Emodin induces hepatocellular carcinoma cell apoptosis through MAPK and PI3K/AKT signaling pathways in vitro and in vivo

WANFU LIN, MAOFENG ZHONG, HUIXIA YIN, YONGAN CHEN, QINGXIN CAO, CHEN WANG and CHANGQUAN LING

Department of Traditional Chinese Medicine, Changhai Hospital, Second Military Medical University, Shanghai 200433, P.R. China

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Correspondence to: Professor Chen Wang or Professor Changquan Ling, Department of Traditional Chinese Medicine, Second Military Medical University, No 800 Xiangyin Road, Yangpu, Shanghai 200433, P.R. China
E-mail: wangchenchina@126.com
E-mail: lingchangquan@hotmail.com

Abbreviations: HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal regulated kinase; JNK, c-Jun-N-terminal kinase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AKP, alkaline phosphatase; GGT, gamma-glutamyltransferase; Cr, creatinine; BUN, blood urea nitrogen

Key words: emodin, human hepatocellular carcinoma, anti-proliferation, apoptosis, molecular mechanisms

Abstract. Emodin is an active ingredient derived from root and rhizome of Rheum palmatum L and many studies have reported that it exhibits anticancer effects in a number of human tumors. However, there is little information demonstrating the possible effects of emodin on the proliferation and apoptosis of hepatocellular carcinoma (HCC). In the present study, we show that emodin may inhibit the proliferation of SMMC-7721 cells in a dose- and time-dependent manner and induced apoptosis of cells in a concentration-dependent manner after treatment for 24 h. Moreover, we further discovered that the possible molecular mechanisms involved may relate to the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways. Emodin may induce the phosphorylation of extracellular-signal-regulated kinase (ERK) and p38 while mildly suppressed the expression of p-c-Jun-N-terminal kinase (p-JNK). However, emodin did not affect the expression of the total (t)-ERK, t-p38 or t-JNK. Furthermore, emodin also suppressed the activation of p-AKT, but not the t-AKT. In vivo, we found that emodin suppressed tumor growth in experimental mice without an obvious change in body weight, which may work through the antiproliferation and apoptosis inducing effects. Moreover, emodin improves the liver and kidney function in mice, revealing that emodin may improve the life quality of the mice with implanted tumors. In conclusion, the above findings indicate that emodin may be a potentially effective and safe drug to induce apoptosis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and 86% of cases occur in developing countries. It ranks fifth for incidence and second for mortality in developing countries and for developed countries it ranks 11th for incidence and seventh for mortality, with an estimated 792,000 incident cases and 818,000 related deaths occurring globally in 2013 (1). Surgery is currently the most effective treatment but many patients are diagnosed in an advanced stage that was not appropriate for surgical treatment. The effects of alternative treatments such as traditional chemotherapeutic agents is still unsatisfactory due to their side-effects and the development of drug resistance (2,3). Therefore, it is necessary and urgent to identify new agents or therapeutic strategies with lower toxicity that are active against HCC for the prevention and treatment of HCC.

Many agents extracted from natural plants have shown their effects in the prevention and treatment of cancer (4). Among these, many of the medicines derive from Traditional Chinese Medicine (TCM) and have been confirmed to be effective in the treatment of a number of tumors via targeting and regulating multiple molecular pathways (5,6). Emodin is an active ingredient derived from root and rhizome of Rheum palmatum L, which is a plant widely used in Chinese medicine as a laxative for thousands of years (7,8). The molecular formula of emodin is C15H10O5 and its molecular weight is 270.24. In recent decades, increased attention was focused on anticancer activities of emodin since studies have reported that it exhibits antiproliferative and apoptosis-inducing effects in a number of human cancers such as colon, cervical and gastric cancer (9-11). It may also suppress migration and metastasis of cancer such as breast cancer and HCC (12,13). However, there is little information demonstrating the possible effects of emodin on the proliferation and apoptosis of HCC at present. Therefore, further interpretations are still needed to elucidate the exact mechanisms.
Thus, in the present study, we examined the effects of emodin on the proliferation and apoptosis of HCC cells in vitro and in vivo, as well as the molecular mechanisms involved.

