

RESEARCH ARTICLE

Anti-metastatic Potential of Ethanol Extract of *Saussurea involucrata* against Hepatic Cancer *in vitro***Munkhzaya Byambaragchaa¹, Joseph de la Cruz², Seung Hak Yang³, Seong-Gu Hwang^{1*}****Abstract**

The rates of morbidity and mortality of hepatocellular carcinoma (HCC) have not lessened because of difficulty in treating tumor metastasis. Mongolian *Saussurea involucrata* (SIE) possesses various anticancer activities, including apoptosis and cell cycle arrest. However, detailed effects and molecular mechanisms of SIE on metastasis are unclear. Thus, the present study was undertaken to investigate antimetastatic effects on HCC cells as well as possible mechanisms. Effects of SIE on the growth, adhesion, migration, aggregation and invasion of the SK-Hep1 human HCC cell line were investigated. SIE inhibited cell growth of metastatic cells in dose- and time-dependent manners. Incubation of SK-Hep1 cells with 200-400 µg/mL of SIE significantly inhibited cell adhesion to gelatin-coated substrate. In the migration (wound healing) and aggregation assays, SIE treated cells showed lower levels than untreated cells. Invasion assays revealed that SIE treatment inhibited cell invasion capacity of HCC cells substantially. Quantitative real time PCR showed inhibitory effects of SIE on MMP-2/-9 and MT1-MMP mRNA levels, and stimulatory effects on TIMP-1, an inhibitor of MMPs. The present study not only demonstrated that invasion and motility of cancer cells were inhibited by SIE, but also indicated that such effects were likely associated with the decrease in MMP-2/-9 expression of SK-Hep1 cells. From these results, it was suggested that SIE could be used as potential anti-tumor agent.

Keywords: *Saussurea involucrata* - liver cancer - metastasis - MMP-2 - MMP-9 - TIMP-1

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Introduction

Most patients with liver cancer die within one year after diagnosis largely because of frequent tumor metastasis (Suresh et al., 2011). Hepatocellular carcinoma (HCC) characterized by its hypervascularity, tendency to invade vasculature and frequently shows early invasion into blood vessels as well as intrahepatic metastasis and later shows extrahepatic metastasis (Mitsunobu et al., 1996; Chan and Yeo, 2012). The prognosis for HCC depends mainly on the clinicopathological characteristic regarding invasion and metastasis (Chung et al., 2003). Metastasis is one of the major causes of mortality in cancer patients and occurs as a complex multistep process that involves many cytophysiological changes, including cancer cell adhesion, invasion and migration (Sternlicht and Werb, 2001; Yeh et al., 2012). One critical characteristic of metastatic cancer cells is the ability to degrade basement membranes and extracellular matrix (ECM). This degradative process is mediated mainly by matrix metalloproteinases (MMPs). The two gelatinases MMP-9 (gelatinase B) and MMP-2 (gelatinase A) plays a key role in degradation of the basement membrane mainly composed of collagen

type IV and gelatin. These gelatinases are expressed in hepatocellular carcinoma cells and are associated with progression, metastasis and invasion of these tumors (Roomi et al., 2012). Thus, the inhibition of MMP activity is important in terms of preventing cell metastasis and invasiveness (Zhang et al., 2013). Interestingly, SK-Hep1 is invasive and expresses high MMP-9 expression levels (Lai et al., 2010) and gelatinase activity required for migration and invasion (Gianelli et al., 2001).

The current trend of cancer research is the investigation of medicines of plant origin because of their affordability and accessibility with minimal side effects. Also some traditional prescriptions were employed for inhibition of tumor metastasis. *Saussurea involucrata* KAR. et KIR., belongs to the Asteraceae family, is a rare and beneficial traditional medicinal herb, grows in the mountains at heights of 4000-4300m in Mongolia and China. The dried aerial parts of *S involucrata* have long been used for the treatment of rheumatoid arthritis, impotence, irregular menses, cough with cold, stomachache, and altitude sickness (Li and Zhao., 1989; Jing et al., 2005). Recent studies have shown that *S involucrata* extract (SIE) has anti-cancer effect however, the antimetastatic activity

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and its detailed mechanism of *Saussurea involucrata* had not yet been demonstrated. *S. involucrata* contains anti-metastatic compounds such as rutin (Lata et al., 1995), hispidulin (Lijun et al., 2010) and apigenin (Hu et al., 2008).

