Ligustrum lucidum Ait. fruit extract induces apoptosis and cell senescence in human hepatocellular carcinoma cells through upregulation of p21

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Abstract. Nü-zhen-zi, the fruit of *Ligustrum lucidum* Ait., is one of the most frequently used liver Yin tonifying Chinese herbs for the treatment of liver cancer. However, the effect of *Ligustrum lucidum* fruit on hepatocarcinoma cells remains unknown. In the present study, we evaluated the effects of a *Ligustrum lucidum* fruit extract (LLFE) on human hepatocellular carcinoma Bel-7402 cells. The results showed that LLFE inhibited the proliferation of the Bel-7402 cells in a dose- and time-dependent manner. LLFE induced apoptosis in Bel-7402 cells accompanied by activation of caspase-3, -8 and -9. LLFE-induced apoptosis was completely abrogated by a pan caspase inhibitor, Z-VAD-FMK. LLFE treatment also caused a large and flat morphologic cellular change, positive SA-β-gal staining, and G_0/G_1 phase cell cycle arrest in the Bel-7402 cells, accompanied by upregulation of p21 and downregulation of RB phosphorylation. Specific knockdown of p21 expression by RNA interference partially abrogated LLFE-induced apoptosis, and significantly abrogated LLFE-induced cell senescence. These observations suggest that Nü-zhen-zi is a potential anticancer herb and support the traditional use of Nü-zhen-zi for hepatocarcinoma treatment.

Introduction

Liver cancer remains the fifth most common cancer in men and the seventh in women worldwide (1). The curative effect of current treatment for liver cancer is less than satisfactory. There is a great need to develop new approaches for liver cancer treatment. In China, Traditional Chinese Medicine (TCM) has long been used for liver cancer treatment, and has been confirmed to effectively control cancer progression, improve the quality of life, and prolong survival times to some extent in liver cancer patients (2-5). Based on different syndrome patterns, TCM therapeutic principles, such as invigorating spleen and regulating Qi, clearing heat-dampness, dissipating stasis, softening hardness, or tonifying liver and kidney can be used to treat cancer (5-8).

The fruit of *Ligustrum lucidum* Ait. (Nü-zhen-zi in Chinese) is one of the most frequently used liver/kidney Yin tonifying herbs in liver cancer patients for clinical syndrome amelioration (9). As a tonic Chinese herb, *Ligustrum lucidum* fruit has been confirmed to be effective in improving chemotherapy-induced myelosuppression, alopecia and immunosuppression (10-12). *Ligustrum lucidum* fruit has also been used to enhance the therapeutic effects of chemotherapy in TCM clinical practice. *Ligustrum lucidum* fruit has been demonstrated to display antiproliferative potential against lung and pancreatic carcinoma, breast and prostate adenocarcinoma, glioma and colorectal carcinoma (13-15). However, the effect of *Ligustrum lucidum* fruit on liver cancer cells remains unknown.

The present study aimed to evaluate the effect of an aqueous extract of *Ligustrum lucidum* fruit on hepatocarcinoma cells. We observed that *Ligustrum lucidum* fruit extract (LLFE) induced apoptosis in human hepatocellular carcinoma Bel-7402 cells through activation of caspases. LLFE also induced cell senescence accompanied by upregulation of p21 and downregulation of RB phosphorylation. In addition, silencing of p21 by RNA interference partially abrogated LLFE-induced apoptosis, and significantly abrogated LLFE-induced cell senescence.

Materials and methods

Chemicals and reagents. The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo (Kumamoto, Japan). Colorimetric CaspACE™ Assay System was the product of Promega (Madison, WI, USA). Z-VAD-FMK, Caspase-8 and Caspase-9 Colorimetric Assay kits were purchased from R&D Systems

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(Minneapolis, MN, USA). Antibodies against p53, p16, p21, RB, pRB and β-actin, and the Senescence β-Gal staining kit were the products of Cell Signaling Technology (Danvers, MA, USA). Small interfering RNA (siRNA) against p21 and control siRNA were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine™ 2000 was from Invitrogen (Carlsbad, CA, USA).

