

Fuzheng Qingjie recipe induces apoptosis in HepG2 cells via P38 MAPK activation and the mitochondria-dependent apoptotic pathway

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Abstract. Fuzheng Qingjie (FZQJ) recipe is a polyherbal Chinese medicine capable of suppressing tumor growth and is used as an adjuvant therapy for various types of cancer. However, its anticancer mechanisms are yet to be fully elucidated. In the present study, we explored whether p38 mitogen-activated protein kinase (MAPK) was involved in FZQJ-mediated mitochondria-dependent apoptosis in human hepatocellular carcinoma cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used to measure the viability of HepG2 cells. 4,6-Diamidino-2-phenylindole (DAPI) and Annexin-V fluorescein isothiocyanate (FITC) were used to analyze the apoptosis of HepG2 cells. The mitochondrial membrane potential ($\Delta\psi$) and phosphorylated P38 MAPK protein were examined by a flow cytometer following 5,5',6,6'-tetrachloro-1,1',3,3'-tetraeth-

ylbenzimidazolcarbocyanine iodide (JC-1) and Alexa Fluor[®] 647 mouse anti-phosphorylated P38 MAPK antibody staining, respectively. The activation of caspase-9 and caspase-3 were measured using colorimetric assays. Additionally, Bcl-2 and Bax expression were examined using reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis. The results demonstrated that water extract of FZQJ was able to induce apoptosis of HepG2 cells *in vitro*. FZQJ-induced apoptosis was accompanied by the loss of $\Delta\psi$, downregulation of Bcl-2 and upregulation of Bax expression, and the activation of caspase-3, -9 and P38 MAPK. These results indicated that FZQJ induced apoptosis in HepG2 cells at least via P38 MAPK activation and the mitochondria-dependent apoptotic pathway.

Introduction

Due to latent onset, rapid progression and poor prognosis, hepatocellular carcinoma (HCC) is the third-leading cause of cancer-related mortality worldwide, particularly in Africa and Asia (1,2). Despite the continuous introduction of numerous new treatments, the overall five-year survival rate remains low (3,4). As the majority of patients are in the advanced stages and have metastasis when they are diagnosed with HCC, they are not referred for surgical resection, liver transplantation or radiofrequency ablation. These patients are only able to be treated with chemotherapy and/or radiotherapy in addition to palliative care. However, the development of cancer cell resistance and severe side effects represent main obstacles to the success of chemoradiotherapy. It is thus urgent to develop new therapeutics with lower toxicity and higher efficiency, or new adjuvant therapies that are able to improve the efficiency or decrease the side effects of the current chemoradiotherapy to improve patient survival and quality of life. Traditional Chinese medicine (TCM) may be a promising candidate.

In accordance with the TCM principle, cancer occurs when Xie is stronger than Zheng in the human body. Xie refers to various pathogenic factors (including viruses, fungi and bacteria). Zheng means the disease-fighting ability of the body, including immune function. Fuzheng (improving

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Abbreviations: TCM, traditional Chinese medicine; HCC, hepatocellular carcinoma; FZQJ, Fuzheng Qingjie; MAPK, mitogen-activated protein kinase; DAPI, 4,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription polymerase chain reaction; $\Delta\psi$, mitochondrial membrane potential; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; caspase, cysteine aspartyl-specific protease; HRP, horseradish peroxidase

Key words: Fuzheng Qingjie recipe, hepatocellular carcinoma, apoptosis, mitochondrial membrane potential, P38 MAPK

Zheng) Quxie (eradicating Xie) is thus the basic theory of fighting cancer in TCM. The clinical manifestations of Zheng deficiency include weakness, night sweating, dizziness, pallor, shortness of breath and anemia. Zheng deficiency is common in patients following chemotherapy and/or radiotherapy. These patients often present with symptoms of Xie, including ache, hyperpyrexia, restlessness and a quick pulse rate. Clearly, Zheng deficiency and Xie often coexist in patients with malignancy. Therefore, doctors of TCM always prescribe Fuzheng herbs and Quxie herbs together for cancer patients.

