Berbamine induces SMMC-7721 cell apoptosis via upregulating p53, downregulating survivin expression and activating mitochondria signaling pathway

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Abstract. Hepatocellular carcinoma (HCC) is a primary malignancy in the liver, which is a global health problem. The present study aimed to observe the apoptotic effects of berbamine on SMMC-7721 cell lines and to investigate the effects of berbamine on induction of the intrinsic apoptotic pathway. The human HCC SMMC-7721 cells were cultured and cell morphology observed using a phase contrast microscope. SMMC-7721 cell apoptosis was examined by employing a flow cytometry assay. The nuclei of SMMC-7721 cells were stained with DAPI and observed by utilizing a laser fluorescence microscope. Cytochrome c (Cyto c) levels were evaluated by using immunofluorescence staining. The reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR) and western blot analysis were used to examine the mRNA and protein levels of B-cell lymphoma 2, (Bcl-2), Bax, Bcl-2-associated X, apoptosis regulator, p53 and survivin, respectively. Berbamine inhibited SMMC-7721 cell growth at 20 and 0 μmol/l, compared with control group (0 μmol/l berbamine). DAPI results demonstrated that berbamine affected the nucleus morphology of SMMC-7721 cells. Berbamine at a concentration of 20 μmol/l (P<0.05) and 40 μmol/l (P<0.01) significantly enhanced apoptosis rate compared with control group. Berbamine triggered Cyto c release from SMMC-7721 cell nuclei to the cytoplasm. Berbamine (10, 20, 40 μmol/l) significantly enhanced Bax and p53 levels and decreased Bcl-2 and survivin levels compared with control group, according to RT-sqPCR and western blot assay findings. In conclusion, berbamine induced SMMC-7721 cell apoptosis, through upregulating p53 expression and downregulating survivin expression, which further triggered mitochondria signaling pathway-mediated apoptosis.

Introduction

Hepatocellular carcinoma (HCC) is a kind of primary malignancy in the liver, which is a global health problem, and characterizes with higher mortality and morbidity rates (1-3). The HCC is the 5th most prevalent type of cancer and the 3rd leading reason for the higher cancer-associated mortality all over the world (2,4). In the worldwide, there are ~650,000 HCC cases were reported annually, in which 75-80% distributes in the Asian countries, and 55% distributes in China (5,6). Meanwhile, in China, the HCC has become the second leading cause for the cancer-related mortality (7). Nowadays, the chemotherapetic treatment is the most common and efficacy strategy for HCC therapy, however, a series of side-effects, such as the drug resistance and the toxicity, limit the application in clinics (8). Therefore, attempting to discover a kind of effective chemotherapeutic agent is urgent and critical for the HCC therapy.

In recent years, many traditional Chinese medicine has been studied and a few of effective and safe antitumor compounds have been screened (9). Berbamine is one of the active components that extracted from the *Berberis amurensis*, a traditional Chinese medicine, which is a kind of alkaloid (10). The berbamine is crystallized and it's structure (molecular formula: $C_{37}H_{48}N_2O_6 \cdot 2HCl$) has been revealed in the past years (10). The berbamine has been applied for centuries, to treat the inflammatory diseases and the other disorders (11-21). The natural compounds and their associated derivatives of the traditional Chinese medicine are the promising candidates for the anticancer drugs (15,16). Actually, a large number of the phytochemicals characterize with anticancer effects in many cancer model in both *in vivo* and *in vitro* levels (17). In recent years, many reported that the berbamine could inhibit proliferation of plenty tumors *in vitro* levels, including breast cancer, non-small cell lung cancer (NSCLC), multiple myeloma and the leukaemia (18-21). Especially, the berbamine could

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significantly inhibit the proliferation of the durg-resistant leukemia *in vivo* (22). Moreover, the berbamine could also therapy the imatinib-resistant chronic myeloid leukemia effectively according to a clinical research (23).

To the best of our knowledge, there are only a few studies (15,24,25) investigating the effects of berbamine on the human HCC. Therefore, further researches are required and necessary to investigate the biological function and protective roles of berbamine in HCC. In the present study, the hepatocarcinoma cell, SMMC-7721 was used, which is a commonly used representative HCC cell line according to the previous studies (6,15,16). The SMMC-7721 was treated *in vitro* with the berbamine at different concentrations. This study aims to observe the apoptotic effects of berbamine on SMMC-7721 cell lines and to investigate the effects of berbamine on induction of intrinsic apoptotic pathway.

