



Emodin inhibits the proliferation and migration of B16F10 cells and induces their apoptosis

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Background: Emodin, extracted from the rhizomes of various Chinese herbs, is a natural anthraquinone derivative with the formula C₁₅H₁₀O₅. Many recent studies have shown that emodin has an antitumour effect. In this study, emodin was investigated in vitro to observe its effect on the proliferation, migration, and apoptosis of the B16F10 melanoma cell line.

Methods: B16F10 cells were treated with 20, 40, 60, or 80 μM emodin for 24 h. A Cell Counting Kit-8 (CCK-8) was used to measure the effect of emodin on cell proliferation. After 24 h of emodin treatment, a scratch test was used to detect the wound healing rate of each group. A Transwell test was used to measure the effect of emodin on cell migration ability. The apoptosis rate of the B16F10 cells was determined by a TUNEL assay. The expression of caspase-3 was measured by western blot analysis.

Results: Compared with the control group, emodin significantly inhibited the proliferation and migration of the B16F10 cells in a concentration-dependent manner. Emodin also inhibited the migration of the B16F10 cells and induced their apoptosis.

Conclusions: Emodin can effectively suppress the viability and migration of B16F10 cells and may induce apoptosis through the mitochondrial pathway or death receptor-mediated pathway.

Keywords: Emodin; B16F10; proliferation; migration; apoptosis

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Introduction

Cutaneous melanoma (CM) is a skin tumour derived from abnormal melanocytes that is prone to migrate in the early stage. It accounts for approximately 75% of skin cancer-related deaths (1). Recently, the incidence of CM is one of the most rapidly increasing among cancers worldwide. The increased incidence varies among populations, ranging from 3% to 7% (2). Studies from Europe and the United States reports increases in the incidence of CM (3,4).

The mortality of melanoma has also increased in men

older than 65 years. This finding may be attributed to their unique biology and/or an altered host immune response (5). Previously, treatment options were limited by modest response rates in patients with advanced-stage melanoma. Despite targeted therapy and immunotherapy raising the response rate in recent years, the long-term survival rate remains low. Therefore, the discovery of efficient therapeutics will be key for treating CM to improve overall survival.

Emodin, extracted from the rhizomes of various Chinese herbal medicines, such as rhubarb, rheum and

polygonum, is a natural anthraquinone derivative with the formula $C_{15}H_{10}O_5$. The chemical name of emodin is 1,3,8-trihydroxyanthraquinone. It forms an orange-yellow long needle-like crystal. In recent years, many studies have confirmed that emodin has many biological effects, such as anti-inflammatory, antibacterial, and immune regulation effects (6-8). With further research on its antitumour activity, emodin was found to inhibit lung cancer, breast cancer and other tumours by inhibiting tumour cell proliferation and promoting apoptosis (9,10).

This study mainly investigated the effect of emodin on the proliferation, migration, and apoptosis of the B16F10 mouse melanoma cell line. The goal was to further understand the mechanism of apoptosis and provide experimental evidence for the treatment of melanoma with emodin.

Methods

Materials

Emodin and dimethyl sulfoxide (DMSO) were purchased from Sigma. DMEM, foetal bovine serum, a CCK-8 kit, and 0.25% EDTA-trypsin were purchased from Gibco (Thermo Fisher Scientific, Inc). A TUNEL kit and DAPI staining kit were purchased from Nanjing Kaiji Biotechnology Co., Ltd.; a penicillin and streptomycin solution were purchased from United Bioresearch, Inc. Six-well cell plates and 96-well cell plates were purchased from Shanghai Jiayuan Biotech Co., Ltd. The polyvinylidene fluoride (PVDF) membranes were purchased from Beijing Solarbio Science & Technology Co., Ltd. The antibodies against β -actin (rabbit anti-mouse RRID: AB_2242334) and caspase-3 (RRID: AB_331439) were purchased from Cell Signalling Technology, Inc. The secondary fluorescence antibody (polyclonal goat anti-rabbit RRID: AB_2801422) was purchased from Yeasen Biotech Co., Ltd.

Cell line and cell culture

Mouse melanoma B16F10 cells (RRID: CVCL_0159) were purchased from the Cell Bank of the Chinese Academy of Sciences. B16F10 cells were grown in DMEM (pH=7.4) with 10% foetal bovine serum and penicillin (final concentration, 100 U/L) and streptomycin (final concentration, 100 mg/L). The cells were cultured in an incubator with 95% air and 5% CO_2 with saturated humidity at 37 °C. The cells in the logarithmic growth phase were selected for experiments and digested with

EDTA-trypsin when their confluence reached 80–90%. The cells were seeded into six 96-well plates. The medium was changed every 2 days.

