Original Article

Apigetrin Inhibits Thyroid Cancer Cell Growth and Proliferation through Down-Regualtion of HIF1α and VEGF Expression

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ABSTRACT

Aim: Thyroid cancer is a commonly detected endocrine malignant tumour which accounts for 1% of the total human cancers. The present study was aimed to investigate the effect of apigetrin on anaplastic thyroid cancer cell viability and understand the mechanism involved. **Materials and Methods:** Fluorescent microscopy using diamidino-2-phenylindole (DAPI)staining was used to examine the nuclear fragmentation. Changes in protein expression were analysed by Western blot analysis and migration potential using wound healing assay. **Results:** Treatment with apigetrin exhibited inhibitory effect on anaplastic thyroid cancer cell viability in dose based manner. The cleavage of PARP and activation of caspase-3/-9 was promoted in anaplastic thyroid cancer cells on treatment with apigetrin. A significant reduction in hypoxiainducible factor1α (HIF1α) and vascular endothelial growth factor (VEGF) expression was caused by apigetrin in anaplastic thyroid cancer cells. The count of Terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL)-positive cells was increased significantly ($p<0.05$) in the cell cultures on treatment with apigetrin. The cell migration was also suppressed by apigetrin in dose based manner. Moreover, treatment of mice with apigetrin inhibited the tumor growth and development markedly in comparison to the untreated group. **Conclusion:** The present study demonstrates that apigetrin plays an important role in the inhibition of thyroid cancer. Therefore, apigetrin needs to be investigated further as therapeutic agent for thyroid cancer treatment.

Keywords: *Scutellaria,* Papillary, Follicular, Terpenoid, Pharmacophore.

INTRODUCTION

Thyroid cancer is one of the commonly diagnosed malignant tumour of endocrine gland comprising of 1% of the total cancers.[1] The papillary and follicular thyroid cancers account for around 95% of thyroid carcinoma cases whereas the anaplastic thyroid cancer accounts for less than 5% of the thyroid cancers.[1] The papillary and follicular thyroid cancers, together known as differentiated thyroid cancers grow at very slow speed and can be treated by using surgery, suppression of thyroid-stimulating hormone and radioiodine ablation.[2,3] On the other hand, anaplastic thyroid cancer is a deadly disease and the patients have average survival period of 6-months following diagnosis.[2,3] At the time of diagnosis anaplastic thyroid cancer has been found to undergo metastasis to the distant organs in more than 75% of the patients.[4] Because of its diagnosis at well advanced stage anaplastic thyroid cancer cases are assigned to the stage IV by the American Joint Commission.[5] So far there has been no effective treatment strategy available in the market for anaplastic thyroid cancer.[6] The lack of adequate and efficient

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treatment strategy demands for discovery of novel therapeutic molecules for thyroid cancer.[6]

The *Labiatae* family comprises of a large number of plants having great importance in traditional system of medicine.[7-10] One of the members of this genus is *Scutellaria* which shows activity against allergy, inflammation, hepatitis, thrombosis and oxidative disorders.[7-10] The major compounds isolated from *Scutellaria* genus include scutellarin, baicalein, baicalin, wogonin, apigetrin, etc. with good activity against carcinoma cells.[11,12] The present study was aimed to investigate the effect of apigetrin obtained from Biorbyt Ltd (5 Orwell Furlong Cowley Road Cambridge, Cambridgeshire CB4 0WY UK) on BHP2-7 and ATC351 cancer cell proliferation and metastasis potential. The study showed that thyroid cancer growth is inhibited by apigetrin treatment *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells and materials

BHP2-7 and ATC351 thyroid cancer cell lineswereprovided by the Shanghai Institute of Biochemistry and Cellular Biology, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) was used for culturing the cell lines. The medium was mixed with fetal bovine serum (10%) and antibiotics [penicillin (100 U/ml) and streptomycin (100 U/ml)] under humidified atmosphere containing 5% CO_2 at 37°C.

