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Emodin inhibits the proliferation of papillary thyroid carcinoma by activating AMPK

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Abstract. Emodin has been demonstrated to serve antitumor roles in a variety of tumor types, but the effect of emodin on papillary thyroid carcinoma and its molecular mechanisms remain unclear. In the current study, the role of emodin on papillary thyroid carcinoma was analyzed *in vitro* and *in vivo*. TPC-1 cells were treated with emodin (0, 10, 25 or 50 μM), and cell viability and apoptosis were detected using Cell Counting Kit-8 and flow cytometry, respectively. The expression levels of AMPK-associated proteins were examined using western blot analysis. To study the effect of emodin on the AMPK pathway, AMPK activator, AICAR and an AMPK inhibitor, Dorsomorphin, were used in TPC-1 cells. *In vivo,* mice were used to confirm the mechanism of emodin on papillary thyroid carcinoma. The results of the current study indicated that emodin treatment induced cell apoptosis and cell cycle arrest in TPC-1 cells. Furthermore, the inhibitory effect increased in a dose dependent manner. Following emodin treatment, the cell viability of TPC-1 cells was significantly decreased, and apoptosis rate increased (P<0.05). Furthermore, the expression levels of AMPK were increased in the emodin group compared with the control group (P<0.05). Similar effects were observed following AMPK activator treatment in TPC-1 cells. Following AMPK activator treatment, cell proliferation and the cell cycle were inhibited. Also, the AMPK inhibitor was demonstrated to mediate the therapeutic effect of emodin. In addition, the results of the present study demonstrated that emodin inhibited the MEK/ERK pathway. Additionally, the *in vivo* results of the current study were consistent with those *in vitro*. In conclusion, the current study demonstrated that the administration of Emodin inhibited the proliferation of papillary thyroid cancer cells via activating AMPK pathway activity.

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Introduction

Thyroid carcinoma is a malignant tumor that is derived from thyroid epithelial cells and is a common endocrine malignancy (1). Compared with other types thyroid cancer, the incident rate of papillary thyroid carcinoma is highest, and is responsible for 90% of thyroid cancers. However, early detection of papillary thyroid carcinoma usually results in successful treatment and a favorable patient prognosis (2,3). The incidence of papillary thyroid carcinoma has increased in the previous few decades, and the 5-year survival rate is 59% in the advanced stage, worldwide (4). Therefore, identifying novel drugs and investigating the underlying mechanisms of this disease are important for improving patient overall survival.

Emodin, which is also known as 1,3,8-trihydroxy-6-methy-anthraquinone, can be isolated from a number of Chinese medicinal herbs, including Rheum and Polygonum (5). A number of studies have demonstrated that emodin inhibits growth in multiple cancer types, including lung (5) and pancreatic cancer (6), and hepatocellular carcinoma (7). However, to the best of our knowledge, no research on the effect of emodin on papillary thyroid carcinoma has been previously performed. Previous reports have indicated that emodin serves an inhibitory role via AMPK and ERK in non-small cell lung cancer (8) and breast cancer (9).

AMPK has been revealed to serve an important role in cellular metabolic processes, including nutrient deprivation, mitochondrial dysfunction and oxidative stress (10). AMPK signaling has been indicated to be associated with cell differentiation, proliferation and the promotion of oncogenes and tumor growth, such as colorectal cancer and lung cancer (11,12). However, whether AMPK serves a role in activation or inhibition during emodin treatment in papillary thyroid carcinoma requires clarification.

In the current study, whether emodin exerts a role in inhibiting the development of papillary thyroid carcinoma via AMPK was investigated further.

