Water extract of *Hedyotis Diffusa* Willd suppresses proliferation of human HepG2 cells and potentiates the anticancer efficacy of low-dose 5-fluorouracil by inhibiting the CDK2-E2F1 pathway

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Abstract. *Hedyotis Diffusa* Willd (HDW), a Chinese herbal medicine, has been widely used as an adjuvant therapy against various cancers, including hepatocellular carcinoma (HCC). However, the underlying anticancer mechanisms are yet to be elucidated. In the present study, the anticancer effects of HDW were evaluated and the efficacy and safety of HDW combined with low-dose 5-fluorouracil (5-FU) were investigated. HepG2 cells were cultured *in vitro* and nude mouse xenografts were established *in vivo*. The proliferation of HepG2 cells was measured using the MTT method and flow cytometry. The mRNA and protein expression levels of cyclin-dependent kinase 2 (CDK2), cyclin E and E2F1 were examined using relative quantitative real-time PCR and western blot analysis, respectively. The results showed that water extract of HDW remarkably inhibited HepG2 cell proliferation in a dose-dependent manner via arrest of HepG2 cells at the G0/G1 phase and induction of S phase delay. This suppression was accompanied by a great decrease of E2F1 and CDK2 mRNA expression. In addition, HDW remarkably potentiated the anti-cancer effect of low-dose 5-FU in the absence of overt toxicity by downregulating the mRNA and protein levels of CDK2, cyclin E and E2F1. Our findings support the use of HDW as adjuvant therapy of chemotherapy and suggest that HDW may potentiate the efficiency of low-dose 5-FU in treating HCC.

Introduction

Due to the high prevalence of hepatitis B and C virus infections in many countries, the incidence of hepatocellular carcinoma (HCC) is increasing and the mortality rate of HCC is extremely high in some countries (1). According to the data published by the International Agency for Research on Cancer, there were 564,000 HCC patients in 2000 and 55% of which were in China (2). Curative treatments for HCC include surgery, local destruction techniques (radiofrequency ablation or percutaneous ethanol injection) and liver transplantation. Unfortunately, only ~40% of patients can benefit from curative treatments and only 1/3 of patients are typically resectable (3). Hence, for most patients with unresectable HCC, palliative treatments, which include transarterial chemoembolization, systemic therapy and radiotherapy are their only choices, and the survival rate and quality of life for them remain dismal (4,5). Low efficacy and severe adverse effects still exist for chemotherapeutics. Development of more effective and less toxic antineoplastic agent remains an urgent task.

A meta-analysis has shown that patients simultaneously receiving chemotherapy and Chinese medicines may have higher 1-, 2- and 3-year survival rates (6). Indeed, many traditional Chinese medicines have long been used to fight various cancers and manage the side effects of chemotherapy for several decades. For instance, astragalus induced cancer cell apoptosis. It reversed the immunosuppressive effects of chemotherapy drugs by stimulating the production of interleukin-6 and TNF (7,8).

Hedyotis Diffusa Willd (HDW) is an ancient Chinese medicine belonging to the Rubiaceae family, which is capable

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Abbreviations: HDW, *Hedyotis Diffusa* Willd; HCC, hepatocellular carcinoma; 5-FU, 5-fluorouracil; CDK2, cyclin-dependent kinase 2; DMSO, dimethyl sulfoxide; MTT, methyl thiazolyl tetrazolium; PCR, polymerase chain reaction; HRP, horseradish peroxidase; PI, proliferating index

of heat-clearing, detoxification and activating blood according to the Chinese medicinal theory (9). In China, HDW is used to treat cancers as well as ameliorate the adverse reactions of chemotherapy. Pharmacological studies showed that it contains compounds with anticancer activities, including anthraquinones, hemiterpenes, flavones, polyphenols, organic acids and polysaccharides (10-12). Our previous study demonstrated that HDW extract inhibited angiogenesis and induced tumor cell apoptosis via the mitochondrion-dependent pathway (13,14). By performing molecular docking simulation, we found that components in HDW (such as quercetin, asperuloside) could bind cyclin-dependent kinase 2 (CDK2) (15). CDK2 and downstream transcription factor E2F1 regulate the transition from G1 to S phase during cell proliferation (16). Inhibition of CDK2 activity can inhibit cell proliferation.

