

Tegillarca granosa extract Haishengsu inhibits tumor activity via a mitochondrial-mediated apoptotic pathway

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Abstract. Haishengsu (HSS) is an active natural extract isolated from *Tegillarca granosa*, which has previously been demonstrated to inhibit the proliferation of several types of cancer cells *in vitro*. Our previous study indicated that HSS may induce apoptosis to suppress growth of human hepatocellular carcinoma BEL-7402 cells by activating Fas pathway. The present study demonstrated that HSS treatment induces the *in vitro* apoptosis of BEL-7402 cells via the mitochondrial-mediated apoptotic pathway detected by DNA fragmentation assay, caspase activity assay and transmission electron microscopy assay, and inhibits tumor xenograft growth *in vivo*. Alterations in apoptotic regulatory proteins were detected, including decreased expression of B-cell lymphoma2 (Bcl-2), upregulation of Bcl-2-associated X protein and mitochondrial cytochrome *c* release, and downstream activation of apoptotic signaling. Furthermore, apoptotic induction was caspase-dependent, as indicated by cleavage of the caspase substrate, poly (ADP-ribose) polymerase. Oral administration of 62.5-250 mg/kg HSS markedly reduced the growth of hepatocellular carcinoma tumor xenografts in nude mice. In addition, immunohistochemical staining for caspase-3 protein and transmission electron microscopy further indicated the induction of apoptosis in these tumor tissues. Taken together, the present study demonstrated that HSS may effectively induce apoptosis to suppress the growth of BEL-7402 cells *in vitro* and *in vivo*, and therefore may hold promise for further development as a novel cancer therapy.

Introduction

The ocean covers ~70% of the Earth's surface; it is a rich source of natural resources and contains ~80% of all the biodiversity

on our planet. It is home to approximately one million multicellular species and one billion unicellular species (1). During the past 30-40 years, >22,000 novel natural products have been identified from marine sources, whereas ~131,000 have been identified from terrestrial sources. These marine products have various biological properties, including anticancer and antiviral activities (2,3). The study of anticancer marine natural products (MNPs) is an important approach to global drug discovery. MNPs have featured significantly in the development of anticancer drugs; furthermore, they are generally free of deleterious side effects and are usually inexpensive (4).

A large proportion of MNPs have been extracted from marine invertebrates, including alkaloids, peptides, terpenes and polyketides (5,6). *Tegillarca granosa* is a type of marine invertebrate widely distributed in China, which has been a traditional Chinese seafood for hundreds of years. Haishengsu (HSS) is an extract isolated from *T. granosa*, and its major chemical constituents are small molecular weight proteins that range between 15 and 23 kDa. Previous clinical and experimental studies regarding HSS have investigated its anticancer properties, and the possible mechanisms associated with its activity. Clinical studies have indicated that HSS, used in combination with other chemotherapeutic agents, may display significant activity against human cancers, such as non-small-cell lung cancer and advanced renal cell cancer; therefore, it may have the potential to improve the quality of life for these patients (7,8). Experimental studies have demonstrated that HSS may have an inhibitory effect on several human cancer cells, including ovarian cancer cell lines (SKOV-3 and OVCAR-3), lung carcinoma cell lines (A549 and NCI-H292), leukemia cell lines (K562 and K562/ADM) and renal carcinoma cell lines (OS-RC-2) (9-13). Furthermore, murine studies have indicated that HSS may inhibit the growth of Ehrlich ascites tumors and improve immunological functions (14). Our previous study demonstrated that HSS was able to induce the apoptosis of human hepatocellular carcinoma (HCC) cells in a concentration-dependent manner, through the Fas signaling pathway, with caspase-8 and caspase-3 activation (15).

As a type of programmed cell death, apoptosis is a genetically controlled suicide mechanism that enables organisms to control cell number and eliminate cells that threaten survival (16). Classical apoptosis can be initiated via two major pathways: The intrinsic or mitochondrial-mediated pathway,

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and the extrinsic or death receptor-mediated pathway (17). A study from our laboratory indicated that HSS induces apoptosis in HCCBEL-7402 cells via the Fas signaling pathway (15). However, little is currently known regarding the mechanisms responsible for the mitochondrial-mediated apoptotic pathway of HSS against HCC cells. The present study further investigated whether mitochondrial-mediated apoptotic pathways are involved in the inhibitory effects of HSS on BEL-7402 cells, with the aim of providing further experimental evidence to support the HSS-based treatment of HCC.