Materials and methods

Materials. Emodin (1,3,8-trihydroxy-6-methyl-anthraquinine), dimethyl sulfoxide (DMSO) and Cell Counting kit-8 (CCK-8) were purchased from Sigma (St. louis, MO, USA). Emodin was dissolved in 100% DMSO to prepare stock solutions of 5, 12.5, 25, 50 and 100 mmol/l, respectively, which were diluted in high glucose Dulbecco’s modified Eagle’s medium (DMEM) to the indicated concentrations before each assay. DMSO (0.2%) was used as vehicle control for all the experiments. The Annexin V-FITC apoptosis detection kit was purchased from Nanjing KeyGen Biotech. Co., Ltd, Nanjing, China. Rabbit phosphorylated (p)-Akt and total (t)-Akt polyclonal antibody, rabbit p-extracellular-signal-regulated kinase (ERK) 1/2 and t-ERK1/2 monoclonal antibody, rabbit p-p38 and t-p38 monoclonal antibody, rabbit p-c-Jun-N-terminal kinase (p-JNK) and t-JNK monoclonal antibody, rabbit anti-cleaved caspase-3, -9 and pro-caspase-3, -9 monoclonal antibody, mouse anti-β-actin and PCNA monoclonal antibody were all purchased from Cell Signaling Technology (Boston, MA, USA). Rabbit anti-Ki-67 antibody was purchased from Abcam (Cambridge, MA, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay kit was purchased from Roche Diagnostics (Meylan, France). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), gamma-glutamyltransferase (GGT), creatinine (Cr) and blood urea nitrogen (BUN) colorimeter testing kits were obtained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China).

Cell culture. The HCC cell line SMMC-7721 was obtained from the Cell Bank of the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, China) and cultured in DMEM (Thermo Fisher Scientific, Shanghai, China) with 10% fetal bovine serum (Gibco-Life Technologies, Carlsbad, CA, USA). The cells were cultured in an incubator at 37˚C in a humidified atmosphere of 5% CO₂ and sub-cultured when the cell density reached 80-90%.

Cell proliferation assay. Cell proliferation was measured with CCK-8 according to the manufacturer's instructions. Briefly, SMMC-7721 cells were seeded at a density of 3x10⁵ cells/well in a 96-well plate and cultured for 24 h. Emodin was then added to the wells with final concentrations of 0, 10, 25, 50, 100 and 200 μmol/l and incubated for 12, 24 and 48 h. Before detecting the absorbance, 10 μl CCK-8 was added to each well and incubated for an additional 1.5 h at 37°C. Finally, the absorbance at 450 nm was measured using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometric analysis of apoptosis assay. Cell apoptosis was detected using the Annexin V-FITC apoptosis detection kit following the manufacturer’s instructions. In brief, SMMC-7721 cells were seeded into the 6-well plates with 3x10⁵ cells in each well. After incubation at 37°C for 24 h, different concentrations (25, 50 and 100 μmol/l) of emodin were added to the wells. Then, cells of each sample were collected in a centrifuge tube after an additional 24 h. After washed with phosphate-buffered saline (PBS), the cells were suspended in 500 μl of Annexin V binding buffer (1X), 5 μl of Annexin V-FITC and propidium iodide (PI) were added and incubated with for 15 min at room temperature away from the light. The stained cells were analyzed by flow cytometry on FACScalibur (BD Biosciences, San Jose, CA, USA).

Western blot assay. After treatment with 100 μmol/l emodin for indicated time, the cells were washed three times with ice-cold PBS to stop the stimulation. Then, the cells were lysed in RIPA protein lysis buffer containing 1 mM PMSF on ice. The cell lysates were centrifuged at 12,000 x g for 15 min at 4°C and the supernatant was collected as the total proteins and transferred to a new tube. The protein concentration was determined using a BCA assay kit (Beyotime Institute of Biotechnology, Shanghai, China) and equal amounts of proteins (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane. After blocked by Tris-buffered saline and Tween-20 (TBST) buffer containing 5% BSA for 1 h at room temperature, the PVDF membrane was incubated with appropriate concentrations of primary antibodies (dilution, 1:1,000) at 4°C overnight. After washing the membrane with TBST three times for 15 min, the membrane was incubated with corresponding secondary antibody labeled with horseradish peroxidase-conjugated (goat anti-mouse, 1:5,000, goat anti-rabbit, 1:2,000) for 2 h at room temperature. Following three washes with TBST for 15 min, the immunoreactive bands were detected using an enhanced chemiluminescence detection kit (Sigma). β-actin was used as the internal control and the relative values of target protein were corrected in accordance with the absorbency of the internal control.

Antitumor activity in vivo. All work performed with animals was in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Committee on the Ethics of Animal Experiments of the Second Military Medical University. Four-week-old male BALB/c-nu nude mice were obtained from the Shanghai SIPPR-BK Laboratory Animal Co., Ltd., (Shanghai, China) and maintained under standard conditions in the Laboratory Animal Center of the Second Military Medical University. SMMC-7721 cells were harvested and resuspended in PBS. The mice were subcutaneously inoculated with 5x10⁶ cells into the right flank. Tumor volume was calculated using the following formula: Larger diameter x (smaller diameter) ²/2. The mice were randomly divided into three groups (n=5) when the tumor volume reached 75-100 mm³ and then treated every day for two weeks with intraperitoneal injection of either vehicle (DMSO), 25 or 50 mg/kg emodin. Body weights were measured every week. The mice were euthanized at the end of the experiment and the tumor masses were removed and weighed.

