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Original Research Article

Emodin induces osteosarcoma cell apoptosis by promoting ATM protein cleavage

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Abstract

Purpose: *To investigate the effect of emodin on osteosarcoma cell proliferation and apoptosis.*
Methods: *The osteosarcoma cell proliferation ability was determined by CCK-8, apoptosis level and cell cycle were determined via flow cytometry. Honechst staining was used to determine the ratio of nuclear pyknosis, Western blotting was used to determine the expression of AKT, p-AKT and caspase-3 precursor protein in each group of osteosarcoma cells.*
Results: *Emodin significantly inhibited the MG-63 cell proliferation and promoted the apoptosis of osteosarcoma cells in a dose-dependent manner ($p < 0.05$). It further blocked osteosarcoma cells in G1/G0 phase and decreased the expression of AKT, p-AKT, and caspase-3 ($p < 0.05$).*
Conclusion: *Emodin inhibits osteosarcoma cell growth by inhibiting ATM protein cleavage. There is a need to carry out further investigation of the effect of emodin on osteosarcoma cells using animal models in order to ascertain its potential in the management of osteosarcoma.*

Keywords: *Emodin, ATM, osteosarcoma, Proliferation, Apoptosis*

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INTRODUCTION

The pathogenesis of osteosarcoma is complex. It originates from normal osteoblasts or osteoblast precursor cells, and the femur is the most common site with a high degree of malignancy and easy metastasis. It is characterized by fusiform mechanism cells that form bone-like tissue [1]. Due to the lack of effective and specific early diagnostic indicators in clinics, most patients have pulmonary metastasis during treatment. The clinical treatment of osteosarcoma is mainly surgical resection combined with adjuvant chemotherapy. However, the long-term survival rate is not significantly improved [2]. Therefore, further studies on the mechanism of osteosarcoma, and exploring new approaches to management are of great significance in reducing the mortality of patients.

Emodin is an anthraquinone derivative isolated from many plants, with anti-inflammatory, antibacterial, and antiviral activities [3]. In recent years, more attention has been paid to the anti-tumor activity of emodin. Related data has shown that emodin has inhibitory effects on lung cancer, gastric cancer, breast cancer, and other tumors. Its mechanism is mainly related to inhibiting

tumor-related angiogenesis and promoting cell apoptosis [4]. The anti-tumor effect of emodin has the features of multiple targets and multiple pathways, and it has a good synergistic effect with antineoplastic drugs, radiotherapy, and chemotherapy.

The ATM protein is a mutant gene of ataxia telangiectasia, which can repair damaged DNA and regulate the growth of cancer cells, but its mechanism in osteosarcoma cells is unclear [5]. Therefore, this study aimed to investigate the effect of emodin on osteosarcoma cells and explore the molecular mechanism.

EXPERIMENTAL

Cell culture

Osteosarcoma cell line MG-63 purchased from Shanghai Huzheng Biotechnology Co., Ltd was cultured on RPMI-1640 medium (Aantpiden, Wuhan, China) containing 10 % fetal bovine serum (Royacel SERUN, Lanzhou, China) serum at 37 °C and 5 % CO_2.

Cell viability assay

Cells were inoculated into 96-well plates at 1000 cells/well. At the beginning of the experiment, 24, 48, and 72 h after seeding, each well was measured with CCK8 kit (Fuyuanbio, Shanghai, China) according to the manufacturer's instructions.

Apoptosis analysis

The Annexin V/PI kit (Fushen, Shanghai, China) method was used to determine apoptosis rate. Annexin V-FITC was mixed with the cells evenly, incubated without light for 10 min, and centrifuged before the addition of Propidium iodide (PI) stain. Cell apoptosis level was analyzed using flow cytometry.

Cell cycle analysis

Cell lines were inoculated at 3×10^5 (MG-63) cells per well on a 6-well plate and incubated overnight in an incubator. Different concentrations of emodin were added as treatment for 24 h, and 3 parallel groups were set up for each concentration. The cells were washed twice with phosphate-buffered saline (PBS) and stored in a refrigerator containing 70 % alcohol at 20 °C for 24 h. Propidium iodide staining (Fushen, Shanghai, China) was done at 4 °C for 15 min, and the cells were analyzed by flow-type inspection.

