The Role of E-Cadherin/β-Catenin in Hydroxysafflor Yellow A Inhibiting Adhesion, Invasion, Migration and Lung Metastasis of Hepatoma Cells

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Liver cancer is the second leading cause of cancer death. Due to treatments failures from drug resistance and cancer metastasis, discovering more effective treatments is imperative. As an angiogenesis inhibitor extracted from the Chinese herb-Safflower, hydroxysafflor yellow A (HSYA) inhibits the tumor growth in H22-bearing mice. Poorly differentiated hepatoma cells showed the ability to invade and metastasize, which are dependent on the angiogenesis. Accordingly, we hypothesized that HSYA could inhibit the metastasis of liver cancer cells. We investigated the metastasizing potential of human hepatic carcinoma SMMC-7721 cells treated with HSYA. A pulmonary metastatic model of mouse hepatoma H22 cells was established to evaluate the effect and possible mechanism of HSYA on lung metastasis from liver cancer. The results showed that HSYA inhibited the proliferation, invasion and migration of SMMC-7721 cells and reduced its adhesion to the extracellular matrix (ECM). In H22 mice treated with HSYA, the formation of E-cadherin/β-catenin complex resulted in the activation of peroxisome proliferator-activated receptor γ and inhibition of matrix metalloproteinase-2. As a result, the degradation of ECM was reduced and epithelial-mesenchymal transition was prevented. The present findings indicate that HSYA can prevent pulmonary metastasis in liver cancer, which provides strong evidence for the application of HSYA in treatments.

Key words  hydroxysafflor yellow A; E-cadherin; β-catenin; lung metastasis; hepatoma cell

Liver cancer is one of the most prevalent malignant diseases and the second leading cause of cancer death. In the past several years, morbidity of liver cancer has been increasing.¹ Even though current therapies for liver cancer such as surgery and chemotherapy could result in clinical improvements, the long-term effects of these treatments are still unsatisfactory because of high malignancy, fast growth, early metastasis, and high risk of recurrence.²,³ Due to drug resistance and incomplete surgical excision, metastasis and recurrence of liver cancer often occurs and presents a major obstacle in the successful treatment of liver cancer.⁴,⁵ Liver cancer metastasis can be intrahepatic and extrahepatic,⁶ which up to 43.4% of extrahepatic metastatic sites occur in the lungs.⁷ Therefore, the development of drugs which prevent liver cancer from metastasizing is one of major directions for liver cancer treatments. Traditional Chinese Medicine (TCM) has been applied clinically for cancer treatments for thousands of years in China. More and more medical researchers have demonstrated that TCM has a definite therapeutic effect on cancerous tumors with unique advantage.⁸–¹⁰

In patients with advanced tumors and systemic metastasis, chemotherapy treatment combined with TCM has been shown greater therapeutic effects and less toxicity. Moreover, compared to other drugs used to treat cancers, TCM may result in fewer and milder side effects.¹¹ Therefore, TCM could be important in the development of well tolerated and effective anti-metastatic cancer drugs.

According to the angiogenesis theory of Folkman,¹² the growth of all solid tumors is dependent on angiogenesis and neovascularization. In recent years, the inhibition of angiogenesis has become a focus in blocking the growth and metastasis of cancer.¹³ By using chick chorioallantoic membrane,¹⁴ we discovered an angiogenesis inhibitor, hydroxysafflor yellow A (HSYA), which the chemical structure showed in Fig. 1.¹⁵ HSYA was extracted from the Chinese herb-safflower, which was traditionally used in activating blood circulation and dissipating blood stasis. Safflower is also used to treat tumor patients with metastasis as patients often experience platelet aggregation and blood hyper-viscosity. Furthermore, we found that HSYA could inhibit the proliferation of abnormal human umbilical vein endothelial cells (HUVEC) induced by culture supernatant of human hepatic carcinoma HepG2 cells without affecting normally cultured HUVEC.¹⁶ HSYA also inhibited tumor growth and angiogenesis in nude mice bearing human gastric adenocarcinoma BGC-823 cells.¹⁷,¹⁸ Thus, these results indicate that HSYA potentially acts as a tumor angiogenesis inhibitor.

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inhibitor, may exert anti-tumor growth effects.

