Original Research

Scorpion inhibits epithelial–mesenchymal transition and metastasis of hepatocellular carcinoma

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Impact statement

The unfavorable clinical outcome and poor prognosis of hepatocellular carcinoma (HCC) are due to high rates of recurrence and metastasis after treatments. Here we found Scorpion, one of the most important wind calming drugs, has antitumor effect. Scorpion-medicated serum inhibited the proliferation, induced apoptosis, and decreased migration and invasion capacity of Hepa1-6 cells *in vitro*. Water decoction of Scorpion restrained tumor growth and metastasis in nude mouse of HCC metastasis models. Further experiments showed that Scorpion could suppress EMT of HCC both *in vitro* and *in vivo*. Our results suggested that the Scorpion could inhibit Hepa1-6 cells' invasion and metastasis in part by reversing EMT and providing a possible potential approach for preventing HCC metastasis.

Abstract

Hepatocellular carcinoma (HCC) is one of the most malignant diseases worldwide. The unfavorable clinical outcome and poor prognosis are due to high rates of recurrence and metastasis after treatments. Some scholars of traditional Chinese medicine suggested that endogenous wind-evil had played an important role in metastasis of malignant tumor. Therefore, the drug of dispelling wind-evil could be used to prevent cancer metastasis and improve the poor prognosis. So we wondered whether Scorpion, one of the most important wind calming drugs, has antitumor effect especially in epithelial–mesenchymal transition (EMT) and metastasis of HCC in this research. We found that Scorpion-medicated serum could inhibit proliferation, induce apoptosis, and decrease migration and invasion capacity of Hepa1-6 cells *in vitro*. Meanwhile, we observed that water decoction of Scorpion restrained tumor growth and metastasis in nude mouse of HCC metastasis models. Further experiments showed that Scorpion could suppress EMT, which is characterized by increased epithelial marker E-cadherin expression and decreased mesenchymal markers N-cadherin and Snail expression following Scorpion treatment both *in vitro* and *in vivo*. These results suggested that the Scorpion could inhibit Hepa1-6 cells' invasion and metastasis in part by reversing EMT and providing a possible potential approach for preventing HCC metastasis.

Keywords: Scorpion, epithelial–mesenchymal transition, metastasis, hepatocellular carcinoma

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Introduction

HCC is one of the most malignant diseases worldwide because of its aggressive tumor biology and lack of effective therapies.[1] Population-based research showed that it was also identified as the third leading cause of cancer-related death in China.[2] At present, surgical resection is still the standard treatment for most HCC patients, but incidence rates continue to increase rapidly for liver cancer and the survival ratio is still low. The unfavorable clinical outcome and poor prognosis are due to high rates of recurrence and metastasis after treatments.[3–5] Previous studies showed that epithelial–mesenchymal transition (EMT) was an important initial event in the process of cancer metastasis and can conduce to resistance to chemotherapy.[6–8] For this

reason, suppressing the EMT in liver cancer is significant and meaningful to the prevention and treatment of hepatocarcinoma.

Scorpion, the dry body of *Buthus martensii* Karsch, has been widely used in the treatment of various tumors in China long time ago.[9] It has a certain effect against liver cancer in the clinical treatment but its action mechanism remains enigmatic.[10–13] The "wind" is one of the six pathogenic factors in the theory of traditional Chinese medicine (TCM) and it is divided into endogenous wind and external wind. The diseases caused by endogenous wind are characterized as migrant and variable. In recent years, some scholars of TCM suggested that the metastasis of cancer is similar to the wind syndrome, which is described as progressing rapidly and changing frequently. Then they come up with hypotheses that endogenous wind-evil had played an important role in metastasis of malignant tumor for which being secret whirling and changeable, therefore, the drugs of dispelling wind-evil or stopping endogenous wind could be used to prevent cancer metastasis and improve the poor prognosis.[14] The effects of antitumor of wind-dispelling drugs in some clinical studies have been reported these years.[15,16] So we assumed that as one of the liver-pacifying wind-subduing drugs, Scorpion could affect hepatic malignant tumor growth and metastasis.

Most of the previous investigations about the effect of Scorpion on antitumor therapy were focused on the Scorpion venom and its peptide.[17–20] In this study we investigated the Scorpion water extraction, the major method of administration in clinics of Chinese Medicine, to determine its influence on HCC cells *in vitro* and *in vivo*, and explored the potential associated mechanisms. Our study focused on the role of Scorpion in EMT and metastasis of HCC.

Materials and methods

Ethics statements

All animal experimental procedures were conducted in conformity with the guidelines for the care and use of laboratory animals in Fourth Military Medical University.

Reagents

The Chinese herb Scorpion we used in this study was the dry body of *B. martensii* Karsch, which was purchased from EFong Pharmaceutical (Guangzhou, China). The Scorpion was decocted twice with distilled water for 1 h. The decoction was collected, filtered, merged, and concentrated to 1 g/mL (equivalent to crude herb materials) and stored at −20°C for future use.

Cell culture

HCC (Hepa1-6) cells and luciferase-expressing HCC (Hepa1-6-luc$^+$) cells were gifts from Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, China. All the cells were cultured in DMEM (HyClone, Logan, UT) containing 10% FBS (ZETA Life Inc., Menlo Park, CA) and maintained with 5% CO_2 in a humidified chamber at 37°C.

Preparation of Scorpion-medicated serum

Twenty male Sprague-Dawley rats (200 ± 20 g) were purchased from the Center of Animal Experiment of Fourth Military Medical University (license no. scxk2012-0007, Xi'an, China). Based on the preliminary experiments (data not shown), rats in Scorpion group were given administration by intragastrical gavage with Scorpion solution at 0.63 g/200 g d ($n = 10$) and rats in control group were given same volume of saline every day according to body surface area (0.63 mL/200 g d).[21] All rats were maintained with normal diet and blood samples were obtained from heart 1 h after the last administration at the third day.[22] The blood samples were stored at 4°C overnight, centrifuged at 3000 r/min for 15 min, and the supernatant was collected. The serum was inactivated at 56°C water bath for 30 min and sterilized with a 0.22 μm filter membrane and stored at −80°C. In this study, all the cells were cultured in DMEM containing 10% of the serum.