Materials and methods

Cell culture of SMMC-7721. The human normal hepatocyte and the human HCC cell line, SMMC-7721, were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The SMMC-7721 cells were cultured in the Dulbecco's modified Eagle's medium (DMEM) containing 100 μg/ml streptomycin sulfate, 100 U/ml penicillin and 10% fetal bovine serum (FBS) (all from Gibco-BRL, Grand Island, NY, USA). The SMMC-7721 cells were incubated in a humidified incubator (Thermo Electron Corporation, Waltham, MA, USA) and 5% CO_2 atmosphere at 37°C. The SMMC-7721 cells were detached from the cell plates by utilizing the 0.25% trypsin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 3 min when the cells growing to near confluence.

Cytotoxicity on cells (MTT assay) and IC_{50}. The MTT assay was employed to examine the cytotoxicity (IC_{50}) or the cell viability. Briefly, the normal hepatocyte and the SMMC-7721 were placed within the 96-well culture plates at a density of $5x10^3$ cells/well in 200 μl medium at 37°C for 24 h. Total of 20 μl of MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to the 96-well plates, and cultured for 4 h. Then, the supernatant was discarded and the DMSO was added (150 μl/well; Sigma-Aldrich; Merck KGaA). The absorbance was measured by using a Universal microplate reader (SpectraMax Plus 384; Molecular Devices, LLC, Sunnyvale, CA, USA) at the wavelength of 570 nm. The cytotoxicity of normal hepatocyte of SMMC-7721 cells was illustrated as IC_{50} (concentration of 50% cytotoxicity, which was extrapolated form the linear regression analysis of experimental findings).

Cell morphology observation. The SMMC-7721 cells were added to the DMEM medium and the cell concentration was adjusted to $1x10^5$ cells/ml, and the cell were seeded into the 6-wells plate (2 ml/well). The cells were cultured for 24 h, and then treated with the different concentration of crystallized berbamine (10, 20 and 40 μmol/l, cat. no. 6078-17-7; Shanghai Standard Technology Co., Ltd., Shanghai, China) for another 24 h. The berbamine at the concentration of 0 μmol/l was considered as control group (without berbamine treatment, and only with DMEM). The cell morphology was observed and photographed by employing the phase contrast microscope.

Apoptosis examination. SMMC-7721 cells apoptosis was examined by employing FITC-Annexin V/PI cell apoptosis kit (cat. no. C1062; Beyotime Biological Technology Co., Ltd., Shanghai, China) due to the manufacturer's instructions. The SMMC-7721 cells were harvested by utilizing 0.25% trypsin, and rinsed the PBS for three times, then cells were suspended in the 500 μl binding buffer. The suspended SMMC-7721 cells were treated with Annexin V-FITC (5 μl) at 4°C for 10 min. Then, SMMC-7721 cells were treated with 7-ADD solution (10 μl) at 4°C for 5 min. Finally, SMMC-7721 cell apoptosis was evaluated by using FACS flow cytometer (BD Biosciences Inc., Franklin Lakes, NJ, USA).

DAPI staining. The cover glasses were embedded into the 6-well plates, and the SMMC-7721 cells were added for culturing 4 h. Then, the different concentrations of berbamine (0, 10, 20 and 40 μmol/l) were added into the culture medium. Total of 48 h later, the SMMC-7721 cells were fixed with the 4% paraformaldehyde for 15 min and subsequently incubated with the LysoTracker (red) at 37°C for 30 min. The cells were then washed by using the PBS for 3 times, and the nuclei were counter-stained by using the 4'-6-diamidino-2-phenylindole (DAPI; Roche Diagnostics, Basel, Switzerland). The fluorescent images were observed by utilizing a laser fluorescence microscope (Olympus, Tokyo, Japan).

Immunofluorescence staining for cytochrome c (Cyto c). The treatment for cells were performed according to the processes of 'DAPI staining' section. After the PBS washing, the SMMC-7721 cells were incubated by using the 5% BSA (Gibco-BRL) for 0.5 h, and washed with PBS for 3 times. Then, the cells were incubated with the rabbit anti-human Cyto *c* monoclonal antibody (cat. no. ab133504, 1:1,000; Abcam, Cambridge, MA, USA) at room temperature for 2 h. Then, the cells were washed with the PBS for 3 times, and 5 min per time. The cells were incubated with the FITC-conjugated goat anti-rabbit IgG (cat. no. ab6717, 1:2,000; Abcam) at room temperature for 1 h in dark. Finally, the cells were re-stained by using the DAPI for 10 min, and mounting. The fluorescent images were observed by using a fluorescence microscope (Olympus).

Reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR). Total cellular RNAs were extracted by utilizing RNAsimple Total RNA kit (Sigma-Aldrich; Merck KGaA). Then, the First-Strand cDNA Synthesis kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to synthesize cDNA due to manufacturer's instructions. Then, the synthesized cDNA was used to amplify the target genes by utilizing PCR assay under the following conditions: 95°C for 4 min, following with 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and terminated with 72°C for 10 min. The primers for Bcl-2, Bax, p53, survivin and GAPDH (synthesized by Western Biotechnology Inc., Chongqing, China) were listed in Table I. Finally, the amplified products were loaded onto 1.5% agarose gels, and the images were digitally captured

Table I. Sequence of the primers used for reverse transcription-semi-quantitative polymerase chain reaction.

Genes	Sequence (5'-3')
Bax	
Forward	GCTCTGAGCAGATCATGAAG
Reverse	CAAAGTAGAAAAGGGCGACA
Bcl-2	
Forward	CCGGGAGATAGTGATGAAGT
Reverse	ACTCAAAGAAGGCCACAATC
p53	
Forward	ACATGTGTAACAGTTCCTGC
Reverse	TCCATCCAGTGGTTTCTTCT
Survivin	
Forward	CCAGTGTTTCTTCTGCTTCA
Reverse	CGCACTTTCTTCACAGTTTC
GAPDH	
Forward	AAGATCATCAGCAATGCCTC
Reverse	TTGGCAGGTTTTTCTAGACG

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X, apoptosis regulator.

Figure 1. Observation for the IC$_{50}$ and cell viability. (A) IC$_{50}$ examination. **P<0.05 represents the IC$_{50}$ in SMMC-7721 group compared to normal hepatocyte group. (B) Cell viability was examined by using MTT assay. *P<0.05 represents the cell viability compared to control group. #P<0.05 represents the cell viability compared to 10 μmol/l berbamine. &P<0.05 represents the cell viability compared to 20 μmol/l berbamine.

by using the camera and analyzed with the image analysis software.

Western blot analysis. The SMMC-7721 cells were lysed with the lysis buffer. The lysis products were separated by using the 15% SDS-PAGE and the total proteins in the gels were electro-transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with the 5% defatted milk in the PBS supplementing with 0.05% Tween-20 at 4°C for 1 h. The membranes were incubated with the rabbit anti-human Bcl-2 monoclonal antibody (cat. no. ab31124, 1:3,000) and mouse anti-human Bax monoclonal antibody (cat. no. ab77566, 1:3,000), rabbit anti-human survivin polyclonal antibody (cat. no. ab469, 1:2,000) (all from Abcam), mouse anti-human p53 monoclonal antibody (cat. no. 554167, 1:3,000; BD Biosciences Inc.) and rabbit anti-human GAPDH polyclonal antibody (cat. no. ab9385, 1:2,000; Abcam) at 4°C overnight. Then, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. ab6721, 1:2,000) and HRP-conjugated goat anti-mouse (cat. no. ab6789, 1:2,000) (both from Abcam) at room temperature for 1.5 h. The western blot bands were visualized by using the TMB color liquid (PE Applied Biosystems, Foster City, CA, USA).

Statistical analysis. The data were analyzed by using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The data were illustrated as the mean ± standard deviation. The comparisons among multiple groups were analyzed by using the ANOVA test followed by the Tukey's post host test. The comparisons between two groups were analyzed by using the Student's t-test. P-value <0.05 was considered to indicate a statistically significant difference.
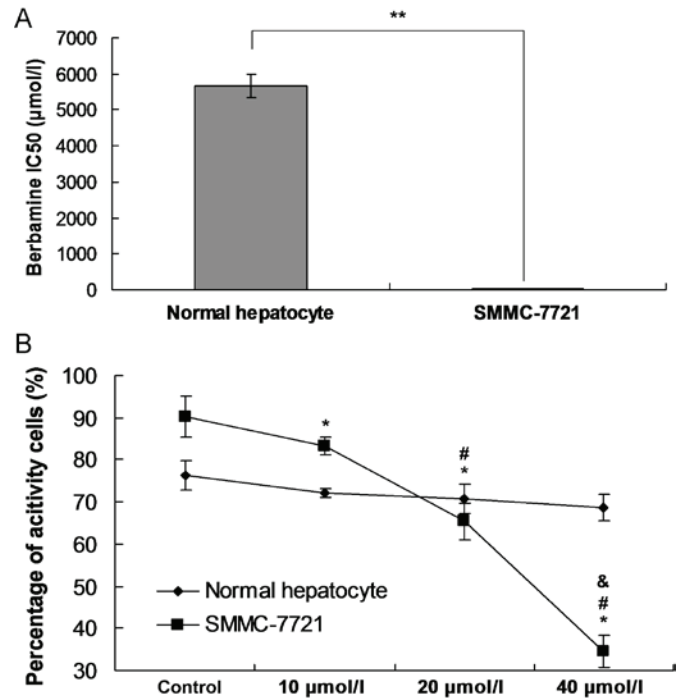
Results