Fuzheng recipes are able to supplement the vital energy, tonify kidney and nourish yin, including *Ginseng* and *Ganoderma*. In clinical trials and basic experiments, these herbs are found to possess the ability to modulate immunity, enhance the efficiency of chemoradiotherapy and improve the quality of life of patients (5-8). Quxie herbs are capable of heat-clearing and detoxification, including *Hedyotis Diffusa* Willd and *Prunella vulgaris*. Pharmacological studies have demonstrated that they contain ingredients that directly induce cancer cell apoptosis, suppress angiogenesis as well as cell invasion and migration (9-12). The combination of Fuzheng and Quxie herbs are not only able to improve the efficiency but also alleviate the adverse effect of chemoradiotherapy.

Fuzheng Qingjie (FZQJ) recipe is a polyherbal formula of Fuzheng and Quxie herbs, which includes *Astragalus membranaceus*, *Ligustrum lucidum*, *Ganoderma lucidum*, *Rhizoma dioscorea*, *Hedyotis Diffusa* Willd and *Prunella vulgaris* (13). The first four are Fuzheng herbs and the other two are Quxie herbs. They have been used for a long time as adjuvant treatments for gastrointestinal malignancies with proven clinical efficacy. Previously, we used an apoptosis microarray (Spring Bioscience, Pleasanton, CA, USA) to screen the pharmacological targets of FZQJ recipe in cancer cells and identified that FZQJ could regulate numerous apoptosis-related genes, including Bax, caspase-3 and -9 and P38 mitogen-activated protein kinase (MAPK; unpublished data). Since the activation of P38 MAPK can result in mitochondria-dependent apoptosis, we investigated whether the activation of P38 MAPK is involved in the apoptotic cell death induced by FZQJ.

Materials and methods

Preparation of water extract of FZQJ herbs. Herbs were purchased from Tongchun Pharmaceutical Co., Ltd (Fuzhou, Fujian, China). Their quality met the criteria described in the Pharmacopoeia of the People's Republic of China. To prepare water extract, *Astragalus membranaceus* (60 g), *Ligustrum lucidum* (60 g), *Ganoderma lucidum* (30 g), *Rhizoma dioscorea* (30 g), *Prunellae vulgaris* (30 g) and *Hedyotis diffusa* Willd (60 g) were pulverized into extremely fine powders with a mortar and pestle and immersed in distilled water, respectively. The mixture was simmered for 2 h and filtered. The solution was concentrated so that it contained 2.66 g of dry herbs per 1 ml and was stored at 4°C until use.

Cell culture. Human HepG2 hepatoma cells were purchased from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 culture medium (Gibco-BRL, Carlsbad, CA, USA) with 100 ml/l of fetal calf serum, 1x10⁵ U/l of penicillin

and 100 mg/l of streptomycin (Gibco-BRL) in an incubator (Thermo Fisher Scientific, Rockford, IL, USA) at 37°C with 5% CO₂. Cell morphology was observed using an inverted microscope (Olympus, Tokyo, Japan).

MTT assay. Cells were cultured in the absence or presence of water extract of FZQJ at the final concentrations of 0.5, 1, 1.5 and 2 mg/ml for 24, 48 and 72 h respectively. Then, 20 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Invitrogen Life Technologies, Carlsbad, CA, USA) was added to each well and incubated for another 4 h prior to discarding the medium. The purple-blue formazan precipitate was dissolved in 100 µl of dimethyl sulfoxide (DMSO). The absorbance (OD) was measured at 490 nm with a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The cell viability ratio was calculated according to the following formula: Cell viability ratio (%) = average OD_{treatment group}/average OD_{vehicle group} x 100%.

DAPI staining. In brief, following treatment with different concentrations of water extract of FZQJ for 24 h, the cells were fixed with 4% paraformaldehyde and then incubated with 1 µg/ml of 4,6-diamidino-2-phenylindole (DAPI) staining solution (Beyotime Inc., Shanghai, China) at room temperature for 5 min and washed with PBS 3 times. The morphology of nuclei in HepG2 cells was observed under a fluorescence microscope (Olympus) at an excitation wavelength of 350 nm.