In this study, we showed that water extract of HDW was able to suppress the expression of CDK2 and E2F1 mRNA in HepG2 cells, which is consistent with molecular docking simulation. More importantly, our findings suggested that water extract of HDW effectively inhibited HepG2 cell growth *in vivo* and promote the anticancer efficiency of low-dose (5-fluorouracil) 5-FU via the inhibition of the CDK2-E2F1 pathway.

Materials and methods

Reagents. 5-FU was purchased from Tianjin King York Aminophenol, Inc. (Tianjin, China). HDW was purchased from Purapharm Co., Ltd. (Hong Kong, China). Specimens were authenticated by the Department of Pharmacy of Fujian University of Traditional Chinese Medicine (Fuzhou, China). To prepare the HDW water extract, HDW was steeped in 10-fold volume of distilled water and decocted two times, 20 min each time. The aqueous extract was then filtered and concentrated to make a decoction, and subsequently stored at 4°C until use. Each milliliter of water extract was equivalent to 1.8 g crude HDW.

Animals. Forty female BALB/c *nu/nu* mice (6-weeks-old, 18-22 g) were purchased from Shanghai Slac Experimental Animal Co., Ltd. (Shanghai, China). The animals were maintained in a pathogen-free facility (23±2°C, 55±5% humidity). Animal care and experiment procedures were approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine.

Cell line and culture. The HepG2 cell line was obtained from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China), and was grown in high glucose DMEM (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Gibco).

MTT assay. To evaluate the effect of HDW on tumor cell proliferation, $1x10^4$ HepG2 cells were seeded in 96-well plates for 24 h and treated with HDW at the final concentrations of 0, 1.25, 2.5, 5 and 10 mg/ml for 24 h. Then, 20 μl of 5 mg/ml methyl thiazolyl tetrazolium (MTT) was added and incubated for another 4 h before it was discarded. The purple-blue MTT formazan precipitate was dissolved in 100 μl dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm. Cell viability was calculated according to the following formula: cell viability (%) = average $OD_{treatment\ group}$/ average $OD_{blank\ group}$ x100%.

Cell cycle assay. Cells were incubated in 6-well plates with either 4.62 mg/ml HDW (IC_{50}) or vehicle. They were referred to as HDW group and control group, respectively. After 24 h, cells were digested and washed. Progression through the cell cycle was measured with flow cytometery (BD Biosciences, Franklin, NJ) according to the instructions of the Cycle-test Plus DNA assay kit (BD Biosciences). The percentages of cells in G0/G1, S and G2/M phase were evaluated by the ModFit software (BD Biosciences). The proliferating index (PI) was calculated according to the following formula: PI=(S+G2/M)/ (G0/G1+S+G2/M) x100%.

Mouse xenograft experiments. BALB/c *nu/nu* mice were inoculated with $1x10^4$ HepG2 cells at the right flank and treatment was initiated when the estimated tumor volumes uniformly reached ~50 mm³. The mice were randomly divided into 4 groups (n=10/group): low-dose 5-FU group, HDW group, combination group (HDW plus low-dose 5-FU) and vehicle control group, which received 5-FU [10 mg/kg/day, intraperitoneally (i.p.)], HDW [6 g/kg/day, intragingivally (i.g.)], 5-FU (10 mg/kg/day, i.p.) plus HDW (6 g/kg/day, i.g.) and normal saline (NS, i.g.), respectively. The mouse body weight as well as tumor width (d) and length (D) were measured once every 3 days. Tumor volume (TV) was calculated by formula: TV (mm³)=d²xDx0.52. After 4 weeks of treatment, blood from each mouse was collected, and the tumors were harvested and weighed. The levels of alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN) and creatinine (CRE) (Kehua, Shanghai, China) were measured by Biochemical Analyzer (Toshiba, Kawasaki, Japan). The tumor inhibitory rate (TIR) was calculated as follows: TIR (%) = [1-average tumor weight$_{(treatment\ group)}$/average tumor weight$_{(vehicle\ group)}$] x100%.

