



Asiaticoside Down-Regulates HIF-1 α to Inhibit Proliferation, Migration, and Angiogenesis in Thyroid Cancer Cells

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Background: Thyroid cancer (TC), the most prevalent endocrine malignancy, has been subjected to various treatment methods. However, the efficacy of asiaticoside (AC) for treating TC remains uncertain.

Aims: To explore the impact of AC on TC and determine its potential mechanisms of action.

Study Design: In vitro and in vivo cell line study.

Methods: We evaluated the effects of AC on human TC cell lines, namely TPC-1 and FTC-133. Both in vitro and in vivo experimental validations were conducted.

Results: AC significantly diminished the viability and proliferation of TC cells based on the CCK-8 assay and Edu staining findings.

Migration and invasion assays revealed that AC effectively curtailed the migration and invasiveness of TC cells. The tube formation assay demonstrated that AC substantially impeded TC cell-induced angiogenesis. Western blot assay revealed that AC significantly reduced the expression levels of TRAF6, HIF-1 α , and VEGFA, indicating that AC could potentially exert its anticancer effect by inhibiting the TRAF6/HIF1 α pathway. Our in vivo experiments, which involved administering AC to BALB/c nude mice injected with TPC-1 cells, demonstrated significant inhibition of tumor growth and reduction in the expression of Ki-67, TRAF6, HIF-1 α , and VEGFA.

Conclusion: Our study highlights the significant inhibitory effect of AC on TC, offering fresh insights and potential drug candidates for TC treatment.

INTRODUCTION

Thyroid cancer (TC) is the most common endocrine malignancy, and its incidence has increased globally in the last two decades. More than 95% of the TCs originate from follicular epithelial cells, with variable metastases to the lymph nodes.¹ Although surgical resection and radiometabolic therapy have been successfully used to treat a majority of TC cases, some TCs become refractory or metastasize. Angiogenesis, an unregulated and unrestricted biological process in tumors, induces tumor cell proliferation, local invasion, and hematogenous metastasis.² The transcription factor hypoxia-inducible factor 1 α (HIF1 α) plays a crucial role in hypoxic responses, activating various downstream effectors, including vascular endothelial growth factor (VEGF). VEGF expression is associated with tumor progression in numerous cancers, including those of the pancreas, breast, cervix, and thyroid. Overexpression of VEGF is associated with an increase in the growth, progression, invasion, and metastasis of TC cells.² TNF receptor-associated factor

6 (TRAF6), a member of the TRAF family, originally identified for its role in inflammatory signaling, has recently been implicated in cancer. Overexpression of TRAF6 regulates tumorigenesis and angiogenesis in several cancers, including those of the lung and pancreas.³ Inhibition of the TRAF6/HIF-1 α /VEGF pathway reportedly mitigates TC angiogenesis and metastasis.⁴ Recent studies have highlighted the therapeutic potential of active compounds from herbs. Asiaticoside (AC), a pivotal biochemical constituent isolated from *Centella asiatica*, reportedly wields significant anticancer properties against certain malignancies. It serves as a prominent inhibitor of the advancement of gastric cancer while simultaneously inducing endoplasmic reticulum stress.⁵ Moreover, AC can suppress the epithelial-mesenchymal transition (EMT) and stem cell-like traits of pancreatic cancer PANC-1 cells, by inhibiting the activation of p65 and p38MAPK.⁶ In triple-negative breast cancers, AC effectively restricts the EMT by enhancing the PPARG expression while simultaneously suppressing the P2RX7-facilitated TGF- β /Smad signaling pathway.⁷ Furthermore, AC directly



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opposes cell proliferation and considerably reduces resistance to chemotherapeutic drugs within hepatocellular carcinoma cells.⁸ Experimental studies have revealed that AC can subvert the malignancy of osteosarcoma cells, which is typically induced by macrophagic polarization of the M2 phenotype, via the inhibition of the TRAF6/NF- κ B pathway.^{9,10} However, the effects of AC on TC and their underlying mechanisms remain elusive. Herein, we aimed to evaluate the impact of AC on TC and determine their potential mechanisms of action.

