

Emodin suppresses angiogenesis and metastasis in anaplastic thyroid cancer by affecting TRAF6-mediated pathways *in vivo* and *in vitro*

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Abstract. Emodin has been recognized to be an anti-cancer agent against a number of types of human cancer. It was demonstrated that TNF receptor-associated factor 6 (TRAF6) was correlated with cancer angiogenesis and metastasis. The present study confirmed the association between TRAF6 and the angiogenesis/metastasis of anaplastic thyroid cancer (ATC). The anti-angiogenesis and metastatic effects of emodin, in addition to its molecular mechanisms in ATC, were investigated. A total of two ATC cell lines, namely 8505c and SW1736, were studied. ATC cells were implanted into nude mice to form xenografts or to establish lung metastasis models. Emodin was used to incubate ATC cells or to treat animals orally. An MTT assay was used to assess cell proliferation. A wound healing assay was employed to evaluate cell migration. ELISA analysis was used to detect the vascular endothelial growth factor (VEGF) content. Western blotting was used to determine the protein expression levels. In the *in vivo* study, cancer angiogenesis was assessed by micro vascular density measurement. The lung metastatic rate was the criterion for cancer metastasis. The results of the present study demonstrated that the proliferation of ATC was inhibited by emodin. The activation of the TRAF6/hypoxia inducible factor (HIF)-1 α /VEGF and TRAF6/basigin (CD147)/matrix metalloproteinase-9 (MMP9) pathways were associated with the angiogenesis and metastasis of ATC. In a concentration-dependent manner, emodin inhibited the TRAF6/HIF-1 α /VEGF and TRAF6/CD147/MMP9 signaling pathways to suppress

angiogenesis and metastasis. In conclusion, emodin exerted anti-angiogenic and anti-metastatic activities in ATC by affecting TRAF6-mediated pathways.

Introduction

As a common malignant tumor of the endocrine system, thyroid cancer accounts for ~1% of human carcinomas. According to pathological characteristics, including the extent of differentiation, thyroid cancer may be divided into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC) (1). PTC and FTC are the well-differentiated types which comprise 95% of the total thyroid cancer cases. Although ATC accounts for only 5% of thyroid cancer cases, it is one of the most lethal types of cancer among human malignant tumors (2). The prognosis of ATC is poor, which is evidenced by its short survival time. The median survival of ATC was reported to be between 3 and 5 months following diagnosis (3). Traditional therapies, including thyroid-stimulating hormone suppressive therapy and radioiodine ablation therapy, are effective and curative in PTC and FTC, and not in ATC. According to the American Joint Commission on Cancer, ATC cases are classified as stage IV when diagnosed (4). The malignancy of ATC is characterized by its invasive and metastatic features. A total of 90% of patients with ATC present with extra-glandular spread and 75% are identified to have distant metastasis when diagnosed (5).

TNF receptor-associated factors (TRAFs) are a family of adaptor proteins which regulate multiple signaling pathways via their interactions with the intracellular domains of a number of receptors (6). Through ubiquitin E3 ligase activity, TRAFs perform interaction-mediated signaling events. According to previous studies, a number of TRAFs have been observed to be associated with malignant tumor occurrence, development and progression (7-9), suggesting a role for TRAF6 in human cancer. A previous study indicated that TRAF6 expression was elevated in tumor cells and further increased the polyubiquitination of hypoxia inducible factor (HIF)-1 via direct binding (10), which was an important regulator of tumor angiogenesis (11,12). Additionally, TRAF6

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was indicated to be correlated with tumor metastasis (8,13). A recent study demonstrated that the important molecular regulator CD147 was the cofactor of TRAF6; TRAF6 knock-down with small interfering RNA significantly decreased the level of CD147-mediated tumor metastasis (14). These reports indicated that the role of TRAF6 in tumor malignancy was characterized by angiogenesis and metastasis.

Emodin, also termed as 1,3,8-trihydroxy-6-methylantraquinone, is a natural anthraquinone extracted from rhizome of *Rheum palmatum* L. Various biological activities of emodin have been reported previously, including anti-inflammatory, anti-proliferative, anti-fibrotic and anti-cancer activities (15). Emodin was observed to be effective in inhibiting a number of types of human cancer, including pancreatic cancer, liver cancer, gastric cancer and leukemia (16-18). However, the anti-cancer activities of emodin in anaplastic thyroid cancer have rarely been reported previously. The present study implemented experiments to observe the anti-cancer activity of emodin in ATC. In addition, the inhibitory effects of emodin against tumor metastasis and angiogenesis were investigated. Therefore, it may be hypothesized that emodin exerts metastasis-and angiogenesis-suppressing abilities. The present study further demonstrated the anti-metastatic and anti-angiogenic effects of emodin in nude mice carrying human ATC *in vivo* and in cultured human ATC cells *in vitro*.