Cell viability assay

The changes in BHP2-7 and ATC351 cell viability on exposure to different apigetrin concentrations was assessed by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay. BHP2-7 and ATC351 cells were put at concentration of 3 x10^5 cells/well in the 96-well plates and cultured overnight. Apigetrin at 50, 100, 150, 200, 250 and 300 µM concentrations was mixed with the medium and cells were incubated in it for 72 hr. MTT solution (5 mg/ml) 30 µl was put into each well of the plate incubation was performed for 5 hr under standard conditions. The supernatant from the plates was decanted followed by addition of 160 µl DMSO to every well. The cell survival was measured by recording absorbance for each well three times by microplate reader at 587 nm.

Western blot analysis

BHP2-7 cells after 72 hr of exposure to 150, 200, 250 and 300 µM concentrations of apigetrin were PBS washed. Then cell lysis was achieved using TNN buffer consisting of Triton X-100 (1.2%), Nonidet P-40 (1.2%), along with 12 mg/ml solution of aprotinin, 12 mg/ml solution of leupeptin, $PhCH_2SO_2F$ (2 mM), sodium floride (110 mM), Na-ortho-vanadate(350 mM) and Na4P2O7(350 mg/ml). The protein separation from lysates was carried out on 10-12% SDS-PAGE and subsequently transferred to the PVDF. The protein incubation was initially performed at 4°C with primary antibodies for overnight followed by membrane washing with PBS. Then incubation was carried out for 2 hr with secondary antibodies conjugated to horseradish peroxidase at room temperature. The immune-stained blots were detected using enhanced chemiluminescence reagent (ELPS, Seoul, Korea). The band visualization was made by the ECL Plus system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The primary antibodies used were against: VEGF, HIF-1α, Bax, Bcl-2, cleaved caspase-3 and cleaved PARP (all from Cell Signaling Technology, Inc., Danvers, MA, USA).

Terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) assay

Onto the18 mm cover glasses BHP2-7 cells in DMEM medium were grown to ~80% confluence for 24 hr. Then cells were treated for 72 hr with 150, 200, 250 and 300 µM concentrations of apigetrin followed by fixing in 2% paraformaldehyde. The cells were washed with PBS and subsequently stained in the 2 µg/ml solution of 4,6-diamidino2-phenylindole (DAPI) at 37°C for 35 min. The nuclear fragmentation in the DAPI stained cells was examined by fluorescent microscope using TUNEL kit (Chemicon, Temecula, CA, USA) in accordance with the manufacturer's instructions.

Immunofluorescence

BHP2-7 cells were put onto 18-mm glass plates and cultured for 24 hr to attain 80% confluency in growth medium. Apigetrin at concentrations of 150, 200, 250 and 300 µM was mixed in the medium and incubation was carried out for 72 hr. After fixing with cold PFA (2%), PBS washed cells were incubated with primary antibodies against VEGF and HIF-1α for overnight at 4°C. Then incubation was carried out with secondary antibodies conjugated to FITC followed by 30 min DAPI staining at 37°C. The slides were examined using confocal laser scanning microscope (Olympus, Tokyo, Japan).

Wound healing assay

BHP2-7 cells were put into 6 well plates and cultured for 24 hr at 3 × 105 cells per well to attain 95% confluency. The monolayer of cells was scratched through the

middle by 100 µl pipette tip to create the wound. The loose cells were cleaned by fresh medium containing 5% FBS. The cellular monolayers were incubated for 72 hr with the 150, 200, 250 and 300 µM concentrations of apigetrin. Images of cell migration were captured by the inverted microscope connected to a digital camera and measurement of the wound area was carried out using Adobe Photoshop software version 7.0 (Adobe Systems, Inc., San Jose, CA, USA).

In vivo study

Fifty male nude mice (6 week old; weighing, 22-25 g) were purchased from the Animal Experimental Centre, Beijing, China. All the mice were put under sterilized conditions free of pathogens and were provided free access to the animal chow and water *ad libitum*. The mice were acclimatized for 1-week under laboratory conditions prior to starting the actual experiment under 12 hr dark/light cycle at 24°C. The experimental procedures involving mice were approved by the Animal Care and Use Committee, Chinese Medical University, China. The experiments were carried out as per the guidelines of National Institute of Health, China. The mice were assigned into five groups of 10 each randomly. Sham, untreated and three treatment groups (100, 200 and 300 mg/kg apigetrin). The mice in the untreated and three treatment groups were inoculated 2 × 10^6 BHP2-7 cells subcutaneously on one of the flanks. The mice in treatment groups were given intraperitoneal injection of 100, 200 and 300 mg/kg doses of apigetrin on day-2 of tumour inoculation. On the day 30th of tumour inoculation, the mice were sacrificed using isoflurane anaesthesia to extract the tumour. The tumour volume was measured using digital caliper and the weight of tumour was also recorded.