Extraction of Ligustrum lucidum fruit. An aqueous extract of *Ligustrum lucidum* fruit was prepared as a lyophilized-dry powder as previously described (16,17). Authentic *Ligustrum lucidum* fruit herb material was obtained from Longhua Hospital. *Ligustrum lucidum* fruit was soaked for 1 h, and decocted twice with an 8-fold volume of boiling distilled water for 2 h. The decoction was filtered and centrifuged twice at 12,000 rpm for 30 min to remove the insoluble ingredients. The supernatants were mixed with an equal volume of ethanol and kept at 4˚C overnight, and centrifuged at 12,000 rpm for 30 min to remove the insoluble ingredients. The resultant supernatants were lyophilized, weighed, dissolved in RPMI-1640 medium and adjusted to a concentration of 400 mg/ml, and were sequentially passed through 0.45- and 0.22-μm filters and sterilized.

Cell culture. Human hepatocellular carcinoma Bel-7402 cells and human hepatocyte HL-7702 cells were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. Bel-7402 and HL-7702 cells were grown in RPMI-1640 medium with 10% FBS and 1% pen-strep, and maintained at 37˚C in a humidified incubator with a 5% CO_2 atmosphere.

Cell proliferation assay. Cells in logarithmic growth phase were seeded into a 96-well plate ($4x10^3$ cells/well) and allowed to attach for 24 h before treatment. The cells were exposed to various doses of LLFE for 72 h, and cell viability was evaluated every 24 h by using the CCK-8 colorimetric assay according to the manufacturer's instructions. The cell survival rate was calculated as follows: Cell survival rate (%) = experimental OD value/control OD value x 100%.

Flow cytometric analysis. LLFE-treated Bel-7402 cells were collected, stained with Annexin V-FITC and PI as recommended by the manufacturer, and detected using a FACSCalibur flow cytometer (Becton-Dickinson). For cell cycle analysis, LLFE-treated Bel-7402 cells were stained with PI (50 μg/ml) and analyzed using a FACSCalibur flow cytometer.

Caspase activity assay. After treatment with different concentrations of LLFE, caspase-3, -8 and -9 activities were measured by the cleavage of the specific chromogenic substrate according to the manufacturer's instructions. For caspase inhibition, cells pretreated with Z-VAD-FMK (50 μmol/l, 2 h) were incubated with LLFE for another 72 h.

Senescence-activated β-galactosidase staining. Bel-7402 cells ($3x10^4$) were plated in 35-mm-diameter plates and treated with different doses of LLFE for 5 days. Senescence-activated expression of β-galactosidase activity (18) was detected by the Senescence β-Gal staining kit according to the manufacturer's protocol, and observed under a microscope.

Western blotting. Western blot analyses were performed as previously described (16,17). Briefly, collected cells were lysed and subjected to 8-12% SDS-PAGE gel, and transferred onto a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). The transferred membranes were blocked with 5% non-fat milk, washed, and probed with the indicated antibodies. Blots were then washed and incubated with IRDye 700- and IRDye 800-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA), and visualized in the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

siRNA treatment. For siRNA transfection, Bel-7402 cells were cultured on a 6-well plate to 60% confluency, and 80 pmol of specific or non-specific control siRNA was introduced into the cells using Lipofectamine™ 2000 according to the manufacturer's recommendations. After 24 h of transfection, cells were treated with 200 μg/ml of LLFE or the same volume of RPMI-1640, and harvested for apoptosis assay or senescence β-Gal staining.

Statistical analyses. Results are expressed as means ± standard deviation of at least two independent experiments, each conducted in triplicate. Differences between the control and LLFE treatment were analyzed by one-way ANOVA. Differences were considered to indicate a statistically significant result at $P<0.05$.