Materials and methods

Cell culture. Human ATC cell lines 8505c and SW1736 were acquired from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and used in the present study. Following thawing and recovery, 8505c cells were cultured in Eagle's minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) while SW1736 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.). The two media were supplemented with fetal bovine serum (10%; Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotic mix (penicillin/streptomycin; Invitrogen; Thermo Fisher Scientific, Inc.). Cells were cultured in an incubator providing a humidified atmosphere composed of 5% CO₂ and 95% fresh air at 37°C.

Cell viability assay. The cell viability of ATC cells was assessed by MTT assay. Cells were seeded into a 96-well plate at density of 7x10³ cells/well and cultured for 12 h. The medium was replaced with either control saline or emodin solutions (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at incremental concentrations (0, 10, 15, 20, 25, 30, 35 and 40 µmol/l) for 12 h. Following washing, MTT solutions were added into each well to incubate the cells for 4 h at 37°C. Dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals. A microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to measure the absorbance values at 540 nm. A total of three replicate readings were recorded and used for further analysis.

ELISA analysis. The concentration of vascular endothelial growth factor (VEGF) in cell culture supernatants was determined by ELISA using a Quantkine Human VEGF ELISA kit

(cat. no. VDE00; R&D Systems, Inc., Minneapolis, MN, USA). Procedures were performed according to the manufacturer's protocol. The recorded absorbance values and plotted standard curves were used to calculate the concentration of VEGF in the cell culture supernatants.

Wound healing assay. The migratory ability of ATC cells was evaluated via a wound healing assay. 5x10⁵ ATC cells were cultured on a 60-mm diameter culture dish (Corning Incorporated, Corning, NY, USA) to reach a confluence of 90%. The wound was made using a 2-mm razor blade and the injury line was marked. The peeled-off cells were removed and the remaining cells were cultured and allowed to migrate to heal the wound for 24 h. Following careful washing, cells were fixed and subjected to DAPI fluorescence labeling using a DAPI kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. The wound closure was observed with a fluorescence microscope at x100 magnification.

In vivo studies. The male Balb/c nude mice (6 weeks old; weight, 19-22 g; n=62) used in the present study were acquired from Charles River Laboratories, Inc. (Wilmington, MA, USA). Mice were kept in an artificial environment providing a 12-h light/dark cycle, a 50% humidity and a temperature of 20-24°C in laminar flow cleanrooms. Animals were had free access to sterile water and standard chow. All animal experimental procedures were performed according to the recommended guidelines for the care and use of laboratory animals issued by the Chinese Council on Animal Research. The protocol was reviewed and approved by the Animal Ethics Committee of the College of Medicine, Zhejiang University (Hangzhou, China).

Angiogenesis study. Harvested ATC cells suspended in culture mediums (1x10⁷ cells in 200 µl per mouse for 10 mice) were inoculated into the left flank of each mouse. Mice continued to survive for 28 days. Mice were orally administered with emodin at 100 mg/kg, once per day for 28 days. Immunohistochemical staining of platelet endothelial cell adhesion molecule (CD31) was used to mark the neovessels in tumor tissues in the present study. The tumor tissues were fixed by 10% neutral buffered formaldehyde for 10 h and processed into sections at 5 µm using paraffin-embedding method. Following blocking by QuickBlock blocking buffer (cat. no. P0620; Beyotime Institute of Biotechnology, Shanghai, China) at room temperature for 15 min, the sections were incubated with a CD31 antibody (cat. no. ab32457; 1:1,000; Abcam, Cambridge, UK) at 4°C for 12 h. Following washing, a secondary antibody (anti-rabbit IgG HRP-conjugated; cat. no. ab191688; 1:2,000; Abcam, Cambridge, UK) was used to incubate the slides at room temperature for 20 min. The images were observed using a light microscope at x200 magnification. Microvessel density (MVD) was evaluated by counting the number of CD31-positive vessels in 10 different fields.

