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Naringin inhibits thyroid cancer cell proliferation and induces cell apoptosis through repressing PI3K/AKT pathway

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ABSTRACT

The present study aimed to investigate the anti-tumor effects of naringin in thyroid cancer (TC), and to explore the underlying mechanisms. TC cell lines TPC-1 and SW1736 were treated with 6, 12 or 25 μg/ml naringin for indicated times. Then, cell proliferation was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, and cell apoptosis was analyzed by flow cytometer. Moreover, cell proliferation and apoptosis related genes (cyclin D1, c-Myc, survivin, Caspase3, Bcl-2, and Bax) were measured by western blot assay and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) respectively. Cleaved Caspase3 was measured using western blot assay. Phosphatidylinositol 3-kinase (PI3K)/AKT pathway was also analyzed in this study. Results indicated that naringin dose- and time-dependently inhibited TPC-1 and SW1736 cell proliferation, and naringin dose-dependently induced TPC-1 and SW1736 cell apoptosis. In addition, we found that naringin dose-dependently enhanced the expression of Caspase3, cleaved Caspase3 and Bax, and reduced the expression of cyclin D1, c-Myc, survivin, and Bcl-2 in TPC-1 and SW1736 cells. Moreover, we found that naringin dose-dependently suppressed PI3K/AKT pathway activation in TC cells. In conclusion, the data of this study suggested that naringin presented anti-tumor effects in TC cells through inhibiting TC cell proliferation and inducing cell apoptosis via regulating the expression of cell proliferation and apoptosis related genes and PI3K/AKT pathway activation. Our study suggested the potential value of naringin in the treatment of TC and provided more theoretical evidence for the treatment of TC.

1. Introduction

Thyroid cancer (TC) is the most common malignant tumor of the endocrine system. In the past 30 years, the incidence of thyroid cancer has increased year by year, and the recurrence rate of thyroid cancer has increased, leading to an increase in thyroid cancer mortality [1–3]. Thyroid cancer can be divided into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), and anaplastic thyroid cancer (ATC). PTC is the most common type of thyroid cancer, accounting for 70%–80% of all types of thyroid cancer [4]. Studies have shown that the prevalence of PTC has increased at an average rate of nearly 4% per year in recent years [5]. At present, surgical treatment, thyroid hormone inhibition therapy, isotope iodine 131 therapy and adjuvant radiotherapy are the main methods for TC treatment [6–8]. As the incidence of TC is increasing year by year, it is increasingly urgent and severe to solve this medical problem. Therefore, the development of new, effective and low-toxic TC inhibitor is of great significance in improving the survival rate of TC patients and improving the quality of life of patients and their families.

Modern pharmacological studies have shown that the active ingredients of traditional Chinese medicine have obvious inhibitory effects on TC, and have the characteristics of small side effects, various targets, and wide therapeutic pathways, which have obvious advantages compared with western medicine [9]. Naringin is a natural flavonoid [10]. Studies have shown that naringin has a wide range of biological activities, including anti-inflammatory [11,12], anti-oxidative stress [11,12], anti-atherosclerosis [13], hypoglycemia [14], myocardial protection [15], and anti-tumor effects [16–18], etc. Chen et al., [16] reported that naringin suppresses cell growth of human non-small cell lung cancer by regulating microRNA-126/vascular cell adhesion molecule 1 (VCAM-1). Results from Cai et al., [17] indicated that naringin inhibits ovarian tumor growth by promoting apoptosis.

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Besides, Raha et al., [18] suggested that naringin induces AGS cancer cell growth inhibition by downregulating the PI3K/Akt/mTOR cascade via activation of MAPK pathways. He et al., [19] suggested that Naringin inhibits osteosarcoma cell proliferation and metastasis through regulating the expression of zinc finger E-box binding homeobox 1 (Zeb1). Another report indicated that naringin inhibits the development of glioblastoma via preventing focal adhesion kinase (FAK) activity [20]. Moreover, naringin can increase the sensitivity of human prostate cancer cells to paclitaxel [21]. However, till now, the effect of naringin on PTC remains unclear.

Therefore, the aim of the present study was to investigate the effect of naringin on TC cells *in vitro*, and further to explore the molecular mechanism.