Materials and methods

HSS preparation. HSS was extracted from the shellfish *Tegillarca granosa* as described previously (15), and dissolved in phosphate-buffered saline (PBS) prior to use.

Cell culture. The human HCC cell line BEL-7402 was obtained from the Shanghai Cell Bank at the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). Cells were maintained in RPMI 1640 medium (GE Healthcare, Chicago, IL, USA) supplemented with 10% (v/v) fetal bovine serum (GE Healthcare), 100 U/ml penicillin and 100 mg/ml streptomycin, under 5% CO₂ at 37°C, in a humidified incubator.

DNA fragmentation assay. DNA fragmentation is a marker of cell apoptosis (18). Briefly, BEL-7402 cells were treated with various concentrations (0–100 µg/ml) of HSS for 48 h at 37°C. Subsequently the cells were centrifuged (500 x g for 5 min at 4°C) and washed with PBS, and the pellets were then resuspended in 500 µl cell lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% SDS, 500 mg/l proteinase K] and incubated overnight at 50°C. Following incubation, the cell lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1 v/v), the DNA was precipitated with sodium acetate (50 µl; 3 M, pH 5.2) and ethanol (1 ml) at -20°C overnight, and subsequently washed with 70% ethanol. The DNA pellet was dissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated with RNase A (20 µg/ml) at 37°C for 30 min. Subsequently, DNA samples were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

Caspase activity assay. The activities of caspase-3 and -9 were detected using a Caspase Colorimetric Assay kit (cat. nos. ab39401 and ab65608; Abcam, Cambridge, UK), according to the manufacturer's protocol. Caspase activity was measured via the cleavage of chromogenic caspase substrates. Briefly, following treatment with various concentrations (0–100 µg/ml) of HSS for 48 h at 37°C, BEL-7402 cells were harvested and lysed in the supplied lysis buffer. The lysed cells were centrifuged at 18,000 x g for 10 min, and protein concentrations were determined using a bicinchoninic acid assay. The proteins were mixed with caspase substrates at 37°C for 2 h, and the p-nitroaniline light emission was quantified using a microplate reader at 405 nm.

Western blot analysis. After treatment, cells were harvested and lysed with ice-cold lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) containing protease inhibitors

(Sigma Aldrich, Merck KGaA, Darmstadt, Germany). The mitochondrial and cytosolic proteins were extracted using a Cell Mitochondria Isolation kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer's protocol. Briefly, the cell pellet was then resuspended in the extraction buffer which was enclosed in the isolation kit. After 30 min incubation on ice, cells were homogenized with a dounce homogenizer, and the homogenate was centrifuged at 600 x g for 10 min at 4°C. The supernatant was spun at 11,000 x g for 10 min at 4°C. The supernatant (cytosolic fraction) was removed and maintained at -80°C. The pellet containing mitochondria was resolved in the lysis buffer which was enclosed in the isolation kit. The protein concentration was determined using the bicinchoninic acid assay (Beyotime Biotechnology). Western blotting was performed as previously described (19). Extracted proteins (50 µg) in lysates were separated by 10–12% SDS-PAGE and then transferred electrophoretically onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk overnight at 4°C. Subsequently, membranes were stained with primary antibodies against poly (ADP-ribose) polymerase (PARP; 1:1,000), caspase-9 (1:1,000), caspase-3 (1:100), B-cell lymphoma 2 (Bcl-2; 1:500), Bcl-2-associated X protein (Bax; 1:1,000), cytochrome *c*, cytochrome *c* oxidase (COX)-IV (1:100), α -tubulin (1:5,000), (cat. nos. ab4830, ab32539, ab4051, ab59348, ab32503, ab14744 and ab7291 respectively; Abcam). A rabbit polyclonal antibody to GAPDH was used as a loading control (1:500; cat. no. ab8245). Bands were detected with SuperSignal West Femto Maximum Sensitivity substrate (Pierce; Thermo Fisher Scientific, Inc.) and imaged with the ChemiDoc™ MP Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Densitometry analysis was performed using Quantity One analysis software (version 4.6.9; Bio-Rad Laboratories, Inc.).