Results

LLFE inhibits the proliferation of Bel-7402 cells. The effect of LLFE on the proliferation of Bel-7402 cells was detected by CCK-8 assay. At final concentrations of 50-800 μg/ml, LLFE significantly inhibited the proliferation of Bel-7402 cells in a dose- and time-dependent manner (Fig. 1A) ($P<0.05$). In contrast, LLFE had no significant effect on the proliferation of human normal hepatocyte HL-7702 cells even at concentrations that were highly toxic to the Bel-7402 cells (Fig. 1B). These observations were consistent with a previous report that aqueous extracts of *Ligustrum lucidum* are inactive on normal human mammary epithelial cells (13).

LLFE induces the apoptosis of Bel-7402 cells. Apoptosis, an evolutionarily conserved cell suicide process elicited by physiological, pathological or pharmacological stimuli, has been recognized as a major anticancer treatment response (19,20). Thus, we determined the effects of LLFE on the apoptosis of Bel-7402 cells. As shown in Fig. 2, treatment with 100-400 μg/ml of LLFE for 72 h induced significant apoptosis in the Bel-7402 cells in a dose-dependent manner ($P<0.01$).

LLFE activates caspases in Bel-7402 cells. To determine whether caspases contribute to the LLFE-induced apoptosis of Bel-7402 cells, activities of caspases were measured by the cleavage of the specific substrate. Caspase activity assays showed that LLFE activated caspase-3, -8 and -9 in the Bel-7402 cells in a dose-dependent manner (Fig. 3A-C) ($P<0.01$). In addi-

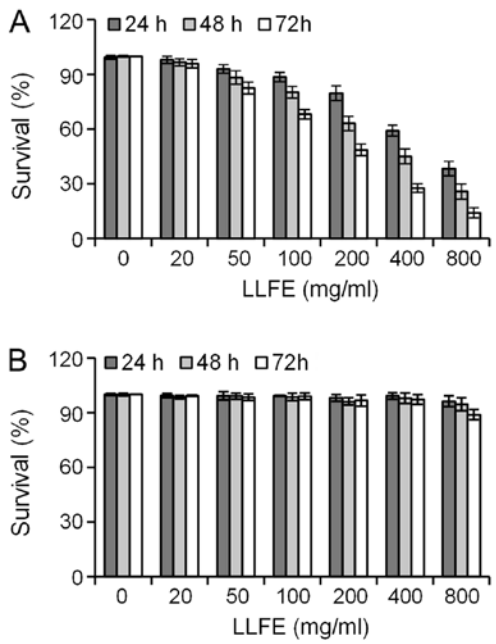

Figure 1. Effects of LLFE on the proliferation of Bel-7402 and HL-7702 cells. (A) Human hepatocellular carcinoma Bel-7402 cells and (B) human normal hepatocyte HL-7702 cells were treated with different concentrations of LLFE for 72 h, and cell viability was evaluated every 24 h by CCK-8 assay. Data shown are representative of three independent experiments. LLFE, *Ligustrum lucidum* fruit extract; CCK-8, Cell Counting Kit-8.

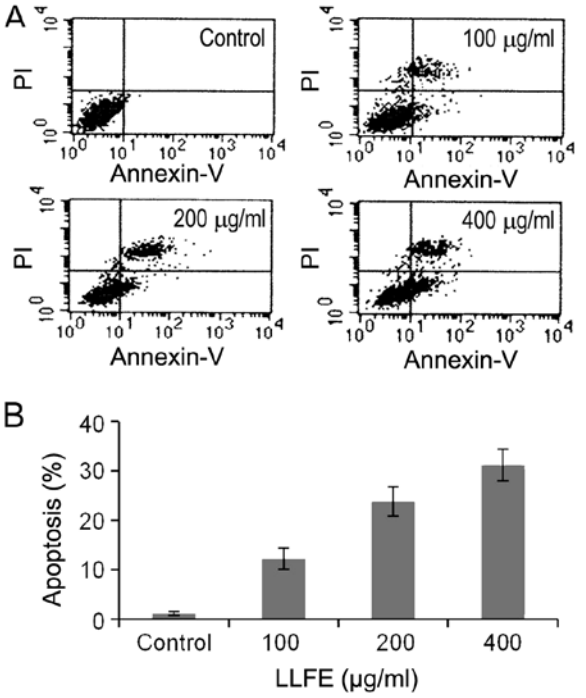

Figure 2. LLFE induces apoptosis. (A) Bel-7402 cells were treated with the indicated doses of LLFE for 72 h. Apoptotic cells were quantified by Annexin V/PI staining and flow cytometric analysis, and (B) expressed as the mean percentage ± SD. LLFE, *Ligustrum lucidum* fruit extract.

tion, LLFE-induced apoptosis of Bel-7402 cells was completely abrogated by the pan-caspase inhibitor Z-VAD-FMK (Fig. 3D) ($P<0.01$), suggesting that LLFE-induced apoptosis is associated with the caspase cascade.