Berbamine exhibits no significant cytotoxic potential to normal hepatocyte. In order to confirm the cytotoxic potential of berbamine to the normal hepatocyte, the IC$_{50}$ was examined, and the MTT assay was also performed in this study. The results indicated that the IC$_{50}$ value in normal hepatocyte group (5670.94 μmol/l) was higher significantly compared to the SMMC-7721 group (35.48 μmol/l) (Fig. 1A; P<0.001). The MTT assay also showed that the there were even not cytotoxic effects of berbamine on the cell viability of normal hepatocyte at different concentrations (Fig. 1B). However, the cell viability of SMMC-7721 group was significantly decreased following with the increased concentration of berbamine (Fig. 1B; all P<0.05).

Berbamine inhibits SMMC-7721 cells growth. According to the cell morphology, the SMMC-7721 cells in control group grew well, and formed the fusion colony cells (Fig. 2A). However, following the increased concentration of berbamine (from 10 to 40 μmol/l), the SMMC-7721 cell amounts were obviously decreased (Fig. 2B-D). Meanwhile, the SMMC-7721 cells in Control group were pleomorphic, and SMMC-7721 cells in other group were round or elliptical (Fig. 2). Moreover, some of the cells also suspended in the medium in 20 and 40 μmol/l berbamine group. These results suggest that the berbamine obviously inhibited the SMMC-7721 cells growth.

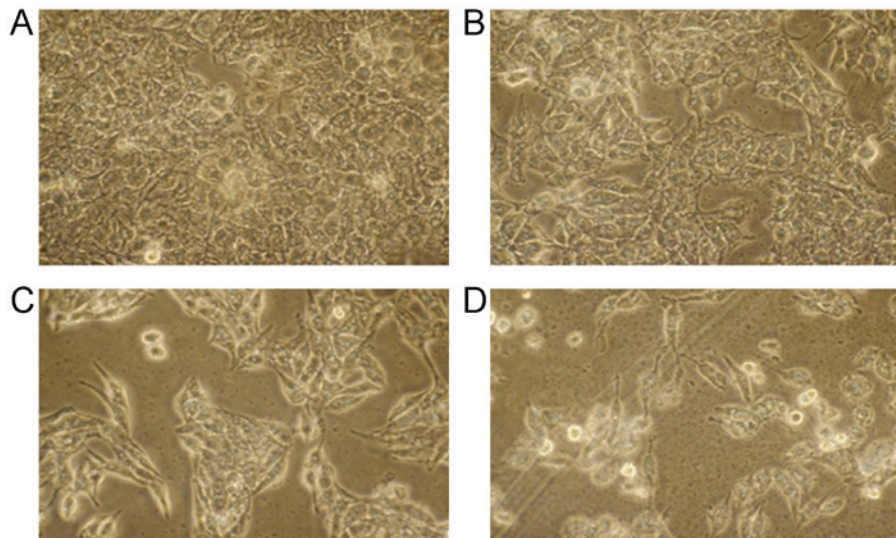*Berbamine affects nucleus morphology.* The SMMC-7721 cells were stained with DAPI and observed under the

Figure 2. Cell morphology observation for SMMC-7721 cells under inverted microscope (magnification, x200). (A) Control group; (B) 10 μmol/l berbamine; (C) 20 μmol/l berbamine; and (D) 40 μmol/l berbamine.
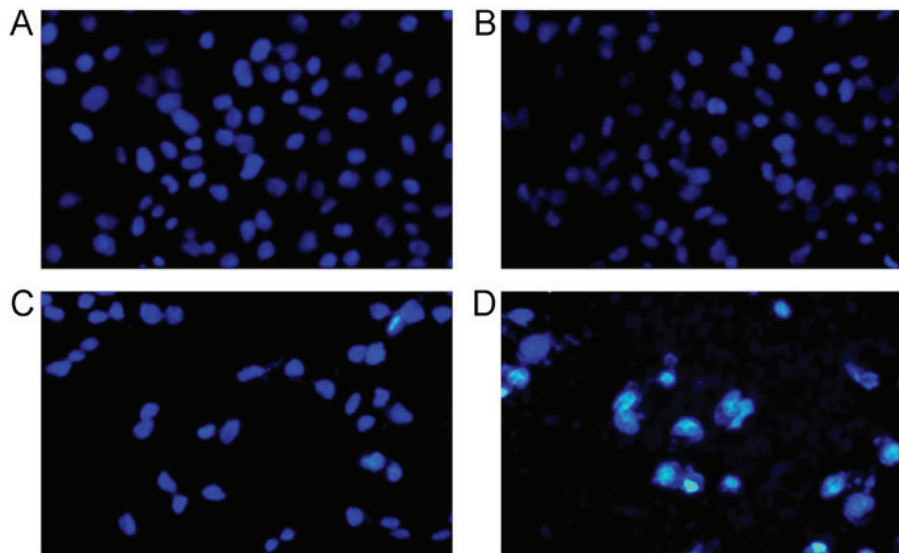

