Emodin Suppresses the Migration and Invasion of Melanoma Cells

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INTRODUCTION

Melanoma arises from dysfunction of normal melanocytes originating from epidermal tissue. Characterized by rapid growth, melanoma is considered as one of the most aggressive malignant neoplasm of skin.13 Melanoma accounts for approximately 80% of all skin cancer deaths.21 The incidence of melanoma has raised dramatically for the past few decades, especially in the western regions where people get less sun protection due to their lighter skin tones.3–5) Great progress has been made in improving treatment of malignant melanoma. However, owing to the high-metastatic potential and drug resistance of melanoma, the therapeutic effects are not satisfactory.5–7) Therefore, exploration of a new therapeutic strategy for melanoma with highly metastatic abilities is extremely urgent for melanoma therapy.

Metastasis involves various complicated processes featured by a chain of stereotypical molecular activities that result in degradation of the extracellular cell matrix (ECM), separation of cancer cells, cell adhesion to endotheliocytes, cell migration, cell invasion, cell motility and reconstruction of growth at distant sites.8,9) In the process described above, the degradation of ECM act as a key step of cancer metastasis and invasion.10) Previous research has demonstrated that numerous proteolytic enzymes are responsible for the degradation of the ECM, involving matrix metalloproteinases (MMPs).11) MMPs, as a family of zinc-dependent endopeptidases, play a significant part in degradation of the ECM related to the migration, invasion and angiogenesis of tumor cells.12) Dysregulation of the MMPs family proteins occurs frequently, which affects cell metastatic spread and invasion of cancer cells. Among the family of MMPs, the expression levels of MMP-2 and MMP-9 increased vastly in a variety of malignant cancer cells.12,13) Besides, MMP-2/-9 act as the most important enzymes in dissolving collagen types IV, the major component of the basement membrane.14) Several experiments have confirmed the important role of two MMPs in migration and invasion.11,15) Emerging treatments with the regulation of MMP-2/-9 activity can thereby block the spread of melanoma.16) Owing to their important functions in regulating cell invasion and migration, pharmacological inhibition of MMP-2/-9 proteins is a promising strategy for anti-metastatic or sensitization of cancer cells to chemotherapy.

In the process of melanogenesis and melanocyte development, the Wnt/β-catenin signaling pathway plays a critical role, and it is considered to be highly conserved in evolution.16,17) Furthermore, recent studies have proved that Wnt/β-catenin is associated with tumor progression of B16F10 and A375 melanoma cells.18,19) β-Catenin, as an essential constituent of Wnt/β-catenin pathway, activates the expression of metastasis-related proteins by interacting with T-cell factor (TCF) or lymphoid enhancing factor (LEF).20,21) Abundant evidence showed Wnt/β-catenin pathway has an impact on the expression of numerous target genes such as MMP-2, MMP-9 and proliferation-associated gene c-Myc.22,23)

During recent years, phytochemicals, which are natural products extracted from Chinese herbs, have arisen attention of researchers on account of their stable therapeutic outcomes and fewer side-effect profiles.24) Emodin is also known as...
1,3,8-trihydroxy-6-methylanthraquinone (Fig. 1A). It is extracted from herbs and has traditionally served as an important ingredient of various herbal cathartic.\(^{25}\) Nevertheless, recent researches highlighted that emodin possesses a wide range of pharmacological roles including anti-bacterial,\(^{26}\) immune modulation,\(^{27}\) anti-osteoporotic,\(^{28}\) anti-inflammatory,\(^{29}\) anti-allergic,\(^{30}\) anti-tumor,\(^{31}\) and enhancing cancer chemotherapy\(^{32}\) activities. Recently, it has been proved in pharmacological researches that emodin could inhibit proliferation and induce apoptosis in numerous tumors, including breast cancer,\(^{33}\) hepatocellular carcinoma,\(^{34}\) gastric carcinoma\(^{35}\) and colon cancer.\(^{36}\) However, the anti-metastatic effect and mechanism of emodin in melanoma remain unclear owing to lack of evidence from effective studies. Hence our study aimed to investigate emodin-mediated anti-proliferative and apoptosis-inducing effects in B16F10 and A375 melanoma cells and whether emodin has anti-metastatic activity on B16F10 and A375 melanoma, thus finding out a novel potential anticancer agent for melanoma with highly metastatic abilities.