In this study, we examined the anti-proliferative, anti-adhesive, anti-migrative and anti-invasive effects of ethanol extracts from *Saussurea involucrata*, and further elucidated the molecular mechanism of action in SK-Hep1 cells.

Materials and Methods

Cell line and culture media

SK-Hep1 (human hepatocarcinoma cells) cells were purchased from Korean Cell Line Bank in South Korea. Chang Liver normal cells were obtained from Konkuk University (South Korea). Fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL (Grand Island, NY, USA). Penicillin/streptomycin mix was purchased from Lonza (Walkersville, MD, USA). CCK-8 reagent was purchased from Dojindo (Kumamoto, Japan). SYBR Green PCR Master Mix was purchased from Applied Biosystems. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Human hepatocarcinoma SK-Hep1 and human normal Chang liver cells were maintained in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 100 µg/mL streptomycin under 5%CO₂ in a humidified incubator at 37°C.

Preparation of SIE

Snow lotus (*Saussurea involucrata*) collected in Mongolia was used in this study. The 10 g air-dried plants were kept in 500 ml 95% ethanol for 48h and then filtered. The ethanol was evaporated to with a rotary vacuum evaporator and freeze-dried to give a powder. The stock solution of *Saussurea involucrata* extract (SIE) was prepared by dissolving snow lotus powder in culture medium (10mg/mL) and the experimental concentrations were diluted in the basal medium.

Cell viability analysis

Cell viability was quantified by CCK-8 assay. Briefly, the cells were plated in 96-well culture plates at a density of 10⁵ cells/mL and allowed to adhere at 37°C for 12h. Then cells were exposed to various doses of SIE. At various times after exposure, the media with various SIE concentrations were discarded; the cells were washed with PBS once. Then fresh media with CCK-8 reagent were added to each well. After 1 hour at 37°C, the absorbance of OD450 was determined using an ELISA plate reader (Tecan, Switzerland). Morphological changes after exposure to SIE were observed by an inverted microscope.

Quantitative real time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen,

MA, USA). Complementary DNA (cDNA) was obtained by following the protocol of THERMOScript RT-PCR System (Life Technologies). PCR was applied to cDNAs prepared from untreated and SIE treated samples. Quantitative PCR was performed using the Power SYBR Green PCR Master Mix from Applied Biosystems in a Bio-Rad C1000TM Thermal Cycler following the manufacturer's protocol. Amplification was carried out in a total volume of 20ml for 40 cycles of 15s at 95°C, 20s at 60°C, and 30s at 72°C. Samples were run in triplicate and their relative expression was determined by normalizing expression of each target GAPDH. These were then compared with the normalized expression in control untreated sample to calculate a change value (%). Primer sequences were as follows: human GAPDH: sense 5'-CGAGATCCCTCCAAAATCAA-3', antisense 5'-AGGTCCACCACTGACACGTT-3'; human PTHrP exonIV: sense 5'-ACCTCGGAGGTGTCCCCTAAC-3', antisense 5'-TCAGACCCAAATCGGACG-3'; human IL-11: sense 5'-AGCGAGTGGATCACTGAAGTCC-3', antisense 5'-TTGCCATGTCTCGCAGGC-3'; human MMP-2: sense 5'-GGCCCT GTCACCTCTGAGAT-3', antisense 5'-GGCATCCAGGTTATCGGGGA-3'; human MMP-9: sense 5'-CGGAGCACGGAGACGGGTAT-3', antisense 5'-TGAAGG GGAAGACGCACAGC-3'; human MT1-MMP: sense 5'-TGGGTAGC GATGAAGTCTTC-3', antisense 5'-AGTAAAGCAGTC GCTTGGGT-3'; human TIMP-1: sense 5'-GATCCA GCGCCCAGAGAGACACC-3', antisense 5'-TTCCAC TCCGGGCAGCATT-3'; human TIMP-2: sense 5'-GGCGTTTTGCAATGCAGATGTAG-3', antisense 5'-CACAGGAGCCGTCACCTTCTCTTG-3'.

