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Saikosaponin-d inhibits proliferation of human undifferentiated thyroid carcinoma cells through induction of apoptosis and cell cycle arrest

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Abstract. – OBJECTIVE: Saikosaponin-d is a triterpene saponin derived from *Bupleurum falcatum*. L and has been reported to exhibit a variety of pharmacological activities such as anti-bacterial, anti-virus and anti-cancer. The aim of the present study was to explore the effect of saikosaponin-d on the proliferation and apoptosis of human undifferentiated thyroid carcinoma.

MATERIALS AND METHODS: Three human anaplastic thyroid cancers cell lines were cultured in the presence of saikosaponin-d and their proliferation was measured by MTT assay. Cell apoptosis and cell cycle distribution were analyzed with flow cytometry. Western blot was performed to determine the proteins expression. The *in vivo* effect of saikosaponin-d was measured with an animal model.

RESULTS: *In vitro*, MTT assay showed that saikosaponin-d treatment inhibited cell proliferation in three human anaplastic thyroid cancers cell lines ARO, 8305C and SW1736. In addition, saikosaponin-d promoted cell apoptosis and induced G1-phase cell cycle arrest as shown by flow cytometric analysis. On the molecular level, our results showed that saikosaponin-d treatment increased the expression of p53 and bax, and decreased the expression of Bcl-2. In addition, saikosaponin-d administration led to a significant up-regulation of p21 and down-regulation of CDK2 and cyclin D1. Xenografts tumorigenesis model demonstrated that saikosaponin-d markedly reduced the weight and volume of thyroid tumors *in vivo*.

CONCLUSIONS: The present study suggested that saikosaponin-d might be a new potent chemopreventive drug candidate for human undifferentiated thyroid carcinoma through induction of apoptosis and cell cycle arrest.

Key Words:
Saikosaponin-d, Proliferation, Human undifferentiated thyroid carcinoma, Apoptosis.

Introduction

Thyroid carcinoma is one of the most common malignancies in the endocrine system, and its incidence is higher in women than in men[1]. It is classified into three types including papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and undifferentiated thyroid cancer (UTC). Among them, PTC and FTC are more differentiated with better prognosis and response to surgery and radioiodine treatment[2,3]. Nevertheless, there are no effective therapies for patients with UTC who do not respond to radioactive iodine therapy and exhibit worse prognosis associated with airway obstruction and distant metastasis[4]. Patients with UTC usually die within 1 year, and combination therapies consisting of radioiodine, surgery, chemotherapy and external radiation cannot reduce the mortality[5]. Thus, it is urgent to develop new and effective therapeutic agents in the treatment of UTC.

Saikosaponin-d is one of the major triterpenoid saponins derived from *Bupleurum falcatum L*, which is a commonly prescribed agent against inflammatory and infectious diseases in China, Japan and other Asian countries[6,7]. Investigations of saikosaponin-d provided evidence that it could sensitize cervical and ovarian cancers to cisplatin through ROS-mediated apoptosis and prevent the carcinogen-induced tumorigenesis, making it a potential chemotherapeutic candidate for tumor treatment[8-10]. Recent study demonstrated that saikosaponin-d enhanced the anticancer potency of TNF-α via overcoming its undesirable response of activating NF-kappa B signaling in cancer cells, indicating that saikosaponin-d has a significant potential to be developed as a combined adjuvant remedy with TNF-α for cancer patients[11]. However, the inhibitory effect of saikosaponin-d on thyroid carcinoma has never been investigated. In the present study,

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we for the first time investigated the anti-proliferative effect of saikosaponin-d on undifferentiated thyroid cancer cells and explored the mechanism of action under the anti-tumor property on human thyroid carcinomas.

Materials and Methods

Reagents

Saikosaponin-d (> 95% purity, HPLC) was purchased from Sigma (St. Louis, MO, USA). Dissolve the saikosaponin-d in dimethyl sulfoxide (DMSO) and stored at room temperature. DMEM (Dulbecco's Modified Eagle Medium), fetal bovine serum (FBS) and penicillin-streptomycin were obtained from Gibco (Gibco, Grand Island, NY, USA).

Cell Culture

The human anaplastic thyroid cancers (ATC) cell lines ARO, 8305C and SW1736 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% FBS streptomycin (100 mg/mL) and penicillin (100 U/mL). Cultured cells were maintained at 37°C and 5% CO_2 in a humid environment and passaged when the confluency reached 80%.