Quantitative real-time polymerase chain reaction (PCR) examination. Total cellular RNA and tissular RNA were isolated using TRIzol one-step method as described in the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Single-stranded cDNA was synthesized using oligo(dt) primer (Promega, Madison, WI) in a 20 μl reaction mixture. The primer pairs of CDK2, E2F1, cyclin E and GAPDH were designed and synthesized as follows: E2F1, 5'-TGATACCCCAACTCCCTCTACC-3' and 5'-TGT CTCCCTCCCTCACTTTCC-3'; CDK2, 5'-CGCAAATG CTGCACTACGACC-3' and 5'-GCCCACCTGAGTCCAAATA GCC-3'; Cyclin E, 5'-TGACTGCCTTGAATTTCCTTATG-3' and 5'-GCACCACTGATACCCTGAAACC-3'; GAPDH, 5'-AC GGATTTGGTCGTATTGGGC-3' and 5'-CTCGCTCCTGGA AGATGGTGAT-3'. Real-time PCR was performed with power SYBR-Green I PCR Master mix kit (Applied Biosystems, Foster City, CA) by a 7500 real-time PCR system (Applied Biosystems). The relative quantitative PCR conditions for 40 cycles were performed as follow: denaturation at 95°C for 15 sec, anneal and extension at 60°C for 1 min. After the amplification phase was completed, the melting curve examination was performed in order to eliminate non-
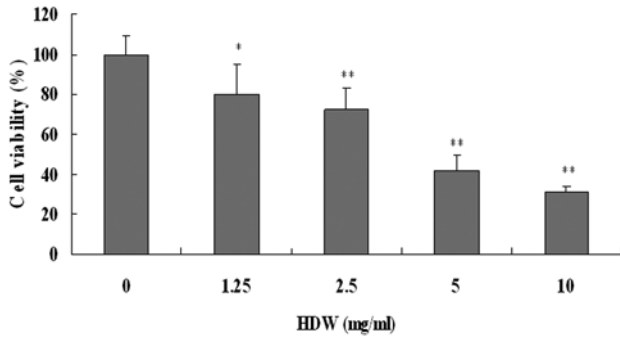
Figure 1. Effect of HDW on the viability of HepG2 cells. HepG2 cells were treated with different concentrations of HDW water extract for 24 h. Data represent mean ± SD of at least triplicate experiments. Compared with control cells (0 mg/ml group), *P<0.05, **P<0.01, one-way ANOVA.

specific products. The fold change in gene expression of each sample was analyzed by SDS software (Applied Biosystems) using the equation $2^{-\Delta\Delta Ct}$ method to calculate the relative level of each mRNA and expressed as a ratio relative to GAPDH housekeeper genes, where Ct value is the fractional cycle number at which the fluorescence exceeds that of background, and $\Delta\Delta Ct=(Ct_{target}-Ct_{GAPDH})_{sample}-(Ct_{target}-Ct_{GAPDH})_{control}$ (17).

Western blot analysis. Tumor cells were put on dry ice for 10 min in lysis buffer. After centrifugation at 11,000 rpm 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 nitrocellulose (NC) membranes. The NC membranes were then put into blocking solution (1% bovine serum albumin) for 1 h and incubated in monoclonal anti-mouse CDK2, E2F1 or cyclin E primary antibody solutions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-β-actin antibody solution (Beyotime, Shanghai, China) overnight at 4°C with shaking, and then in horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime) for at least 1 h. Chemiluminescence was detected using a Chemiluminescence imaging system (Bio-Rad, Hercules, CA).

Statistical analysis. The data were expressed as mean ± SD of at least triplicate experiments. Double and multiple comparisons were performed using independent-samples t-test and one-way ANOVA, respectively. P-value <0.05 was considered significant. All results were analyzed using the SPSS 16.0 statistical software.