Cell adhesion assay

Adhesion assay was performed as described previously (Burg-Roderfeld et al., 2007). The 6 well culture plates were coated with 0.1% gelatin in deionized water overnight at 37°C and left to air-dry for 30 min. SK-Hep1 cells (5×10⁴cells/mL) were harvested by trypsin and resuspended in media with various concentrations of SIE (0, 200 and 400µg/mL). Cells were then dispensed into each well and incubated in 5%CO₂ at 37°C for 2h. After removal of the media, the attached cells were gently washed twice with PBS and counted time-dependently under a microscope (Olympus CK40).

Cell invasion assay

The invasion of cancer cells was assessed in transwell chambers with 6.5mm polycarbonate filters of 8µm pore size (Corning Inc., Corning, NY, USA). The Matrigel (1:20 dilution in cold DMEM, BD Biosciences) was added to the filter to form a thin gel layer and dried in a laminar hood overnight. SK-Hep1 (5×10⁵cells/mL) and various concentrations of SIE (0, 50, 100, 200 and 400µg/mL) were suspended in 100µL of serum free medium and added to the upper compartment of the invasion chamber. The lower chamber was filled with 500µL of the conditioned medium. The matrigel invasion chambers were incubated at 37°C for 24h in 5%CO₂. Then the cells on the upper surface of the filter were removed using a cotton swab and the cells that had moved to the lower surface of the

filter were fixed with 10% methanol and stained with 5% Giemsa. Invasion was quantified by visual counting under microscope (Olympus CK40). The values obtained were calculated by averaging the total number of cells from three filters.

Wound closure assay

Cell motility was examined using the wound healing assay. SK-Hep1 cells were seeded at 5×10^5 cells/mL in 6 well plates. After cells reached 100% confluency, an injury line was made with a plastic pipette tip in the central area of culture. Cells were washed with PBS and exposed to various doses of SIE (0-200 μ g/mL). Wound closure was monitored and photographed at the same position of the wound at 0, 24, 48 and 72h after scrapping under a microscope (Olympus, $\times 100$).

Cell aggregation assay

SK-Hep1 cells were cultured as spheroids in the DMEM medium by hanging drop method (Kelm et al., 2003). Subconfluent cells were trypsinized, counted and resuspended in medium in the presence or absence of various concentrations of SIE (0, 200 and 400 μ g/mL). 20 μ L of cell suspension (5×10^5 cells/mL) was then placed onto the undersurface of 60mm tissue culture dish lids which were then placed over PBS and incubated at 37°C in 5% CO₂. Cells were then analyzed for aggregates under the microscope (Olympus) at 0, 2, 4, 24 and 48h after incubation. Single cell, 2 cells or multiple cell aggregations were counted as single particle. The experiments were performed 3 times.

Statistical analysis

All *in vitro* experiments were done in triplicate, and the experiments were repeated at least thrice. The intensities of the mRNA bands were normalized to the GAPDH bands and quantified by comparing with those of control cells. Data were expressed as means \pm standard deviations. The difference between control and SIE-treated cells was evaluated using Student's t test. The p value <0.05 was considered statistically significant.

Results

Inhibition of cell proliferation

The anti-proliferative activity of the SIE on the SK-Hep1 cells was evaluated using CCK-8 cell proliferation assay kit. The result showed that SIE exerts dose- and time-dependent anti-proliferative effects against SK-Hep1 cells (Figure 1). This data also revealed that SIE has a strong anti-proliferative effect on SK-Hep1 cells in certain concentration (IC₅₀=172.95 μ g/mL).