5-Ethynyl-2′-deoxyuridine (EdU) assay

The EdU assay kit (RiboBio, Guangzhou, China) was used to evaluate the cellular proliferative activity. The Hepa1-6 cells were cultured in 24-well plates with Scorpion-medicated serum and appropriate control serum, respectively, for 24 h. The cells were incubated after the addition of 200 μL 50 μM EdU at 37°C for 2 h and the cells were fixed in 4% paraformaldehyde for 30 min. After permeabilizing with 0.5% Triton X-100 for 10 min, 200 μL Apollo reaction liquid was added to the reaction for 30 min at room temperature. Two hundred microliters Hoechst 33342 was added in each well for 30 min to stain the cell nucleus. The images were then obtained under a fluorescence microscope (Olympus FV1000, Japan) at a 200× magnification, and the number of proliferative cells was calculated and analyzed in five fields selected randomly for each sample. Each sample was conducted in duplicate and the experiment was repeated three times.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

The TUNEL (Promega, USA) staining was used to detect the apoptotic Hepa1-6 cells. The experiments were carried out according to the manufacturer's instructions. 5×10^4 Hepa1-6 cells were seeded per well in six-well plates and treated with Scorpion-medicated serum or control serum in 5% CO_2 at 37°C for 24 h. Implanted cells were fixed in 4% paraformaldehyde for 15 min and after permeabilizing in 0.1% Triton X-100, TUNEL reaction liquid was filled at 37°C for 1 h in the dark. The cells were stained with DAPI for 20 min at last and the cells were washed with PBS three times. Fluorescence microscope at 200× magnification was used to observe the number of apoptotic cells and to calculate the mean value of five areas chosen randomly to make a statistical analysis of each sample.

Wound healing assay

Straight lines were drawn across each well on the reverse side of six-well culture plates with a marker pen. Hepa1-6 cells were seeded at 5×10^5 per well into six-well plates and pipette tip was used to scratch confluent cell monolayer when each well was coated with the cells. The scratches were perpendicular to the horizontal lines. The plates were washed with serum-free medium to remove detached cells and cultured in DMEM medium supplemented with 10% Scorpion-medicated serum or the appropriate control serum for 24 h. The images were captured at 0 and 24 h with an inverted microscope (Olympus IX2-ILL100, Japan). The wound width was measured by Image J software. The experiment was repeated three times.

Transwell assay

Plated Hepa1-6 cells were pretreated with Scorpion-medicated serum or the appropriate control serum for 24 h at 1×10^5 cells per well in 150 μL of serum-free medium in 24-well transwell chambers with 8 μm fiber membrane pore size coated with matrigel (BD Biosciences, San Jose, CA). The outer compartment was filled with 600 μL medium containing 10% FBS. The cells were incubated in a humid 5% CO_2 atmosphere at 37°C for 24 h. The upper surface of the membrane was removed softly and the lower surface was fixed in 4% paraformaldehyde for 15 min and stained with crystal violet for 15 min. The images were captured with an inverted microscope. Selected five random fields of each sample and the area of cells that had invaded through the membranes were analyzed statistically by Image-Pro Plus 6.0. The experiment was conducted three times.

HCC metastasis mouse models

Twenty-four 4-week-old male nude mice were bought from the Center of Animal Experiment of Fourth Military Medical University (Xi'an, China) and were kept in Lab of Experimental Animal Centre with pathogen-free conditions. Mice were randomly divided into three groups ($n = 8$ per group) with difference treatment: tumor bearing with PBS treated (control), tumor bearing with Scorpion water extraction treated (Scorpion), non-tumor bearing with PBS treated (normal). Hepa1-6-luc$^+$ cells (1×10^7cells/mouse) were used to establish liver cancer metastatic models in nude mice via tail intravenous injection. After seven days accommodation with no treatment, control group and normal group received PBS 0.2 ml/day by intragastric administration; the Scorpion group was treated with the same volume of Scorpion solution (containing Scorpion 0.088 g/20 g d) by intragastric administration. Mouse body weight was measured in each group every two or three days and bioluminescent imaging was conducted by using the IVIS Lumina II System (Caliper Life Sciences, Boston, MA) twice a week. Five minutes before imaging, 3 mg D-luciferin (Yeasen Biotech, Shanghai, China) was administered into each mouse by intraperitoneal injection. The imaging time of exposure ranged from 30 to 60 s which depended on the total number of luciferase-expressing cells. The light emitted from the tumor tissues containing luciferase was evaluated by Living Image Software (Caliper Life Sciences, Boston, MA). For each mouse, the metastatic tumor burden was estimated by photons. After four weeks, all mice were sacrificed and their lung and liver tissues were collected for histological analysis and further experiments.

Histological analysis

The lungs and livers were fixed with 4% paraformaldehyde for 24 h and then placed into graded ethanol to dehydrate before embedding in paraffin. The tissue samples were sectioned at 5 μm thickness for each slide and stained with hematoxylin and eosin (H&E) for valuation of tumor metastasis.

Immunohistochemical staining

Immunohistochemical staining on paraffin-embedded tissue sections for E-cadherin, N-cadherin, and Snail expression was performed by a standard protocol (San Ying Biotechnology, Wuhan, China). Deparaffinized slides in two changes of xylene with 20 min for each and then transferred slides to graded ethanol to dehydrate. The slides were placed in boiling antigen retrieval buffer and maintained at boiling temperature for 15 min and then retrieval solution was allowed to cool at room temperature. Tissue endogenous peroxidase activity was blocked by incubating sections in 0.3% H_2O_2 solution in methanol for 10 min. After rinsing twice with PBS, the sections were blocked in 3% goat serum for 60 min at room temperature. Primary antibodies (E-cadherin, N-cadherin, Snail [San Ying Biotechnology, Wuhan, China] all at a dilution of 1:50) were applied diluted in PBS containing 3% goat serum at 4°C overnight. The slides were rinsed with PBS and incubated with envision secondary antibody for 30 min at room temperature. Developed with DAB chromogen at room temperature and observed under a microscope. The slides were counterstained in a hematoxylin bath for 20 s and then washed for 5 min. Negative controls were established by replacing the primary antibody with normal rabbit serum. No detectable staining was evident in the negative controls.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was used to detect the expression level of E-cadherin, N-cadherin, and Snail mRNA which were EMT-related factors in Hepa1-6 metastasis models. The typical EMT-induced model was yielded as described before.[23] Total RNA was extracted from established EMT-induced Hepa1-6 cells which had already been treated with TGF-β1(10 ng/mL) in Scorpion-medicated serum or control serum, respectively, for 72 h in TRIzol reagent (Life Technologies, New York, NY) and followed by using chloroform and isopropanol to extraction and precipitation. Complementary DNA (cDNA) was synthesized with random primers. PCRs were performed in a 25 μL reaction system containing 12.5 μL SYBR Premix Ex Taq (Takara, Tokyo, Japan), 1 μL each primer, 0.5 μL cDNA template, and on a BioRad Real-Time PCR System at 95°C for

5 s and 60°C for 34 s for 40 cycles. Triplicate samples were normalized to β-actin and specific primers for E-cadherin, N-cadherin, Snail, and β-actin were synthetized by BioSune Company (Shanghai, China). The primers sequences were described in Table 1.

