that berberine inhibit CCA cell proliferation and migration. In colony formation, the proliferation of CCA cells decreased upon berberine treatment dose-dependently (berberine 1 μM, 5 μM and 10 μM). In order to explore whether berberine suppress the migration on CCA cells, wound healing assay was performed. In wound healing assay, SNU-308, -478, -1079, and SNU-1196 cells exhibited difference between migration with berberine treated at the concentration of 10 μM

and 20 μM for 24 h and 48 h. These data suggested that the berberine suppression on proliferation and migration on CCA cells.

In the present study, berberine increased Bax, Caspase-9 and Caspase-3 at the mRNA level in SNU-478 cells and xenograft tumor tissue, indicating that maybe berberine induces apoptosis via intrinsic pathway. However, berberine increased pro-apoptotic Bax and anti-apoptotic Bcl-2 at the mRNA level in xenograft tumor tissue. Therefore, further research is needed to validated in more xenograft tumor tissue. Previous researches revealed berberine induces apoptosis through intrinsic and extrinsic pathways in numerous types of cancers [41,51,52]. In the previous report demonstrated that berberine increased pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl-2 and Bcl-xl of protein expression in QBC939 CCA cell line. Further studies are required with protein level of CCA cell lines.

Berberine has a wide range of autophagy activities in other cancers [53,54]. However, little is known about the autophagy mechanism of berberine in CCA. Accordingly, we examined whether berberine is involved in autophagy in this study. In berberine-treated SNU-478 cells, we discovered an increase in Beclin-1 and LC3-II mRNA expression. The results of the SNU-478 xenografts mice also validated the effect of berberine on Autophagy. Thus, suggesting that berberine can induce autophagy (at least partially) in vitro and in vivo. More studies are required to fully explain how berberine may modulate autophagy in CCA.

EMT has been considered the critical mechanism involved in cancer metastasis and invasion. In order to determine whether or not berberine is involved in EMT, we measured mRNA of EMT associated factor. Snail1 is one of the important transcription factors for the regulation of the EMT. In this work, there's no significant reduction in SNU-478 cells in mRNA level; however, we found that Snail1 expression decreased following berberine in SNU-478 xenograft tumor tissue in mRNA level. In previous study, berberine significantly decreased the protein expression of snail1 in SiHa cells (Cervical cancer cell) [38]. But there were no results of protein level in our data, therefore, future studies are required that berberine decrease of Snail1 in protein level. Due to only one EMT associated factor was used in this study, future studies will need to experiment other EMT factors in

order to verify the berberine effects of EMT.

Furthermore, the expression and activation of MMPs, such as MMP-2 and MMP-9, which are associated with cancer migration and invasion. We tested whether berberine suppress MMP-2 and MMP-9 activities and mRNA level. According to our data, MMP-9 activity suppressed and the mRNA level of MMP-9 also decreased in SNU-308 cells. Previously studies have demonstrated that treatment with berberine significantly inhibited MMP-2 and MMP-9 such as human ovarian cancer cells in enzymatic activities [55], human tongue squamous cancer cells [56]. However, our results did not show a berberine effect on mRNA levels and enzyme activity of MMP-2 in SNU-308 cells. Therefore, the protein level and mRNA of MMP-2 and MMP-9 are required further investigation.

In this experiment, SNU-478 xenografted mouse model was established for the investigation of anticancer effects of berberine. The results showed that berberine inhibited of tumor growth in xenografted mice without any influence on body weight. These results were consistent with the data of previous studies that demonstrated the anticancer effects of berberine *in vivo* of other cancers [38, 50]. H&E staining showed that the anticancer effect of berberine in SNU-478 xenograft tumor is closely associated to apoptosis, as shown by the extent of DNA fragmentation and nuclear condensation (Fig. 8E). The pro-apoptotic genes and autophagy associated genes increased and EMT associated gene decreased in CCA xenograft tumor. In addition, previous studies have only *in vitro* results. However, this present study evaluated the efficacy of berberine in the treatment of CCA at the animal level by establishing CDX (cell-derived xenografts). Actually, PDX (patient-derived xenografts) model is closer to the characteristics of clinical tumor samples. In the future, we well further evaluate the efficacy of berberine in the treatment of CCA based on the PDX model. Our data for the first time showed that berberine decreased tumor size of CCA xenograft mouse model.

In conclusion, our findings showed that berberine inhibits cell proliferation, migration, colony formation, EMT through apoptosis and autophagy. This study provides first evidence that berberine is associated with autophagy and EMT in Korean patients-derived cell lines. Furthermore, berberine increased Bax, Bcl-2,

Caspase-9, Caspase-3, and decreased Snail1 in SNU-478 xenograft tumor. These findings suggest berberine can be a potential therapeutic agent to treat patients with this devastating malignancy.