2. Materials and methods

2.1. Cell culture

Two human TC cell lines (papillary thyroid cancer cells TPC-1 and anaplastic thyroid cancer cells SW1736) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These two cell lines were all grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 μg/ml streptomycin, and 100 units/ml penicillin (Gibco). All the cells were incubated at 37 ℃ with 5% CO_2.

2.2. Cell treatment

TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin [16] for 24 h, 48 h, or 72 h at 37 ℃. Naringin was purchased from Beyotime Biotechnology (Shanghai, China).

2.2.1. MTT assay

MTT assay was used to measure cell proliferation. TPC-1 and SW1736 cells were seeded into 96-well plates at a density of 2000 cells/well and cultured for 24 h. Then, the cells were treated with 6, 12 or 25 μg/ml naringin for 24 h, 48 h, or 72 h at 37 ℃ respectively. Subsequently, 20 μl MTT reagent (Beyotime Biotechnology, Shanghai, China) was added into each well and incubated for another 4 h at 37 ˚C. Finally, the culture medium was replaced by 150 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). To assess cell proliferation, the optical density (OD) values were detected at 490 nm using a microplate reader (BioTek Instruments, Inc.).

2.3. Flow cytometry assay

To analyze cell apoptosis, the Annexin-V/propidium iodide (PI) Apoptosis Detection Kit (Beyotime, Shanghai, China) was performed. In brief, a total of 1×10^6TPC-1 and SW1736 cells/well were seeded in 6-well plates and then treated with 6, 12 or 25 μg/ml naringin for 48 h at 37 ℃. Then, the cells were washed twice with cold phosphate buffer saline (PBS), and collected through centrifugation (2,000 × g; 5 min; 4 ˚C). Subsequently, the cells were stained with 5 μl ANNEXIN-V-FITC/10 μl PI for 15 min in the dark. At the end of the experiment, cell apoptosis was detected by using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed by Flowjo 7.6.1 (FlowJo, LLC, Ashland, OR, USA).

2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from TPC-1 and SW1736 cells was isolated by using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The RNA was then reverse transcribed into cDNA by using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Finally, FastStart SYBR Green Master Mix (Invitrogen; Thermo Fisher Scientific, Inc.) was performed to analyze the cDNAs per as the manufacturer's protocols. The PCR thermocycler conditions were as follows: 95 ˚C for 5 min, followed by 38 cycles of amplifcation at 95 ˚C for 45 sec, 60 ˚C for 45 sec and 72 ˚C for 60 sec. GAPDH was used as the internal control. The primer sequences for qPCR were as follows:

GAPDH forward, 5′-CTTTGGTATCGTGGAAGGACTC-3′;
reverse, 5′-GTAGAGGCAGGGATGATGTTCT-3′;
Bcl-2 forward, 5′-GGGACGCGAAGTGCTATTGGTA-3′;
reverse, 5′-CAGGCTGGAAGGAGAAGATGC-3′;
Bax forward, 5′-ATTGAGAAACGATTTGCCTACA-3′;
reverse, 5′-GGGAAATGGCTTATTCTCCTTTGCTT-3′;
Caspase3 forward, 5′-TGTCGATGCAGCAAACCTCA-3′;
reverse, 5′-GACTTCTACAACGATCCCCTC-3′;
Cyclin D1 forward, 5′-TCTACACCGACAACTCCATCC-3′;
reverse, 5′-GCATTTTGGAGAGGAAGTGTTC-3′;
c-Myc forward, 5′-CCTCCACTCGGAAGGACTATC-3′;
reverse, 5′-TGTTCGCCTCTTGACATTCTC-3′;
survivin forward, 5′-CACCGCATCTCTACATTCAAGA-3′;
reverse, 5′-CAAGTCTGGCTCGTTCTCAGT-3′. The $2^{-\Delta\Delta Ct}$ method [22] was used to determine the gene expression. Each experiment was performed in triplicate.

