Int J Clin Exp Med 2016;9(11):21327-21333
www.ijcem.com /ISSN:1940-5901/IJCEM0036068

Original Article

Inhibition of emodin on growth of human bladder cancer cell BIU87 xenograft in nude mice

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Received July 18, 2016; Accepted September 10, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Objective: This study aims to investigate the inhibiting effects of emodin on the growth of human bladder cancer cell BIU87 and nude mice with transplanted tumor as well as its mechanism. Methods: The human bladder cancer model was established, which was cell BIU87 xenograft nude mice model. The nude mice were divided into 4 groups, including control group, emodin group, Z-VAD-FMK group and emodin + Z-VAD-FMK group. We measured the tumor diameter and its growth curve. 4 weeks later, all the mice were sacrificed and the tumor tissue was weighted. The apoptosis of the tumor cells was detected by transferase-mediated nick end labeling (TUNEL) method. Immunohistochemistry (IHC) was used to detect NF-Kβ and XIAP expression. RT-PCR was conducted to measure NF-Kβ and XIAP mRNA expression. Western blot (WB) was used to analyze the expression of NF-Kβ and XIAP at the protein level. Results: The tumor was (0.41±0.05) g in emodin group, which was lighter than emodin + Z-VAD-FMK (0.69±0.07) g, Z-VAD-FMK (1.04±0.09) g and control (1.08±0.13) g groups, significantly (F=90.56/27.49, P<0.01). The cell apoptosis level was higher in emodin group than the other three groups. The results of IHC, RT-PCR and WB showed that emodin could down-regulate the expressions of NF-Kβ and XIAP. Conclusions: Emodin could significantly inhibit the in vitro and in vivo growth of human bladder cancer cell BIU87, probably through down-regulating NF-Kβ and XIAP to induce cell apoptosis.

Keywords: Emodin, bladder cancer, nuclear factor, apoptosis inhibitor, apoptosis

Introduction

Bladder cancer is common in urogenital system. The incidence was 7.49/10^4 in China, which was 2.5% of all malignant tumors [1]. Transitional-cell carcinoma was the main pathologic types in bladder cancer, and with the characteristics of high recurrence rate, metastatic rate and effective prevention for tumor in I or II types, which made it the ideal model in chemical prevention and intervention [2]. However, the effect of chemotherapy alone was poor for the tumor in advanced stages. Some of the traditional Chinese medicines have the anti-tumor effects. Emodin, which was the effect component in rhubarb, polygonum cuspidate, polygonum multiflorum Thunb and so on, had the effects of antibacterial, antivirus, immunosuppression, protecting liver and so on [3].

Recently, many researches showed that emodin had functions of inhibiting tumor growth, promoting tumor apoptosis, inhibiting tumor invasion and migration and so on [4, 5].

In our study, we established the model of human bladder cancer cell BIU87 xenograft in nude mice, and investigated the effects of emodin on bladder tumor growth and its mechanisms to provide clinically anti-tumor therapy with the experimental basis.

Material and methods

Animals

35 SPF BALB/c female nude mice weighted 20-22 g were obtained from experimental animal center of Hubei Province (SCXK (Hubei province) 2003-0005), and raised in experimental animal center of Huazhong University of Science and Technology. Human bladder cancer cell BIU87 was obtained from Institute of Urology Peking University. Emodin, mass fraction >98%, PBS and RPMI-1640 medium

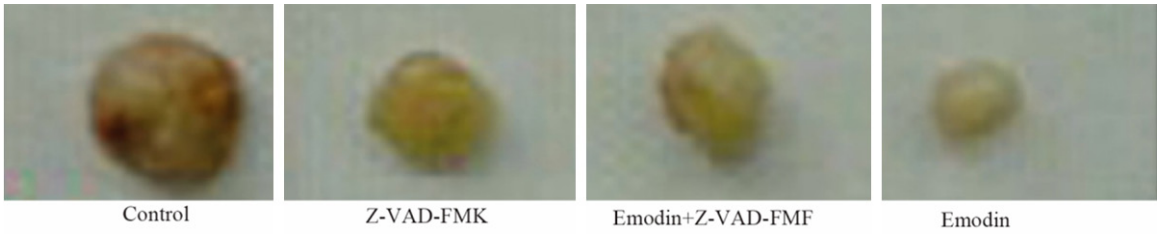

Figure 1. Inhibition of emodin on bladder cancer xenograft.