Results

HDW inhibits proliferation of HepG2 cells in vitro. The effect of HDW on the proliferation of HepG2 cells was evaluated by MTT method. As shown in Fig. 1, in the presence of various concentrations of HDW, the proliferation of HepG2 was remarkably inhibited in a dose-dependent manner. The concentration of HDW to inhibit 50% cell growth (IC_{50}) was estimated to be 4.62 mg/ml. The results demonstrated that water extract of HDW could markedly inhibit the proliferation of HepG2 cells.
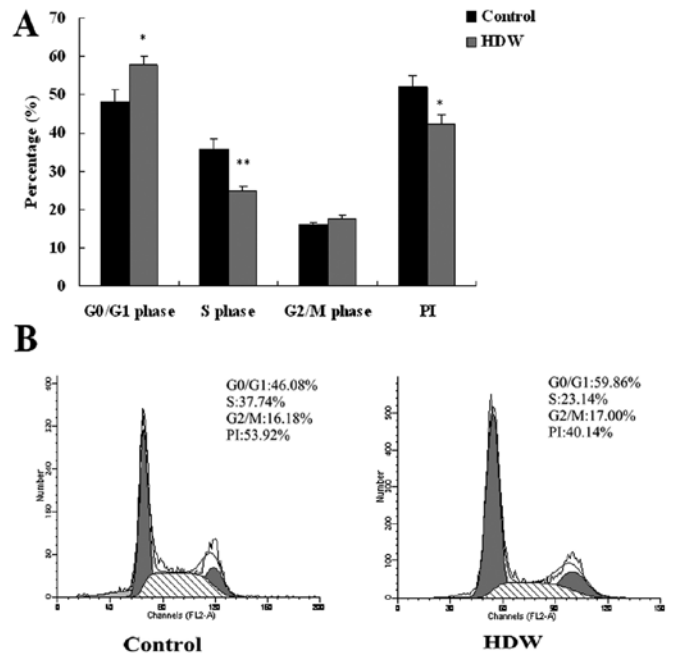

Figure 2. Effect of HDW on the cell cycle of HepG2 cells. Cells were treated with HDW water extract at a concentration of 4.62 mg/ml for 24 h and examined using flow cytometry with PI dye. (A) Compared with the control group, *P<0.05 and **P<0.01, independent-samples t-test and (B) is representative of 3 experiments.
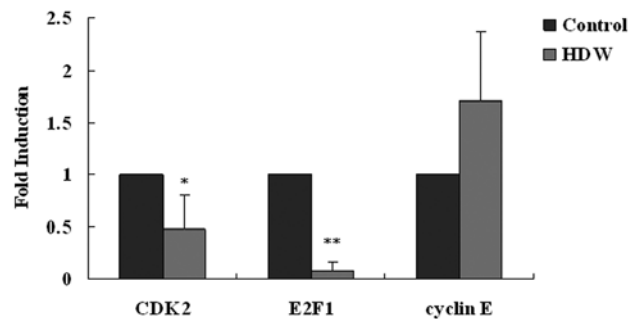

Figure 3. Effect of HDW on the mRNA expression of cell cycle regulators. HepG2 cells were cultured in the presence or absence of 4.62 mg/ml HDW water extract for 24 h *in vitro*. Real-time PCR was performed with GAPDH as an internal positive control. The data for HDW-treated samples were expressed relative to the average value of the control samples ± standard deviations of the means. Compared with the control group, *P<0.05 and **P<0.01, independent-samples t-test.