Statistical analysis

The data are presented as the mean ± SD of triplicate experiments. Analysis of the data was made statistically using ANOVA and an unpaired Student's *t*-test. The differences were taken significant statistically at $P<0.05$ values. Statistical analysis of the data was performed by the SPSS software for Windows operating system (version 10.0; SPSS, Chicago, IL, USA).

RESULTS

BHP2-7 and ATC351 cell viability inhibition by apigetrin

Exposure of BHP2-7 and ATC351 cells to apigetrin was followed by MTT assay to determine changes in cell viability (Figure 1). Apigetrin was added to the cells at 50, 100, 150, 200, 250 and 300 µM concentrations and viability was assessed at 48 hr. The suppression of BHP2-7 and ATC351 cell viability by apigetrin was concentration dependent. At 50, 100, 150, 200, 250 and 300 µM concentrations of apigetrin BHP2-7 cell viability was reduced to 87, 71, 58, 43, 35 and 22%, respectively. Exposure to 50, 100, 150, 200, 250 and 300 µM concentrations of apigetrin suppressed ATC351 cell viability to 89, 74, 55, 41, 32 and 18%, respectively.

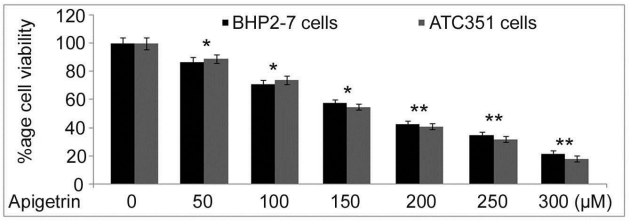

Figure 1: Effect of apigetrin on BHP2-7 and ATC351 cell viability. The cytotoxicity of apigetrin in BHP2-7 and ATC351 cells at 72 hr wasassessed by MTT assay. The presented values are the mean ± SD of experiments performed independently in triplicates. **P*<0.05 and ***P*<0.02 vs. untreated cells.

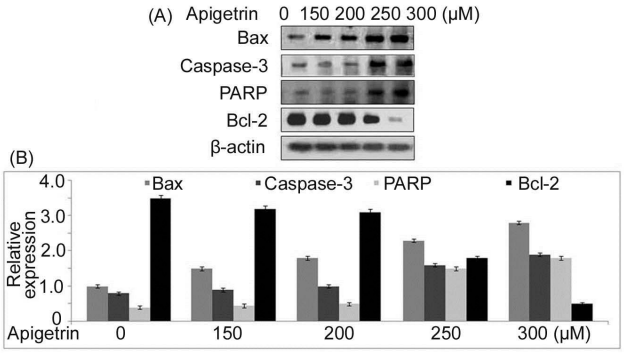

Figure 2: Effect of apigetrin on pro-apoptotic proteins in BHP2-7 cells. (A) Exposure of BHP2-7 cells to apigetrin for 48 hr was followed by western blotting to assess expression of Bax Bcl-2, cleaved PARP and cleaved caspase-3. (B) The expression level was quantified taking β-actin as control. **P*<0.05 and ***P*<0.01 vs. untreated cells.

Apigetrin up-regulates pro-apoptotic proteins in BHP2-7 cells

Changes in Bax, PARP cleavage, Bcl-2 and caspase-3 cleavage in BHP2-7 cells on exposure to apigetrin was assessed by western blotting (Figure 2). Exposure of BHP2-7 cells to apigetrin for 48 h at 150, 200, 250 and 300 µM concentrations increased expression of Bax and cleavage of PARP and caspase-3. The expression of Bcl-2 was suppressed in BHP2-7 cells on exposure to apigetrin for 48 hr.