It is well known that liver cancer is a typically angiogenesic tumor. Generally, some of poorly differentiated hepatocellular carcinoma cells (HCC) were found in arterial hyper-vascularity. This kind of HCC has the ability to invade and metastasize, the process depends upon angiogenesis. It has been reported that HSYA can prevent tumor growth in H22-bearing mice through its ability to inhibit angiogenesis. Thus, we hypothesize here that HSYA can inhibit metastasis of liver cancer cells.

The process of liver cancer metastasis consists of several key steps, including basement membrane degradation, epithelial–mesenchymal transition (EMT), tumor migration and invasion (Fig. 2). These steps are modulated by cell adhesion molecules (CAM) and signaling pathways (Fig. 2). The adhesion and invasion mediated by E-cadherin, a key CAM, is due to the formation of a complex with the intracellular catenins, E-cadherin/β-catenin/α-catenin. This complex directly enables the actin cytoskeleton to sustain the intercellular adherent junctions. The decrease in E-cadherin expression in liver cancer cells can destroy the intercellular adhesion and extracellular matrix (ECM). EMT, which is characteristic of low E-cadherin expression, occurs with the induction of the tumorigenesis, invasion and metastasis. In the cytoplasm and nucleus of less-differentiated cancer tissues, β-catenin expression is significantly increased, while cell membrane β-catenin expression is decreased. The entry of β-catenin into the nucleus activates matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), while inhibiting peroxisome proliferator-activated receptor γ (PPARγ). MMP-2 and MMP-9 degrade the extracellular matrix and promote the cell migration. Furthermore, adhesion molecular E-cadherin/catenins, MMP-2, and angiogenesis factors could be together applied to the normal diagnosis and the prognostic assessment of liver cancer as the tumor metastatic markers. Our previous study showed that HSYA could inhibit the expression of MMP-9 in transplanted tumor tissue. Therefore, we also hypothesize that HSYA may inhibit liver cancer metastasis by preventing the degradation of basement membrane and EMT of hepatic cancer cells.

To demonstrate these two hypotheses, in the present study we evaluated the effect of HSYA on metastasis of hepatic cancer in vivo and in vitro, and explored its mechanism at cellular and molecular levels. Firstly, the effect of HSYA on the metastasizing potential of human hepatic carcinoma SMMC-7721 cells was investigated in vitro through cell adhesion, invasion and wound healing experiments. Secondly, the effect and mechanism of HSYA in a pulmonary metastatic model of liver cancer was evaluated through hematoxylin and eosin stain. The expressions of E-cadherin, β-catenin, MMP2 and PPARγ in metastatic pulmonary tissue were also measured to examine HSYA therapeutic mechanisms.

**MATERIALS AND METHODS**

**Animals** Forty eight male KM mice weighing 20±2g were supplied by Si Pei Fu Laboratory animal technology Co., Ltd. (Beijing, China, Certificate No. SCXK [Jing] 2011-0004). Mice were allowed to access food and water freely, and housed in a constant temperature (22±1°) and humidity (65±5%) environment under a 12h light/dark cycle. The mice were randomly divided into 6 groups (n=8): control group; liver cancer lung metastasis model group; model groups with 5-fluorouracil (5-FU); model groups treated with 0.57mg/kg...
HSYA, 1.13 mg/kg HSYA, or 2.25 mg/kg HSYA.

To establish a mouse liver cancer lung metastasis model, KM mice were injected H22 cells through the vena caudal. Controls were injected with saline. From the first day of establishing the lung metastasis model, treated groups of mice were given 100 µL intraperitoneally with 0.57 mg/kg, 1.13 mg/kg, or 2.25 mg/kg HSYA (National Institutes for Food and Drug Control, Beijing, China) twice per day. The mice in the 5-FU group were given 100 µL intraperitoneal injections of 20 mg/kg 5-FU (Sigma-Aldrich, MO, U.S.A.) every other day. Control mice and model mice were given 100 µL intraperitoneal injections of saline twice per day. All of the experiment procedures involving animal maintenance and treatments were carried out according to the guidelines Principles of Laboratory Animal Care established by National Institutes of Health, China. The experiment protocol was approved by the institutional animal ethics committee of Beijing University of Chinese Medicine, China.