LLFE induces cell senescence in Bel-7402 cells. Upon treatment with low doses of LLFE, the Bel-7402 cells gradually exhibited a large and flattened morphology, indicative of cell senescence (Fig. 4). Thus, we further performed senescence-activated β-galactosidase (SA-β-gal) staining. As shown in Fig. 4, LLFE treatment resulted in a higher percentage of cells with SA-β-gal-positive staining, compared with the controls ($P<0.01$). In addition, flow cytometric analysis revealed that the cell cycle of LLFE-treated Bel-7402 cells was

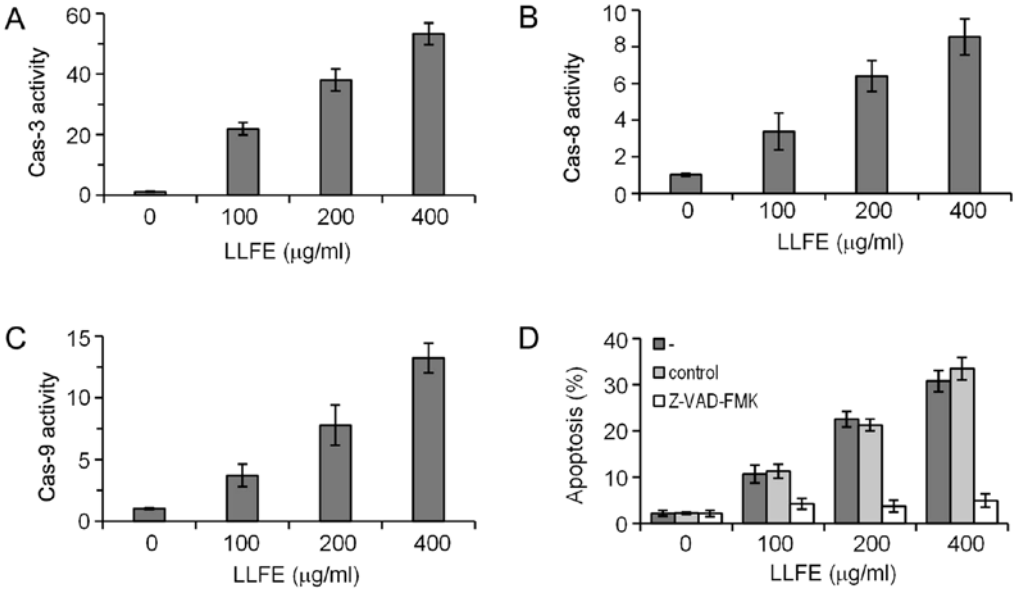

Figure 3. LLFE activates caspase activity. Bel-7402 cells were treated with 100-400 μg/ml of LLFE for 72 h. (A) Caspase-3, (B) caspase-8 and (C) caspase-9 activities were measured by the cleavage of the specific chromogenic substrate. (D) After pretreatment with Z-VAD-FMK (50 μmol/l) for 2 h, Bel-7402 cells were exposed to 100-400 μg/ml of LLFE for 72 h, and subjected to apoptosis detection by flow cytometric analysis. LLFE, *Ligustrum lucidum* fruit extract.