Metastasis study. In the lung metastasis experiment, 52 mice were used. A total of 5 control animals received injection of cell culture medium. An ATC cell suspension at a density of

2×10^7 cells/ml was injected into the tail vein of the mice. Mice continued to survive for 28 days. Mice were orally administered with emodin at 100 mg/kg, once per day for 28 days. Lung tissue samples were obtained and then fixed with 10% formalin at room temperature for 10 h and then embedded in paraffin wax. Tissues were processed into 5 μ m tissue sections. H&E staining was applied; the slides were stained with hematoxylin for 5 min and eosin for 30 sec at room temperature. Stained slides were subsequently observed with a light microscope at $\times 200$ magnification. Metastatic rate was calculated by comparing number of mice with metastasis to the number of mice without metastasis.

Western blotting. Total proteins were extracted from tumor tissues and ATC cells using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and a protein extraction kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The concentration of proteins was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Vertical electrophoresis was performed on SDS-PAGE gels loaded with protein samples. The gel was 10% and 40 μ g protein sample was loaded per lane. Proteins were electro transferred to polyvinylidene fluoride/nitrocellulose membranes. Membranes were blocked by 10% defatted milk (in TBST) at 37°C for 30 min. The PVDF membranes were incubated with primary antibodies against TRAF6 (cat. no. ab33915; 1:2,000; Abcam), HIF-1 α (cat. no. 79233; 1:2,000; Cell Signaling Technology, Danvers, MA, USA); VEGF (cat. no. ab53465; 1:2,000; Abcam); MMP9 (cat. no. sc21733; 1:2,000; Santa Cruz Biotechnology, Inc.); basigin CD147 (cat. no. 13287; 1:2,000; Cell Signaling Technology); GAPDH (cat. no. ab9485; 1:4,000; Abcam) at 4°C for 8 h. Membranes were subsequently washed with TBST 3 times. Secondary antibodies conjugated to HRP (cat. nos. 193651 and 99817; 1:2,500; Abcam and cat. no. sc2351; 1:2,500; Santa Cruz Biotechnology, Inc.) were used to incubate the membranes at room temperature for 20 min. The immunoblots were visualized with an Enhanced Chemiluminescence Prime Detection kit (GE Healthcare, Chicago, IL, USA) and the densities of the immunoblots were analyzed by ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

Statistics. Data collected in the present study were presented as the mean \pm standard error of the mean. Performed using the software SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA), Student's t-test and one-way ANOVA were used to analyze the significant differences between groups. Post hoc tests were carried out by SNK tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Emodin inhibits the proliferation of ATC cells in a concentration-dependent manner. A total of two different human ATC cells, namely 8505c and SW1736 cells, were incubated with emodin solutions at incremental concentrations. An MTT assay was performed to identify the non-cytotoxic concentrations. The results are presented in Fig. 1. Emodin

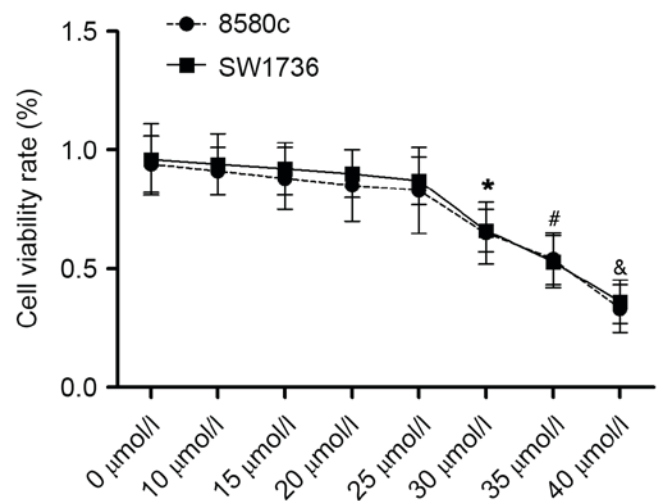


Figure 1. Effects of emodin incubation on ATC cell proliferation. The cell viability of ATC cells was calculated following incubation with emodin solutions at incremental concentrations (0, 10, 15, 20, 25, 30, 35 and 40 μ mol/l). * $P < 0.05$ vs. 25 μ mol/l; # $P < 0.05$ vs. 30 μ mol/l; & $P < 0.05$ vs. 35 μ mol/l. ATC, anaplastic thyroid cancer.

began to inhibit the proliferation of 8505c and SW1736 cells at 30 μ mol/l. Therefore, concentrations < 30 μ mol/l (0, 10, 15, 20 and 25 μ mol/l) were chosen for the subsequent experiments to avoid the anti-proliferative effect of emodin on the metastatic and angiogenic abilities of ATC.