Table 1. Inhibition of emodin on bladder cancer xenograft

Groups	Cases	Tumor size before reatments (mm^3)	Tumor weight after treatments (g)	Inhibition rate (%)
Control	8	55.36±18.73	1.08±0.13	-
Z-VAD-FMK	8	57.21±15.14	0.854±0.09	20.7
Emodin + Z-VAD-FMK	8	55.09±16.23	0.69±0.07	36.11
Emodin	8	56.98±14.77	0.41±0.05	62.04
F		3.88	2.88	
P		0.016	0.027	

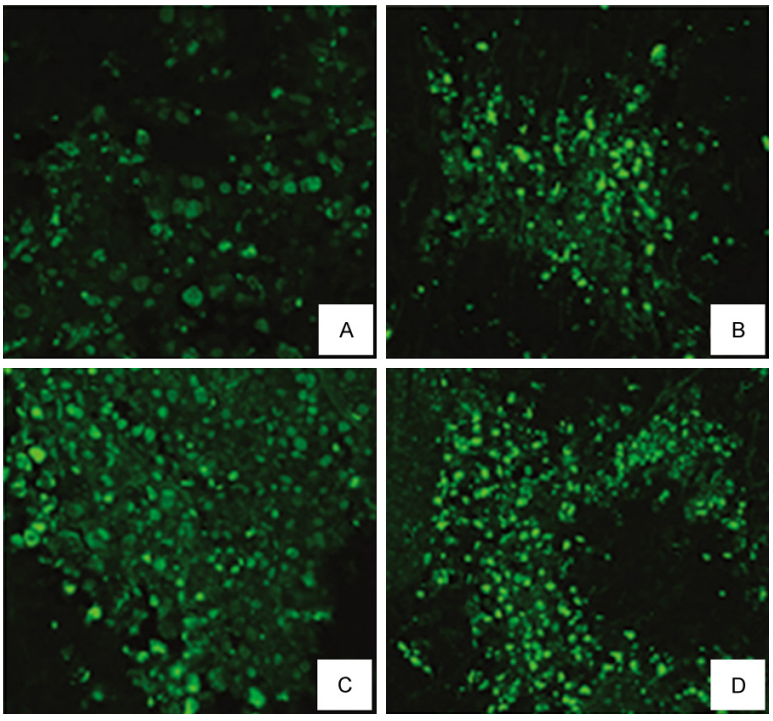

Figure 2. Bladder cancer cell apoptosis by TUNEL (yellow-green fluorescence showing the positive cells). A: Control group, B: Z-VAD-FMK group, C: Emodin + Z-VAD-FMK group, D: Emodin group.

were obtained from Sigma-Aldrich Inc. (USA). ECL agent, TaqDNA polymerase, Caspase inhibitor (Z-VAD-FMK) and Annexin v/FITC kit were obtained from Wuhan Good-Time Biotechnology Co., Ltd. (Wuhan, China). Trizol was obtained from Thermo Fisher Scientific Inc. (Shanghai, China). Reverse transcription kit was obtained from Fermentas (Thermo). Rabbit anti-NF-Kβ and rabbit anti-XIAP primary antibodies, goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from Sant Cruz Biotechnology, Inc. (Shanghai, China). Gel image system (1550IW) was obtained from Leica Microsystems (Germany). Microplate reader was obtained from Stratagene, Agilent Technologies (USA). Flow cytometry (FACSC alibur) was obtained from Becton Dickinson (USA).

Methods

Cell culture and xenograft tumor: BIU87 cells were cultured in RPMI-1640 containing 10% FBS, 100 U/ml ampicillin and 100 μg/ml streptomycin, placed in the conditions of 95% humidity, 37°C and 5% CO_2. BIU87 cells were with passage culture, and those in logarithmic growth phase were used for experiments. Adjusted the cell density to 2×10^7/ml. 0.2 ml of the cell suspension were injected subcutaneously in each mouse. The obvious tumor was observed at the 4th week with size of 0.4~0.5 cm. 32 mice were successfully established as the model.