In vivo antitumor efficacy. Female BALB/c nude mice (6–8 weeks of age, weight 18–22 g) were provided by the Laboratory Animal Institute, Chinese Academy of Medical Sciences (Beijing, China). Animal experiments were performed in accordance with the Chinese Animal Protection Network, and laboratory protocols were approved by the Animal Care and Use Committee of Qingdao University (Qingdao, China). The mice were housed in cages in a room with an artificial 12-h light/dark cycle at a constant temperature range (24±2°C) and relative humidity (55±10%). The mice were acclimated for one week prior to the study, and fed standard chow and water *ad libitum*. In particular, mice were maintained in specific-pathogen-free-conditions. Animals were subjected to surgical procedures in accordance with Institutional guidelines. Tumors were established by subcutaneous injection of 2x10⁶ BEL-7402 cells into the flank. Tumor volumes were estimated according to the formula: Tumor volume (mm³) = (length x width²)/2. When tumors reached a size of ~100 mm³, the mice were randomly assigned to 4 groups (n=6/group): Control and 3 treatment groups. The mice in the HSS-treatment groups were intragastrically administered 0.5 ml HSS daily, at doses of 62.5, 125 or 250 mg/kg in a solution containing 0.5% sodium carboxymethylcellulose. The vehicle treated control mice received 0.5% sodium carboxymethylcellulose. The mice were monitored closely

and weighed twice a week. The mice were sacrificed 26 days and then tumor xenografts, liver and kidney samples were collected for the following experiments.

Transmission electron microscopy (TEM) assay. Tumor tissues from HSS-treated mice were examined using transmission electron microscopy. Briefly, 1 mm³ tumor tissue sections were fixed in 4% (w/v) glutaraldehyde followed by 1% (w/v) osmic tetroxide, and then embedded in epoxy resin. Serial 0.5 μm sections were sliced and stained with uranyl acetate-lead citrate, and examined under an H-7650 transmission electron microscope at 80 kV (Hitachi, Ltd., Tokyo, Japan).

Histological evaluations. Tissue sections (4 μm) were generated from the paraffin-embedded tissue specimens, and were stained with hematoxylin and eosin (H&E). Endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol at room temperature for 15 min after deparaffinization and rehydration. Antigen retrieval was performed by heating the slides in 0.01 M sodium citrate buffer at 95°C for 3 min. After blocking with 10% bovine serum albumin in PBS for 60 min, the slides were incubated with a primary antibody against caspase-3 (1:10; cat. no. ab4051; Abcam) at 4°C overnight. After three washes in PBS, the slides were incubated with horseradish peroxidase-conjugated secondary antibodies (1:200; cat. no. ab6728; Abcam). The immune complex was visualized using 3,3'-diaminobenzidine-chromogen (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. Counterstaining was performed on representative sections with hematoxylin, and then the slides were observed under a light microscope (Olympus Corporation, Tokyo, Japan). Quantitative analysis was performed using Image-Pro Plus software (version 4.5; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Statistical analyses were performed with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). All data were presented as the mean ± standard deviation. The differences between groups were evaluated using one-way analysis of variance followed by Student Newman-Keuls test. P<0.05 is considered to indicate a statistically significant difference.

Results

HSS induces apoptosis of BEL-7402 cells. Our previous research demonstrated that HSS induces apoptosis of BEL-7402 cells, as determined by flow cytometric analysis and Hoechst 33258 nuclear staining (15). To further confirm these results, a DNA fragmentation assay was conducted, since DNA fragmentation is a classical hallmark of apoptosis. Apoptosis of BEL-7402 cells was observed following treatment with HSS for 48 h at 25, 50 and 100 μg/ml. HSS treatment resulted in increased DNA fragmentation in BEL-7402 cells, when treated with increasing HSS concentrations (Fig. 1). These results indicated that HSS exhibited a significant apoptosis-inducing effect on BEL-7402 cells.

HSS induces caspase activation in BEL-7402 cells. Activation of the caspase cascade is an important mechanism in apoptotic cell death in most cell systems (20). The enzymatic activities

