

Gallic acid from *Terminalia chebula* inhibited the growth of esophageal carcinoma cells by suppressing the Hippo signal pathway

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ABSTRACT

Objective(s): To explore the molecular mechanism of gallic acid (GA) from *Terminalia chebula* in suppressing the growth of esophageal carcinoma (EC).

Materials and Methods: Human EC cells (EC9706 and KYSE450) were treated with different concentrations of GA (10, 20, and 40 µg/ml), which were subjected to 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, plate clone formation assay, annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining, and Western blotting. EC mice were divided into model, 0.3% GA, and 1% GA groups to observe the tumor volume and the expressions of YAP, TAZ, Ki-67, and caspase-3 in tumor tissues.

Results: GA decreased cell viability and colony formation of EC9706 and KYSE450 cells, which was more obvious as the concentration increased. In addition, GA promoted cell apoptosis in a concentration-dependent manner with the up-regulation of pro-apoptotic proteins (Bax, cleaved caspase-3, and cleaved caspase-9) and nuclear YAP/TAZ, as well as the down-regulation of anti-apoptotic protein Bcl-2 and the levels of p-YAP and p-TAZ. Moreover, GA decreased the growth of xenograft tumor *in vivo*, with the reduction in the tumor volume and the reduction of YAP and TAZ expressions in the tumor tissues. In addition, Ki-67 expression in GA groups was lower than those in the Model group, with the increase in caspase-3 expression in the tumor tissues. Changes aforementioned were obviously shown in the 0.3% GA group.

Conclusion: GA blocked the activity of the Hippo pathway to suppress cell proliferation of EC and facilitate cell apoptosis, which is expected to be a novel strategy for treatment of EC.

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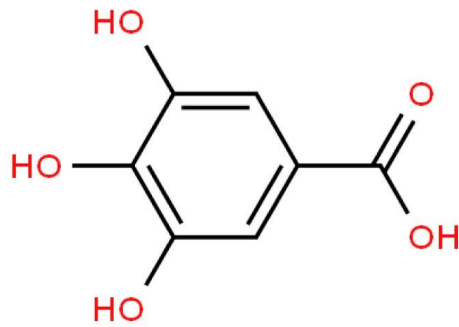
Introduction

Esophageal carcinoma (EC) is the common cause of malignant dysphagia and tumor-related death, which ranks eighth in the prevalence and sixth in the mortality rate in all malignancies due to high malignancy (1). As estimated, more than 4.50 million people were afflicted by the EC worldwide, and its prevalence keeps rising in recent years (2). In China, the 5-year survival rate of EC was only 3.8%, since most patients have progressed into middle/advanced stage, or even metastasis when diagnosed, owing to the difficulties in early diagnosis (3). In the meantime, surgical treatment remains the top option for EC, which would lead to relatively poor prognosis, and patients are susceptible to recurrence and metastasis (4). In addition, the current available anti-tumor drugs may also bring about the deleterious side effects on patients (5), which may greatly encourage find new anti-tumor agents for EC with fewer adverse effects.

The natural botanical anti-tumor drugs have gained increasing attention among scholars in China and other countries. For example, *Terminalia chebula* (*T. chebula*), a traditional medicine belonging to the genus *Terminalia*, family Combretaceae, consists of tannin, polyphenol, triterpene, flavone, lipids,

and amino acids (6). Gallic acid (GA), as the major component of *T. chebula*, has been reported to have various biochemical and pharmacological properties, including anti-inflammation, anti-virus, anti-oxidation, and cardiovascular protection (7, 8). GA (3,4,5-trihydroxybenzoic acid) is a common organic compound in the form of polyphenol with molecular weight of 170.12 and molecular formula of C₇H₆O₅ (9), as shown in Figure 1. As research progresses, it is important to emphasize that GA has been identified to be cytotoxic to cancer cells, exerting promising anti-tumor effects on different types of cancer cells, such as lung cancer, gastric cancer, and ovarian cancer (10-12). Besides, GA was found to be able to decrease the secretion of leptin from the oral squamous carcinoma cells, with the decrease of p44/42 MAPK, thereby altering the biological activities of tumor cells (13). However, the evidence focusing on the role and mechanism of GA in EC remains scant, requiring more effort. Furthermore, several lines of evidence support the causal role of GA in cancers through modulation of a certain signal pathway (14, 15). Hippo, a highly conservative signal pathway that can modulate organ volume and cellular proliferation and apoptosis, has been reported to be involved in the development of multiple diseases and