Western blot

The Hepa1-6 cells were washed from each group with ice-cold PBS twice and solubilized in RIPA buffer (Beyotime Biotechnology, Shanghai, China) containing 1 mmol/L PMSF on ice. The protein concentration of each sample was detected by the BCA Protein Assay kit (Thermo Scientific, Fremont, CA). Hepa1-6 cells lysates were divided by SDS-PAGE, with 20 μg amount of protein charged in each lane and transferred to PVDF membrane. Then blocked the membranes with 5% skim milk for 2 h and incubated with the primary antibodies (E-cadherin, N-cadherin, Snail, and β- actin 1:2000 diluted in 5% skim milk) at 4°C overnight. The membranes were treated with appropriate peroxidase-conjugated secondary antibodies at room temperature for 1 h and washed with PBS containing 0.1% Tween-20 (PBST) three times. The signals were visualized with chemiluminescent HRP substrate reagent (Millipore, MA, USA) and Image J software was used to analyze images. For Western blot analysis of mouse lung samples, samples from the control group, normal group, and Scorpion group were homogenized into 200 μL RIPA containing 1 mmol/L PMSF, respectively. The tissue protein concentration was determined by the BCA Protein Assay and 20 μg amount of protein was loaded in each lane for Western blot analysis.

Table 1. Primers list for quantitative real-time PCR analysis.

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
β-actin	GTGACGTTGACATCC GTAAAGA	GCCGGACTCATC GTACTCC
E-cadherin	CAGTTCCGAGGTCTA CACCTT	TGAATCGGGAGTCTT CCGAAAA
N-cadherin	AGGCTTCTGGTGAAA TTGCAT	GTCCACCTTGAAA TCTGCTGG
Snail	CACACGCTGCCTTGT GTCT	GGTCAGCAAAAG CACGGTT

PCR: polymerase chain reaction.

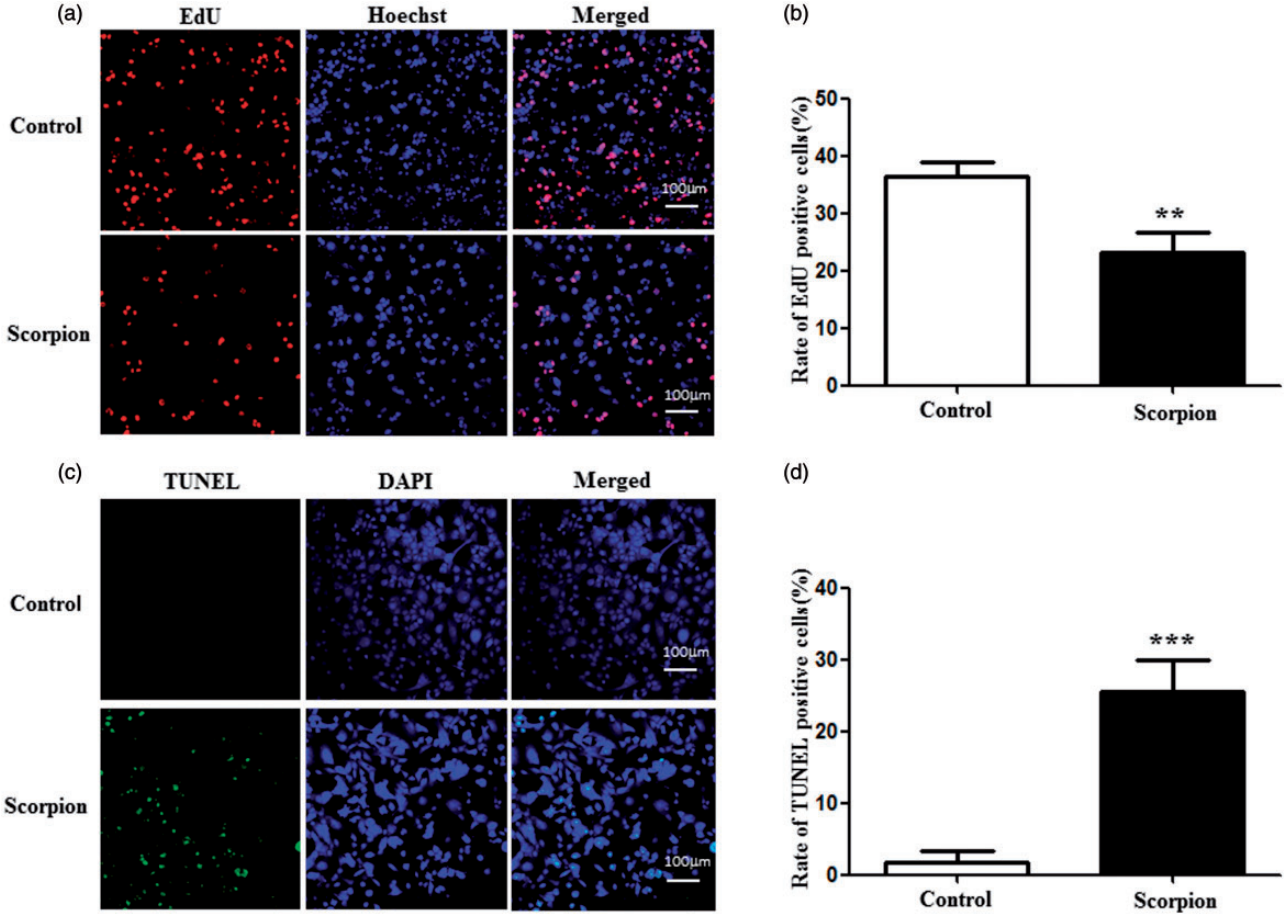

Figure 1. Scorpion-medicated serum affected proliferation and apoptosis of hepatoma carcinoma cells. (a) EdU staining of Hepa1-6 cells cultured in 24-well plates with Scorpion-medicated serum and control serum, respectively, for 24 h. Scale bars, 100 μm. (b) Rate of EdU positive cells was calculated and compared. Bars = means ± SD, **$P < 0.01$. (c) TUNEL staining of Hepa1-6 cells cultured in 24-well plates with Scorpion-medicated serum and control serum, respectively, for 24 h. Scale bars, 100 μm. (d) Rate of TUNEL positive cells was calculated and compared. Bars = means ± SD, ***$P < 0.001$. DAPI: 4,6-diamino-2-phenyl indole; EdU: 5-ethynyl-2′-deoxyuridine; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. (A color version of this figure is available in the online journal.)