Materials and methods

Cell lines. TPC-1 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). TPC-1 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Thermo Fisher Scientific,
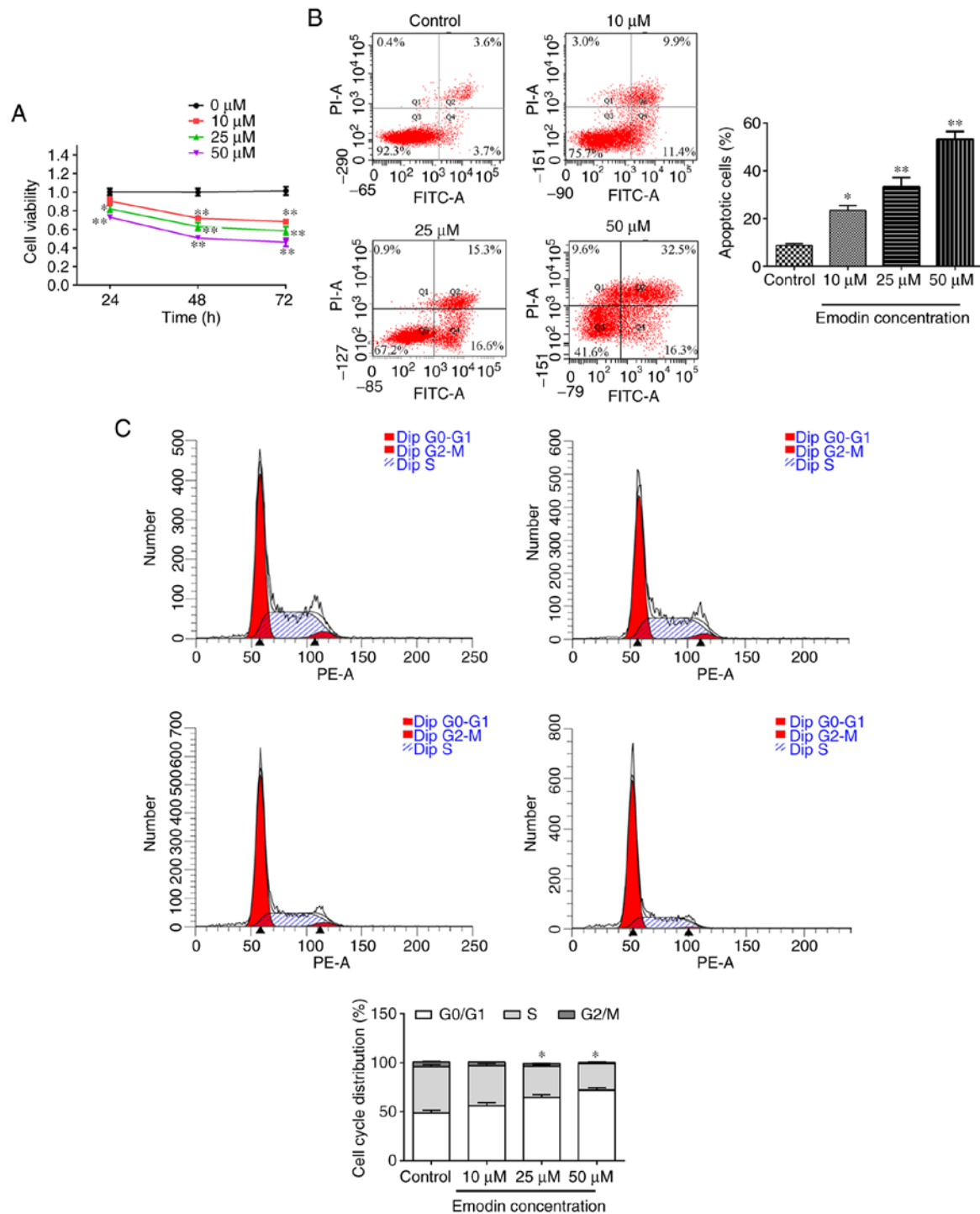
Figure 1. Emodin inhibits the proliferation and cell cycle of TPC-1 cells. (A) Cell counting kit-8 detection of cell viability of TPC-1 cells. (B) Flow cytometry to detect apoptosis. (C) Flow cytometry to detect the cell cycle. Data are presented as mean ± standard deviation and analyzed by ANOVA. There were three parallel samples in each group. *P<0.05, **P<0.01 vs. control.

Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in 5% CO_2.

Cell grouping. To study the effect of emodin on the proliferation of TPC-1 cells, 10, 25 and 50 μM emodin were added to TPC-1 cells for 24, 48 and 72 h at 37°C, respectively.

To further study the underlying mechanism of emodin on TPC-1 cells, cells were treated with different concentrations

of emodin (0, 5, 10, 20, 40 or 80 μm) for 48 h at 37°C, and the IC_{50} was calculated to be 49.01 μM using SPSS 20.0 (IBM Corp.). Thus, emodin at a concentration of 50 μM was used for subsequent experiments. The cells were divided into the control group, emodin treatment group (Emodin), AMPK activator group (AICAR), and emodin + AMPK inhibitor group (Combine). The cells of the control group were treated with PBS. In the emodin group, 50 μM emodin was added to cells for 48 h at 37°C. Cells in the AICAR group were treated with
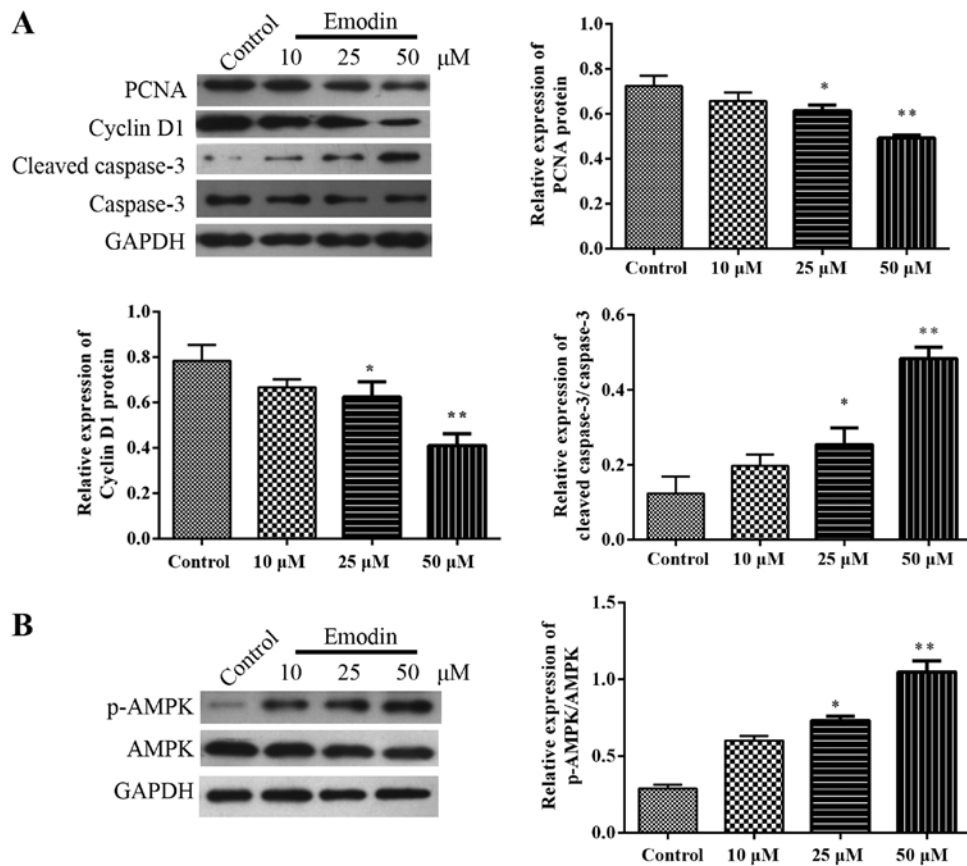
Figure 2. Emodin decreased the expression of PCNA, Cyclin D1, increased the expression of Cleaved-caspase-3, p-AMPK/AMPK in TPC-1 cells. (A) Western blot analysis to detect PCNA, Cyclin D1 and Cleaved-caspase-3/caspase-3; (B) Western blot analysis to detect p-AMPK/AMPK protein expression. Data are presented as mean ± standard deviation and analyzed by ANOVA. There were three parallel samples in each group. $^{*}P<0.05$, $^{**}P<0.01$ vs. control. PCNA, proliferating cell nuclear antigen; p, phosphorylated.

