

Gambogic acid inhibits the growth of ovarian cancer tumors by regulating p65 activity

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Abstract. Ovarian cancer patients often have poor prognosis, therefore, it is important to search for more effective therapeutic strategies to treat them. Gambogic acid (GA) exhibits an anti-tumor effect through various mechanisms, and has multiple targets in tumor cells. The present study aimed to elucidate the efficacy of GA in the treatment of ovarian cancer both *in vivo* and *in vitro* by analyzing its impact on cell survival and tumor growth through cell cycle and apoptosis analysis. GA inhibited the growth of ovarian cancer cells in a dose and time dependent manner, and arrested the cell cycle in ovarian cancer cells. Furthermore, GA increased caspase-3 and caspase-9 activity and inhibited RELA/NF- κ B p65 (p65) DNA binding activity. Finally, GA suppressed tumor growth *in vivo*. Therefore, the current study suggests that GA inhibits the growth of ovarian cancer by regulating p65 activity, and may be developed as a novel therapeutic strategy to treat ovarian cancer.

Introduction

Ovarian cancer is one of the most serious malignant tumors in the female reproductive system. In the worldwide more than 125,000 cases died of ovarian cancer (1), while in China, the incidence of ovarian cancer has risen by 30% in recent years (2). At present, the primary clinical problem is to improve early diagnosis of patients and the efficacy of ovarian cancer treatment. In recent years, surgery combined with drug treatment has improved patient prognosis, however the 5-year survival rate of advanced ovarian cancer remains <30% (3,4). Therefore, there is a strong imperative to search for novel therapeutic strategies to treat ovarian cancer.

Efforts are being made to develop a novel anti-cancer drug from natural compounds. Some compounds used in traditional Chinese medicine are known to exhibit antitumor activity with few side effects (5). Gambogic acid (GA), which is used in traditional medicine, exerts its anti-tumor effect through various mechanisms (6). Previous studies have suggested that GA induces cell cycle arrest and apoptosis (7), inhibits the activity of telomerase, angiogenesis and tumor metastasis, and decreases resistance to multiple chemotherapy drugs (8,9). The effect of GA is thought to be highly selective, in one study it was found to induce apoptosis only in tumor cells (10). However, the mechanism of action of GA on tumor cells remains unclear.

Evidence from previous studies suggests that the NF- κ B signaling pathway is closely related to tumor formation via different mechanisms including cell apoptosis, cell cycle arrest, cell differentiation and migration (11). The NF- κ B family consists of the transcription factors RELA/NF- κ Bp65 (p65), RELB, c-Rel, NF- κ B1 (p50), and NF- κ B2 (p52), of which p65 is the most widely studied member. In resting cells, p65 remains in the cytoplasm combined with the inhibitory protein I κ B. However, some factors such as mitogen are able to stimulate the movement of p65 into the nucleus, where it promotes the transcription and translation of important genes that regulate cell proliferation and apoptosis (12). The aim of the present study was to investigate whether p65 exerts an important role in the action of GA on ovarian cancer tumors.

Materials and methods

Cell culture. Human ovarian adenocarcinoma cancer cells from the SKOV3 cell line were obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in the RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA) including 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in an environment of 5% CO₂ at 37°C. For cell cycle analysis, the cells were synchronized to G₀ by serum starvation for 72 h prior to treatment with GA.

Reagents. Propidium iodide (PI) and RNAase used in the cell cycle analysis were obtained from Sigma-Aldrich (St. Louis,

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MO, USA). The cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). The p65 DNA activity kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Caspase-3, caspase-9, p65, phospho-p65 and β -actin antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell survival analysis. The cell count kit analysis was detected by the protocol of methods as described previously (13). i) After cultivation in an incubator, the cell suspension was inoculated in 96-well plates (100 μ l/well), ii) 10 μ l of CCK-8 reagent was added to each well. iii) The culture plate was incubated in the incubator for 2 h. iv) The absorbance value was measured at 450 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.). v) Calculation of cell survival rate was carried out using the formula: Cell survival (%) = $(V_{\text{drug}} - V_{\text{control}}) / (V_{\text{drug=0}} - V_{\text{control}}) \times 100\%$.