Cell Proliferation Human hepatic carcinoma SMMC-7721 cells (Chinese Academy of Sciences, Beijing, China) were cultured at 37°C and 5% CO2 in a humidified atmosphere in RPMI-1640 medium (BD, New Jersey, NJ, U.S.A.), supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Hangzhou, China). Cells were seeded in 96-well culture plates at a density of 3×103 well. Twenty four hours later, the cells were treated with either saline or HSYA. After 24 or 48 h of incubation, 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco, Solon, OH, U.S.A.) was added to each well and the cells were incubated for 4 h at 37°C. The medium in each well was then replaced with 150 µL of dimethyl sulfoxide (DMSO). Rate of cell proliferation was detected via optical density (OD) using a microplate reader at 570 nm.

Cell Adhesion Ninety-six-well culture plates were coated with 50 µL of Matrigel for 30 min at 37°C, then SMMC-7721 cells (5×104/well) treated with saline or HSYA were seeded into the 96-well plates. Seeded cells were further treated with 100 µL of saline or of their respective concentrations of HSYA. The plates were incubated at 37°C and 5% CO2 for 1 or 2 h, and no-adherent SMMC-7721 cells were washed away with phosphate-buffered saline (PBS). The adhesive potential were measured via OD values using the MTT assay as explained above.

Cell Invasion A total of 30 µL of 2 mg/mL Matrigel was added to the top chamber of each well in a Transwell plate (8.0 µm pore size) (Coring Costar, NY, U.S.A.). The plates were left at 37°C for 30 min, allowing the Matrigel to solidify. SMMC-7721 cells (3×104 cells/100 µL) were seeded into the upper chamber and 100 µL of saline or HSYA were added. The bottom well was filled with 600 µL RPMI-1640 medium (supplemented with 10% fetal bovine serum). After incubation at 37°C for 24 h, the top chamber was removed and the membranes was fixed with ethanol for 20 min. Non-migrated cells on the membranes were removed using cotton swabs and migrated cells were stained with Giemsa for 15 min. Images of 9 random fields (20×objective) for each membrane were captured, and the cell counts for all 9 fields of each membrane were averaged to give a mean cell count.

Wound-Healing SMMC-7721 cells were plated into 24-well plates with a density of 2×104 cells in each well. After the cells incubated at 37°C, 5% CO2 for 6 h, the plates were then scratched linearly in multiple areas with a 20 µL micropipette tip. The detached cells were washed with PBS and cultured by using a new medium with their respective concentration of HSYA. The same wound margins were photographed by phase contrast microscopy at 0, 12, and 24 h, respectively. Then the wound healing rate was calculated according to the following formula: (the average width of wound in 0 h – the average width of wound in 12 or 24 h)/the average width of wound in 0 h.

H22 Cells-Induced Lung Metastasis Model and Treatment Mouse hepatoma H22 cells (Beijing Cancer Hospital, Beijing, China) were taken out from liquid nitrogen. The cells were centrifuged and the supernatant was discarded. The cells were diluted with normal saline and injected into abdominal cavity to proliferate. When the mouse abdomen was bulged (about 4–5 d), the liver ascites were extracted and diluted with normal saline. 8.0×105 cells were injected into KM mice by vena caudal to establish a mouse liver cancer lung metastasis model. The control group received the same volume of saline by tail intravenous injection.

Sampling Mice inoculated with H22 cells were treated with HSYA for 11 d and body weight was measured every second day. On the 12th days, mice were sacrificed and the lung tissues were taken out. The left lobe of each lung tissue was fixed in 4% paraformaldehyde while the remaining lung tissue was preserved in liquid nitrogen for subsequent experiments.

Immunohistochemistry The fixed lung tissues were paraffin-embedded and serial sections at 6 µm were prepared and stained with hematoxylin/eosin. The stained tissues were observed and imaged using an optical microscope. Antigen retrieval was performed by boiling the slides in citrate buffer (pH 6.0) for 30 min. The slides were incubated with 3% H2O2 for 10 min, followed by incubation with primary antibody against β-catenin (rabbit anti-mouse) in 1:50 dilution overnight at 4°C. Then a poly-HRP anti-Rabbit immunoglobulin G (IgG) was applied and signals were visualized with 3,3’-diaminobenzidine (DAB). The slides were photographed under a light microscope.