2 mM AMPK activator (AICAR, Cell Signaling Technology, Inc.) for 48 h at 37˚C (13). In the combination group, TPC-1 cells were treated with 50 μM emodin and 10 μM AMPK inhibitor (Dorsomorphin; cat. no. HY-13418A; MedChemExpress) for 48 h at 37˚C, as previously described (13). After 48 h, the cells were collected and analyzed using Cell Counting Kit-8 (CCK-8), flow cytometry and western blot analysis.

CCK-8 assay. TPC-1 cells ($2x10^4$ cells/ml) in each group were seeded into 96-well plates and cultured at 37˚C at 5% CO_2. After incubation for 24, 48 and 72 h, cells were incubated with 10 μl CCK-8 (Dojindo Molecular Technologies, Inc.) at 37˚C for 4 h according to the manufacture's protocol. The supernatant was carefully discarded and the absorbance of each hole was measured at 450 nm. Cell viability=[Treatment (OD)]-Blank (OD)/[Control (OD)-Blank (OD)].

Flow cytometry. Following incubation for 48 h, a AnnexinV-FITC/PI cell apoptosis detection kit (Meilunbio) was used to measure apoptotic cells. The cells ($2x10^5$) were centrifuged at 800 x g for 5 min at 4˚C, collected and washed with PBS twice at 4˚C. The cells ($1x10^6$/ml) were resuspended with 250 μl 1X binding buffer. The cells (100 μl) were subsequently added to a pipe with 5 μl Annexin V-FITC and 10 μl propidium iodide (PI; 20 μg/ml). The mixture was cultured at 25˚C for 15 min in darkness. The apoptotic rate was analyzed using a flow cytometer (Cytoflex3L8C; Beckman Coulter,

Inc.) and the results were analyzed using Cell Quest software version 2.0 (BD Biosciences).

Animals. A total of 36 specific pathogen free Balb/c female nude mice, (age, 5 weeks; weight, 18-20 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. [license no. scxk (Jing) 20160006]. Food, water and bedding materials were sterilized, and the animals were provided with food and water *ad libitum*. Mice were housed in specifc pathogen free conditions, at 22-26˚C with 40-70% humidity and 12 h light/dark cycles.