Preparation of emodin

Emodin was dissolved in DMSO at a concentration of 0.2 mmol/L as a stock solution and stored in a refrigerator at –20 °C. The stock solution was diluted to a desired concentration in DMEM medium at the time of use. The mass fraction of DMSO in the emodin solution was less than 0.1%.

Cell proliferation assay

The cells in the logarithmic growth phase were washed twice with PBS, and 0.25% trypsin was used to detach the cells at room temperature, and the cells was transferred to a centrifuge tube. After resuspending the cells, the cells were counted by a haemocytometer under the microscope and seeded into a 96-well plate at 10^4 cells/well. The seeded cells were cultured for 24 h. The 0.1% DMSO group was simultaneously prepared as a control, and the experimental group was treated with 20, 40, 60, or 80 μ M emodin for 24 h. The blank zero group was treated with equal amounts of PBS. After adding 10 μ L of CCK-8 reagent to each well, the cells were incubated for 4 h in an incubator. The absorbance was measured at a wavelength of 450 nm, and the viability of the B16F10 cells in different concentrations of emodin was calculated.

Cell scratch test

The cells in the logarithmic growth phase were added to 6-well plates. Three parallel lines were drawn as a marker underneath the 6-well plate. When the confluence reached 100%, a 100 μ L pipette tip was used to place a scratch vertically and slowly into the 6-well plate. The wells were washed with PBS three times, and serum-free medium with 20, 40, 60, and 80 μ M emodin was added. Serum-free (0.1% DMSO) medium was simultaneously added to a well as the control. The cells were observed under an inverted microscope at 0 h and 24 h, and ImageJ (RRID:SCR_003070) was used to calculate the migration rate.

Transwell test

Cells in the logarithmic growth phase were added to the

upper chamber at a density of 2×10^5 /mL (150 μ L) and treated with 20, 40, 60, or 80 μ M emodin in serum-free medium for 24 h. The control group was treated with 0.1% DMSO. Then, 800 μ L of medium containing 10% foetal bovine serum was added to a 24-well plate placed in the lower chamber. The 24-well plate was placed in an incubator for 24 h. The upper chamber liquid was blotted and carefully wiped with a cotton swab after 24 h. The cells on the surface of the upper chamber were removed, and the bottom surface was fixed with 4% paraformaldehyde and stained with crystal violet for 20 min. The number of migrating cells was counted in different fields of view under a microscope. Five visual fields were randomly selected for statistical analysis, and the differences between the groups were statistically analysed.

TUNEL assay

Cell slides were washed with deionized water and sterilized for use. B16F10 cells were seeded onto 6-well plates at a density of 2×10^5 /well and cultured overnight. After the cells were attached, emodin (60 μ M) was added, and incubation was continued for 24 h. The cells attached to the cell slides were naturally dried and fixed with 4% paraformaldehyde for 30 min and washed with PBS 3 times. The TUNEL reaction solution was prepared according to the instructions, and the whole process was performed on ice. The reaction solution was added dropwise to each cell slide to allow the reaction solution to completely cover the cell area. The reaction was incubated at 37 °C for 1 h in the dark and washed 3 times with PBS for 5 min each time. The DAPI solution was diluted to 1 mg/L and then added dropwise to the cell slide. The cells were then incubated at 37 °C for 15 min in the dark and then washed 3 times with PBS for 5 min each time. The image was observed under an inverted fluorescence microscope after a quenching agent was added, and the surrounding area was devoid of cells. Three fields of view were randomly selected and photographed under a laser confocal microscope.

Western blot analysis

Proteins were collected and separated through SDS-polyacrylamide gel electrophoresis from cells that had reached 80–90% confluence and treated with 20, 40 and 60 μ M emodin for 24 h. The control group was treated identically. The separated proteins were transferred to a

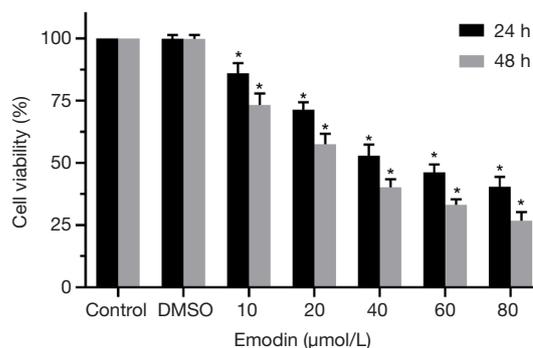


Figure 1 Effect of emodin on the proliferation of the B16F10 cells. There was no significant difference in the proliferation rate between the 0.1% DMSO group and the control group. Cells treated with different concentrations of emodin showed an inhibitory effect on the proliferation of the B16F10 cells compared with the control group (*, $P < 0.05$).