Cell cycle and apoptosis analysis. SKOV3 cells were treated with varying concentrations of GA for 24 h. After the cells were harvested, 70% alcohol was used to fix the cells overnight. The fixed cells were washed with phosphate-buffered saline (PBS), then 150 μ l RNAase (0.1 mg/ml) was added for 30 min at 4°C. Following this, 120 μ l PI (0.1 mg/ml) was added and annexin V staining was carried out for 10 min, at 4°C. Finally, the cells were analyzed for DNA content distribution by flow cytometry (FCM).

Western blot analysis. SKOV3 cells were harvested following treatment with varying concentrations of GA. Protein separation was carried out by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (14,15). The specific antibodies caspase-3 (1:800) and caspase-9 (1:800) came from the Apoptosis Antibody Sampler kit (cat. no. 9915T; Cell Signaling Technology, Inc.), the specific antibodies p65 (1:800) and phospho-p65 (1:800) came from the NF- κ B p65 Antibody Sampler kit (cat. no. 4767T; Cell Signaling Technology, Inc.), and were used to detect the protein levels of indicated cells. β -actin served as an internal reference.

Analysis of p65 DNA activity. p65 DNA activity analysis was performed following the previously defined protocol (16). i) 10 μ g of nuclear extract was added to the cell culture plate, which had already been incubated with 100 μ l of p65 DNA sequence overnight at 4°C. ii) Each well was washed five times with 200 μ l wash buffer. iii) 100 μ l diluted goat anti-rabbit secondary antibody (1:1,500; cat. no. 7074; Cell Signaling Technology, Inc.) was added for 1 h. iv) Each well was washed five times with 200 μ l wash buffer. v) 100 μ l of developing solution per well was added for 45 min. vi) 100 μ l of stop solution per well was added for 5 min. vii) Absorbance was measured at 450 nm.

Murine xenograft model. Equal numbers of SKOV3 cells (2×10^6) were harvested at the log growth phase. Tumor xenografts were established by subcutaneous fat pad injections into athymic mice. The tumor volumes were monitored as described previously (17). After sixty days, the mice were sacrificed.

GA therapy was initiated ten days after subcutaneous fat pad injections were performed. 3.2 μ M of GA was administered to the mice intraperitoneally every 2 days. All animal procedures were performed according to the 1998 Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, Beijing, China), and with the ethical approval of the Dongda Hospital Animal Care and Use Committee (Xi'an, China; permit no. SYXK 2013-0024) and the Ethical Committee of Southeast University (Nanjing, China; permit no. 2013008).

Statistical analysis. All statistical analyses were performed using Graph Pad software (version 5; GraphPad Software Inc., La Jolla, CA, USA). A one-way ANOVA statistical test was also carried out. $P < 0.05$, was considered to indicate a statistically significant difference.

Results

GA inhibited the growth of ovarian cancer cells in a dose and time dependent manner. The SKOV3 cells were treated with varying concentrations of GA (0, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2 μ M). After 24 h, the cell survival rate was analyzed using the CCK-8 assay. The results demonstrated that GA inhibited SKOV3 cell growth in a dose-dependent manner (Fig. 1A). The SKOV3 cells were then treated with 3.2 μ M GA, and cell survival rate was analyzed at set times (0, 30, 60, 120 and 240 min) by CCK-8. GA was also found to inhibit SKOV3 cell growth in a time-dependent manner (Fig. 1B).

GA arrested the cell cycle in ovarian cancer cells. To further study the mechanism of GA on the SKOV3 cells, cell cycle analysis was performed using FCM. The cells were treated with varying concentrations of GA (0, 3.2, and 6.4 μ M). After 24 h, the cells were harvested. The results of the cell cycle analysis indicated that a higher proportion of the cells treated with GA were arrested in the S-phase compared to control cells (Fig. 2). This demonstrates that treatment of GA inhibited cell growth by arresting ovarian cancer cells in the S-phase.