MATERIALS AND METHODS

Cells and culture

Two TC cell lines (TPC-1 and FTC-133) were obtained from the Cell Resource Center, Peking Union Medical College, which is a part of the National Science and Technology Infrastructure (NSTI-BMCR; <http://cellresource.cn>). The cells were cultured in high-glucose DMEM (Invitrogen-Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin G, and 100 U/ml of streptomycin, and incubated at 37 °C in 5% CO₂. Based on the treatment to be administered, the cells were divided into the control or AC-treated groups, with AC concentrations of 5, 10, and 20 μ M/ml of medium.

Animals

Male BALB/c nude mice (8-12 weeks old) were procured from Beijing Viton Lever Laboratory Animal Technology Co., Ltd. The mice were housed in a pathogen-free facility with ad libitum access to food and water, under controlled temperature conditions of 22 °C. All experimental procedures were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the Second Affiliated Hospital of Wenzhou Medical University. The mice were allocated into two experimental groups: a control group and an AC-treated group (10 mg/kg).

Cell proliferation assay

For the cell proliferation assay, cells in the logarithmic growth phase were dispensed at 2,000 cells/well into 96-well plates and incubated overnight. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Solarbio, Beijing, China). In each well, 10 μ l of the CCK-8 reagent was added, and the plates were further incubated at 37 °C in a humidity-controlled environment. Absorbance at 450 nm was quantified for three days post-seeding using a microplate spectrophotometer (BioTek, Winooski, VT, USA).

5-Ethynyl-2'-deoxyuridine assay

Cell proliferation was determined using the 5-ethynyl-2'-deoxyuridine (Edu) assay. The cells were seeded on glass coverslips in 24-well plates at a density of 2 x 10⁴ cells/well and incubated at 37 °C for 24 h. Subsequently, the cell nuclei were stained with 4',6-diamidino-2-phenylindole and visualized using a fluorescence microscope (Olympus, Tokyo, Japan).

Cell migration and invasion assays

For the migration assay, a scratch test was performed. Initially, a confluent monolayer of cells was scratched with a sterile pipette

tip to create a wound. The cells were then washed with phosphate-buffered saline to remove detached cells and debris, and fresh serum-free medium was added. Cell migration into the wound area was monitored and photographed at 0, 12, and 24 hours after wounding.

For the invasion assay, Transwell chambers were utilized. The Transwell's upper compartment was pre-coated with 50 μ l of ECM gel (Matrigel; Matrigel: serum-free medium ratio, 1:5). After 4 h, 6 x 10⁴ cells in 200 μ l of serum-free medium were seeded in the upper chamber. The lower compartment was filled with 800 μ l of medium containing 10% FBS. After 24 h, non-invading cells in the upper chamber were removed. Cells that invaded through the membrane to the lower surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai, China). The invaded cells in five visual fields were counted using an inverted microscope (IX50; Olympus Tokyo, Japan) at 100 x magnification.

Tube formation assay

The impact of TC cells on angiogenesis in HUVECs was assessed using a tube formation assay. ECM gel (Matrigel; Sigma-Aldrich) was added to chilled 96-well plates and incubated at 37 °C for 2 h for polymerization. Subsequently, HUVECs (PromoCell, Heidelberg, Germany) were co-cultured with thyroid cell lines at 3.5 x 10⁴ cells/well and incubated for 5 h at 37 °C. Thereafter, the tube formation was imaged under an inverted microscope (AxioObserver D1; Zeiss). Tubule length and organization were analyzed using an angiogenesis analyzer (Image J plug-in) (NIH).