Cell Proliferation Assay

The anaplastic thyroid cancers cells were seeded onto 24-well plate at the density of $1x10^6$ cells/mL per well and cultured overnight. The cells were treated with various concentrations (5 μmol/L, 10 μmol/L, 15 μmol/L and 20 μmol/L) of saikosaponin-d for different time points (12 h, 24 h and 48 h). Then, cell proliferation was determined by MTT [3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

Flow Cytometric Analysis

Cells were exposed to different concentrations of saikosaponin-d (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24 h. The cells were washed with phosphate buffered saline (PBS), detached with trypsin and harvested. Apoptosis cells were detected with annexin V-FITC/PI according to the protocol of Annexin V-FITC cell apoptosis detection kit (BD, Franklin Lakes, NJ, USA).

Cell Cycle Distribution

Cells were seeded at the density of 1.0×10^6 cells and exposed to various concentrations of saikosaponin-d (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24 h. Cells were washed twice with PBS, collected by centrifugation, and fixed in ice-cold 70% ethanol at –20°C overnight. Then, cells were stained with 100 μl propidium iodide (PI) staining solution for 30 min in the dark followed by cell cycle analysis.

Western Blot Analysis

The anaplastic thyroid cancers ARO cells were harvested by trypsinization, lysed in buffer and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). After immunoblotting, the membranes were blocked in PBS/0.1% Tween-20 with 5% nonfat dry milk, and primary antibodies were incubated in PBS/0.1% Tween-20 with 0.1%-5% nonfat dry milk. Antibodies directed against p53, bax, bcl-2, p21, CDK2, cyclin D1 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) with GAPDH used as a loading control.

Animals

Male athymic nude mice, ages 8 to 12 weeks, were housed and maintained in laminar flow cabinets under specific pathogen-free conditions. The mice were treated in accordance with the animal Care and Use Guidelines under a protocol approved by the Institutional Animal Care and Use Committee.

In vivo Xenografts

Tumor response to saikosaponin-d was gauged using a nude mouse model of thyroid cancer. The anaplastic thyroid cancers ARO cells were harvested from sub-confluent cultures by trypsinization and then washed. Using a 30-gauge needle under direct visualization, 5×10^6 cells were injected subcutaneously into the cervical area. Tumors were allowed to grow for one week. Then, 24 mice were randomly divided into four groups: control group with PBS treatment, and three groups with oral gavage of saikosaponin-d (5 mg/kg, 10 mg/kg and 20 mg/kg body weight/day). Tumor volume was calculated using the formula (A) (B^2) $\pi/6$, where A was the length of the longest aspect of the tumor, and B was the length of the tumor perpendicular to A. After 4-week treatment, mice were killed and the actual tumor weight was also measured.

Statistical Analysis

Data are expressed as the mean ± SEM and statistical analysis was carried out with SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Comparison between groups was made using ANOVA analysis. Statistical significance was accepted as $p < 0.05$.

Results

Saikosaponin-d Inhibited the Proliferation of Anaplastic Thyroid Cancers Cells

In order to investigate the anti-proliferative effect of saikosaponin-d, ATC cells were cultured in the presence of various concentrations of saikosaponin-d (5 μmol/L, 10 μmol/L, 15 μmol/L and 20 μmol/L). MTT assay showed that saikosaponin-d treatment significantly inhibited the proliferation of ARO (Figure 1A), 8305C (Figure 1B) and SW1736 (Figure 1C) in a time- and dose-dependent manner. These data suggested that saikosaponin-d had an anti-proliferative effect on anaplastic thyroid cancers cells.

Saikosaponin-d Induced Apoptosis and Cell Cycle Arrest of Anaplastic Thyroid Cancers Cells

Three anaplastic thyroid cancers cells lines were treated with different concentrations of saikosaponin-d (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24 h and the apoptosis rate was analyzed using Annexin V/PI staining. Results showed that treatment with saikosaponin-d for 24 h significantly increased the apoptosis rates of ARO (Figure 2A), 8305C (Figure 2B) and SW1736 (Figure 2C) in a concentration-dependent manner compared with the control group. Analysis of ARO cell cycle distribution showed that saikosaponin-d administration significantly increased the proportions of cells in G1 phase. Meanwhile, the number of cells in S phase decreased compared with the levels in the controls (Figure 3A and B). Similar results were also observed in 8305C (Figure 3C and D) and SW1736 (Fig. 3E and F) after treatment with saikosaponin-d. Together, these results demonstrated that saikosaponin-d administration promoted apoptosis and induced G1-phase cell cycle arrest of anaplastic thyroid cancers cells.