GA increased the activity of caspase-3 and caspase-9 in ovarian cancer cells. To study whether caspase activity was involved in the GA-induced growth arrest in SKOV3 cells, protein levels of active caspase-3 and active caspase-9 were measured before and after GA treatment. The preceding results suggested that the vehicle could not exert an influence on the survival of SKOV3 cells, therefore western blot analysis was performed to compare the levels of active caspase-3 and caspase-9 before and after GA treatment (Fig. 3). The results obtained show that GA treatment increased the expression of active caspase-3 and active caspase-9, whilst overall caspase-3 and caspase-9 levels remained unchanged, demonstrating that GA inhibited cell growth by upregulating caspase-3 and caspase-9 activity.

GA inhibited p65 DNA binding activity in ovarian cancer cells. Evidence from previous studies suggests that the NF- κ B signaling pathway is closely related to tumor formation via different mechanisms including cell apoptosis, cell cycle arrest, cell differentiation and migration (11). In the current study, phosphorylated p65 (p-p65) expression decreased after treatment

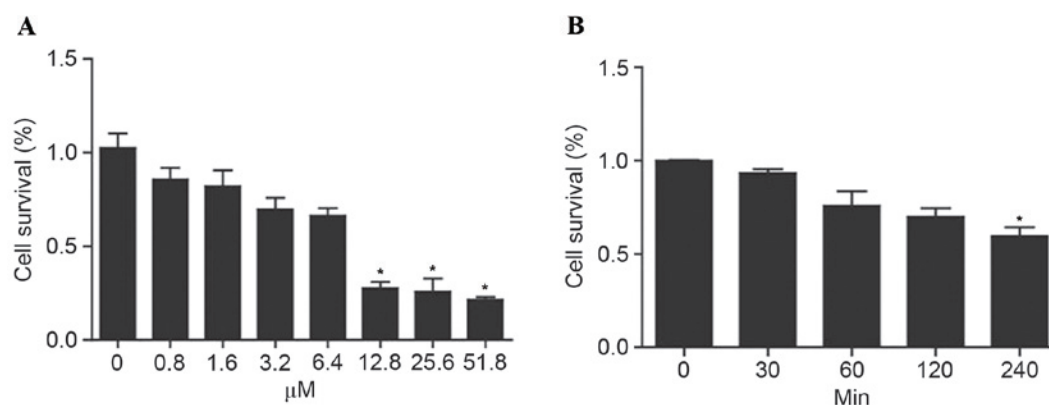


Figure 1. Gambogic acid (GA) inhibited the growth of ovarian cancer cells in a dose and time dependent manner. The percentages of cell viability were detected using the Cell Count Kit-8 (CCK-8) assay. (A) The percentage of SKOV3 cells that survived following exposure to increasing doses of GA. (B) The percentage of SKOV3 cells that survived following treatment with GA for differing periods of time. The data shown represent cell counts at 0, 30, 60, 120 and 240 min following exposure to GA ($3.2 \mu\text{M}$). * $P < 0.05$ vs. untreated cells. The results shown on the graphs are the means of three independent experiments; bars, means \pm SD. The experiments were repeated three times.