Figure 3. DAPI staining for SMMC-7721 cell nucleus observed under immunofluorescence microscope (magnification, x200). (A) Control group; (B) 10 μmol/l berbamine; (C) 20 μmol/l berbamine; (D) 40 μmol/l berbamine. The light stained cells represent the DAPI positively stained cells.

microscope. The results indicated that the cells illustrated the normal cell morphology and the nucleus were round and stained homogeneously in control group (Fig. 3A). The nucleus were narrowed and deeply stained 48 h post the 10 μmol/l berbamine treatment (Fig. 3B). In 20 μmol/l berbamine treatment group, we found the deeply stained nucleus, chromatin condensation in most SMMC-7721 cells (Fig. 3C). However, the nucleus illustrated as strong fluorescence staining, fragmentary and polymorphonuclear in 40 μmol/l berbamine treatment (Fig. 3D). The nucleus in Fig. 3B-D illustrated the classical apoptotic morphology.

Berbamine induces SMMC-7721 cells apoptosis. Forty-eight hours post the berbamine treatment, the SMMC-7721 cells were stained with the Annexin V/PI. The results indicated that the berbamine (10 μmol/l) increased the apoptosis rate, however, no significant difference was discovered compared to Control group (Fig. 4A and B, $P>0.05$). The berbamine at concentration of 20 μmol/l ($P<0.05$) and 40 μmol/l ($P<0.01$) significantly enhanced the apoptosis rate compared to the Control group, respectively (Fig. 4A, C and D).

Berbamine triggers Cyto c release from SMMC-7721 nucleus. The immunofluorescence staining was used to examine the Cyto *c* release from the SMMC-7721 nucleus. The results indicated that the Cyto *c* was slightly stained in the control group (Fig. 5), and which mainly distributed in the nucleus. However, the staining of Cyto *c* in berbamine treatment was significantly strengthened in 20 and 40 μmol/l compared to control group (Fig. 5), and the Cyto *c* was transferred from the nucleus to the cytoplasm.