Adhesion of SK-Hep1 cells in the presence of SIE

Since adhesion of tumor cells to endothelial cells is mediated by ECM (extracellular matrix) proteins, we investigated the adhesion of SK-Hep1 cells to gelatin coated surfaces either in the presence or absence of SIE. Relative adherence was measured by setting the number of adherent cells at 6h to 100%. The result show that SIE reduces the adhesion of SK-Hep1 cells on gelatin coated

surfaces. The adhesion of SK-Hep1 cells on gelatin was reduced by 71.3 \pm 4.5% (200 μ g/mL of SIE) and 83.7 \pm 2.9% (400 μ g/mL of SIE) at 6 hours of incubation (Figure 2).

SIE decreases motility of SK-Hep1 cells

Cell motility is a critical metastatic event that occurs in various epithelial cells during cancer progression. In wound closure assay, the figure showed that migration of SK-Hep1 was clearly inhibited by the treatment of 200 μ g/mL SIE (Figure 2A). The wound sizes between the wound line (grey) were compared between SIE treated and untreated cells. As shown in Figure 2B, the motility of SK-Hep1 cells was inhibited by 70.3 \pm 3.97% (200 μ g/mL of SIE) at 48h.

SIE inhibits cell aggregation of SK-Hep1 cells

SIE at a concentration of 400 μ g/mL significantly inhibited the colony forming ability (aggregation) of SK-Hep1 cells (Figure 4) compared to untreated control

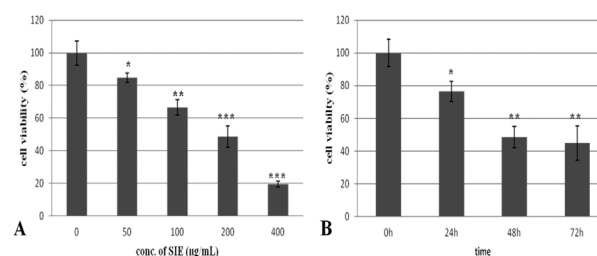


Figure 1. Effect of SIE on Viability of SK-Hep1 Cells. A) Proliferation of increasing dose of SIE on SK-Hep1 cells incubated for 24 hours; B) proliferation inhibition of 100 μ g/mL SIE on SK-Hep1 cells incubated at different time intervals. Data represents the means \pm SD (n=4), *(p<0.05); ***(p<0.001) Student's t test

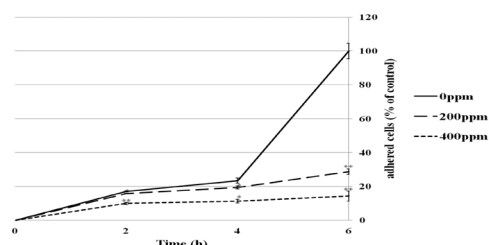


Figure 2. The Effect of SIE on the Adhesion of SK-Hep1 Cells on Gelatin Coated Surfaces. Each point represents the mean \pm SD of at least 3 values; *p<0.01; **p<0.001

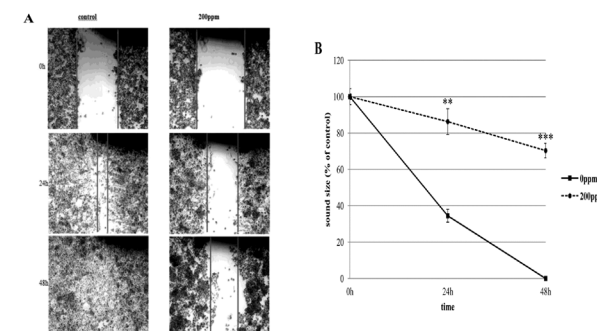


Figure 3. The Effect of SIE on SK-Hep1 Cell Motility. A) Motility of SK-Hep1 cells by wound closure assay. Representative pictures from three experiments are shown; B) The wound sizes were measured at the indicated time points. Results are presented as mean \pm SD of 3 independent experiments. **p<0.01; ***p<0.001

group. Total particle numbers in control group at 24h after treatment was 11.37 ± 2.35 and was reduced in the treated group ($400 \mu\text{g/mL}$) at 94.7 ± 7.03 . This implies that SIE inhibits colony formation of SK-Hep1 cells. Lower concentrations of SIE (0 - $200 \mu\text{g/mL}$) did not have any significant effect on colony forming ability of SK-Hep1 cells in later time periods (24 - 48h). The results demonstrate that the SIE has anti-aggregation activity against liver cancer cells.