PVDF membrane with the application of 90 V and 200 mA for 60 min. Next, PVDF membranes were incubated overnight in a diluted antibody solution (rabbit anti-mouse caspase-3 and anti- β -actin; 1:3,000) at 4 °C. The blots were then incubated with the secondary antibody (1:3,000) for 1 h at 37 °C. The results were detected using an Odyssey infrared imaging system.

Statistics

After all the experiments were repeated 3 times, the measurement data were expressed as the means \pm standard deviation ($X \pm SD$), and the data were processed by SPSS 19.0 statistical software (RRID:SCR_002865). The two groups were compared by *t*-test, and $P < 0.05$ was used to indicate a statistically significant difference.

Results

Effect of emodin on the proliferation of the B16F10 cells

B16F10 cells were treated with different concentrations of emodin (20, 40, 60, 80 μ M) for 24 h and 48 h, and the viability of the B16F10 cells showed a decreasing trend. There was a significant difference compared with the control group ($P < 0.05$) (Figure 1). As the emodin concentration increased, the cell viability rate decreased, showing that emodin inhibited the proliferation of B16F10 cells in a concentration-dependent manner. Changes in

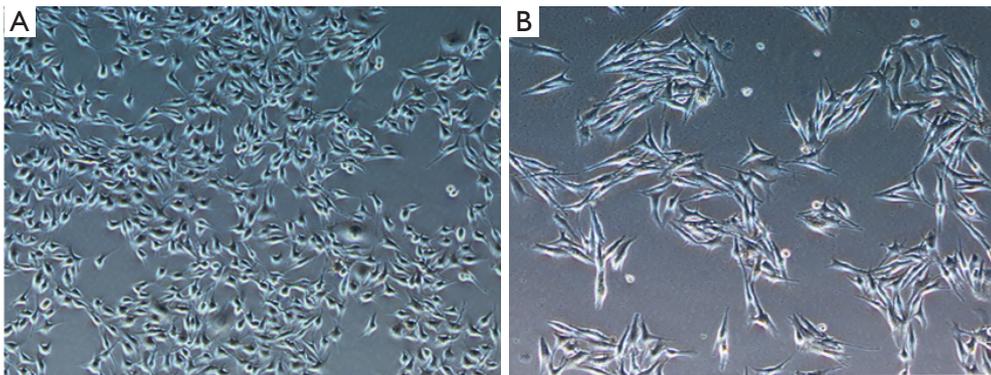


Figure 2 Changes in the morphology of the B16F10 cells treated with 60 μM emodin for 24 h (inverted microscope, $\times 100$). (A) B16F10 cells in the control group grew efficiently and showed a fusiform shape. (B) After treatment with 60 μM emodin, the number of B16F10 cells decreased, and the morphology of the cells began to show reticular dendritic structures. Some cell volumes decreased, and the shape became round.

the cell morphology of the B16F10 cells treated with 60 μM emodin for 24 h were observed under an inverted microscope (Figure 2). After treatment with 60 μM emodin, the number of B16F10 cells decreased, and the morphology of the cells began to show reticular dendritic structures. Some cell volumes decreased, and the shape became round.

Effect of emodin on the migration of the B16F10 cells

After treatment of the B16F10 cells with gradient concentrations of emodin (20, 40, 60, and 80 μM) for 24 h, cell migration changes were observed under an inverted microscope (Figure 3A). B16F10 cells were treated with the same gradient concentration of emodin solution for 24 h in the Transwell test. The number of cells migrating to the bottom surface of the membrane decreased in a concentration-dependent manner (Figure 3B). The migration ratio of the cells the experimental group was significantly reduced compared with that of the cells in the control group ($P < 0.05$) (Figure 4A). The number of cells migrating to the bottom surface of the membrane was significantly different from that of the control group ($P < 0.05$) (Figure 4B).