Berbamine enhances Bax and p53 levels and decreases Bcl-2 and survivin levels. In this study, four apoptosis associated
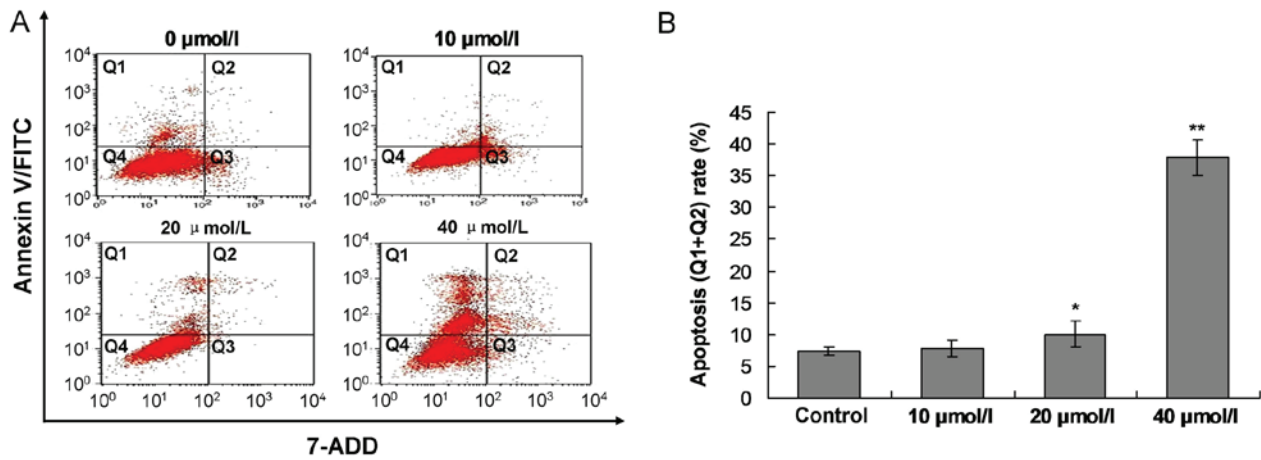
Figure 4. Observation for the Berbamine induced SMMC-7721 cell apoptosis by using flow cytometry assay. (A) Images for flow cytometry assay in every groups. (B) Statistical analysis for flow cytometry assay. Q1 quadrant represents the living cells; Q2 quadrant represents the early apoptosis cells; Q3 quadrant represents the late apoptosis cells; and Q4 quadrant represents the death cells. The apoptosis rate was composed of percentage of Q1 plus Q2. *P<0.05 and **P<0.01 represent apoptosis rate compared to the control group.
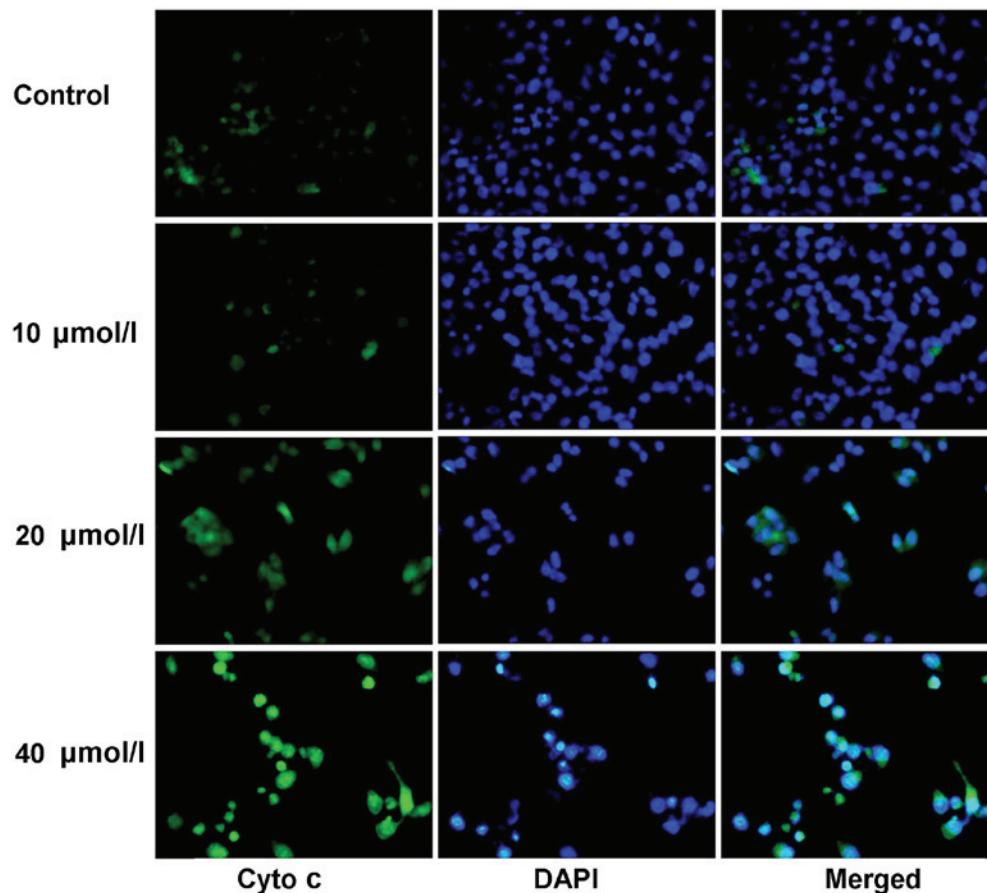

Figure 5. Examination for the Cyto C expression by using immunofluorescence assay at 48 h post berbamine treatment (magnification, x200). The green fluorescence illustrates Cyto C staining, the blue fluorescence illustrates DAPI staining, the merged images are the Ctyo C and DAPI double stained SMMC-7721 cells.

biomarkers, including Bax, Bcl-2, p53 and survivin, were examined by using both of RT-sqPCR and western blot analysis. The RT-sqPCR results showed that the Bax and p53 mRNA levels were significantly increased in 10, 20 and 40 μmol/l berbamine treatment group compared to control group (Fig. 6; P<0.05). Meanwhile, the Bcl-2 and survivin

mRNA levels were significantly decreased in 10, 20 and 40 μmol/l berbamine treatment group compared to control group (Fig. 6; P<0.05).