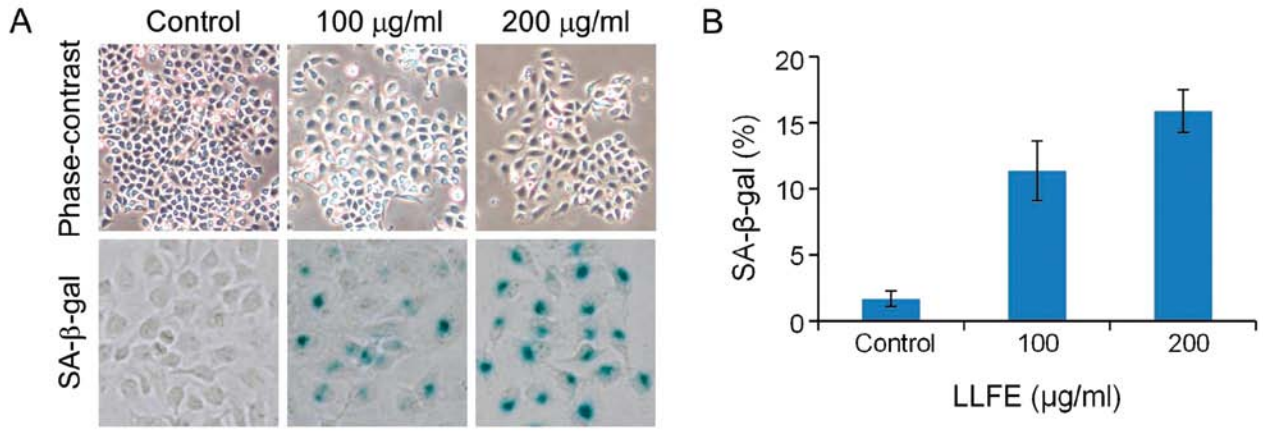

Figure 4. LLFE activates SA-β-gal. (A) Bel-7402 cells were treated with 100 and 200 μg/ml LLFE for 5 days. Cell morphological changes were observed under a microscope (upper panels, x400), and cells were subjected to SA-β-gal staining and observed under a microscope (lower panels, x1,000). (B) Percentage ± SD of SA-β-gal-positive cells from a representative experiment. LLFE, *Ligustrum lucidum* fruit extract.

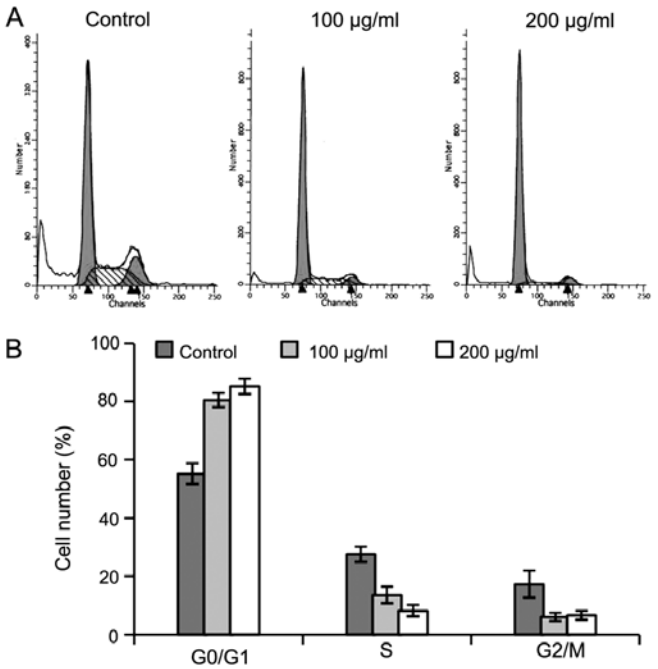

Figure 5. LLFE induces cell cycle arrest. Bel-7402 cells were treated with 100 and 200 μg/ml LLFE for 5 days. (A) Cell cycle distribution of LLFE-treated Bel-7402 cells was analysis by flow cytometric analysis, and (B) expressed as means ±SD. LLFE, *Ligustrum lucidum* fruit extract.

