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Original Article

The inhibition effect of Honokiol in liver cancer

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Abstract: *Objective:* The goal of this study was to investigate the effect of Honokiol and the possible mechanism on human hepatoma cells HepG2. *Methods:* CCK8 assay was used to test the effect of Honokiol on proliferation of HepG2 cells. Flow cytometry was employed to test the apoptosis effect of Honokiol on HepG2 cells. Quantitative Western blot was used to detect the protein level of Bad and Bcl-2. Western blot assay was conducted to determine the level of β-catenin. β-catenin antibody combined with Honokiolwasadded to investigate the effect on proliferation and apoptosis. *Results:* Compared with the control group, Honokiol could inhibit HepG2 cell proliferation and induce apoptosis at the concentration of 1.0 μmol/L, up-regulate the level of Bad and down-regulatethe level of Bcl-2. Western blot results showed that Honokiol could attenuate expression of β-catenin in a time and concentration dependent manner, while β-catenin antibody could strengthen the effect of Honokiolon HepG2. *Conclusion:* Honokiol can inhibit HepG2 proliferationand induce apoptosis, which may be mediated by the Wnt signaling pathway.

Keywords: Honokiol, human hepatoma cells, proliferation, apoptosis, Wnt signal pathway

Introduction

Honokiol is one of the main active ingredients extracted from Magnolia officinalis and belongs to the deciduous tree plant of Magnoliaceae [1]. Modern pharmacological studies have shown that Honokiol has significant and sustained central muscle relaxation, central nervous system inhibition, anti-inflammatory, antibacterial, anti-pathogenic microorganisms, anti-ulcer, anti-oxidation, anti-aging, cholesterol lowering, and other pharmacological effects [2, 3]. In addition, more and more studies have shown that Honokiol can inhibit growth of tumor cells at low concentrations, and it has a good anti-tumor effect on osteosarcoma, colon cancer, and cervical cancer [4]. However, its specific molecular mechanism is not clear so far.

This study reports thatHonokiol can inhibit the proliferation of human hepatoma cells HepG2 and induce apoptosis, and the inhibition effect can be promoted by β-catenin antibody. This suggests that the anti-tumor effect of Honokiol might be related to the Wnt signaling pathway. Therefore, β-catenin might be used as a target to regulate the growth of tumor cells. However, the specific mechanism remains to be studied.

Materials and methods

Cell culture

Human hepatoma cells HepG2 were purchased from the American Type Culture Collection (ATCC). The cells were incubated in were 37°C and the CO_2 concentration was 5%.

Reagents

Honokiolwaspurchased from Xi'an Yuxuan Biotech Co., Ltd. (purity: 98%). Aantibodies used in this study (Bad and Bcl-2) were purchased from Santa Cruz Biotechnology, and β-catenin was purchased from Abcam. Apoptosis staining kits, protein extraction kits, CCK8 kits, and ECL kits were provided by Biyuntian Biotech Co., Ltd. DMEM medium was purchased from Semimic Biotech Co., Ltd.

CCK8 test

Approximately 3,000 Human Hepatoma cells per well were seeded in 96-well plates. After the cells were adhered, the cells were treated with Honokiol in different concentrations. At 24 and 48 hours after treatment, the upper medi-

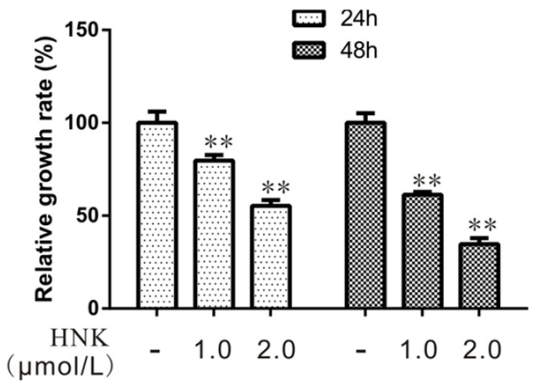

Figure 1. Effect of Honokiol on proliferation in HepG2 cells. CCK8 assay showed the relative growth rate of HepG2 cells ($^{*}P<0.05$, $^{**}P<0.01$ vs control).

um was aspirated and 100 ml of fresh medium containing 10 ml of CCK8 reagent was added to each well (do not containany air bubbles). After 2-3 hours of reaction, cell viability was measured at 450 nm using an ELISA reader.