**MATERIALS AND METHODS**

**Cell Line and Culture** B16F10 and A375 melanoma cells were purchased from ATCC (Manassas, VA, U.S.A.) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), streptomycin (100 µg/mL) and penicillin (100 U/mL). Cells were incubated in a humidified atmosphere with 5% CO\(_2\) at 37°C. Each test was implemented on cells in logarithmic growth phase.

**Materials** Emodin was bought from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.) and its purity was more than 99%. Emodin was dissolved in dimethyl sulfoxide (DMSO) with a concentration of 200 mM and reserved at −20°C. BML-284 was bought from MedChemExpress and 10 µM BML-284 was used in this study. In each test and cell culture, we kept the DMSO concentration no more than 0.1% to make sure there is no noticeable impact on growth or death of cells. Phosphate buffered saline (PBS), FBS, DMEM, streptomycin and penicillin were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.). Cell counting kit-8 (CCK-8) reagent, Transwell chamber, Matrigel and Annexin V-propidium iodide (PI) Apoptosis Detection kit were bought from Sigma-Aldrich. DMSO was purchased from Amresco Corp. (Solon, OH, U.S.A.). Radio immunoprecipitation assay (RIPA) lysis buffer kit was obtained from BestBio (Shanghai, China). Enhanced chemiluminescence (ECL) kit and polyvinylidene difluoride (PVDF) membrane were bought from BIORAD (Hercules, CA, U.S.A.). Protease inhibitor cocktail was obtained from Sigma-Aldrich. Rabbit antibodies specific to MMP-2 (Catalogue No. YT2798), MMP-9 (Catalogue No. YT1892) and Bcl-2 (Catalogue No. YT0470) and mouse antibody specific to Bax (Catalogue No. YM3619) were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). The rabbit antibodies against β-catenin (Catalogue No. 8480S), c-Myc (Catalogue No. 5605S), TCF (Catalogue No. 2203S) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Catalogue No. 5174S) were provided by Cell Signaling Technology (Danvers, MA, U.S.A.).

**Cell Counting Kit-8 Test** CCK-8 method was used to examine cell viability. Primarily, B16F10 or A375 cells in logarithmetic growth phase were cultured in 96-well plates at a density of 1 × 10\(^4\) cells/well for 12 h. Four wells were set for each concentration group. Following different treatments, we added 10 µL of CCK-8 reagent to each well. After incubation in the dark for 2 h, we measured the light absorbance at 450 nm on a micro-plate reader. Relative inhibition rate was calculated via...
the following formula: the relative inhibition rate (%) = (the average absorbance of the control group—the average absorbance of each emodin group)/the average absorbance of the control group × 100%.

**Colony Formation Test** Colony formation test was used to further detect cell proliferation. B16F10 or A375 cells were cultivated in 6-well plates at a density of 1 × 10⁵ cells/well overnight. After treatment of DMSO/ emodin, we cultured the cells for 10 d at 37°C. After visible clones were formed, the cell clones were fixed with 4% paraformaldehyde for 20 min, and stained with 0.5% crystal violet for 30 min. The stained clones were pictured with microscopy.

**Analysis of Apoptosis by Flow Cytometry** Cell apoptosis was detected by flow cytometry. At first, we treated Cells with different doses of emodin (20, 40, and 60 µM). Only 0.1% DMSO was added to the control group. After 24 h, we harvested the cultured cells and washed them twice with PBS. Next, the cells were centrifuged and washed with pre-chilled PBS. After that, the cell pellets were resuspended in 500 µL binding buffer containing fluorescein isothiocyanate (FITC)-labelled Annexin-V and PI dye and incubated for 20 min on ice. Cell apoptosis was detected by BD FACS Aria II cytometer and analyzed by Flow jo_V10.

