Ethyl acetate extract from Jiedu Xiaozheng Yin inhibits the proliferation of human hepatocellular carcinoma cells by suppressing polycomb gene product Bmi1 and Wnt/β-catenin signaling

XU-ZHENG CHEN¹, ZHI-YUN CAO¹, JIN-NONG LI¹, HAI-XIA HU¹, YOU-QUAN ZHANG², YUN-MEI HUANG¹, ZHI-ZHEN LIU¹, DAN HU¹, LIAN-MING LIAO¹ and JIAN DU²

¹Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou 350122;
²The Second Affiliated Hospital, Fujian University of Traditional Chinese Medicine, Fuzhou 350003, P.R. China

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Correspondence to: Dr Lian-Ming Liao, Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, 1 Qiuyang Road, Minhou Shangjie Town, Fuzhou, Fujian 350122, P.R. China
E-mail: llm@fjtcm.edu.cn
Professor Jian Du, The Second Affiliated Hospital, Fujian University of Traditional Chinese Medicine, Hudong Road, Gulou, Fuzhou, Fujian 350003, P.R. China
E-mail: dujian@fjtcm.edu.cn

Abbreviations: EE-JXY, ethyl acetate extract from Jiedu Xiaozheng Yin; MTT, methyl thiazolyl tetrazolium; HCC, hepatocellular carcinoma; TCM, traditional Chinese medicine; APL, acute promyelocytic leukemia; HDW, Hedyotis diffusa Willd; SF, Sophora flavescens; PC, Pseudobulbus cremastrae; DMSO, dimethyl sulfoxide; IF, immunofluorescence; IHC, immunohistochemistry; PcG, polycomb group; RT-PCR, reverse transcription-polymerase chain reaction; DAPI, 4,6-diamidino-2-phenylindole; PCNA, proliferating cell nuclear antigen

Key words: Jiedu Xiaozheng Yin, hepatocellular carcinoma, Bmi1, Wnt, proliferation

Abstract. Jiedu Xiaozheng Yin (JXY) is a Chinese herbal decoction used to treat hepatocellular carcinoma (HCC). Previous studies have demonstrated that JXY can inhibit HCC cell proliferation via induction of G0/G1 phase arrest. In this study, we investigated whether the inhibitory effect of JXY on HCC cells is associated with the inhibition of the Wnt/β-catenin pathway and the polycomb gene product Bmi1. Ethyl acetate extract from JXY (EE-JXY) was prepared. Methyl thiazolyl tetrazolium (MTT) and colony formation assays were used to measure cell proliferation. Immunofluorescence was used to analyze the expression and location of β-catenin and Bmi1. Immunohistochemistry was used to examine the expression of proliferating cell nuclear antigen (PCNA), c-myc and cyclin D1, β-catenin, Bmi1, c-myc, cyclin D1 and p16INK4A mRNA levels were detected by RT-PCR. The results demonstrated that EE-JXY inhibited the expression of PCNA, c-myc, cyclin D1 and Bmi1, and upregulated the expression of p16INK4A. We also found that EE-JXY could facilitate β-catenin translocation from the cytoplasm and nuclei to the cytomembrane. Finally, suppression of cell proliferation and expression of Bmi1 and Wnt/β-catenin by EE-JXY was confirmed in a mouse xenograft model of HCC. Thus, EE-JXY can inhibit the proliferation of HCC partially via suppression of the Bmi1 and Wnt/β-catenin signaling pathways.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China due to the high prevalence of chronic HBV infection (1,2). According to the data published by the Chinese Society of Liver Cancer in 2009, HCC in China accounts for ~55 and 45% of the annual new cases of and deaths attributed to global HCC, respectively (3). Despite the fact that surgical resection, transplantation, local ablation (percutaneous ethanol injection, radiofrequency ablation, percutaneous acetic acid injection and microwave ablation, and transcatheter arterial chemoembolization) and immunotherapy are potentially curative modalities (4), many patients with HCC cannot be treated by these therapies due to the metastasis of cancer cells, severe adverse effects of antineoplastic drugs, or the expensive costs. Seeking alternative therapies to improve the curative rate of HCC has been an urgent task for oncologists.

