Effect of Yanggan Jiedu Sanjie formula on human hepatocellular carcinoma Bel-7402 cells

Hu Bing, Zhang Tong, An Hongmei, Zheng Jialu, Yan Xia, Huang Xiaowei, Tian Jianhui, Li Miao

Abstract

OBJECTIVE: To observe the effect of Yanggan Jiedu Sanjie (YGJDSJ) formula on human hepatocellular carcinoma Bel-7402 cells.

METHODS: Bel-7402 cells were treated with YGJDSJ. Cell proliferation was detected by cell counting kit-8 assay. Cell apoptosis was identified by Hoechst 33258 staining and flow cytometric analysis. Cell cycle distribution was quantified by flow cytometric analysis. Caspase activities were measured by commercial kit. Cell senescence was detected by senescence-associated β-galactosidase (SA-β-gal) staining. Protein expression and phosphorylation were identified by Western blot. Protein expression was knocked-down by siRNA.

RESULTS: YGJDSJ inhibited proliferation of Bel-7402 cells in a dose- and time-dependent manner. YGJDSJ induced apoptosis and activated caspase-3, 8, and 9 in Bel-7402 cells. YGJDSJ-induced apoptosis was completely abrogated by a pan caspase inhibitor, Z-VAD-FMK. YGJDSJ also induced cell senescence, up-regulated cyclin-dependent kinase inhibitor 1a (CDKN1a) and CDKN2a expression and down-regulated retinoblastoma protein (RB) phosphorylation in Bel-7402 cells. Specific knockdown of CDKN1a and CDKN2a significantly reduced YGJDSJ-induced cell senescence in Bel-7402 cells.

CONCLUSION: YGJDSJ inhibited cell proliferation, induced caspase-dependent apoptosis and CDKN1a/CDKN2a-RB signalling mediated cell senescence in Bel-7402 cells. Our findings suggest that YGJDSJ might be potential for hepatocellular carcinoma treatment.

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Keywords: Carcinoma, hepatocellular; Chinese herbal formula; Apoptosis; Cell senescence; Cell cycle; Cyclin-dependent kinase inhibitor p21; Cyclin-dependent kinase inhibitor p16; Retinoblastoma protein
geted therapy. Of these, surgical treatment, including liver transplantation and resection, is the only method that possibly cures hepatocellular carcinoma. However, due to its characteristics of concealed onset and rapid progression, a considerable proportion of the patients are in the advanced stages when diagnosed, and are ineligible for surgery. Meanwhile, TACE, radiofrequency ablation, and other topical treatments present a poor long-term efficacy. Targeted therapies even with drug combinations can only prolong the overall survival time by several months in patients. Currently, hepatocellular carcinoma is still the main cause of cancer deaths. Therefore, there is an urgent need to develop new therapeutic approaches for hepatocellular carcinoma treatment.

Traditional Chinese Medicine (TCM) has played an active role in the treatment of hepatocellular carcinoma. Hepatocellular carcinoma-associated clinical manifestations and treatment were described as early as in Huang Di Nei Jing (400 BC). Successive TCM doctors have contributed to the development of new strategies for liver cancer management. In recent decades, a new understanding of the pathogenesis of liver cancer, and development in its prevention and treatment has been achieved in the field of TCM. Also some new effective Chinese herb resources have been discovered for its treatment. A combination of TCM therapy with surgery, TACE and targeted therapy, or TCM monotherapy, has become a feature of liver cancer treatment in China. TCM treatment effectively alleviates the clinical symptoms, improves quality of life and immune function, delay the progression of the disease, and prolong the survival time of patients with hepatocellular carcinoma.

TCM treatment, based on its symptom pattern identification, is the basic principle for the intervention of liver cancer. This involves merging of different Chinese herbs for the treatment according to the clinical manifestations, staging, and other specific conditions of the patients. This treatment is a kind of personalized therapy, but is also empirical. It is necessary to develop a specific TCM formula for liver cancer treatment. Based on clinical practice and relevant studies, we have proposed a regimen for liver cancer treatment, which is nourishing the liver (Yanggan, YG), detoxification (Jiedu, JD) and resolving tumor masses (Sangjie, SJ), and accordingly an YGJDSJ herbal formula was established. In this study, we aimed to evaluate YGJDSJ’s effectiveness on hepatocellular carcinoma cells, and the possible mechanism underlying its action.