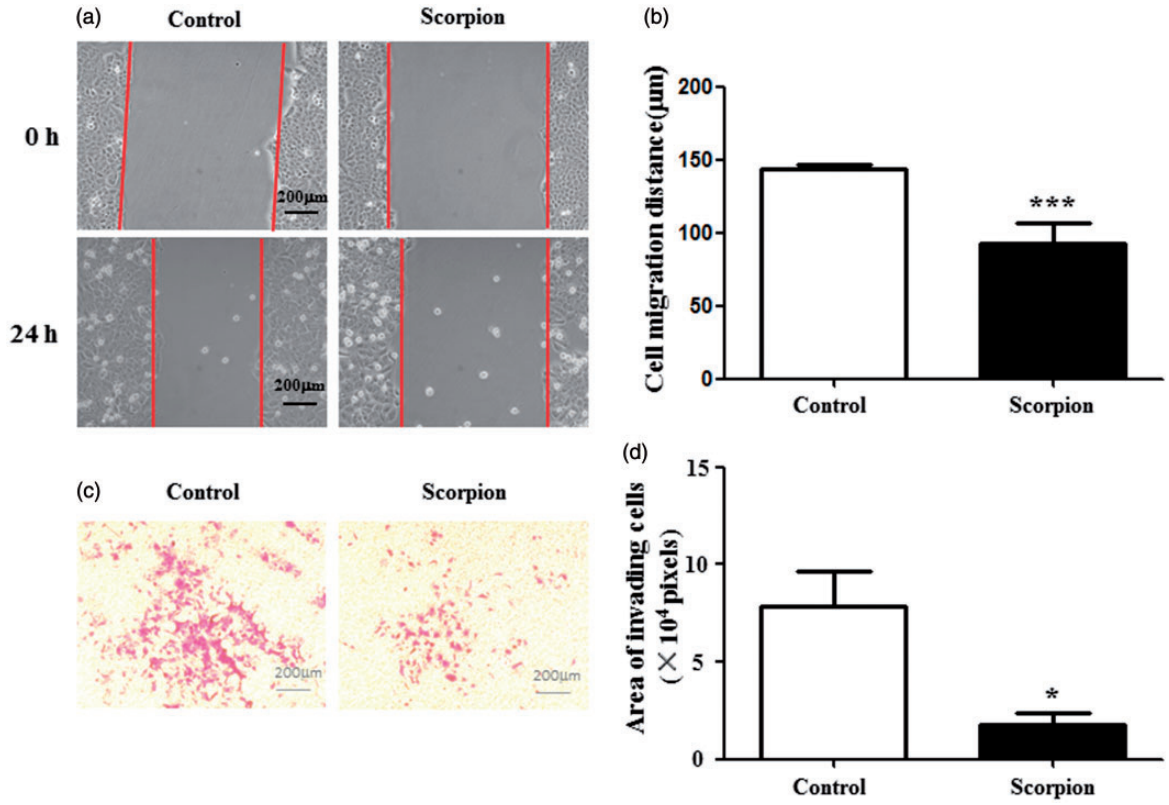

Figure 2. Scorpion-medicated serum inhibited the migration and invasion of hepatoma carcinoma cells. (a) Representative images of the wound healing assay in Hepa1-6 cells treated with Scorpion-medicated serum and control serum. Scale bars, 200 μm. (b) Cell migration distance was calculated and compared. Bars = means ± SD, ***P < 0.001. (c) Representative images of transwell assay in Hepa1-6 cells treated with Scorpion-medicated serum and control serum. Scale bars, 200 μm. (d) Area of invading cells was calculated and compared. Bars = means ± SD, *P <0.05, ***P < 0.001. (A color version of this figure is available in the online journal.)

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The t-test was used to examine the differences in statistics between each group and ANOVA analysis was used to compare the differences in mice body weights. Statistical analyses were performed using SPSS 22.0. P values <0.05 indicate statistical significance.

Results

Scorpion-medicated serum affected proliferation and apoptosis of hepatoma carcinoma cells

The effect of Scorpion on proliferation of Hepa1-6 hepatoma carcinoma cells was evaluated by the EdU incorporation assay. Proliferation rate of Hepa1-6 cells was suppressed distinctly after 24 h of Scorpion-medicated serum exposure. The proliferation rate of hepatoma carcinoma cells was about 36% in control, while 23% in Scorpion-medicated serum group (Figure 1(a) and (b)). Then, TUNEL assay was employed to detect the effect of Scorpion-medicated serum on apoptosis of Hepa1-6 cells. Hepa1-6 cells with Scorpion-medicated serum treated 24 h resulted in a remarkable increase in the rate of apoptosis relative to the control group (Figure 1(c) and (d)). The apoptosis rate of Hepa1-6 cells was about 2% in control group, while reached to 26% in Scorpion-medicated serum group. These results suggested that Scorpion-medicated serum could inhibit the proliferation and induce the apoptosis of hepatoma carcinoma cells.

Scorpion-medicated serum inhibited the migration and invasion of hepatoma carcinoma cells

The influence of Scorpion-medicated serum on hepatoma carcinoma cell migration and invasion was investigated by wound healing assay and transwell analysis. The scratched images were collected at 0 and 24 h after the wounding and the width of the wound was measured. The migration distance of Hepa1-6 was about 143 μm in control group while only about 92 μm in Scorpion-medicated serum group (Figure 2(a) and (b)). Moreover, the results of transwell assay showed that the invasion cells of Hepa1-6 with Scorpion-medicated serum treatment were less than that of control group obviously (Figure 2(c) and (d)). These results indicated that the migration and invasion abilities of Hepa1-6 cells were weakened by Scorpion-medicated serum *in vitro*.

Scorpion-medicated serum suppressed TGF-β1-induced EMT in Hepa1-6 cells in vitro

The EMT plays a critical role in tumor metastasis process and TGF-β1 is regarded as an effective inducer in the development of EMT.[24] We investigated the expression of

E-cadherin, N-cadherin, and Snail in TGF-β1-induced Hepa1-6 EMT models after treatment of Scorpion-medicated serum for 72 h by qRT-PCR and Western blot analysis. The results of qRT-PCR analyses showed that stimulating with TGF-β1 led to down-regulation of epithelial marker E-cadherin mRNA expression and up-regulation of mesenchymal markers and transcription factors N-cadherin and Snail mRNA expression in Hepa1-6 cells. Scorpion-medicated serum dramatically repressed TGF-β1-induced EMT process. The mRNA expression of E-cadherin was increased in Scorpion-medicated serum treatment while the mRNA expression of N-cadherin and Snail was decreased (Figure 3(a)). The changes in the protein expression of E-cadherin, N-cadherin, and Snail were evaluated by Western blot and the results were in good agreement with the PCR conclusions from above (Figure 3(b) and (c)). These results suggested that Scorpion-medicated serum suppressed TGF-β1-induced EMT in Hepa1-6 cells *in vitro*.

Scorpion restrained the growth and metastases of Hepa1-6 in vivo

For the Scorpion-medicated serum had shown inhibiting effect on Hepa1-6 cells *in vitro*, we moved on to explore the therapeutic effect of Scorpion in metastasis model of liver cancer. Hepa1-6-luc cells were injected into the tail vein of nude mice. The mice were then treated with Scorpion decoction or PBS by intragastric administration. Bioluminescent imaging results showed that Scorpion can restrain the formation and growth of metastasis tumor *in vivo* (Figure 4(a)). Meanwhile, photon counts statistic indicated that the mice which were treated with Scorpion decoction had fewer metastatic tumors (Figure 4(b) and (c)) and the metastasis ability on Hepa1-6 cells in the mice of Scorpion group was weaker than the mice in control group. There was no significant difference in body weights between the mice in each group during the whole experimental stage (Figure 4(d)). H&E staining demonstrated Hepa1-6 cell metastasis in lung and liver (Figure 5(a)). Statistic results showed that both the metastasis number and relative area of lung decreased significantly in Scorpion group than that of control group (Figure 5(b) and (c)). These data suggested that Scorpion restrained the growth and metastases of Hepa1-6 *in vivo*.