HDW arrests HepG2 cells at G0/G1 phase and induces S phase delay. Progression through the cell cycle was examined using flow cytometry and the results are shown in Fig. 2. When HepG2 cells were treated with 4.62 mg/ml HDW (IC_{50}), the percentage of cells in G0/G1 phase increased from 48.16±3.11-57.71±2.29% (P<0.05). On the contrary, the percentage of cells in S phase decreased from 35.73±2.56-24.71±1.43% (P<0.01). As expected, PI in the HDW group was lower than that of the control group (42.29±2.30% vs. 51.85±3.11%, P<0.05). These results demonstrated that HDW inhibited the proliferation of HepG2 cells by inducing G0/G1 phase arrest and S phase delay.
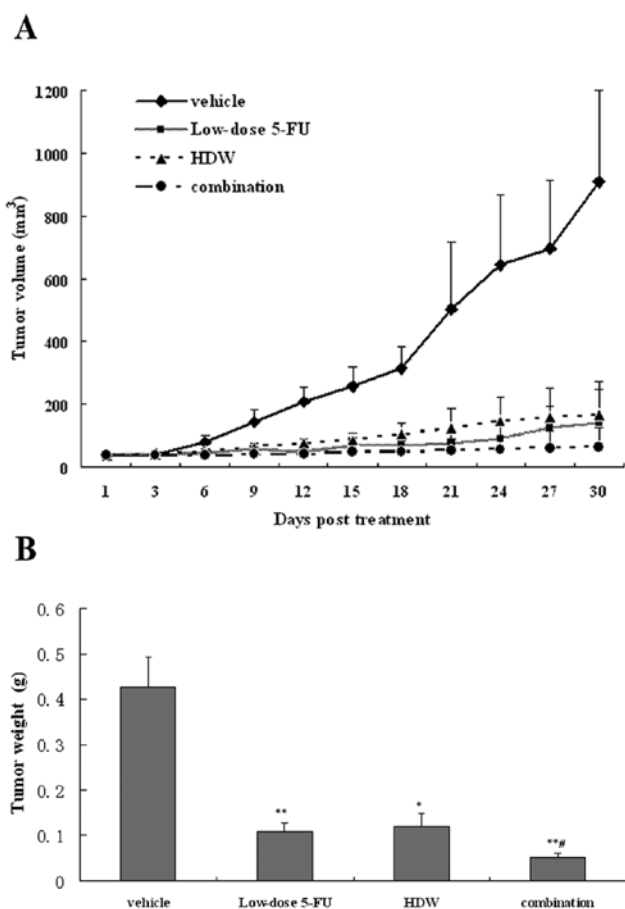
Figure 4. Effects of HDW on tumor growth. The tumor-bearing nude mice were treated with low-dose 5-FU (low-dose 5-FU group), HDW (HDW group), low-dose 5-FU plus HDW (combination group) and normal saline (vehicle control group) respectively. (A) Tumor volume analysis once every 3 days and (B) tumor weight at sacrifice. The values were expressed as mean ± SD, *P<0.05 and **P<0.01 vs. the vehicle group, #P<0.05 vs. the low-dose 5-FU group.
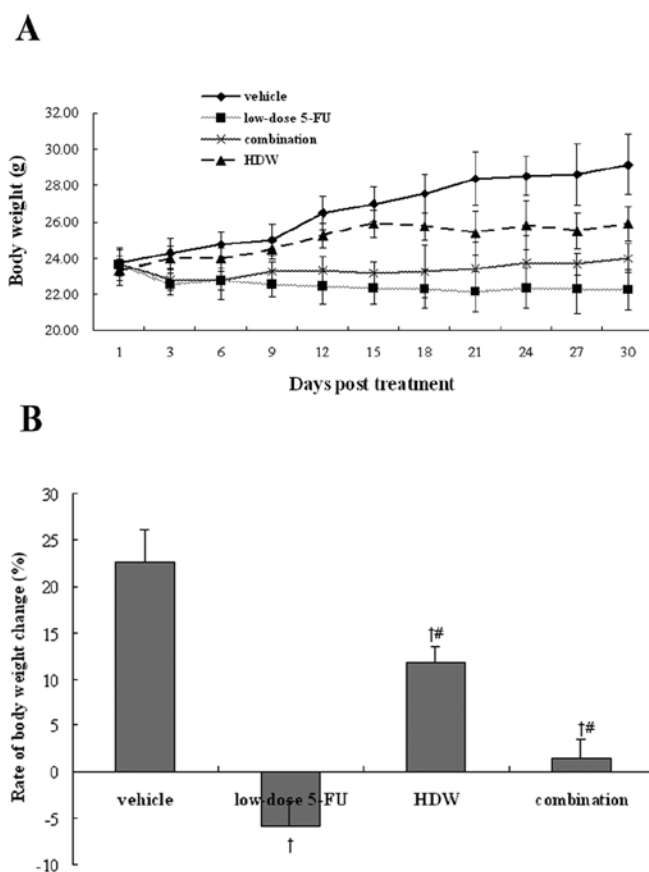