**MATERIALS AND METHODS**

**Chemicals and reagents**

DMEM medium and fetal bovine serum were obtained from Hyclone (Logan, UT, USA). Cell Counting Kit-8 (CCK8) was obtained from Dojindo (Kumamoto, Japan). Annexin V-FITC apoptosis detection kit was purchased from BD Pharmingen™ (Franklin Lakes, NJ, USA). Caspase activity detection kits, Hoechst 33342 and Z-VAD-FMK were purchased from Beyotime (Haimen, Jiangsu, China). Antibodies against cyclin-dependent kinase inhibitor 1a (CDKN1a), phospho-retinoblastoma protein (pRB), and senescence β-Gal staining kit were the products of Cell Signaling Technology (Danvers, MA, USA). Antibodies CDKN2a, retinoblastoma protein (RB) and β-actin were procured from Bioworld Technology (St. Louis Park, MN, USA). Small interfering RNAs (siRNAs) targeting CDKN1a and CDKN2a, and control siRNA were produced by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine™ 2000 was from Thermo Fisher Scientific (Waltham, MA, USA). 5-Fluorouracil (5-Fu) was from Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China).

**Cell culture**

Human hepatocellular carcinoma cells Bel-7402 cells and human hepatocytes HL-7702 cells were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Bel-7402 and HL-7702 cells were grown in the DMEM medium with 10% FBS and 1% Pen-Strep, and maintained at 37 °C in a humidified incubator with 5% CO₂ atmosphere.

**Herb preparation**

The main herbs in YGJDSJ formula (Chinese patent ZL201110145109.0) include Nuzhenzi (Fructus Ligustri Lucidi) 12 g, Banzhilian (Herba Scutellariae Barbatae) 30 g, Shemei (Herba Euphorbiae Heliocapae) 15 g, Maozhuacao (Radix Ranunculi Ternati) 15 g, Yujin (Herba Solani Nigri) 15 g, Zeqi (Herba Euphorbiae Heliocapae) 15 g, Mazzhuacuo (Radix Ranunculi Ternati) 15 g, Yujin (Radix Carunculacae Wenyujin) 15 g and Huzhanggen (Radix Polygoni Cuspidati) 15 g. Authenticated herbs were obtained from the Longhua Hospital according to the original proportion. Herb extraction was performed as described previously. Briefly, herbs were decocted twice with 8-fold volume of distilled water for 1 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 DMEM medium and adjusted to a concentration of 400 mg/mL, and was sequentially passed through 0.45 and 0.22 μm filters for sterilization.

**Cell proliferation assay**

Cells in the logarithmic growth phase were seeded into 96-well plate (Corning Inc., Corning, NY, USA) (4 x 10³ cells/well) and allowed to attach for 24 h before treatment. The cells were exposed to various doses of YGJDSJ or 5-Fu (50 μg/mL) for 72 h, and cell viability was evaluated every 24 h by using CCK-8 assay ac-
According to the manufacturer’s instructions. The cell survival rate was calculated as follows: cell survival rate (%) = [experimental OD value / control OD value] × 100%.

Observation of apoptotic morphology
Morphological changes of apoptosis were detected by Hoechst 33258 staining. Briefly, 2 × 10⁴ Bel-7402 cells were seeded into 24-well plates (Corning Inc, Corning, NY) and incubated for 24 h. Bel-7402 cells were treated with different doses of YGJDSJ or 5-Fu (50 μg/mL) for 72 h, and stained with Hoechst 33258 for 5 min at room temperature. The cells were observed and photographed using an inverted fluorescence microscope (AFM010-2, Nikon, Japan).

