Synergisic effect of APRIL knockdown and Jiedu Xiaozheng Yin, a Chinese medicinal recipe, on the inhibition of hepatocellular carcinoma cell proliferation

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Abstract. It is well documented that A proliferation-inducing ligand (APRIL), a member of the tumor necrosis factor superfamily, plays a crucial role in the occurrence and development of tumors. In the present study, we evaluated the synergistic effect of APRIL knockdown and Jiedu Xiaozheng Yin (JXY), a Traditional Chinese Medicinal recipe, on the inhibition of hepatocellular carcinoma (HCC) cell proliferation and elucidated the underlying mechanism. The results demonstrated that both APRIL knockdown using small interfering RNA (siRNA) and JXY treatment could trigger cell cycle arrest and cell apoptosis, and suppress HCC cell proliferation through an NF-κB-related pathway. Synergism was further demonstrated between APRIL knockdown and JXY treatment. In conclusion, these results indicate that APRIL is a target gene for HCC and combination of siRNA-APRIL and JXY application holds great promise as a novel approach for the treatment of APRIL-positive HCC.

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Abbreviations: HCC, hepatocellular carcinoma; APRIL, A proliferation-inducing ligand; JXY, Jiedu Xiaozheng Yin; NF-κB, nuclear factor-κB; VEGF, vascular endothelial growth factor; HDW, *Hedyotis diffusa* Willd; SF, *Sophora flavescens*; PC, *Pseudobulbus cremastrae*; TNF, tumor necrosis factor; TNFSF13, tumor necrosis factor superfamily 13; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RIPA, radioimmunoprecipitation; MTT, methyl thiazolyl tetrazolium; ECL, electrochemiluminescence; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ANOVA, analysis of variance

Key words: A proliferation-inducing ligand, Jiedu Xiaozheng Yin, hepatocellular carcinoma, nuclear factor-κB

Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies, represents the third leading cause of cancer-related deaths worldwide (1). Although screening in high-risk populations has increased the detection of early-stage HCC and consequently improved survival, most cases still present with advanced and unresectable stages due to intrahepatic metastasis and vascular invasion (2,3). Seeking alternative therapies to improve the survival of HCC patients has been an urgent task for oncologists.

A proliferation-inducing ligand (APRIL), also known as TALL-2 and TNFSF13, was identified via database mining in 1998 by Hahne *et al* (4). APRIL is a member of the TNF family located on human chromosome 17p13. APRIL expression is low in normal cells, including monocytes, dendritic and T cells (5,6). However, studies have demonstrated that it is overexpressed in many tumors, which suggest that APRIL plays a crucial role in the occurrence and development of these tumors (7-9).

Heat-clearing and detoxifying Chinese herbs, which play an important role in antitumor therapy in China, involve the use of extracts from these herbs for the treatment of different types of cancer (10-12). Jiedu Xiaozheng Yin (JXY), a polyherbal Traditional Chinese Medicine (TCM) recipe, is composed of *Hedyotis diffusa* Willd (HDW), *Sophora flavescens* (SF), *Prunella* and *Pseudobulbus Cremastrae* (PC) and is used as a heat-clearing and detoxicating adjuvant therapy for HCC. Our previous study demonstrated that JXY can inhibit the angiogenesis of tumors via the downregulation of vascular endothelial growth factor-A (VEGF-A) and vascular endothelial growth factor receptor-2 (VEGFR-2) expression (13). Moreover, an ethyl acetate extract from JXY inhibits the cell proliferation of HCC by suppressing polycomb gene product Bmi1 and Wnt/β-catenin signaling (14,15). A randomized control trial showed that the addition of JXY to the standard treatment of stage III HCC patients could improve the immune function of patients, decrease recurrence and increase overall survival (16).

In the present study, we evaluated the synergistic effect of APRIL knockdown and JXY treatment on the proliferation of HCC cells and elucidated the underlying mechanism.