Cell apoptosis and mitochondrial membrane potential assays. Cells were incubated in 6-well plates for 24 h in the absence or presence of different concentrations of FZQJ water extract. Then cells were digested by trypsinase and washed twice with cold PBS. A final concentration of 1x10⁶ cells/ml of single-cell suspension was prepared. Apoptosis and mitochondrial membrane potential (Δψ) were measured by a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with Annexin-V fluorescein isothiocyanate (FITC) or 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; BD Biosciences). Cells (10,000) were acquired and analyzed using CellQuest software (BD Biosciences). Each determination was performed as three parallel assays.

Reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA was isolated using the TRIzol one-step method according to the manufacturer's instructions (Invitrogen Life Technologies). Single-stranded cDNA was synthesized using oligo (dT) primer (Takara, Dalian, China) in a 20 µl reaction mixture. Bcl-2 and Bax mRNA were evaluated using PCR. The primer pairs of Bcl-2, Bax and GAPDH were as follows: Bcl-2, forward 5'-CAG CTG CAC CTG ACG CCC TT-3' and reverse 5'-GCC TCC GGT ATC CTG GAT CC-3'; Bax, forward 5'-TGC TTC AGG GTT TCA TCC AGG-3' and reverse 5'-TGG CAA AGT AGA AAA GGG CGA-3'; GAPDH, forward 5'-AGA AGG CTG GGG CTC ATT TG-3' and reverse 5'-AGG GGC CAT CCA CAG TCT TC-3'. DNA amplification was performed for 40 cycles following an initial denaturation step at 94°C for 5 min in a thermo cycler by using the following program: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 10 min. PCR reagent kit was obtained from

Takara. Finally, the amplified products were separated on a 1.2% agarose gel and examined using a Gel Doc 2000 Imaging System (Bio-Rad, Hercules, CA, USA).

Western blot analysis. HepG2 cells were collected and lysed in lysis buffer (Beyotime Inc.) for 10 min following treatment with different concentrations of FZQJ water extract for 24 h. Following centrifugation at 12,000 x g at 4°C for 20 min, the supernatant was collected and the protein concentration determined using the Bradford assay. Equal amounts of denatured protein were separated on SDS-PAGE gels and transferred onto PVDF membranes. These membranes were then put into blocking solution for 1 h and incubated in solution with either monoclonal anti-human Bax or Bcl-2 primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C with agitation and then in horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime Inc.) for at least 1 h. Protein was detected with ECL solution (Beyotime Inc.) using a chemiluminescence imaging system (Bio-Rad).

Caspase-3 and -9 activation analysis. The activities of caspase-3 and -9 were examined using caspase-3 and -9 colorimetric assay kits (KeyGen Biotech, Nanjing, Jiangsu, China). Briefly, HepG2 cells were collected and lysed on ice with 100 μ l of lysis buffer (containing 1% dithiothreitol) following treatment with different concentrations of FZQJ water extract for 24 h. Following centrifugation at 11,000 x g for 1 min, the protein concentration of the supernatant was determined using the Bradford assay. Equal amounts of protein were incubated with specific substrates of caspase-3 or -9 at 37°C in the dark for 4 h. Finally the samples were determined at 405 nm by a microplate reader (BioTek Instruments Inc.). The activities of caspase -3 or -9 were calculated according to the formula: average OD_{treatment}/average OD_{vehicle}.

Phosphorylated P38 MAPK assay. Phosphorylated P38 MAPK (p-P38 MAPK) was evaluated using flow cytometry. The cells were digested and rinsed with PBS following treatment with different concentrations of FZQJ water extract for 48 h. Then cells were suspended, fixed with fixation buffer for 10 min at 37°C, permeabilized on ice for 30 min and stained with Alexa Fluor[®] 647 mouse anti-p-P38 MAPK antibody (BD Biosciences). The cells were analyzed with the flow cytometer.