Flow cytometric analysis
For apoptosis detection, YGJDSJ or 5-Fu treated Bel-7402 cells were collected, stained with Annexin V-FITC and PI as recommended by the manufacturer, and detected in a FACS caliber flow cytometer (Becton Dickinson, San Jose, CA, USA). For cell cycle analysis, YGJDSJ treated Bel-7402 cells were stained with PI (50 μg/mL) and analyzed in FACS caliber flow cytometer.

Caspase activity detection
After treatment with different concentrations of YGJDSJ or 5-Fu (50 μg/mL), caspase-3, 8, and 9 activities were measured by cleavage of the specific chromogenic substrate according to the manufacturer’s manual. For caspases inhibition, cells preincubated with Z-VAD-FMK (50 μmol/L, 2 h) were treated with YGJDSJ for another 72 h.

Senescence-associated β-galactosidase (SA-β-gal) staining
Bel-7402 cells (3 × 10⁴) were plated in 6-well plates and treated with different doses of YGJDSJ for 5 d. Cell senescence were detected by senescence β-Gal staining kit according to the manufacturer’s protocol, and observed under microscope. Positive β-Gal staining was analyzed by Image-Pro Plus software and expressed as fold of control.

siRNA transfection
siRNA transfection was performed as described previously. Briefly, Bel-7402 cells were cultivated in 6-well plates until 60% confluence, and 80 pmols specific or non-specific control siRNA were introduced into the cells using Lipofectamine™ 2000. After 24 h of transfection, cells were treated with 100 μg/mL of YGJDSJ or same volume of DMEM for 5 d, and subjected to Senescence β-Gal staining and Western blot.

Western blot
Western blot analysis was performed as described previously. Briefly, the 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 Immunocyticals, Gilbertsville, PA), and visualized in Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Statistical analysis
Statistical analysis was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± standard deviation (x ± s) of at least two independent experiments, each performed in triplicate. Differences between groups were analyzed by one-way analysis of variance or student’s test. A P < 0.05 was considered significant.

RESULTS

YGJDSJ inhibits proliferation of Bel-7402 cells
Cell proliferation was detected by CCK-8 assay. As shown in Table 1, YGJDSJ significantly inhibited proliferation of Bel-7402 cells in a dose- and time-dependent manner (P < 0.05). The proliferation was completely inhibited by treatment with 1600 μg/mL of YGJDSJ for 72 h. The inhibitory effects of YGJDSJ on proliferation of Bel-7402 cells were lower than 5-Fu at same dose (P < 0.01). YGJDSJ demonstrated no significant effect on the proliferation of normal human hepatocytes HL-7702 cells at these concentrations (P > 0.05).

YGJDSJ induces apoptosis in Bel-7402 cells
Hoechst 33258 staining showed that after treatment with YGJDSJ, the nuclei of some Bel-7402 cells were densely stained or fragmented, suggesting the occurrence of apoptosis. Further Annexin V-FITC/PI double labeling and flow cytometry analysis revealed that YGJDSJ significantly induced Bel-7402 cell apoptosis in a dose-dependent manner (P < 0.01). The apoptosis inducing effects of YGJDSJ in Bel-7402 cells were lower than 5-Fu at same dose (P < 0.01) (Figure 1).

YGJDSJ activates caspases in Bel-7402 cells
Apoptosis is a process of caspase-mediated cell death. We also observed the effect of YGJDSJ on caspases in Bel-7402 cells. As shown in Figure 2, YGJDSJ significantly activated caspase-3, 8, and 9 in Bel-7402 cells in a dose-dependent manner (P < 0.01). The effects of YGJDSJ on caspase-3 and 9 were lower than 5-Fu at same dose (P < 0.01). However, the effects of YGJDSJ on caspase-8 cells were similar to 5-Fu at same dose (P > 0.05). Meanwhile, Z-VAD-FMK, a pan caspase inhibitor, significantly abrogated YGJDSJ-induced apoptosis in Bel-7402 cells. These observations suggest that YGJDSJ induces caspase-dependent apoptosis in Bel-7402 cells.
Table 1 Effects of YGDSJ on the proliferation of Bel-7402 cells and HL-7702 cells (x ± s)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bel-7402 cells</th>
<th>HL-7702 hepatocytes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Control</td>
<td>100.0±2.1</td>
<td>100.0±2.5</td>
</tr>
<tr>
<td>5-Fu (50 μg/mL)</td>
<td>69.2±3.7*</td>
<td>47.6±2.8*</td>
</tr>
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<td>YGDSJ (25 μg/mL)</td>
<td>95.7±2.4a</td>
<td>92.4±1.9a</td>
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<td>74.7±3.9a</td>
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<td>YGDSJ (800 μg/mL)</td>
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<tr>
<td>YGDSJ (1600 μg/mL)</td>
<td>13.9±2.4a</td>
<td>6.4±0.7a</td>
</tr>
</tbody>
</table>