Emodin suppresses VEGF expression by inhibiting the TRAF6/HIF-1 α signaling pathway in cultured ATC cells in a concentration-dependent manner, within non cytotoxic concentrations. In the present study, it was observed that in cultured 8505c and SW1736 cells, the TRAF6/HIF-1 α /VEGF signaling pathway was activated. As a result, the concentration of VEGF in the culture supernatant was significantly elevated. VEGF is thought to be an important effector in the initiation and promotion of tumor angiogenesis. Following incubation with emodin at non-cytotoxic concentrations, however, the activation of TRAF6/HIF-1 α /VEGF was markedly suppressed in a concentration-dependent manner. Subsequently, the VEGF content in the culture supernatant was decreased in a concentration-dependent manner following incubation with emodin. These results are presented in Fig. 2.

Emodin suppresses the metastatic ability of ATC cells by inhibiting the TRAF6/CD147/MMP9 signaling pathway in cultured ATC cells in a concentration-dependent manner, within non-cytotoxic concentrations. As demonstrated in Fig. 3, the TRAF6/CD147/MMP9 signaling pathway was significantly activated in cultured 8505c and SW1736 cells. However, following incubation with emodin at non-cytotoxic concentrations, the activation of TRAF6/CD147/MMP9 signaling was markedly inhibited in a concentration-dependent manner. Additionally, a wound healing assay was performed to evaluate the metastatic ability of ATC cells. Presented in Fig. 3, the results indicated that incubation with emodin markedly impaired the motility of 8505c and SW1736 cells in a concentration-dependent manner.