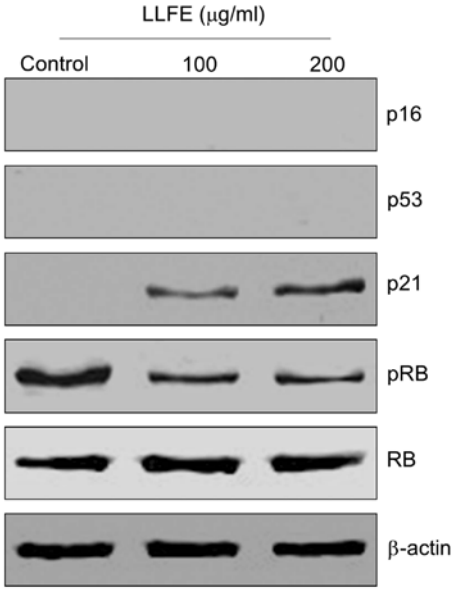

Figure 6. Effects of LLFE on expression of cell senescence regulatory genes. Bel-7402 cells were collected after 5 days of treatment with LLFE (100 and 200 μg/ml), and subjected to western blot analyses using antibodies against p16, p53, p21, RB and pRB. β-actin was used as a loading control. LLFE, *Ligustrum lucidum* fruit extract.

arrested in the G_0/G_1 phase (Fig. 5) ($P<0.01$). These observations suggest that LLFE induces senescence in Bel-7402 cells.

Effects of LLFE on the expression of senescence regulatory genes. It has been reported that cell senescence is regulated by the CDKN1a ($p21^{WAF-1/Cip1}$)/pRB or the CDKN2a ($p16^{INK4A}$)/pRB signaling pathway (21,22). We examined the effects of LLFE on the expression of senescence regulatory genes in the Bel-7402 cells by western blotting. As shown in Fig. 6, treatment with low doses of LLFE caused an upregulation in the p21 expression, and downregulation of RB phosphorylation. However, expression of p53 and p16 was not detected in the Bel-7402 cells.

Role of p21 in LLFE-induced apoptosis and cell senescence. Since p21 expression is upregulated by LLFE, we further determined the role of p21 in LLFE-induced apoptosis and cell senescence. As shown in Fig. 7, the expression of p21 was significantly inhibited by specific siRNA. Specific knockdown of p21 expression partially abrogated LLFE-induced apoptosis (Fig. 7A), and significantly abrogated LLFE-induced cell senescence (Fig. 7B). These observations suggest that p21 may contribute to LLFE-induced apoptosis and cell senescence.

Discussion

The present study demonstrated that LLFE inhibited proliferation in a dose- and time-dependent manner, and induced apoptosis in Bel-7402 cells. The initiation and execution of

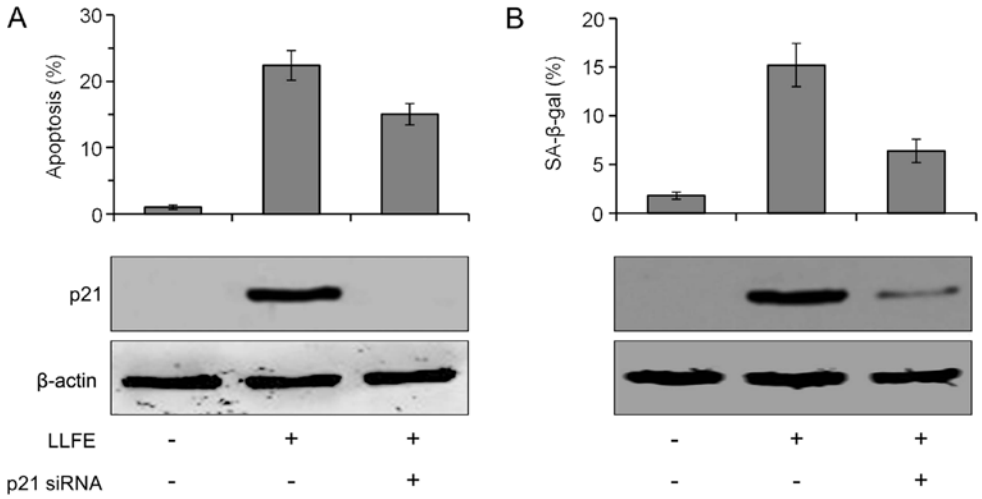

Figure 7. Role of p21 in LLFE-induced apoptosis and cell senescence. After 24 h of transfection with p21 siRNA or control siRNA, Bel-7402 cells were treated with 200 μg/ml of LLFE for 3 or 5 days, and subjected to western blot analyses and (A) apoptosis detection, and (B) SA-β-gal staining. LLFE, *Ligustrum lucidum* fruit extract.