Materials and methods

Preparation of the herbal medicine. JXY is composed of HDW (30 g), *Prunella* (15 g), PC (15 g) and SF (15 g). These four herbs of JXY were purchased from the Guo Yi Tang Hospital of Fujian University of Traditional Chinese Medicine (Fuzhou, China). The quality of the 4 medicinal plants met the criterion of the Pharmacopoeia of the People's Republic of China. To prepare the crude water extract, 300, 150, 150 and 150 g of the 4 medicinal plants, HDW, *Prunella*, PC and SF, respectively, were crushed into powders. Then, they were mixed together and immersed in 10 l of distilled water. The mixture was simmered for 2 h and filtered. Subsequently, the solution was concentrated by a rotary evaporator and stored at 4˚C until use.

Animals. The clean grade level male Sprague-Dawley (SD) rats (licence no. SCXK (Hu) 2007-0005), with a body weight of 180-220 g, were purchased from Shanghai SLAC Experimental Animal Co., Ltd. (Shanghai, China). All rats were maintained under a regulated temperature (18-22˚C) and a relative constant humidity (50-60%). All animal handling and experimental procedures were approved by the Animal Care and Use Committee of Fujian University of TCM.

Preparation of JXY-containing serum. The 20 SD rats were divided into 4 groups using a random digit table method (n=10/group) and were administered JXY solution orally at a dose of 9 g/kg/day or saline respectively twice a day for 7 days. One hour after the last treatment, blood was collected from the main ventral artery under aseptic conditions, and centrifuged at 3,000 rpm for 15 min. Serum from the 2 groups was referred to as JXY and control serums, respectively. All sera were filtered through a 0.22-μm filter membrane and inactivated at 56˚C water for 30 min and stored at -20˚C until its use in pharmacological studies.

Cell culture. The HepG2 HCC cell line was purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and incubated in 5% CO_2 at 37˚C.

Real-time-quantitative polymerase chain reaction (RT-qPCR). Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was used to synthesize the first strand of cDNA. qPCR was performed using an Applied Biosystems 7500 Real-Time PCR system and SYBR® Premix Ex Taq™ kit (Perfect Real-Time) (Takara Bio Inc., Shiga, Japan). Primers used were as follows: Tumor necrosis factor receptor-associated factor 6 (TRAF6) sense, 5'-CCCGCGCA CTAGAACGAGCAA-3' and antisense, 5'-GCCATGGCCAC ACAGCAGTCA-3'; NF-κB sense, 5'-CGCGCCGCTTAGGA GGGAGA-3' and antisense, 5'-GGGCCATCTGCTGTTGGC AGT-3'; β-actin sense, 5'-CAATGAAGATCAAGATCATTG CTCCTCC-3' and antisense, 5'-TCAAGAAAGGGTGTAAC GCAACTAAGTC-3'. The expression of β-actin served as the input reference. The relative mRNA expression of TRAF6 and NF-κB were calculated with the comparative threshold cycle (Ct) ($2^{-\Delta\Delta Ct}$) method.

Protein extraction and western blotting. Western blotting was performed as previously described (17). In brief, cell pellets were lysed in a radioimmunoprecipitation assay (RIPA) buffer with 1 mM phenylmethanesulfonyl fluoride (both from Beyotime Institute of Biotechnology, Haimen, China). The cell lysate was centrifuged at 10,000 x g for 10 min at 4˚C and the supernatant was transferred to a microcentrifuge tube. The protein concentration was determined with a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology) and 20 μg proteins were loaded/lane. The protein samples were separated on SDS-PAGE at 10-15%, and electrotransferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Zurich, Switzerland). The membranes were blocked by 5% non-fat milk in Tris-buffered saline Tween-20 (TBST; pH 7.6) for 60 min at room temperature, followed by incubation in primary antibodies overnight at 4˚C and secondary antibodies (Thermo Scientific Pierce, Rockford, IL, USA) for 90 min at room temperature. After the membranes had been washed 3 times, the proteins were detected using electrochemiluminescence (ECL; Amersham Pharmacia Biotech). The antibodies used for western blot analyses were as follows: APRIL, NF-κB and β-actin (Cell Signaling Technology, Beverly, MA, USA).