Effect of saikosaponin-d on the expression of apoptosis-related proteins

In order to further explore the molecular mechanism underlying saikosaponin-d -induced cell apoptosis, the expression levels of apoptosis-related proteins were assessed by western blot analysis. The

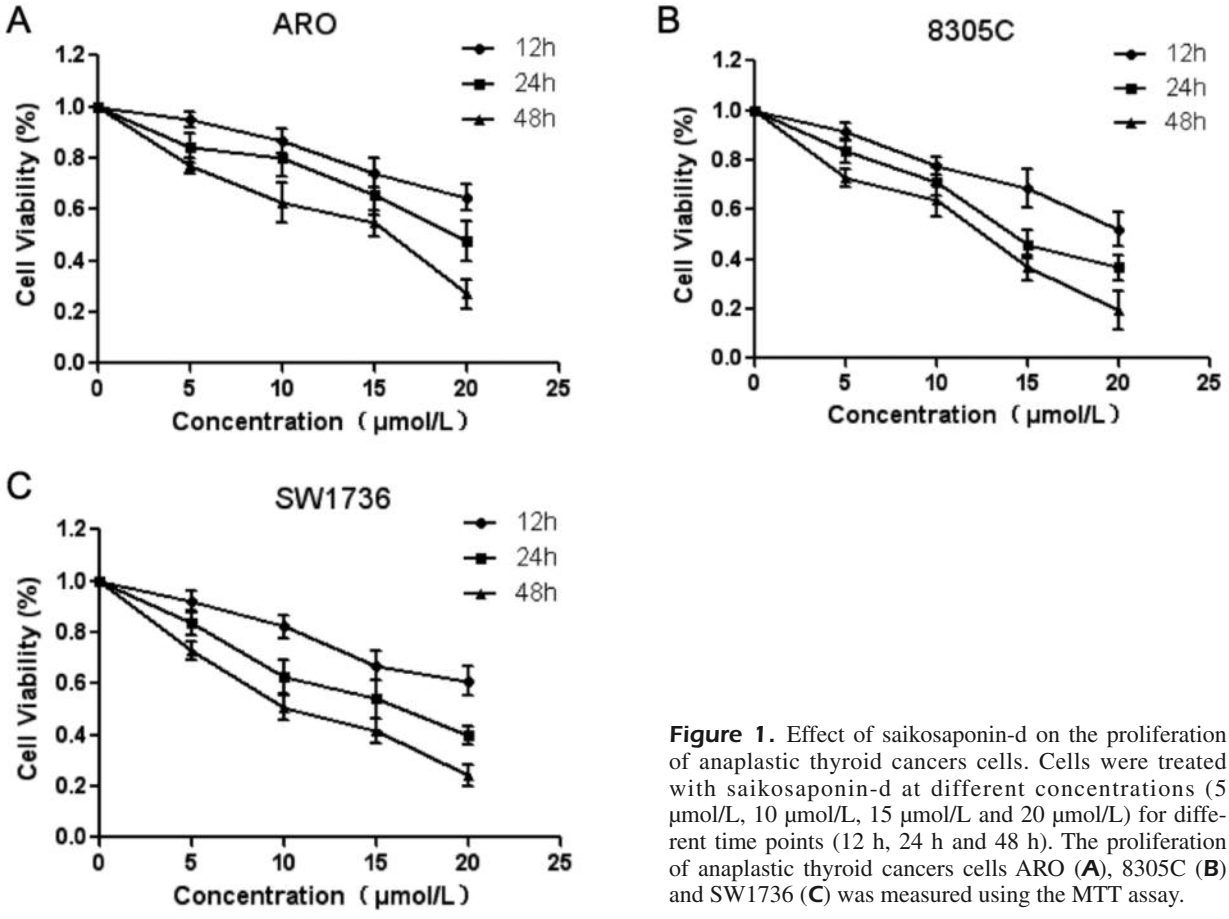

Figure 1. Effect of saikosaponin-d on the proliferation of anaplastic thyroid cancers cells. Cells were treated with saikosaponin-d at different concentrations (5 μmol/L, 10 μmol/L, 15 μmol/L and 20 μmol/L) for different time points (12 h, 24 h and 48 h). The proliferation of anaplastic thyroid cancers cells ARO (**A**), 8305C (**B**) and SW1736 (**C**) was measured using the MTT assay.