Statistical analysis. Data were analyzed using the SPSS 16.0 statistical package. All results are expressed as the mean \pm standard deviation (SD) of at least three experiments. The data for multiple comparisons were performed by one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

Water extract of FZQJ recipe inhibits HepG2 cell proliferation. FZQJ made according to the patent (13). The growth of HepG2 cells was evaluated using an MTT assay. As shown in Fig. 1, in the presence of indicated concentrations of water extracts, cell proliferation was inhibited in a dose- and time-dependent manner. The inhibitory rate of HepG2 cells was able to reach as high as 92.57% at the highest concentration

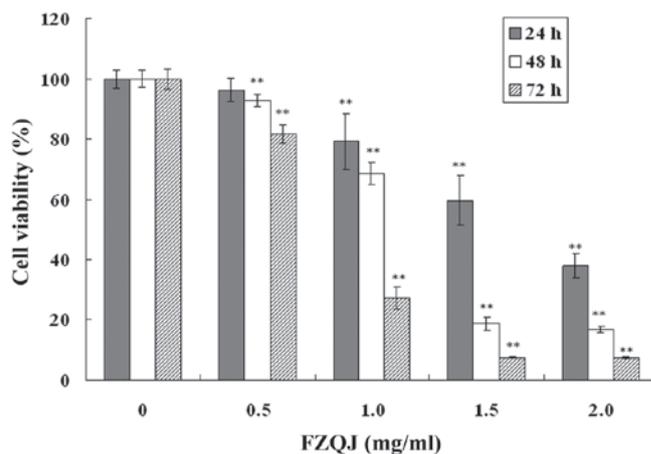


Figure 1. Effect of water extract of FZQJ on the proliferation of HepG2 cells. HepG2 cells were treated with different concentrations of FZQJ water extract for 24, 48 and 72 h, respectively. Data represent the mean \pm SD of at least three experiments. **P<0.01, compared with the vehicle control. FZQJ, Fuzheng Qingjie; SD, standard deviation.

for 72 h. The results demonstrated that water extract of FZQJ markedly inhibited the proliferation of HepG2 cells.

Water extract of FZQJ induces apoptosis of HepG2 cells. We first evaluated apoptosis of HepG2 cells treated with indicated doses of FZQJ for 24 h through observing the morphological changes using an inverted fluorescence microscope. As shown in Fig. 2A, a decreased cell number was associated with morphological changes of cells following the addition of FZQJ to the medium; polygon- or spindle-shaped cells became round, shrunk and collapsed. Following staining of the nuclei with DAPI (a fluorescent DNA-binding agent), more brightened nuclei were observed in the FZQJ-treated cells, with chromatin pyknosis and fragmentation (Fig. 2B). Clearly, FZQJ water extract induced marked apoptotic morphological alterations.

Next, cells were evaluated flow cytometrically by Annexin-V staining. Annexin-V is a cellular protein used as a probe to detect cells that have expressed phosphatidylserine on the cell surface (an event identified in apoptosis). Therefore, Annexin-V positive cells are considered to be apoptotic cells. As displayed in Fig. 2C, the percentage of apoptotic cells stained with Annexin V markedly increased in FZQJ-treated cells in a dose-dependent manner. Taken together, these data revealed that FZQJ extract induced apoptosis of HepG2 cells.

Water extract of FZQJ induces a decrease in the mitochondrial membrane potential. Apoptosis is often accompanied by a decrease of $\Delta\psi$. Loss of $\Delta\psi$ is important in the early apoptotic process (14). JC-1 is a lipophilic fluorochrome that is found to be sensitive to $\Delta\psi$ and is used as an indicator of $\Delta\psi$ during apoptosis. JC-1 has two different formations, aggregates and monomers. In normal cells with a polarized $\Delta\psi$, JC-1 aggregates stay in the mitochondria and emit red fluorescence (red channel) and monomers stay in the cytoplasm and exhibit green fluorescence (green channel). Therefore, JC-1-treated normal cells demonstrate green and red channels on flow cytometers. When the mitochondria $\Delta\psi$ depolarizes, JC-1 does not form aggregates in the