Scorpion inhibited EMT of Hepa1-6 in vivo

To investigate whether Scorpion regulates EMT of HCC *in vivo*, we examined the expression change of epithelial marker E-cadherin and mesenchymal markers N-cadherin and Snail with immunohistochemical staining and Western blot. The area of positive staining for E-cadherin of lung tissue samples in Scorpion group was mainly identified in the tumor cells membrane and adjacent benign tissues which was increased significantly than control group.

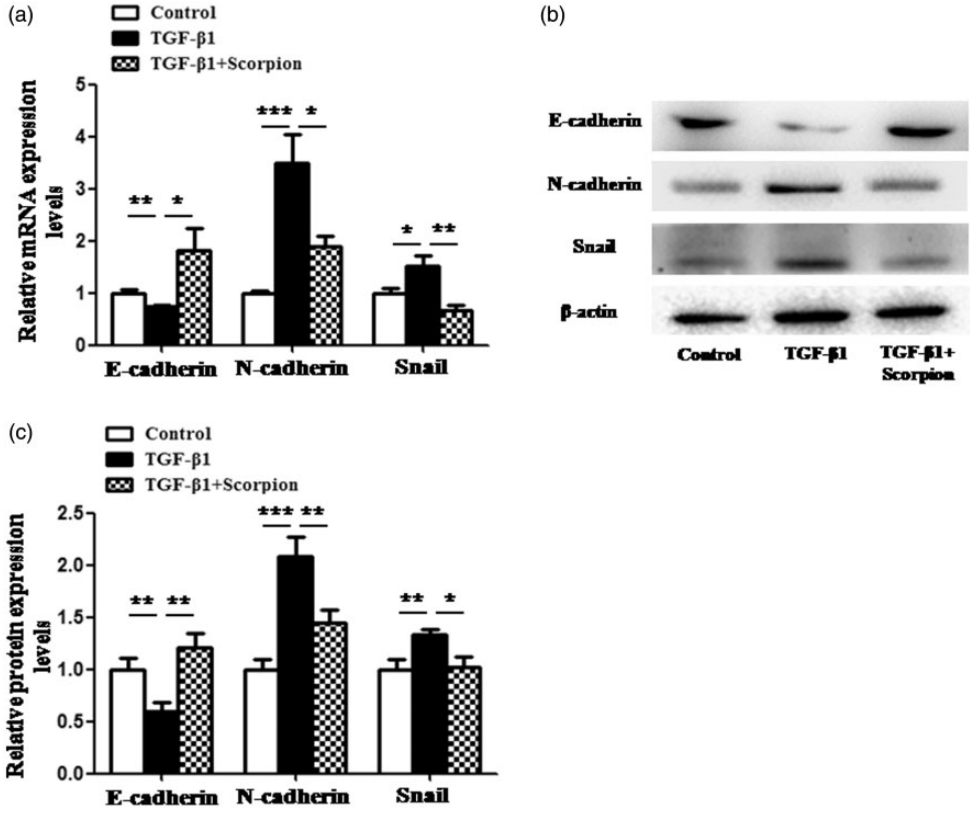

Figure 3. Scorpion-medicated serum suppressed TGF-β1-induced EMT in Hepa1-6 cells *in vitro*. (a) Relative mRNA expression levels of E-cadherin, N-cadherin, and Snail in Hepa1-6 cells (induced by TGF-β1) treated with Scorpion-medicated serum and control serum for 72 h were measured by qRT-PCR. Bars = means ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (b) Protein levels of E-cadherin, N-cadherin, and Snail in Hepa1-6 cells (induced by TGF-β1) treated with Scorpion-medicated serum and control serum for 72 h were measured by Western blot. (c) Relative protein expression levels of E-cadherin, N-cadherin, and Snail were calculated and compared. Bars = means ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. TGF-β1: transforming growth factor-β1.