Moreover, the western blot assay also showed that the Bax and p53 protein levels were significantly increased, and Bcl-2 and survivin protein levels were significantly decreased in 10,
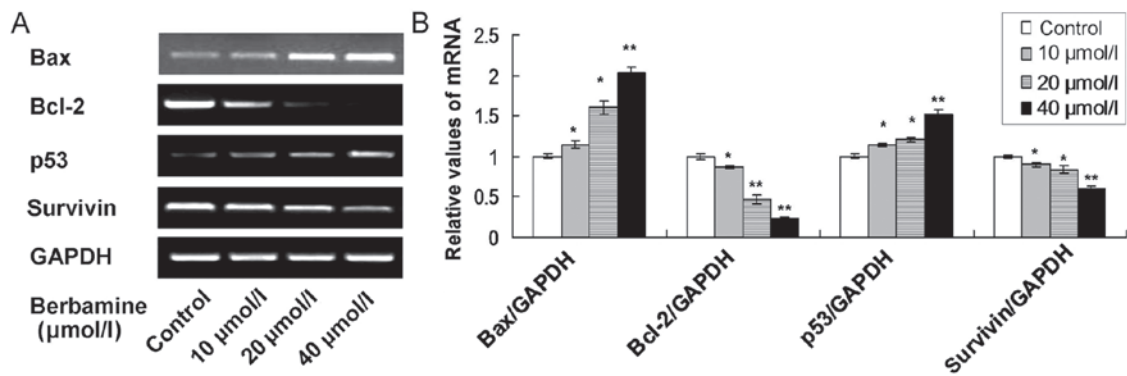
Figure 6. RT-sqPCR examination for the apoptosis associated genes in berbamine treated SMMC-7721 cells. (A) RT-sqPCR bands for the Bax, Bcl-2, p53 and survivin mRNA expression. (B) Statistical analysis for the mRNA expression. *P<0.05 and **P<0.01 represent the mRNA expression compared to the control group. The data are presented as the mean ± standard deviation (n=6).
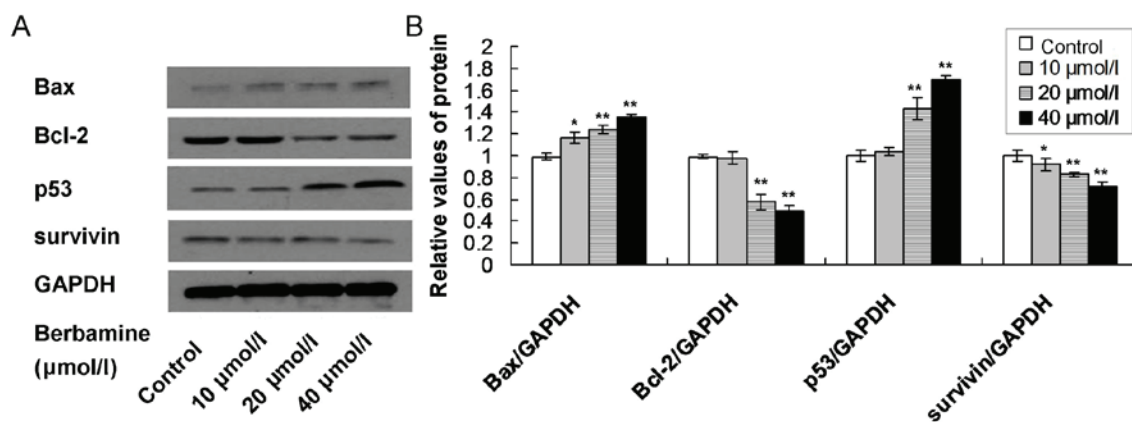

Figure 7. Western blot analysis examination for apoptosis associated proteins in berbamine treated SMMC-7721 cells. (A) Western blot bands for Bax, Bcl-2, p53 and survivin protein expression. (B) Statistical analysis for the protein expression. *P<0.05 and **P<0.01 represent the protein expression compared to the control group. The data are presented as the mean ± standard deviation (n=6).

20 and 40 μmol/l berbamine treatment group compared to control group (Fig. 7; P<0.05).

Discussion

Although the berbamine has been extensively applied for many cancer treatment, however, the anticancer role of berbamine in HCC therapy has not been fully investigated. In this study, we studied the anticancer effects of berbamine on the SMMC-7721 cells proliferation and apoptosis.

Anti-apoptosis is the typical characteristic for the cancer cells, therefore, induction of cancer cell apoptosis is a key end for developing antitumor drugs (26). Therefore, this study observed the nucleus morphology of the SMMC-7721 cells, and the nucleus illustrated as fragmentary and polymorphonuclear in 20, 40 μmol/l berbamine treatment, which suggests that the classical apoptotic morphology was appeared in the berbamine treatment cells. Meanwhile, the apoptosis rate was also evaluated by using the flow cytometry assay, and results revealed that berbamine at concentration of 20 and 40 μmol/l significantly induced apoptosis compared to the control group, which suggests that berbamine processes the function of anti-apoptosis in SMMC-7721 cells, and is consistent with previous study (19,27).