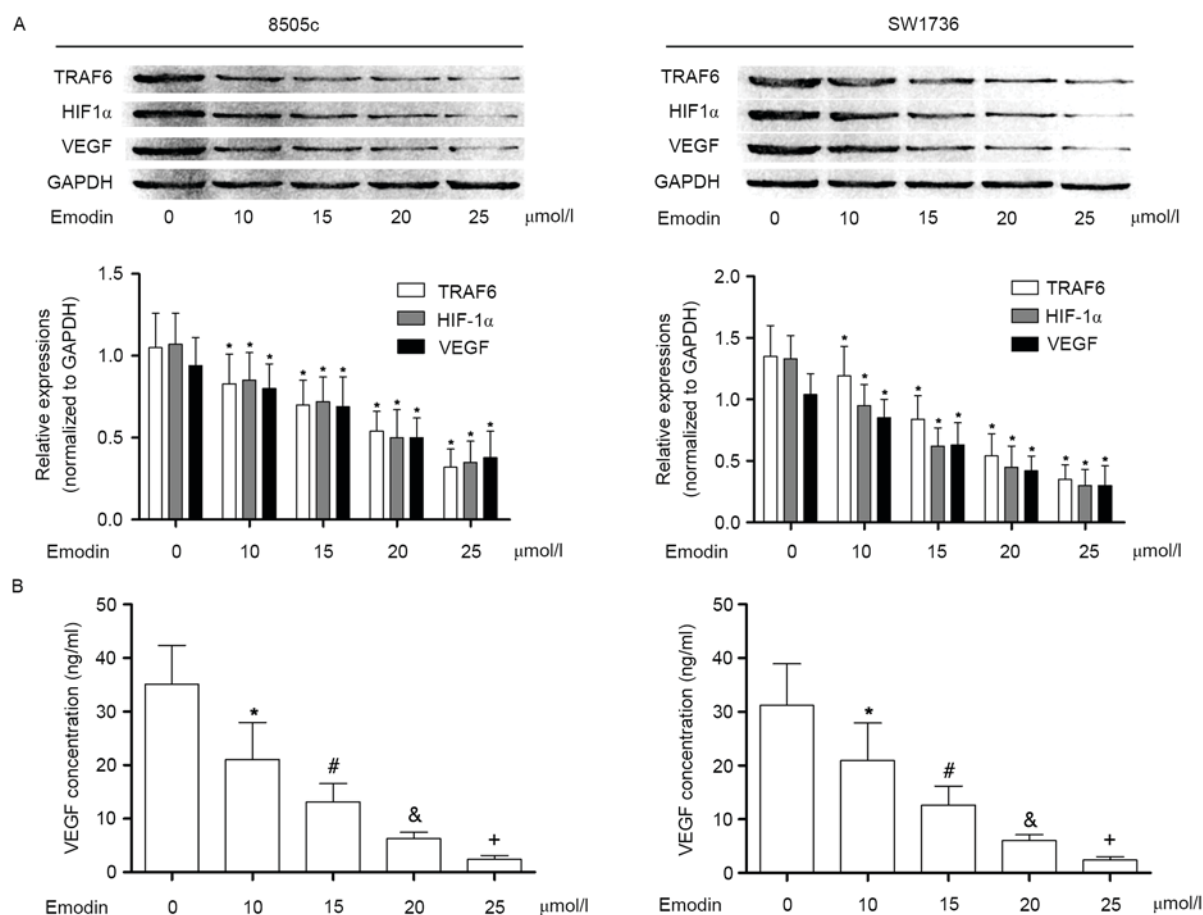


Figure 2. Effects of emodin on TRAF6/HIF1 α /VEGF pathway in anaplastic thyroid cancer cells. (A) Immunoblotting of TRAF6, HIF1 α , VEGF and GAPDH in 8505c (left) and SW1736 cells (right) following incubation with emodin at concentrations of 0, 10, 15, 20, 25, 30, 35 and 40 μ mol/l, respectively. The lower panels indicate the relative expression levels of TRAF6, HIF1 α and VEGF (GAPDH was used as an internal reference), in 8505c (left) and SW1736 cells (right) incubated with emodin at concentrations of 0, 10, 15, 20, 25, 30, 35 and 40 μ mol/l, respectively. (B) Results of ELISA analysis. Columns indicate the detected concentrations of VEGF in the cell supernatants of 8505c (left) and SW1736 cells (right) treated with emodin at incremental concentrations. * $P < 0.05$ vs. 0 μ mol/l; # $P < 0.05$ vs. 10 μ mol/l; & $P < 0.05$ vs. 15 μ mol/l; + $P < 0.05$ vs. 20 μ mol/l. TRAF6, TNF receptor-associated factor 6; HIF1 α , hypoxia-inducible factor 1- α ; VEGF, vascular endothelial growth factor.

Emodin abates ATC angiogenesis and metastasis by suppressing TRAF6-mediated signaling pathways in vivo. In the present study, *in vivo* experiments were performed to reinforce the conclusion derived from the *in vitro* studies. ATC cells were used to inoculate the nude mice. In harvested ATC tissues, the TRAF6/HIF-1 α /VEGF signaling pathway was activated. However, following treatment with emodin, the activation of the TRAF6/HIF-1 α /VEGF and TRAF6/CD147/MMP9 signaling pathways in ATC tissues was significantly inhibited. The MVD assay demonstrated that emodin administration suppressed angiogenesis in ATC. In addition, a lung metastasis model was established by tail vein injection of ATC cells. Emodin administration decreased the lung metastatic rate. These results are presented in Fig. 4.

Discussion

As one of the undifferentiated human carcinomas, ATC is causes frequent mortality; patients diagnosed with ATC exhibit a mean survival of ~6 months (1). ATC is aggressive and characterized by highly invasive behavior and early distant metastasis (19). The 5-year survival rate of ATC has been reported to be <5% (2). Additionally, conventional therapies

have proven to be unable to improve the clinical outcomes. Thus, investigations into novel anti-cancer agents against ATC are of clinical significance. In the present study, an herbal extract termed emodin was used to treat ATC. The results of the *in vitro* and *in vivo* experiments indicated that emodin inhibited angiogenesis, in addition to suppressing the metastatic ability of ATC. Further examination demonstrated that TRAF6-mediated angiogenesis and metastasis were affected by emodin, indicating that TRAF6 may be the molecular target of emodin.

It has been reported that emodin exerts anti-cancer activities by inducing cancer cell apoptosis, cell cycle arrest, and by suppressing the cellular abilities of proliferation, angiogenesis, migration and invasion (20-22). Although emodin has been proven to be effective in a number of human cancer types, the anti-cancer effect of emodin on ATC has rarely been reported in previous studies. In the present study, two ATC cell lines, 8505c and SW1736, were treated with serially diluted emodin solutions. It was observed that the proliferation of ATC was markedly inhibited when concentrations of emodin were above 30 μ mol/l. In addition, the suppressive effect of emodin effects on the angiogenesis and metastasis of ATC was investigated.

