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Dihydromyricetin Induces Apoptosis by Reducing TGF-β via P53 Activation in HepG2 Cells

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Abstract

Di-hydromyricetin extracted from the stems and leaves of *Ampelopsis grossedentata* is a bioactive flavonoid compound. It exhibits antithrombotic anti-inflammatory and anticancer activity according to our previous research. Di-hydromyricetin could induce human hepatocellular carcinoma cells apoptosis. In our study we demonstrated that DHM could significantly inhibit HepG2 cells proliferation and induce HepG2 cells apoptosis by flow cytometry and MTT methods. Furthermore, we found DHM could regulate TGF-β signal pathway (P53 SMAD3 and P-SMAD2/3) proteins. Moreover, we confirmed that the antitumour activity of DHM was regulated through the activation of p53 (MDM2 P-MDM2 BAX and Bcl-2). Our findings defined that DHM could induce HepG2 cells apoptosis by reducing TGF-β via p53 pathway.

Key words:

Di-hydromyricetin; Anti-cancer; Proliferation; Apoptosis

Introduction

Dihydromyricetin (DHM Figure 1A) a bioactive flavonoid compound extracted from the stems and leaves of *Ampelopsis grossedentata* Vitaceae has oxidation resistance antitumour and free radical scavenging capability [1,2]. It was reported that DHM could promote hepatocellular carcinoma apoptosis via the P53 activation-dependent pathway [3,4]. Meanwhile it could induce Hep G2 cells autophagy involved inhibition of m-TOR [5]. Transforming growth factor β (TGF-β) a multifunctional cytokine regulates cellular proliferation differentiation and extracellular matrix production [6]. TGF-β a major pro-fibro genic cytokine and a common drugs target of liver disease treatment [7]. TGF-β initiates its diverse cellular responses by binding and activating specific cell surface receptors having intrinsic serine-threonine kinase activity. These activated TGF-β receptors stimulate the SMAD proteins phosphorylation [8]. In the nucleus SKI proteins repress SMAD ability to activate TGF-β target genes by disrupting active complexes of SMAD 2 or SMAD 3 with SMAD 4 [9]. TGF-β secreted abundantly by tumour cells as well as by the local microenvironment promotes invasion and metastases of various neoplasms through autocrine and paracrine mechanisms [10]. The P53 tumour suppressor belongs to a protein family regulating cell proliferation apoptosis and differentiation. Mesoderm differentiation is inhibited in P53-depleted embryos. In mammalian cells the full transcriptional activation of the CDK inhibitor p21WAF1 by TGF-β requires P53 [11]. SMAD2/3 and the tumour suppressor protein P53 physically interact and jointly regulate the transcription of several TGF-β target genes. Activated RAS may well have general growth-promoting effects. However, in the presence of wild-type P53 this would be balanced by the positive role played on P53/SMAD cooperation that would sustain TGF-β growth control and thus limit neoplastic transformation [12,13]. According to our previous study results we verified that DHM could inhibit HCC cell proliferation and induce cell apoptosis. In the present study we firstly found DHM could regulate TGF-β pathway in HepG2 cells. Moreover, we proved that targeting TGF-β via P53 activation play an important role in DHM induced HepG2 cell apoptosis.

Materials and Methods

Medicine and reagents

DHM purchased from Sigma-Aldrich Inc. (St. Louis MO) was dissolved to 50 mM in di-methylsulfoxide (DMSO; MP Bio-medicals USA) as a stock solution stored at -20°C and then stock solution was subsequently diluted in culture medium (RPMI-1640; Gibco-BRL Carlsbad CA USA) to the desired concentration for experiments. Pifithrin-α (PFT-α) purchased from Sigma-Aldrich Inc. is the P53 inhibitor (St. Louis MO). The monoclonal human anti-rabbit antibodies were purchased from Cell Signalling Technology (TGF-β SMAD3 P-SMAD2/3 P-MDM2 MDM2 P53 Bcl-2 BAX and GAPDH). The HRP-conjugated purchased from Earth Ox (San Francisco CA USA) is the second antibody.

Cell culture and DHM treatment

The HepG2 cell line was donated by Affiliated Hospital of Guangdong Medical University (Zhanjiang China). RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL Carlsbad CA USA) 100 U/ml penicillin and 100 U/ml streptomycin (Hyclone Laboratories Inc. USA) were used to culture cells which were maintained in the 95% air with 5% CO2 at 37°C. HepG2 cells (proliferation reached 50-60%) were treated with different DHM concentrations.

Cell morphological observation

The 1 × 10^6/mL cell suspension were prepared. Cells were treated with DHM or PFT-α (After PFT-α treated cells for 30 min and then DHM treated cells for 24 hrs) respectively inverted microscope (Leica Wetzlar Germany) was used to observe cell morphology.