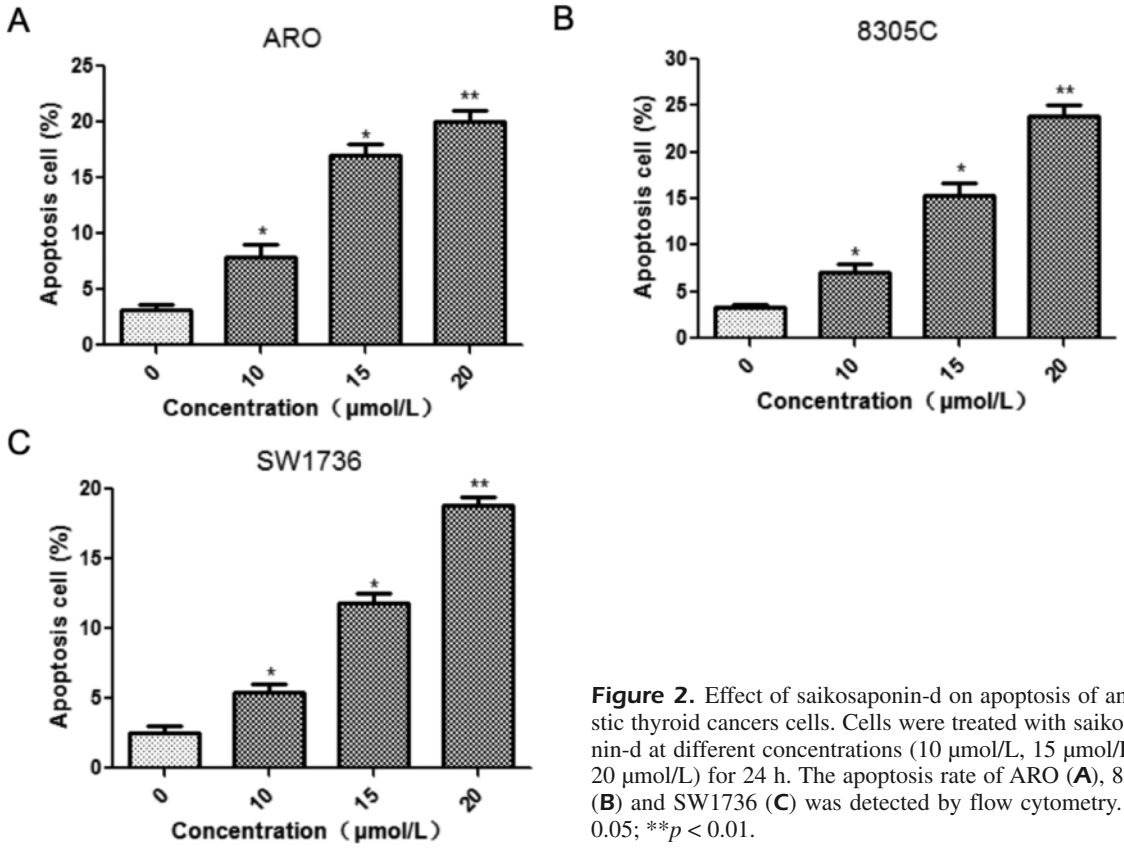

Figure 2. Effect of saikosaponin-d on apoptosis of anaplastic thyroid cancers cells. Cells were treated with saikosaponin-d at different concentrations (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24 h. The apoptosis rate of ARO (**A**), 8305C (**B**) and SW1736 (**C**) was detected by flow cytometry. *$p < 0.05$; **$p < 0.01$.

expression of tumor suppressor gene p53 was increased significantly (Figure 4A and B). As shown in Figure 4C and Figure 4D, cells treated with saikosaponin-d showed a decrease in Bcl-2 expression and an increase in bax in a dose-dependent manner. Also, the bax/Bcl-2 ratio was significantly increased in a dose-dependent manner after treatment with saikosaponin-d (Figure 4E).

Effect of Saikosaponin-d on the Expression of Cell Cycle-Related Proteins

Next, we examined the cell cycle-related proteins in anaplastic thyroid cancers cells by western blot. We found that the expression of p21 was increased after treatment with saikosaponin-d (Figure 5A and B). The expression of the G1 phase cell cycle regulatory proteins, including CDK2 and cyclin D1, were decreased in a concentration-dependent manner following treatment with saikosaponin-d (Figure 5C and D).

Saikosaponin-d Reduced the Growth of Anaplastic Thyroid Cancers Cells in vivo

To further investigate the effect of saikosaponin-d on tumor growth *in vivo*, a nude mouse model of thyroid cancer was established by subcutaneously injection of three dose levels of saikosaponin-d. Our results showed that the volume (Figure 6A) and weight (Figure 6B) of tumors in the treatment groups was significantly decreased in a dose-response manner, compared with tumors in the control group. Thus, these results indicated that saikosaponin-d reduced the growth of anaplastic thyroid cancers cells *in vivo*.