Knockdown of APRIL. The short hairpin RNA targeting APRIL mRNA was constructed by GeneChem Biotechnology (Shanghai, China). The shRNA sequences were: 5'-GATCCGCAACCTTCTTCCCTTCTGCTTCAAGA GAGCAGAAGGGAAGAAGGTTGTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAACAACCTTCTTCCCTTCTGCT CTCTTGAAGCAGAAGGGAAGAAGGTTGCG-3'; the negative control sequences were: 5'-GATCCGTTCTCCGAA CGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAG AATTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAAT TCTCCGAACGTGTCACGTTCTCTTGAAACGTGACAC GTTCGGAGAACG-3'.

Transfection with shRNA was carried out using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen). Cells transfected with APRIL-shRNA were seeded into 6-well culture plates at a density of $1x10^5$ cells/well. Cells were allowed to grow for 24, 36 and 72 h and were then harvested for analysis. Insignificant control siRNA was used as a negative control under similar conditions.

Cell viability assay. To analyze the viability of cells treated with JXY and/or APRIL-shRNA, $1x10^4$ cells/well were seeded in 96-well plates containing 0.2 ml of medium. After treatment, MTT (5 mg/ml; Sigma, St. Louis, MO, USA) was added to each well (including the control well) and the mixture was incubated at 37˚C for 4 h. The culture medium was then replaced with an equal volume of dimethyl sulfoxide (Sigma). After shaking at room temperature for 15 min, the 490-nm absorbance (A490) of each well was determined on a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability was calculated according to the following formula: Cell viability (%) = A490 (sample)/A490 (control) x 100.

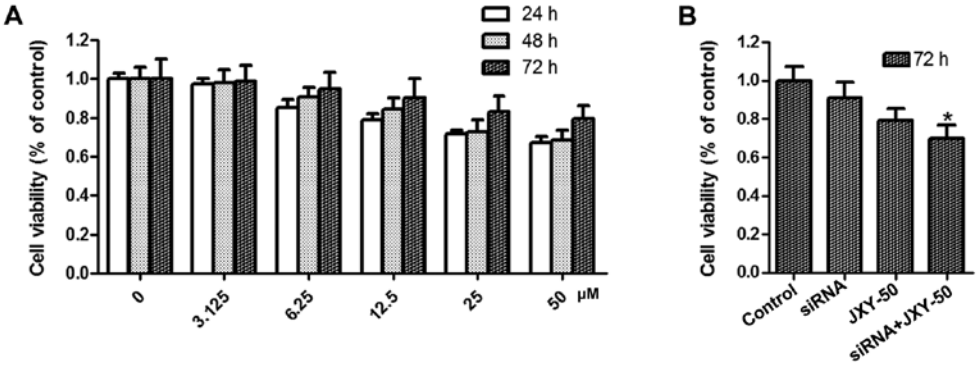

Figure 1. (A) JXY-containing serum at different concentrations inhibits the proliferation of HCC cells *in vitro*. (B) Comparison of the levels of cell viability after treatment of JXY at the indicated concentrations for 72 h. The viability of the HCC cells was analyzed using methyl thiazolyl tetrazolium (MTT) method. Although JXY treatment had no effect on HCC cell proliferation (P>0.05), the combination of APRIL knockdown and JXY treatment significantly suppressed cell proliferation in a dose-dependent manner (*P<0.05). Data shown are expressed as the mean ± standard deviation. JXY, Jiedu Xiaozheng Yin; HCC, hepatocellular carcinoma; APRIL, A proliferation-inducing ligand.

TUNEL assay of cell apoptosis. Cells were seeded onto slides and cultured before being treated with JXY and/or APRIL-shRNA. The cells were then fixed in 4% paraformaldehyde for 60 min and permeabilized with 0.1% Triton X-100 on ice for 2 min. Cell apoptosis was determined using a TUNEL Apoptosis Assay kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions 48 h post-transfection. Briefly, TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture was added to the cells for 15 min and the slides were then rinsed in phosphate-buffered saline (PBS) before being incubated in a humidified chamber at 37˚C for 60 min in the dark. Anti-fluorescence quenching solution was added before examination of the cells under a confocal laser-scanning microscope (TCS SP5) at an excitation wavelength of 450-500 nm and an emission wavelength of 515-565 nm (green fluorescence) in order to evaluate the proportion of apoptotic cells.