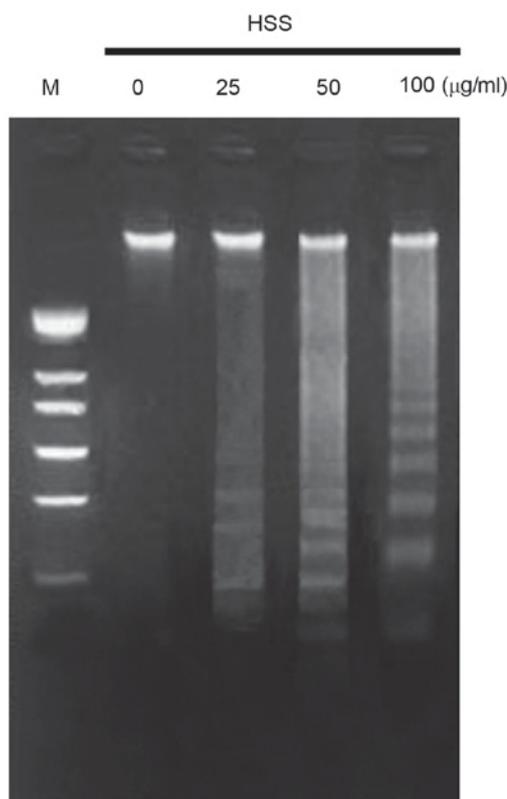


Figure 1. HSS induces DNA fragmentation in hepatocellular carcinoma-derived BEL-7402 cells. Cells were pretreated with 0, 25, 50 or 100 μg/ml HSS for 48 h, and DNA fragmentation was analyzed by agarose gel electrophoresis. Results are representative of three independent experiments. M, DNA markers of 2 and 1 kb, 750, 500 and 200 bp; HSS, Haishengsu.

of caspase-3 and caspase-9 were detected using a Caspase Colorimetric Assay kit. Following treatment with HSS, the enzymatic activities of caspase-3 and caspase-9 were significantly increased (Fig. 2A). The relative activities of caspase-3 and caspase-9 were increased to 2.438±0.219 (P<0.01) and 1.834±0.163 (P<0.01), respectively, following treatment with 100 μg/ml HSS. To further confirm the role of caspase, western blot analysis was performed to detect the protein levels of caspase-3 and caspase-9. In agreement with the caspase activity assay results, caspase-9 activation accompanied the proteolytic degradation of the inactive pro-enzyme (47 kDa) into the active form (37/35 kDa) (Fig. 2B). Furthermore, HSS exhibited a concentration-dependent effect on caspase-9 activation (Fig. 2C). Similarly, caspase-3 activation, via proteolytic degradation of the 32 kDa pro-enzyme into the 19/17 kDa activate form, was detected in the presence of HSS in a concentration-dependent manner (Fig. 2B and C).

HSS regulates apoptosis-related protein expression in BEL-7402 cells. To further investigate the involvement of the HSS-induced apoptotic pathway in BEL-7402 cells, the expression of apoptosis-related proteins: Bax, Bcl-2, cytochrome c, PARP and cleaved PARP, was determined by western blot analysis (Fig. 3). BEL-7402 cells treated with HSS for 48 h exhibited a concentration-dependent upregulation of the proapoptotic protein Bax, as compared with the control group, whereas the anti-apoptotic protein Bcl-2 was downregulated (Fig. 3A and C). The ratio of Bax/Bcl-2 was also significantly