2.5. Western blot assay

TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 48 h and then total cellar proteins were harvested by using radioimmunoprecipitation assay lysis solution (Solarbio, Beijing) in line with the manufacturer's protocols. Protein concentration was measured by using the Enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology) following the manufacturer's protocols. A total of 50 mg of protein lysates were separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocked with 5% non-fat milk at room temperature for 2 h, the membranes were incubated with primary antibodies: Caspase3 (Cat no. 14220; 1: 1000; Cell Signaling Technology, Inc.), cleaved Caspase3 (Cat no. 9664; 1: 1000; Cell Signaling Technology, Inc.), Bcl-2 (Cat no. 4223; 1: 1000; Cell Signaling Technology, Inc.), Bax (Cat no. 5023; 1: 1000; Cell Signaling Technology, Inc.), cyclin D1 (Cat no. 2978; 1: 1000; Cell Signaling Technology, Inc.), c-Myc (Cat no. 18583; 1: 1000; Cell Signaling Technology, Inc.), survivin (Cat no. 2808; 1: 1000; Cell Signaling Technology, Inc.), p-AKT (Cat no. 4060; 1: 1000; Cell Signaling Technology, Inc.), AKT (Cat no. 4685; 1: 1000; Cell Signaling Technology, Inc.), and β-actin (Cat no. 4970; 1: 1000; Cell Signaling Technology, Inc.) at 4 ˚C overnight, and then incubated with HRP-conjugated secondary antibody (Cat no. 7074; 1: 2000; Cell Signaling Technology, Inc.) at room temperature for 2 h. At last, the protein bands were observed by using BeyoECL Star (Special Supersensitive ECL Chemiluminescence Kit) (Cat no. P0018AM; Beyotime Institute of Biotechnology).

2.6. Statistical analysis

SPSS 19.0 (IBM Corp., Armonk, NY, USA) was performed for statistical analysis. Data were displayed as mean ± standard deviation (SD). Differences between groups were determined by Student's t-test or One-way analysis of variance with Bonferroni correction post-hoc. $p < 0.05$ indicated a significant difference.

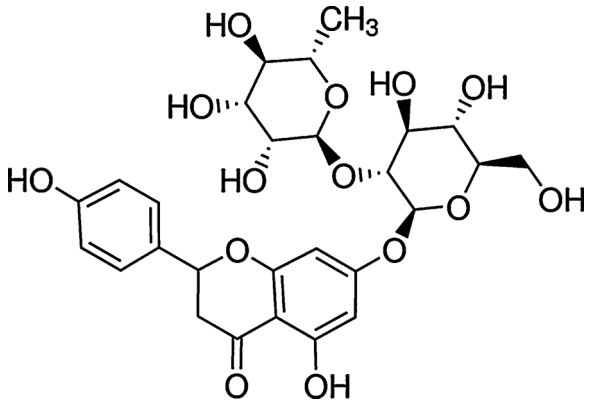

Fig. 1. The molecular formula of naringin.

3. Results

3.1. Naringin inhibited TPC-1 and SW1736 cell proliferation in a dose- and time-dependent manner

The molecular formula of naringin was shown in Fig. 1. To investigate the effect of naringin on TC cells, we firstly studied the effect of naringin on TC cell proliferation using MTT assay. TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 24 h, 48 h, or 72 h at 37 °C respectively. Then, cell proliferation was determined using MTT assay, and the results indicated that naringin inhibited TPC-1 and SW1736 cell proliferation in a dose- and time-dependent manner (Fig. 2 A and B).

3.2. Naringin induced TPC-1 and SW1736 cell apoptosis in a dose-dependent manner

TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 48 h. We then determined the effect of naringin on TC cell apoptosis. Results showed that compared with the control group, naringin treatment dose-dependently induced TPC-1 (Fig. 3A and B) and SW1736 (Fig. 3C and D) cell apoptosis.

3.3. Naringin dose-dependently affected cell proliferation and apoptosis related gene expression in TPC-1 and SW1736 cells

Next, we identified the potential molecular mechanisms by which naringin affected the TPC-1 and SW1736 cells. TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 48 h. Then, the protein expression of cyclin D1, c-Myc, survivin, Caspase3, cleaved Caspase3, Bcl-2, and Bax in TPC-1 and SW1736 cells was measured using western blot assay, and the data indicated that compared with the control group, naringin treatment dose-dependently inhibited the protein expression of cyclin D1, c-Myc, survivin, and Bcl-2, and enhanced Caspase3, cleaved Caspase3 and Bax protein expression in TPC-1 (Fig. 4A) and SW1736 cells (Fig. 5A). Similar results were obtained from qRT-PCR assay. Compared with the control group, naringin treatment dose-dependently inhibited the mRNA expression of cyclin D1, c-Myc, survivin, and Bcl-2, and enhanced Caspase3 and Bax mRNA expression in TPC-1 (Fig. 4B-G) and SW1736 cells (Fig. 5B-G).