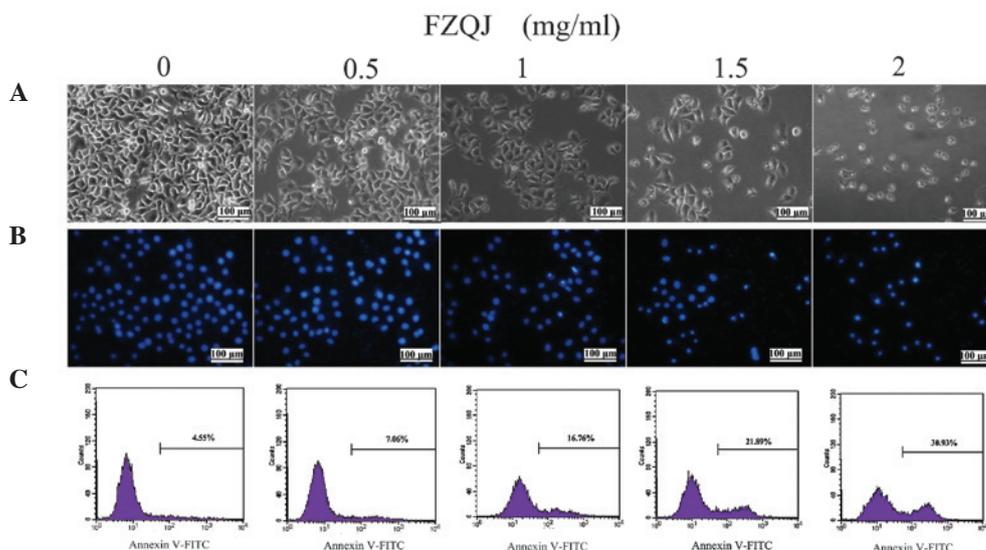


Figure 2. Water extract of FZQJ induces apoptosis of HepG2 cells. (A) Vehicle and FZQJ-treated cells at the indicated time points were visualized by phase-contrast microscopy and (B) fluorescence microscope (DAPI staining) and images were captured at a magnification of x200. (C) The representative apoptotic profiles of FZQJ extract-induced apoptosis using flow cytometric analysis. FZQJ, Fuzheng Qingjie; DAPI, 4,6-Diamidino-2-phenylindole.

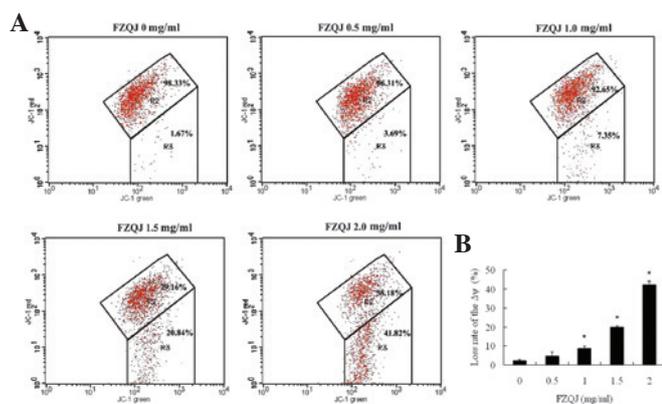


Figure 3. Water extract of FZQJ induces loss of the $\Delta\psi$. After the cells were treated with indicated concentrations of FZQJ water extract for 24 h, $\Delta\psi$ was measured using JC-1 fluorescence dye by a flow cytometer. (A) A typical chart is shown. (B) Loss rate of $\Delta\psi$ from three experiments. * $P < 0.05$ and ** $P < 0.01$, compared with the vehicle control. FZQJ, Fuzheng Qingjie; $\Delta\psi$, mitochondrial membrane potential; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarboyanine iodide.

mitochondria but remains as monomers in the cytoplasm, resulting in increased numbers of cells with reduced JC-1 fluorescence in the red channel. As shown in Fig. 3, JC-1 fluorescence was observed in red and green channels (R2 region) in the vehicle-treated cells, indicating that the majority of cells were alive. In the presence of increasing concentrations of FZQJ, there was a significant increase in the number of cells with lowered red fluorescence (R3 region), indicative of a depolarized $\Delta\psi$. Thus, the data indicate that FZQJ-induced apoptosis was associated with the depolarization of $\Delta\psi$.