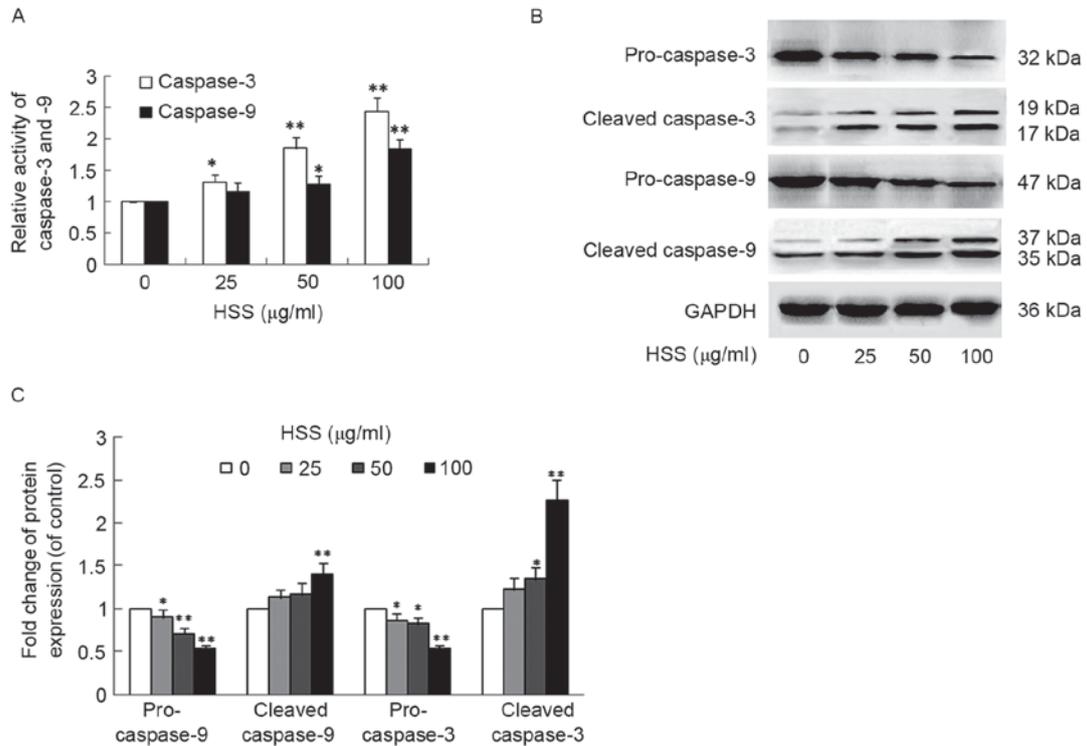


Figure 2. HSS induces caspase activation in hepatocellular carcinoma-derived BEL-7402 cells. (A) Cells were treated with 0, 25, 50 or 100 $\mu\text{g/ml}$ HSS for 48 h, and the caspase-3 and caspase-9 activities were detected using a Caspase Activity kit. (B) Protein levels of pro-caspase-3 and -9 in BEL-7402 cells were determined by western blot analysis and (C) quantified using Quantity One analysis software. GAPDH served as the loading control. Data are presented as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$, compared with the control group. HSS, Haishengsu.

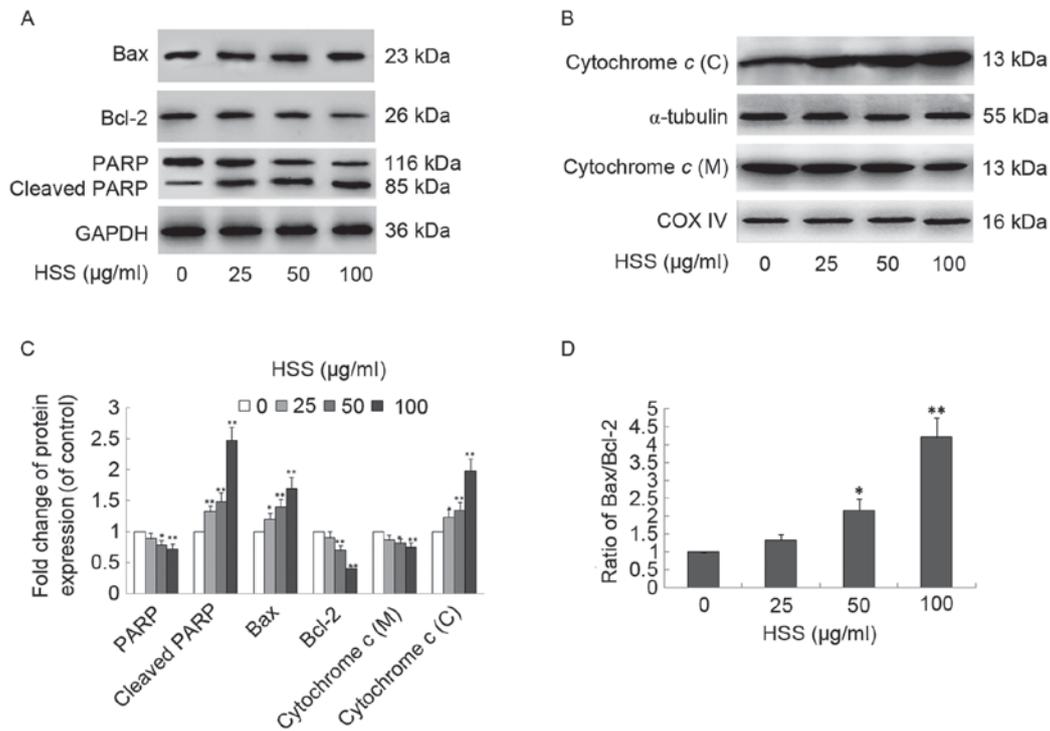


Figure 3. HSS induces alterations in apoptosis-related proteins in hepatocellular carcinoma-derived BEL-7402 cells. BEL-7402 cells were treated with 0, 25, 50 or 100 $\mu\text{g/ml}$ HSS for 48 h, and western blot analysis was performed on whole cell lysates, and cytosolic and mitochondrial fractions. (A) Expression of Bcl-2, Bax, PARP and cleaved-PARP was analyzed by western blot analysis. GAPDH from the same sample served as the loading control. (B) Cytochrome *c* localization assay. Cytochrome *c* was measured in mitochondrial and cytosolic fractions by western blot analysis. COX IV and α -tubulin were used as internal controls for the mitochondria and cytosol, respectively. (C) Protein expression levels of Bcl-2, Bax, PARP, cleaved PARP and cytochrome *c* were quantified using Quantity One analysis software in BEL-7402 cells. (D) Bax/Bcl-2 ratios following treatment with various concentrations of HSS were compared. Data are presented as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$, compared with the control group. HSS, Haishengsu; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly (ADP-ribose) polymerase; COX IV, cytochrome *c* oxidase IV; C, cytosolic; M, mitochondrial.