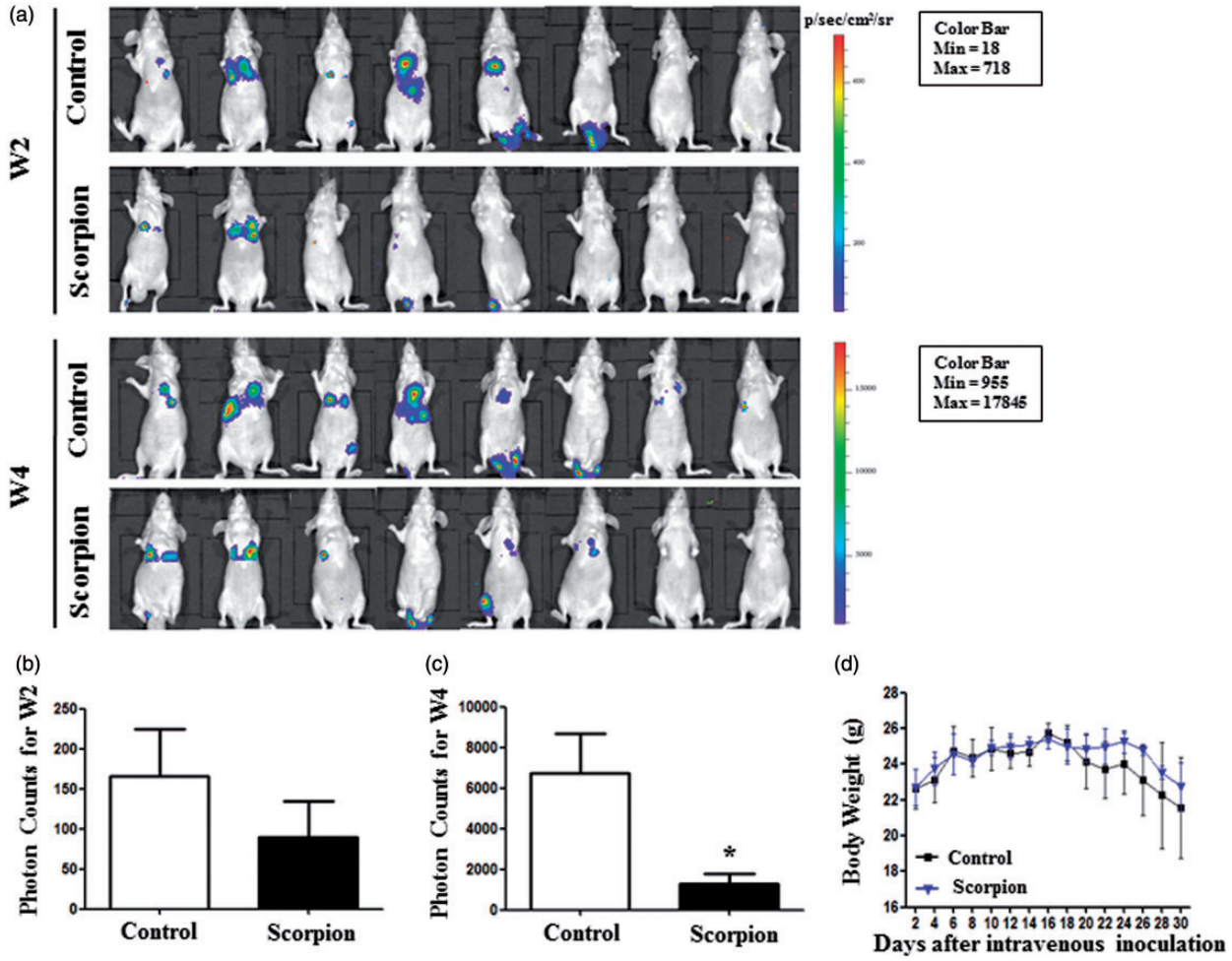

Figure 4. Scorpion restrained the growth and metastasis of HCC *in vivo*. (a) Bioluminescent imaging of nude mice transplanted with 1×10^7 Hepa1-6-luc$^+$ cells and treated with Scorpion decoction and PBS by intragastric administration. (b) Photon counts of nude mice after transplanted with Hepa1-6-luc$^+$ cells two weeks were compared. Bars = means ± SD. (c) Photon counts of nude mice after transplanted with Hepa1-6-luc$^+$ cells four weeks were compared. Bars = means ± SD, *$P < 0.05$. (d) Body weight of nude mice after transplanted with Hepa1-6-luc$^+$ cells were compared. (A color version of this figure is available in the online journal.)

And compared with the control, area of staining for N-cadherin and Snail decreased significantly in Scorpion-treated group (Figure 6(a)). To further confirm the above observation, we investigated the protein expression of E-cadherin, N-cadherin, and Snail in lung samples from different groups with Western blot. The statistics showed that there was an increased expression of E-cadherin in the samples from Scorpion group; meanwhile the expression of N-cadherin and Snail was all declined with Scorpion-treated group (Figure 6(b) and (c)). These data point to as a sign that Scorpion could change the statuses of E-cadherin, N-cadherin, and Snail of lung tissues in the mice tumor metastasis models. Altogether, these results suggested that Scorpion could inhibit EMT process of HCC and then inhibit metastasis of HCC.

Discussion

The HCC metastasis is the main cause of HCC-related mortality.[25] However, how to inhibit metastasis remains poorly understood. In this study, we report that Scorpion, a kind of TCM, could inhibit proliferation, induce apoptosis, suppress migration and invasion of HCC cells. Scorpion could restrain the metastasis and EMT process of HCC both *in vitro* and *in vivo*, thereby it has a possible therapeutic effect in treatment with HCC.

The primary process of tumor metastasis is the ability of tumor cells to acquire motor ability and invasion phenotype. Studies have shown that EMT makes the tumor cells win the movement characteristics.[26] On the other hand, HCC cells can also get stem cell properties through EMT and have the ability of self-renewal and differentiation eventually to form a new metastasis.[27,28] Now many kinds of methods for the treatment of HCC may even induce EMT process of HCC to metastasis faster while slowing tumor growth. So, EMT is one of the important aspects for the prevention and control of HCC, and the inhibition of EMT has important therapeutic value in the treatment of HCC.[29,30]

Some researchers of TCM consider that the formation of tumor metastasis was endogenous wind-evil which being secret whirling and changeable and it also was the determining factor of metastasis of HCC.[14] Based on this theory, the method of calming endogenous wind is the primary principle in Chinese medicine treatment for HCC and its

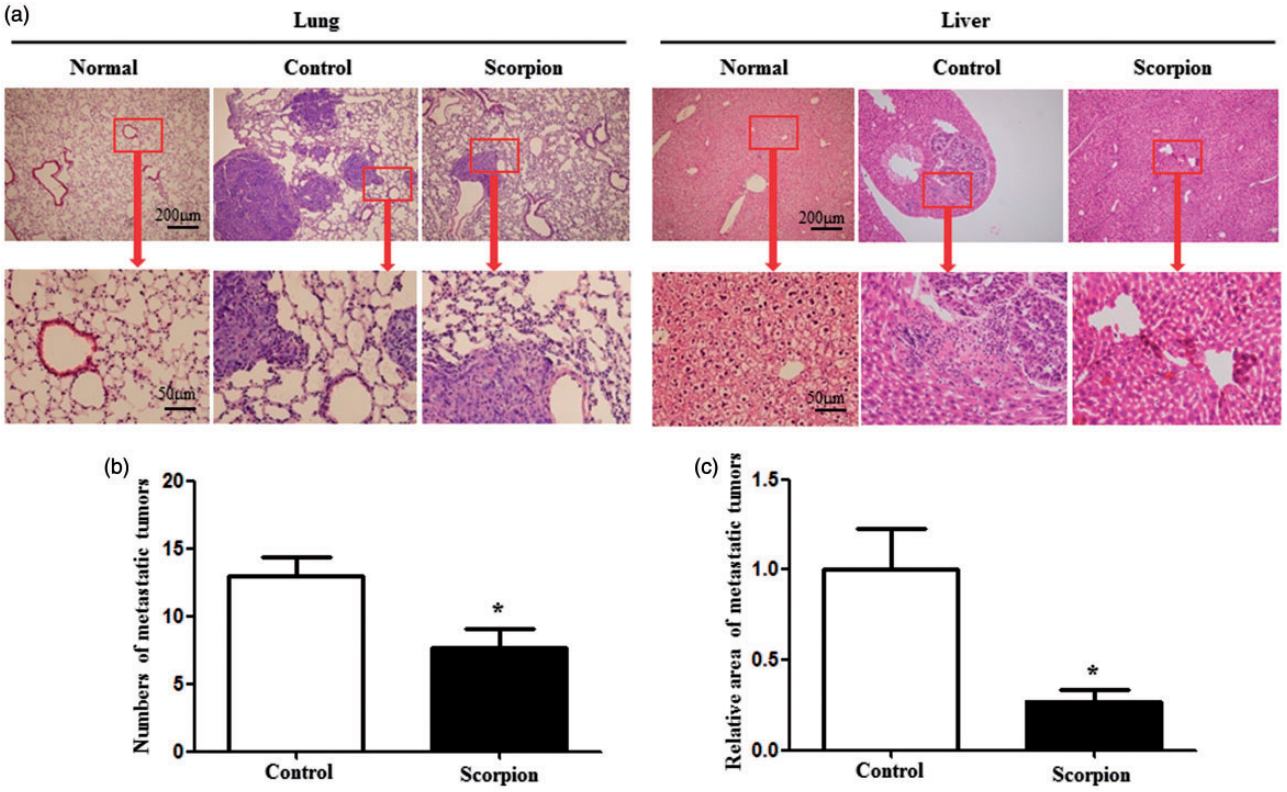

Figure 5. Metastasis of HCC detected by H&E staining. (a) Representative images of H&E staining of lung and liver sections from nude mice transplanted with Hepa1-6 cells and treated with Scorpion decoction and PBS by intragastric administration. The bottom row pictures are magnified from the red box of the top row. Scale bars, 200 μm. (b) Numbers of metastatic tumors in lung of nude mice were calculated and compared. Bars = means ± SD, *P < 0.05. (c) Relative area of metastatic tumors in lung of nude mice was calculated and compared. Bars = means ± SD, *P < 0.05. (A color version of this figure is available in the online journal.)