Xenograft model. TPC-1 cells were centrifuged at 800 x g for 5 min at 4˚C, washed with sterile PBS three times and diluted. A total of 40 μl cell suspension was added to 10 μl phenol blue stain and cells were counted under a microscope. A cell suspension with a concentration of $5x10^8$ cells/ml was made. Cells (~0.2 ml/$1x10^7$ cell/ml) were inoculated subcutaneously under the right rear armpit of mice. The nude mice were randomly divided into four groups: Model group, emodin group, AICAR group and the combined group. In the emodin group, mice were intraperitoneally injected with emodin at 40 mg/kg (14). In AICAR group, mice were intraperitoneally injected with emodin at 40 mg/kg (14) and AICAR at 50 mg/kg (15). In the combination group, mice were intraperitoneally injected with emodin at 40 mg/kg and dorsomorphin at 0.2 mg/kg (16). In each group, mice were treated three times a week for a period
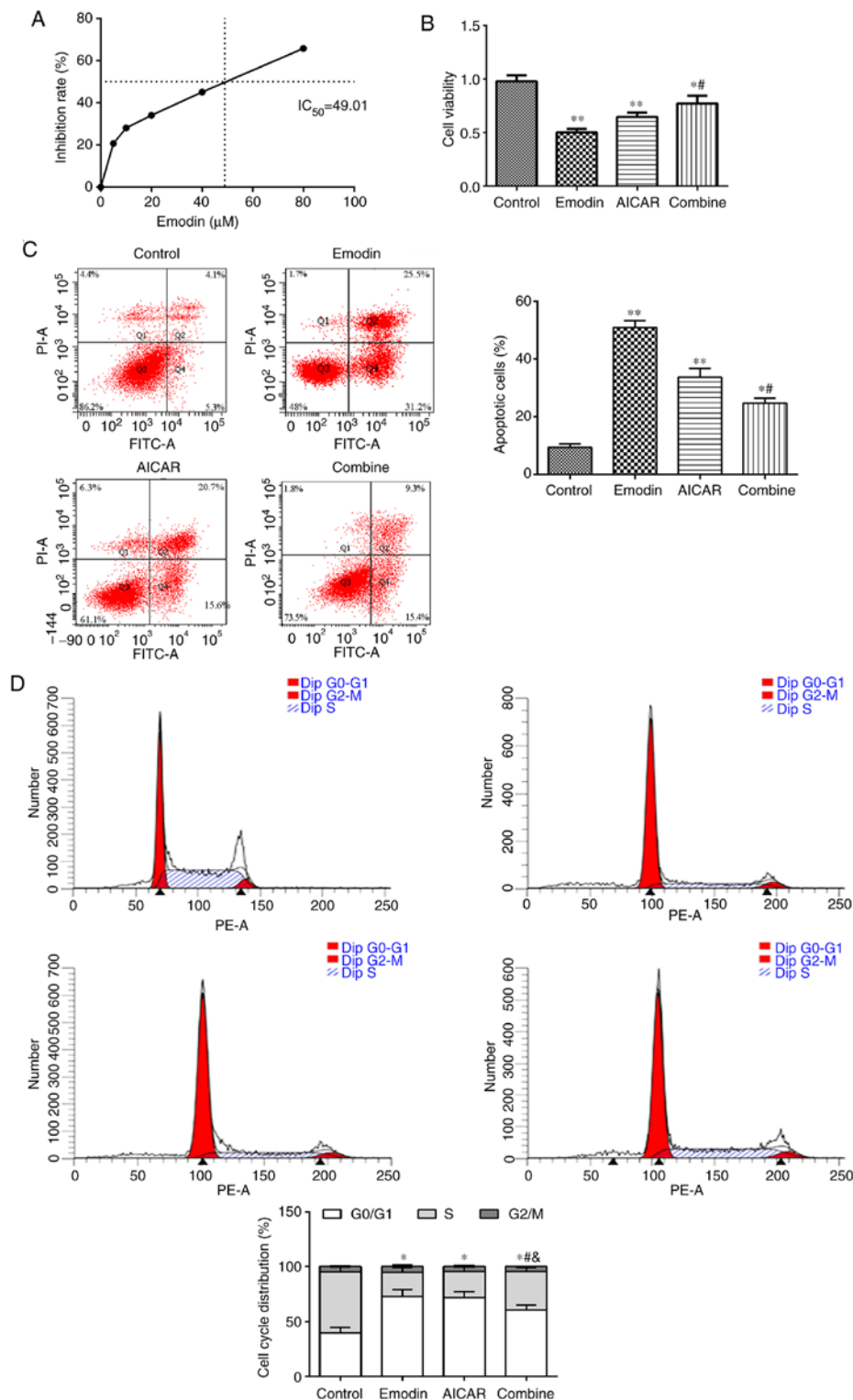
Figure 3. Emodin activates AMPK to affect the proliferation and cell cycle of TPC-1 cells. (A) The inhibition rate of emodin analyzed using CCK-8. (B) The cell viability analyzed using CCK-8. (C) Flow cytometry to detect apoptosis. (D) Flow cytometry to detect cell cycle. Data are presented as mean ± standard deviation and analyzed by ANOVA. There were three parallel samples in each group. The Combine group comprised cells treated with emodin and dorsomorphin. *P<0.05, **P<0.01 vs. control group, #P<0.05 vs. emodin group; &P<0.05 vs. AICAR group. CCK-8, Cell Counting Kit-8.