3.4. Naringin dose-dependently suppressed PI3K/AKT pathway in TPC-1 and SW1736 cells

PI3K/AKT pathway plays an important role in cancer cell growth. We then explored whether naringin affected PI3K/AKT pathway in TPC-1 and SW1736 cells. TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 48 h. Then, the protein expression of p-AKT and AKT in TPC-1 and SW1736 cells was measured using western blot assay, and the ratio of p-AKT/AKT was calculated. We found that naringin treatment dose-dependently reduced the protein expression of p-AKT and the ratio of p-AKT/AKT in TPC-1 (Fig. 6A and B) and SW1736 (Fig. 6C and D) cells.

4. Discussion

The present study demonstrated that naringin showed anti-tumor effects in thyroid cancer cells through inhibiting thyroid cancer cell proliferation and inducing cell apoptosis via regulating the expression of cell proliferation and apoptosis related genes and the activation of PI3K/AKT pathway. These results suggested that naringin is a potential agent for the treatment of TC.

Thyroid cancer is the most widespread endocrine malignancy, and PTC is the most common human thyroid cancer [4,23]. As the overall incidence of TC is increasing year by year, it is extremely urgent to find a new and effective TC treatment strategy. Naringin, one of the most abundant flavonoids in citrus, has been found to exert anti-cancer effects in a variety of tumors [16–21]. Thus, we suspected that naringin might also have anti-tumor effect on TC as well.

Firstly, we studied the effect of naringin on TC cell proliferation, and TPC-1 and SW1736 cells were treated with different concentrations of naringin for 24 h, 48 h, or 72 h. Then, TC cell proliferation was determined, and the results indicated that naringin inhibited TC cell proliferation in a dose- and time-dependent way. Meanwhile, we found that naringin dose-dependently increased TC cell apoptosis. Then, to explore the molecular mechanism by which naringin affected TC cells, the expression of cell proliferation and cell apoptosis related genes (cyclin D1, c-Myc and survivin, Caspase3, cleaved Caspase3, Bcl-2, and Bax) were detected in TPC-1 and SW1736 cells. Cyclin D1, which is highly expressed in cancer cells, is a cell cycle regulatory protein that promotes cell proliferation and participates in tumorigenesis [24]. c-Myc plays a key role in the occurrence and development of tumors and is involved in the regulation of cell proliferation and apoptosis [25,26]. The survivin gene, a novel inhibitor of apoptosis, plays an important role in regulating cell growth and proliferation [27]. Caspase-3, a

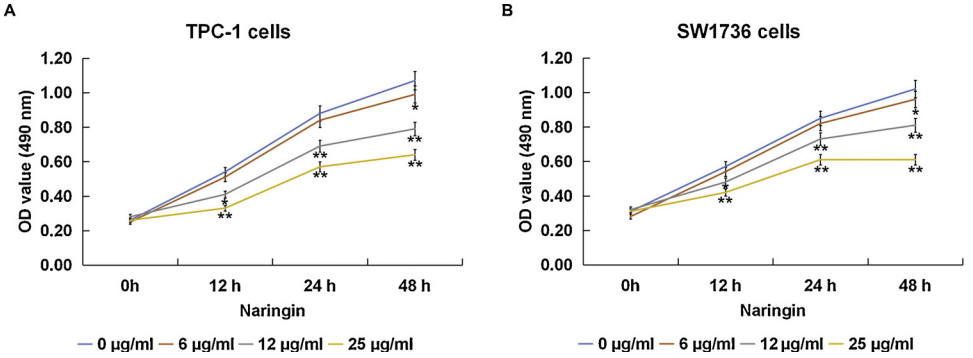

Fig. 2. Effect of naringin on thyroid cancer cell proliferation.
TPC-1 (A) and SW1736 (B) cells were treated with 6, 12 or 25 μg/ml naringin for 12 h, 24 h, or 48 h at 37 °C respectively. Then, cell proliferation was determined using MTT assay. Data were displayed as mean ± SD. *, **p < 0.05, 0.01 vs. 0 μg/ml naringin treatment group.