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Molecular formula: $C_7H_6O_5$

Figure 1. The molecular structural formula of GA
GA: gallic acid

tumors, including EC (16-19). Nevertheless, whether GA can affect EC by mediating the Hippo pathway remains unclear. In this regard, this study analyzed the effect of GA with different concentrations on proliferation and apoptosis of EC9706 and KYSE450 cells via mediating the Hippo pathway. Meanwhile, the EC mice models were constructed to further clarify the effect of GA on tumor growth and Hippo signal pathway.

Materials and Methods

Ethics statement

All the experiments of this study gained the approval of the Ethics Committee of this Hospital, and were carried out in accordance with the Ethical decision making about animal experiments (20).

Cell culture

The human EC cell lines (EC9706 and KYSE450), purchased from American Type Culture Collection (ATCC, USA), were cultured in RPMI-1640 medium, supplemented with 10% deactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C and 5% CO₂.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay

Cells in logarithmic phase were inoculated on a 96-well plate (5×10^3 /well), followed by culture at 37 °C and 5% CO₂. GA at different concentrations (0, 10, 20, and 40 µg/ml) was added to the wells for incubation. At 12, 24, 36, 48, and 72 hr, cells were further incubated with 10 µl of MTT (Sigma, USA) for 4 hr without light, and then 100 µl of DMSO (Sigma, USA) on a shaker for 10 min without light. The optical density (OD) of each well was determined at wavelength of 570 nm by using a microplate reader in triplicate. As per the method mentioned in a previous study, high performance liquid chromatography (HPLC) was carried out to determine the level of GA from *T. chebula* (21).

Plate clone formation assay

Cells were inoculated onto the 96-well plate supplemented with GA at different concentrations (0, 10, 20, and 40 µg/ml), and after confluence, cells were digested in the EDTA-supplemented trypsin that was terminated by the addition of serum. Thereafter, cells

were centrifuged at 1500 rpm for 5 min to remove the supernatant, and the sediment was resuspended in 1 ml medium, followed by cell counting through trypan blue staining. Then, cells at density of 2000/well were seeded evenly on a 6-well plate, where they were further incubated in 2 ml medium at 37 °C and 5% CO₂. Six days later, the number of colonies was counted (22, 23). This experiment was conducted in triplicate.

Flow cytometry to measure cell apoptosis

Seeded on a 96-well plate, cells were treated with GA in different concentrations (0, 10, 20, and 40 µg/ml) for 48 hr. Thereafter, 1 ml trypsin was added for cell digestion for 5 min, and the cells were transferred into the centrifuge tubes for centrifugation at 5000 rpm at 4 °C for 5 min. Sediment, instead of supernatant, was rinsed in PBS twice. Following that, sediment was labeled in 5 µl of Annexin V-fluorescein isothiocyanate (FITC) for 20 min, and 5 µl of propidium iodide (PI) for 20 min. After incubation, a flowcytometer was used for analysis, and cells located in the right upper quadrant (AnnexinV-FITC⁽⁺⁾/PI⁽⁺⁾) and lower quadrant (Annexin V-FITC⁽⁺⁾/PI⁽⁻⁾) were taken as the apoptotic cells. This experiment was conducted in triplicate.