of two weeks. In the model group, mice were intraperitoneally injected with normal saline. The tumor volume $(V)=(axb^2)/2$ was calculated every week. The tumor volume was observed every week for 4 weeks. The health and behaviors of animals were monitored every day, including diet, weight, mental states and death. There was no death observed during the experiment. The maximum percentage of body weight loss was 10.2% in animals from start to endpoint. After a period of 28 days, 3% pentobarbital sodium (40 mg/kg) was intraperitoneally injected, and the nude mice were sacrificed via cervical dislocation. Five minutes after cardiac arrest, death was confirmed. The tumor was subsequently separated and weighed. Partial tumor sections were
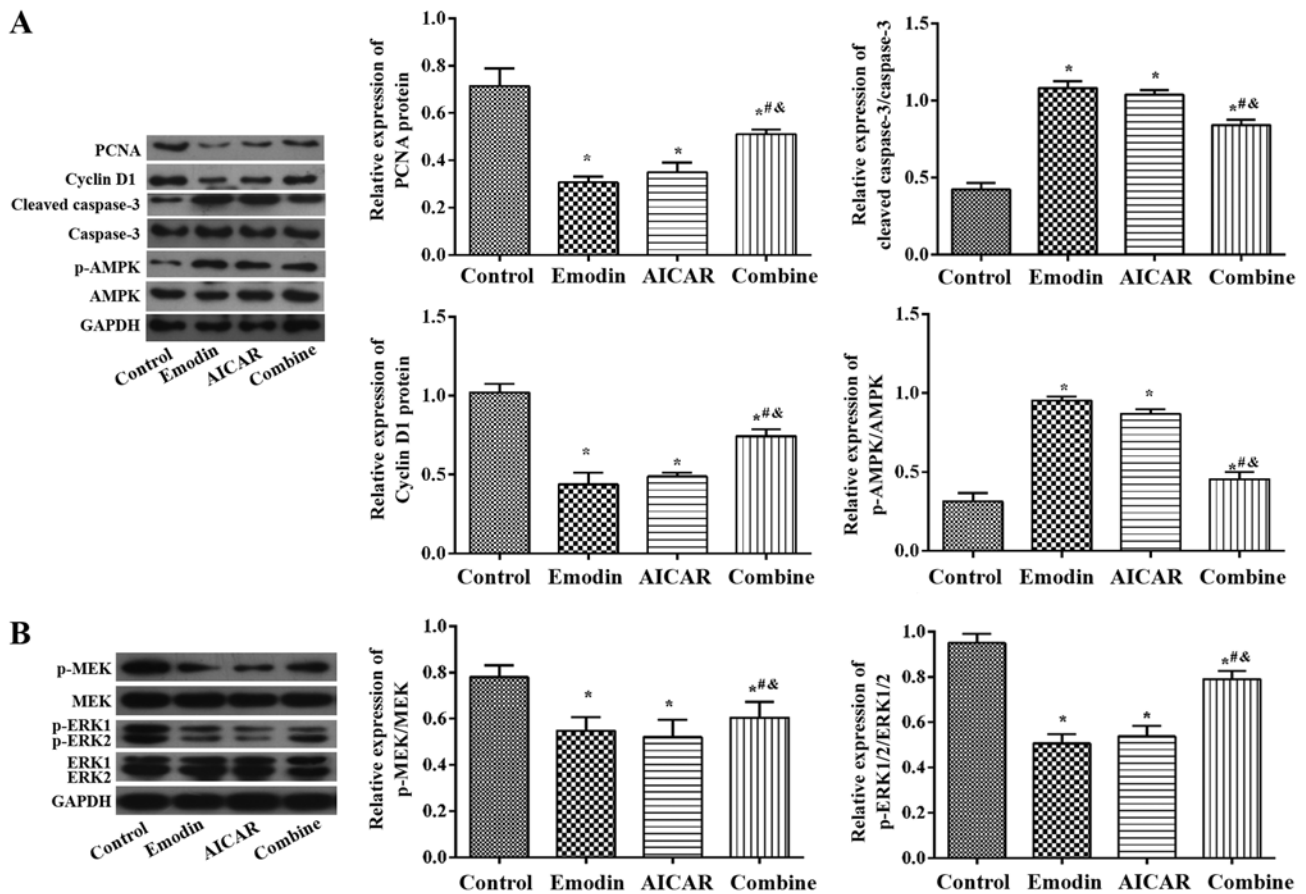
Figure 4. Emodin activates AMPK to affect the MEK-ERK pathway in TPC-1 cells. (A) Western blot analysis to detect PCNA, Cyclin D1, Cleaved-caspase-3/caspase-3, p-AMPK and AMPK proteins expression. (B) Western blot to detect p-MEK/MEK, p-ERK1/2/ERK1/2 proteins expression. GAPDH was used as the internal control. Data are presented as mean ± standard deviation and analyzed by ANOVA. There were three parallel samples in each group. *P<0.05 vs. control group, #P<0.05 vs. emodin group; &P<0.05 vs. AICAR group. PCNA, proliferating cell nuclear antigen; p, phosphorylated.

stored at -80°C for use in western blot analysis, and partially tumor sections were fixed with 4% paraformaldehyde at 25°C for 24 h for use in the TUNEL assay.

TUNEL. Tumor sections were 4 μm thick. After regular dewaxing with xylene and gradient ethanol, the sections were assessed using a Situ TUNEL Apoptosis Detection kit (cat. no. G001-2-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. In brief, the sections were treated with 50 μl TdT reaction solution for 60 min at 37°C, then washed with PBS for in triplicate. A total of 50 μl streptavidin-HRP solution was added for 30 min at 37°C in the dark, after which the solution was washed three times with PBS for 3 times. Samples were then treated with 80 μl DAB solution at room temperature for 10 min. At the end of staining procedure, the number of TUNEL positive cells was observed under the microscope. A total of five fields of view were randomly selected, and the number of TUNEL positive cells was counted. The nuclei of apoptotic cells were stained brown. Apoptotic index=(number of apoptotic positive cells/total cells) x100%.

Western blot analysis. Total protein of the TPC-1 cells or tissues in each group was extracted using a Tissue or Cell Total Protein Extraction kit (cat. no. BC3710; Beijing Solarbio Science & Technology Co., Ltd.) and the concentration of proteins was assessed using a BCA protein quantification