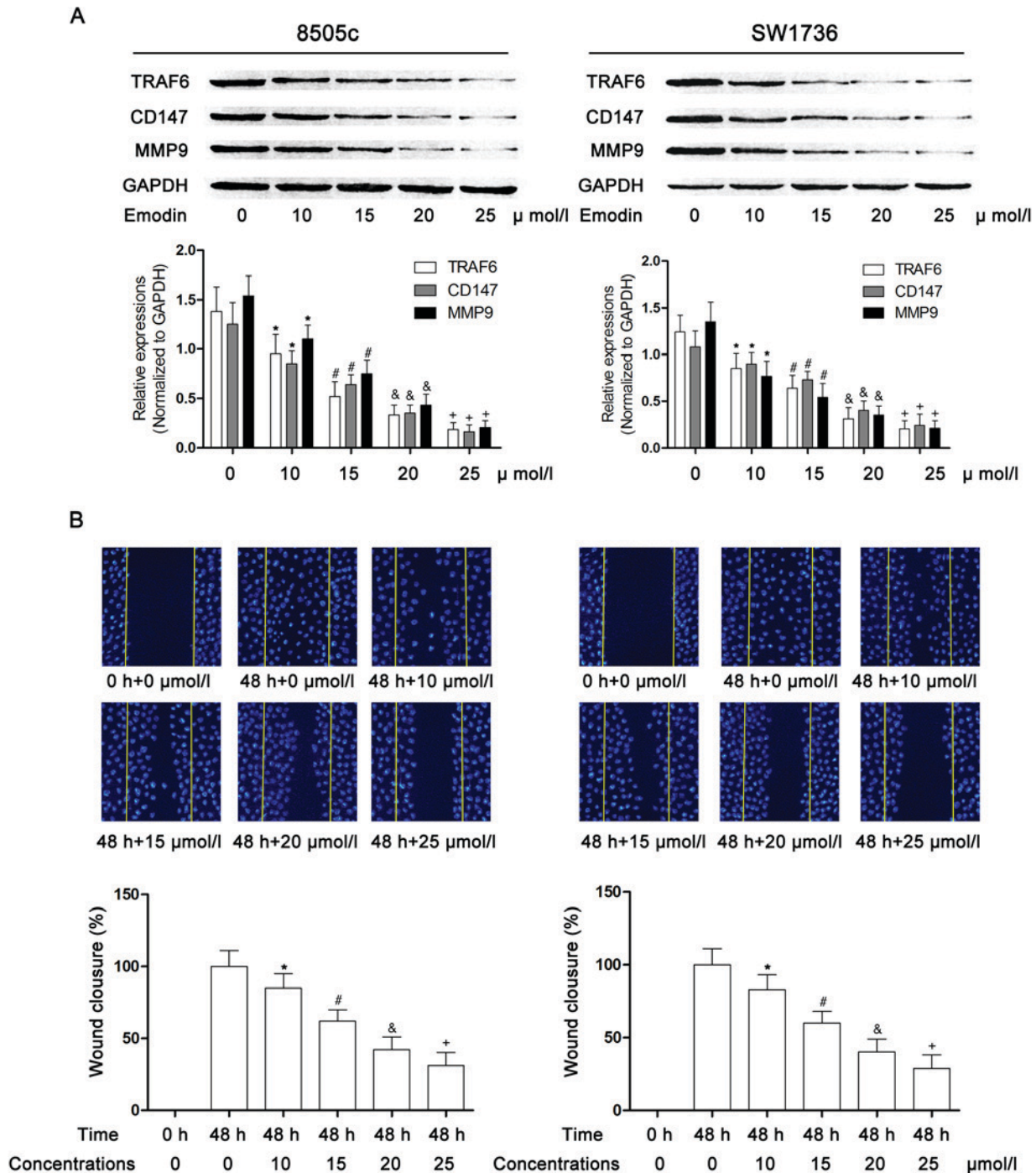


Figure 3. Effects of emodin on the TRAF6/CD147/MMP9 pathway in anaplastic thyroid cancer cells. (A) Immunoblotting of TRAF6, CD147, MMP9 and GAPDH in 8505c (left), and SW1736 cells (right), following incubation with emodin at concentrations of 0, 10, 15, 20, 25, 30, 35 and 40 $\mu\text{mol/l}$, respectively. The lower panels indicate the relative expression levels of TRAF6, CD147 and MMP9 (GAPDH was used as an internal reference) in 8505c (left) and SW1736 cells (right) incubated with emodin at concentrations of 0, 10, 15, 20, 25, 30, 35, 40 $\mu\text{mol/l}$ respectively. (B) Captured fluorescent images (magnification, x200) of the wound healing assay in 8505c (left) and SW1736 cells (right) following incubation with emodin at incremental concentrations. Cells were stained with DAPI. Yellow lines indicate the edges of wound. The lower panels indicate the wound closure in 8505c (left) and SW1736 cells (right), respectively. * $P < 0.05$ vs. 0 $\mu\text{mol/l}$; # $P < 0.05$ vs. 10 $\mu\text{mol/l}$; & $P < 0.05$ vs. 15 $\mu\text{mol/l}$; + $P < 0.05$ vs. 20 $\mu\text{mol/l}$. TRAF6, TNF receptor-associated factor 6; CD147, basigin; MMP9, matrix metalloproteinase 9.

TRAFs are the intracellular protein family which transduce signals from intracellular part of tumor necrosis factor receptors. Previous studies have linked one of the family members, TRAF6, to the oncogenesis, development and metastasis of numerous types of human cancer (23). Protein and mRNA overexpression of TRAF6 has been observed in human cancer

tissues (24,25). *In vitro*, increased and consistent expression of TRAF6 has been identified in multiple human cancer cell lines (9). In the present study, a similar phenomenon was observed: TRAF6 was highly expressed in two different ATC cell lines *in vitro* and their xenografts *in vivo*. A previous study indicated that TRAF6 enhanced cancer angiogenesis

