Ellagic acid inhibits cell proliferation, migration, and invasion of anaplastic thyroid cancer cells via the Wnt/β-catenin and PI3K/Akt pathways

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INTRODUCTION

Thyroid cancer is the most common malignant endocrine tumor with an increasing incidence worldwide every year (Hsu et al., 2014; Haymart, 2021). Anaplastic thyroid cancer (ATC), also known as undifferentiated thyroid cancer, is a rare subtype of thyroid cancer. Although this subtype accounts for 1–2% of all thyroid cancers, it is the most lethal subtype (Molinaro et al., 2017). Unfortunately, there is currently no effective treatment for ATC. Previous studies have shown that traditional cancer treatments, including surgery, chemotherapy and radiotherapy, do not significantly improve the prognosis of ATC patients (Tuttle et al., 2017; Tiedje et al., 2018). Therefore, it is important to explore novel ATC treatment strategies. Attenuation of proliferation, migration, and invasion of ATC cells is considered an effective strategy for suppressing the development of ATC.

Ellagic acid (EA) (molecular formula C14H8O6; Fig. 1A) is a natural polyphenolic compound and dimeric gallic acid derivative that is widely distributed in various fruits and nuts. EA possesses a wide range of biological activities, including antioxidant, antiviral, and antitumor activities (Shakri et al., 2018). In the field of antitumor therapy, EA has been reported to exert inhibitory effects on various cancers, such as colorectal, breast, and prostate cancers (Ceci et al., 2018). Additionally, in our previous study, we demonstrated that EA inhibited the proliferation and migration of hypertrophic scar fibroblasts (Liu et al., 2021). However, the role of EA in ATC remains unclear.

The canonical Wnt/β-catenin and PI3K/Akt pathways play key roles in tumor development. Unphosphorylated β-catenin enters the nucleus and initiates the transcription of downstream target genes; the nuclear β-catenin level represents the activation level of the Wnt/β-catenin pathway (Rao & Kühl, 2010).Akt is a key molecule involved in the PI3K/Akt pathway, and its phosphorylation level represents the activation level of this pathway (Jiang et al., 2020). Aberrant activation of the Wnt/β-catenin and PI3K/Akt pathways is associated with ATC progression (Saji & Ringel, 2010; Sastre-Perona & Santisteban, 2012; Jiang et al., 2022; Zhang et al., 2022).

In this study, we evaluated the effects of EA on proliferation, migration, and invasion of ATC cells. Furthermore, we investigated whether Wnt/β-catenin and PI3K/Akt signaling pathways are involved in EA-induced regulation.
MATERIALS AND METHODS

Cells culture

The normal human thyroid cell line Nthy-ori3-1 (cat. no. CTCC-003-0031) was obtained from Meisen Cell (Zhejiang, China), and ATC cell line BHT-101 (cat. no. BCNC-S59868) was obtained from the BeNa Culture Collection (Beijing, China). Both cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C with 5% CO2. All cell culture reagents were purchased from Gibco (Thermo Fisher Scientific, MA, USA).

Antibodies and drugs

The primary antibodies used for western blotting were as follows: anti-β-catenin (1:5000, cat. no. ab32572; Abcam, Cambridge, UK), anti-Histone H3 (1:2000, cat. no. ab1791; Abcam), anti-phospho-pan-Akt (1:500; cat. no. AF0016; Affinity, OH, USA), anti-pan-Akt (1:500; cat. no. AF6261; Affinity), and anti-β-actin (1:2000; 60008-1-Ig; Proteintech, IL, USA). The secondary antibodies (1:5000; goat anti-mouse cat. no. SA00001-1, goat anti-rabbit cat. no. SA00001-2; Proteintech) were used for western blotting analysis. EA (cat. no. B21073; Yuanye Biotech, Shanghai, China) was dissolved in 1 M NaOH. The final concentration of NaOH was ≤ 0.1% (v/v), which did not induce toxicity (Duan et al., 2019). Lithium chloride (LiCl) (cat. no. L9650; Sigma-Aldrich, MO, USA), an agonist of the Wnt/β-catenin pathway, was dissolved in sterile double-distilled water and used at a concentration of 25 mM. Recombinant human insulin-like growth factor-1 (IGF-1) protein (cat. no. 291-G1; R&D Systems, MN, USA), an agonist of the PI3K/Akt pathway, was dissolved in RPMI 1640 and used at a concentration of 100 ng/mL.