Effect of emodin on the apoptosis of the B16F10 cells

Compared with those of the control group, the B16F10 cells treated with emodin at a concentration of 60 μM for 24 h showed an increased number of fluorescently stained cells (Figure 5). Caspase-3 has an important impact on

apoptosis. In our study, caspase-3 was detected and analysed by western blotting, which indicated that the expression levels of caspase-3 had increased in cells treated with 40 μM and 60 μM emodin (Figure 6). These results confirmed that emodin may promote B16F10 cell apoptosis.

Discussion

The incidence of CM has been one of the most rapidly increasing among cancers worldwide. The proliferation and invasive ability of CM are the main factors leading to early lymphatic and distant metastasis. The poor chemotherapy effect on melanoma has led to a 5-year survival rate of patients with advanced melanoma that is still low (11). It is gratifying that with further research of melanoma performed in recent years, some advancements in targeted therapy and immunotherapy have been achieved (12,13). Recently, several studies have shown that emodin can inhibit tumour growth by inhibiting tumour cell proliferation and inducing apoptosis (14-16). Emodin can also synergize with various antitumour drugs to reduce tumour resistance (17). Aloe-emodin, reported as a potential anticancer agent, has a molecular structure similar to that of emodin. Aloe-emodin can reduce tumour cell viability in a dose-dependent manner and inhibit the metastasis of tumours formed by B16F10 cells (18). The antitumour effects of emodin may similar to drugs with a similar anthraquinone structure (Figure 7). Additionally, emodin can upregulate the expression of caspase-3 to induce the apoptosis of HeLa cells via the mitochondrial pathway

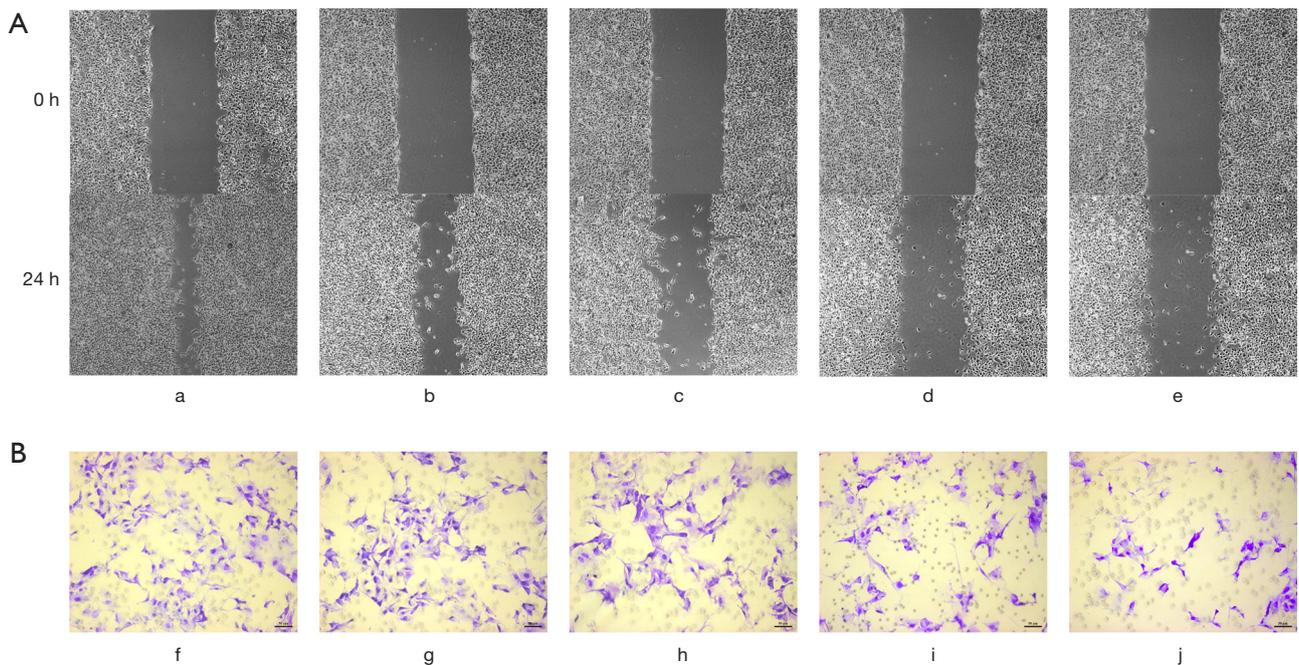


Figure 3 Scratch test and Transwell test of the B16F10 cells treated with different concentrations of emodin for 24 h. (A) Cell scratch test. Panel (a) shows the control group (0.1% DMSO). Panel (b,c,d,e) the 20, 40, 60 and 80 μM emodin groups, respectively. The cells on both sides of the scratches were uniform at 0 h. After treatment with emodin for 24 h, the scratch wounds in the control group monolayer had obviously healed. The scratch wounds in the cells of the 20 μM emodin group also had obviously healed. The scratch wound healing in the 40 μM emodin group was less obvious. The scratch wound healing in the 60 μM emodin group showed mild healing. Scratch wound healing in the 80 μM emodin group was not obvious (inverted microscope, $\times 40$).