Discussion

Anaplastic thyroid carcinoma is one of the most aggressive tumors in human. Even though it is rare and represents a small part of all malignant thyroid diseases, it accounts for approximately 40% of thyroid carcinoma related deaths[3]. Previous reports showed that saikosaponin-d possessed immune-modulatory, anti-inflammatory and anticancer activities[12,13]. In the present study, we for the first time investigated the effects of saikosaponin-d on proliferation, apoptosis and cell cycle distribution in anaplastic thyroid cancers cells.

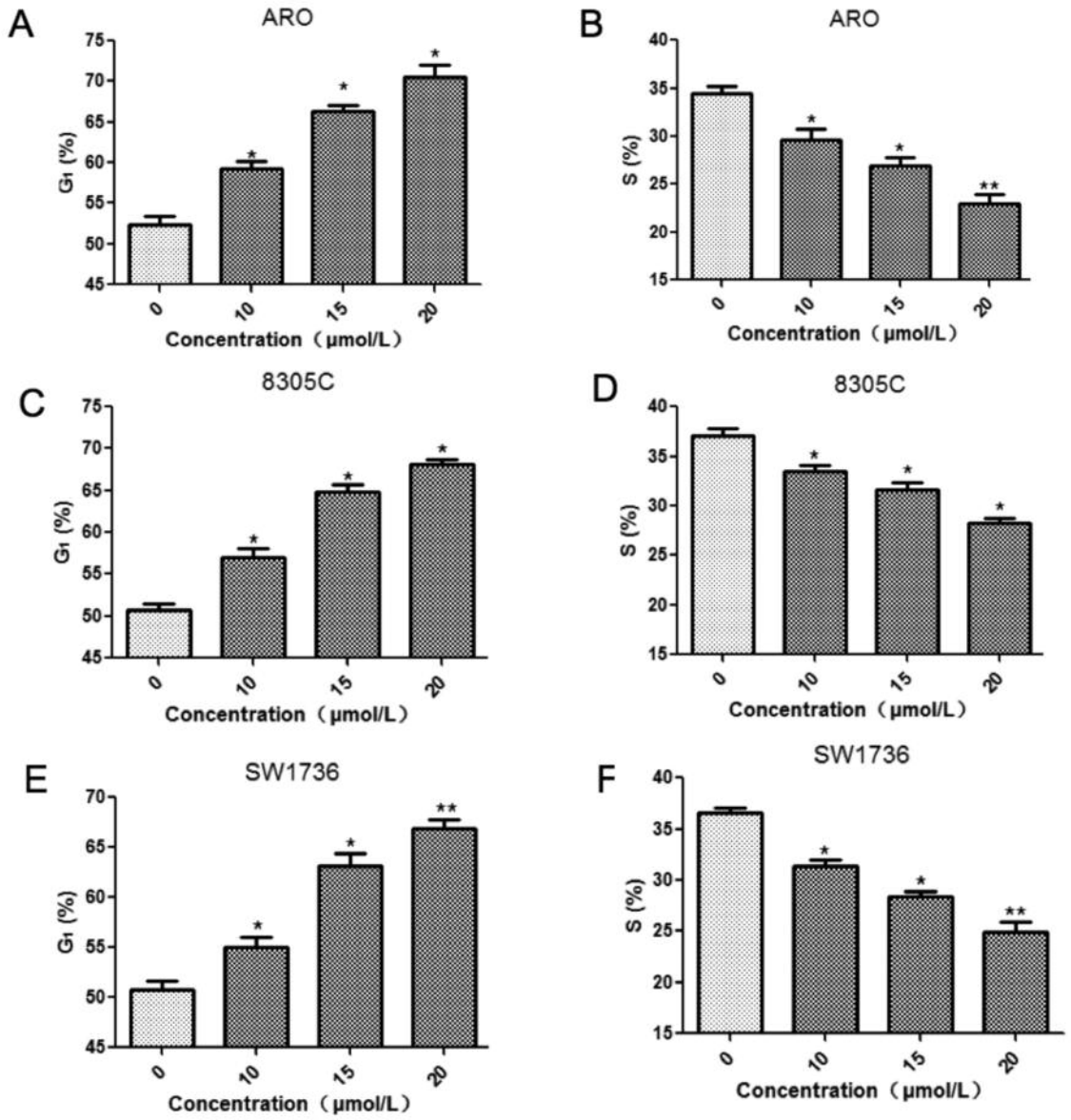

Figure 3. Effect of saikosaponin-d on cell cycle distribution of anaplastic thyroid cancers cells. Cells were treated with saikosaponin-d at different concentrations (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24 h. Cell cycle distributions at G1 and S phase of ARO (**A** and **B**), 8305C (**C** and **D**) and SW1736 (E and F) were analyzed by flow cytometry. *$p < 0.05$; **$p < 0.01$.