Water extract of FZQJ induces activation of caspase-3 and -9 in HepG2 cells. The activities of caspase-3 and -9 are closely associated with the mitochondria-dependent apoptosis pathway. We next examined whether caspase-3 and -9 were involved in FZQJ-induced depolarization of $\Delta\psi$ in

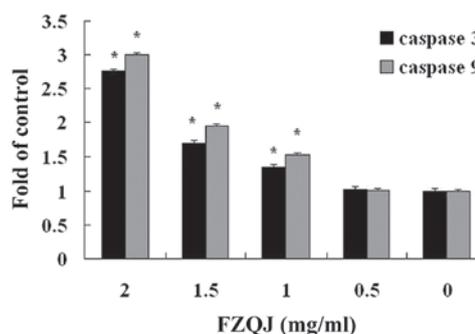


Figure 4. Water extract of FZQJ increases activities of caspase-3 and -9 in HepG2 cells. After the cells were treated with indicated concentrations of FZQJ water extract for 24 h, activities of caspase-9 and -3 were examined and the data are expressed as the mean \pm SD of at least three experiments. * $P < 0.01$, compared with the vehicle control. FZQJ, Fuzheng Qingjie; SD, standard deviation.

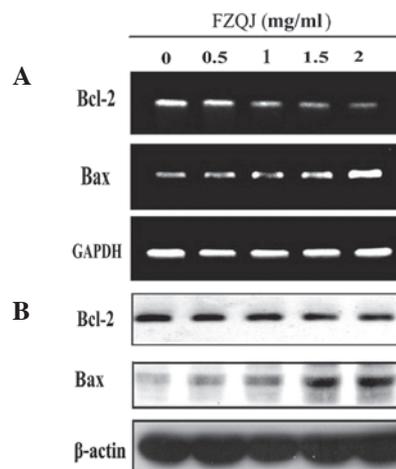


Figure 5. Water extract of FZQJ downregulates Bcl-2 expression and upregulates Bax expression. After HepG2 cells were treated with FZQJ water extract for 24 h, Bax and Bcl-2 expression were analyzed by (A) RT-PCR and (B) western blot analysis. FZQJ, Fuzheng Qingjie; RT-PCR, reverse transcription polymerase chain reaction.

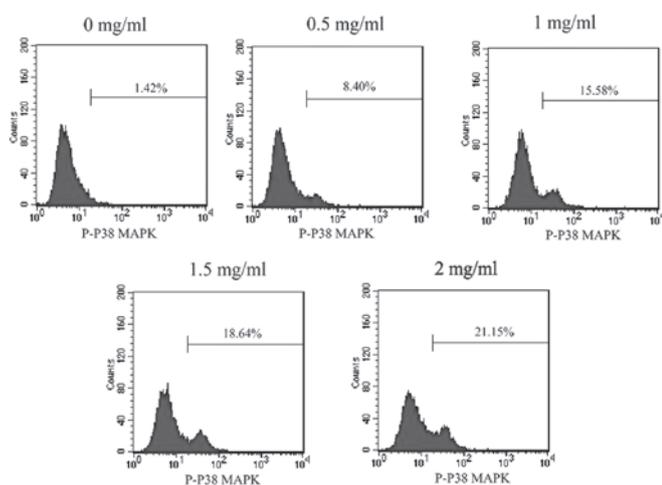


Figure 6. Water extract of FZQJ activates P38 MAPK. After HepG2 cells were treated with FZQJ water extract for 24 h, p-P38 MAPK levels were quantified by measuring FITC fluorescence intensity using a flow cytometer. The markers in the histograms represent the percentage of p-P38 MAPK positive cells. FZQJ, Fuzheng Qingjie; MAPK, mitogen-activated protein kinase; FITC, fluorescein isothiocyanate.