Cytotoxicity assay

The Cell Counting kit (CCK-8) assay was used to perform the cytotoxicity assay. The assay was performed as previously described (Liu et al., 2021). Briefly, cells were seeded at a density of 5×104 cells per well in a 96-well plate (n=3). After cell attachment, the cells were treated with varied concentrations of EA (0–160 µM). After treatment for 24 hours, 10 µL of CCK-8 (cat. no. 40203ES60; Yeasen, Shanghai, China) reagent was added to each well. After incubation for 2 hours at 37°C, the absorbance was measured at 450 nm wavelength using a microplate reader (Thermo Fisher Scientific, MA, USA). The results were expressed as cell survival rates. Cell survival rate (%) = (OD value of treated group – OD value of blank) / (OD value of control group – OD value of blank) × 100.

Proliferation assay

A CCK-8 assay was used to assess cell proliferation. BHT-101 cells were seeded at 5×103 cells per well in a 96-well plate (n=3), and the OD values were measured at 24, 48, and 72 h after treatment according to the experimental requirements. In addition, the OD value detection method was the same as that of the cytotoxicity assay. The results are expressed as OD values.

A plate colony formation assay was performed to assess cell proliferation. BHT-101 cells were seeded at a density of 200 cells/well. After incubation for 12 days at 37°C, colonies were fixed with 4% paraformaldehyde (cat. no. P1110; Solarbio, Beijing, China) for 20 min at room temperature and stained with 0.5% crystal violet stain solution (cat. no. 60506ES60; Yeasen) for 10 min at room temperature, and the number of colonies was counted using ImageJ software (version 1.51w).

Migration assay

A wound healing assay was used to assess cell migration. This assay was performed as previously described (Liu et al., 2021). Briefly, BHT-101 cells were cultured in a 12-well plate (n=3) with RPMI 1640 containing 10% FBS until the cell confluence reached 100%. A scratch wound was made in the middle of each well using a 200-µL pipette tip. The culture medium was then replaced with serum-free RPMI 1640 medium. At 0 and 24 h after scratching, images were captured in five random fields of view using an inverted light microscope (magnification 100×; Olympus Corporation, Tokyo, Japan). The wound area was measured using ImageJ software. The results were expressed as migration rates. Migration rate (%) = (initial wound area – wound area at 24 h)/initial wound area ×100.

Transwell invasion assay

Transwell invasion assay was used to assess cell invasion. Transwell plates (24-well, 8 µm; Labgic, Beijing, China) were pre-coated with Matrigel (BD Biosciences, CA, USA) for 30 min at 37°C. BHT-101 cells were seeded in the upper chambers of Transwell plates (n=3) at a density of 5×104 cells/well and incubated in serum-free RPMI 1640 medium. The lower chambers were supplied with a culture medium containing 10% FBS. After 48 h, non-invading cells on the upper face of the membrane were removed with a cotton swab, and the invaded cells were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.5% crystal violet stain solution for 10 min. Images were captured in five random fields of view using an inverted light microscope (magnification 200×). The number of cells was counted using ImageJ software.

Western blotting

Western blotting was used to assess protein expression levels. The harvested cells were processed using a nuclear and cytoplasmic protein extraction kit (cat. no. P0028; Beyotime, Shanghai, China) to extract nucleoproteins and RIPA lysis buffer (cat. no. 9806S; CST, MA, USA) to extract the total protein. Western blotting was performed to detect the levels of β-catenin and Histone H3 (loading control) in nucleoprotein, and total Akt (t-Akt), phosphorylated Akt (p-Akt), and β-actin (loading control) in total protein, as described previously (Liu et al., 2021). Samples were separated using 10% SDS-PAGE gels, and subsequently electro-transferred to PVDF membranes (Millipore, MA, USA) for immunoblotting analysis. After incubation with primary antibodies, the membranes were incubated with the corresponding secondary antibodies. Protein chemiluminescence was detected using the Super ECL Detection Reagent (cat. no. 36208ES60; Yeasen) using a KETA GL Imaging System (Wealtec, NV, USA), and the gray values of the bands were quantified using ImageJ software.

Statistical analysis

The quantified data are presented as mean ± standard deviation (S.D.). The data were assessed for normality
using the Shapiro-Wilk test and were analyzed by one-way ANOVA followed by post hoc Dunnett’s or Sidak test using the GraphPad Prism (version 7.03) statistical package. A P-value of <0.05 indicated a statistically significant difference.