Reporter gene transfection and luciferase activity assay. Cells at 80-90% confluence growing on a 35-mm dish were co-transfected with the firefly luciferase reporter of NF-κB containing a TA promoter (1 μg, pNF-κB-TA-luc; Beyotime Biotechnology, Shanghai, China) along with the *Renilla* luciferase reporter (0.1 μg; Promega Co., Madison, WI, USA) for 6 h using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen). Some cells were further treated with JXY and/or APRIL shRNA. The luciferase activity was assessed in the cellular extracts using a dual-luciferase reporter gene assay kit (Beyotime Biotechnology. Briefly, the relative fluorescence light unit (RLU) at 560 nm of the mixture consisting of 50 μl total cell lysate and 100 μl of the firefly luciferase assay reagent was evaluated using a multimode microplate reader (Tecan Infinite M200; Tecan, Männedorf, Switzerland) for a total period of 10 sec. Then, 100 μl of *Renilla* luciferase assay reagent was added into the aforementioned mixture and its fluorescence at 465 nm was measured. The relative activity of the reporter gene was calculated by dividing the RLU at 560 nm by that at 465 nm.

Statistical analysis. Data are the results from at least 3 independent experiments. All data are presented as the means ± SD.

Table I. Synergistic effect of APRIL knockdown or/and JXY treatment on cell cycle distribution in HCC cells.

Treatment	G1 (%)	G2/M (%)	S (%)
Blank	42.46±2.35	38.93±2.03	18.16±1.97
Control	43.39±4.18	33.06±3.17	23.55±2.52
shRNA	49.68±4.76	37.09±3.63	13.23±2.98[a]
Herb	44.69±3.89	22.06±3.53[a,b]	33.25±3.43
shRNA + Herb	59.01±5.01[a,b]	20.6±2.99[a,b]	20.39±3.05

Combination of APRIL knockdown and JXY treatment significantly induced G0/G1 cell cycle arrest. Values are the mean ± standard deviation; [a]P<0.05 vs. the control cells; [b]P<0.05 vs. the cells treated with shRNA alone. APRIL, proliferation-inducing ligand; JXY (Herb), Jiedu Xiaozheng Yin; HCC, hepatocellular carcinoma.

Significance was assessed using one-way ANOVA among groups or unpaired t-test for 2 groups. All P-values were two-sided, and the differences were considered significant at a value of P<0.05. All statistical analyses were carried out using SPSS 18.0.

Results

Synergistic inhibitory effect of APRIL knockdown and JXY-containing serum on HCC cell proliferation. To investigate the effect of APRIL knockdown and/or JXY treatment on HCC cell proliferation, an MTT assay was performed in HCC cells following treatment with JXY-containing serum at different concentrations. As shown in Fig. 1, in comparison with the control groups, APRIL knockdown had no effect on HCC cell proliferation while APRIL knockdown significantly enhanced the inhibitory effect of JXY on cell proliferation (P<0.05). Collectively, these results indicated that the synergistic effect of APRIL knockdown and JXY suppressed HCC cell proliferation.