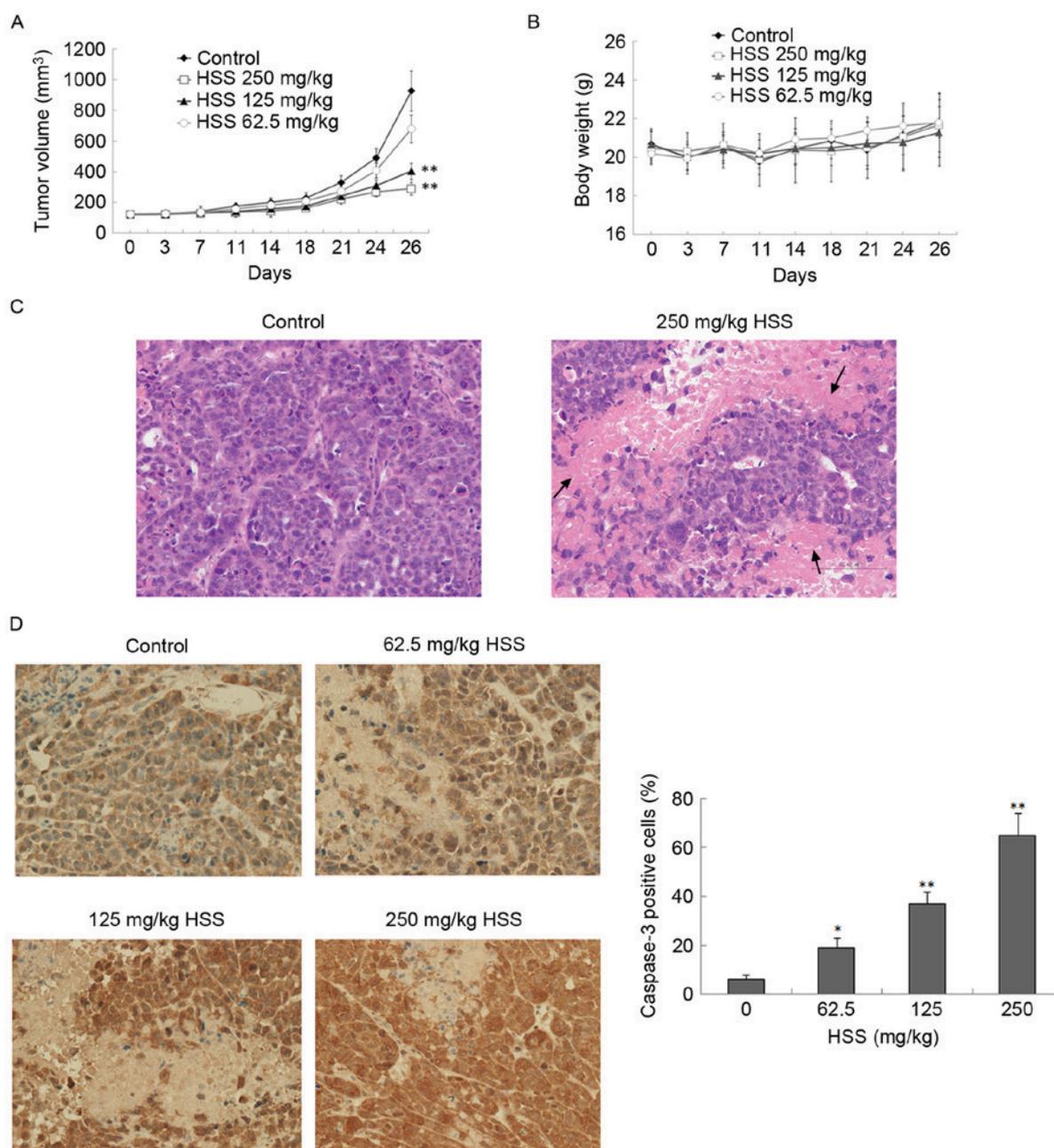


Figure 4. HSS treatment inhibits tumor growth of hepatocellular carcinoma-derived BEL-7402 cell xenografts and induces cell apoptosis in nude mice *in vivo*. (A) BEL-7402 tumors were established in the flank of mice. The mice were received daily intragastric administrations of 0.5% sodium carboxymethylcellulose, containing HSS doses of 62.5, 125 or 250 mg/kg, as indicated. (B) Bodyweight of the mice in the control and treatment groups. (C) Tumors were harvested after the mice were sacrificed (n=6/group), and were examined histologically following hematoxylin and eosin staining (magnification, x200). The small arrow indicates the necrotic regions. (D) Cells expressing caspase-3 were counted to calculate the apoptotic index (magnification, x200). Assay was conducted in triplicate. *P<0.05 and **P<0.01, compared with the control group. HSS, Haishengsu.