Notes: *P < 0.01, **P < 0.05, ***P > 0.05 vs the control group; *P < 0.05, **P < 0.01, ***P > 0.05 vs the 5-Fu group. YGDSJ: Yanggan Jiedu Sanjie formula.

Figure 1 YGDSJ induces apoptosis in Bel-7402 cells
A-E: Bel-7402 cells were treated DMEM, 50 μg/mL of 5-Fu, 50, 100 and 200 μg/mL of YGDSJ for 72 h, stained with Hoechst 33258 and observed under fluorescence microscope (×400); F: apoptotic cells were quantified by Annexin V/Pt staining and by flow cytometric analysis and expressed as mean ± standard deviation. *P < 0.01 vs the control group; **P < 0.01, ***P < 0.05 vs the 5-Fu group. YGDSJ: Yanggan Jiedu Sanjie formula.

Figure 2 YGDSJ activates caspases in Bel-7402 cells
Bel-7402 cells were treated with 50 μg/mL of 5-Fu and 50-200 μg/mL of YGDSJ for 72 h. A: caspase-3; B: caspase-8; C: caspase-9; D: after pretreatment with Z-VAD-FMK (50 μmol/L) for 2 h. Bel-7402 cells were exposed to 50-200 μg/mL of YGDSJ for 72 h, and subjected to apoptosis detection by flow cytometric analysis. *P < 0.01 vs the control group; **P < 0.01, ***P > 0.05 vs the 5-Fu group; **P < 0.01 vs the corresponding dose of YGDSJ-treated Z-VAD-FMK (-) group. YGDSJ: Yanggan Jiedu Sanjie formula.
YGJDSJ promotes senescence in Bel-7402 cells
Apart from cell apoptosis, cell senescence is also an important effective mechanism of anti-cancer therapy. Senescent cells usually exhibit large and flattened morphology, cell-cycle arrest, and are SA-β-gal-positive. We observed that after YGJDSJ treatment, some Bel-7402 cells presented senescent morphology and SA-β-gal-positive staining accompanied by cell cycle-arrest in G0/G1 phase, suggesting that YGJDSJ induces Bel-7402 cell senescence (Figure 3). However, 5-Fu failed to induce cell senescence in Bel-7402 cells (data not shown).

Effect of YGJDSJ on the expression of cell senescence-associated proteins
Western blot assay was used to detect the effect of YGJDSJ on the expression of cell senescence related proteins. After treatment with YGJDSJ, expression of CDKN1a and CDKN2a was elevated, RB phosphorylation was decreased, but RB expression was unchanged (Figure 4).

Role of CDKN1a and CDKN2a in YGJDSJ induced cell senescence
siRNA was used to address the relation between CDKN1a and CDKN2a and YGJDSJ induced cell senescence in Bel-7402 cells. As shown in Figure 5, specific knockdown of CDKN1a and CDKN2a expression by siRNA significantly abrogated YGJDSJ induced cell senescence. These observations suggest that up-regulation of CDKN1a and CDKN2a contributed to the induction of cell senescence by YGJDSJ.

DISCUSSION
In general, cancer cells present with uncontrolled prolif-
Cell apoptosis is a protein-regulated and autonomous process that contributes to the anti-cancer effect of YGJDSJ. The analysis revealed that YGJDSJ induced apoptosis in Bel-7402 cells, suggesting that cell apoptosis contributes to the anti-cancer effect on hepatocellular carcinoma cells.