MTT assay

MTT assay was performed to determine the cytotoxicity of DHM. Cell density was adjusted to 5 × 10^4 cells/mL. The 96-well plate (200 μL medium/well -5×10^3 cells/well) was placed overnight. DHM (10, 50 and 100 μM) treated cells for 6, 12 and 24 h; 20 μL MTT (5 mg/mL) solution was transferred to per well. 96-well plate was incubated for 4 h in cell culture box. After supernatants were removed 150 μL DMSO were added to per well. The plate was shacked for 5 minutes and the absorbance was detected with reader (MULTISKAN MK3 Thermo) at 595 nm. Data are calculated according to the formula: Inhibitory rate (%) = [A595 (control–A595 treated)]/[A595 (control)–A595 (blank)] × 100%.

Western blot

Western blotting analysis method was performed according to the Cell Signalling Technology provided protocol. Briefly cells were lysed with the lysis buffer with the instruction book. In order to measure the sample protein level BCA Protein Assay kit was purchased (Beyotime Institute of Biotechnology Jiangsu China) and 50-200 mg/lane were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and 5% milk were used to blocked poly-vinylidene fluoride (PVDF Millipore) containing the protein for 3 h at room temperature. Antibodies (P-MDM2 P53 Bcl-2 BAX TGF-β SMAD3 and P-SMAD2/3) were used at the 1:1000. The anti-GAPDH antibody was used at a 1:3000 dilution; The HRP-conjugated secondary antibody was used at a 1:3000 dilution. HRP-conjugated secondary antibodies were used to incubate the PVDF membranes for 2 h which were washed with TBST 10 min for 3 times at room temperature. Chemiluminescent HRP substrate (Millipore) was used to on the PVDF membrane which following was exposed to Kodak Image Station 4000 MM (USA)

Statistical analysis

Graph Pad Prism 5 was used to perform statistical analysis. Data are appeared as the mean ± SD; error bars represent the SD in all figures. Student's t-test method was used to evaluate statistical differences marked with the *P<0.05,** P<0.01 or *** P<0.001. At least three independent experiments were performed and figures were obtained from that.

Results

DHM inhibits cell proliferation and promotes cell apoptosis in HepG2 cells

HepG2 cells treated with DHM became round and floating which is different from the control DHM inhibitory effects of HepG2 cells was evaluated by MTT assay. Result demonstrated that could HepG2 cells proliferation was inhibited by DHM in a concentration–time dependent manner (Figure 1B). Flow cytometry was used to detect cell apoptosis and data show cell apoptosis was induced by DHM in a concentration-dependent manner (Figure 1C) accompanied with normal cells decreased and sloughed cells increased (Figure 1D). These data verify that DHM exerts a remarkable HepG2 cells growth inhibition and promotion on apoptosis. 100 μM for 24 h treatment is IC50 value.

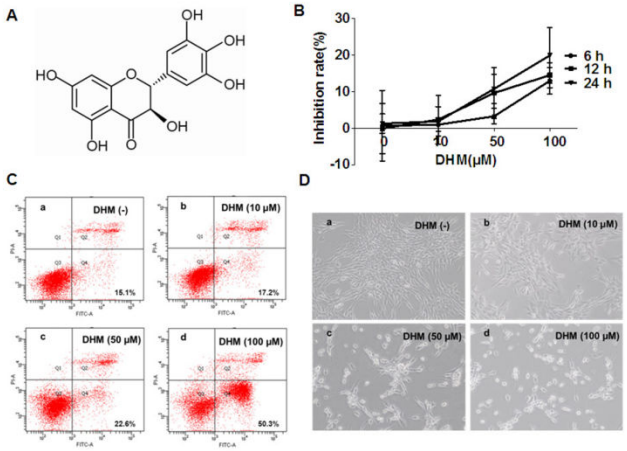

Figure 1: DHM promotes HepG2 cells apoptosis; A. The DHM chemical structure; B. HepG2 cells were treated with DHM different concentrations (10, 50 and 100 μM) treatment for 6, 12 and 24 h and cell growth inhibition were analysed by MTT method; C. DHM-induced cell apoptosis of HepG2 cells treated with DHM different concentrations (10, 50 and 100 μM) treatment for 6 h which were visualized by flow cytometry; D. DHM-induced apoptosis of HepG2 cells treated with DHM different concentrations (10, 50 and 100 μM) treatment for 24 h which were visualized by microscopy (×100).