Flow cytometry

About 5 × 10^5 cells were plated each well in 6-well plates, and treated with different concentrations of Honokiol. Then cells were harvested 48 hours later and resuspendedin PBS and dilutedto 1 mL. Staining reagent was then added and test the cells were analyzedby flow cytometryimmediately.

Western blot

About 5 × 10^5 cells per well were placedin 6-well plates. After the cells were adhered, they were treated with different concentrations of Honokiol. After 24 and 48 hours, the supernatant medium was discarded and the cells were washed three times in PBS, following the manufacture's instructions. After electrophoresis, the proteins were transferred to nitrocellulose (NC) membranes and blocked with 5% BSA for 2 h at room temperature. The primary antibodies were blocked for 2 hours and washed with TBST. The secondary antibodies were blocked for 1 hour, washed with TBST, and finally the Western blot was analyzed using an ECL kit.

Statistical analysis

SPSS 18.0 software was used for statistical analysis. The experimental data areexpressed as $\bar{x} \pm s$. Variance analysis was better for three groups and comparisons were made.

Results

Effect of Honokiol on the proliferation of HepG2 cells

The results of CCK8 showed that Honokiol can inhibit growth of HepG2 cells in a concentration-dependent manner (**Figure 1**), and the inhibition of Honokiol was more pronounced at 48 hours than at 24 hours.

Effect of Honokiol on the apoptosis of HepG2 cells

Flow cytometry results show that Honokiol can promote apoptosis of HepG2 cells compared with the control group (**Figure 2A**); Western blot results show that expression of the apoptotic protein Bad was up-regulated and the protein level of Bcl-2 was decreased by increasing the concentration of Honokiol (**Figure 2B**, **2C**).

The effect of Honokiol on expression of β-catenin protein in the Wnt signaling pathway

Western blot quantitative analysis showed that the β-catenin protein expression level gradually decreased by increasing the concentration of Honokiol and duration of action (**Figure 3**). This result suggests that the the role of Honokiol on HepG2 cells may be related to the Wnt signaling pathway.

Effect of β-catenin antibody on cell proliferation

CCK8 results show that β-catenin antibody can enhance the effect of Honokiol and further inhibit cell proliferation (**Figure 4**).

Effects of β-catenin antibody on apoptosis

Western blot quantitative analysis showed that β-catenin antibody can enhance Honokiol-induced apoptosis, increase the level of Bad protein, and down-regulate Bcl-2 protein levels (**Figure 5A**, **5B**).

Discussion

Liver cancer is one of the most common malignancies in the world and it seriously endangers human health. Primary liver cancer is very com-