Synergistic effect of APRIL knockdown and JXY on cell cycle arrest. A DNA cell cycle analysis was performed, in order to

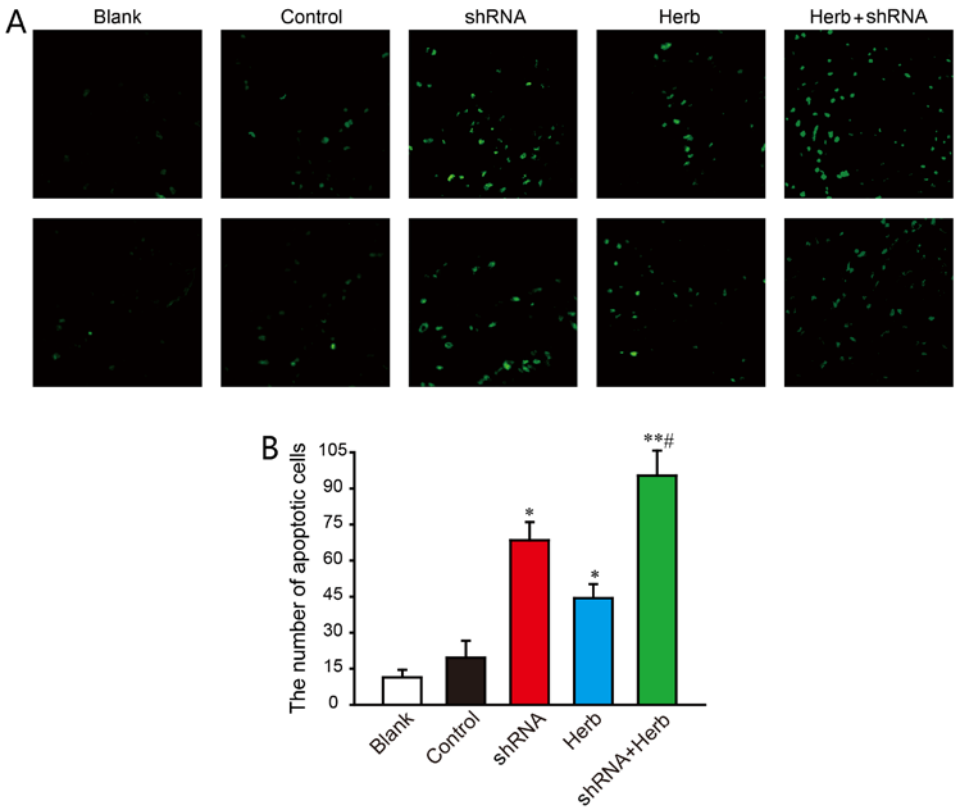

Figure 2. Effect of APRIL knockdown and JXY treatment on HCC cell apoptosis as confirmed by TUNEL assay. (A) The TUNEL assay was carried out by One Step TUNEL Apoptosis Assay kit. The images of TUNEL-positive cells were captured by a fluorescence microscope (magnification, x200). (B) The quantitative result of the TUNEL assay was analyzed. Data are represented as the mean ± standard deviation; *P<0.05 vs. the control cells; **P<0.05 vs. the cells treated with JXY (Herb) alone; $^{\#}$P<0.05 vs. the cells treated with shRNA alone. APRIL, A proliferation-inducing ligand; JXY, Jiedu Xiaozheng Yin; HCC, hepatocellular carcinoma; TUNEL, TdT-mediated dUTP nick end labeling.

assess the effects of APRIL knockdown and/or JXY treatment on cell cycle distribution. As shown in Table I, treatment with JXY or APRIL knockdown resulted in an accumulation of cells in the G0/G1 phase of the cell cycle. Notably, the combination of APRIL knockdown and JXY treatment significantly induced G0/G1 cell cycle arrest (shRNA + Herb 59.01±5.01% vs. Herb 44.69±3.89%; shRNA + Herb 59.01±5.01% vs. shRNA 49.68±4.76%) (P<0.05).

Next, we detected the percentage of cell apoptosis using TUNEL staining. The results showed that both JXY treatment and APRIL knockdown increases the percentage of cell apoptosis (P<0.05; Fig. 2). In addition, combination of APRIL knockdown and JXY treatment significantly enhanced the number of apoptotic cells (P<0.05; Fig. 2). Collectively, these results indicated the synergistic effect of APRIL knockdown and JXY treatment on inhibition of HCC cell growth possibly through the induction of G0/G1 cell cycle arrest and the promotion of cell apoptosis.