**Cell Scratch-Wound Healing Test** Scratch-wound healing test was applied to estimate cell migration. After culturing B16F10 or A375 cells in 6-well culture plates to confluence, we used an aseptic pipette tip of 100 µL to scratch the cells. Next, we washed the plates twice with PBS to remove floating cells. After incubation with different treatments for 24 or 48 h, the cells were observed by a microscope at ×100 magnification. At 0, 24, or 48 h after scraping, the typical areas were pictured. We expressed the migration rate of cells as the following formula: the rate of migration (%) = (average scratch-wound areas—average no migration areas)/average scratch-wound areas × 100%.

**Transwell Migration Test** Cell migration was also detected by Transwell migration test. B16F10 or A375 cells (1 × 10⁵ cells) under different treatment were plated into the Transwell upper chambers with medium involving 1% FBS. At the same time, we added medium containing 20% FBS into the lower chambers. Then we cleared out the cells on the upside of the membrane after 24 h. The cells migrated into the other side of the filter were fixed with 4% paraformaldehyde for 20 min, and stained with 0.5% crystal violet. The stained cells were pictured and counted in three randomly selected fields with microscopy at ×200 magnification.

**Transwell Invasion Test** Cell invasion was examined by Transwell invasion test. Firstly, we added Matrigel (artificial basement membrane) to the upper surface of the Transwells for 12 h. B16F10 or A375 cells (1 × 10⁵ cells) under different treatment were cultured onto the Matrigel in the Transwell upper chambers with medium involving 1% FBS. At the same time, we added medium containing 20% FBS into the lower chambers. Then we cleared out the cells on the upside of the membrane after 24 h. The cells migrated into the other side of the filter were fixed with 4% paraformaldehyde for 20 min, and stained with 0.5% crystal violet. The stained cells were pictured and counted in three randomly selected fields with microscopy at ×200 magnification.

**Western Blotting** The cells (3 × 10⁶ cells per well) under different treatments were harvested and lysed in lysis buffer on ice for 30 min. After centrifugation at 18000 rpm at 4°C for 20 min, we collected the supernatant and detected its protein Fig. 2. Apoptosis-Inducing Effect on B16F10 and A375 Melanoma Cells by Emodin
Annexin V-FITC/PI assay was performed to detect the melanoma B16F10 and A375 cell apoptosis rates after different emodin treatments for 24 h. Only 0.1% DMSO was added to the control group. (A, B) B16F10 melanoma cell apoptosis rates were detected among different groups. (C, D) B16F10 melanoma cell apoptosis rates were detected among different groups. (E) Protein expressions of Bcl-2 and Bax were measured in B16F10 melanoma cells after treatment of emodin overnight in a dose-dependent manner. (F) The analysis of Bax/Bcl-2 ratio in B16F10 cells with emodin treatment. (G) Protein expressions of Bcl-2 and Bax were measured in A375 melanoma cells after treatment of emodin overnight in a dose-dependent manner. (H) The analysis of Bax/Bcl-2 ratio in A375 cells with emodin treatment. We repeated the experiment in three separate tests. The outcomes are expressed as mean ± S.D. Error bars are used to present S.D. * p < 0.05 vs. control group, ** p < 0.01 vs. control group.
concentration with a protein assay kit. Then we separated the proteins with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred them from the gel onto PVDF membrane. After blocking the membranes in skim milk, we incubated the membranes for 12 h at 4 °C using indicated primary antibodies against MMP-2 (1 : 500), MMP-9 (1 : 500), Bcl-2 (1 : 500), Bax (1 : 500), β-catenin (1 : 1000), c-Myc (1 : 1000), TCF (1 : 1000) and GAPDH (1 : 5000), followed by labelling with the appropriate secondary antibodies at 1 : 5000 dilution for 1 h at room temperature. An enhanced chemiluminescence detection system was used to determine the immunoreactive bands. Blots were imaged in a Molecular Imager System (Bio-Rad) and the results were analyzed.