Figure 5. Evaluation of toxicity of HDW and combination therapy. (A) Body weight of the mice was measured once every 3 days after initial treatment and (B) the rate of body weight change (RBW) was calculated by the formula: RBW (%) = [(BW on sacrifice) - (BW on initial treatment)] / (BW on initial treatment) x100%. †P<0.01 vs. the vehicle group. #P<0.01 vs. the low-dose 5-FU group.

HDW downregulates the expression of CDK2 and E2F1 mRNA of HepG2 cells. CDK2, cyclin E and E2F1 transcription factor play critical roles in cell cycle progression from G0/G1 to S phase. To investigate how HDW interferred G1/S transition of HepG2 cell cycle, expression of CDK2, E2F1 and cyclin E mRNA was evaluated with relative quantitative real-time PCR. As shown in Fig. 3, the levels of CDK2 and E2F1 mRNA decreased to 48 and 0.08%, respectively by HDW. However, HDW did not affect the transcription of cyclin E mRNA. These results suggested that HDW induced G0/G1 phase arrest and S phase delay of HepG2 cells at least partly through the CDK2-E2F1 pathway *in vitro*.

Assessment of anticancer effects of HDW alone or in combination with low-dose 5-FU in vivo. The reason of testing HDW for treatment of HCC is to see if HDW can be used as an adjuvant therapeutic agent in combination with low-dose chemotherapy, which is not as effective as standard-dose therapy. To evaluate the effects of HDW on tumor growth and if it could enhance the effect of low-dose 5-FU, the leading chemotherapeutic drug for advanced HCC, we examined tumor xenograft volume once every 3 days after initial treatment and tumor weight at sacrifice. As shown in Fig. 4A, all treatment groups showed

tumor growth delay as assessed by tumor volume changes. At sacrifice, the tumor volume was 909.92±289.99 mm³ in the vehicle group, 139.73±104.46 mm³ in the low-dose 5-FU group, 165.35±106.17 mm³ in the HDW group and 65.92±59.34 mm³ in the combination group. Thus, either HDW or low-dose 5-FU could significantly inhibit the tumor growth. When they were simultaneously administered, a higher inhibition was achieved (P<0.01). A similar tendency was observed when the tumor weights were compared among the 4 groups (Fig. 4B). The TIR was 74.51% in the low-dose 5-Fu group, 71.83% in the HDW group and 87.87% in the combination group, respectively. The anticancer efficacy of HDW was comparable to low-dose 5-FU. Furthermore, HDW potentiated the effect of low-dose 5-FU.

Evaluation of toxicity of HDW and combined therapy. We also investigated the gross toxicity of HDW alone or HDW combined with low-dose 5-FU in tumor-bearing mice. As tumor growth progressed, mice in all groups gradually showed signs of dullness, inertia, dim skin color, etc. The body weights of tumor-bearing mice in each group are shown in Fig. 5. At sacrifice, body weight decreased by 5.92±2.79% in the low-dose 5-FU group. On the contrary, the body weight increased by 22.65±3.42% in the vehicle group, 11.79±1.81% in the HDW

Table I. Hepatorenal toxicity of low-dose 5-FU, HDW and a combination of low-dose 5-FU and HDW.