kit (cat. no. 23225; Thermo Fisher Scientific, Inc.). Samples (50 μg/lane) were separated using 12% SDS-PAGE (Bio-Rad Laboratories, Inc.) and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). Skim milk powder (5%) was used to block membranes for 1 h at 4°C. The primary antibodies were diluted with 5% BSA and incubated with samples at 4°C overnight. The primary antibodies used were as follows: Rabbit anti-human proliferating cell nuclear antigen (PCNA) antibody (1:700; cat. no. orb386383; Biorbyt, Ltd.), anti-Cleaved caspase-3 antibody (1:500; cat. no. ab49822; Abcam), caspase-3 antibody (1:500; cat. no. ab13847; Abcam), anti-Cyclin D1 antibody (1:200; cat. no. ab16663; Abcam), anti-phosphorylated (p-)AMPKa1 (Thr172) Antibody (1:1,000; cat. no. orb99303; Biorbyt, Ltd.), anti-AMPKa1 Antibody (1:1,000; cat. no. orb338932; Biorbyt, Ltd.), anti-ERK1/2 antibody (1:600; cat. no. orb106403; Biorbyt, Ltd.), anti-p-ERK1/2 antibody (1:600; Biorbyt, Ltd.), anti-MEK antibody (1:600; cat. no. orb38774; Biorbyt, Ltd.), anti-p-MEK antibody (1:600; cat. no. orb106207; Biorbyt, Ltd.) and GAPDH antibody (1:8,000; cat. no. orb555879; Biorbyt, Ltd.). The secondary antibody was HRP-labeled anti-rabbit IgG (1:1,800; cat. no. ab6721; Abcam) for 1 h at 4°C. GAPDH was used as the internal control. Blots were visualized on X-ray film using the enhanced chemiluminescence detection system (Cytiva). Densitometric analysis of the bands for the expression of protein was performed using Image J 6.0 (National Institutes of Health).
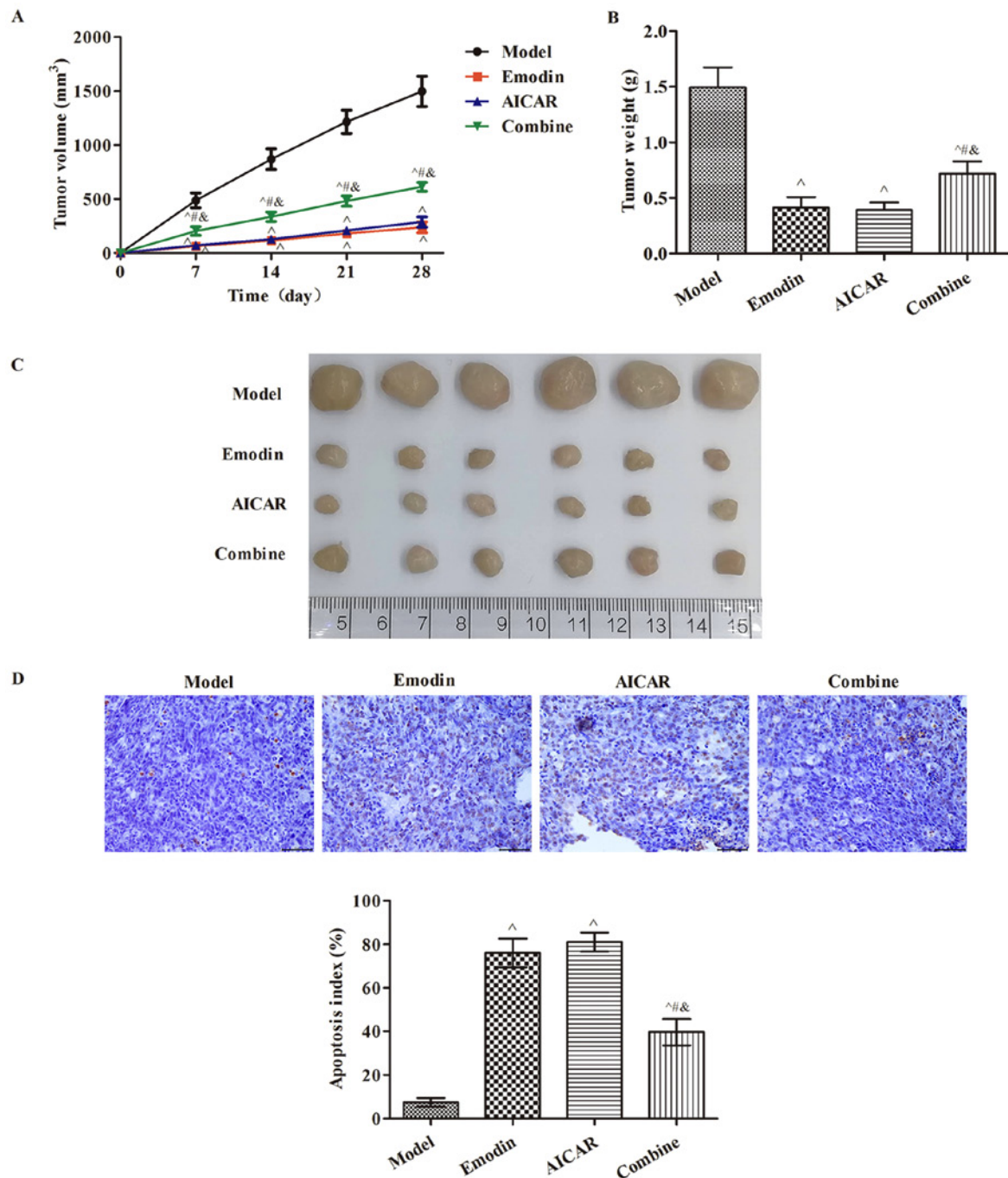
Figure 5. Emodin activates AMPK to suppress tumor growth. (A) Tumor volume. (B) Tumor weight. (C) Tumor images. (D) Apoptosis cells were analyzed using a TUNEL kit in tumor tissues (magnification, x400). Data are presented as mean ± standard deviation and analyzed by ANOVA. There were six mice in each group. The Combine group comprised cells treated with emodin and dorsomorphin. $^{\wedge}P<0.05$ vs. model group, $^{\#}P<0.05$ vs. emodin group; $^{\&}P<0.05$ vs. AICAR group.

Statistical analysis. The statistical data in the current study was analyzed using SPSS 20.0 statistical software (IBM Corp.). The data are presented as mean ± standard deviation. Among groups, differences were analyzed using ANOVA and followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Emodin inhibits proliferation of papillary thyroid cells. Compared with the control group, cell viability was significantly decreased following treatment with varied doses of emodin (Fig. 1A). Additionally, the proportion of cells in the apoptosis and G_0/G_1 phase increased significantly, and the proportion of cells in the S phase decreased (P<0.05; Fig. 1B and C). These data indicated that emodin inhibited TPC-1 cell proliferation and the cell cycle.