Hochest staining

Cultured osteosarcoma cells (MG-63) in good growth and logarithmic growth phases were mixed with 0.25 % trypsin to facilitate digestion and prepare cell suspension. After counting, 3×10^4 cells per well was inoculated on a 6-well plate, and then saturated with humidified air for 24 h. Hochest33258 (Sigma) was added to achieve a final concentration of 10 mg/L of the culture medium. The mixture was incubated in the dark for 30 min and the ratio of cell pyknosis was observed under a fluorescent microscope and photographed.

Western blot

Total protein was determined with radioimmunoprecipitation assay (RIPA) and protein concentration was quantified by Bicinchoninic acid (BCA) kit. The protein was separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene fluoride (PVDF) membrane. The sample was sealed with 10 mL 5 % skimmed milk powder at room temperature for 2 h, and then incubated with primary antibodies: AKT (#9272, CST), p-AKT, and caspase-3 (#9662, CST) overnight at 4 °C. Tris-buffered saline with 0.1 % Tween® 20 detergent (TBST) was used to wash off non-specifically bound primary antibodies on membrane.

Secondary antibodies were incubated at room temperature for 1 to 2 h. The TBST was also used to wash off non-specifically bound secondary antibodies on membrane and samples were examined using electro-generated chemiluminescence (ECL) method.

Statistical analysis

Data were analyzed using SPSS 21.0 software, and the measurement data are expressed as mean ± standard deviation (SD). Variance was used to compare data between multiple groups. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of emodin on osteosarcoma cells proliferation

As shown in Figure 1, Emodin significantly inhibited MG-63 cell proliferation in a concentration dependent manner.

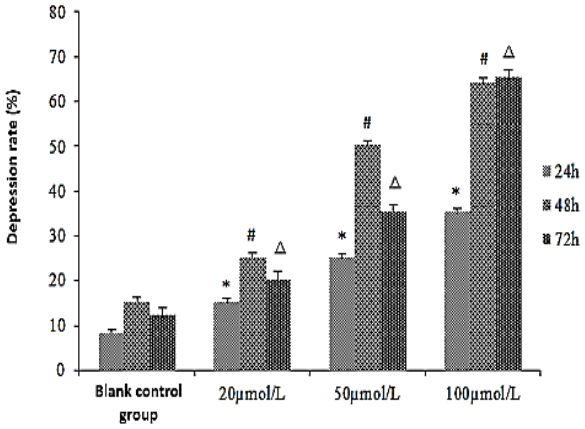

Figure 1: Proliferation activity of osteosarcoma cells MG-63. *P < 0.05, compared with blank control group at 24 h; $^{\#}p$ < 0.05, compared with blank control group at 48 h; $^{\triangle}p$ < 0.05, compared with blank control group at 72 h

Effect of emodin on apoptosis

Figure 2 reveals that emodin promotes apoptosis also in a dose dependent manner with 100 μm/L exhibiting the highest percentage apoptosis rate.

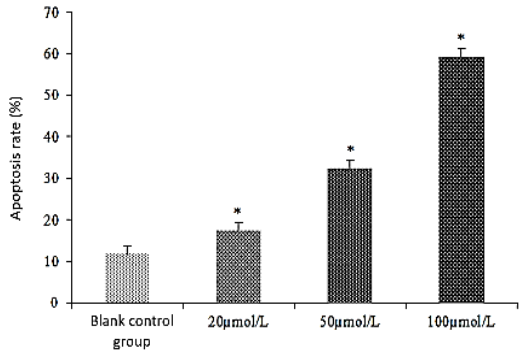

Figure 2: Emodin effect on MG-63 cell apoptosis. *P < 0.05, compared with blank control group

Effect of emodin on cell cycle of osteosarcoma

Cell cycle analysis indicated that emodin significantly inhibits osteosarcoma cells in G1/G0 stage (Table 1).