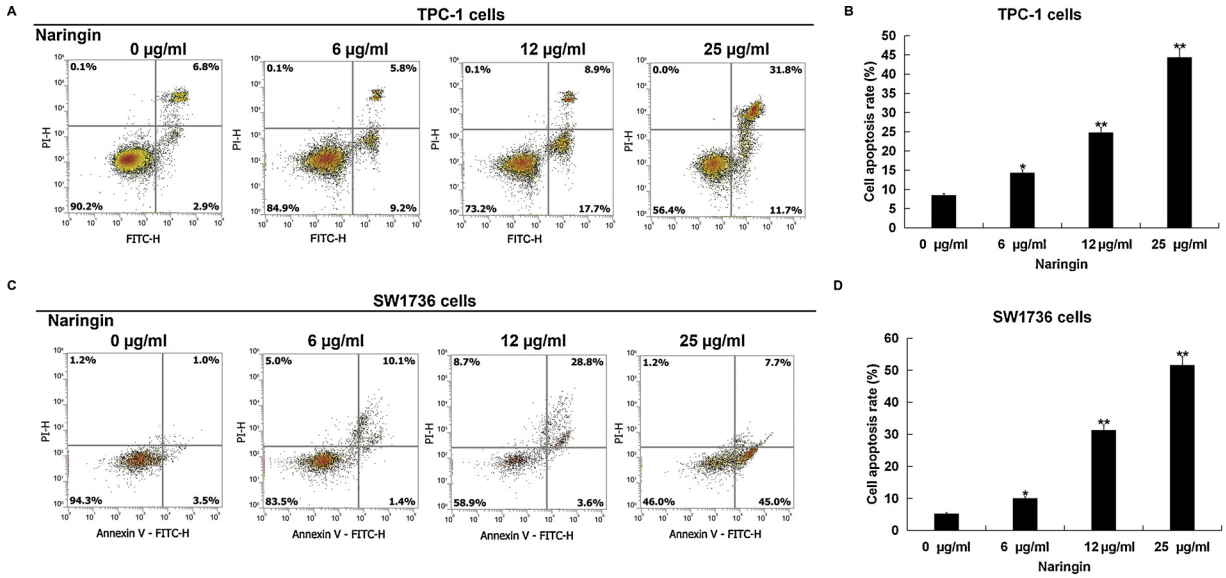

Fig. 3. Effect of naringin on thyroid cancer cell apoptosis.
TPC-1 (A and B) and SW1736 (C and D) cells were treated with 6, 12 or 25 μg/ml naringin for 12 h, 24 h, or 48 h at 37 ℃ respectively. Then, cell apoptosis was determined using flow cytometer assay. Data were displayed as mean ± SD. *, **p < 0.05, 0.01 vs. 0 μg/ml naringin treatment group.