HepG2 cells. The activities of caspase-3 and -9 were examined by colorimetric assay. The results demonstrated that the activities of caspase-3 and -9 were markedly increased in a dose-dependent manner. Following treatment with 2 mg/ml of FZQJ extract for 24 h, 2.77-fold and 2.99-fold increases were observed for caspase-3 and -9 activity compared with the vehicle control, respectively (Fig. 4). The data suggested that water extract of FZQJ could decrease $\Delta\psi$ in HepG2 cells via the activation of caspase-3 and -9.

Water extract of FZQJ downregulates Bcl-2 and upregulates Bax. Antiapoptotic Bcl-2 and proapoptotic Bax are members of the Bcl-2 family and are critical in apoptosis controlled by mitochondria. To further investigate how FZQJ induces mitochondria-dependent apoptosis, the expression of Bcl-2 and Bax were evaluated in mRNA and protein levels by RT-PCR and western blot analysis, respectively. As shown in Fig. 5A, compared with the vehicle control, FZQJ decreased Bcl-2 mRNA and increased Bax mRNA expression in a dose-dependent manner. Similar results were observed in western blot analyses (Fig. 5B), indicating that FZQJ induced depolarization of $\Delta\psi$ in HepG2 cells through regulating the expression of members of the Bcl-2 family.

Water extract of FZQJ activates P38 MAPK. Activation of P38 MAPK has been demonstrated to lead to cell death via stimulating mitochondrial Bax translocation and activating caspase -9 and -3 (15). To investigate whether FZQJ induces mitochondrial apoptosis via activation of P38 MAPK, phosphorylation of P38 MAPK was evaluated. As shown in Fig. 6, FZQJ treatment caused a dose-dependent increase of p-P38 MAPK level in HepG2 cells, indicating that FZQJ could activate P38 MAPK.

Discussion

In previous years, TCMs have been used in an increasing number of countries to treat tumors and the underlying

mechanisms are being investigated (16,17). TCMs are drawing more and more attention from oncologists as, in addition to direct inhibition on tumor growth, TCMs may improve the anticancer response and reduce the side effects of chemotherapy. Moreover, TCMs have few and mild adverse effects (18,19).

FZQJ recipe has been prescribed to treat cancer and manage the side effects of chemotherapy in China. It is composed of six medicinal herbs. Our previous study demonstrated that these 6 herbs are commonly prescribed for patients with malignant tumors according to cluster analysis (20). These herbs contain compounds with anti-cancer activities, including polysaccharides, saponins, flavones, anthraquinones and polyphenols. By molecular docking simulation, we revealed that Bcl-x1, tumor necrosis factor (TNF)- α , interleukin (IL)-2, and cyclooxygenase 2 are possible targets of *Hedyotis diffusa* Willd and *Prunella vulgaris* in the FZQJ recipe (21). Pharmacological studies have demonstrated that compounds in herbs of FZQJ recipe can inhibit the proliferation of tumor cells, induce apoptosis of tumor cells, improve the sensitivity of cancer cells to chemotherapy and modulate immune function. For instance, *Astragalus* polysaccharides were demonstrated to enhance the chemosensitivity of H22 hepatoma cells resistant to adriamycin (22). *Astragalus* polysaccharides can inhibit the proliferation of the basal-like breast cancer cell line MDA-MB-468 via regulating p53/MDM2 positive and negative feedback loops (23). 2-Hydroxy-3-methylantraquinone from *Hedyotis diffusa* Willd was revealed to be able to induce apoptosis in different tumor cells through the modulation of MAPK pathways (24), the Ca^{2+} /calpain/caspase-4 pathway (25), as well as the alteration of Fas/FasL and the activation of caspase-8 (26). *Ganoderma lucidum* polysaccharides can enhance immunity via reducing the levels of serum IL-6 and TNF- α and increasing those of serum IL-2, IL-4 and IL-10 (27). Oleanolic acid from *Prunella Vulgaris* was demonstrated to induce apoptosis of lung adenocarcinoma cells through downregulating Bcl-2 expression and upregulating Bax and Bad expression (28). An oleanolic acid-enriched extract of *Ligustrum Lucidum* were found to have potential immunomodulatory effects through enhancing the proliferative activity of blood lymphocytes and upregulating the CD4⁺CD8⁺ and CD4⁺CD8⁻ cell populations as well as regulating the expression of Th1- and Th2-related cytokines (29). In addition, a polysaccharide from *Dioscorea opposita* Thunb roots was demonstrated to be able to enhance the immunological activity via stimulating ConA-induced T lymphocyte proliferation (30). Thus, it is possible the recipe containing these herbs according to the TCM theory may have additive anticancer effects.