Traditional Chinese medicine (TCM) herbs are widely used as complementary and alternative medicines for cancers in China (5-9). The underlying anticancer mechanisms of TCM have been extensively investigated for several decades. TCM can induce cell apoptosis and differentiation, inhibit cell division and angiogenesis, and promote immune function (reviewed in refs. 10,11). In addition, several randomized controlled trials have shown that TCM therapy can improve the quality of life of patients and alleviate chemoradiotherapy-induced adverse effects (12). TCM is considered as a valuable
addition to standard cancer therapy, despite the fact that the ingredients of TCM are not completely understood.

According to the TCM theory, the combined use of several herbs may have synergistic effects by acting on multiple targets. Typically, a polyherbal formula has a principal component, and others serve as adjuvant agents to enhance the pharmacological actions or facilitate the delivery of the principal component. This principle of formulation has been practiced for more than 5,000 years and is unanimously accepted by TCM physicians. For example, Realgar-Indigo naturalis formula has been verified to be effective in treating human acute promyelocytic leukemia (APL). The formula is composed of Realgar-Indigo naturalis, Salvia milliorrhiza and Radix pseudostellariae. In this polyherbal formula, Realgar is a principal element and is capable of clearing heat and removing toxicity to fight APL cells. Indigo naturalis can cool the blood and detoxify the body. Salvia milliorrhiza and Radix pseudostellariae have the ability to tonify Qi and invigorate blood circulation. The latter three herbs enhance the effect of Realgar in clearing heat and removing toxicity. Indeed, a pharmacological study showed that the formula has the strongest effect on fighting cancer cells compared to Realgar alone or in combination with one of the other three herbs (13).

Jiedu Xiaozheng Yin (JXY) is a polyherbal decoction to treat HCC and is composed of Hedoytes diffusa Wild (HDW), Sophora flavescens (SF), Prunella and Pseudobulbus Cremasdrae (PC). In this compound decoction, HDW is the principal component intended to clear heat and toxins and resolve hard mass (including cancer). Prunella acts to clear liver fire. SF and PC can assist HDW to clear heat, detoxify the body and resolve hard mass. Pharmacological studies have shown that the four herbs are capable of inducing apoptosis and inhibiting proliferation and angiogenesis of tumor cells (14-17). A randomized control trial showed that addition of JXY to standard treatment of stage III HCC patients can improve the immune function of patients, decrease recurrence and increase overall survival (18). Experimental studies have also demonstrated that JXY can inhibit the angiogenesis of tumors via downregulation of VEGF-A and VEGFR-2 expression (19) and inhibit tumor cell proliferation via induction of G0/G1 phase arrest (9). In addition, we also found that JXY can downregulate the expression of cancer stem cell-related markers CD133 and c-kit (20). In the present study, we investigated whether the antitumor effects of JXY involve the Wnt/β-catenin pathway and the polycomb gene product, which are important regulators of cancer stem cells (21,22).

Materials and methods

**EE-JXY preparation.** JXY is composed of HDW (30 g), Prunella (15 g), PC (15 g) and SF (15 g). The ethyl acetate extract from JXY (EE-JXY) was prepared. Briefly, JXY (7.5 kg) was refluxed with 75% ethanol for 2 times, 3 h each time. The extract was pooled. The alcohol was removed under vacuum using a rotary evaporator. The residue was dissolved with water. The solution was partitioned sequentially with petroleum ether, chloroform, ethyl acetate and n-BuOH. The extract was evaporated in vacuum and stored at 4°C prior to use. EE-JXY was diluted using dimethyl sulfoxide (DMSO) into 200 mg/ml for the in vitro experiments. For the in vivo study, EE-JXY was dissolved in normal saline to a final concentration of 6 mg/ml.

**Cell lines and culture.** Human hepatocellular carcinoma PLC/PRF/5 and Huh7 cell lines were purchased from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China), and grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (both from Gibco, Carlsbad, CA, USA).

**Cellular growth assay.** The growth of cells was evaluated using the methyl thiazolyl tetrazolium (MTT) method. Briefly, adherent PLC/PRF/5 or Huh7 cells in 96-well plates were treated with EE-JXY with final concentrations of 0 (control group), 0.0625, 0.125, 0.25 and 0.5 mg/ml for 24, 48 and 72 h, respectively. Then the culture medium was discarded and MTT (Invitrogen-Life Technologies, Carlsbad, CA, USA) was added. After incubation for another 4 h, the purple-blue MTT formazan precipitate was dissolved using DMSO. The OD was measured at 570 nm. Cell growth was represented by cell viability as: Cell viability (%) = average OD JXY group/average OD control group x 100%.