The cell apoptosis mainly includes endogenous apoptosis and exogenous apoptosis, and the mitochondira-associated apoptosis is the key process for endogenous apoptosis. For the mitochondria-associated apoptosis, the Cyto *c* transferred from the nuclues to mitochondria and cytoplasm, and the Bcl-2 protein family plays an important role in the whole apoptotic processes (28). Our result indicated that Cyto *c* levels in berbamine treatment was significantly increased in 20 and 40 μmol/l compared to control group, which suggest that the Cyto *c* was transferred from the nucleus to the cytoplasm and mitochondria, and induced the apoptosis. Among the Bcl-2 family proteins, the Bcl-2 belongs to the anti-apoptotic protein and Bax belongs to the pro-apoptotic protein (29), both of which regulate the activity of the caspase protein and induce the cascade cell apoptosis (30). In our study, berbamine significantly enhanced Bax levels and decreased Bcl-2 levels compared to the un-treated with berbamine group, which suggests that the apoptosis of SMMC-7721 was triggered by the mitochondria-associated signaling pathway.

p53 is the most common antitumor gene of 20 kb, which includes 11 exons and 10 introns, and participates in the processes of cell cycle regulation, DNA damage repair and induction of apoptosis (28,31). Normally, the p53 remains the low levels, however, when DNA damaged p53 was

overexpressed, which upregulated pro-apoptotic genes, down-regulated anti-apoptotic genes through multiple-signaling pathways (32). In our study, the p53 expression was significantly increased in berbamine treatment group compared to no berbamine treatment group. The over-expressed p53 may trigger the increased expression of Bax and decreased expression of Bcl-2 in SMMC-7721 cells. Survivin is a recent years discovered anti-apoptotic protein, which participates in the progression and development of malignant tumors (33). The survivin inhibits the cell apoptosis by inhibiting apoptotic factors, caspase-3 and -7 activity directly (34). The present results indicated that berbamine significantly inhibited the survivin mRNA and protein expression in SMMC-7721 cells. The above results suggest that the p53 and survivin play critical role in berbamine triggered apoptosis.

Recent years, there were many studies based on berbamine derivatives have been published. Nam *et al* (35) reported that the berbamine derivatives could inhibit the Jak2/Stat3 signaling and induce apoptosis of human melanoma cells. Xu *et al* (36) showed that the berbamine derivatives affect the Ca^{2+}-stimulated Mg^{2+}-dependent ATPase in erythrocyte membranes. Liang *et al* (37) reported that 4-chlorbenzoyl berbamine as a novel derivative of berbamine, could inhibit the growth of human myeloma cells by modulating the NF-κB and JNK signaling pathway. Meanwhile, a few previously published studies (15,24,25) also reported the berbamine induced SMMC-7721 cell apoptosis in the other signaling pathways. Wang *et al* (24,25) proved that the berbamine induced apoptosis was mediated by the mitochondrial transmembrane potential and Fas-mediated mediated signaling pathway. Meng *et al* (15) also found that the berbamine induced the apoptosis and inhibited tumor growth by activating Ca^{2+}/calmodulin-dependent protein kinase II. In the present study, berbamine induced SMMC-7721 cells apoptosis through upregulating p53 expression and downregulating survivin expression, which is distinguish from the above studies (15,24,25).

However, the specific pathways that mediated the role of berbamine have not been fully clarified. Therefore, we thought that the mechanism for the anticancer effects of berbamine in HCC cell lines may also involve many other signaling pathways. In the further studies, we would attempt to investigate the mechanism of action of berberine further by examining the crosstalk between different signaling pathways, such as the p53-dependent signaling pathway, MAPK-survivin signaling pathway and PI3K-Akt pathway.

Totally, the present findings fully proved that the berbamine exhibited higher anticancer activity and trigger-proliferation activity, by activating p53 and survivin expression and triggering the mitochondria signaling pathway. We speculated that interfering the p53 and survivin may control the progression of the HCC. Meanwhile, the extraction process of the berbamine is simple and the price is low. Therefore, the we believed that the present results are significant for clinics.

Although this study received some interesting results, there were also a few limitations. Firstly, the cancer-specific effects of berbamine on the cancer progression has not been fully explored. Secondarily, we have not compared the compound of berbamine to the standard of care, may be there have been a synergistic effect. Thirdly, the extrinsic apoptotic pathway

proteins, such as caspase-8 and -10, were not examined. Fourthly, the commercially available p53 and survivin inhibitors were not employed to confirm the effect of berbamine on the intrinsic apoptosis signaling pathway in the present study. Fifthly, although the total levels of Cyto *c* was examined, the Cyto *c* transfer from nucleus to cytoplasm has not been observed.

In conclusion, berbamine induced SMMC-7721 cells apoptosis, through upregulating p53 expression and down-regulating survivin expression, which further triggers mitochondria signaling pathway mediated apoptosis. In the following researches, we would investigate the molecular mechanisms for berbamine triggered antitumor activity, and evaluate it's security.

Acknowledgements

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