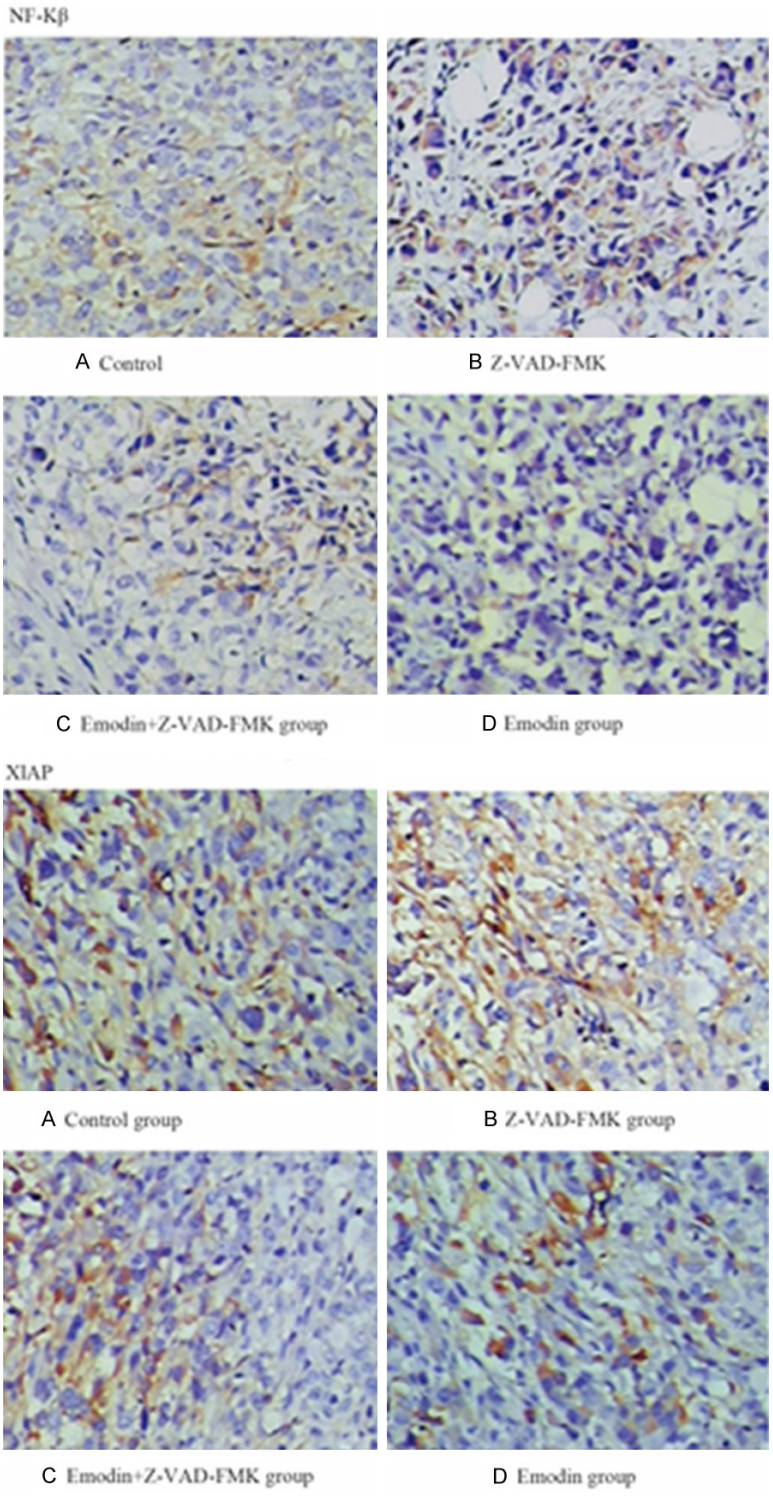

Figure 3. RT-PCR detecting NF-xB and XIAP mRNA expressions (claybank granules were positive for protein expression).

Groups and treatments: 32 mice were divided into 4 groups randomly, 8 in each. ① control group: intraperitoneal injecting with 0.2 ml saline; ② emodin group: intraperitoneal injecting with emodin (50 mg/kg/d) in 0.2 ml saline; ③ Z-VAD-FMK group: intraperitoneal injecting with Z-VAD-FMK (6 mg/kg/d) in 0.2 ml saline; ④ emodin + Z-VAD-FMK group: intraperitoneal injecting with emodin (50 mg/kg/d) and Z-VAD-FMK (6 mg/kg/d) in 0.2 ml saline. Mice were injected with drugs once a day at a period of 4 weeks.