Transcriptional level and activity of MMP-2 and MMP-9

Analysis of mRNA, reverse transcription-PCR and quantitative real time assays evaluated the inhibitory effects of SIE on MMP-2 and MMP-9 expression in SK-Hep1 cells. MMP-2 and MMP-9 expression levels decreased considerably in a dose-dependent manner after treatment with various concentrations of SIE (Figure 5). These results show that SIE regulates the expression of MMP-2 and MMP-9.

Metastasis related gene expressions

Since SIE down-regulated metastasis mediator genes

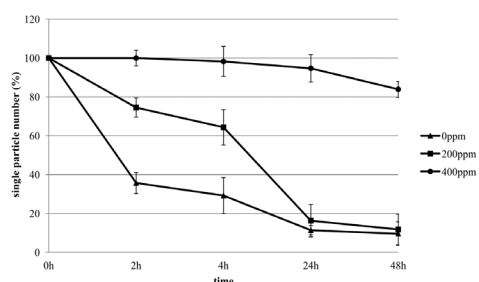


Figure 4. Effect of SIE on Aggregation of SK-Hep1 Cells. SK-Hep1 cells were treated with 200 - $400 \mu\text{g/mL}$ of SIE for 0 - 48 hours. Adhesion was assessed at 0 , 2 , 4 , 24 and 48h after treatment as described in Methods. Each bar represents the mean \pm SD of 8 experiments

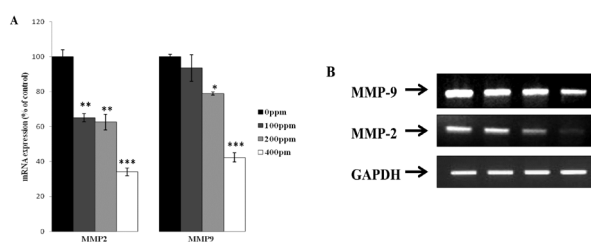


Figure 5. Age-specific Suppression of MMP-2 and MMP-9 Expression at the Transcriptional Level. A) quantitative real-time PCR; and B) Reverse Transcription-PCR to analyze the mRNA expression of MMP-2 and MMP-9. The values represented the means \pm SD of at least 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

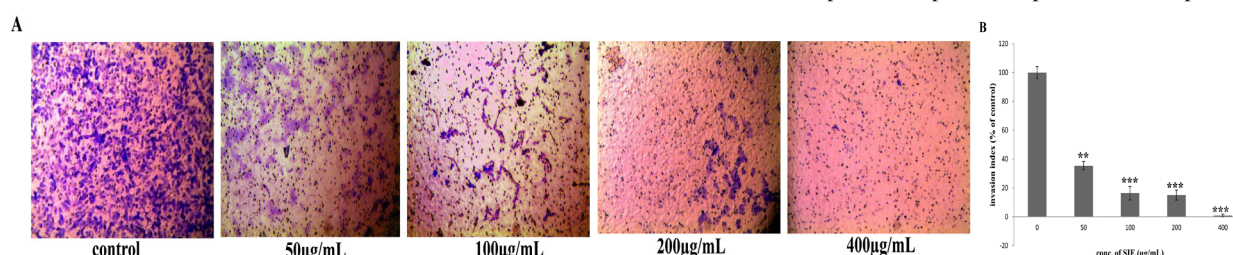


Figure 7. A) The invaded cells were fixed and then stained. Representative pictures from three experiments are shown; and B) The invasive activity was determined by counting invaded cells in three filters. The data are represented as the means \pm SD ($n=3$). ** $p < 0.01$; *** $p < 0.001$ compared with untreated cells

MMP-2/-9, we also examined the effect of SIE on the expression of inhibitor and activator genes of MMPs. Among them, our results show that SK-Hep1 cells treated with 200 - $400 \mu\text{g/mL}$ SIE reciprocally up-regulated the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) by 84.43% ($200 \mu\text{g/mL}$) and 107.08% ($400 \mu\text{g/mL}$) (Figure 6) and suppressed the membrane type-1-MMP (MT1-MMP) expression to 14.62% compared to the levels of expression in untreated control group.