Western blotting

Cells were lysed with a nuclear and cytoplasmic protein extract kit (Biotেকে, Shanghai, China), and the concentration of proteins was determined by using a bicinchoninic acid (BCA) kit (Thermo, Rockford, IL, USA). Proteins were then separated by performing the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred on the poly(vinylidene fluoride) (PVDF) membranes. Then, the unoccupied sites on the membrane, except the proteins, were blocked by incubating with 5% skimmed milk at 4 °C overnight, followed by incubation with anti-Bax (1/2000, ab32503, Abcam, USA), anti-cleaved-caspase-3 (ab2302, 1 µg/ml, Abcam, USA), anti-cleaved-caspase-9 (ab2324, 1 µg/ml, Abcam, USA), anti-Bcl-2 (ab32124, 1/1000, Abcam, USA), anti-YAP (phosphor, ab226760, 1/5000, Abcam, USA), anti-YAP (ab52771, 1/5000, Abcam, USA), anti-TAZ (phosphor, AF4315, 1/1000, Affinity Biosciences, USA), anti-TAZ (DF4653, 1/1000, Affinity Biosciences, USA), anti-β-actin-loading control (ab8227, 1/1000, Abcam, USA), and anti-TATA binding protein TBP-nuclear loading control (ab51841, 1 µg/ml, Abcam, USA). After three washes (5 min/wash) with PBS at room temperature, the secondary antibody (anti-rabbit IgG, HRP-linked, ab97051, 1/2000, Abcam, USA) was added for incubation at 37 °C for 1 hr, which was terminated by 3 washes in PBS. Membrane was then immersed in the electrochemiluminescence (ECL) reagent (Pierce, Waltham, MA, USA) for 1 min at room temperature, and later, covered by plastic film with the residual liquid being discarded. In a dark room, the protein bands were developed. Expressions of target proteins were normalized to that of the β-actin or TBP.

Construction of EC models on mice

The EC models on mice were constructed according to the previous study (24). Trypsin (2 ml) was added for the digestion of KYSE450 cell for 5 min at 37 °C with 5% CO₂, which were centrifuged and collected, with the

supernatant being discarded. They were resuspended in the fresh medium, and inoculated into the 6-week-old male BALB/cA nude mice at $1 \times 10^7/100 \mu\text{l}$ ($n = 15$) at the dorsum of right-sided thigh, with $100 \mu\text{l}$ Matrigel. From the 7th day, these mice were divided into three groups randomly, *i.e.*, Model group (normal drinking water), 0.3% GA group (0.3% GA w/v in drinking water), and 1% GA group (1% GA w/v in drinking water), with 5 mice in each group. Treatment was repeated once per week for 4 weeks continuously.

Immunohistochemistry staining

Twenty-eight weeks later, mice were euthanized under anesthesia to obtain the tumor tissues that were fixed in 4% neutral formaldehyde. Thereafter, samples were embedded in paraffin and then sliced into sections for hydration. Sections were blocked in PBS containing 50% goat serum for 30 min. Then, sections were incubated with anti-YAP (ab52771, 1/50, Abcam, USA), anti-TAZ (ab224239, 1/50, Abcam, USA), anti-Ki-67 (ab15580, 5 $\mu\text{g/ml}$, Abcam, USA), and anti-caspase-3 (ab31325, 1/25, Abcam, USA) at 4 °C overnight, followed by two washes with PBS (5 min/wash). Incubation was then performed with anti-rabbit IgG (HRP-linked, ab97051, 1/2000, Abcam, USA), and ended by three washes with PBS (3 min/wash). At last, sections were mounted in

neutral balsam following color development in DAB, counterstaining in hematoxylin, dehydration in ethanol in gradient concentrations, and clearing in xylene.

Statistical methods

Data were analyzed by using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). Measurement data were presented by mean \pm standard deviation, and compared among groups by using One-way ANOVA followed by Tukey's honest significant difference (HSD) test in post-hoc comparisons. $P < 0.05$ suggested the statistical significance of difference.

Results

GA inhibits the proliferation and colony formation of EC cells

In comparison with the Control group, GA resulted in no significant changes in the cell viability of EC9706 and KYSE450 cells at 12 hr, but obvious decline was found in the cell viability of EC cells from 24 hr to 72 hr with the increase of the GA concentrations (all $P < 0.05$, Figure 2). Furthermore, the inhibitory effect of GA on the EC cells was detected by the plate colony formation assay, and consequently, GA treatment could significantly restrict the colony formation of EC9706 and KYSE450 cells in a concentration-dependent manner (Figure 3).