Tumor size and inhibition rate detection: Tumor size was measured and obtaining the maximun diameter (a) and transverse diameter (b). The tumor size (volume) was calculated using formula of $(a \times b^2)/2$, and then drawing the tumor growth curve. After sacrifice, the tumors were collected and weighted. Inhibition rate = (1-average weight in treating groups/average weight in control group) × 100%.

TUNEL was used to detect tumor cell apoptosis: According to the specification of TUNEL kit, TUNEL agent was added and incubated at 37°C for 1 h. Seal the slides and observed under laser scanning confocal microscope (LSCM) (400 ×). 10 fields of each slide were selected. The blank slides without samples were taken as control. Detecting NF-Kβ and XIAP expressions by IHC: Slides were obtained from each sample and carried on embedding and dewaxing. The slides were dewaxed with xylene and hydrated with gradual ethanol, then sealed with 0.3% H_2O_2. After antigen retrieval with microwave, the slides were blocked with 10% goat serum, then incubated with rabbit anti-NF-Kβ/XIAP antibody (1:5000) at 4°C, overnight. Washed with PBS and added secondary antibodies (1:500) labeled by HRP at 37°C for 0.5 h. Stained with DAB for 3~5 min. Washed

Table 2. IHC detecting NF-xB and XIAP expressions in bladder cancer tissue

Groups	NF-Kβ	XIAP
Control	0.55±0.065	55.36±18.73
Z-VAD-FMK	0.62±0.035	57.21±15.14
Emodin + Z-VAD-FMK	0.41±0.081*	55.09±16.23
Emodin	0.39±0.11#,*	56.98±14.77
F	5.21	7.73
P	0.029	0.018

Note: *comparing to the control, P<0.01; #comparing to Z-VAD-FMK and emodin + Z-VAD-FMK groups, P<0.05.

and stained with hematoxylin. Washed and dehydrated with gradual ethanol. Sealed the slides and observed.

Detecting NF-Kβ and XIAP mRNA expressions by RT-PCR: 50 mg of tissues were obtained and treated by Trizol. Total RNA was obtained and carried on reverse transcription. cDNA was obtained and carried on PCR. The primers were synthesized according to previous references [6] by Invitrogen (Thermo). PCR products were analyzed by 1.5% agarose gel and observed under ultraviolet.

Detecting NF-Kβ and XIAP protein expressions by Western blot: 50 mg of tissues were obtained and lysis buffer were added at a rate of 5 times. The tissue homogenate was centrifuged at 12,000 × g, 4°C for 15 min. The supernatant was obtained and the total protein concentration was detected by Bradford method. The protein sample was analyzed with 8% separating gel by SDS-PAGE. The loading volume was 30 μg. After SDS-PAGE, the proteins were transferred into PVDF membrane, then blocked with 5% nonfat milk at 25°C for 2 h. Added rabbit anti-AIF (1:500), rabbit anti-Endo G (1:200) and rabbit anti-β-actin (1:200) polyclonal antibodies and incubated at 25°C for 1 h. ECL system was for coloring.

Images analysis

The integral absorbance (IA) of the mRNA or protein bands were analyzed by image analyzer according to IA = average absorbance × area.

Statistical analysis

All the data were analy-zed by SPSS 11.5 software. The data were showed as mean ± standard deviations (X±S). The comparisons between were analyzed by chi-square. P<0.05 was considered as statistical significance.

Results

Inhibition of emodin on bladder cancer xenograft

There was no significant difference in tumor size among groups before treatments (F=0.15, P>0.05), indicating the comparability of tumor size in each group. The tumor growth was slower in emodin group than other 3 (**Figure 1**). After treatments, the tumor were lighter in emodin and emodin + Z-VAD-FMK groups, significantly (F=90.56/27.49, P<0.01). There were significant differences in tumor size between emodin and emodin + Z-VAD-FMK group (t=10.01, P<0.01), which indicated that emodin might inhibit tumor growth by non-Caspase apoptosis pathway (**Table 1**).

Emodin inducing tumor apoptosis

The nucleus in the positive cell by TUNEL method was yellow-green fluorescence. By comparing the IA between groups, the IA was higher in emodin group than other 3 groups, signifcantly (P<0.05) (**Figure 2**).