Western blotting

The cells were lysed to form debris and collect proteins. These proteins were denatured by mixing it with a loading buffer and boiling it for 5 min. Subsequently, they were subjected to SDS-PAGE and transferred to a PVDF membrane at 300 mA for 60-90 min. The membranes were blocked with 5% milk for 2 h and subsequently incubated overnight at 4 °C with primary antibodies against TRAF6 (Cat No. CL647-66498, 1:1000), HIF-1 α (Cat No. 66730-1-Ig, 1:1000), VEGFA (Cat No. 66828-1-Ig, 1:1000), and β -actin (Cat No. Biotin-60008, 1:1000) (all obtained from Proteintech). Thereafter, the membranes were treated with HRP-conjugated secondary antibody (Cat No. SA00001-2, 1:10,000; Proteintech). Bands were visualized using ECL solution and quantified by densitometry.

Statistical analysis

Data have been obtained from at least three independent experiments and are expressed as means \pm standard deviations. Normality of data distribution was assessed using the Shapiro-Wilk test. For comparing more than two groups, one-way ANOVA was used for independent groups and repeated measures ANOVA was used for dependent groups. Post-hoc analysis was performed using Tukey's HSD. Non-parametric data was analyzed using the Kruskal-Wallis and Dunn's tests. Differences in protein levels between normal thyroid and PTC samples were analyzed using the Mann-Whitney U test. A p-value of < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism (version 6.0;) and SPSS (version 20.0;).

RESULTS

AC inhibits the TC cell proliferation

Changes in cell viability in the TPC-1 and FTC133 cell lines were examined 24, 48, and 72 h after AC treatment using CCK-8 (Figure 1a). Compared to the control group, cell viability significantly reduced when AC concentrations reached 10 μM ($p < 0.01$) and 20 μM ($p < 0.001$). EDU staining revealed a significant decrease in the number of EDU-positive cells at 10 μM ($p < 0.001$) and 20 μM ($p < 0.001$) AC concentrations, indicating that AC inhibited TC cell proliferation in a dose-dependent manner (Figure 1b).

AC inhibits TC cell migration and invasion

The number or proportion of cells undergoing migration and invasion was significantly lower in the AC-treated groups than in the control group. Specifically, at 10 μM ($p < 0.001$) and 20 μM ($p < 0.001$) AC concentrations, there was a significant decrease in the number of cells undergoing migration and invasion (Figures 2a and 2b). The inhibitory effect of AC on TC cell migration and invasion was dose-dependent.

AC inhibits TC cell angiogenesis

The angiogenic ability of AC on TPC-1 and FTC133 cell lines was examined. After HUVEC inoculation, tube formation was