**Flat colony formation assay.** After cells were exposed to 0.25 mg/ml EE-JXY for 24 h, cells were detached by trypsin, seeded in a 6-well plate at a final concentration of 3x10^3 cells/ml and incubated for 10 days. Then colony formation was examined using a crystal violet cell colony staining kit (Genmed Sciences Inc., Manassas, VA, USA) according to the manufacturer’s instructions. The colony formation ability was evaluated via the OD value at 570 nm.

**Mouse xenograft model.** Six-week old female BALB/c nu/nu mice were housed at 23±2°C in a humidified pathogen-free facility. Food and water were provided ad libitum. Huh7 cells at a concentration of 5x10^6 cells/ml were mixed with Matrigel basement membrane matrix (1:1; BD Biosciences, Franklin Lakes, NJ, USA) on ice. The 200-µl cell mixture was injected subcutaneously. When the tumor volume (TV) reached ~100 mm³, the mice were randomized into two groups and received 0.13 g/kg/day EE-JXY and normal saline, respectively by gavage. After 3 weeks, the tumors were removed for analysis. The tissue was fixed in 10% formalin and received paraffin embedding. Immunohistochemistry (IHC) assays were performed according to the Animal Care Guidelines issued by the Ministry of Science and Technology of P.R. China, and the Animal Care Committee of Fujian University of Traditional Chinese Medicine approved our protocol.

**Reverse transcription-PCR (RT-PCR) assay.** Total RNA from the tumor xenografts was extracted and subjected to reverse transcription according to the manufacturer’s recommended protocol (Invitrogen-Life Technologies and Promega, Madison, WI, USA, respectively). A 20-µl RT-PCR reaction mixture contained 10 µl of 2X Taq MasterMix (containing dNTPs, DNA polymerase, buffer, Mg²⁺), 400 nmol/l of each primer and 0.4 µg template DNA. Amplification conditions were as follows: 2 min at 94°C followed by 35 cycles for 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C. The primer pairs are shown
in Table I. The amplified products were size-fractionated on 1.5% agarose gel and detected by a gel imaging system (Bio-Rad, Hercules, CA, USA). The mRNA levels of samples were normalized with that of GAPDH as follows: mRNA level = (gray-scale value)sample/(gray-scale value)GAPDH.

**IF analysis.** The cells were seeded in a 96-well plate for 24 h. After treatment with 0 and 0.25 mg/ml EE-JXY, the cells were fixed with 4% paraformaldehyde for 10 min and incubated in blocking buffer containing Triton X-100 for 20 min. The antibody anti-β-catenin and Bmi1 antibodies (1:250, ab32572 and ab38295; Abcam, Cambridge, MA, USA) were added and incubated at 4°C overnight. After rinsing in PBS 3 times for 10 min, the cells were incubated in the Alexa Fluor 555-labeled donkey anti-rabbit secondary antibody (1:1,000, A0453; Beyotime, Shanghai, China) for 2 h in dark. After rinsing another 3 times, 300 ng/ml 4',6-diamidino-2-phenylindole (DAPI; C1006; Beyotime, Shanghai, China) was added to the wells. After 5 min, fluorophore was detected using a high content analysis system (BD Biosciences).

**IHC analysis.** The tumor xenografts were paraformaldehyde-fixed, and embedded in paraffin following standard protocols. The deparaffined tumor sections were subjected to immunostaining for proliferating cell nuclear antigen (PCNA), c-myc and cyclin D1 with appropriate antibodies (Maxin, MAB-0145, RMA-0664 and RMA-0541; Fuzhou, Fujian, China). The average percentage of positive cells was determined by counting the brown-colored cells under a microscope (Leica Microsystems, Bensheim, Germany).

**Statistical analysis.** Data were analyzed and processed using the SPSS 18.0 statistical software, and are presented as the mean ± standard deviation (SD) of at least three samples. Statistical comparisons between two groups were performed using the independent samples t-test, and between multiple groups were analyzed by one-way ANOVA analysis. A P-value <0.05 was considered significant.