Table 1: Emodin effect on the cell cycle of osteosarcoma MG-63

Group	G1/G0 stage	S stage	G2 stage
Blank control	29.52±0.38	26.22±0.76	44.01±1.25
Emodin 20μmol/L	38.26±0.96*	47.58±0.19*	14.15±1.06*
Emodin 50μmol/L	43.10±1.28*	51.62±0.71*	8.51±0.38*
Emodin 100μmol/L	50.26±3.15*	54.36±1.58*	3.23±0.41*
F	240.13	882.57	4082.10
P-value	<0.001	<0.001	<0.001

Compared with blank control group at *p < 0.05

Effect of emodin on ratio of nuclear shrinkage of osteosarcoma cells

Hoechst staining showed that the proportion of nuclear pyknosis in emodin treated groups was significantly raised (Figure 3).

Effect of emodin on key proteins in MG-63 cells

Western blot analysis showed that with increase in emodin concentration, the expression levels in AKT, caspase-3, and p-AKT protein in osteosarcoma cells decreased significantly. These results suggest that emodin may accelerate MG-63 apoptosis by inhibiting AKT (Figure 4).

DISCUSSION

Osteosarcoma, has a high rate of metastasis, about 80 % of which metastasized to the lung. The 5-year survival rate of osteosarcoma metastatic patients is still less than 20 % [6]. Furthermore, chemotherapeutic drugs have unpleasant side effects such as gastrointestinal reactions, toxicity of important organs and myelosuppression thereby causing significant harm to the patient's body during treatment. It is therefore important to continue in the effort to develop potent anti-tumor drugs with minimal toxicity.

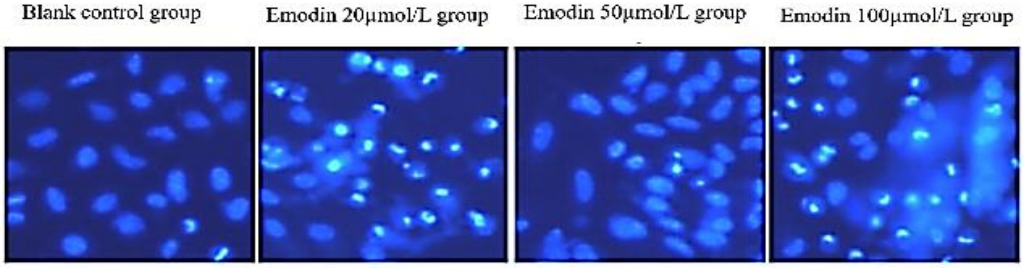

Figure 3: Hoechst staining diagram of ratio of nucleus shrinkage of MG-63 osteosarcoma cells