Immunofluorescence Test Immunochemistry test was performed to examine the expression of MMP-2/-9. In brief, B16F10 or A375 cells under different treatment were washed twice with PBS and fixed by ice-cold 100% methanol for 20 min at −20 °C. After blocking the membranes in skim milk, cells in different groups were incubated with primary antibodies against MMP-2 (1 : 100), MMP-9 (1 : 100) at 37 °C for 2 h. Next, cells were washed with TBST, followed by labelling with the appropriate secondary antibodies (Abcam; ab150080) at 1 : 500 dilution for 1 h at 37 °C. Then, the cell nuclei were stained by 4′-6-diamidino-2-phenylindole (DAPI) (Beyotime). Finally, we observed the cells with Confocal laser scanning microscope (LEICA TCS SP8, Germany).

**RESULTS**

**Emodin Suppressed Growth of B16F10 and A375 Cells**

CCK-8 tests and colony formation test were applied to detect the functional role of emodin on the growth of melanoma B16F10 and A375 cells. The outcomes shown in Figs. 1B and C proved that emodin inhibited significantly the proliferation of B16F10 and A375 cells after treatment for 2 and 48 h. What is more, the inhibitory role on the growth of B16F10 and A375 cells improved with the increasing doses of emodin (20, 40, 60, and 100 µM) compared to the control group (0.1% DMSO only). When the concentration of emodin was 60 µM, the 24 and 48 h inhibitory rates of B16F10 cells were (34.8 ± 8.7) and (52.2 ± 6.2)%, and the 24 and 48 h inhibitory rates of A375 cells were (37.1 ± 8.3) and (50.3 ± 5.7)%, respectively. However, as the dose reached 80 µM, the inhibition rates of B16F10 at the time point of 2 and 48 h increased...
to (50.2 ± 4.6) and (66.9 ± 6.7)%, and the inhibition rates of A375 at the time point of 24 and 48 h increased to (45.5 ± 6.0) and (53.3 ± 5.5)%, respectively, showing severe cytotoxic reactions. Based on the outcomes of the CCK-8 test, we divided B16F10 or A375 cells into 4 groups for further scientific researches: Control group with 0.1% DMSO only, low concentration emodin (20 µM) group, moderate concentration emodin (40 µM) group and high concentration emodin (60 µM) group. Likewise, our outcomes of colony formation test informed that the cell proliferation of B16F10 and A375 cells was suppressed by emodin in a dose-dependent manner (Figs. 1D–G). Collectively, the results suggested that emodin inhibited the proliferation ability of B16F10 and A375 cells.

Emodin Induced Cell Apoptosis in B16F10 and A375 Cells

To test the apoptosis inductivity of emodin on B16F10 and A375 melanoma cells, Annexin V-FITC/PI double staining test was then performed. Annexin V-FITC/PI quadrant distribution of B16F10 cells in all 4 groups was measured (Figs. 2A–D). As shown in Fig. 2B, we found that the apoptosis rate of B16F10 in the control group was (1.51 ± 0.26)%, while those of the emodin groups increased to (5.46 ± 0.53), (15.97 ± 1.28) and (22.83 ± 4.55)%, respectively. In Fig. 2D, the apoptosis rate of A375 in the control group was (3.91 ± 1.30)%, while those of the emodin groups increased to (14.72 ± 0.86), (20.88 ± 1.59) and (34.44 ± 2.83)%, respectively. We found meaningful differences (p < 0.05) between these emodin-treated groups and the control group. To further examine the apoptosis potential of emodin on the B16F10 and A375 cells, apoptosis-related protein expressions were detected, including Bax and Bcl-2 (Figs. 2E–H). Emodin suppressed the protein expression of Bcl-2, and promoted protein expression of Bax. In addition, the Bax/Bcl-2 ratio increased in a dose-dependent manner, which is in connection with cell apoptosis. The outcomes revealed the induced role of emodin on apoptosis of B16F10 and A375 cells.