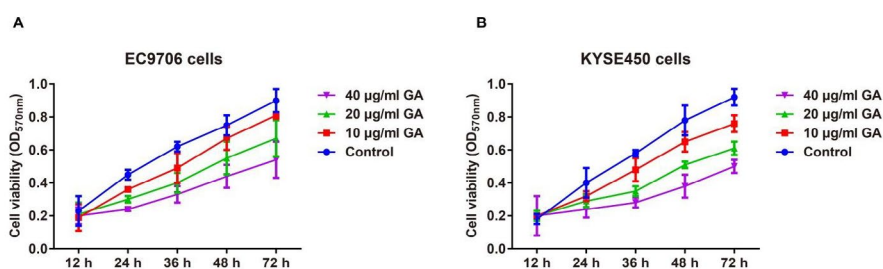


Figure 2. Effect of different concentrations (10, 20, and 40 $\mu\text{g/ml}$) of GA on the proliferation abilities of EC9706 (A) and KYSE450 (B) cells by MTT assay
GA: gallic acid

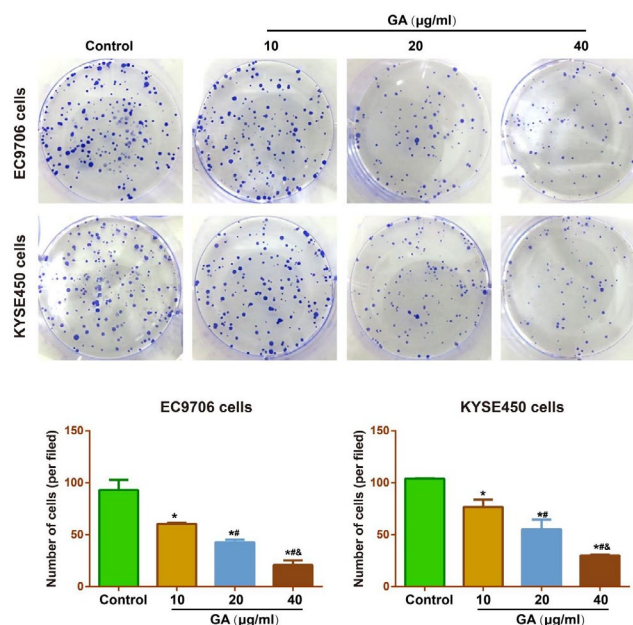


Figure 3. Effect of different concentrations (10, 20, and 40 $\mu\text{g/ml}$) of GA on the colony formation of EC9706 (A) and KYSE450 (B) cells by plate colony formation assay
Note: * $P < 0.05$ vs Control group; # $P < 0.05$ vs 10 $\mu\text{g/ml}$ GA; & $P < 0.05$ vs 20 $\mu\text{g/ml}$ GA
GA: gallic acid

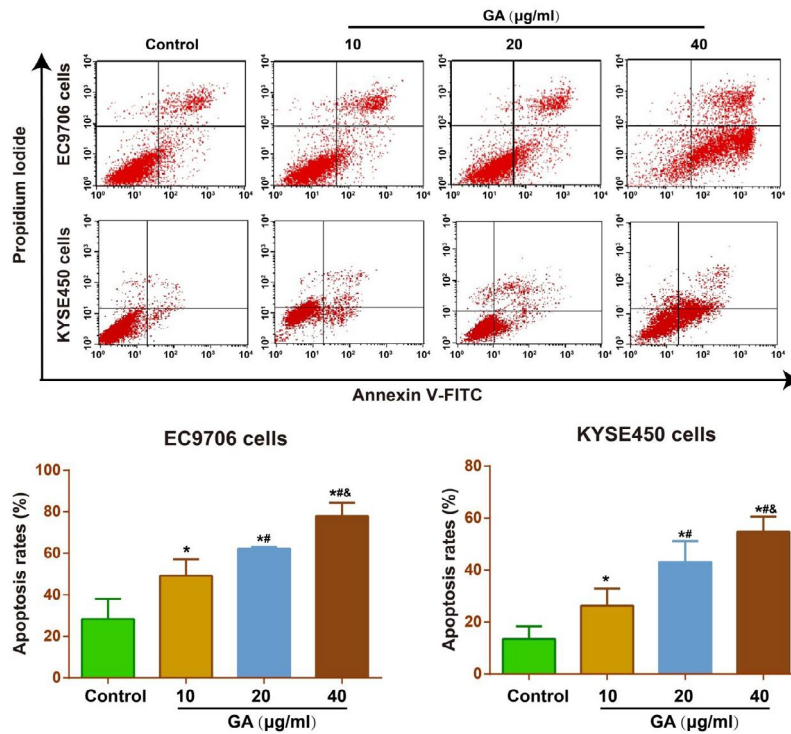


Figure 4. Effect of different concentrations (10, 20, and 40 µg/m) of GA on the apoptosis of EC9706 and KYSE450 cells by Annexin V-FITC/PI staining
 Note: * $P < 0.05$ vs Control group; # $P < 0.05$ vs 10 µg/ml GA; & $P < 0.05$ vs 20 µg/ml GA
 GA: gallic acid