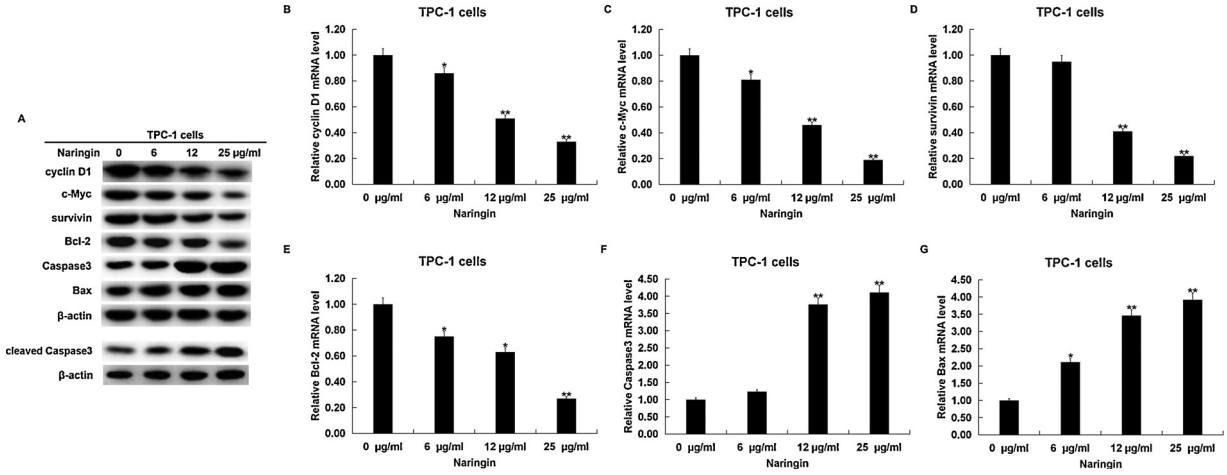

Fig. 4. Effect of naringin on the expression of cyclin D1, c-Myc, survivin, and Bcl-2, Caspase3 and Bax in TPC-1 cells.
TPC-1 cells were treated with 6, 12 or 25 μg/ml naringin for 12 h, 24 h, or 48 h at 37 ℃ respectively. Then, the protein and mRNA expression of cyclin D1, c-Myc, survivin, and Bcl-2, Caspase3, cleaved Caspase3 and Bax in TPC-1 cells were detected using western blotting (A) and qRT-PCR (B-G). Data were displayed as mean ± SD. *, **p < 0.05, 0.01 vs. 0 μg/ml naringin treatment group.

regulator of cell death, plays an important role in the process of apoptosis and its inhibition contributes to tumorigenesis [28]. Bax is well known pro-apoptotic gene, while Bcl-2 can inhibit the apoptosis of tumor cells [29,30]. As expected, in the current study, we found that naringin treatment dose-dependently inhibited the expression of cyclin D1, c-Myc, survivin and Bcl-2, and enhanced Caspase3, cleaved Caspase3 and Bax expression in TC cells. These results indicated that naringin inhibited TC cell proliferation and induced TC cell apoptosis at least partly through regulating the expression of cyclin D1, c-Myc and survivin, Caspase3/cleaved Caspase3, Bcl-2, and Bax.

PI3K/AKT pathway plays an important role in cancer cell growth and it is activated in cancers including TC [31,32]. And a previous study has shown that naringin inhibits AGS cancer cell proliferation via suppressing PI3K/AKT pathway [18]. Therefore, we finally explored whether naringin affected PI3K/AKT pathway in TPC-1 and SW1736 cells, and the results showed that naringin repressed PI3K/AKT pathway in TC cells. However, whether naringin acts directly or indirectly through regulation of the PI3K/AKT pathway in TC cells remain to be explored. And further experiments are needed to verify whether the inhibited expression of cyclin D1, c-Myc, survivin, Bcl-2 And the enhanced expression of Bax, Caspase3 and cleaved Caspase3 were result from Akt inhibition. We will perform this in the future project.

In summary, our study demonstrated for the first time the anticancer effect of naringin on TC. Our research provides more theoretical basis for the treatment of TC. However, this study is only a preliminary study of the effect naringin on TC. More experimental research is needed to confirm the current results. For instance, the effects of naringin on other TC cell lines should be provided. The effect of naringin on TC cell migration, invasion and epithelial-mesenchymal transition (EMT) should be studied. Besides, the present study only conduct experiments *in vitro*, and the effects of naringin on TC should be studied *in vivo*. Moreover, rescue experiments are needed to verify whether naringin acts directly or indirectly through regulation of the PI3K/AKT pathway in TC cells. We will perform all these issues in the future.