The xenograft tumors were isolated and fixed in a 10% formalin solution, dehydrated and embedded in paraffin. The samples were then sectioned at a 5-μm thickness and either stained with hematoxylin and eosin (H&E) to reveal tumor
tissue necrosis or immunohistochemistry (IHC) stained using antibodies against Ki-67 (1:400) or PCNA (1:500). The apoptosis of paraffin-embedded sections of the tumors was detected by a TUNEL assay kit according to the manufacturer's protocol. All slides were detected under a phase-contrast microscope (magnification, x200).

Function tests of the liver and kidney. In order to determine the safety of emodin in treating the nude mice bearing liver cancer, we collected 1 ml of blood through eye-bleeding at the time of necropsy. The blood was centrifuged at 3,000 rpm for 10 min to obtain sera and the sera were analyzed for the levels of ALT, AST, AKP, GGT, Cr and BUN using the respective colorimeter testing kits following the manufacturer's protocol.

Statistical analysis. All experiments were performed at least three independent times and the results are presented as the mean ± standard deviation (SD). Statistical analysis was performed with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The statistical analysis was performed using a one-way analysis of variance (ANOVA) and Dunnett's test. P<0.05 was considered statistically significant.

Results

Emodin inhibits the proliferation of SMMC-7721 cells. In order to investigate the anticancer activity of emodin on liver cancer, we first investigated the effect of emodin on the proliferation of SMMC-7721 cells using the CCK-8 assay. As shown in Fig. 1, the viability of the cells decreased evidently as the concentrations of emodin increased from 10 to 200 µmol/l. In addition, emodin also showed the antiproliferative effect on SMMC-7721 cells in a time-dependent manner compared with the control.

Emodin induced apoptosis of SMMC-7721 cells. To determine the effect of emodin on apoptosis induction in SMMC-7721 cells, flow cytometry was used to assess the effect after treatment with emodin at different concentrations (25, 50 and 100 µmol/l) for 24 h. As shown in Fig. 2, the percentage of apoptotic cells (including early and late apoptotic cells) was shown to significantly increase as the emodin concentration increased (2.75±0.88, 17.40±2.81, 28.30±2.40 and 60.19±4.62, respectively). These data clearly showed that emodin treatment may significantly induce apoptosis of SMMC-7721 cells in a dose-dependent manner.

Effect of emodin on the expression of proteins associated with tumor apoptosis. To further determine the probable mechanism(s) underlying the decrease in cell viability caused by emodin, we used western blotting to detect the protein expression of MAPK and PI3K/AKT pathways since they act as key regulators of cellular survival and apoptosis in various human cancers. The results indicate that emodin may significantly promote the expression of p-ERK and p-p38 after treatment of 100 µmol/l emodin for 0, 15, 30 and 60 min, respectively. However, the protein lever of p-JNK was suppressed mildly by emodin after treatment for more than 30 min. Whereas, the total ERK, p38 and JNK remained unchanged. On the other hand, emodin also inhibited the expression of p-AKT.
but did not affect the total AKT under the same condition (Fig. 3). In addition, because caspase activation is considered to be a hallmark of apoptosis, we further examined and found that cleaved caspase-3 and cleaved caspase-9 were clearly increased in emodin-treated cells in a time-dependent manner (30, 60, 120 and 240 min). However, pro-caspase-3 and -9 were mildly decreased with treatment of 100 µmol/l emodin after 120 min (Fig. 4).

**Emodin inhibits the growth of SMMC 7721 cell xenografts in nude mice.** Based on the above *in vitro* results, we next investigated whether emodin has the potential anticancer effect *in vivo* by using a xenograft tumor model of liver cancer. As expected, emodin suppressed the tumor growth in a dose-dependent manner, while little influence on the body weight of mice was observed (Fig. 5). To detect cell necrosis and the expression of Ki-67 and PCNA in tumor tissues, H&E staining and IHC analysis were performed. H&E staining showed that emodin may induced cell death and caused symptoms of necrosis in the tumor masses. In IHC analysis, Ki-67 and PCNA, which are markers of cell proliferation, showed significant reduction with treatment of emodin in a dose-dependent manner. Moreover, TUNEL staining was used to detect the apoptosis of the tumor sections and the result indicated that the apoptotic index was significantly increased as determined by the percentage of TUNEL stained nuclei (Fig. 6).