increased with HSS treatment (Fig. 3D). Furthermore, cytochrome *c* was released from the mitochondria to the cytosol; a decrease in the relative density of mitochondrial cytochrome *c* was observed, with an increase in the relative density of cytosolic cytochrome *c*, at 25, 50 and 100 $\mu\text{g/ml}$ HSS (Fig. 3B and C). PARP is a downstream target of active caspase-3 during the induction of the apoptotic signaling pathway, and previous research has indicated that it is cleaved into two fragments (21). HSS was also able to induce PARP cleavage into its active form, as indicated by a reduction in the full-length form (116 kDa) and an increase in the cleaved form (85 kDa) in a concentration-dependent manner (Fig. 3A and C).

HSS administration inhibits tumor growth in nude mice. The effects of HSS on tumor growth were further investigated using a xenograft model. BEL-7402 cells were implanted into the flank of female BALB/c nude mice. The experiment was terminated after 26 days and the tumor size was measured. Tumors in the control group developed faster than the HSS treatment groups, and were $928.9 \pm 131.4 \text{ mm}^3$ in size 26 days post-injection (Fig. 4A). HSS administration inhibited tumor growth in a concentration-dependent manner in xenograft nude mice (Fig. 4A). There were no significant differences in overall body weight between the control and the HSS-treated groups (Fig. 4B). Furthermore, H&E staining revealed an

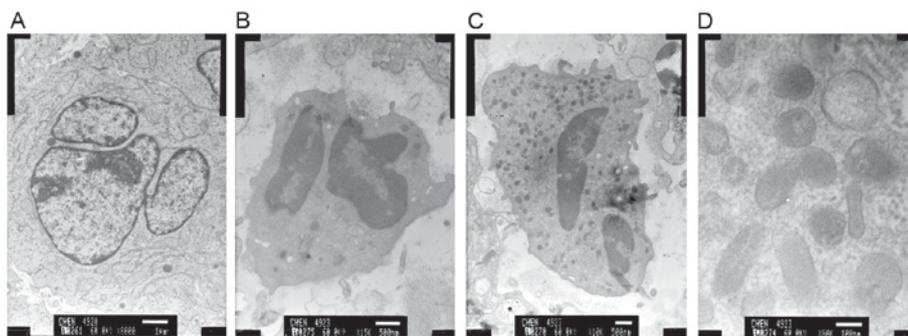


Figure 5. HSS administration alters the cellular ultrastructure in nude mice. Ultrastructural features of BEL-7402 cells induced by HSS were examined using a transmission electron microscope. (A) Control group (magnification, x8,000), (B) HSS group (62.5 mg/kg; magnification, x15,000), (C) HSS group (125 mg/kg; magnification, x10,000), and (D) HSS group (250 mg/kg; magnification, x80,000). HSS, Haishengsu.

increase in necrotic regions in the tumors of HSS treatment groups, compared with the control (Fig. 4C). Histological analysis of liver and kidney samples from both the control and HSS-treated mice revealed no obvious pathological alterations (data not shown), indicating that HSS did not exhibit any strong toxicity.

HSS administration increases apoptosis in nude mice. Caspase-3 expression was investigated in xenograft models by immunohistochemical staining. Treatment with HSS resulted in a significant increase in the proportion of caspase-3-positive cells, in a dose-responsive manner (Fig. 4D). HSS treatment significantly increased the ratio of caspase-3-positive cells by 13, 31 and 59%, at concentrations of 62.5, 125 and 250 mg/kg, respectively, compared with the control ($P < 0.05$).