Cysteine aspartyl-specific proteases (caspases), a family of cysteine proteases, act in concert in a cascade-triggered manner and are activated via three main pathways when cells receive apoptotic signals (31). The three pathways are death receptors signaling, the mitochondrial pathway and the endoplasmic reticulum stress pathway. The second pathway is an intrinsic pathway, in which mitochondria are the central organelle governed by pro- and anti-apoptotic members of the Bcl-2 family (32). Numerous molecules in mitochondria are found to be closely associated with cell apoptosis, including

cytochrome c (cyt-c), apoptosis-inducing factor and reactive oxygen species (33). When a mitochondrion is damaged, mitochondrial outer membrane permeabilization occurs and $\Delta\psi$ collapse. Then cyt-c is released from the mitochondrial intermembrane space into the cytoplasm. Subsequently, cyt-c couples with apoptosis protease activating factor-1 and triggers pro-caspase-9 assembly to promote production of active caspase-9 (34,35). Active caspase-9 further activates the downstream proteases of the caspase cascade, including caspase-3, -6 and -7 (36). These proteases may lead to DNA mismatch repair dysfunction, and even fragmentation, finally causing inevitable apoptotic cell death (37). Caspase-3 and -9 are key proteases responsible for the execution of apoptosis.

The mitochondria-dependent cell death cascade is regulated by the Bcl-2 family. The members of the Bcl-2 family include anti-apoptotic (Bcl-2) and pro-apoptotic members (Bax). Bax can promote the openness of mitochondrial permeability transition pores (MPTP) and release cyt-c, subsequently activating the caspase cascade and triggering apoptosis. Inversely, Bcl-2 can inhibit the openness of MPTP and protect cells from apoptosis. Previous studies have provided evidence that overexpression of the Bcl-2 protein or decreased expression of the Bax gene was associated with poor prognosis in various diseases (38). In the present study, we demonstrated that FZQJ may downregulate Bcl-2 expression and upregulate Bax expression and activate caspase-3 and -9, indicating that FZQJ-induced apoptosis of HepG2 cells is at least through the mitochondria-dependent apoptotic cascade.

P38 MAPK is a member of the MAPK family. It is crucial in regulating cellular proliferation, differentiation and apoptosis (39-41). The upregulation of P38 MAPK activity is essential for apoptotic induction in tumor cells. Apoptosis mediated by Fas/Fas-ligand (42), c-myc (43), p53 (44) as well as c-jun and c-jos (41) are associated with the enhanced activation of p38 MAPK. In addition, numerous studies also demonstrated that P38 MAPK activity could activate the mitochondria-dependent cell death pathway by stimulating Bax translocation from the cytosol to the mitochondria, further promoting the release of cyt-c and thereby triggering the cell death cascade (45,46,15). Our findings demonstrated that water extract of FZQJ could activate P38 MAPK, which may be responsible for the activation of the mitochondria-dependent cell death cascade.

In conclusion, our results demonstrate that the anti-cancer effect of FZQJ recipe on HepG2 cells may involve the activation of P38 MAPK and the subsequent mitochondria-dependent apoptotic cascade. These data provide scientific basis for the clinical application of FZQJ recipe as an adjunct for chemoradiotherapy.

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