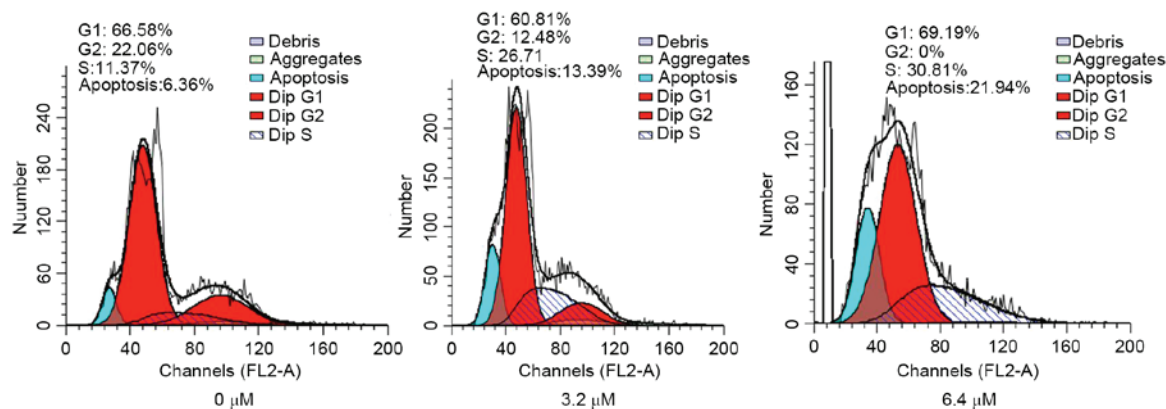


Figure 2. Gambogic acid (GA) arrested the cell cycle in ovarian cancer cells. Cell cycle analysis was carried out on SKOV3 cells treated with GA (3.2 and $6.44 \mu\text{M}$) for 24 h. The cells were harvested for analyzed DNA content distribution and annexin V staining for apoptosis by flow cytometry (FCM).

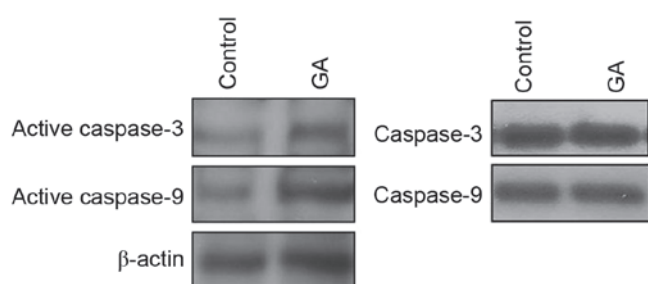


Figure 3. Treatment of SKOV3 cells with gambogic acid (GA) increased the activity of caspase-3 and caspase-9 in ovarian cancer cells. SKOV3 cells were treated both with and without GA. Levels of active caspase-3 and caspase-9 were assessed by western blot analysis. Though levels of active caspase-3 and active caspase-9 increased in cells treated with GA, overall levels of caspase-3 and caspase-9 remained the same. The data were collected from three independent experiments. β -actin acted as a control.

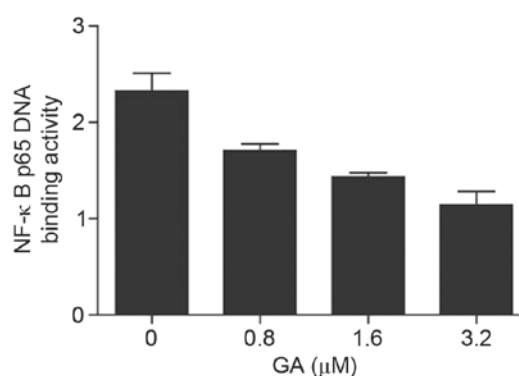


Figure 4. Gambogic acid (GA) inhibited the RELA/NF- κ B p65 DNA binding activity of ovarian cancer cells. SKOV3 cells were treated with increasing concentrations of GA. NF- κ B DNA binding activity was analyzed using a NF- κ B p65 transcription factor assay kit. Data show the means \pm SD.

with GA. Furthermore, p65 DNA binding activity markedly decreased when treated with increasing concentrations of GA (Fig. 4). Taken together, these results suggest that GA inhibited the cell survival of ovarian cancer cells by suppressing p65 DNA binding activity.