Groups	ALT (IU/l)	AST (IU/l)	BUN (mmol/l)	CRE (μmol/l)
Vehicle	58.44±15.00	217.88±53.20	8.07±1.05	31.36±6.50
Low-dose 5-FU	58.70±17.70	206.10±55.40	7.89±1.70	35.96±8.30
HDW	72.00±15.19	216.50±54.87	7.13±0.76	31.48±5.17
Combination	62.00±12.65	202.00±26.44	9.78±2.20	36.08±4.16

ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; CRE, creatinine.
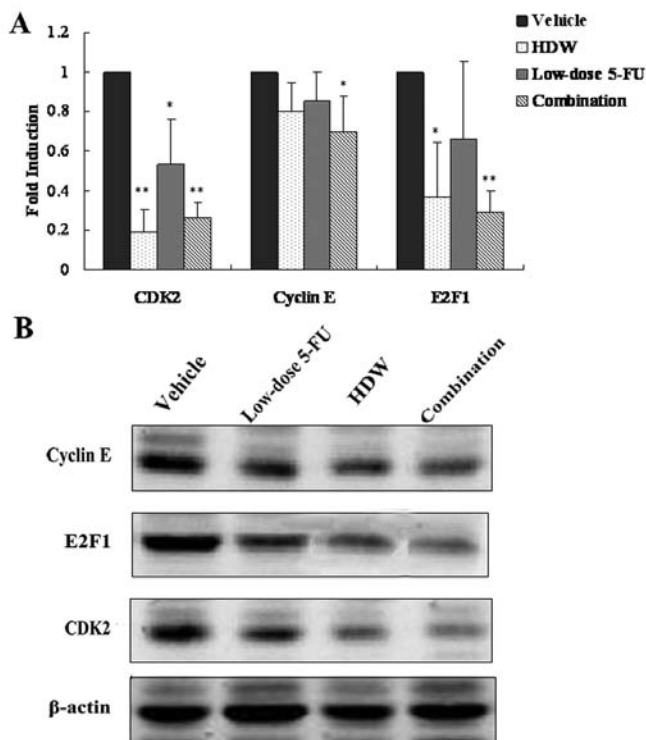

Figure 6. Effect of HDW on expression of cell cycle regulators. The levels of CDK2, E2F1 and cyclin E were determined with (A) relative quantitative real-time PCR and (B) western blot analysis. The values were expressed as mean ± SD, *P<0.05 and **P<0.01 vs. the vehicle group.

group and 1.39±2.15% in the combination group. No overt toxicity in liver and kidney function was observed in each group (Table I). These results indicate that addition of HDW to low-dose 5-FU does not increase the toxicity of the latter.

HDW and combination therapy downregulate the expressions of cell cycle regulators in vivo. As shown above, HDW treatment downregulated the expressions of CDK2 and E2F1 mRNA of HepG2 cells *in vitro*. Here we further investigated HDW alone and HDW combined with low-dose 5-FU on the expression of CDK2, E2F1 and cyclin E *in vivo* using both western blot analysis and real-time PCR. The results are shown in Fig. 6. Compared with the vehicle group, HDW markedly downregulated the expression levels of CDK2 and E2F1 mRNA. Low-dose 5-FU significantly decreased the expression level of CDK2 mRNA but showed no effects on E2F1 and cyclin E mRNA expression.

As expected, a combination of HDW and low-dose 5-FU caused a greater decrease in the expression levels of CDK2 and E2F1 mRNA (Fig. 6A). Expression of cyclin E was also decreased in the combination group. These results were further supported by western blot analysis (Fig. 6B), indicating that HDW enhanced the efficacy of low-dose 5-fluorouracil by further inhibiting the CDK2-E2F1 pathway.

Discussion

5-FU-based regimens as standard treatment have been widely used clinically in the treatment of various solid tumors, such as colorectal cancer (18), HCC (19), breast cancer (20) and glioma (21). Clinical trials have proven that 5-FU-based chemotherapy significantly improves overall and disease-free survival of such patients (22). Despite these favorable results, low response rate (13%) (23), resistance (24), short half-life (10 min) (25), severe cytotoxic and immunosuppressive adverse reactions (26-29) limit its clinical application. Recently, the regimens of low-dose-based chemotherapy have been used against cancers with relatively mild toxicities (30-37). For example, regimen with low-dose 5-fluorouracil and cisplatin has been reported to improve the median survival time of patients with advanced HCC (36). Patients with unresectable squamous cell carcinoma of the esophagus also had favorable overall survival when treated with low-dose cisplatin and continuous infusion of 5-FU (37). Unfortunately, low-dose chemotherapy regimens are still undoubtedly less effective than standard-dose therapy regimens.