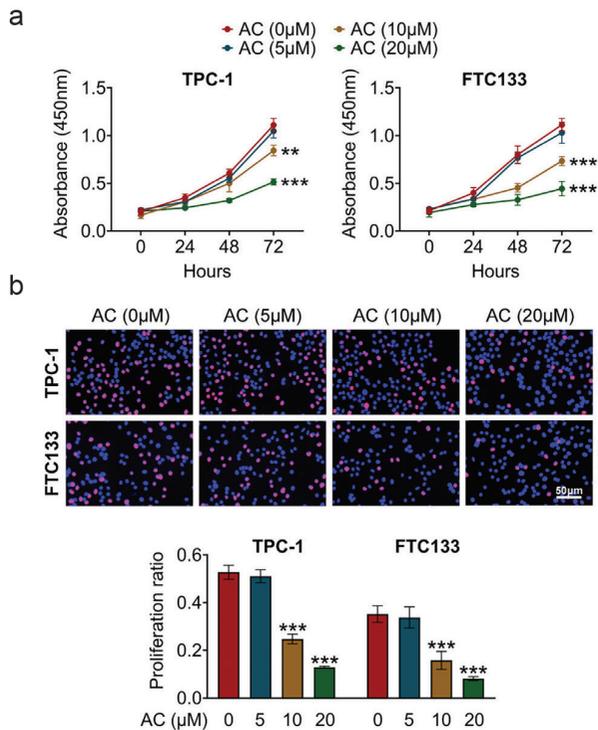


FIG. 1. Changes in cell viability and proliferation of TPC-1 and FTC133 cells after administration of AC (0, 5, 10, and 20 μM). (a) Changes in cell viability 24, 48, and 72 h after AC administration. (b) Cell proliferation (expressed as proliferation rate). Red color represents EDU-positive cells (N = 3). $**p < 0.01$ and $***p < 0.001$ vs. the control group. AC, Asiaticoside; EDU, 5-Ethynyl-2'-deoxyuridine.

observed in the control group and the group treated with 5 μM AC. However, when the AC concentration reached 10 μM ($p < 0.001$) or even 20 μM ($p < 0.001$), the number of tubes formed decreased significantly in a dose-dependent manner (Figure 3).

AC inhibits the TRAF6/HIF1 α pathway

AC may exert its inhibitory effect through the TRAF6/HIF1 α pathway. Thus, the expression levels of TRAF6, HIF-1 α , and VEGFA were examined using Western blot analysis. Compared to the control group, the relative protein expression of TRAF6, VEGFA (Figure 4a), and HIF-1 α (Figure 4b) in the TC cell lines significantly reduced in a dose-dependent manner when the AC

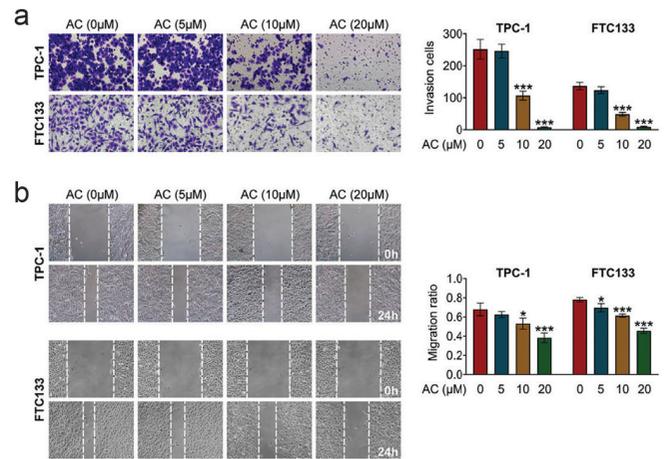


FIG. 2. Changes in invasion and migration of TPC-1 and FTC133 cells after administration of AC (0, 5, 10, and 20 μM). (a) Number of cells undergoing invasion in 24 h. (b) Distance migrated by the cell migration in 24 h (expressed as migration rate) (N = 3). $*p < 0.05$ and $***p < 0.001$ vs. the control group. AC, Asiaticoside.

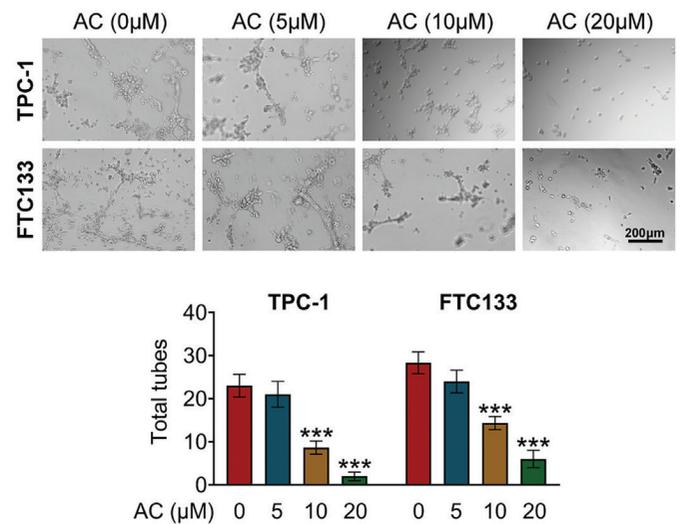


FIG. 3. Tube formation after administration of AC (0, 5, 10, and 20 μM) in TPC-1 and FTC133 cells inoculated with HUVEC (N = 3). $***p < 0.001$ vs. the control group. AC, Asiaticoside; HUVEC, human umbilical vein endothelial cell.

concentrations reached 10 μM ($p < 0.05$ or $p < 0.001$) and 20 μM ($p < 0.001$).