Abnormal thyroid cell proliferation plays an important role in human diseases. Its deregulation causes goiter, thyroid adenomas, and carcinomas[14,15]. MTT assay showed that saikosaponin-d treatment inhibited the proliferation of three anaplastic thyroid cancers cell lines. Furthermore, data from *in vivo* xenograft tumorigenesis model suggested that saikosaponin-d also markedly reduced the growth of anaplastic thyroid cancers cells. Taken together, these results demonstrated that saikosaponin-d suppress the proliferation of anaplastic thyroid cancers cells both *in vitro* and *in vivo*.

Apoptosis, the programmed cell death, is characterized by typical cellular and molecular features such as cell shrinkage, externalization of phosphatidylserine and condensation of chromatin[16]. It plays an important role in organ development and homeostasis. Cell cycle is controlled by cell cycle checkpoint which ensures the fidelity of cell divi-

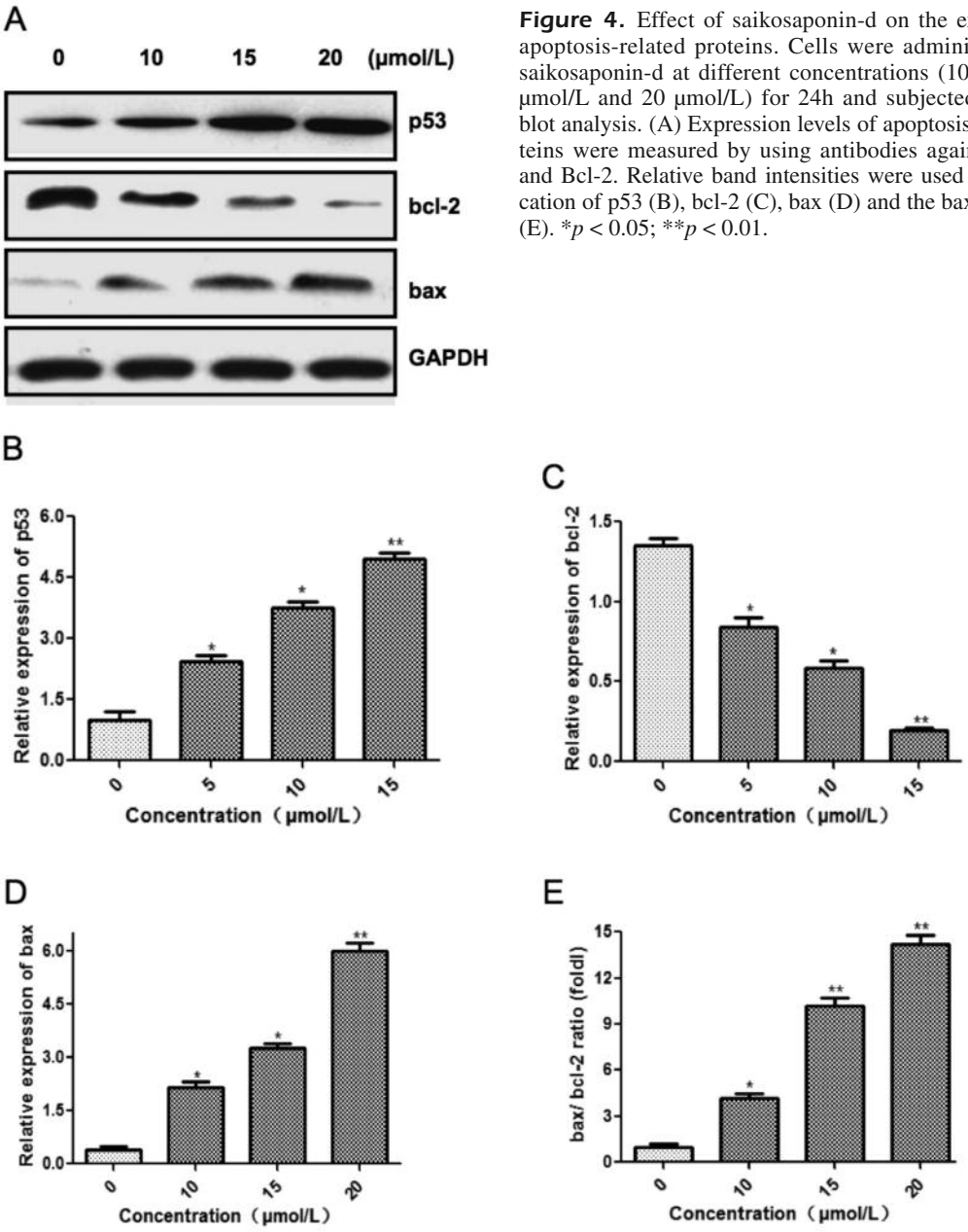

Figure 4. Effect of saikosaponin-d on the expression of apoptosis-related proteins. Cells were administrated with saikosaponin-d at different concentrations (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24h and subjected to western blot analysis. (A) Expression levels of apoptosis-related proteins were measured by using antibodies against p53, bax and Bcl-2. Relative band intensities were used for quantification of p53 (B), bcl-2 (C), bax (D) and the bax/bcl-2 ratios (E). $*p < 0.05$; $**p < 0.01$.