Western Blot Lung tissue was homogenized in RIPA lysis buffer containing a protease inhibitor. The total proteins (8 µg/lane) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride (PVDF) membranes through semi-dry transfer. The membranes were blocked for 2 h with 5% nonfat-dried milk in Tris buffered saline with Tween 20 (TBST) and incubated overnight at 4°C in primary antibodies (E-cadherin diluted at 1:200, 98 kDa; β-catenin diluted at 1:200, 88 kDa; PPARγ diluted at 1:400, 57 kDa; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) diluted at 1:400, 37 kDa) (Wuhan Boster Biological Technology, Wuhan, China). The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Labeled proteins were visualized with ECL detection reagents and exposed to Kodak X-OMAT film. Band intensities were measured by densitometry and densities of E-cadherin, β-catenin, PPARγ were normalized to GAPDH as an internal control.

Gelatin Zymography The total proteins were lysed with RIPA lysis buffer. Protein concentrations were analyzed by
the BCA kit. A gelatin zymography kit (Shanghai Genmed Gene Medicine Technology, Shanghai, China) was used to detect the MMP2 activity. MMP2 activity was identified as unstained bands against the background of Coomassie blue-stained gelatin.

**Real-Time RT PCR** The total RNA was isolated with Trizol reagent, and the integrity of RNA was examined by agarose gel electrophoresis. Three micrograms of RNA was reverse transcribed using M-MLV at 42°C (Promega, Wisconsin, U.S.A.). Real-time quantitative PCR was based on SYBR Green (BIO-RAD, CA, U.S.A.), and GAPDH was used as an internal reference gene. The specific oligonucleotide primers as follows: mouse E-cadherin [BC098501]: sense, 5′-GCTCTACTGTCTTACGG-3′, antisense, 5′-TCTTCTCCA CCTCTCTCTT-3′; mouse PPARγ [U01664]: sense, 5′-TCTGCAAGTATGTTCTC-3′, antisense, 5′-TAGCAGTCTGACCATCAT-3′; GAPDH [NM_008084.3]: sense: 5′-CTTCTATGTA CACAACCTC-3′, antisense: 5′-TAGACTATGACGACAT-3′. The PCR amplification conditions as follows: denaturation at 95°C for 3 min; followed by 40 cycles of 95°C for 15 s, 55 (PPARγ and GAPDH) or 59°C (E-cadherin) for 10 s, and 72°C for 20 s. Amplification and data acquisition were performed using BIO-RAD CFX system. The relative quantity of gene expression of the sample was calculated by dividing the target gene expression level in each sample by that of the internal reference gene.

**Statistical Analysis** All results were expressed as mean±standard deviation (S.D.). The results of cell proliferation and cell adhesive assay were analyzed by one-way ANOVA and results of cell invasion and wound-healing assay were analyzed by Independent Student’s t-tests. For the analysis of mice body weights and the expression of proteins and mRNAs, Independent Student’s t-test was applied between control group and tumor group. The difference between model group and tumor treated groups, such as 5-FU group and three HSYA groups, was assessed by one-way ANOVA followed by post hoc Student–Newman–Keuls multiple comparison analysis. A probability value of p<0.05 was considered to be significant.

**RESULTS**

**HSYA Inhibited the Viability of Human Hepatic Carcinoma SMMC-7721 Cells** In this study, we first investigated the effects of HSYA on the proliferative rate of SMMC-7721 cells. MTT assay showed that the growth of SMMC-7721 cells was suppressed after treating with 0.375, 0.75, 1.5, 3.0, 6, 12 and 24 µM HSYA for 24 h ($F_{6,48}=49.325$, $p<0.001$). After SMMC-7721 cells were treated with HSYA for 48 h, 0.75, 1.5, 3.0, 6, 12 µM of HSYA significantly inhibited the growth of SMMC-7721 cells ($F_{6,48}=38.515$, $p<0.001$) (Fig. 3A), while 24 µM of HSYA had no effect.

**HSYA Inhibited the Invasion and Migration of SMMC-7721 Cells and Its Adhesion to the Extracellular Matrix** In order to simulate the metastatic process of hepatic carcinoma cells in the body, Matrigel matrix was used as the substitute for the extracellular matrix. Matrigel matrix consists of laminin, type IV collagen and other extracellular matrix components and closely resembles the complex extracellular environment. The results showed that SMMC-7721 cells treated with HSYA for 1 h had significantly decreased adhesive capacity to the Matrigel matrix compared to the saline group ($F_{4,20}=6.935$, $p=0.001$) (Fig. 3B). In the SMMC-7721 cells treated with HSYA for 2 h, only 3.0 µM and 4.5 µM of HSYA significantly reduced the adhesive capacity compared to saline-treated cells ($F_{4,20}=3.080$, $p=0.040$) (Fig. 3B).