**Emodin treatment improves the liver and kidney function in mice.** Previous studies have shown that emodin may inhibit lung metastasis in mice with no obvious changes in liver and kidney functions (14). In the present study, we also demonstrated that emodin does not cause obvious toxicity in nude mice since there were no obvious changes in their body weight. Furthermore, as showed in Fig. 7, emodin may decrease the levels of serum ALT, AST, AKP, GGT, Cr and BUN, which indicated the improvement of the liver and kidney function in mice.

**Discussion**

Despite increasing progress in treatment methods in recent years, the prognosis of HCC has not substantially improved
since many patients were detected in an advanced stage when there are limited therapeutic options. Sorafenib is the only drug for treating advanced HCC that has been approved by the USA Food and Drug Administration in the past decade (15). However, many patients may not benefit from this drug due to its side-effects and rapidly development of drug resistance. Therefore, it is still imperative to identify novel drugs for HCC treatment.

Increased evidence shows that many Chinese herbs have antitumor properties and induction of apoptosis is one of the mechanisms (5). Emodin, which was extracted from traditional Chinese medicine *Rheum palmatum* L., was found effective in suppressing cancer proliferation, invasion and metastasis in different types of cancer (16). Although Subramaniam *et al.* (17) have demonstrated that emodin may inhibit growth and induce apoptosis of HCC in vitro and in vivo through the inhibition of the STAT3 signaling cascade, further studies of the underlying molecular target and mechanism are still necessary.

In the present study, we assessed and validated the efficacy of emodin on HCC in vitro and in vivo. Emodin inhibited the proliferation of SMMC-7721 cells in a dose- and time-dependent manner and induced apoptosis of cells after treatment for 24 h. In vivo, we found that emodin may suppress the tumor growth in experimental mice without an obvious change in body weight, which may work through the antiproliferation and apoptosis inducing effects. Moreover, emodin may also improve the liver and kidney function in mice, revealing that emodin may improve the quality of life of the mice with implanted tumors. Thus, these findings indicate that emodin may be a potential effective and safe drug to induce apoptosis of HCC.

Mitogen-activated protein kinase (MAPK) is an important signaling pathway, which is mainly composed
of three subfamilies such as ERK, p38 and JNK signaling pathways (18,19). Significant attention has been focused on the important role of the MAPK pathway since it is critically involved in tumor cell proliferation, apoptosis, invasion and tumor metastasis (14,20). Thus, we investigated whether emodin could mediate its anticancer effects in part through the MAPK signaling pathway by detecting the protein expression of ERK, p38 and JNK and their phosphorylation events. We found that emodin induced the activation of ERK and p38 via promoting their phosphorylation, which is contrary to some cases that activation of the ERK or p38 pathway has been associated with proliferation and the apoptotic signaling pathways of HCC (21,22). Nevertheless, the involvement of ERK or p38 MAPK pathway in the proliferation and apoptosis remains somewhat controversial. In an experiment performed by Zhao et al (23), they found that Methyl CpG-binding protein 2 (MeCP2) promotes cell proliferation by activating ERK1/2 and inhibiting p38 activity in HCC. Lu et al(24) also discovered that the suppression of AAA domain containing 2 (ATAD2) increased interactions of MKK3/6 with p38 that led to p38 activation and subsequently apoptosis. Emodin may also induce apoptosis of colorectal cancer cells through activating p53/p38/Puma pathway by triggering ROS production (25). However, according to the study by Liu et al (26), aspafilioside B may induce G2 phase arrest and apoptosis by upregulate ERK and p38 phosphorylation, which is consistent with our result that the activation of ERK and p38 may induce apoptosis of HCC. We think the roles of the ERK and p38 are partly associated with cell type. On the contrary, JNK signaling pathway was considered to induce HCC cell cycle arrest and induced HCC apoptosis (27). In the present study we also found that emodin may suppress the activation of JNK mildly, this indicates the JNK pathway may not play a major role in emodin’s effect.

The phosphoinositide 3-kinase (PI3K) signaling cascade is another critical pathway in cancer as it promotes cell survival and growth (28,29). According to the clinical research, p-AKT was higher in tumor (53%) than in cirrhotic tissues (12%) while it was absent in normal liver. Inhibitors of this pathway are under active development as anticancer therapeutics (30). As expected, the phosphorylation of AKT was suppressed by emodin in a time-dependent manner, which may reveal partly the possible mechanism of apoptosis inducing effect of emodin on HCC.

In summary, the present study demonstrated that emodin inhibited proliferation and induced apoptosis of HCC in vitro and in vivo.
and in vivo. The mechanisms may be transduced through MAPK and PI3K/AKT signaling pathways. Our results shed some light on the mechanisms behind the effect of emodin on HCC and suggested that emodin could be a potential safe candidate for the treatment of HCC due to its high efficacy and less systemic side-effects.

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References