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# 국문요약

**연구배경**: 담관암은 담관에서 발생하는 드문 상피 세포 악성 종양으로 화학요법에 내성을 가지고 있다. 따라서 담관암에 매우 효과적인 치료제는 없다. 최근 베르베린을 포함한 많은 천연 화합물은 연구자들의 관심을 끌었으며, 지난 수십 년 동안 그 사용이 증가했다. 베르베린은 *coptis chinensis* 와 같은 여러 식물의 줄기, 뿌리줄기와 뿌리에서 분리되어 박테리아 감염, 당뇨병, 심혈관 및 염증성 질환의 치료에 치료제로 사용되어 왔다. 이전의 다른 연구들에서 베르베린이 증식, 이동 침윤 억제를 통해 유방암, 자궁경부암, 결장암, 간암, 폐암, 난소암 등 다양한 종양에 미치는 항암 효과가 꾸준히 보고되어져 왔다. 그러나 담관암에 대한 베르베린의 항암 활성에 대해서는 알려진 바가 거의 없었다. 이 연구의 목적은 담관암에 대한 베르베린의 효과와 기본 메커니즘을 조사하는 것이었다.

**방법**: 본 연구에서는 인간 담관암 세포주 (SNU-308, SNU-478, SNU-1079, SNU-1196)를 사용하였다. 세포 생존력은 MTT assay 에 의해 알아냈고. 세포의 장기 증식은 colony formation assay 에 의해 알아냈다. 또한, 세포의 이동은 wound healing assay 에 의해 조사되었다. MMP-9 및 MMP-2 의 활성은 zymography 로 조사하였다. MMP-9, MMP-2, Bax, Bcl-2, Capsae-9, Caspase-3, Beclin-1, LC3-II 및 Snail1 의 mRNA 발현을 qRT-PCR 로 조사하였다.

**결과**: 베르베린이 다양한 담관암 세포주에서 세포 증식, 이동 및 군집 형성을 억제하고 SNU-478 세포에서 Bax, Caspase-9 과 -3 를 증가시켰다. 베르베린은 SNU-478 세포에서 MMP-9 활성을 차단했다. 이종이식 모델에서 베르베린은 종양 성장을

억제하였고, SNU-478 이종이식 종양 모델에서 Bax, Bcl-2, Caspase-9, Caspase-3 를 증가시켰고, Snail1 을 감소시켰다.

**결론**: 이 연구는 베르베린이 *in vitro* 및 *in vivo* 모델에서 세포자연사 및 자가포식의 기전을 통해 세포 증식, 이동, 군집 형성을 억제함을 시사하였다. 따라서 베르베린은 치명적인 악성 종양 환자를 치료할 수 있는 잠재적 치료제가 될 수 있다.

**핵심주제어**: 담관암, 베르베린, 세포사멸사, 세포이동

# 감사의 글

2022년 8월을 끝으로 2년간의 석사과정을 마치게 되었습니다. 학업을 시작한 날부터 논문을 완성하기까지 많은 도움과 응원해 주신 분들에게 감사의 마음을 전하고자 합니다.

먼저, 지도교수님이신 황신 교수님께 이번 기회를 통해서 깊은 감사와 존경의 인사를 올립니다. 논문이 완성될 수 있도록 세심히 지도해 주시고, 늘 든든한 길잡이가 되어 주신 이경진 교수님께도 깊은 감사를 드립니다. 대학원 입학부터 졸업까지 한결같이 따뜻한 격려와 도움을 주신 여명구 교수님께 감사드립니다. 또한 바쁘신 와중에도 학위 논문을 심사해주신 정동환 교수님께 감사드립니다.

실험실에서 많은 시간을 함께 했던 실험실 식구들에게도 감사의 마음을 전합니다. 제가 친 모든 사고의 뒷수습을 묵묵히 해주셨던 김윤규 선생님. 사고 칠 사람 한 명 줄어들어 앞으로 심심하실 거예요. 어리바리 어디로 튈지 모르는 저를 졸업까지 잘 이끌어 주셔서 감사합니다. 실험실의 든든한 살림꾼이자 동물실 메이트 (구) 훈지 (현) 유진, 양유진 선생님. 세심한 배려와 관심 항상 고마웠어요. 언제나 몸과 마음이 평안하길 기도할게요. 그리고 졸업 가보자고.. 나의 멘탈지킴이이자 내 웃음의 팔 할을 차지했던 다은이. 덕분에 재미있게 대학원 생활할 수 있었고, 지금까지 잘 버틸 수 있었어 항상 고마워. 다른 실험실이었지만, 말동무가 되어 주시고 잘 챙겨 주셨던 이상은 박사님께도 감사의 말씀을 전합니다.

힘들 때마다 큰 힘이 되어 주었던 은서. 너의 센스는 그 누구도 따라 오지 못할 거야. 언제나 고맙고, 은서의 앞날이 언제나 빛나길 응원할게. 그리고 존재만으로도 든든한 은별이, 지원이. 응원 덕분에 큰 힘이 됐어 고마워. 기도와 따뜻한 사랑을 보여 주었던 사론이 언니 고마워요!

마지막으로 2년여 간의 과정을 잘 마무리 할 수 있도록 사랑과 기도로 지지해주었던 우리가족, 감사하고 사랑합니다. 일일이 다 언급하지는 못하지만 이 외에도 저를 위해 기도해주시고 응원해주신 모든 분들께 감사의 마음을 전하며, 항상 행복하고 건강하길 기원합니다.

무엇보다도 제 작은 발걸음마다 함께 하시며, 항상 선한 길로 인도해주시는 하나님께 이 모든 영광 돌립니다.