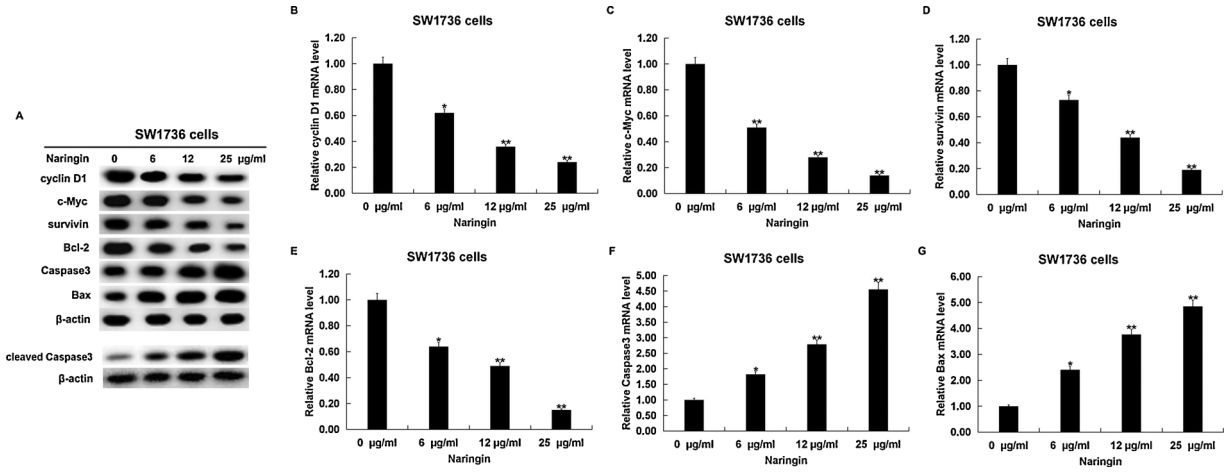

Fig. 5. Effect of naringin on the expression of cyclin D1, c-Myc, survivin, and Bcl-2, Caspase3 and Bax in SW1736 cells.
SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 12 h, 24 h, or 48 h at 37 ℃ respectively. Then, the protein and mRNA expression of cyclin D1, c-Myc, survivin, and Bcl-2, Caspase3, cleaved Caspase3 and Bax in SW1736 cells were detected using western blotting (A) and qRT-PCR (B-G). Data were displayed as mean ± SD. *, **p < 0.05, 0.01 vs. 0 μg/ml naringin treatment group.

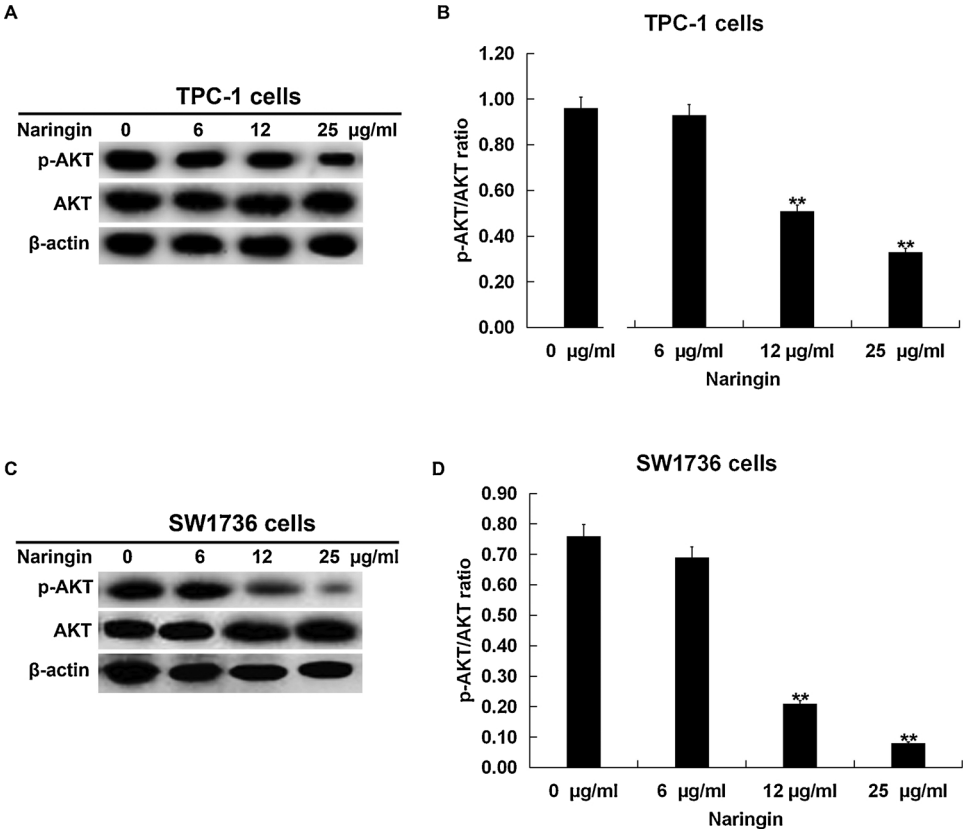

Fig. 6. Effect of naringin on PI3K/AKT pathway in thyroid cancer cells.
TPC-1 and SW1736 cells were treated with 6, 12 or 25 μg/ml naringin for 12 h, 24 h, or 48 h at 37℃ respectively. Then, the protein expression of p-AKT and AKT in TPC-1 (A) and SW1736 (C) cells was measured using western blot assay. The ratio of p-AKT/AKT in TPC-1 (B) and SW1736 (D) cells was calculated. Data were displayed as mean ± SD. **p < 0.01 vs. 0 μg/ml naringin treatment group.

Disclosures

All authors declare no financial competing interests.
All authors declare no non-financial competing interests.

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