The result from the cell invasion assay showed that 1.5 µM of HSYA significantly reduced the invasion of SMMC-7721 cells when compared to the cells treated with saline ($t=2.281$, $p=0.027$) (Fig. 4). Furthermore, results from the wound-healing assay showed that the SMMC-7721 cells treated with saline exhibit spread morphology by 12 and 24 h of plating. In contrast, HSYA was able to significantly reduce SMMC-7721 cell migration when compared to the cells with saline treatment at 12 h ($t=2.625$, $p=0.028$) (Fig. 5).

**HSYA Inhibited the Pulmonary Metastasis Induced by H22 Liver Cancer** To investigate the effect of HSYA on the metastasis of hepatic cancer in vivo, the pulmonary metastatic...
model of liver cancer was established. During the course of the experiment, the weights of mice in all groups increased, with the greatest gain noted in the control group (Fig. 6A). The weights of mice between the pulmonary metastatic model group and the three HSYA-treated groups were not statistically significant. However, mice treated with 5-FU weighed significantly less than both the model mice without treatment and mice treated with HSYA.

In control mice, the pulmonary alveoli were intact without any signs of hyperplasia (Fig. 6B). In contrast, in pulmonary metastatic mice, significant tumor growth was observed, resulting in the formation of a tumor thrombus. Further, the liver sinusoid consisted of hepatocyte-like cells that were consistent with the morphology observed in liver cancer. In addition, mono bi- and multi-nucleated tumor cells were observed.

Zero point fifty-seven microgram/kilogram HSYA significantly reversed the pathological feature in the lungs of metastatic mice, as the lung tissues showed smaller and fewer solid tumors and less prominent hyperplasia. Although mice treated with 1.13 mg/kg HSYA and 2.25 mg/kg HSYA showed a solid tumor in the lungs, mice treated with 1.13 mg/kg HSYA retained intact pulmonary alveoli with less obvious hyperplasia and significantly smaller solid tumor than the pulmonary metastatic mice. While there were no solid tumors in the pulmonary metastatic mice treated with 5-FU, the pulmonary alveoli were damaged and severe hyperplasia was observed (Fig. 6B). As shown in Fig. 6C, there were many white spots in the gross photos of lung tissues except for the control mice. In addition, the pulmonary white spots of model group were much more and bigger than control and treated groups. However, the pulmonary metastatic mice treated with 0.57 mg/kg HSYA had little and smaller white spots in their lungs (Fig. 6C).

**HSYA Induced the Expression of E-Cadherin and Inhibited the Entry of β-Catenin to Nucleus in Lung Tissues of Liver Cancer Mice**

Initial processes of metastasis involve the degradation of ECM, followed by the migration and invasion of mesenchymal cells. The migratory and invasive properties have been attributed to the modulation of multiple cell adhesion molecules such as E-cadherin and β-catenin, and their downstream molecules, including matrix metalloproteinase. Hence, we examined the expression of E-cadherin and the activity of matrix metalloproteinase 2 (MMP 2) in the metastatic site (lung tissues) of mice with induced liver cancer. The expression of E-cadherin at protein and mRNA levels were significantly decreased in the model group compared to the control group (for protein: \( t=8.666, p=0.001 \); for mRNA: \( r=3.687, p=0.006 \)). When mice were administered with 0.57 mg/kg HSYA and 1.13 mg/kg HSYA, the expres-
Fig. 6. HSYA Inhibited the Pulmonary Metastasis in Mice with H22 Liver Cancer

(A) The weight change curve of pulmonary metastatic mice over period of the treatment. The mice were weighted every other day for 11 d. Values represent means±S.D.; \( *p<0.05 \) versus tumor group; \( #p<0.05 \) versus pulmonary metastatic mice treated by 5-FU. (B) Histological examination of lung tissue was performed after the pulmonary metastatic mice were treated with saline, 5-FU or HSYA for 11 d. Lung tissues were stained with hematoxylin/eosin solution (magnification ×200). (C) Representative lung samples from pulmonary metastatic mouse sacrificed 11 d after normal saline, 5-FU and HSYA treatment. a: control group; b: tumor group; c: tumor + 5-FU; d: tumor + 0.57 mg/kg HSYA; e: tumor + 1.13 mg/kg HSYA; f: tumor + 2.25 mg/kg HSYA.
pression of E-cadherin in lung tissues was regained (for protein: $F_{2,10}=6.643$, $p=0.007$; for mRNA: $F_{2,17}=6.631$, $p=0.002$) (Figs. 7A, B).