AC promotes TC progression via HIF1 α

To test the hypothesis that AC exerts its effects via HIF-1 α , different treatment groups were established: the control group, 20 μM AC alone treatment group, and treatment group expressing HIF-1 α and 20 μM AC. Compared to the control group, treatment with AC significantly decreased cell viability ($p < 0.001$) (Figure 5a), cell invasion ($p < 0.001$) (Figure 5b), migration ratio ($p < 0.001$) (Figure 5c), and tube formation ($p < 0.001$) (Figure 5d). However, in the group where HIF-1 α was expressed in addition to AC treatment, cell viability ($p < 0.001$), the number of EDU-positive cells ($p < 0.001$), migration ratio ($p < 0.001$), invasion ($p < 0.001$) and tube formation ($p < 0.01$) were significantly higher than in the group treated with AC alone.

AC inhibits tumor growth in vivo

An in vivo model of TC was established by injecting TPC-1 cells into BALB/c nude mice, and the effects of 10 mg/kg AC treatment were observed. In the group administered 10 mg/kg AC, the tumor size (Figure 6a), tumor volume (Figure 6b) at various time points, and tumor weight (Figure 6c) had reduced compared to in the control group. Immunohistochemical analysis revealed a decrease in the number of Ki-67 positive tumor cells in the AC-treated group ($p < 0.001$) (Figure 6d). Furthermore, the expression levels of TRAF6, HIF-1 α , and VEGFA were lower in the AC-treated group than in the control group ($p < 0.001$) (Figures 6e, f).

DISCUSSION

TC is an endocrine tumor with a high global incidence, which has been continuously increasing over the past decades.¹¹ It encompasses multiple subtypes, including papillary, follicular, medullary, and undifferentiated TC, each contributing to its diverse pathobiology and clinical presentation. Although a favorable prognosis is expected in most patients with TC, certain subtypes and advanced stages are associated with poorer outcomes. Currently available therapeutic options, though numerous, require improvements in both efficacy and tolerability. Hence, the exploration of novel therapeutic strategies and medications remains a significant clinical endeavor. Increasing evidence suggests that AC plays an important role in several diseases. AC demonstrates ROS-reducing effects, such as inhibiting TGF- β 1-induced MMT and ROS by activating Nrf2,¹² which protects the peritoneum and prevents peritoneal fibrosis.¹³ AC also demonstrates an anti-inflammatory effect, which modulates cutaneous allergic inflammation and could be developed as a therapeutic drug for atopic dermatitis.¹⁴ Recently, the antitumor effects of AC have been identified in several cancers. AC reportedly inhibits gastric cancer progression and induces endoplasmic reticulum stress.⁵ Additionally, AC inhibits triple-negative EMT in breast cancer.⁷ A terpene glycyrrhetic acid extract, a triterpenoid, reportedly reduces specific protein expression in TC cells. Thus, it could be used for the treatment of TC and other endocrine tumors.¹⁵ However, the effect of AC on TC remains unclear.

