

# Apoptosis is induced by shikonin through the mitochondrial signaling pathway

XUXIA TANG<sup>1</sup>, CHEN ZHANG<sup>1</sup>, JIONGZHOU WEI<sup>1</sup>, YUTING FANG<sup>1</sup>, RONGXIANG ZHAO<sup>1</sup> and JIANXIN YU<sup>2</sup>

<sup>1</sup>Department of Otolaryngology, The First Affiliated Hospital of Zhejiang Traditional Chinese Medical University;

<sup>2</sup>Department of Plastic Surgery, The Second Affiliated Hospital of Zhejiang University Medical College, Hangzhou, Zhejiang 310000, P.R. China

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**Abstract.** The aim of the present study was to investigate the effects of shikonin (SHI) on the induction of apoptosis in human TT medullary thyroid carcinoma cells, and to explore the role of mitochondrial signaling in this process. MTT, Annexin V-phycoerythrin/7-aminoactinomycin D staining, electron microscopy and JC-1 probe staining were performed to analyze mitochondrial membrane potential, and western blot analysis was used to examine the activation of the mitochondrial signaling pathway, and the changes in mitochondrial apoptosis pathway-associated protein expression. Following culture for 24-72 h, treatment with various concentrations of SHI inhibited the proliferation of TT cells, in a dose- and time-dependent manner. Transmission electron microscopy demonstrated the presence of typical apoptotic structures, as well as mitochondrial structural changes. The expression levels of apoptosis-associated proteins caspase-9, caspase-3 and poly adenosine triphosphate ribose polymerase increased in a dose-dependent manner following treatment with SHI. In addition, the mitochondrial membrane potential of the experimental group was significantly decreased, and the mitochondrial apoptosis pathway-associated proteins were altered. A possible mechanism underlying SHI-induced apoptosis through the mitochondrial signaling pathway is the regulation of B cell lymphoma 2 (Bcl-2)/Bcl-2-associated protein X expression levels, resulting in the decrease in mitochondrial membrane potential and the activation of the caspase-9/caspase-3 enzyme-associated reactions.

## Introduction

Medullary thyroid cancer (MTC) originates from the thyroid parafollicular cells (also termed C cells), accounting for 5-10% of all thyroid malignancies (1). MTC is a type of malignant tumor, the prevalence of which is marginally higher among the female population compared with the male population. At present, surgical removal of the tumor remains the preferred method for the treatment of MTC. Unlike differentiated thyroid cancer, treatment with radioiodine has no therapeutic effect on MTC, and the effects of radiotherapy, radionuclide therapy and chemotherapy remain poor. Biological immune treatments, including molecular targeted drugs, cancer vaccines, monoclonal antibodies, and suicide or immune genes have demonstrated therapeutic effects in clinical or pre-clinical trials (2,3). In recent years, natural medicine has had an important role in cancer treatment, and numerous natural monomer compounds, such as paclitaxel and matrine, have been shown to exhibit antitumor effects (4,5). Therefore, investigating novel therapeutic targets for the treatment of MTC is important for the identification of effective drugs.

*Lithospermum erythrorhizon* is the dry root of the perennial herb Comfrey *Arnebia*, and has a long medicinal history in China. Studies have demonstrated that shikonin (SHI), the main effective ingredient of *L. erythrorhizon* exhibits anti-tumor effects towards HL60 human promyelocytic leukemia cell lines (5), liver cancer (6), prostate cancer (7), colorectal cancer (8), oral squamous cell carcinoma (9), basal cell carcinoma (10) and osteosarcoma (11). SHI effects the metabolism, proliferation, differentiation, signal transduction and gene expression of tumor cells, and inhibits the activity of DNA topoisomerase, oxidative stress, and proteasomes, thereby inhibiting the growth of tumor cells (12,13). SHI has also been demonstrated to increase the sensitivity of tumor cells towards chemotherapeutic drugs, and may therefore serve as an effective chemical sensitizer (12,13). The death of tumor cells is predominantly induced via three mechanisms: Necrosis, apoptosis and autophagy. Previous studies reported that SHI induced tumor cell death through the apoptotic signaling pathway, and may act by inhibiting the activation of nuclear factor (NF)- $\kappa$ B (14); upregulating caspase proteases (15); inhibiting the expression of survivin (16); regulating the mRNA and protein expression of B cell lymphoma 2 (Bcl-2)