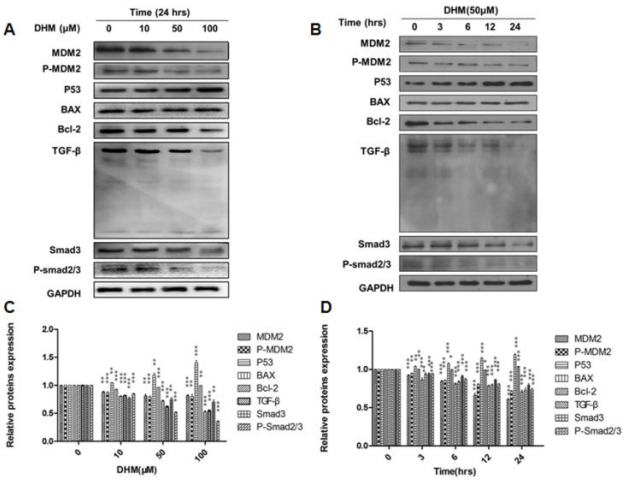

Figure 2: DHM regulates TGF-β and apoptosis-related proteins. A. Different concentration (10, 50 and 100 μM) DHM treated HepG2 cells for 24 h TGF-β and apoptosis-related proteins expression were checked; B. 50 μM DHM treated HepG2 cells for 3, 6, 12 and 24hrs and then TGF-β signal and apoptosis-related proteins expression were checked; C D. Column diagrams of A and B.

DHM induce apoptosis accompanied with TGF-β signal pathway proteins regulation

HepG2 cells were treated with different DHM concentrations (0, 10, 50 and 100 μM) for 24 h; 50 μM DHM treated cells for schedule time (3, 6, 12 and 24hrs) P53 and Bcl-2 expressions were evaluated. Meanwhile with the increasing DHM concentration and treatment time MDM2 P-MDM2 TGF-β SMAD3 and P-SMAD2/3 expressions were down-regulated but the BAX expression alteration did not change significantly (Figure 2).

Cell growth recovers gradually after DHM were withdrawn

To confirm whether DHM cell growth inhibition will restore after DHM were withdrawn 50 μM DHM pre-treated HepG2 cells for 12 h and fresh culture medium were replaced moreover, at schedule time (0, 3, 6, 12 and 24hrs) cell growth was observed. Cells treated with 50 μM DHM for 12 h which cell growth was inhibited and morphology changed showing became round and floating. However, cell growth restored at 12 h after DHM was withdrawn (Figure 3A).

TGF-β pathway proteins expression of HepG2 cells increased gradually after DHM were withdrawn

In this study we evaluated TGF-β expression after DHM were withdrawn. Cells were treated with 50 μM DHM for 6 h and supernatant was replaced with fresh culture medium. P53 and Bcl-2 decreased after DHM were withdrawn with the incubation extension. Meanwhile TGF-β SMAD3 MDM2 P-MDM2 and P-SMAD2/3 proteins expression levels increased after DHM were withdrawn. The BAX expression change was not obvious. These results strongly indicated that DHM could induce cell apoptosis by targeting TGF-β signal pathway proteins (Figure 3B).

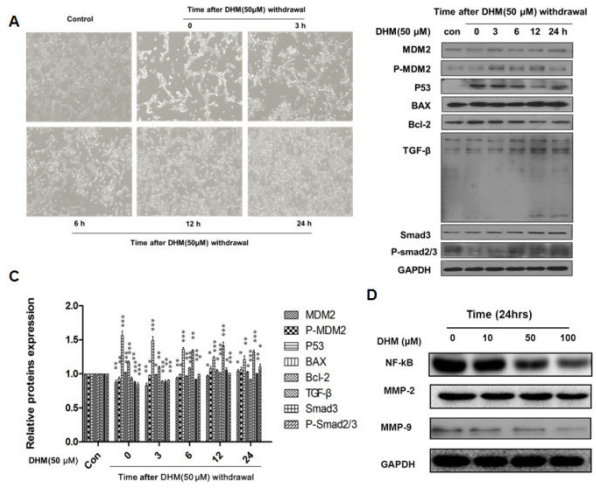

Figure 3: TGF-β signal pathway and apoptosis-related proteins expressions recovered after DHM were withdrawn.

TGF-β and P53 play key roles in DHM-triggered cell apoptosis

DHM treatment leaded HepG2 cell line apoptosis. However, cell apoptosis of cells co-treated with PFT-α and DHM (PFT-α pre-treated cells for 30 min) were alleviated (Figure 4A). The HepG2 cells treated by DHM and PFT-α apoptosis rate was lower than that of cells treated only by DHM which was confirmed by Annexing V/PI assay (Figure 4-C). DHM treatment co-incubated with PFT-α induced a strong P53 down-regulation and down-regulation of TGF-β MDM2 P-MDM2 Bcl-2 SMAD3 and P-SMAD2/3. Moreover, the apoptosis-promoting effect induced by co-treatment with PFT-α and DHM was rather weaker than that of DHM alone treated cells. The BAX expression change was still not obvious (Figure 4B).