GA facilitates the apoptosis of EC cells

After treatment with GA (10, 20, and 40 µg/ml) for 24 hr, the apoptosis rate of EC9706 and KYSE450 cells was increased in a concentration-dependent manner (Figure 4). To better understand its underlying mechanism, we carried out the Western blotting assay to determine the expressions of apoptosis-related proteins in EC9706 and KYSE450 cells. As a result, a gradual rise in the expressions of pro-apoptotic proteins (including Bax, cleaved caspase 3, and cleaved caspase 9), and a decrease in the anti-apoptotic protein (Bcl-2) were observed with increment in the concentrations of GA (all $P < 0.05$, Figure 5).

GA suppresses the Hippo signal pathway in EC cells

Western blotting assay was also performed to detect the expressions of Hippo signal pathway, and the results indicated that in comparison with the Control group, GA with the concentrations of 10, 20, and 40 µg/ml had gradually decreased YAP and TAZ phosphorylation with increased nuclear YAP/TAZ (all $P < 0.05$), but no significant changes were discovered in the total levels of YAP and TAZ (all $P > 0.05$), suggesting that GA could suppress the activity of YAP-Hippo signal pathway (Figure 6).

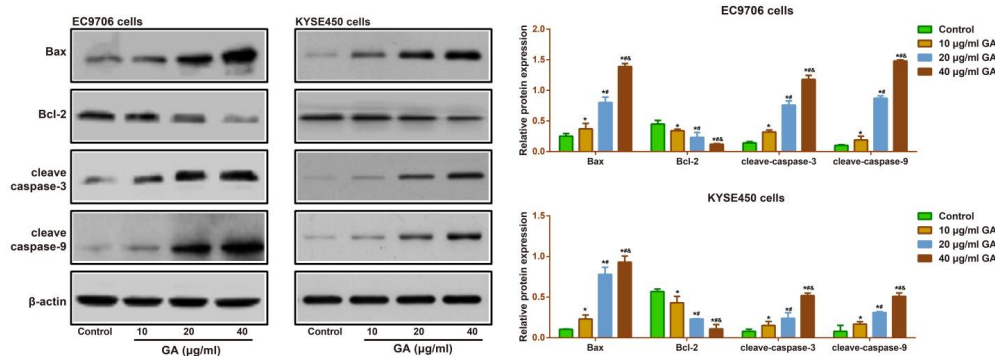


Figure 5. Effect of different concentrations (10, 20, and 40 µg/ml) of GA on the expressions of the apoptosis-related proteins (Bax, cleaved caspase 3, cleaved caspase 9, and Bcl-2) in EC9706 and KYSE450 cells by Western blotting assay
 Note: * $P < 0.05$ vs Control group; # $P < 0.05$ vs 10 µg/ml GA; & $P < 0.05$ vs 20 µg/ml GA
 GA: gallic acid