**Correspondence to:** Professor Jianxin Yu, Department of Plastic Surgery, The Second Affiliated Hospital of Zhejiang University Medical College, 388 Yu Hangtang Road, Hangzhou, Zhejiang 310000, P.R. China  
E-mail: jianxinyucn@126.com

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family-associated genes (8), p53 (8), c-myc (7) and Fas (9); and altering the mitochondrial membrane potential (17). Previous studies have also reported that SHI induced cell death through the non-apoptotic pathway (autophagy and necrosis-like programmed cell death) (14,15).

Studies have yet to report SHI-induced cell death of human TT medullary thyroid carcinoma cells or its underlying mechanism. Thus, the present study investigated SHI-induced cell death of TT cells.

## Materials and methods

**Materials and apparatus.** SHI, bovine serum albumin and lead citrate were obtained from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 was obtained from Gibco Life Technologies, (Carlsbad, CA, USA). MTT was obtained from Fuzhou Maixin Biotechnology Development Co., Ltd. (Fuzhou, China). Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) apoptosis detection kit, cell cycle kit and MitoScreen (JC-1) were obtained from BD Biosciences (San Diego, CA, USA). The following primary antibodies: Rabbit monoclonal anti-Bcl-2 (cat. no. 2870), rabbit monoclonal anti-myeloid cell leukemia 1 (Mcl-1) (cat. no. 5453), rabbit monoclonal anti-Bcl-extra large (xL) (cat. no. 2764), rabbit monoclonal anti-Bcl-2-associated X protein (Bax) (cat. no. 5023), rabbit polyclonal anti-Bcl-2 interacting protein (Bid) (human-specific; cat. no. 2002), mouse monoclonal anti- $\beta$ -actin (cat. no. 3700), rabbit polyclonal anti-caspase 3 (cat. no. 9662), rabbit polyclonal anti-cleaved caspase 3 (Asp175) (cat. no. 9661), rabbit polyclonal anti-caspase 9 (cat. no. 9502) and rabbit polyclonal anti-cleaved caspase 9 (cat. no. 9501) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyvinylidene difluoride membrane was obtained from EMD Millipore (Billerica, MA, USA). BD FACSCanto™ flow cytometer was obtained from BD Biosciences Inc.

**Cell culture.** Human TT medullary thyroid carcinoma cells (Sigma-Aldrich) were cultured in F-12K medium supplemented with 10% fetal bovine serum (Gibco Life Technologies) in a humidified incubator at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**MTT assay.** The TT cells were seeded into a 96-well culture plate at a density of 8–10 × 10<sup>3</sup> cells/well, and the supernatant was discarded when the cells became adherent to the wall. A total of 200  $\mu$ l SHI at various final concentrations (0.5, 1, 1.5, 2, 3, 4, 6 and 8  $\mu$ g/ml) was subsequently added, with five repeated wells per treatment group. A negative control group, consisting only of RPMI-1640 solution, was also established. Each treatment group was set up in three parallel wells, and in the control group an equal volume of complete media was added. Following drug-cultivation for 24, 48 and 72 h, an MTT assay was performed to analyze the impact of SHI on TT cell growth. The absorbance of each well at 570 nm was measured by iMark microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the inhibition rates were calculated according to the following equation: Inhibition rate = 1 - (OD<sub>dosing</sub> - OD<sub>blank</sub>) / (OD<sub>negative</sub> - OD<sub>blank</sub>) × 100. Where OD is the optical density. The experiment was repeated three times.

**Flow cytometry.** The cells were inoculated in 6-well culture plates at 20–40 × 10<sup>4</sup> cells/well. When the cells became wall adherent, 2 ml culture medium containing 2 or 4  $\mu$ g/ml drug was added to each well, and for the control group an equal volume of complete medium was added. The cells were collected after 24 h culture, and washed twice with phosphate-buffered saline (PBS), then resuspended in 500  $\mu$ l of 1X binding buffer. A total of 100  $\mu$ l cell suspension was then added into 5  $\mu$ l Annexin V-PE/7-AAD apoptosis detection solution, prior to being mixed and incubated in the dark at room temperature for 15 min. Following the addition of 400  $\mu$ l of 1X binding buffer, the apoptosis rate was detected using a Guava® EasyCyte™ Plus flow cytometer (EMD Millipore).