sion. Exogenous or endogenous stimulating factors can induce cell cycle arrest and resulted in the breakdown of cell division, cell death and apoptosis[17]. Because uncontrolled proliferation is an essential step in tumorigenesis, induction of apoptosis and cell cycle arrest could provide a possible mechanism for anti-tumor therapy[18]. Previous study showed that saikosaponin-d inhibited the proliferation and promoted cell apoptosis thorough induction of p53 and the Fas/FasL apoptotic system in human non-small cell lung cancer A549 cells[19]. Another study demonstrated that saikosaponin-d induced cell apoptosis and cell cycle arrest associated with the simulation of p53 and further induction of Bax and p21 expression in human hepatoma cell lines, suggesting that saikosaponin-d may be useful as a liver cancer chemopreventive agent[20]. In our study, we found that saikosaponin-d administration promoted cell apoptosis and induced G1-phase cell cycle arrest, indicating the possible mechanism by which saikosaponin-d inhibited the proliferation of anaplastic thyroid cancers cells.

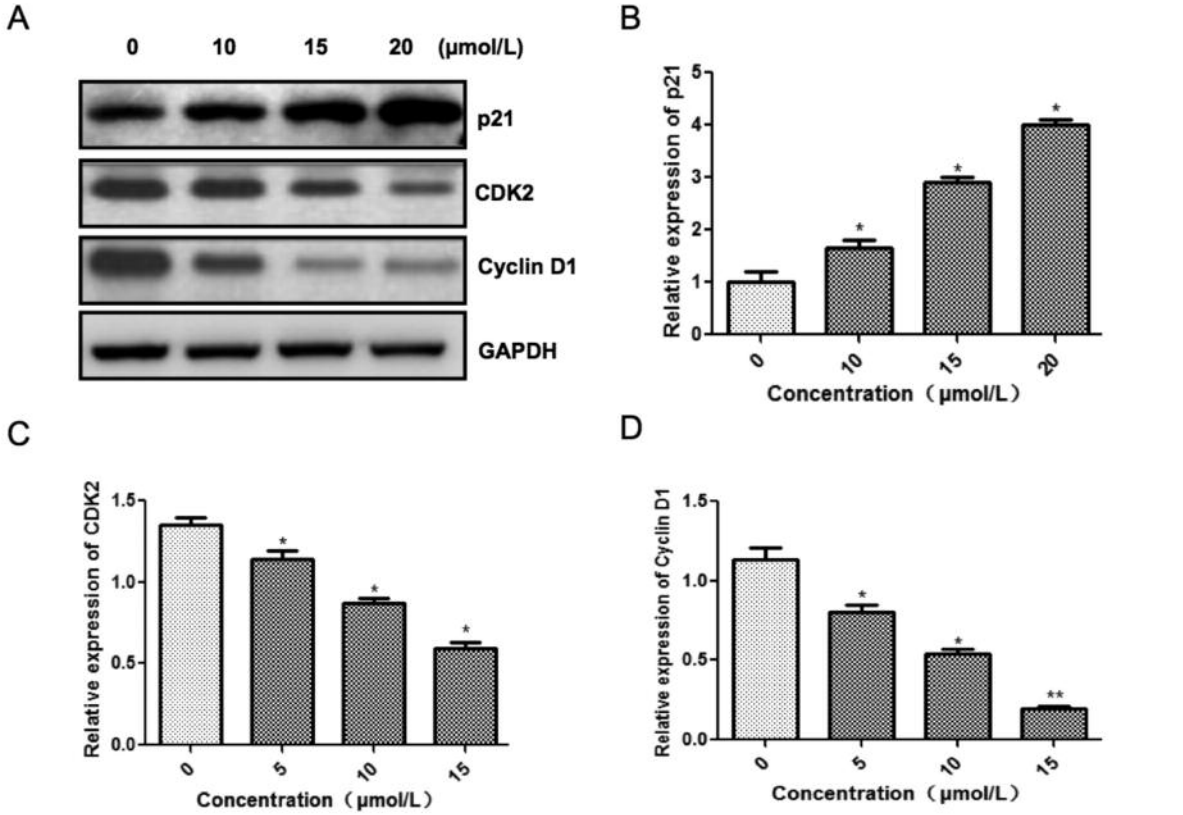

Figure 5. Effect of saikosaponin-d on the expression of cell cycle-related proteins. Cells were administrated with saikosaponin-d at different concentrations (10 μmol/L, 15 μmol/L and 20 μmol/L) for 24h and western blot was performed to examine the expression of p21, CDK2 and Cyclin D1 (A). Relative band intensities were used for quantification of p21 (B), CDK2 (C) and cyclin D1 (D). *$p < 0.05$; **$p < 0.01$.