To explore the effect of AC on TC cells, we treated two TC cell lines (TPC-1 and FTC133) with AC in vitro. We found that AC treatment significantly reduced the viability, proliferation, migration, and invasiveness of the TC cells; it also significantly reduced its angiogenic ability. Jiang et al.¹⁶ identified the inhibitory effects of Rh2 (an anticancer-like molecule) on the migration and proliferation of TC cells. Deng and Sun¹⁷ found that Platycodonopsis saponin D effectively blocks PTC progression and prevents cell proliferation by arresting the cell cycle and enhancing apoptosis. These findings are consistent with the inhibitory effects of AC identified in this study, including the inhibition of cell migration, invasion, and proliferation. Furthermore, we found that AC inhibited the tube-forming ability of TC cell lines. Tumor angiogenesis is defined as the proliferation of a vascular network that provides a supportive microenvironment rich in oxygen and nutrients for the optimal growth of tumors. He et al.¹⁸ established that AC inhibited osteoclast formation and function, which is consistent with the findings in our study. However, recently AC has been found to demonstrate promote tubule formation when used as a dressing.¹⁹ This difference in the effect of AC on tubule formation may depend on the application scenario; in cancer, AC's ability to inhibit tubule formation is utilized.

In our study, we found that the expression levels of TRAF6, HIF-1 α , and VEGFA were significantly decreased after treatment with AC. This suggests that AC may exert its inhibitory effect on TC via the TRAF6/HIF1 α pathway. In the in vivo experiments, we injected TPC-1 cells into BALB/c nude mice and treated them with AC. AC significantly reduced the tumor volume and weight and

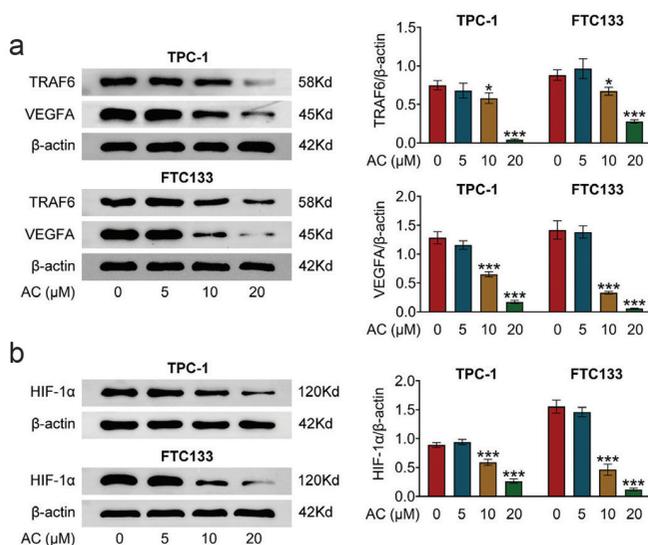


FIG. 4. Changes in the expression levels of TRAF6, VEGFA, and HIF-1 α in TPC-1 and FTC133 cells inoculated with HUVEC after administration of AC (0, 5, 10, and 20 μM) (values are expressed relative to the expression of β -actin). (a) Relative expression level of TRAF6 and VEGFA. (b) Relative expression level of HIF-1 α (N = 6).

* $p < 0.05$ and *** $p < 0.001$ vs. the control group.

VEGFA, Vascular endothelial growth factor-A; HUVEC, human umbilical vein endothelial cell.