**Preparation of transmission electron microscopy (TEM) samples and observation of apoptotic morphology.** The cells in the logarithmic growth phase were seeded into the 6-well plates at a density of 1 × 10<sup>6</sup> cells/well, and the treatment drug was added 24 h after inoculation. The blank control group (RPMI-1640-treated cells) and the 24 h treatment groups (2 and 4  $\mu$ g/ml SHI-treated TT cells) samples were prepared and were sectioned using a Reichert ultramicrotome (Reichert-Jung Inc., Vienna, Austria), in order to obtain 70–90 nm sections, which were stained with lead citrate solution and uranyl acetate (Sigma-Aldrich) 50%-saturated ethanol solution for 15 min, respectively. Samples were then observed using a TEM (JEM-1400 Plus; JEOL USA, Inc., Peabody, MA, USA).

**Impact of SHI on mitochondrial membrane potential ( $\Delta\Psi_m$ ).** Cell culture was conducted as previously described in the flow cytometry method, and once the cells became wall adherent, they were washed twice with PBS, prior to treatment with 2 and 4  $\mu$ g/ml SHI-containing medium, and equal volume of complete medium for 16 h culture. The cells were collected, and a JC-1 probe was added prior to 15 min incubation at room temperature in the dark. The cells were then washed twice with MitoScreen (JC-1) kit, and the  $\Delta\Psi_m$  change was detected with a flow cytometer at 490 nm excitation wavelength, and mitochondrial morphology was observed using TEM.

**Western blot analysis.** The cell cultures and drug treatments were conducted as previously described in the flow cytometry methods section. The total protein of the 2 and 4  $\mu$ g/ml SHI-treated groups was extracted, and protein concentration was measured using the DC Protein Assay kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. The protein samples were subsequently separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc.), then transferred onto polyvinylidene fluoride membranes using the wet method. The membranes were then blocked with 3% bovine serum albumin for 1 h, prior to being incubated with primary antibodies (1:1,000; Cell Signaling Technology, Inc.) at room temperature for 3 h. The membranes were then washed three times with Tris-buffered saline with Tween® 20 (TBST), and incubated with the horseradish peroxidase-labeled secondary antibody at room temperature for 2 h, and washed three times with TBST. The membranes were treated with ECL reagents (GE Healthcare, Milwaukee, WI, USA) prior to analysis. Images of

Table I. Human TT medullary thyroid carcinoma cell growth inhibition rate following treatment with various concentrations of SHI (%).

Time (h)	Blank control group	Growth inhibition rate following SHI treatment ( $\mu\text{g}/\text{mg}$ )			
		0.5	1	2	4
24	-	11.3 $\pm$ 5.2	18.9 $\pm$ 5.5	29.3 $\pm$ 4.3 <sup>a</sup>	51.4 $\pm$ 4.1 <sup>a</sup>
48	-	15.8 $\pm$ 4.7	30.2 $\pm$ 3.1 <sup>a</sup>	47.2 $\pm$ 5.1 <sup>a</sup>	62.7 $\pm$ 3.5 <sup>a</sup>
72	-	28.4 $\pm$ 4.8 <sup>a</sup>	40.4 $\pm$ 3.6 <sup>a</sup>	66.0 $\pm$ 2.5 <sup>a</sup>	84.3 $\pm$ 2.7 <sup>a</sup>

<sup>a</sup>P<0.05, vs. the blank control group. SHI, shikonin.

Table II. Changes in the 24 h TT human medullary thyroid carcinoma cell cycle following treatment with various concentrations of SHI.