Emodin Decreased Migration and Invasion Ability of B16F10 and A375 Cells

In order to investigate effects of emodin on B16F10 and A375 cell migration and invasion ability, scratch-wound healing test and Transwell test were performed. After incubation with complete media without emodin overnight, B16F10 or A375 cells were scratched and DMEM with various emodin concentrations (20, 40, and 60 µM) were added. Only 0.1% DMSO was added into the control group. After incubation for 24 and 48 h, the cells were pictured by a microscope at ×100 magnification. Microscopy imaging and the quantitative analysis of the cells revealed that B16F10 and A375 cells migrated slower in the emodin-treated group compared to the control group (Figs. 3A–F). Furthermore, as shown in the results of Transwell migration and invasion test, the number of B16F10 or A375 cells migrating or invading into the other side of the Transwell decreased obviously in the emodin-treated groups (Figs. 4A–F). To figure out the mechanisms of emodin on melanoma B16F10 and A375 cells, Western blot analysis and immunofluorescence assay were applied to investigate the effects of emodin on protein expression of MMP-2/-9, which are closely concerned with cell invasion and migration. The results of Western blotting and immunofluorescence assay indicated that compared with
control group (0.1% DMSO only), MMP-2/-9 protein expressions were obviously reduced in B16F10 and A375 cells with emodin treatment (20, 40, and 60 µM) for 24 h (Figs. 5A–J). These observations suggest that emodin had a significant dose-dependent inhibitory effect on the B16F10 and A375 cell migration and invasion abilities.

Wnt/β-Catenin Pathway Was Suppressed by Emodin in B16F10 and A375 Cells In order to find out the mechanisms by which emodin suppresses migration and invasion of B16F10 and A375 cells, we examined the protein expression in the Wnt/β-catenin pathway, which are related to the cell metastasis. Western-blot results suggested that the protein expression in Wnt/β-catenin pathway (β-catenin, c-myc, TCF) were inhibited by emodin (Figs. 6A, B), and this may explain the possible mechanisms of how emodin suppressed melanoma B16F10 and A375 cell migration and invasion abilities.

Emodin Inhibited Migration and Invasion of B16F10 Cells by Down-Regulating of Wnt/β-Catenin Pathway To further detect the role of emodin in the migration and invasion of B16F10 cells, we employed BML-284 as a Wnt agonist. In the research, we explored the role of emodin or together with BML-284 on the migration and invasion of B16F10 cells. As shown in Fig. 7A, the protein expressions of β-catenin, c-myc, TCF, MMP-2, MMP-9 were inhibited by emodin compared to control group, while the results were reversed by combining with BML-284. Next, CCK-8 test was used to examine the

Fig. 5. Inhibitory Roles of Protein Expression of MMP-2/-9 in B16F10 and A375 Melanoma Cells by Emodin

(A) Representative western blots for MMP-2/-9 in B16F10 melanoma cells under different emodin doses (20, 40, and 60 µM) for 24 h. Only 0.1% DMSO was added to the control group. (B) Representative western blots for MMP-2/-9 in A375 melanoma cells under different emodin doses (20, 40, and 60 µM) for 24 h. Only 0.1% DMSO was added to the control group. (C, D) Statistical analysis of the protein expression of MMP-2/-9 in different B16F10 cell groups. (E, F) Statistical analysis of the protein expression of MMP-2/-9 in different A375 cell groups. The expression of GAPDH was taken as a reference antibody. (G, H) The results of immunofluorescence staining of MMP-2/-9 in B16F10 melanoma cells with different emodin treatment. (I, J) The results of immunofluorescence staining of MMP-2/-9 in A375 melanoma cells with different emodin treatment. Scale bar, 20 µm. Red: staining for MMP-2/-9 proteins, Blue: staining for cell nuclei by DAPI. We repeated the experiment in three separate tests. The outcomes are expressed as mean ± S.D. Error bars are used to present S.D. *p < 0.05 vs. control group, **p < 0.01 vs. control group.

Fig. 6. Inhibitory Roles of Wnt/β-Catenin Signaling Pathway in B16F10 and A375 Melanoma Cells by Emodin

(A) After treatment of a series of emodin doses (20, 40, and 60 µM), the protein expressions of β-catenin, c-Myc, and TCF in B16F10 melanoma cells in different groups. (B) The protein expressions of β-catenin, c-Myc, and TCF in A375 melanoma cells in different groups. The expression of GAPDH was taken as a reference antibody.
role of emodin on the viability of B16F10 cells. Our data suggested that the cell viability of B16F10 cells was suppressed in response to emodin. However, the treatment of BML-284 reversed the inhibitory effect of emodin (Fig. 7B). In addition, as demonstrated by scratch-wound healing test and Transwell test, B16F10 cell migration and invasion abilities was restrained by emodin, while BML-284 ameliorated these effects (Figs. 7C–G). Collectively, the results indicated that emodin hindered the migration and invasion abilities of B16F10 cells by down-regulation of Wnt/β-catenin pathway.