Emodin activates the phosphorylation of the AMPK pathway. Fig. 2 indicated that the expression levels of PCNA (Fig. 2A) and Cyclin D1 (Fig. 2A) protein were decreased, and the expression of Cleaved-caspase-3/caspase-3 (Fig. 2A) and
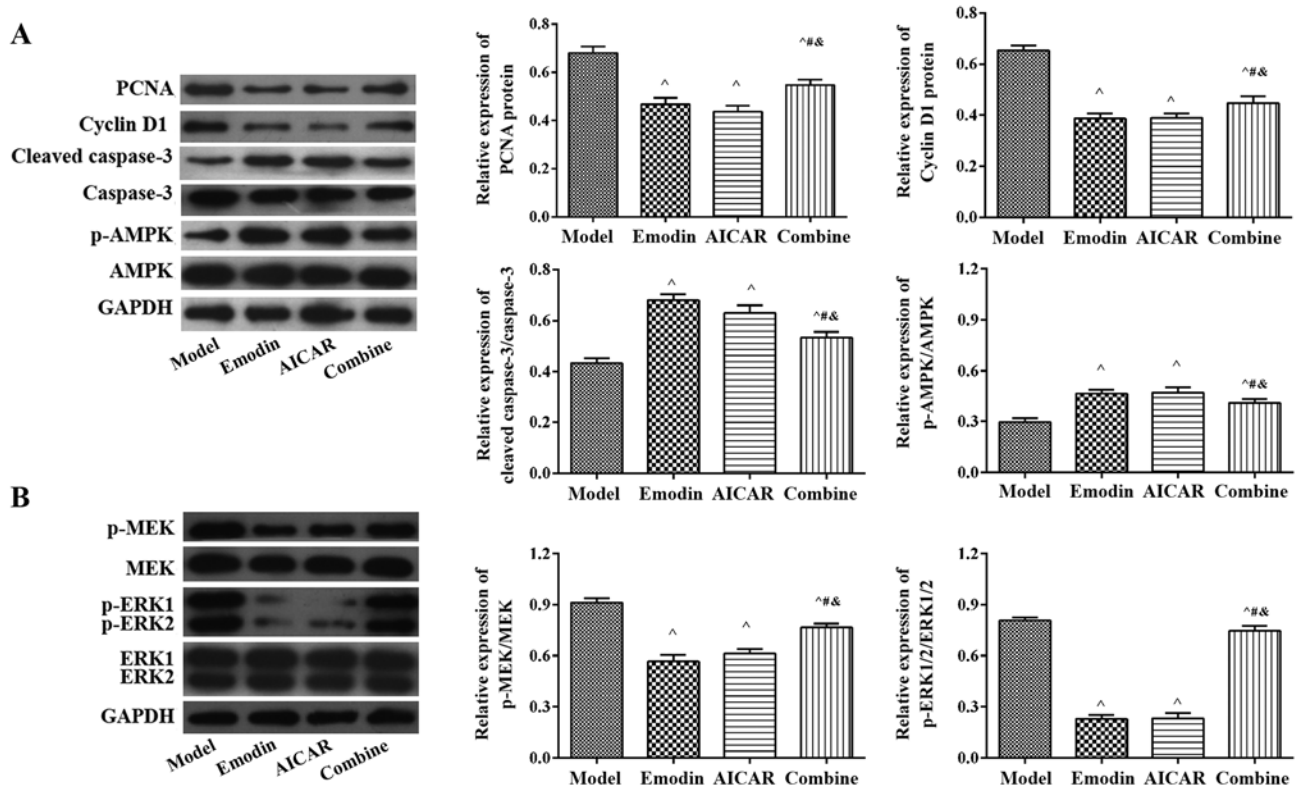
Figure 6. Emodin activates AMPK to suppress the MEK-ERK pathway *in vivo*. (A) Western blot analysis to detect PCNA, Cyclin D1, Cleaved-caspase-3/caspase-3 and p-AMPK/AMPK proteins expression. (B) Western blot analysis to detect p-MEK/MEK, p-ERK1/2/ERK1/2 protein expression. Data are presented as mean ± standard deviation and analyzed by ANOVA. There were six mice in each group. The Combine group comprised cells treated with emodin and dorsomorphin. ^P<0.05 vs. model group; #P<0.05 vs. emodin group; &P<0.05 vs. AICAR group. PCNA, proliferating cell nuclear antigen; p, phosphorylated.

p-AMPK/AMPK protein (Fig. 2B) were upregulated following treatment with 25 and 50 μM emodin when compared with the control group (P<0.05). These data suggested that emodin may serve a role in the expression of the AMPK pathway and the inhibition of TPC-1 cell proliferation and the cell cycle.

Emodin activates the AMPK pathway to affect proliferation papillary thyroid cells. In order to elucidate whether emodin affects proliferation and the cell cycle in TPC-1 cells via the AMPK pathway, AMPK inhibitor (Dorsomorphin) and activator (AICAR) were used to perform subsequent experiments. The experimental results are presented in Fig. 3. The IC$_{50}$ of emodin was analyzed in Fig. 3A. When compared with the control group, the proliferation of cells in the emodin and AICAR group were reduced (Fig. 3A), the apoptosis rate in TPC-1 cells was significantly increased and the distribution was also markedly raised in the G$_0$/G$_1$ phase (P<0.05; Fig. 3C and D). Compared with the emodin group, the malignant biological behavior of the combined group was increased (P<0.05).