Group	M1 (G <sub>0</sub> /G <sub>1</sub> phase)	M2 (S phase)	M3 (G <sub>2</sub> /M phase)	M4 (sub-G <sub>0</sub> phase)
Negative group	68.8	5.7	25.0	0.2
2 $\mu\text{g}/\text{ml}$	22.2	2.2	10.3	21.0
4 $\mu\text{g}/\text{ml}$	13.3	1.3	3.0	52.3

Changes in TT cell cycle following 24 h culture with various concentrations of SHI. The percentages of M1, M2 and M3 were not identified to be significantly increased when compared with the negative control group (P>0.05). SHI, shikonin.

the target protein were captured and the expression levels were quantified using ChemiDoc™ XRS<sup>+</sup> system and Image Lab™ software (Bio-Rad Laboratories, Inc.). The following protein expression levels were detected in treated cells: Bcl-2, Bax, Bid, Bcl-xL, Mcl-1, cleaved caspase 9, cleaved caspase 3, and cleaved poly (ADP-ribose) polymerase, with  $\beta$ -actin serving as an internal control.

**Statistical analysis.** Comparisons between treatment groups were conducted by one-way analysis of variance, Student's t-test and Pearson correlation analysis. When the intergroup analysis showed no homogeneity, a rank sum test was performed. The SPSS 13.0 statistical package (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Impact of SHI on the proliferation of TT cells.** The effect of treatment with SHI on the proliferation of TT cells as determined by the MTT assay demonstrated that various concentrations of SHI affected the proliferation of TT cells after 24-72 h of culture. TT cell proliferation was shown to increase following treatment with SHI in a dose-dependent manner (Table I).

**TT cell cycle changes.** In the negative control group, flow cytometry demonstrated the absence of a sub-diploid peak (apoptosis peak), which appeared prior to the G<sub>0</sub>/G<sub>1</sub> phase, whereas in the 2  $\mu\text{g}/\text{ml}$  SHI-treated cells, the sub-diploid peak appeared after 24 h of culture. The 4  $\mu\text{g}/\text{ml}$  SHI-treated cells exhibited a marked pre-G<sub>0</sub>/G<sub>1</sub> phase sub-diploid peak (Fig. 1).

The following results in the cell cycle were observed following 24 h of treatment with various concentrations of SHI. The percentages of cells in the M1 (G<sub>0</sub>/G<sub>1</sub>), M2 (S), and M3 phase (G<sub>2</sub>/M) in the 24 h treatment groups (2 and 4  $\mu\text{g}/\text{ml}$  SHI-treated TT cells) showed no significant increase when compared with the negative control group (P>0.05; Table II), thus the effects of SHI on the percentages of cells in the various stages of the cell cycle were not significant.

**TT cell apoptosis.** 7-AAD and Annexin V-PE dual-labeling staining was performed to measure apoptosis levels. Compared with the negative control group, the 2  $\mu\text{g}/\text{ml}$  SHI-treated group exhibited a higher percentage (8.03%) of single-positive cells in early apoptosis (Annexin V-PE<sup>+</sup>/7-AAD<sup>-</sup>) after 24 h of treatment, compared with the control group (0.1%). The percentage of double positive cells in late apoptosis, namely the secondary necrosis phase (Annexin V-PE<sup>+</sup>/7-AAD<sup>+</sup>), increased to 11.33%, compared with the control group (0.1%). After 48 h of treatment, the percentage of single-positive cells in early apoptosis (Annexin V-PE<sup>+</sup>/7-AAD<sup>-</sup>) was increased to 24.99%, and that of the cells in late apoptosis (Annexin V-PE<sup>+</sup>/7-AAD<sup>+</sup>) was increased to 14.19%, compared with the control. After 72 h treatment, the double positive cells (late apoptosis) were the predominant cells, and the ratio markedly increased to 68.73%, whereas single-positive cells accounted for only 6.1%. Intergroup comparison revealed that these results were statistically significant (P<0.01) (Fig. 2).

**Apoptotic morphology observation.** TEM was used to observe the cell morphology of the control group after 24 h treatment (Fig. 3A). TEM observation revealed that the apoptotic morphology of the 2  $\mu\text{g}/\text{ml}$  SHI-treated group exhibited