Inhibition of invasion by SIE

As shown in Figure 7, the total number of cells that invaded to the underside of the filters were significantly decreased to $35.4\% \pm 2.87$ ($50 \mu\text{g/mL}$); $16.3\% \pm 4.65$ ($100 \mu\text{g/mL}$); $15\% \pm 3.43$ ($200 \mu\text{g/mL}$) and $0.84\% \pm 0.96$ ($400 \mu\text{g/mL}$) by SIE treatment, in a dose-dependent manner. In $400 \mu\text{g/mL}$ treatment group, the invasion was totally inhibited by SIE. These results show that SIE has strong anti-invasive effect in SK-Hep1 cells.

Discussion

Hepatocellular carcinoma (HCC) is a common malignant neoplasm and cause of cancer-related death in Asian countries. The mortality rate of HCC has not decreased, mainly because of the difficulty of treatment related to invasion and metastasis (Weng et al., 2008). Metastasis is therefore a multistep process involving numerous tumor cell-host interactions. These interactions are defined by the invasive phenotype that can be separated into three steps: attachment, local proteolysis, and migration (Stetler-Stevenson et al., 1993).

Generally, metastasis of cancer cells involves

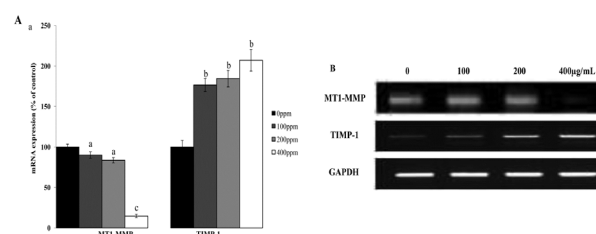


Figure 6. The Effect of SIE on mRNA Expressions of Metastasis Related Genes of SK-Hep1 Cells. A) The histogram shows that there were significant increase of TIMP-1 and decrease of MT1-MMP mRNA expressions in SIE treated cells; and B) Gel images of MT1-MMP, TIMP-1 and GAPDH mRNA. The expression levels of MT1-MMP and TIMP-1/GAPDH mRNA of treated group were compared with those of the control, ($p < 0.01$), Student's t test. Results are mean values \pm SD of independent experiments performed in triplicate

multiple processes and various cytophysiological changes, including altered adhesive capability between cells and the extracellular matrix (ECM) and damaged intercellular interaction. Degradation of ECM by cancer cells through proteases such as serine proteinase, matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator (PA) may lead to the separation of the intercellular matrix to promote cancer cell mobility, and may eventually lead to metastasis (Yeh et al., 2013). Among the involved proteases, MMP-2/-9 are most vital for the degradation of base membranes, and are therefore deeply involved in cancer invasion and metastasis (Yang et al., 2010; Gialeli et al., 2011).

In cancer research, one of the most active fields is the development of novel antimetastatic drugs with low toxicity and high efficacy. Chemoprevention involves the use of natural or synthetic chemicals to block, reverse, and suppress the process of carcinogenesis but still efficacy and safety remains a primary concern as toxicity and other side effects of chemotherapy are severe (Kelloff et al., 1994; Tan et al., 2011). Because of this, the quest and interest in the identification of medicinal plants and derived natural products for developing cancer therapeutics increased steadily (Dai et al., 2011). *Saussurea involucrata* extract (SIE) has several therapeutic effects on cancer cells, including inhibition of cell proliferation, cell-cycle blockage and induction of cell apoptosis. However, no studies on anti-invasion and anti-metastasis in hepatoma exist, and the mechanisms of SIE remain unclear. Thus, we designed this study to verify whether SIE has anti-metastatic effects in hepatocarcinoma.