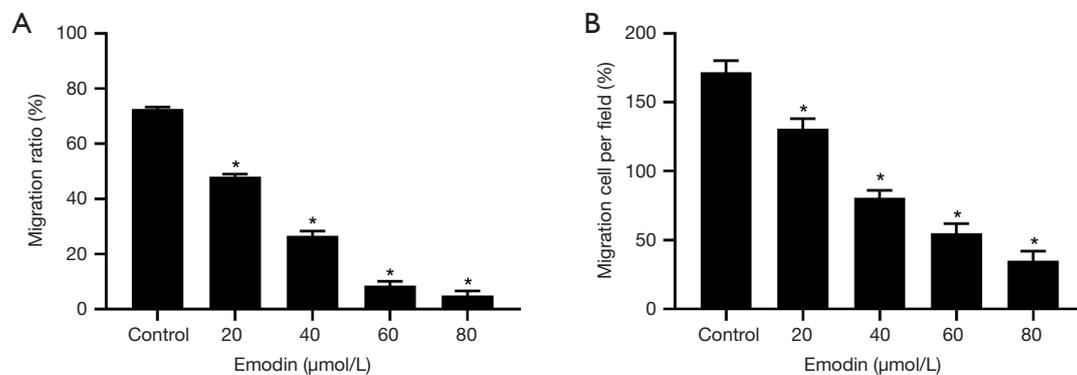


Figure 4 The migration ratio of cell scratch test and migration cells per field in the Transwell assay. (A) After 24 h, the migration ratio of the cells in the 20, 40, 60 and 80 μM emodin treatment groups was significantly reduced compared with that of the cells in the control group (*, $P < 0.05$). (B) After 24 h, the number of cells that migrated to the bottom of the membrane in the 20, 40, 60 and 80 μM emodin treatment groups was significantly reduced compared with that of the control group (*, $P < 0.05$).