As mentioned above, E-cadherin can form a complex with β-catenin and α-catenin to commonly maintain the intracellular adhesion and cell polarity (D’Souza-Schorey C 2005). In the present study, the Western blot results showed that there was no difference in the total protein expression of β-catenin among all of the groups ($t=-1.984$, $p=0.118$; $F_{2,10}=2.533$, $p=0.106$) (Fig. 7A). However, the expression of β-catenin was significantly decreased in the plasma membrane and increased in the cytoplasm of lung tissues of mice with liver cancer. This pattern of β-catenin expression was reversed by 0.57 mg/kg HSYA or 1.13 mg/kg HSYA (Fig. 7C).

It is also well known that a lower expression of E-cadherin and higher activity of MMP2 occur when tumor cells invade,25,31 These changes destroy the stability between the tumor cells and the extracellular matrix by activating transcription factors, such as PPARγ. PPARγ is a nuclear transcription factor important in cell apoptosis, angiogenesis and metastasis and serves as a major barrier to tumor cells during metastasis. The expression of PPARγ were decreased in lung tissues of liver cancer mice when compared to control mice (for protein: $t=11.951$, $p<0.001$; for mRNA: $t=4.897$, $p=0.001$) (Figs. 7A, B). When mice with liver cancer were treated with HSYA, the mRNA expression of PPARγ increased significantly, especially at relatively low doses of 0.57 mg/kg and 1.13 mg/kg HSYA ($F_{2,21}=12.843$, $p<0.001$). The protein expression of PPARγ also increased in mice treated with HSYA ($F_{2,10}=18.381$, $p<0.001$) (Figs. 7A, B).

The activity of MMP2 in the lung tissues increased significantly in the pulmonary metastatic mice with liver cancer when compared to control mice, which was observed by Gelatin zymography. Zero point fifty-seven, 1.13, and 2.25 mg/kg of HSYA reduced the activity of MMP-2 in the lung tissues of pulmonary metastatic mice with liver cancer (Fig. 8), which helped to protect the damage of the extracelluar matrix barrier.

DISCUSSION

As mentioned above, safflower was used to treat liver cancer in TCM.36) The effective component extracted from Safflower, HSYA, has been shown to have neuroprotective effects,37 as well as act antagonistically against ischemic brain damage38 and cardiovascular disease.39) Our previous studies, however, show that HSYA can have direct antitumor action on liver and gastric cancers through inhibiting tumor angiogenesis. In the present study, we have demonstrated, for the first time, that HSYA inhibited both the proliferation of SMMC-7721 cells and their adhesion to ECM. SMMC-7721 cells even treated with a lower dose of HSYA for a short duration of time already exhibited less invasion and migration. Furthermore, a lower dose of HSYA played a remarkable role in the resistance of pulmonary metastasis in H22 liver cancer through the formation of the E-cadherin/β-catenin complex. The E-cadherin/β-catenin complex increased the expression of E-cadherin and inhibited the entry of β-catenin into nuclear from cytoplasm. These changes were associated with activating the expression of PPARγ and inhibiting the activity of MMP-2, ultimately leading to a reduction in ECM degradation and prevention of pulmonary metastasis.

Patients with liver cancer exhibit blood stasis as a result of microcirculatory disturbances, which increased of blood viscosity, thrombi formation and blood agglutination.40) Therefore, in Chinese Medicine, increasing blood circulation and dissipating blood stasis are important therapies for treating primary liver cancer in the clinic. Intriguingly it was reported that safflower possessed the ability to inhibit the pulmonary metastasis of mice hepatoma cells,41 even when HSYA only accounted for 0.70–1.85% of the total content (2.25 mg/kg), which is a much lower dose than the dose used in the present study. Furthermore,42) the dose of HSYA we applied to mice in the present study was also reference to the previous experiments of HSYA treating transplanted human gastric adenocarcinoma BGC-823 mice.42) It stands to reason that the resistance of HSYA on pulmonary metastasis in mice liver cancer may be achieved at a low dose. Encouragingly, a low dose of HSYA was found to be efficacious when examining SMMC-7721 cell proliferation, adhesion, invasion and wound-healing assays. The results observed are potentially due to the fast metabolism of HSYA within the body43) or the special keto-enol tautomerism equilibrium the compound undergoes.44) One hour after HSYA treatment, the adhesive ability of SMMC-7721 cells to Matrigel matrix was significantly inhibited when compared to control. However, the inhibition decreased at 2h, which may be due to the Matrigel matrix increasing the adherence of SMMC-7721 cells to plate over time.