RESULTS

Effect of EA on the proliferation of ATC cells

To determine the appropriate concentration of EA for use in ATC cells, we performed a cytotoxicity assay for EA on Nthy-ori3-1 cells, a normal human thyroid cell line, using the CCK-8 assay. The results showed that EA did not cause significant cytotoxicity in normal cells at a concentration ≤100 μM, which suggested the concentration range used in the following experiments (Fig. 1B). We also examined the cytotoxicity of EA in BHT-101 cells, an ATC cell line, and found that ATC cells were more tolerogenic (Fig. 1B). Next, we evaluated the effect of EA on ATC cell proliferation using CGK-8 and colony formation assays. As shown in Fig. 1C, ATC cell viability was significantly reduced in the EA-treated group (40 or 80 μM) at all time points. Notably, as the concentration of EA increased, cell viability significantly decreased. Additionally, the results of the colony formation assay showed that the colony number of ATC cells in the EA-treated group (40 or 80 μM) was significantly lower than that in the control group (0 μM), and a higher EA concentration resulted in lower colony number (Fig. 1D and 1E), which exhibited a similar trend to the CCK-8 assay. These data indicated that EA inhibited the proliferation of ATC cells in a dose-dependent manner.

Effect of EA on the migration of ATC cells

To evaluate the effect of EA on ATC cell migration, we performed a wound healing assay. As shown in Fig. 2A and 2B, the migration rate of ATC cells from the treated group (40 or 80 μM) was significantly lower than that of the control group (0 μM). As the concentra-
tion of EA increased, the migration rate of ATC cells decreased. These data indicated that EA inhibited the migration of ATC cells in a dose-dependent manner.

Effect of EA on the invasion of ATC cells

To evaluate the effect of EA on the invasion of ATC cells, we performed the Transwell invasion assay to assess cell invasion ability. As shown in Fig. 3A and 3B, the number of invaded ATC cells in the treated group (40 or 80 µM) was significantly lower than that in the control group (0 µM). With an increase in EA concentration, the invasion of ATC cells decreased. These data indicated that EA inhibited the invasion of ATC cells in a dose-dependent manner.

Role of Wnt/β-catenin pathway in EA-induced regulation of ATC cells

We further investigated the role of the Wnt/β-catenin pathway in EA-induced inhibition of ATC cells. First, we evaluated the effect of EA (80 µM) on this signaling pathway in ATC cells. The nucleoprotein level of β-catenin, a marker of the Wnt/β-catenin signaling pathway, was examined by western blotting. As shown in Fig. 4A, EA treatment significantly reduced the level of β-catenin, suggesting that EA inhibited the Wnt/β-catenin pathway in ATC cells. Next, we used LiCl (25 mM), an agonist of the Wnt/β-catenin pathway, to verify whether this signaling pathway was involved in EA-induced inhibition of ATC cells. The results of western blotting showed that LiCl significantly up-regulated the level of β-catenin, indicating that EA inhibited the PI3K/Akt pathway in ATC cells. Next, we used IGF-1 (100 ng/mL), an agonist of the PI3K/Akt pathway, to investigate whether this pathway was involved in the EA-induced regulation of ATC cells. The results of western blotting showed that IGF-1 significantly increased Akt phosphorylation levels (IGF-1 treated group vs. untreated group), and it significantly reversed EA-induced inhibition of Akt phosphorylation level (EA+IGF-1 group vs. EA treated group), which proved the validity of IGF-1 as an agonist of the PI3K/Akt pathway (Fig. 5A).

Role of the PI3K/Akt pathway in EA-induced regulation of ATC cells

In addition to the Wnt/β-catenin pathway, we evaluated the role of the PI3K/Akt pathway, another ATC-associated signaling pathway, in the EA-induced antitumor effects. First, we assessed the effect of EA (80 µM) on the PI3K/Akt pathway by examining the phosphorylation level of the Akt protein, which was determined by the p-Akt/t-Akt ratio. As shown in Fig. 5A, EA treatment significantly decreased Akt phosphorylation levels, indicating that EA inhibited the PI3K/Akt pathway in ATC cells. Next, we used IGF-1 (100 ng/mL), an agonist of the PI3K/Akt pathway, to investigate whether this pathway was involved in the EA-induced regulation of ATC cells. The results of western blotting showed that IGF-1 significantly increased Akt phosphorylation levels (IGF-1 treated group vs. untreated group), and it significantly reversed EA-induced inhibition of Akt phosphorylation level (EA+IGF-1 group vs. EA treated group), which proved the validity of IGF-1 as an agonist of the PI3K/Akt pathway (Fig. 5A). We then evaluated the effects of activating the PI3K/Akt pathway on the EA-induced suppression of cell proliferation, migration, and invasion of ATC cells. The results showed that activation of the PI3K/Akt pathway significantly reversed the EA-induced inhibition of cell proliferation (Fig. 5B and 5C), migration (Fig. 5D), and invasion (Fig. 5E). These data demonstrated that EA attenuated the proliferation, migration, and invasion of ATC cells by inhibiting the PI3K/Akt pathway.