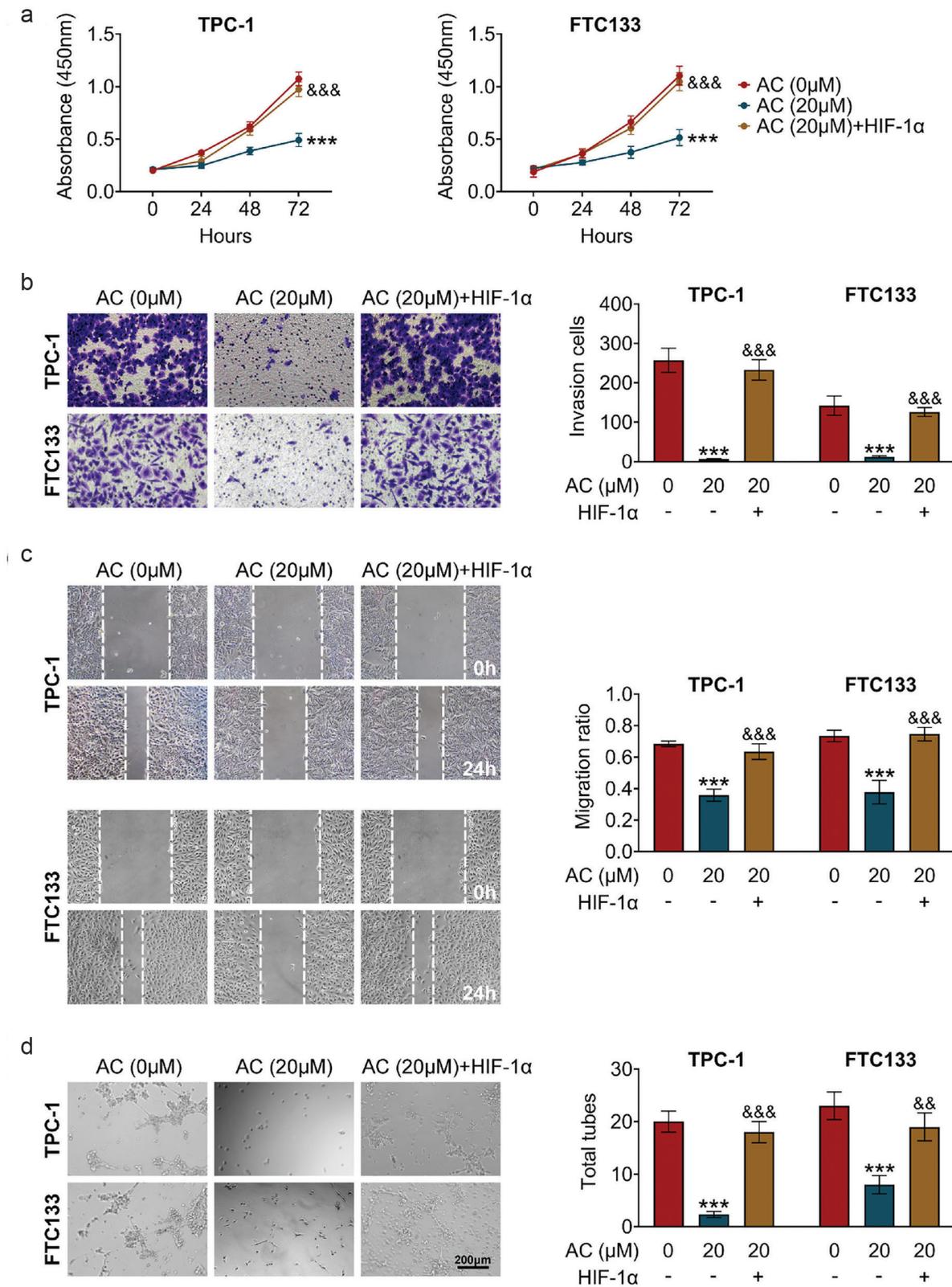


FIG. 5. Effects of AC (0 μM and 20 μM) treatment on cells were observed after overexpression of HIF-1α. (a) Changes in cell viability after 24, 48, and 72 h. Effect on (b) cell invasion, (c) cell migration, and (d) tube formation.