In this study, we explored the anti-metastatic effect of *Saussurea involucrata* in human hepatocarcinoma SK-Hep1 cells. SIE has strong cytotoxic activity on SK-Hep1 cells, and its IC_{50} was 172.95 μ g/mL. Since migration is a critical event in cancer progression and especially in metastasis, the inhibitory effect of SIE on cell migration was evaluated. We used the wound healing assay *in vitro* to observe the effect of SIE on cellular migration. SIE potently retarded the migration of cells towards the wounded area. The results of this study demonstrated that SIE inhibited cell migration which is vital during the early phase of wound healing. Moreover, SIE significantly inhibited the invasion (assessed using the transwell assay) of SK-Hep1 cells at the concentration of 50 μ g/mL to 35.4% \pm 2.87. In this study, using adhesion, wound healing, and invasion assays, we have shown that SIE effectively inhibits the metastasis of tumor cells *in vitro*.

To further explore the mechanisms underlying the antimetastatic effect of SIE, we detected the alteration in levels of several genes by lysing SK-Hep1 cells after they had been incubated in the presence or absence of SIE. In the present study, the SIE was analyzed for the effect on MMP-2/-9 expressions. SIE showed a significant inhibitory effect on the expression of MMP-2/-9 dose-dependently. There is increasing evidence that a specific inhibition of MMP-9 activity might prevent metastasis. Yeh et al. (2012) concluded that MMP-9 is the most important protease in metastasis of human hepatoma. Two of the three central steps of the metastatic cascade, cell adhesion and cell migration were inhibited by MMP-9-

PEX in highly metastatic SW620 cells (Burg-Roderfeld et al., 2007). Especially MMP-9 expression correlated with local tumor growth, invasion, and intrahepatic metastasis, e.g. of hepatocellular carcinomas (Sato et al., 1994; Liu et al., 2011). MMPs gene expression are primarily regulated at the transcriptional and posttranscriptional levels and at the protein level through their activators and inhibitors and cell surface localization (Aguirre Ghiso et al., 1999; Yang et al., 2010). MMPs are subject to inhibition by endogenous tissue inhibitors of metalloproteinases (TIMPs). TIMPs are believed to play important roles in the inhibition of growth and migration, especially in hepatocellular carcinomas (Hwang and Lee, 2006). SK-Hep1 cells treated with SIE reciprocally up-regulated tissue inhibitor of metalloproteinase-1 (TIMP-1) gene expression.

To exert its enzymatic activity, the zymogen of MMP-2 requires cleavage and activation by membrane type MMPs, including MT1-MMP (Sato et al., 1994). MT1-MMP is one of the key enzymes among MMPs, and its over expression seems to have a major effect on tumor growth (Maatta et al., 2000). MT1-MMP is responsible for the activation of MMP-2 (Ogata et al., 1999; Tam et al., 2004). We further demonstrated that SIE notably inhibited the mRNA expressions of MMP-2, MMP-9 and MT1-MMP with real time PCR. These results indicated that antimetastatic effect of SIE was related to the inhibition of enzymatically degradative processes of tumor metastasis. Gene expression of MT1-MMP is reported to decrease in confluent cultures of mouse mammary gland epithelial cells (Tanaka et al., 1997), suggesting the regulation of MT1-MMP expression by cell-cell contact in normal epithelial cells. Therefore, the cell colony formation assay was carried out; then we arrived with the result that SIE caused a large reduction in cell aggregation. The reduction in aggregation may be associated with the significant decreases in migration and invasion by SIE treatment.

To our knowledge, this is the first demonstration of the anti-metastatic effect of SIE on HCC. In conclusion, this study showed that SIE exerts an inhibitory effect on several essential steps of metastasis, including cell adhesion, colony formation, invasion and migration, by regulating the activities of metastasis-associated proteases and their natural inhibitors and activators. The results show that SIE may be a powerful candidate for developing preventive agents against cancer metastasis. The present study suggests that *Saussurea involucrata* may be developed into a promising agent for cancer therapy.

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