**Results**

**EE-JXY inhibits the proliferation of HCC cells in vitro.** Our previous study demonstrated that EE-JXY inhibits the proliferation of HepG2 cells (9). To further evaluate the anticancer potential of EE-JXY for HCC, we investigated the effects of EE-JXY on the proliferation of Huh7 and PLC/PRF/5 cells by MTT assay. EE-JXY inhibited the proliferation of both HCC cell lines in dose- and time-dependent manners (Fig. 1A and B). The half maximal inhibitory concentration (IC50) was estimated to be 0.29 mg/ml for both Huh7 and PLC/PRF/5 cells at 24 h, which is similar to our previous finding for HepG2 cells (0.30 mg/ml) (9). Cells exposed to EE-JXY showed marked morphological changes: originally polygon- or spindle-shaped cells became round and collapsed, and many vacuoles appeared in the cytoplasm (Fig. 1C and D).

Colony formation assay have been used to identity cancer stem cells in vitro (23). We next evaluated the colony formation capacity of Huh7 and PLC/PRF/5 cells under the treatment of EE-JXY. Colony formation assay showed that the numbers of colonies formed by both EE-JXY-treated Huh7 and EE-JXY-treated PLC/PRF/5 cells were significantly fewer than the number in the untreated cells (Fig. 1E-H). Taken together, these data revealed that a significant growth inhibition was exerted by EE-JXY on the two HCC cell lines and possibly on the cancer stem cells.

In the following experiments, Huh7 cells were used to evaluate the anticancer mechanisms of EE-JXY. EE-JXY regulates the Wnt/β-catenin signaling pathway. IF analysis showed that β-catenin mostly accumulated in the cytoplasm or nuclei in the untreated Huh7 cells (Figs. 2A and 3A). After treated with EE-JXY, β-catenin was mostly observed in the cytomembrane. Obviously, EE-JXY could facilitate β-catenin translocation to the cytomembrane. Interestingly, mRNA expression of β-catenin was not affected by EE-JXY. We then investigated the mRNA expression levels of β-catenin downstream genes c-myc and cyclin D1. The mRNA levels were markedly decreased in the EE-JXY-treated mice compared with the control (Fig. 3B).

As PCNA is an indicator of cell proliferation and regulated by cyclin D1 (24,25), we evaluated the expression of PCNA in tumor tissues. The findings demonstrated that PCNA protein expression was inhibited in the EE-JXY-treated mice compared with the control, which is consistent with the smaller tumor volume in the EE-JXY-treated mice (Fig. 3B and D).

**EE-JXY downregulates Bmi1 and upregulates p16INK4A expression.** As the polycomb group (PcG) transcriptional
repressor Bmi1 is a key regulator in liver cancer cell proliferation (26-29), we next examined the effect of EE-JXY on expression of Bmi1 by IF. Huh7 cells treated with EE-JXY were observed under a fluorescence microscope (Fig. 4A). Results showed that the fluorescence intensity of Bmi1 in the EE-JXY-treated cells was lower than that in the vehicle-treated
cells (Fig. 4B), indicating that EE-JXY downregulated the expression of Bmi1. The result was further confirmed by RT-PCR (Fig. 4C and E).

The cyclin-dependent kinase inhibitor 2A (CDKN2A) gene product, p16\(^\text{INK4A}\), is a tumor-suppressor protein that inhibits cyclin dependent kinases 4 and 6. p16\(^\text{INK4A}\) is regulated by Bmi (30). As silencing of p16\(^\text{INK4A}\) is a common event in HCC (31), we investigated the expression of p16\(^\text{INK4A}\) in the EE-JXY-treated cells. As expected, expression of p16\(^\text{INK4A}\) was upregulated in the EE-JXY-treated cells (Fig. 4C and D). Most importantly, similar results were observed in the tumor tissues from mice treated with EE-JXY (Fig. 5).

### Discussion

In the present study we demonstrated that JXY suppressed the proliferation of human hepatoma cells (PLC/PRF/5 and Huh7) at least partially by inhibiting the polycomb gene product Bmi1 and Wnt/\(\beta\)-catenin signaling. Due to the safety profile of JXY, JXY is a valuable adjuvant therapy for standard cancer therapy in patients with HCC.