IHC results of NF-Kβ and XIAP expressions

IPP software was used to analyze the IHC images, and the NF-Kβ and XIAP expression results were showed as average optical density (OD) value (**Figure 3** and **Table 2**).

RT-PCR detecting NF-Kβ and XIAP mRNA expressions

According to **Figure 4** and **Table 3**, NF-Kβ mRNA expression (0.29±0.10) was higher in emodin group than emodin + Z-VAD-FMK (0.3±0.08), Z-VAD-FMK (1.21±0.41) and control (1) groups, significantly (F=303.22, P<0.01). XIAP mRNA expression (0.36±0.12) was higher in emodin group than emodin + Z-VAD-FMK (0.48±0.11), Z-VAD-FMK (1.36±0.23) and control (1) groups, significantly (F=319.32, P<0.01).

WB results of NF-Kβ and XIAP protein expressions in bladder cancer tissue

According to **Figure 5**, NF-Kβ protein expression (2.42±0.13) was higher in emodin group than emodin + Z-VAD-FMK (1.73±0.11), Z-VAD-FMK (0.75±0.08) and control (0.78±0.07) groups, significantly (F=409.38, P<0.01). XIAP mRNA expression (3.13±0.25) was higher in emodin group than emodin + Z-VAD-FMK

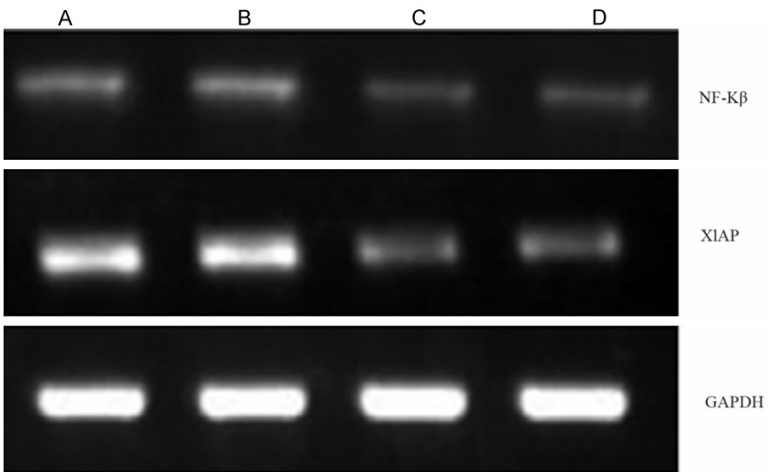

Figure 4. RT-PCR detecting NF-Kβ and XIAP mRNA expressions in bladder cancer tissue (A: Control group, B: Z-VAD-FMK group, C: Emodin + Z-VAD-FMK group, D: Emodin group).

Table 3. RT-PCR detecting NF-Kβ and XIAP mRNA expressions in bladder cancer tissue (x±s)

Groups	NF-Kβ	XIAP
Control	1	1
Z-VAD-FMK	1.21±0.41	1.36±0.23
Emodin + Z-VAD-FMK	0.3±0.08*	0.48±0.11*
Emodin	0.29±0.10*	0.36±0.12*
F	9.24	3.08
P	0.006	0.0046

Note: *comparing to the control, P<0.01.

(2.15±0.18), Z-VAD-FMK (0.81±0.14) and control (0.85±0.09) groups, significantly (F= 258.53, P<0.01).

Discussion

In many mechanisms of tumor pathogenesis, abnormal apoptosis was critical in the cancer generation and development. A lot of anti-tumor drugs could induce tumor cell apoptosis by activating on apoptosis pathway at different positions [7], which provided the treatments with the new pathway for bladder cancer. Apoptosis is the cell death, which participates in many pathophysiological processes. In human body, one of the signal pathways, with high conservation and relation to apoptosis, was the endogenous death receptor pathway, and the other was exogenous mitochondrial pathway. Caspase family played an important role in two pathways, as well as in the apoptosis [8].