Emodin activates AMPK to affect the MEK-ERK pathway. Fig. 4A indicated that PCNA and Cyclin D1 proteins levels were downregulated, and the levels of Cleaved-caspase-3/caspase-3 and p-AMPK/AMPK proteins were upregulated in the emodin and AICAR group when compared with the control group (P<0.05). However, there was no significant difference between the two groups. Fig. 4B indicated that when compared with the control group, p-ERK1/2 and p-MEK expression in

the emodin and AICAR group were decreased (P<0.05). In contrast to the emodin and AICAR group, the two proteins in the combined group were markedly increased (P<0.05). These results indicate that emodin activated AMPK to down-regulate the MEK-ERK pathway in TPC-1 cells.

Emodin suppresses tumor growth via activating AMPK. The mice model of tumor xenograft was established using TPC-1 cells. Nude mice were randomly divided into the model, emodin, AICAR and combined group. The tumor volume and tumor weight in each group are presented in Fig. 5A-C. Consistent with the *in vitro* experiment, the results revealed that emodin significantly suppressed tumor growth *in vivo* when compared with the model mice (P<0.05). The AMPK activator served a similar role to emodin, which suppressed tumor growth. However, administration of the AMPK inhibitor weakened the suppression of tumor growth caused by emodin (P<0.05). Furthermore, the apoptosis index in tumor tissues of each group indicated that emodin increased the number of apoptotic cells compared with the model mice (P<0.05; Fig. 5D). These data suggested that emodin may suppress papillary thyroid carcinoma via activating AMPK.

Emodin suppresses the MEK-ERK pathway via activating AMPK. As presented in Fig. 6A, the expression of PCNA and Cyclin D1 significantly decreased, while the expression of Cleaved-caspase-3/caspase-3 and p-AMPK/AMPK increased in the emodin and AICAR group compared with the model

group (P<0.05). In addition, the expression of p-ERK/ERK and p-MEK/MEK was significantly downregulated following emodin or AICAR administration (P<0.05; Fig. 6B). In contrast to the emodin group, the effect of emodin was significantly lower in the combined group (P<0.05). These data revealed that emodin may suppress the MEK-ERK pathway via activating AMPK.

Discussion

It is well known that emodin inhibits tumor growth in a number of different cancer types, such as pancreatic cancer, hepatocellular carcinoma and breast cancer (6,7,9). It has also been reported that emodin can suppress PCNA expression in some tumor cells, including those in cervical cancer (17). PCNA binds with human DNA polymerase δ to serve a role in DNA duplication and repair, and is usually a marker for cell proliferation and DNA replication (7). Consistent with a previous study (17), the current study demonstrated that emodin suppressed PCNA expression in TPC-1 cells. Cell cycle interruption is a major feature of tumor transformation and the status of the cell cycle represents a prognostic index for cancer (18). Cyclin-D1, which is a regulator of the G_1/S cell cycle, serves an essential role in tumor progression, contributes to tumor growth and resists anticancer drugs (18). It was therefore hypothesized that emodin would reduce the expression of Cyclin-D1 expression in TPC-1 cells and in a xenograft model.

A number of studies have demonstrated that AMPK is a cellular energy and nutritional status sensor in eukaryotic cells that is associated with the mechanism targets of rapamycin complex-1 (19-21). AMPK is likely to be the earliest signaling pathway in a number of evolutionary processes within eukaryotes (20). AMPK activation raises catabolism production (ATP production) and represses anabolic rate (ATP utilization). AMPK not only maintains the energy balance within the cell, but also regulates energy metabolism throughout the body (20). Due to its pivotal role in maintaining energy homeostasis, AMPK has the potential to become a underlying target for the treatment of metabolic diseases, which makes it an interesting study target (21). In the present study, emodin was indicated to be a cancer suppressor, and was revealed to suppress the proliferation and cell cycle of TPC-1 cells, and to promote cell apoptosis. Furthermore, the current study suggested that emodin may activate the AMPK pathway to inhibit cell proliferation and the cell cycle in human papillary thyroid carcinoma cells.

The MEK-ERK signaling cascade is activated by tyrosine kinase, which is linked by its homologous growth factor receptor under normal physiological conditions (22). MEK, which is a tyrosine-threonine kinase, is being widely studied as a potential drug target (22). A number of previous studies have demonstrated that emodin may inhibit cancer proliferation in a number of cancers via the ERK pathway, including in hepatocellular carcinoma (23), non-small cell lung cancer (8) and breast cancer (9). The results of the current study revealed that in human papillary thyroid carcinoma cells, emodin decreased p-ERK1/2 and p-MEK expression by activating AMPK, both *in vitro* and *in vivo*.

The association between emodin and AMPK pathway remains to be further elucidated in future research. Although the present study suggested that emodin may activate AMPK to inhibit the activity of MEK-ERK pathway, the specific mechanism governing this is still unclear and requires further study. In conclusion, the results of the current study suggested that emodin inhibited the proliferation of papillary thyroid cancer cells, and the mechanism may be associated with the activation of AMPK.

Availability of data and materials

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

Authors' contributions

WL and DW carried out the experimental work and the data collection and interpretation. WL, ML and DW participated in the design and coordination of experimental work, and acquisition of data. WL, DW and BL participated in the data collection, analysis of data and preparation of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were approved by The Animal Protection and Use Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University (approval no. 2020-161).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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