To further evaluate the effects of HSS on apoptosis, the ultrastructural morphology of xenografts was observed by TEM. Irregular contours of cells and nuclei, nucleolar margination and reduced presence of cytoplasm were observed in the control group (Fig. 5A). Conversely, cells treated with HSS exhibited apoptotic phenomena; the nucleus decreased in size and demonstrated irregular indented edges and variable shape. The karyotheca was crumpled, the karyoplasm was dense and conglomerated, and the chromosomes were condensed and bound to the karyotheca (Fig. 5B and C). Furthermore, the mitochondria were swollen, and the mitochondrial membrane was reduced in thickness or partially ruptured (Fig. 5D). The mitochondrial alterations indicated that the HSS-induced apoptosis in BEL-7402 cells was associated with the mitochondrial-mediated apoptotic pathway.

Discussion

HCC is one of the most common malignancies worldwide, with ~630,000 new cases reported annually (22). Late diagnosis, recurrence and metastasis often result in a poor prognosis for patients with HCC, and the 5-year survival rate of patients undergoing surgical treatment is <5% (23). Chemotherapy with conventional cytotoxic agents is often very toxic and insufficiently efficacious (24). Therefore, novel chemotherapeutic agents and more effective therapies for the treatment of HCC are urgently required.

Natural products may provide promising sources of novel anticancer therapies. The limited side effects of natural

products in anticancer therapies has already been recognized, and >60% of drugs used to treat cancer are derived from natural sources (25). HSS is a natural extract from *T. granosa*, which has been used in clinical cancer therapy in China. Our previous research indicated that HSS may induce apoptosis of HCC cells in a concentration-dependent manner, via the Fas signaling pathway (15); however, the mechanisms involved in HSS-induced mitochondrial-mediated apoptosis in HCC cells remain unclear. In the present study, it was observed that HSS effectively induced the apoptosis of BEL-7402 cells via the mitochondrial-mediated pathway *in vitro*, and inhibited tumor xenograft growth *in vivo*.

Two major pathways are known to be involved in the regulation of apoptosis, the death receptor (extrinsic) pathway and the mitochondrial-mediated (intrinsic) pathway (26). Increasing evidence has indicated that these two apoptotic pathways are not isolated; crosstalk occurs between them. In the intrinsic pathway, mitochondria are the critical mediators of apoptosis (27-29). Certain stimuli, such as anticancer drugs, can induce permeabilization of the outer mitochondrial membrane and activate the mitochondrial pathway. The present study indicated that HSS treatment may induce the apoptosis of BEL-7402 cells through the intrinsic pathway. HSS treatment promoted the release of cytochrome *c* into the cytosol, which in turn cleaved and activated caspase-9. This resulted in the activation of caspase-3, as evidenced by an increase in the levels of the cleaved caspase-3 protein, and a decrease in the pro-caspase-3 form. Following caspase-3 activation, caspase-3 subsequently cleaves PARP into two fragments, and this leads to DNA fragmentation and the induction of apoptosis (29). The present study also demonstrated that HSS was able to induce the cleavage of PARP into its active form, as evidenced by a reduction in the full-length p116 form, and an increase in the cleaved p85 form. In agreement with the findings of the present study, Li *et al* demonstrated that HSS treatment resulted in caspase-3 activation and Bcl-2 downregulation in K562/ADM cells, demonstrating that HSS treatment could induce apoptosis in these tumor cells (12). Furthermore, our previous study reported that HSS treatment activated caspase-8 and upregulated Fas protein and mRNA expression in HCC cells (15), thus suggesting that HSS acts through the extrinsic and the intrinsic apoptotic pathways.

Bcl-2 family proteins regulate the activation of caspases during mitochondrial-mediated apoptosis. The Bcl-2 family of

proteins consists of anti-apoptotic proteins, such as Bcl-2, and a number of proapoptotic proteins, such as Bax; overexpression of Bcl-2 blocks mitochondrial outer membrane permeabilization and inhibits apoptosis (30). It has been proposed that an agent that could enhance the expression of proapoptotic proteins and/or inhibit the expression of anti-apoptotic proteins may induce apoptosis in cancer cells. Therefore, the balance between Bax and Bcl-2 serves a critical role in the intrinsic pathway of apoptosis (31). Downregulation of Bcl-2 and upregulation of Bax also stimulates the release of cytochrome *c* from the mitochondria into the cytosol. The present study indicated that HSS treatment upregulated the expression of Bax and downregulated the expression of Bcl-2, leading to an increase in the Bax/Bcl-2 protein ratio.

In conclusion, the results of the present study indicated that HSS could induce the apoptosis of HCC-derived BEL-7402 cells *in vitro* and *in vivo*, via intrinsic apoptotic pathways. These findings may provide the basis for further investigations into the potential clinical effects of *T. granosain* HCC.

Acknowledgements

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