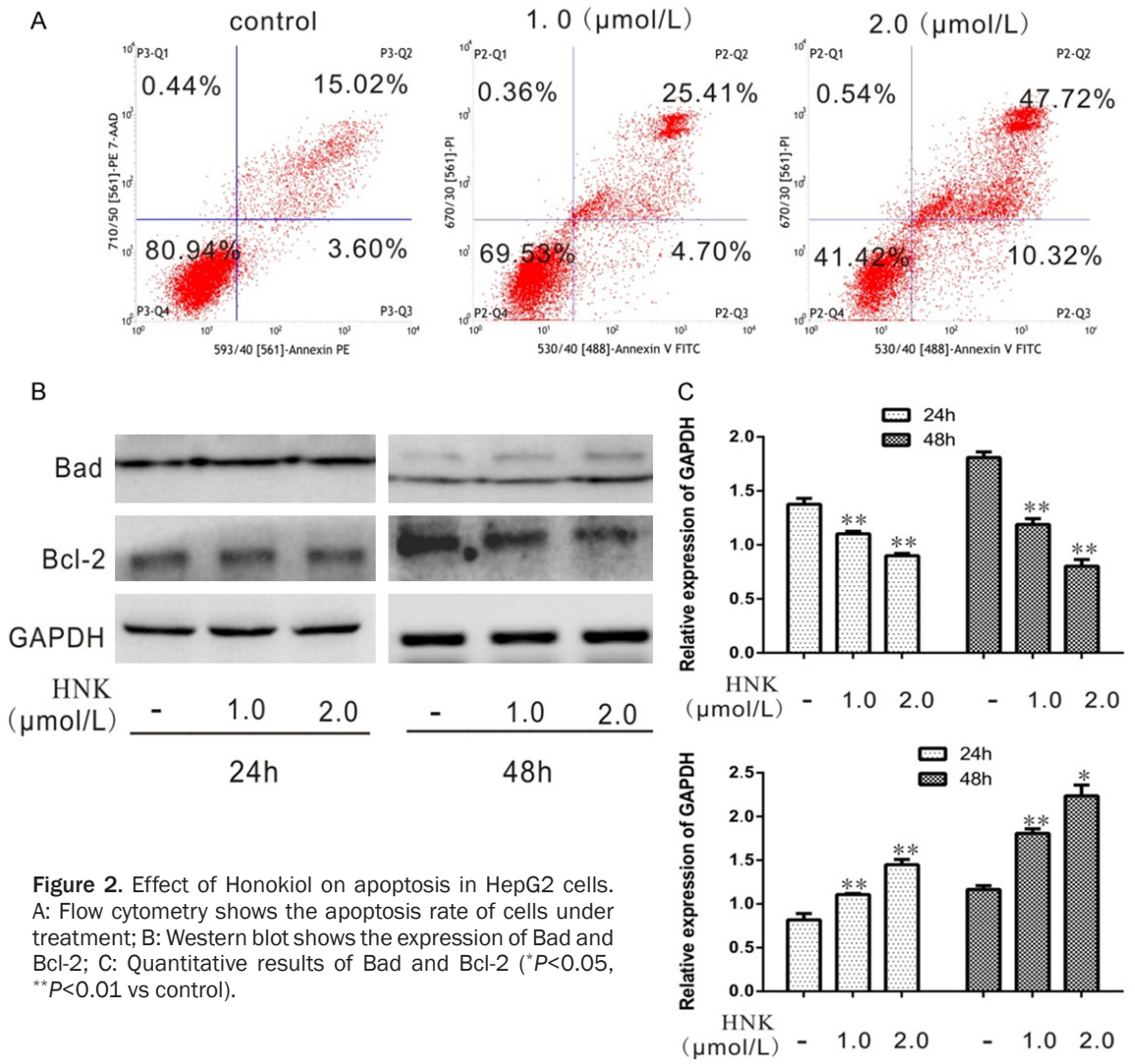

Figure 2. Effect of Honokiol on apoptosis in HepG2 cells. A: Flow cytometry shows the apoptosis rate of cells under treatment; B: Western blot shows the expression of Bad and Bcl-2; C: Quantitative results of Bad and Bcl-2 (*P<0.05, **P<0.01 vs control).

mon in China even in the world. It originates from the epithelial or mesenchymal tissue of the liver and has a high incidence of morbidity and mortality. According to previous results, about 782,000 new cases liver cancer patients were diagnosed in 2012 alone [5]. Conventional treatments are radiation therapy and chemotherapy. Chemotherapy drugs for the treatment of liver cancer are 5-fluorouracil, oxaliplatin, paclitaxel and "green chemotherapy drugs" [6]. However, due tothe severe adverse reactions and poor prognosis of those drugs, the quality of life of the patients is very poor. Therefore, extracting effective ingredients from natural plants to treat tumors has become popular.

Studies have found that many traditional Chinese herbs can control and even kill liver cancer cells by inhibiting the proliferation of liver cancer cells, inducing apoptosis of liver cancer cells, inhibiting invasion and metastasis, and reducing the resistance of liver cancer cells [5]. Honokiol, which is one of effective ingredients in those herbs has a bright future, because of its wide range of pharmacological effects, and inhibiting function onthe proliferation of tumor cells [7]. Liu et al. showed that Honokiol at a concentration of 0.5 μmol/L could significantly inhibit the proliferation of colon cancer cells [8]. Liu Chang et al. reported that Honokiol can inhibit the proliferation and apoptosis of human lung cancer cell A2 metastasis and invasion [9]. Yu Lihong et al. reported that Honokiol can inhibit the proliferation of melanoma B16 cells and the synthesis of melanin in cells [10].