The main members of the Wnt/\(\beta\)-catenin signaling pathway include Wnt (an extracellular factor rich in cysteine), frizzled (a transmembrane protein) and \(\beta\)-catenin (a cytoplasmic protein) (22,32-35). In the absence of Wnt, \(\beta\)-catenin normally...
locates adjacent to cell membranes. When it forms a β-catenin destruction complex with adenomatous polyposis coli (APC), axin and glycogen synthase kinase-3β (GSK-3β), β-catenin is phosphorylated and degraded. Therefore, β-catenin normally remains at a lower level in cells. In the presence of the Wnt signal, Wnt associates with frizzled, unphosphorylated β-catenin translocates from cell membranes to the cytoplasm and subsequently to the nucleus, where it activates various downstream events involved in cell apoptosis and proliferation, such as cyclin D1, c-myc and E-cadherin (34,35).

The Wnt/β-catenin signaling pathway is involved in the carcinogenesis and metastasis of a number of human cancers, such as colorectal carcinoma, breast cancer and HCC (22,31-33,36). Cui et al (37) found overexpression and abnormal

Figure 3. EE-JXY regulates the Wnt/β-catenin signaling pathway in vivo. (A) β-catenin fluorescence in HuH7 cells was visualized using IHC staining by an IF microscope at a magnification of x200. Immunofluorescent labeling of β-catenin (red) and nuclear DAPI staining (blue) was merged. (C) The β-catenin, c-myc and cyclin D1 mRNA levels were detected by RT-PCR analysis. (B) c-myc, cyclin D1 and PCNA staining is indicated by brown color using IHC staining and images were captured at a magnification of x200. (D) Tumor volume was measured by a vernier caliper. EE-JXY, ethyl acetate extract from Jiedu Xiaozheng Yin; DAPI, 4,6-diamidino-2-phenylindole; IHC, immunohistochemistry; IF, immunofluorescence; RT-PCR, reverse transcription-polymerase chain reaction.
accumulation in the cytoplasm or nuclei of β-catenin in HCC. Not surprisingly, pharmacological agents inhibiting the Wnt/β-
catenin pathway such as sorafenib can inhibit the growth of HCC cells (38). Berberine from *Coptis chinensis* Franch was
also reported to inhibit β-catenin/Tcf4 reporter activity and exert an antineoplastic effect (39). In the present study, cells treated with EE-JXY showed less nuclear accumulation of β-catenin, which may be responsible for reduced c-myc and cyclin D1 gene and protein expression in vitro and in vivo, and subsequent decreased expression of PCNA (40) and cell proliferation. One possible ingredient responsible for this effect is matrine in SF. Matrine was reported to regulate the Wnt/β-catenin signaling pathway in human acute erythroleukemia cell line TF-1 (41) and hepatic precancerous lesions (42).

PcG proteins are a group of transcriptional repressors regulating targeted gene expression through chromatin modifications. The PcG proteins have two core multiprotein complexes, polycomb repressive complex (PRC)-1 and -2 (43,44). Bmi1, one member of PRC-1, has been shown to function as an oncogene in multiple tumor types (43,45,46). It regulates cell proliferation, apoptosis and senescence by repressing downstream p16INK4a and p14ARF, which are suppressor genes of the INK4A/ARF locus (43,45,46). Overexpression of Bmi1 has been reported to be associated with the progression and aggressive biological behavior of many cancers, including HCC (47-50). Therefore Bmi1 has been a target for anticancer drug development (51). In this study, decreased expression of Bmi1 and increased expression of p16INK4a were observed in the EE-JXY-treated cells, supporting our previous finding that EE-JXY may regulate HCC cell proliferation via the cyclin D-CDK4 pathway (9).

In addition to inhibiting HCC cell proliferation and colony formation in vitro, EE-JXY markedly suppressed the expression of PCNA in vivo, an indicator of cell proliferation. As our previous study showed that EE-JXY has a strong antitumor effect in HepG2 tumor-bearing mice (9), we did not evaluate the anticancer effect of EE-JXY by comparing the tumor volumes of the EE-JXY-treated mice and the control mice, which requires a higher number of mice. To spare the animals, only 2 mice were used in each group in the present study. Notably, with the dose used in our previous study (9), stronger inhibition of PCNA expression and a smaller tumor volume were observed in the EE-JXY-treated mice.

In conclusion, EE-JXY inhibits the proliferation of PLC/PRF5 and Huh7 cells at least in part by suppressing the Bmi1 and Wnt/β-catenin signaling pathways. Although the knowledge of the mechanism of EE-JXY action is still limited, EE-JXY may have multiple anticancer mechanisms that may deal with multiple targets in cancer cells.

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