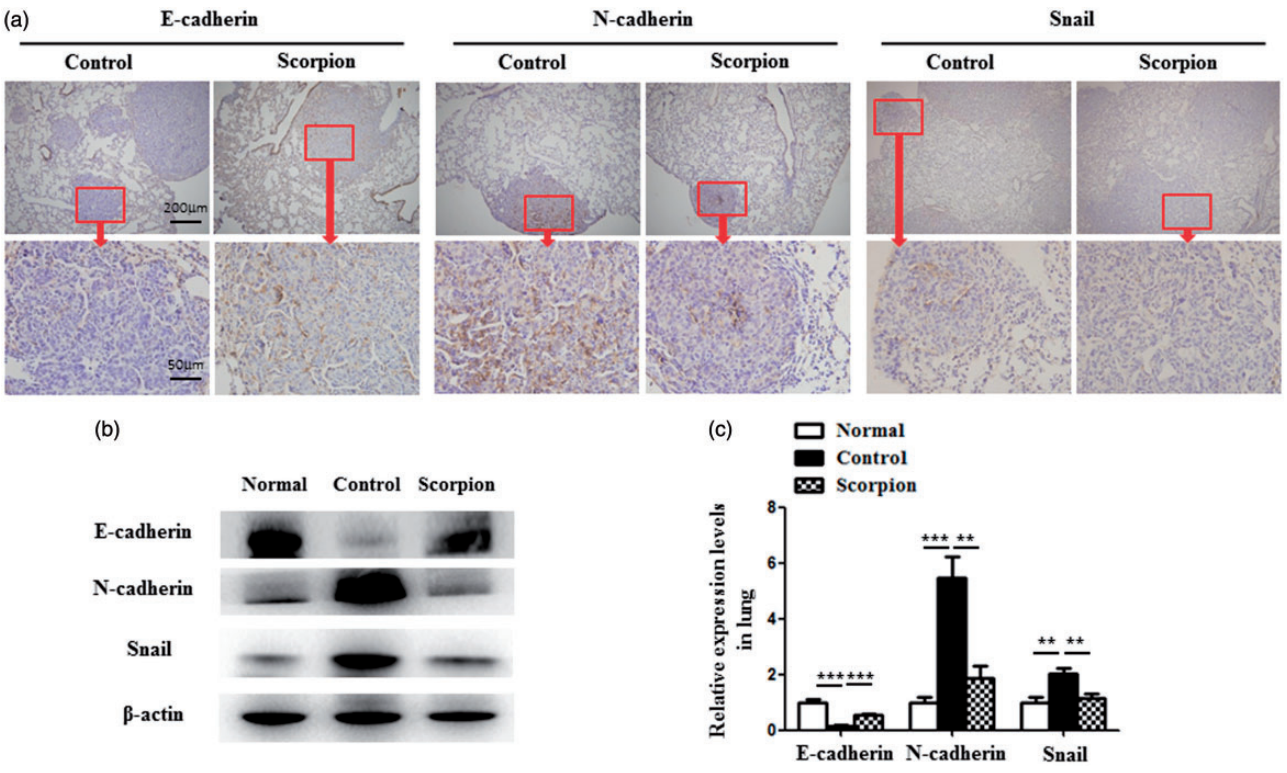

Figure 6. Scorpion inhibited EMT of HCC *in vivo*. (a) Representative images of immunohistochemical staining of lung sections from nude mice transplanted with Hepa1-6 cells and treated with Scorpion decoction and PBS by intragastric administration. The bottom row pictures are magnified from the red box of the top row. Scale bars, 200 μm. (b) Protein levels of E-cadherin, N-cadherin, and Snail in lungs of nude mice transplanted with Hepa1-6 cells and treated with Scorpion decoction of PBS by intragastric administration were measured by Western blot. (c) Relative protein expression levels of E-cadherin, N-cadherin, and Snail were calculated and compared. Bars = means ± SD, **P < 0.01, ***P < 0.001. (A color version of this figure is available in the online journal.)

metastasis, and the liver-pacifying wind-subduing drugs are important components in clinical settings. Scorpion, as one of the most important wind calming drugs, has antitumor effect in various types of tumors in clinical treatment in China. So, we explored the role of Scorpion in EMT and metastasis of HCC in this study.

Our research indicated that the therapeutic effect of Scorpion on HCC included at least two aspects. Scorpion inhibited proliferation and induced apoptosis of HCC cells, suggesting that Scorpion has toxic effect on HCC cells. On the other hand, Scorpion could inhibit EMT and metastasis of HCC cells both *in vitro* and *in vivo*. Now that Scorpion has toxic effect on HCC cells, we wondered whether Scorpion has toxic effect on normal cells. In HCC metastasis model, we also weighed body weight of mice after administration of Scorpion and PBS (Figure 4(d)). The results showed the body weight of two groups had no significant difference, suggesting that Scorpion may have no toxic effect on normal cells.

In majority of liver sections, there were no metastases. In mouse liver H&E staining results, we showed pictures with necrotic foci but not metastasis (Figure 5(a)), and immunohistochemistry staining results of liver sections were not shown. We considered the metastasis capacity of Hepa1-6 cells was relatively low.

There are many active ingredients in Scorpion, including antimicrobial peptides Mucroporin-M1,[31] analgesic peptide BmKBTx,[32] antiepilepsy peptide AEP,[33] antitumor peptide HS-1,[34] etc. We only detected antitumor effect of Scorpion; however, it remains unclear which active ingredient has the role in inhibiting tumor EMT and metastasis. This is the limitation of our study. We also did not explore the target genes of Scorpion. In the next study, we will explore exactly the effect component of Scorpion in inhibiting HCC EMT and metastasis and the key genes regulated by Scorpion involved in HCC EMT and metastasis.

Authors' contributions: XCZ, JX, and YPD supervised the project. Y-QY, JFW, ZFS, and XZ conducted experiments. XCZ and Y-QY wrote the paper. Y-QY and JX contributed equally to this paper.