Synergistic effect of APRIL knockdown and JXY treatment inhibits NF-κB expression. TRAF6 is a unique adaptor protein of the tumor necrosis factor receptor-associated factor family that mediates both tumor necrosis factor receptors and interleukin-1 receptor/Toll-like receptor signaling. TRAF6 plays a very important role in NF-κB activation (18,19). Recent studies have shown that TRAF6/NF-κB signaling pathways play an important role in tumorigenesis and invasion (20,21). Next, we explored whether APRIL knockdown and/or JXY treatment could affect TRAF6 and NF-κB expression. RT-qPCR (Fig. 3) and western blotting (Fig. 4) results showed that APRIL knockdown decreased NF-κB mRNA and protein expression levels (P<0.05), but no difference was found in the expression level of TRAF6 (P>0.05). In addition, the expression levels of both TRAF6 and NF-κB were not altered after JXY treatment. However, the combination of APRIL knockdown and JXY treatment significantly reduced the expression of NF-κB (P<0.05; Figs. 3 and 4). These results suggest that the synergistic effect of APRIL knockdown and JXY treatment in the inhibition of NF-κB expression was dependent on TRAF6 signaling.

Synergistic effect of APRIL knockdown and JXY treatment decreases the activity of NF-κB. A previous study revealed that APRIL could upregulate the activity of nuclear factor NF-κB (22). Therefore, we speculated that APRIL knockdown and/or JXY treatment may downregulate the activity of nuclear factor NF-κB directly. Luciferase activity of pNF-κB-TA-Luc that contained multiple copies of the NF-κB responsive element was used for monitoring NF-κB activity (23). As shown in Fig. 5, the result of NF-κB-controlled luciferase reporter assay revealed that after the knockdown of APRIL, the activity of NF-κB was significantly decreased (P<0.05). In addition, the activity of NF-κB in the JXY-treated cells was also lower than that in the non-treated cells (P<0.05). Among the groups, the activity of NF-κB was the lowest in the combination group

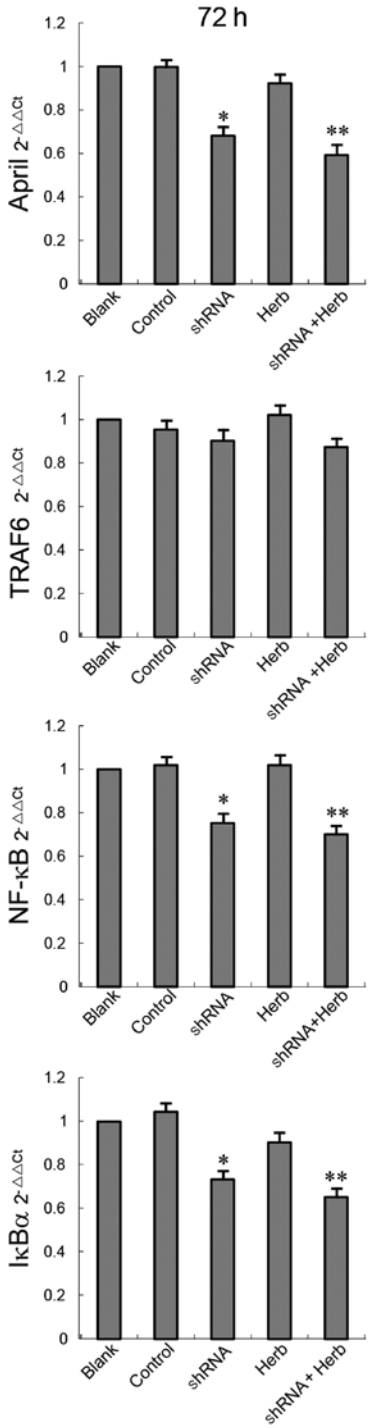

Figure 3. Effect of APRIL knockdown and JXY treatment on mRNA expression levels in HCC cells. The mRNA expression levels of APRIL, TRAF6, NF-κB and IκBα were determined by reverse transcription-polymerase chain reaction and quantified by densitometric analysis. Images are representative of 3 independent experiments. The data were normalized to the mean mRNA expression level of the untreated control; *P<0.05 vs. the control cells; **P<0.05 vs. the cells treated with Herb alone. APRIL, A proliferation-inducing ligand; JXY (Herb), Jiedu Xiaozheng Yin; HCC, hepatocellular carcinoma; TRAF6, tumor necrosis factor receptor-associated factor 6; NF-κB, nuclear factor-κB.