DISCUSSION

Emodin is the major active constituent extracted from rhubarb, which has been applied in traditional Chinese medicine (TCM) for the treatment of diseases like sores and diarrhea. Medicinally, emodin is extensively implemented for its comprehensive physiological and pharmacological roles containing antiviral activities,27 anti-inflammatory,29 anti-pain30 and antitumor31 functions. However, its antitumor activity has caught the attention of more investigators. Recently, researchers tried to detect the medicinal potential and possible mechanisms in the prevention and treatment for a variety of cancers. In the study, we focused on anti-migration and anti-invasion effects of emodin on melanoma. Because of resistance to present drugs and high-aggressive potential, the metastatic melanomas contributed to 80% of melanoma patient deaths. Metastasis is considered to be a feature of tumors with bad clinical prognosis.39 As a result, metastasis is also a major clinical challenge for medical workers on curing melanoma. The metastatic characteristics of melanoma are related to reconstruction of the extracellular cell matrix (ECM) and degradation of the basement membranes. Besides, proteases are believed to play an important part in the process.40 MMPs, as a family of zinc-binding proteolytic enzymes, play a significant part during multiple stages of cancer development such as mi-
migration, invasion and angiogenesis. Among them, MMP-2/-9 exert a vital role in migration and invasion of cancer cells via degrading ECM components such as collagen type IV, which is a main constituent of the basement membrane. In some researches, it has been suggested that protein expression of MMP-2/-9 elevated in cutaneous melanomas. In addition, it has been reported that increased expression levels of MMP-2 and MMP-9 are correlated with angiogenesis and metastasis of choroidal malignant melanoma (CMM). MMP-2/-9 targeted by specific microRNA (miRNA) or long noncoding RNA (lncRNA) could exert tumor-suppressing effects in CMM. Moreover, the Wnt/β-catenin signaling pathway is highly related to melanogenesis and melanocyte development. Researchers have approved that the MMP-2 and MMP-9 are the target genes in Wnt/β-catenin pathway in various cancer cells. Therefore, the inhibition of MMP-2/-9 by down-regulating Wnt/β-catenin signaling pathway could be a significant step in searching potential therapeutic targets for melanoma cells with strong invasive and metastatic abilities.

In previous researches, it has been reported that emodin inhibits cancer cell growth via down-regulation of CD155 in melanoma B16F10 cells and showed strong anti-proliferative effects on B16F10 cells as a mitochondrial uncoupler both in vitro and in vivo. Besides, Gu et al. have reported the anti-migration and anti-invasion effects of emodin on colon cancer cells via the Wnt/β-catenin pathway. Nevertheless, the targets of drug action for migration and invasion of melanoma cells by emodin are still unidentifiable. Until now, few studies have revealed conclusive evidence about the function of emodin on migration and invasion ability of melanoma. In our study, a new cell model (B16F10 and A375 melanoma cells) was used to detect the cytotoxic effect of emodin with a focus on melanoma cell invasion and migration and the underlying molecular mechanisms. In accordance with the previous researches, the results of CCK-8 (Figs. IB, C), colony formation test (Figs. 1D–G) and Annexin V-FITC/PI staining assays (Figs. 2A–D) confirmed that treatment with gradient concentrations of emodin, is able to exert anti-proliferative effect dose-dependently in B16F10 and A375 melanoma cell line in vitro, which is mediated through the induction of apoptosis. Furthermore, wound healing assay (Figs. 3A–F) and Transwell methods (Figs. 4A–F) proved the inhibitory effects on the proliferation of melanoma cells by emodin. In addition, this study is the first one to prove that emodin could be a potential medicine for highly metastatic melanoma treatment.

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**Conflict of Interest** The authors declare no conflict of interest.

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