GA suppressed tumor growth *in vivo*. Finally, tumor xenograft experiments were conducted in nude mice in order to detect whether GA could suppress tumor growth *in vivo*. The results showed that the tumors in GA-treated mice were significantly smaller than in control mice or mice treated with a vehicle

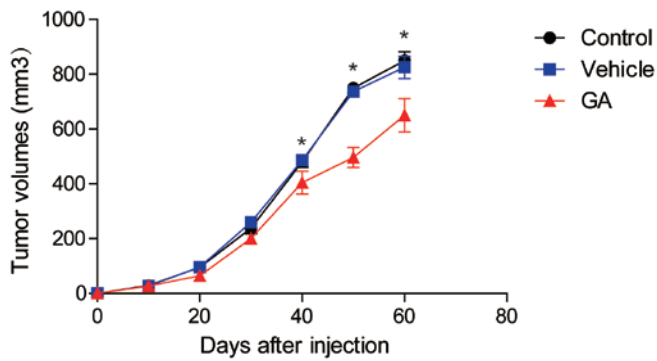


Figure 5. Gambogic Acid (GA) suppressed tumor growth *in vivo*. Tumor volumes of xenografts were dissected from nude mice (n=5) 60 days after subcutaneous inoculation with GA. The data are reported as the mean \pm SD. *P<0.05 GA vs. the control.

(Fig. 5; P<0.05). These results indicated that GA inhibited tumor growth in a mouse model.

Discussion

Due to the high incidence of patient relapse and adverse reactions caused by the chemotherapy drugs currently used to treat ovarian cancer, discovering new therapeutic strategies to improve treatment efficacy is important. Our previous study demonstrated that ovarian cancer was a serious cancer of the female reproductive system (18,19). However, some compounds used in traditional Chinese medicine may play an important role in treating cancer. GA is one such compound with multiple targets that exhibits anti-tumor activity (20,21). In the current study, GA was identified to inhibit the growth of ovarian cancer tumors both *in vivo* and *in vitro*. Furthermore, GA also inhibited the growth of ovarian cancer tumors by arresting the cell cycle. The results indicate that GA inhibits p65 DNA binding activity, thus inhibiting cell proliferation in ovarian cancer tumors.

Future studies should focus on uncovering the mechanism of action of GA on ovarian cancer tumors. Normal cell survival depends on the cell cycle operating normally and is controlled by the precise mechanism of cell cycle regulators (22). Tumor growth is thought to arise from dysregulation of the cell cycle.

GA induced apoptosis in tumor cells with few adverse reactions, unlike radiotherapy and chemotherapy. The present study suggests that GA may be important in regulating the cell cycle of tumor cells by arresting at S-phase. Additionally, these results also suggest that some compounds used in traditional Chinese medicine may be candidates for the development of novel therapeutic strategies for a variety of diseases.

GA exhibits antitumor activity within the scope of the effective dose and seems to cause few side effects in the hemopoietic system and does not seem to adversely affect the immune function of normal cells. These advantages are currently not possessed by other tumor chemotherapy drugs. In the current study, GA inhibited the cell survival of SKOV3 cells by arresting the cell cycle in G2/M phase and inducing apoptosis, suggesting that GA could be developed as a novel treatment of ovarian cancer.

NF- κ B is a class of dimer transcription factor that recognizes and binds to DNA. Stimulated of the cells by various internal and external factors activated NF- κ B, promoting the expression of a series of anti-apoptotic genes. Previous studies have demonstrated that the NF- κ B signaling pathway plays a crucial role in the development of various tumors (23-25). However, it remains unknown whether GA acts on ovarian cancer tumor cells by stimulating the NF- κ B signaling pathway, to induce apoptosis. The current study demonstrated that GA inhibited p65 DNA binding activity, suggesting p65 plays a key role in the mechanism of action of GA.

In conclusion, the present study demonstrates that GA inhibited the growth of ovarian cancer *in vivo* and *in vitro* by arresting the cell cycle and inducing apoptosis. The results indicate that GA may act by inhibiting p65 DNA binding activity, thus inhibiting cell proliferation in ovarian cancer tumors.

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