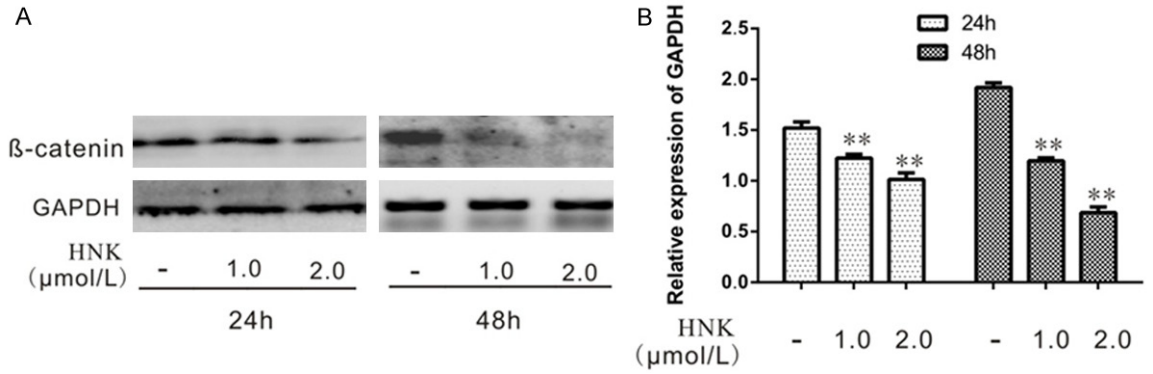

Figure 3. Effect of Honokiol on the level of β-catenin in HepG2 cells. A: Western blot shows the level of β-catenin; B: Quantitative results of β-catenin (*P<0.05, **P<0.01 vs control).

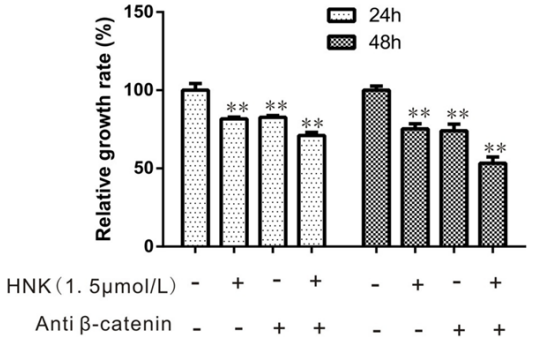

Figure 4. Effect of Honokiol and anti β-catenin on proliferation in HepG2 cells. CCK8 assay shows the relative growth rate of cells (*P<0.05, **P<0.01 vs control).

Studies have confirmed that Honokiol can inhibit the proliferation and induce apoptosis of human osteosarcoma cells in a dose-dependent manner, and its mechanism is related to the reduction of the expression level of mir21 gene [11]. It can cause G0/G1 phase retardation, and induce bone sarcoma cells apoptosis and autophagythrough the ROS/ERK1/2 signaling pathways [12]. Furthermore,Honokiol can effectively down-regulate phosphorylation and activation of renal cell receptor tyrosine kinase and Ras, and inhibit the expression of cell protective enzyme heme oxygenase-1 induced by receptor tyrosine kinase and calcineurin inhibitor, thus promoting the apoptosis of cancer cell [13]. It can inhibit bladder cancer cells by down-regulating the invasion of SRC623 and its relative genes [14]. It can achieve the anti-prostate cancer effect by inhibit the mRNA expression of c-myc [15]. In addition, local application of Honokiol also has a significant preventive effect on UV-induced immuno-suppression, which is conducive to hindering development of malignant skin tumors. Prasad R [16] showed that Honokiol can also induce the degradation of targeted gene AML1-ETO, which has therapeutic effect on acute myeloid leukemia [17]. In brief, Honokiol has great potential for anti-tumor therapy.

Many pathogenesis mechanisms of hepatocellular carcinoma were demonstrated, including abnormal conduction of TGF-β, BMP/Smad, Wnt/β-catenin, PI3K/Akt, deletion of p53, and mutation of PTEN [18, 19]. Wnt signaling plays an important role in embryonic development and cancer progression, in addition to participating in normal physiological function regulation of individuals [20]. Wnt signaling pathway can be divided into canonical Wnt signaling pathway and non-canonical Wnt signaling pathway. Lots of evidence has proven thatabnormally activated classical Wnt signaling is the main pathogenesis of liver cancer. Among members of the classical Wnt signaling pathway which regulate Wnt signaling, β-catenin is the most important protein. When the Wnt protein binds to the Frizzled receptor on the cell membrane surface, it inhibits the formation of the Axin-APC-GSK3β degradation complex, thereby preventing degradation of intracellular β-catenin, resulting in accumulation of β-catenin and further translocation into the nucleus, down-regulate gene regulation, and tumor cell proliferation, apoptosis, migration, and invasion [21]. Therefore, β-catenin gene expression can be used as a very useful indicator of the degree of development of liver cancer.