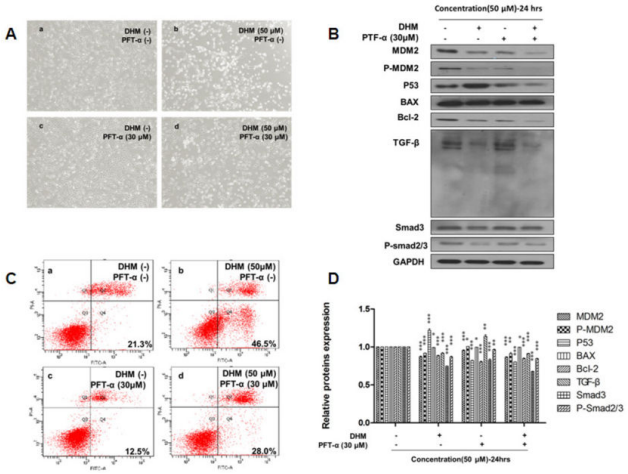

Figure 4: DHM induces apoptosis by P53 activation in HepG2 cells; A: HepG2 cells were co-treated with 50 μM DHM or/and 30 μM PFT-α for 24 h. Cells was exposed as following: a. DHM (-) PFT-α (-); b. DHM (50 μM) PFT-α (-); c. DHM (-) PFT-α (30 μM) d. DHM (50 μM) PFT-α (30 μM); B. The correlation between TGF-β signal pathway and apoptosis-related proteins of HepG2 cells co-treated with 50 μM DHM or/and 30 μM PFT-α for 12hrs were detected with Western blotting assay C. DHM-induced HepG2 cell apoptosis treated with 50 μM DHM or/and 30 μM PFT-α for 12 h which were visualized by flow cytometry; D. Column diagram of B.

Discussion

In the human cancer progression TGF-β signalling pathway proteins show the significant and dual role. During the early stage of tumour progression in the components of the TGF-β signalling pathway TGF-β acts as a tumour suppressor had been exemplified. On the other hand TGF-β also play critical role in promoting tumour progression for example tumour cell invasion and metastasis [14,15]. In our study we verified that TGF-β acts as an important role related in DHM-induced cell apoptosis. The P53 tumour suppressor regulates cell proliferation apoptosis and differentiation [16]. SMAD and P53 protein complexes converge on separate cis binding elements on a target promoter and synergistically activate TGF-β induced transcription. P53 can physically interact *in vivo* with SMAD2 in a TGF-β-dependent fashion. TGF-β resistance was correlated with increased MDM2 expression levels of breast tumour cells. The MDM2 protein is deregulated in many human cancers and exert their oncogenic activity predominantly by inhibiting the P53 tumour suppressor [17]. Thus by interference with the independent tumour suppressors-P53 MDM2 may confer TGF-β resistance in tumours and promote tumour genesis [18]. In our study we evaluated TGF-β signal

pathway major proteins including TGF-β SMAD2 and P-SMAD2/3 in HepG2 cell apoptosis induced by DHM. In addition P53 MDM2 P-MDM2 and Bcl-2 family proteins were also assessed by western blotting. TGF-β pathway and apoptotic proteins altered showing the concentration-time dependent manner. Meanwhile after DHM withdrawal TGF-β signal and apoptosis proteins were recovered according to the treatment. Our data proved that DHM could induce cell apoptosis by inducing P53 protein expression and regulate MDM2 a P53 negative regulator. The cooperation between P53 and TGF-β signalling is regulated by the formation of a P53/SMAD specific interaction [11]. Previous research have shown the possibility that TGF-β resistance involved with MDM2 expression [19,20]. We found DHM could regulate MDM2 and P-MDM2 and then induced P53 protein expression. After PTF-α was added TGF-β signal proteins expression changed according to the treatment which demonstrated that DHM could regulate TGF-β signal proteins through P53. Our study confirmed that TGF-β/P53 pathway may act as new targets which play important roles in DHM-induced cell apoptosis.

DHM treated cells for 6 h and fresh culture medium were replaced. A. Cell growth were observed by microscopy (x100) at 3, 6, 12 and 24 hrs; B.TGF-β pathway and apoptosis-related proteins of cells were checked at 0, 3, 6, 12 and 24 hrs with Western blotting assay after DHM were withdrawn; C. Column diagram of B.

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R.Z.Z. wrote the article. BL. and T.M.L. performed the experiments. J.L. and Q.Y.Z. prepared Figures 1–4. S.T.B. participated in data and statistical analyses. All authors reviewed the final version of the manuscript.

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