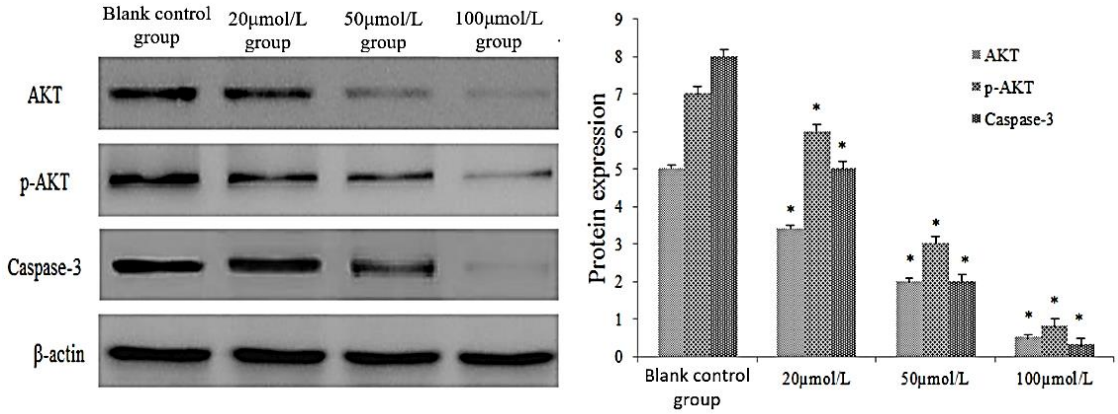

Figure 4: Effect of emodin on key proteins in osteosarcoma cells MG-63

Emodin is the main effective monomer of rhubarb, which is found in rich quantities in some traditional Chinese medicines like *Polygonum cuspidatum* and *Polygonum multiflorum*. It exerts various effects such as antibacterial, antioxidant, hepato and nephron-protective, as well as neuro and myocardial protection. Recent research on emodin at cellular and molecular levels show that it may become an important anti-tumor drug. Its main mechanisms include inhibiting tumor cell adhesion, migration, invasion, and tumor-related blood vessel formation [7].

Inhibiting tumor cell migration and invasion is considered a significant strategy for the prevention and treatment of tumor metastasis. Emodin can inhibit the reduction of adhesion complexes by inhibiting the recruitment of FAK to β1 integrin and the phosphorylation of FAK in many kinds of tumor cells [8]. Relevant data has shown that emodin can significantly inhibit the expression of epidermal growth factor in various cancer cells [9]. The malignant proliferation of osteosarcoma cells involves many regulatory pathways, and emodin inhibits the proliferation of cancer cells by multiple targets. In colon cancer cells [10] emodin can directly inhibit the regulatory molecule, TWIST1 of epithelial-mesenchymal transition, and promote tumor cell apoptosis. Jin and Zhao [11] found that emodin promotes apoptosis of colorectal cancer cells by mitochondrial pathway, release cytochrome C into cytoplasm, down-regulate Bcl-2 and up-regulate Bax, thereby reducing Bcl-2/Bax ratio, and ultimately causing colorectal cancer apoptosis. Studies have also shown that emodin blocks cell cycle progression in S (DNA synthesis) and G2/M phases [12]. This study showed that emodin inhibited the proliferation of osteosarcoma cells and induce cell apoptosis in a concentration dependent manner and duration of action.

Many studies have shown that several caspases in mammalian cells play important roles in regulating cell apoptosis [13,14]. During cell apoptosis, caspase-3 is activated under apoptosis-initiating protein signal. Whether it is receptor-mediated apoptosis characterized by the death of membrane receptor INF family or the mitochondrial pathway, it finally combines in the activation of caspase-3, to induce cell apoptosis. Therefore, the activation of caspase-3 is considered an important indicator of cell apoptosis [15]. ATM protein kinase is mainly located in nucleus of proliferating cells, and regulates cell cycle arrest, DNA repair and cell apoptosis. It may become a new target for the treatment of malignant tumors. As an important part of the mitochondrial apoptosis signal pathway and downstream of AKT, caspase-3 can reflect the severity of apoptosis [16]. Relevant data show that emodin can activate the expression of caspase-3 and 9 by inhibiting the activity of AKT and mitochondrial-dependent apoptosis pathway, in order to promote the apoptosis of cancer cells [17].

In this study, with the increase in emodin concentration, the expression levels in AKT, p-AKT, and caspase-3 protein in osteosarcoma cells decreased significantly. Emodin inhibited the expressions of AKT and p-AKT proteins in osteosarcoma, thereby affecting the activity of caspase-3 and promoting cell apoptosis.

CONCLUSION

Emodin effectively inhibits the proliferation of osteosarcoma cells, induces osteosarcoma cells apoptosis, and inhibits osteosarcoma cells in G1/G0 stage. Thus, it exerts anti-osteosarcoma effect by inhibiting AKT. Further investigation on the effect of emodin on osteosarcoma cells using animal models are required to confirm its potential in the treatment of osteosarcoma.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ran Liao and Jianfeng Cao conceived and designed the study, collected, analyzed and interpreted the experimental data, drafted the manuscript and revised the manuscript for important intellectual content. Both authors read and approved the final manuscript.

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