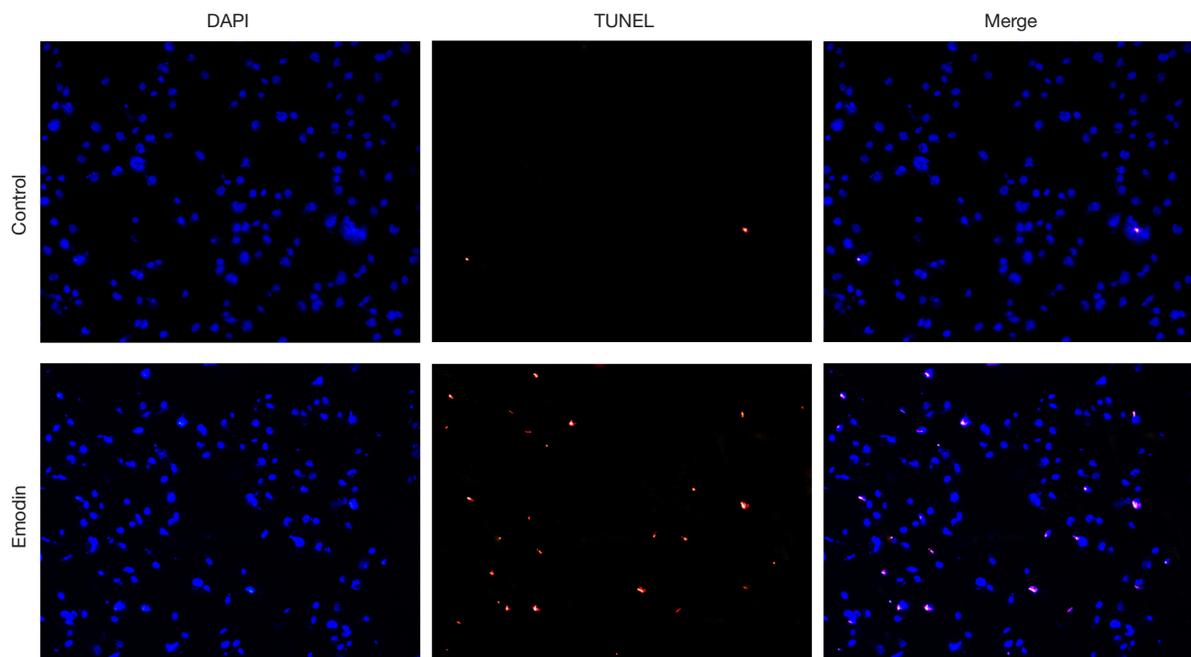


Figure 5 Fluorescence staining of B16F10 cells treated with 60 μ M emodin for 24 h (fluorescence confocal imaging microscope, $\times 100$). Compared with that of the control group, the number of fluorescently stained B16F10 cells was increased after treatment with emodin at a concentration of 60 μ M for 24 h.

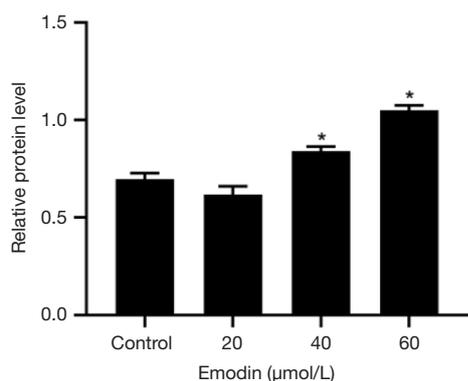
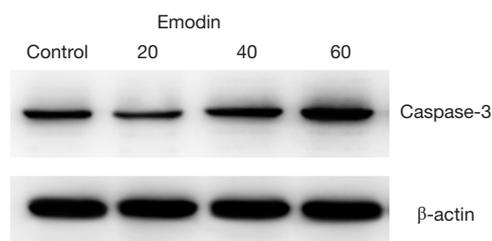


Figure 6 Western blot analysis of caspase-3. Compared with the control group, the 20 μ M group showed no significant difference in caspase-3 levels. The 40 and 60 μ M groups presented with significant differences in the expression level of caspase-3.

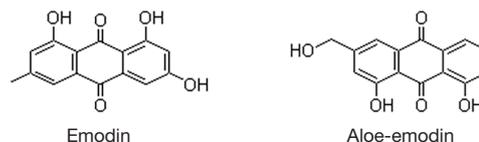


Figure 7 Structural formulas of emodin and aloe-emodin.

and upregulate the expression of Fas and FasL to induce apoptosis through the extracellular pathway (19). Therefore, we explored the effect of emodin on B16F10 cells to provide evidence of its potential clinical application in the treatment of melanoma.

In this study, it was found that the viability of the B16 cells decreased significantly with increasing emodin concentration, indicating that emodin inhibited the proliferation of B16F10 cells in a concentration-dependent manner. The changes in the morphology of B16F10 cells treated with 60 μ M emodin suggest the possible induction of apoptosis. The results from the cell scratch test and Transwell test showed that emodin can significantly inhibit tumour migration. Apoptosis, also known as programmed cell death, is the basic mechanism that maintains the dynamic balance of the number of cells in the body.

The suppression of apoptosis is an important cause of melanoma development. Apoptosis is mainly triggered by four pathways: the mitochondrial pathway, death receptor-mediated pathway, endoplasmic reticulum pathway and caspase-independent pathway. Caspase-3 is a protein shared in the first two pathways. It is a key factor that executes apoptosis, making it an important protein in the process of apoptosis. The activation of the mitochondrial pathway or death receptor-mediated pathway can cause an increase in caspase-3 expression. Its expression level also reflects the extent of apoptosis. The TUNEL assay showed an increased number of fluorescently stained cells, suggesting that emodin promoted the apoptosis of the melanoma cells. Moreover, the results of the western blot analysis showed that the expression of caspase-3 increased in the cells treated with 40 and 60 μ M emodin, supporting the hypothesis that emodin may cause the induction of apoptosis through the mitochondrial pathway or death receptor-mediated pathway.

Conclusions

In conclusion, the results showed that emodin effectively inhibited the proliferation of B16F10 cells, inhibited tumour cell migration and promoted cell apoptosis. Emodin may be one of the compounds for improving the therapeutic methods used for melanoma.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (<http://dx.doi.org/10.21037/tcr-20-1040>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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