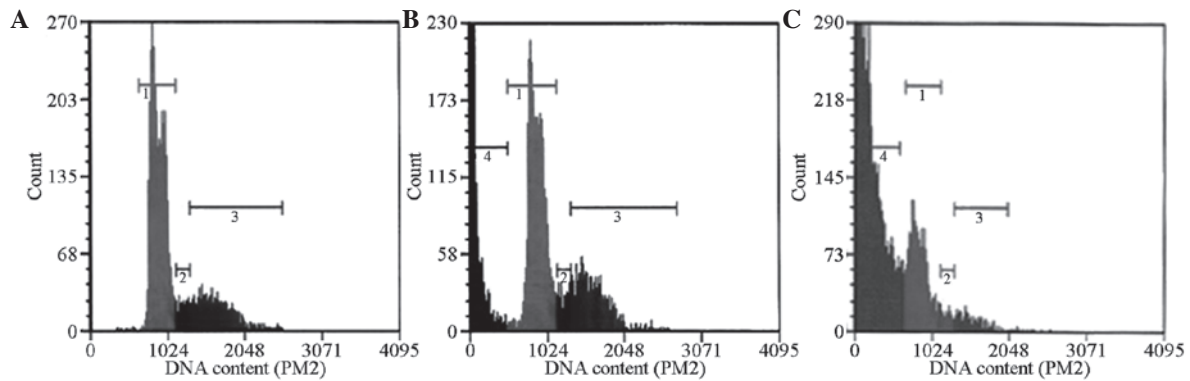


Figure 1. Effect of SHI on TT human medullary thyroid carcinoma cell cycle, as determined by flow cytometric analysis. (A) Negative control, (B) 2  $\mu\text{g/ml}$ , and (C) 4  $\mu\text{g/ml}$  SHI-treated groups. SHI, shikonin.

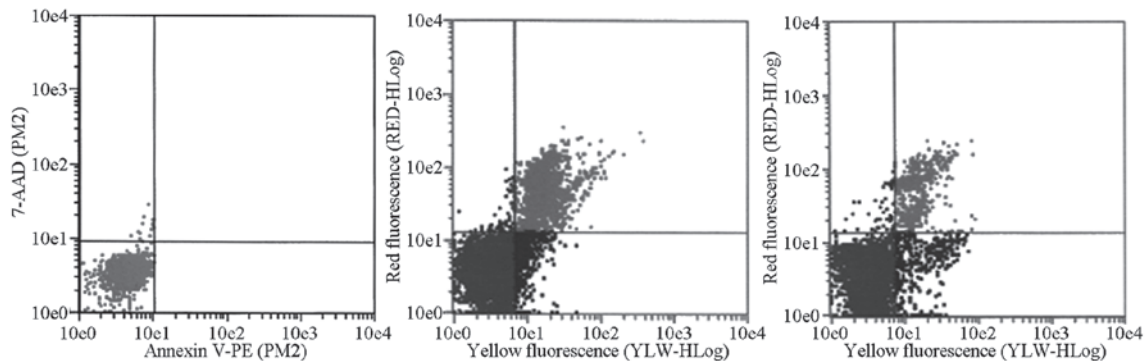


Figure 2. Effect of SHI on TT human medullary thyroid carcinoma cell apoptosis. The TT cell cycle of the experimental groups analyzed by flow cytometry 24 h following treatment. The 2 and 4  $\mu\text{g/ml}$  SHI-treated groups exhibited marked sub-diploid peaks after 24 h treatment. The cells treated with  $>2 \mu\text{g/ml}$  SHI exhibited apoptotic peaks (sub-diploid peaks), and the ratio of sub-diploid peaks was positively correlated with concentration, and exhibited statistically significant differences with the control group ( $P<0.01$ ), in which no cell cycle blockage was observed. SHI, shikonin.