apoptosis are dependent on the activation of the extrinsic and/or intrinsic death pathways (19,20,23). The extrinsic or death receptor pathways are associated with the oligomerization of cell-surface death receptors by their ligands, resulting in recruitment and activation of caspase-8 followed by activation of executioner caspase-3. On the other hand, intrinsic or the mitochondrial pathway involves signals to the mitochondria that lead to the release of cytochrome *c* and Apaf-1, forming an apoptosome that activates the initiating protease caspase-9, which in turn activates caspase-3, causing the cell to undergo apoptosis. The present study showed that LLFE activates caspase-8, -9 and -3, and LLFE-induced apoptosis was blocked by a caspase inhibitor. These observations indicate that LLFE-induced apoptosis in Bel-7402 cells is through the extrinsic and intrinsic pathways.

In addition to apoptosis, cell senescence plays an important role in suppressing tumorigenesis, and may contribute to the outcome of cancer therapy (19,24-26). Cell senescence is a state of stable irreversible cell cycle arrest provoked by a variety of stimuli. Senescent cells maintain some metabolic activity, but can no longer proliferate, even stimulated with mitogens. Cell senescence is usually characterized by large and flattened morphology, an increase in intracellular granules, elevated SA-β-gal activity, and cell cycle arrest (18,24). It has been reported that chemotherapeutic agents such as cisplatin, doxorubicin, SN-38, and camptothecin can inhibit cancer cell growth via cell senescence (26-29). In the present study, we observed that a low dose of LLFE treatment caused large and flat morphologic cellular changes, positive SA-β-gal staining, and G_0/G_1 phase cell cycle arrest, suggesting that LLFE treatment induces cell senescence in Bel-7402 cells.

Cell senescence is closely related to the activation of the CDKN1a ($p21^{WAF\text{-}1/Cip1}$)/pRB or the CDKN2a ($p16^{INK4A}$)/pRB signaling pathway (21,22). p21, an important cell cycle regulator, can inhibit a variety of cyclin/CDK complexes and induce the hypophosphorylation or dephosphorylation of protein Rb. Hypophosphorylated Rb binds to E2F and prevents it from activating target genes that are essential in the cell cycle, usually leading to cell cycle arrest. Overexpression of p21 may induce a senescence-like state in cancer cells (30). In addition, p21 also contributes to drug-induced apoptosis in cancer cells (31,32). Expression of p21 in cancer cells may be dependent or independent of p53 (33-36). The present study showed that LLFE treatment resulted in cell senescence accompanied by upregulation of p21 and downregulation of RB phosphorylation, suggesting that p21/RB may contribute to LLFE-induced cell senescence. Since p53 was not expressed in the Bel-7402 cells, p21 induced by LLFE may be independent of p53. Further observations revealed that knockdown of p21 expression significantly abrogated LLFE-induced cell senescence, suggesting that the effects of LLFE on cell senescence may depend on p21. LLFE-induced apoptosis was partially abrogated by p21 silencing suggesting that the effects of LLFE on apoptosis may partially involve p21.

In summary, the present study demonstrated that LLFE activates caspases to induce apoptosis in human hepatocellular carcinoma Bel-7402 cells with the participation of p21. LLFE also induced cell senescence in Bel-7402 cells, which may correlate with upregulation of p21 and downregulation of RB phosphorylation. These observations suggest that Nü-zhen-zi is a potential anticancer herb and support the traditional use of Nü-zhen-zi for liver cancer treatment. Nevertheless, further studies are needed to determine the upstream signal transduction of p21 upregulation, and to address which chemical(s) are responsible for the LLFE-induced anticancer effects.

Acknowledgements

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