One of the important characteristics of tumor was the tolerance to the inducer in human body, which induced apoptosis in sensitive cells. This phenomenon led to the inhibition on cell apoptosis and infinite proliferation. Cascade reaction was the critical step in cell apoptosis, which was induced by Caspase. Following with its activation by death receptor and mitochondrial pathways, the Caspases in the downstream resulted in cell apoptosis by cutting special substrate [9]. Therefore, Caspases were considered as the main factors mediating cell apoptosis. However, in many mammal apoptosis models induced by stress, the inhibition on Caspases could not prevent from cell apoptosis totally [10], which indicated that there was another apoptosis pathway independent of Caspases signal pathway.

NF-Kβ was the common nuclear transcription factor, which was related to immunity, inflammation, cell growth, proliferation, differentiation and so on. More and more researches showed that NF-Kβ played a critical role in transformation and proliferation of malignant tumor cells [11]. XIAP was the inhibitor of apoptosis protein, which could inhibit Caspase family activity appearing as anti-tumor. Previous, XIAP over-expression was one main reason in the poor chemotherapeutic effect [7].

XIAP could influence Caspase-3 expression to antagonize cell apoptosis as the most powerful Caspase inhibitor [12], and effect on GIST pathogenesis and development. XIAP, also named as BIRC4 (baculoviral IAP repeat containing 4), was with two specially structural domains including Baculovirus IAP repeats (BIR) at N-terminal, such as BIR1, BIR2 and BIR3, and the annular zinc finger domain at C-terminal, and translated by 4 open reading frames [13]. XIAP could activate NF-κB, while NF-κb could up-regulate XIAP, which suggested that XIAP was positive related to NF-κB (r= 0.289, P<0.05). The mechanism might be that

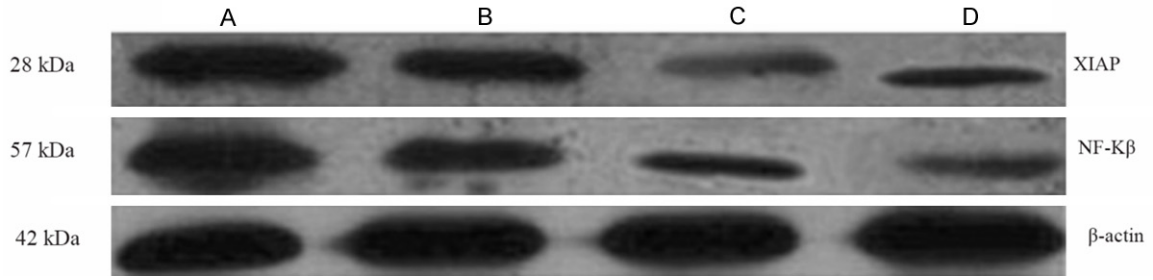

Figure 5. NF-Kβ and XIAP protein expressions in bladder cancer tissue (A: Control group, B: Z-VAD-FMK group, C: Emodin + Z-VAD-FMK group, D: Emodin group).

XIAP combined with TAK1 and TAB1 as the complex, and then the complex activated JNK1; further, JNK1 could activate NF-κB [14]. p65 was the subunit of NF-κB and transferred into nucleus, which was promoted by XIAP [15]. Additionally, XIAP could promote the degradation of NF-κB, as the inhibitor of NF-κB, which resulted in the inhibition on NF-κB [16]. XIAP was the activator of NF-κB, and it could promote NF-κB transferring into nucleus, leading to the expression of the genes related to inhibiting cell apoptosis by NF-κB [17].

In our study, emodin had obvious anti-tumor functions, and it could inhibit tumor growth, induce tumor apoptosis, significantly. In emodin group, the tumor cell apoptosis was higher than other groups, and the NF-Kβ and XIAP expressions decreased, which could up-regulate Caspase-3 expression for anti-tumor. Otherwise, the anti-tumor function of emodin was still obvious in Caspase-dependent apoptosis pathway after adding Z-VAD-FMK that was the inhibitor of Caspase, which indicated that emodin could induce bladder tumor cell apoptosis by non-Caspase pathway.

In conclusions, emodin could inhibit the growth of human bladder cancer cell BIU87 and transplanted tumor in nude mice, and the mechanisms might be related to down-regulating NF-Kβ and XIAP expressions, as well as activating Caspase-dependent apoptosis pathway. In the same time, we found that emodin was also with important anti-tumor function in non-Caspase pathway. However, the mechanisms were needed further investigations.

Disclosure of conflict of interest

None.

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