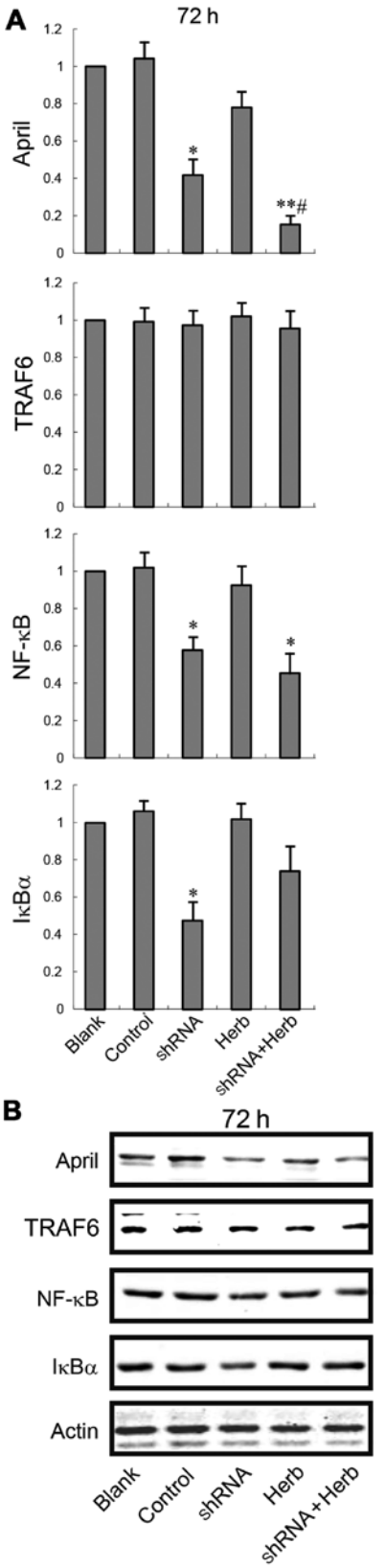

Figure 4. Effect of APRIL knockdown and JXY treatment on protein expression levels in HCC cells. (A) The expression level of all proteins was quantified by densitometric analysis. (B) The expression levels of proteins associated with the NF-κB pathway were assessed by western blot analysis. Data shown are expressed as the mean ± standard deviation of 3 repeated experiments; *P<0.05 vs. the control cells; **P<0.05 vs. the cells treated with the Herb alone; #P<0.05 vs. the cells treated with shRNA alone. APRIL, A proliferation-inducing ligand; JXY (Herb), Jiedu Xiaozheng Yin; HCC, hepatocellular carcinoma; TRAF6, tumor necrosis factor receptor-associated factor 6; NF-κB, nuclear factor-κB.

(shRNA + Herb; P<0.05). Therefore, the synergistic effect of APRIL knockdown and JXY treatment can trigger cell cycle arrest and cell apoptosis and subsequent suppression of HCC cell proliferation through an NF-κB-related pathway.

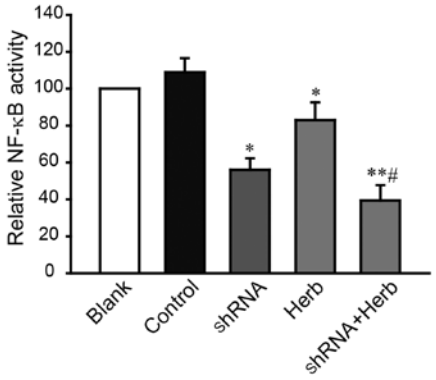

Figure 5. APRIL knockdown and JXY treatment synergistically decrease the activity of NF-κB in HCC cells. The NF-κB activity in HCC cells was analyzed by an NF-κB reporter luciferase assay. Data shown are from representative experiments where electroporations were performed in triplicate. Activation is indicated by the mean ± standard deviation; $^{*}P<0.05$ vs. the control cells; $^{**}P<0.05$ vs. the cells treated with the Herb alone; $^{\#}P<0.05$ vs. the cells treated with shRNA alone. APRIL, A proliferation-inducing ligand; JXY (Herb), Jiedu Xiaozheng Yin; HCC, hepatocellular carcinoma.