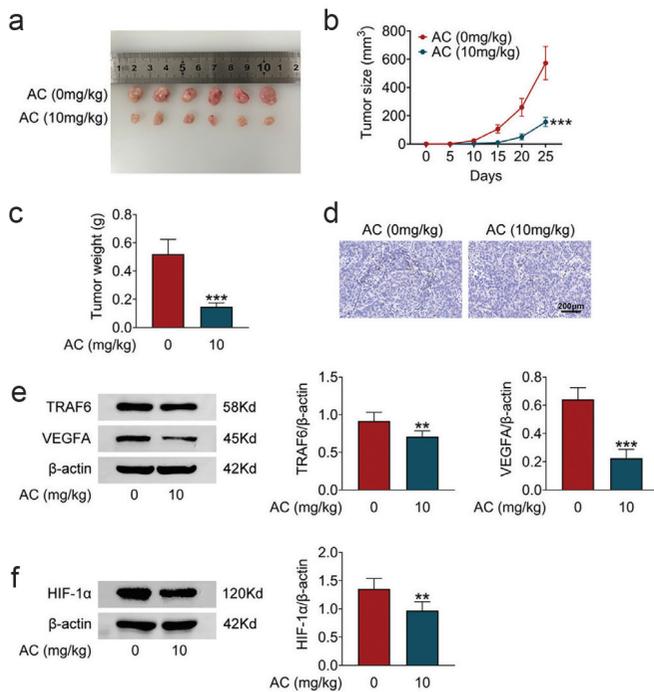


FIG. 6. In vivo model of TC was constructed by xenografting TPC-1 cells in Balb/c nude mice to observe the effect of AC (0 μ M and 10 mg/kg) on tumor growth. (a) Change in tumor size, (b) tumor volume at different time (0, 5, 10, 15, 20, and 25 days), and (c) tumor weight. (d) Immunohistochemistry. (e) Relative expression level of TRAF6 and VEGFA. (f) Relative expression level of HIF-1 α (N = 3). VEGFA, Vascular endothelial growth factor-A.

improved the survival rate of the mice. Additionally, AC decreased the expression of TRAF6, HIF-1 α , and VEGFA in tumor tissues. Zhang et al.²⁰ reported that KDM1A regulates TC stemness and promotes TC progression via the demethylation of HIF-1 α . Song et al.²¹ reported that the inhibition of HIF-1 α /YAP signaling alone or in combination with other potential markers was effective against aggressive PTC. These findings are consistent with those of our study. Additionally, overall, AC effectively inhibited TC development both in vivo and in vitro. These results suggest that AC suppresses TC progression and induces a stress response in vivo by inhibiting the TRAF6/HIF1 α pathway. This finding provides new clues and directions for subsequent in-depth studies.

Although our findings provide new insights into the understanding of AC as a potential anti-TC therapy, it has some limitations that need to be further addressed. First, our study relied primarily on two TC cell lines (TPC-1 and FTC133). Although these cell lines are widely used in research, they may not fully represent the entire biology of TC. Future studies should include a wider variety of TC cell lines or patient-derived cells to verify the applicability of our results in a wider range of situations.²² Second, our study relied heavily on in vitro experiments and in vivo models in nude mice. Although these models can mimic some of the properties of tumors in living organisms, they cannot fully simulate the complex physiological environment of the human body.²³ Therefore, our

findings need to be further confirmed through clinical studies. Furthermore, we mainly focused on the role of the TRAF6/HIF1 α pathway in the inhibition of TC by AC. However, there may be other pathways that are yet to be identified. Future studies should further explore the mechanism of action of AC, including other possible signaling pathways and molecular targets. Finally, although we found that AC has an inhibitory effect on TC, we could not clarify its dose-effect relationship in humans, possible side effects, and toxicity. This information is crucial for evaluating the potential of AC as an anticancer drug. However, our findings indicate that AC may be a promising anti-TC therapy. Further studies are needed to externally validate this and address the limitations of our study.

Our results indicate that AC has a significant inhibitory effect on TC, which was confirmed by both in vivo and ex vivo experiments. AC significantly inhibited the viability, proliferation, migration, invasion, and angiogenesis of TC cells. This inhibitory effect may be exerted by the inhibition of the TRAF6/HIF1 α pathway. These findings provide new evidence for the application of cumarins as an anti-TC drug. However, future studies that perform more in-depth evaluations and externally validate AC's mechanism of action are required.

Ethics Committee Approval: Ethical approval was obtained from the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Informed Consent: Not applicable.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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