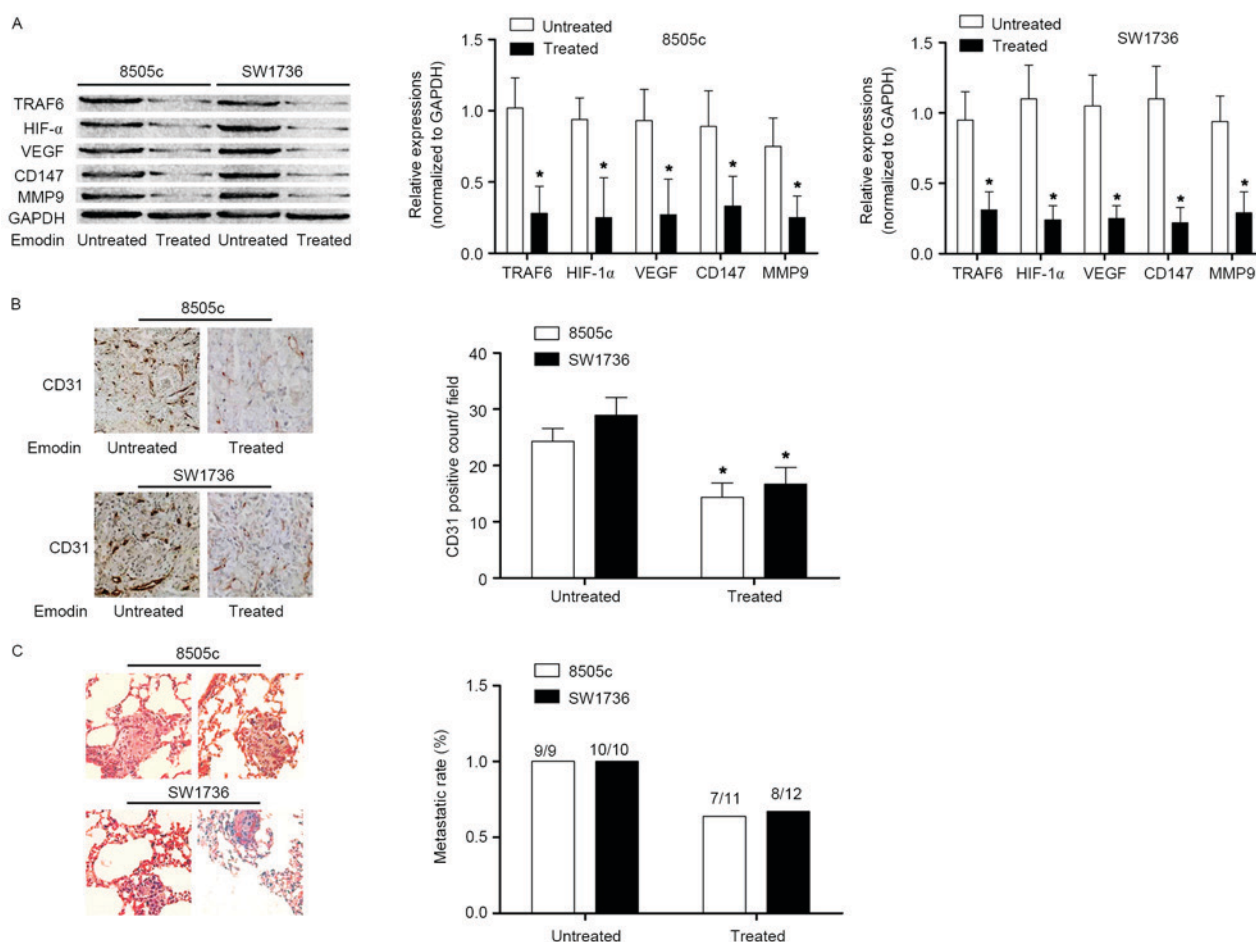


Figure 4. Effects of emodin on angiogenesis and metastasis in ATC *in vivo*. (A) Left Immunoblotting of TRAF6, HIF1 α , VEGF, CD147, MMP9 and GAPDH in ATC xenograft tissues harvested from nude mice treated or untreated with emodin. The graphs indicated the relative expression levels of TRAF6, HIF1 α , VEGF, CD147 and MMP9 (normalized to GAPDH) in 8505c and SW1736 xenografts, respectively. (B) Immunohistochemical staining of CD31 in harvested xenograft tissues of nude mice treated or untreated with emodin. The graph indicates the CD31-positive count. (C) Captured images (magnification, x200) of hematoxylin and eosin staining of lung metastatic lesions of nude mice receiving ATC cell injections. The graph indicates the metastatic rate of ATC lung metastasis in nude mice treated or untreated with emodin. *P<0.05 vs. respective untreated group. ATC, anaplastic thyroid cancer; TRAF6, TNF receptor-associated factor 6; CD147, basigin; MMP9, matrix metalloproteinase 9; HIF1 α , hypoxia-inducible factor 1- α ; VEGF, vascular endothelial growth factor; CD31, platelet endothelial cell adhesion molecule.

by upregulating the expression of HIF-1 α in the presence of dimethylxaloylglycine (10). Under certain pathological conditions, HIF-1 α translocates to the nucleus and binds to the hypoxia responsive element, initiating target gene transcription. VEGF has been demonstrated to be one of the important targets of HIF-1 α and a regulator of cancer angiogenesis (26). In the *in vitro* part of the present study, following emodin incubation, TRAF6/HIF-1 α signaling was markedly suppressed in a concentration-dependent manner. As a result, intra- and extracellular levels of VEGF were decreased. In the *in vivo* part, MVD was observed to be decreased following treatment with emodin, which additionally inhibited TRAF6/HIF-1 α /VEGF in the ATC xenografts.