Furthermore, cell morphology and flow cytometry analysis revealed that YGJDSJ induced apoptosis in Bel-7402 cells in a dose- and time-dependent manner, and the proliferation of Bel-7402 cells was completely inhibited by treatment with 1600 μg/mL of YGJDSJ for 72 h. These results suggest that YGJDSJ has an appreciable anti-cancer effect on hepatocellular carcinoma cells. Furthermore, cell morphology and flow cytometry analysis revealed that YGJDSJ induced apoptosis in Bel-7402 cells, suggesting that cell apoptosis contributes to the anti-cancer effect of YGJDSJ.

Cell apoptosis is a protein-regulated and autonomous cell-death process, which mainly includes two pathways: extrinsic pathway and intrinsic pathway. The extrinsic pathway, FasL, TRAIL, TNF-α, and other death ligands bind to the corresponding death receptors, and these subsequently activate caspase-8 and 3, initiating apoptosis. In the intrinsic pathway, cytotoxic signals promote conformational changes of BAX and BAK, which bind to the mitochondria. These down-regulate the mitochondrial membrane potential to release cytochrome c, subsequently activating caspase-9 and 3, and triggers apoptosis. Activation of caspase-8, 9, and 3 is an indicative molecular event of the apoptosis process. In the present study, it was found that after treatment with YGJDSJ, activities of caspase-8, 9, and 3 in Bel-7402 cells were increased, while blocking of these caspase activities abrogated YGJDSJ-induced apoptosis. These observations suggest that...
YGJDSJ can induce apoptosis in Bel-7402 cells through intrinsic and extrinsic pathways. Cell senescence is a stable state where cells irreversibly withdraw from the cell cycle and lose their proliferative capacity. Senescent cells can no longer proliferate but maintain metabolic activity, exhibit a large and flattened morphology, cell cycle arrest, and are SA-β-gal-positive.\(^{1,3,4}\) Cell senescence is an important mechanism for anti-cancer drug treatment. It has been confirmed that chemotherapeutic drugs, such as cisplatin, camptothecin, and doxorubicin can induce senescence in various cancer cells.\(^{5,6,7}\) Natural products, such as resveratrol, ganoderol F, pseudolaric acid B, β-Asarone, and Androdia camphorate extract can promote cancer cell senescence through different mechanisms.\(^{8,9,10}\) Our results showed that Bel-7402 cells upon YGJDSJ treatment exhibited a large and flattened cell morphology, cell-cycle arrest in the G0/G1 phase, and were SA-β-gal-positive, suggesting that YGJDSJ can promote Bel-7402 cell senescence.

Cell senescence is associated with CDKN1a/CDKN2a-RB signaling pathway.\(^{11,12}\) CDKN1a can inhibit cyclin/CDK activities, while CDKN2a can inhibit CDK4/6 activity, which thereby inhibits RB phosphorylation. Hypophosphorylated RB binds to E2F transcription factors, which prevents E2F1 from activating cell cycle essential genes that initiate chromosome replication to complete the cell proliferation cycle. This is in turn results in the cell-cycle arrest and initiates cell senescence. The present study demonstrated that YGJDSJ up-regulated the expression of CDKN1a and CDKN2a as well as inhibited RB phosphorylation. In addition, specific knockdown of CDKN1a and CDKN2a expression significantly abrogated YGJDSJ induced cell senescence. These observations suggest that YGJDSJ induces Bel-7402 cell senescence in association with CDKN1a/CDKN2a-RB signal transduction. In conclusion, our study showed that YGJDSJ inhibited proliferation and induced caspase-dependent apoptosis in Bel-7402 cells. YGJDSJ induced cell senescence in Bel-7402 cells was associated with up-regulation of CDKN1a and CDKN2a, as well as down-regulation of RB phosphorylation. These findings suggest that it might be a potential for hepatocellular carcinoma treatment.

REFERENCES