Conclusion

Honokiol can inhibit the growth of HepG2 cells and induce apoptosis. The mechanism may be

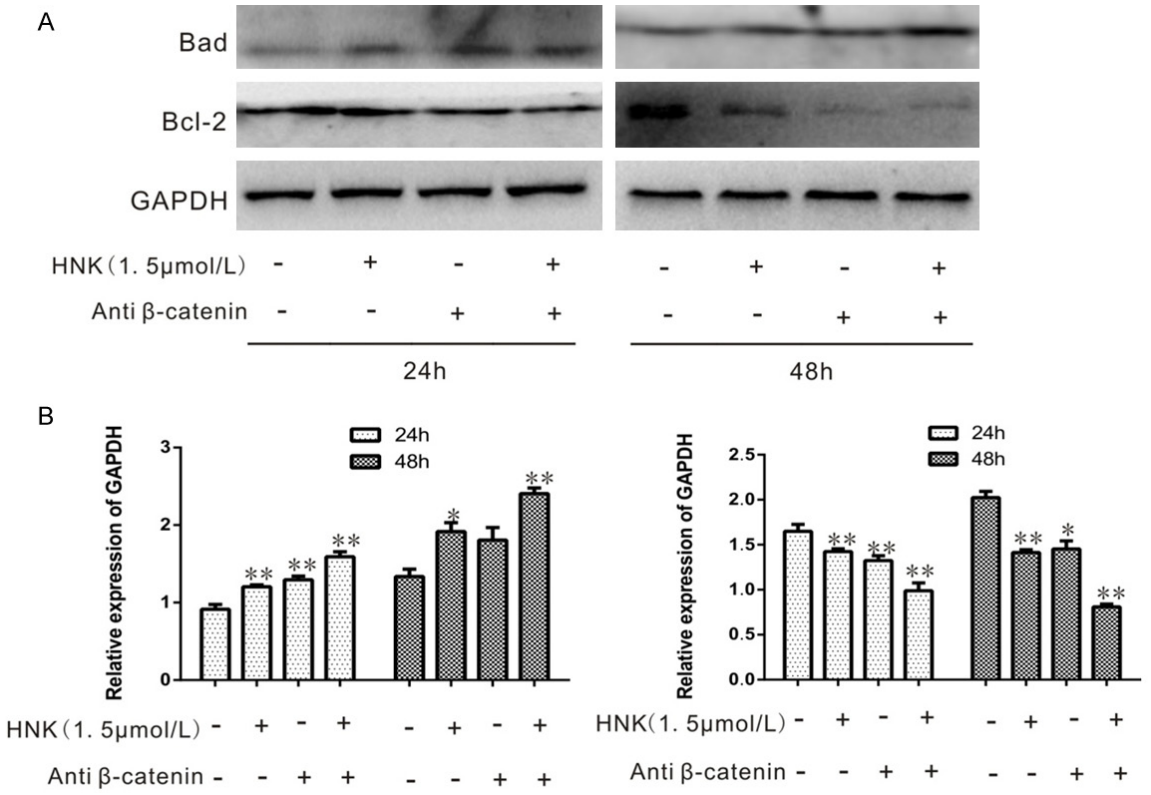

Figure 5. Effect of Honokiol and anti β-catenin on apoptosis in HepG2 cells. A: Western blot showsexpression of Bad and Bcl-2; B: Quantitative results of Bad and Bcl-2 ($^{*}P<0.05$, $^{**}P<0.01$ vs control).

related to the classic Wnt signaling pathways. Combined all the reports on the effect of honokiol on various tumor cells, Honokiol has potential in the treatment of colorectal cancer, lung cancer, prostate cancer, bladder cancer, skin cancer, and leukemia, and can have an anti-tumor effect on multiple biological targets of cancer cells. Honokiol can be used as a very effective antineoplastic candidates in treating cancers.

Further research to analyze other proteins in the Wnt signaling pathway are planned. This work overall provides a theoretical and experimental basis for better application of Honokiol in clinical practice.

Disclosure of conflict of interest

None.

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