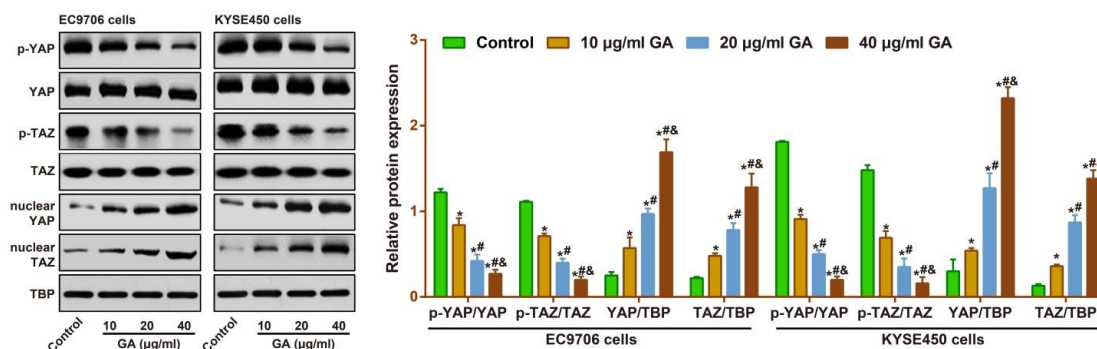


Figure 6. Effect of different concentrations (10, 20, and 40 µg/ml) of GA on the Hippo signal pathway in EC9706 and KYSE450 cells by Western blotting assay

Note: * $P < 0.05$ vs Control group; # $P < 0.05$ vs 10 µg/ml GA; & $P < 0.05$ vs 20 µg/ml GA

GA: gallic acid

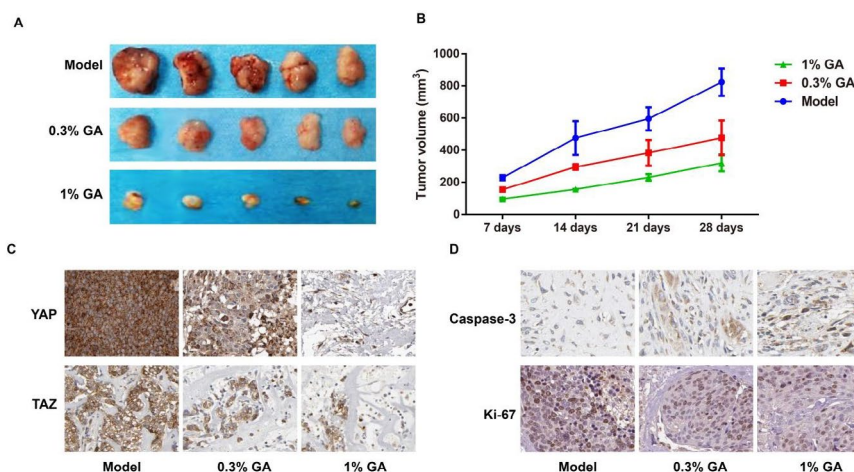


Figure 7. GA suppresses the growth of xenograft in nude mice by blocking the Hippo signal pathway

Notes, A: xenograft in nude mice; B: tumor volume in nude mice; C-D: expression of YAP, TAZ, Ki-67 and Caspase-3 in xenografts of nude mice by immunohistochemistry staining

GA: gallic acid

GA blocks the growth of xenograft in the nude mice by blocking the Hippo signal pathway

When compared to the Model group, GA (0.3% and 1%) treatment significantly reduced the growth of xenograft in nude mice, with decrease in the tumor volume (Figure 7A-B). To further clarify the role of the Hippo signal pathway in the inhibitory effect of GA on the growth of xenograft in mice, we employed the immunohistochemistry method to determine the expressions of YAP and TAZ in the tumor tissues. Consequently, the positive expressions of YAP and TAZ in the GA groups were decreased, as compared with the Model group (Figure 7C). Simultaneously, the levels of Ki-67 (a proliferation indicator) and caspase-3 (an apoptosis indicator) were also tested, and as a result, GA down-regulated Ki-67 but up-regulated caspase-3 in the tumor tissues (Figure 7D). And these changes were obviously shown in the 1% GA treatment group.

Discussion

As we know, chemotherapeutic drugs in clinical practice are potently destructive towards the

cancer cells, but the lack of specificity also results in tremendous damage to normal cells (5). GA, as a kind of natural phenolic compound, has been revealed to have the potential in the tumor treatment (25) that could suppress the generation of tumor cells by slowing down the tumor cell growth and proliferation (26).