Discussion

JXY is a Chinese Traditional Medicinal recipe from Chinese herbs exhibiting heat-clearing and detoxification activity. *Hedyotis diffusa* Willd and *Sophora flavescens* have been demonstrated to exhibit antiproliferative properties, promote apoptosis and inhibit cell invasion through an NF-κB-related pathway in a number of cancer cell lines (24,25). In the present study, we demonstrated that APRIL knockdown and JXY treatment synergistically triggered cell cycle arrest and cell apoptosis and subsequent suppression of HCC cell proliferation through an NF-κB-related pathway. Due to its safety profile, JXY is a valuable adjuvant therapy for HCC patients.

APRIL has been implicated in the development of tumors (26). Exogenous APRIL confers a survival advantage to tumor cells from apoptosis (27,28). Thus, APRIL plays a key role in human tumor growth (29). RNA interference (RNAi) has been widely used in the current research of gene therapy for tumors. Recent progress has demonstrated that the siRNA technique is an efficient method for silencing specific genes (30,31). For siRNA to be applied therapeutically, however, several problems need to be solved, such as the short half-life and delivery method of siRNA-mediated silencing. Despite these problems, siRNA remains a potentially promising technique for the treatment of human cancer, particularly given that it can be more selective and less toxic than traditional approaches (32). Furthermore, combination of the siRNA technique with other medications may be a more effective treatment for HCC.

JXY, a TCM polyherbal decoction, is used to treat HCC. In the present study, we evaluated the synergistic effect of APRIL knockdown and JXY treatment on the proliferation of HCC cells. The results showed that transfection with siRNA-APRIL resulted in significant inhibition of APRIL mRNA and protein levels in HepG2 cells. Moreover, APRIL depletion also inhibited cell proliferation and induced G0/G1 cell cycle arrest and apoptosis in HepG2 cells, whereas no inhibitory effect was observed in non-transfected and siRNA control transfected cells. Although the inhibitory effect of cell growth in HepG2 cells was not obvious using JXY treatment alone, the combination of siRNA-APRIL and JXY application had a synergistic inhibitory effect.

Next, we explored the underlying mechanisms. Previous studies revealed that constitutive activation of NF-κB could play an important role in the regulation of genes involved in tumorigenesis, migration and invasion. In contrast, inhibition of NF-κB activation suppressed migration and invasion (33,34). TRAF6 has been found to play an important role in tumorigenesis, metastasis and invasion by NF-κB activation (35,36). Herein, we detected TRAF6 and NF-κB expression after APRIL knockdown and/or JXY treatment. The results revealed that APRIL knockdown, not JXY treatment, reduced NF-κB expression. In addition, the combination of APRIL knockdown and JXY treatment significantly reduced NF-κB expression. However, all of the treatments had no effect on TRAF6 expression. These results suggest that the synergistic effect of APRIL knockdown and JXY treatment on the inhibition of NF-κB expression are not TRAF6-dependent. Furthermore, we found that APRIL knockdown and/or JXY treatment could downregulate the activity of nuclear factor NF-κB directly. Collectively, we propose that the synergistic effect of APRIL knockdown and JXY treatment triggers cell cycle arrest and cell apoptosis and subsequent suppression of HCC cell proliferation through the inhibition of the NF-κB signaling pathway.

In conclusion, the present study suggests that APRIL is a feasible RNAi target gene for HCC and the combination of siRNA-APRIL and JXY holds great promise as a novel therapeutic approach for APRIL-positive HCC.

Acknowledgements

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