It is well known that chemotherapy drugs have terrible side effects. It is almost impossible to treat cancer without negatively impacting the function of targeted and no-targeted tissues and organs. A treatment for advanced hepatocellular carcinoma which metastasizes to the lungs can destroy normal pulmonary function.7) The present study demonstrated that HSYA could inhibit the pulmonary metastasis of H22 liver cancer with less damage to the lungs than chemotherapy drug 5-FU. Thus, HSYA is safer for treating the pulmonary metastasis.

More important, we revealed the key molecular mechanism of HSYA. HSYA was found to increase the expression of E-cadherin, and inhibit the entry of β-catenin to nucleus, while also inhibit the activity of MMP2 in the lung tissues of liver cancer mice. Previously we found that HSYA inhibited angiogenesis in BGC-823 transplanted tumor tissues and as well as the expression of vascular endothelial growth factor (VEGF) in endothelial cells with abnormal proliferation.16,25 Thus, our results indicated that the ability of HSYA to prevent pulmonary metastasis may be due to its actions on the EMT.

In this study, HSYA was also shown to promote the expression of PPARγ. As a nucleic transcription factor, PPARγ is regulated by the complex E-cadherin/β-catenin32) and is a promising therapeutic target46) for lung47) and for liver cancer.48) More evidence shows that when activated by its ligand, PPARγ could increase the apoptosis of tumor cells, and inhibit cell proliferation and tumor angiogenesis.49,50) Recently, it was reported that HSYA inhibited liver fibrosis induced by oxidative stress through activating PPARγ.51) All of these suggest that HSYA perhaps is an agonist of PPARγ. Nevertheless, further studies are required to confirm this indication.
Fig. 7. The Effect of HSYA on the Protein Expression and mRNA Level in Lung Tissues of Pulmonary Metastatic Mice

(A) The total protein expressions of E-cadherin, β-catenin and PPARγ were detected using Western blot, with GAPDH as the loading control. (B) The mRNA levels of E-cadherin and PPARγ in lung tissues of pulmonary metastatic mice, which were quantified by real-time reverse-transcription PCR (qRT-PCR). (C) Expression of β-catenin in the lung tissues of pulmonary metastatic mice were detected by immunohistochemistry. All sections were counterstained with hematoxylin (magnification ×200). Brown staining in the plasma membrane and extracellular tissue represents β-catenin expression. a: control group; b: tumor group; c: tumor +5-FU; d: tumor +0.57 mg/kg HSYA; e: tumor +1.13 mg/kg HSYA; f: tumor +2.25 mg/kg HSYA. *p<0.05, **p<0.01 versus model group.
CONCLUSION

In summary, the results suggest that HSYA may inhibit the growth and metastasis of liver cancer. A relatively low dose of HSYA can inhibit the proliferation, adhesion, invasion and migration of SMMC-7721 cells, and can also inhibit liver cancer from metastasizing to the lungs. The effect of HSYA may promote the E-cadherin expression by blocking the entry of β-catenin into nucleus. By up-regulating the expression of PPARγ and reducing the activity of MMP-2, HSYA may ultimately decrease the degradation of extracellular matrix and prevent EMT from occurring (Fig. 2). However, a high dose of HSYA promotes pulmonary metastasis of liver cancer,59 which may have the same mechanism with its clinical effect on coronary heart disease and stable angina. Obviously, the concrete mechanism needs to be explored in the future.

VEGF is a negative regulator in the invasion of tumor cells. The angiogenesis inhibitor Bevacizumab can induce tumor growth of liver cancer while still inhibiting the metastasis of hepatoma cells. More encouragement, HSYA had no effect on the normal cells.16 These outcomes are the ideal treatment for patients with glioblastoma to metastasize.

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Conflict of Interest The authors declare no conflict of interest.

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