Evidence has indicated the close association between TRAF6 and cancer metastasis in a number of human malignant tumors (8,13). CD147 belongs to the immunoglobulin family. In a recent study, CD147 was recognized to be a cofactor of TRAF6 which promoted membrane recruitment of CD147 (14). It was hypothesized that CD147 was an inducer of MMPs which served an important role in cancer metastasis and invasion, by degrading the extracellular matrix to promote

cancer cell invasion through vessel walls (27). Overexpression of MMP9 was demonstrated to be correlated with cancer distant metastasis (28). In the present study, it was observed that TRAF6/CD147 signaling was activated in ATC cells and their xenografts. The wound healing assay revealed that treatment with emodin impaired the migratory and invasive abilities of ATC cells. The results of the *in vivo* experiments demonstrated that emodin significantly decreased the lung metastatic rate of ATC. In addition, it was observed that emodin significantly suppressed the activation of the TRAF6/CD147/MMP9 pathway in ATC cells *in vivo* and *in vitro*.

In conclusion, increased expression of TRAF6 was observed in ATC cell lines and xenografts. Emodin exerted its anti-cancer activity against ATC by inhibiting proliferation, angiogenesis and metastasis. In particular, the results of the present study indicated that emodin suppressed ATC angiogenesis by inhibiting the TRAF6/HIF-1 α /VEGF pathway; in addition, emodin suppressed ATC metastasis by inhibiting the TRAF6/CD147/MMP9 pathway. The results of the present study indicated the potential therapeutic benefits of targeting TRAF6 in ATC.

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