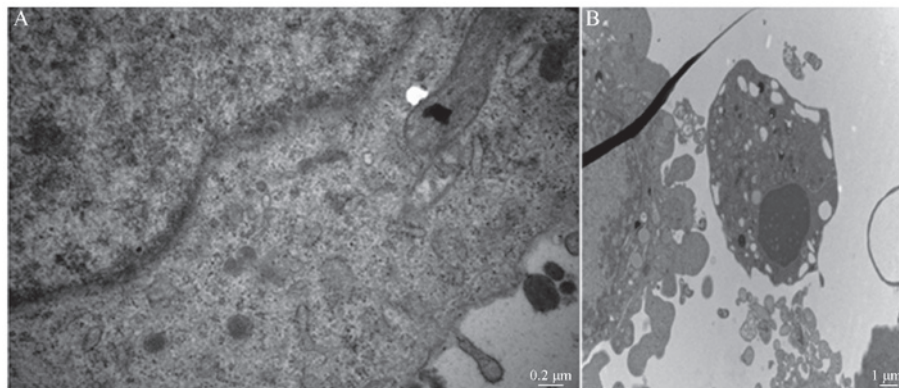


Figure 3. TEM observation of the TT cell-apoptotic morphologies in each group after 24 h of treatment with SHI. (A) Apoptotic morphology of the negative control group after 24 h treatment, as determined by TEM. The inner organelles, nucleus, chromosomal morphology and distribution are all normal. (B) Apoptotic morphology of the 2  $\mu\text{g/ml}$  SHI-treated groups after 24 h treatment as determined by TEM. The cells are shrunken, and although the membrane structure is intact, the nuclei are condensed or disintegrated and dispersed in the cytoplasm; the chromatin is condensed and marginalized. TEM, transmission electron microscopy; SHI, shikonin.

morphological changes typical of apoptosis after 24 h treatment (Fig. 3B).

**Detection of  $\Delta\Psi_m$ .** Detection of the  $\Delta\Psi_m$  of the TT cells by flow cytometry revealed that after 4 h of treatment, the percentage of 2  $\mu\text{g/ml}$  SHI-treated cells with impaired mitochondria increased from 10.1 to 23.4% ( $P<0.05$ ), and that of

the 4  $\mu\text{g/ml}$  SHI-treated cells increased to 42.1% ( $P<0.05$ ), compared with the control group. These results suggest that SHI may decrease the  $\Delta\Psi_m$  in the TT cells, thereby damaging the mitochondrial membrane (Fig. 4).

TEM was used to observe mitochondrial morphology (Fig. 5), and demonstrated that the mitochondria of the negative control group were stained, with a clearly defined

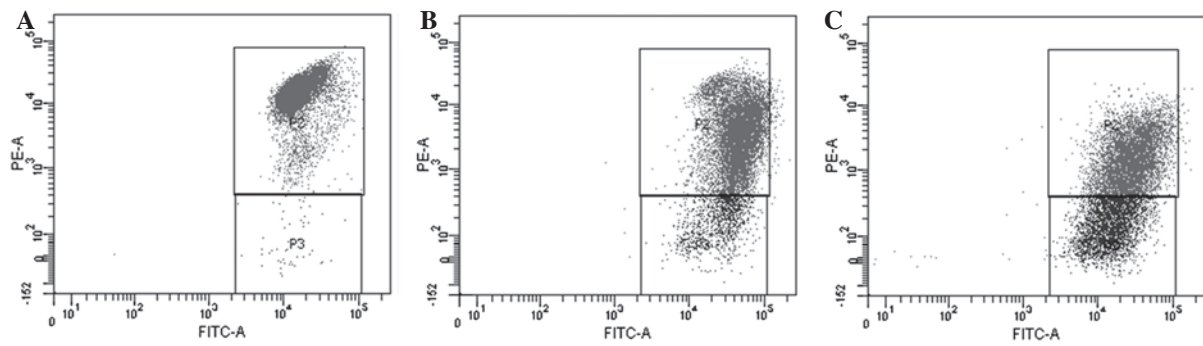


Figure 4. SHI induced the loss of mitochondrial membrane potential ( $\Delta\psi_m$ ). TT cells were treated with the indicated concentration of SHI for 16 h.  $\Delta\psi_m$  was determined by flow cytometry using JC-1 Staining. (A) Control; (B) 2  $\mu\text{g/ml}$  SHI-treated cells; (C) 4  $\mu\text{g/ml}$  SHI-treated cells. SHI, shikonin.