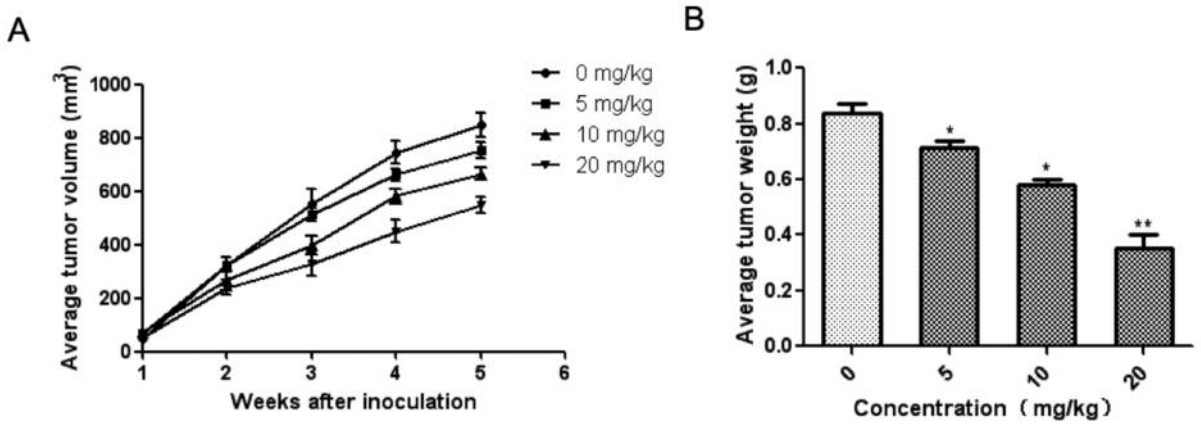

Figure 6. Effect of saikosaponin-d on the growth of anaplastic thyroid cancers cells *in vivo*. Xenograft tumorigenesity model was established and treated with saikosaponin-d at different concentrations (5 mg/kg, 10 mg/kg and 20 mg/kg body weight/day). A, Tumor size of subcutaneous xenografts measured with 7-day interval. B, After 4-week treatment, mice were killed and the actual tumor weight was also measured. *$p < 0.05$; **$p < 0.01$.

We further investigated the underlying molecular mechanism of saikosaponin-d induced-apoptosis and cell cycle arrest in human thyroid carcinoma. It is well known that the protein p53 plays a critical role in multi-cellular organisms, regulating cell proliferation, differentiation and apoptosis[21]. Upon stimulation, p53 could modulate various downstream target genes involved in apoptosis and cell cycle arrest, such as bax, Bcl-2 and p21[22]. In particular, the protein p21 acts as an inhibitor of cell cycle progression and its up-regulation will result in cell cycle arrest in G1 phase[23]. In our study, we found that saikosaponin-d treatment resulted in an increase in p53 and bax expression and a decrease in Bcl-2. The protein expression of p21 was up-regulated after saikosaponin-d administration. In addition, the cyclin-dependent kinase 2 (CDK2) is a member of the cyclin-dependent kinase family of Ser/Thr protein kinases, which functions as a crucial regulator of S-phase progression[24]. Meanwhile, cyclin D1 promotes G1-S phase transition and its over-expression is associated with many cancers including colon cancer, breast cancer and melanoma[25,26]. Our data showed that the expression of cyclin D1 and CDK2 were significantly decreased, suggesting that saikosaponin-d treatment induced G1-phase cell cycle arrest of anaplastic thyroid cancers cell.

Conclusions

The present study for the first time demonstrated that saikosaponin-d inhibited the proliferation of human anaplastic thyroid cancers cells both *in vitro* and *in vivo*. Furthermore, the anti-proliferative effects of saikosaponin-d may be mediated through induction of apoptosis and cell cycle arrest of anaplastic thyroid cancers cells. Therefore, saikosaponin-d might be a new potential medicine that could be used for the treatment of anaplastic thyroid cancers in the future.

Conflict of interest

The Authors declare that there are no conflicts of interest.

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