In the present work, GA was found to be able to suppress the proliferation of EC9706 and KYSE450 cells in a concentration-dependent manner, which was similar with the previous findings (14, 26, 27). Worth mentioning, the blocked cell cycle induced by GA was credited as a partial cause affecting the proliferation. For instance, both Agarwal *et al.* (28) and Chen *et al.* (29) have shown that GA could delay the growth of human prostate carcinoma cells at the G2/M phase. In breast cancer cells, a study also exhibited the arrest of MCF-7 cell in the G2/M phase caused by GA, beneficial for the prognosis of breast cancer and other cancers with p27 deficiency (30). Besides, evidence provided another possibility for the inhibition of cell proliferation by GA through inhibition of tumor neovascularization, which was the necessary process to supply oxygen and nutrients

for tumor growth (31). As suggested by other research, Ehrlich ascites tumor growth in mice was reduced with decreased angiogenesis after GA therapy (32), and suppressed tumor angiogenesis was also identified by tube formation assays in cervical cancer and glioma with GA treatment (33, 34). In this research, GA was observed to inhibit the colony formation of EC9706 and KYSE450 cells, and the colonies were remarkably decreased with the increased concentrations of GA. Current *in vivo* evidence shows that GA restricted the gastric cancer cell growth via expression of RhoB in nude mice (35), and GA feeding inhibited the growth of DU145 and 22Rv1 PCa xenografts in nude mice (36). Furthermore, GA at concentration of 0.3% and 1% in our study reduced tumor volume of EC with down-regulation of Ki-67, which is a nuclear antigen associated with proliferation, mitosis and even indispensable in proliferation; and a higher positive rate of Ki-67 represents the rapid tumor growth of tumor and poorer differentiation (37, 38). All these observations indicated that GA can suppress the cell proliferation to affect tumor growth, including in EC.

Meanwhile, GA also facilitated the tumor cell apoptosis through activation of the mitochondria-controlled apoptotic pathway (39, 40). Bax overexpression triggered apoptosis via promoting the release of Cytochrome C to activate Caspases (caspase-3 and Caspase-9), confirming the key role of the Bax/Bcl-2 ratio in the regulation of cell apoptosis (41, 42). After determination, the levels of Bax, cleave-caspase-3, and cleave-caspase-9 were significantly increased with the increasing concentrations of GA accompanying the decreased Bcl-2 expression. Other reports have also proven that GA led to apoptosis with impaired mitochondrial function as the consequence of imbalanced Bcl-2/Bax ratio as well as elevated caspase-3 (39, 40). The activation of caspase-3, represents apoptosis entering into the irreversible stage, since it is the critical executioner of apoptosis responsible for regulation of multiple important cellular activities (42). Thus, we further detected the expression of caspase-3 in mice with EC, which was similar to the *in vitro* results, showing that GA may affect the apoptotic pathway to facilitate the apoptosis of carcinoma cells.

In addition, according to the current evidence, the therapeutic effect of GA was also realized by mediating the down-stream signal pathways (14). The Hippo pathway plays a key regulatory role in the biological features, having close associations with multiple molecules and pathways (43). YAP/TAZ, as the nuclear effector of Hippo signal pathway, are the oncoproteins critical to the development of tumors (44), which are activated or highly expressed in many cancers (45). Of note, YAP overexpression brought about the poorer prognosis, which was believed as an independent risk factor of EC (46). As for TAZ, researchers found a relation between its expression and the risk of EC recurrence, and TAZ overexpression was relevant to the lower survival rate (18), indicating the activation of the Hippo pathway in EC. Additional evidence demonstrated the relation between the Hippo pathway and proliferation and apoptosis of tumor cells, and increased proliferation, inhibited apoptosis and suppressed tissue overgrowth was presented with the activation of the Hippo pathway (47). The *in vivo* and

in vitro experiments in this study revealed that GA can suppress YAP and TAZ phosphorylation with increased nuclear YAP/TAZ in a concentration-dependent pattern, suggesting that GA might reduce the proliferation of EC cells, while facilitating apoptosis by inhibition of the Hippo signal pathway. However, there existed a limitation in our experiment: the normal mice treated with/without GA would be used to compare with the model group in the future, thus avoiding the potential toxicity of GA.

Conclusion

GA could suppress cell proliferation of EC, decrease the expression of Ki-67, and promote cell apoptosis, which might be associated with the inhibition of the Hippo pathway. Thus, GA would be expected to be a new strategy for treatment of EC.

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No financial source was disclosed.

Conflicts of Interest

No potential conflicts of interest were disclosed.

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