DISCUSSION

In recent years, an increasing number of natural plant extracts have attracted the attention of researchers owing to their low toxicity and few side effects. Some natural extracts have been reported to possess anti-ATC activities. Yu and others found that chrysin (Yu et al., 2013b), a natural flavonoid, and resveratrol (Jaskula-Sztul et al., 2013a), a natural polyphenol phytoalexin, suppressed the growth and Notch1 signaling pathway in multiple ATC cell lines. Patel and others found that hesperetin, a natural flavonoid, exerted antitumor effects on ATC cells by inducing cell apoptosis and cellular differentiation (Patel et al., 2014). Lepore et al. found that cynaropicrin, a natural sesquiterpene lactone, inhibited the proliferation of ATC cells by reducing NF-κB expression and STAT3 phosphorylation (Lepore et al., 2019). Our study, for the first time, demonstrated that EA inhibited the tumor phenotypes of ATC cells and identified associated downstream signaling pathways, which expands our knowledge in the field.
Uncontrollable proliferation is an important characteristic of tumor cells. EA has been reported to inhibit the proliferation of multiple cancers. Malik and others reported that EA treatment inhibited the proliferation of prostate cancer cells (Malik et al., 2011). In ovarian carcinoma, EA was found to inhibit the growth of ES-2 and PA-1 cells (Chung et al., 2013). Wang and others suggested that EA exerts an inhibitory effect on the proliferation of glioblastoma cells (Wang et al., 2016). Similar to the above results, our study demonstrated that EA treatment effectively suppressed the proliferation of ATC cells (Fig. 1). Cell migration and invasion play key roles in the metastasis of malignant tumor. It has also been shown to inhibit cell migration and invasion in some cancers. Xu and others reported that EA inhibits the migration and invasion of osteosarcoma cells by down-regulating c-Jun expression (Xu et al., 2018). In melanoma, EA was found to be able to inhibit cell migration and invasion of WM115 and A375 cells via the epidermal growth factor receptor pathway (Wang et al., 2020). Our results were consistent with these studies, and we found that EA attenuated the migration and invasion of ATC cells (Fig. 2 and 3). In addition, the antiproliferative activity of EA was verified in these two studies. These findings may reveal the broad suppressive effects of EA on cancer cell proliferation, migration, and invasion. However, this has yet to be experimentally confirmed in other cancers.

Previous studies on EA against cancers have revealed that regulation of signaling pathway is involved. Anitha and others found that EA treatment suppressed the Wnt/β-catenin pathway in an animal model of oral oncornogenesis (Anitha et al., 2013). In another in vitro study, EA inhibited the Wnt/β-catenin pathway in colon cancer cells (Fang et al., 2015). Similar to these two studies, our work showed the inhibitory effect of EA on the Wnt/β-catenin pathway, and further demonstrated that this pathway was involved in EA-induced suppression of the pathological phenotypes of ATC cells (Fig. 4). As for the PI3K/Akt pathway, another ATC-associated signaling pathway, Liu et al. reported that EA exerted an inhibitory effect on this pathway in A549 lung cancer cells (Liu et al., 2018). Our study also showed that EA treatment inhibited the PI3K/Akt pathway, by which EA attenuated the proliferation, migration, and invasion of ATC cells (Fig. 5). These results enrich the mechanistic research on the antitumor activity of EA. Our study elaborated on specific signaling mechanisms for...
The EA-induced inhibition of ATC cells (Fig. 6, created with BioRender.com). The mechanisms underlying the EA-induced regulation of ATC remain to be elucidated. The present study has several limitations; the effects of EA on other phenotypes of ATC cells, such as drug resistance, remain to be investigated. Further in vivo animal

Figure 5. Role of the PI3K/Akt pathway in EA-induced regulation of ATC cells. (A) The level of the PI3K/Akt pathway was assessed by detecting the phosphorylation level of Akt using western blotting. Cell proliferation assay was performed using the (B) CCK-8 assay and (C) colony formation assay. (D) Cell migration assay was performed using the wound healing assay (yellow dashed lines denote the wound edge; scale bar =100 µm). (E) Cell invasion assay was performed using the Transwell invasion assay (scale bar =50 µm). EA was used at 80 µM; IGF-1 was used at 100 ng/mL as a pathway agonist. Data are expressed as mean ± S.D; *P<0.05 and **P<0.01.

Figure 6. Schematic illustration of this study. EA inhibits cell proliferation, migration, and invasion of ATC cells via the Wnt/β-catenin and PI3K/Akt pathways.
experiments are required to validate the antitumor effect of EA on ATC. These issues will be explored in future research.

In conclusion, this study demonstrated that EA inhibits the proliferation, migration, and invasion of ATC cells by inhibiting the Wnt/β-catenin and PI3K/Akt signaling pathways. These findings support EA as a candidate drug for ATC treatment and provide a theoretical basis for further in vivo experiments and clinical applications.

Declarations

We thank Editage team for language editing.

REFERENCES