The use of Chinese medicine as adjuvant treatment of chemotherapy has many advantages over chemotherapy alone, such as enhancement of the anticancer effects and reduction of the side effects of chemotherapy and improvement of patients' quality of life (38). HDW, an important component in many anticancer formulas of traditional Chinese medicine, has been verified by many researchers (7,39). However, its anticancer mechanism still remains largely unknown. Our findings showed that HDW could inhibit the growth of HepG2 cells both *in vitro* and *in vivo*. It can arrest HepG2 cells at G0/G1 phase and induce S phase delay at least partly through the CDK2-E2F1 pathway. In addition, we found in animal experiments that although its antineoplastic effect was lower than low-dose 5-FU, it might enhance the antitumor effect of the latter in the absence of overt toxicity, indicating that HDW might be a promising adjuvant therapy for chemotherapy.

The CDK2-E2F1 pathway is critical in regulating the transition of G1 to S phase of cell cycle. CDK2 is one of

the members of cyclin-dependent kinases (CDKs). When CDK2 associates with cyclin E, its serine-threonine kinase activity is activated (40,41) and the conjunction facilitates phosphorylation of retinoblastoma protein with the release of the E2F1 transcription factor (42). The release of the E2F1 transcription factor drives cells through G1 into S phase and promotes cell cycle progression (43). The control of the pathway is disrupted in virtually all human cancers (44), including HCC (45). Several studies have demonstrated that the growth of cancer cells could be inhibited via downregulating the mRNA or protein levels of CDK2, cyclin E or E2F1 transcription factor. For example, Tin *et al* (46) reported that artemisinin inhibited the proliferation of breast cancer cells via selectively downregulating the levels of the CDK2 and CDK4, cyclin E, cyclin D1 and the E2F1 transcription factor. Fang *et al* (47) reported that acetylbritannilactone inhibited the growth of HT-29 human colon cancer cells by inducing cell cycle arrest in G0/G1 phase and this suppression was accompanied by a marked decrease of cyclin E and CDK4 protein levels.

In this study, we observed the effect of HDW on the transcription and protein levels of CDK2, cyclin E and E2F1 involved in CDK2/Rb/E2F signaling. Our results showed that HDW interfered the G1/S transition of HepG2 cells at least via downregulation of the transcript and protein levels of CDK2 and E2F1 transcription factor. The results are consistent with our data of molecular docking simulation (15). Furthermore, we also found *in vivo* low-dose 5-FU could inhibit the expression of CDK2, as reported by others (18). However, it had no effect on the levels of cyclin E and E2F1 transcription factor. Combined with HDW, low-dose 5-FU more remarkably downregulated not only CDK2 but also E2F1 transcription factor and cyclin E. This may partially explain why HDW can enhance the antiproliferative effect of low-dose 5-FU.

In the present study, we also found that administration of low-dose 5-FU alone caused diarrhea in the mice (data not shown) and suppressed body weight gain. Indeed, studies showed that the use of low-dose 5-FU was still associated with many adverse effects, such as mucositis (18%) and diarrhea (39%) (48), which may be the possible reason of weight loss. However, when the mice were treated with the combination of HDW and low-dose 5-FU, the effect was reversed. Wang *et al* (49) reported that HDW could protect the gastrointestinal mucosa. We thus hypothesize that HDW could protect against low-dose 5-FU-induced weight loss by preventing injury to the gastrointestinal mucosa that otherwise would cause diarrhea.

In conclusion, HDW remarkably enhanced the antitumor effect of low-dose 5-FU by inhibiting the CDK2-E2F1 pathway in the absence of overt toxicity. These findings provide important data for further testing the possibility of using HDW as adjuvant therapy for clinical HCC patients. Further studies are necessary to elucidate the compounds in HDW which are responsible for such synergism.

Acknowledgements

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