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DECLARATION OF CONFLICTING INTERESTS

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REFERENCES

1. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012;**379**:1245–55
2. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016;**66**:115–32
3. Ministry of Health of the People's Republic of China. [Updated standards for the diagnosis and treatment of primary liver cancer]. *Zhonghua Gan Zang Bing Za Zhi* 2012;**20**:419–26
4. Chun JM, Kwon HJ, Sohn J, Kim SG, Park JY, Bae HI, Yun YK, Hwang YJ. Prognostic factors after early recurrence in patients who underwent curative resection for hepatocellular carcinoma. *J Surg Oncol* 2011;**103**:148–51
5. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin* 2017;**67**:7–30
6. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011;**331**:1559–64
7. Seton-Rogers S. Epithelial-mesenchymal transition: untangling EMT's functions. *Nat Rev Cancer* 2016;**16:1**
8. Wu L, Feng H, Hu J, Tian X, Zhang C. Valproic acid (VPA) promotes the epithelial mesenchymal transition of hepatocarcinoma cells via transcriptional and post-transcriptional up regulation of Snail. *Biomed Pharmacother* 2016;**84**:1029–35
9. Yingliang W. Research of pharmacological toxicology and clinical application of Scorpio and its effective components. *Chin Tradit Herbal Drugs* 1995;**26**:97–100
10. Feng H. Zhu Liangchun's experience in treating malignant tumor using strengthening body resistance and softening hard tissue method. *J Tradit Chin Med* 2015;**56**:1453–5
11. Chen HZC, Jia Y. Jia Yingjie's experience in treating malignant tumor using insect drugs. *Hunan J Tradit Chin Med* 2015;**31**:32–61
12. Cheng RCJ, Jia Z, Xu N. The research for the treatment of tumor with Chinese herbal scorpion. *Chin J Surg Integr Tradit Western Med* 2002;**8**:360
13. Yang GJD, Makabel B. Clinical utility of animal drug scorpion. *World Clin Drugs* 2006;**27**:564–7
14. He YHHJ. Relationship between "the wind" and tumor metastasis. *Chin J Basic Med Tradit Chin Med* 2006;**12**:124–6
15. Jia WZH, Chen G, Zhang C. Using drug thinking of traditional Chinese medicine on liver cancer. *Chin J Basic Med Tradit Chin Med* 2017;**23**:121–3
16. Li YXB, He Y. The research of antitumor effect on wind medicine. *China J Chin Mater Med* 2011;**36**:3375–9
17. Chen H, Zhidan W, Xia R, Zhaoxia W, Qing J, Qiang G, Haipeng Y, Hengxiao W. Scorpion venom activates natural killer cells in hepatocellular carcinoma via the NKG2D-MICA pathway. *Int Immunopharmacol* 2016;**35**:307–14
18. Dardevet L, Rani D, Aziz TA, Bazin I, Sabatier JM, Fadl M, Brambilla E, De Waard M. Chlorotoxin: a helpful natural scorpion peptide to diagnose glioma and fight tumor invasion. *Toxins* 2015;**7**:1079–101
19. Ding J, Chua PJ, Bay BH, Gopalakrishnakone P. Scorpion venoms as a potential source of novel cancer therapeutic compounds. *Exp Biol Med* 2014;**239**:387–93
20. Sun X, Zhang Y, Jia Q, Wang Z, Wang Z, Zhang W. Effect of polypeptide extract from scorpion venom (PESV) with chemotherapy inhibited angiogenesis of Lewis lung carcinomas. *China J Chin Mater Med* 2011;**36**:1644–9
21. Wei W. *Experimental methodology of pharmacology*. Beijing: People's Medical Publishing House, 2010, p.1698.
22. Li YWJ. The discussion of "ordinary method" of obtaining serum in serum pharmacology method. *Chin Pharmacol Bull* 1999;**15**:569–70
23. Araki K, Shimura T, Suzuki H, Tsutsumi S, Wada W, Yajima T, Kobayahi T, Kubo N, Kuwano H. E/N-cadherin switch mediates cancer progression via TGF-beta-induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma. *Br J Cancer* 2011;**105**:1885–93

24. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* 2003;**15**:740–6
25. Chen P, Wang F, Feng J, Zhou R, Chang Y, Liu J, Zhao Q. Co-expression network analysis identified six hub genes in association with metastasis risk and prognosis in hepatocellular carcinoma. *Oncotarget* 2017;**8**:48948–58
26. Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A, Sarkar S. EMT and tumor metastasis. *Clin Transl Med* 2015;**4**:6
27. Wang X, Dong J, Jia L, Zhao T, Lang M, Li Z, Lan C, Li X, Hao J, Wang H, Qin T, Huang C, Yang S, Yu M, Ren H. HIF-2-dependent expression of stem cell factor promotes metastasis in hepatocellular carcinoma. *Cancer Lett* 2017;**393**:113–24
28. Hu K, Huang P, Luo H, Yao Z, Wang Q, Xiong Z, Lin J, Huang H, Xu S, Zhang P, Liu B. Mammalian-enabled (MENA) protein enhances oncogenic potential and cancer stem cell-like phenotype in hepatocellular carcinoma cells. *FEBS Open Bio* 2017;**7**:1144–53
29. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Gasanovas O. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009;**15**:220–31
30. Xiong W, Ren ZG, Qiu SJ, Sun HC, Wang L, Liu BB, Li QS, Zhang W, Zhu XD, Liu L, Wang WQ, Tang ZY. Residual hepatocellular carcinoma after oxaliplatin treatment has increased metastatic potential in a nude mouse model and is attenuated by Songyou Yin. *BMC Cancer* 2010;**10**:219
31. Zhao Z, Hong W, Zeng Z, Wu Y, Hu K, Tian X, Li W, Cao Z. Mucroporin-M1 inhibits hepatitis B virus replication by activating the mitogen-activated protein kinase (MAPK) pathway and down-regulating HNF4alpha in vitro and in vivo. *J Biol Chem* 2012;**287**:30181–90
32. Lin S, Wang X, Hu X, Zhao Y, Zhao M, Zhang J, Cui Y. Recombinant expression, functional characterization of two scorpion venom toxins with three disulfide bridges from the Chinese scorpion *Buthus martensii* Karsch. *Protein Pept Lett* 2017;**24**:235–40
33. Wang Z, Wang W, Shao Z, Gao B, Li J, Ma J, Che H, Zhang W. Eukaryotic expression and purification of anti-epilepsy peptide of *Buthus martensii* Karsch and its protein interactions. *Mol Cell Biochem* 2009;**330**:97–104
34. Uawonggul N, Sukprasert S, Incamnoi P, Patramanon R, Thammasirirak S, Preecharram S, Bunyatratchata W, Kuaprasert B, Daduang J, Daduang S. Bacterial overexpression of recombinant heteroscorpine-1 (rHS-1), a toxin from *Heterometrus laoticus* scorpion venom: trends for antibacterial application and antivenom production. *Biochem Genet* 2014;**52**:459–73

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