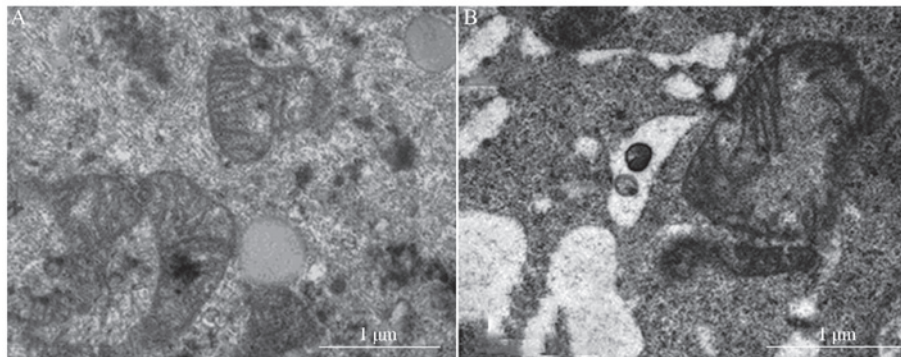


Figure 5. Observation of the mitochondrial morphologies of each group 24 h following SHI treatment by TEM. (A) Observation of the mitochondrial morphology of TT cells of the negative control group by TEM after 24 h treatment with SHI. The mitochondria is markedly stained, and the mitochondrial ridge structure is clearly defined. (B) Observation of TT cells of the 2  $\mu\text{g/ml}$  SHI-treated group by TEM after 24 h of treatment: The mitochondria are abnormally enlarged, and the mitochondrial ridge and membrane are ruptured. SHI, shikonin; TEM, transmission electron microscopy.

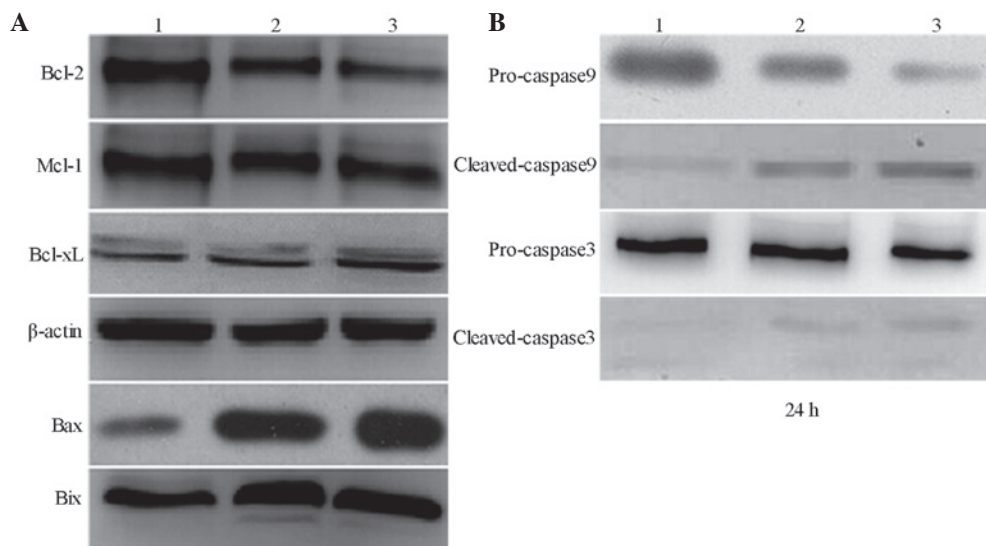


Figure 6. Effect of treatment with various concentrations of SHI on the expression levels of Bax, Bcl-xL, Bcl-2 and Bik. (A) Effect of SHI on the expression of Bax, Bcl-xL, Bcl-2 and Bik. (B) Effect of SHI on the expression of caspase-9 and 3. Lane 1, control; lane 2, 2  $\mu\text{g/ml}$  SHI-treated cells; lane 3, 4  $\mu\text{g/ml}$  SHI-treated cells. SHI, shikonin; Bcl-2, B cell lymphoma 2; Bcl-xL, B cell lymphoma extra large; Bax, Bcl-2-associated protein X; Bid, Bcl-2 interacting protein; Mcl-1, myeloid cell leukemia 1.

mitochondrial ridge structure (Fig. 5A). Conversely, after 24 h treatment, the 2  $\mu\text{g/ml}$  SHI-treated group exhibited abnormally enlarged mitochondria, and the mitochondrial ridge and membrane were both ruptured (Fig. 5B).

Western blotting determined that SHI alters the expression levels of the Bcl-2 family proteins. Following SHI treatment, western blotting demonstrated that the expression levels of anti-apoptotic proteins Bax and Bid were markedly increased,

whereas those of Bcl-2, Bcl-xL and Mcl-1 were markedly decreased. This was shown to occur in a dose-dependent manner (Fig. 6A). In addition, following TT cell treatment with 2 and 4  $\mu\text{g/ml}$  SHI, the levels of caspase-9 and 3 increased in a dose-dependent manner (Fig. 6B).

## Discussion

SHI is the predominant active ingredient of the traditional Chinese medicine *Lithospermum erythrorhizon*. Its antitumor effects were first reported in 1977 by Sankawa *et al* (18), who demonstrated that treatment with 5-10 mg/kg/day SHI was able to effectively inhibit the proliferation of mouse S180 ascites sarcoma cells. Since then, further research has determined that SHI may be used as an anticancer drug with multiple targets, exhibiting its antitumor effects by inducing tumor cell apoptosis, inhibiting cell proliferation, inducing cell differentiation and inhibiting tumor cell metastasis (8,9).

A previous study demonstrated that mitochondria had an important role in the apoptosis of malignant tumor cells (19). Decreased  $\Delta\Psi\text{m}$  is an early manifestation of apoptosis, and is followed by mitochondrial structural damage, during which small molecules are released, including cytochrome *c* and apoptosis-inducing factors, and the caspase-9 and 3 enzyme-linked reactions are activated (8). An association between SHI-induced TT cell apoptosis and mitochondrial signaling has yet to be reported.

The present study used TEM to observe mitochondria, and demonstrated that following treatment with 2  $\mu\text{g/ml}$  SHI for 24 h, the mitochondria of the TT cells were abnormally enlarged and swollen, and the mitochondrial ridge and membrane were ruptured. Following 4 h of treatment with SHI, the  $\Delta\Psi\text{m}$  of the TT cells decreased, resulting in damage to the mitochondrial membrane. These results suggested that SHI-induced apoptosis was associated with a decrease in  $\Delta\Psi\text{m}$  and changes in mitochondrial morphology.

The induction and regulation of mitochondrial outer membrane permeabilization involved numerous proteins, specifically those belonging to the Bcl-2 family. The pro-apoptotic Bcl-2 family members, Bax and Bak, contributed to the mitochondrial outer membrane permeabilization, thereby causing the activation of caspase-9, whereas the anti-apoptotic Bcl-2 and Bcl-xL proteins exhibited inhibitory effects. During the process of SHI-induced apoptosis, the mitochondrial changes increased in a dose-dependent manner, upregulating the expression of Bax and downregulating the expression of Bcl-2, thereby increasing the Bax/Bcl-2 ratio. A previous study also revealed that SHI was able to induce the upregulation of Bax and the downregulation of Bcl-2 in other tumor cells (20).

The increased Bax/Bcl-2 ratio indicated that the TT cells underwent apoptosis via the mitochondrial signaling pathway, and as the  $\Delta\Psi\text{m}$  was decreased, the apoptotic cascade was activated. These results demonstrated that SHI was able to increase the Bax/Bcl-2 expression ratio. Therefore, the SHI-induced decrease in  $\Delta\Psi\text{m}$  may be viewed as the cause of the change in expression levels of the Bcl-2 family proteins.

Previous studies demonstrated that in numerous cell types, such as human bladder cancer cells and oral squamous cell carcinoma cells, SHI may activate caspase-9 and caspase-3 via the mitochondria-dependent signaling pathway (21-23),

thereby demonstrating their roles in the promotion of tumor cell apoptosis. In the present study, western blot analysis demonstrated that the expression levels of caspase-9 and caspase-3 in the SHI-treated cells were downregulated. These results demonstrated the important role of caspases in the SHI-induced TT apoptosis process. The SHI-induced regulation of TT cell apoptosis was mediated by the mitochondrial signaling pathway.

The results of the western blot analysis demonstrated that the expression levels of caspase-9 and caspase-3 in the SHI-treated cells were downregulated, and RT-qPCR analysis also revealed similar results. These results suggested that the caspases had important roles in SHI-induced apoptosis of TT cells.

In conclusion, SHI induced apoptosis of TT cells through the mitochondrial signaling pathway. This was shown by an increase in the expression levels of Bcl-2 apoptotic precursor proteins Bax and Bid and decreased expression levels of anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1, thereby increasing the Bax/Bcl-2 ratio. This resulted in a decrease in the  $\Delta\Psi\text{m}$ , activation of caspase-9 and caspase-3, and thus apoptosis.

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