Apigetrin leads to apoptosis in BHP2-7 cells

The results from DAPI staining showed characteristic apoptotic changes in BHP2-7 cell morphology

onexposure to apigetrin (Figure 3). Apigetrin treatment led to fragmentation of chromatin material and formation of apoptotic bodies in BHP2-7 cells at 48 hr. The percentage of apoptotic BHP2-7 cells showed a marked increase with the increase in apigetrin concentration from 150 to 300 μM.

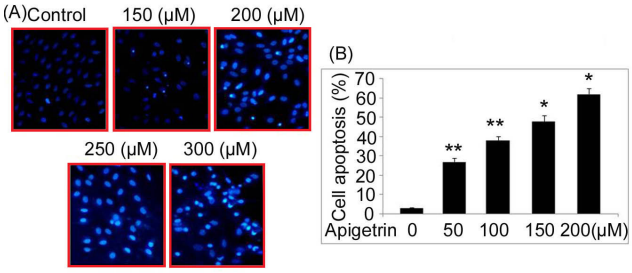

Figure 3: Induction of apoptosis in BHP2-7 cells by apigetrin. After 48 hr of apigetrin exposure BHP2-7 cells were assessed by TUNEL assay using DAPI staining. Magnification x200.

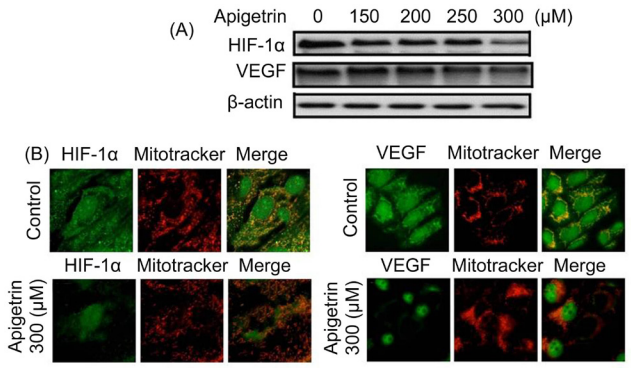

Figure 4: Inhibitory effect of apigetrin on angiogenesis molecules in BHP2-7 cells. (A) BHP2-7 cells at 48 hr of apigetrin exposure were analysed for HIF-1α and VEGF expression by western blotting. (B) Expression of HIF-1α and VEGF was determined using immunofluorescence.

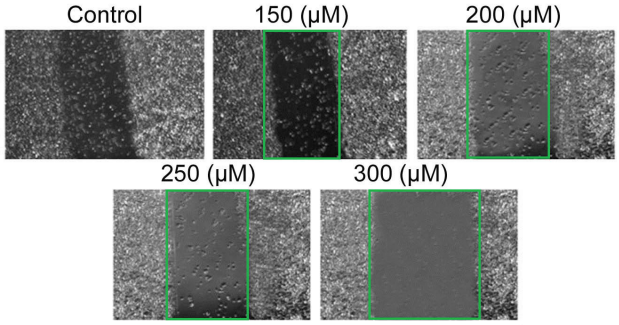

Figure 5: Effect of apigetrin on BHP2-7 cell migration. The cells were exposed for 48 hr to indicated concentrations of apigetrin and then examined by wound healing assay. Magnification, x400.

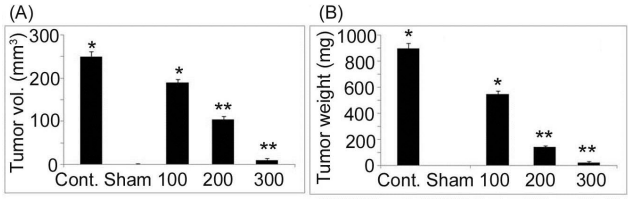

Figure 6: Effect of apigetrin on thyroid cancer growth *in vivo*. (A) Suppression of thyroid tumor volume by apigetrin in BHP2-7 xenograft mice model. (B) Inhibition of tumor weight in the mice by apigetrin treatment. **P*<0.05 vs. control.

Apigetrin inhibits HIF-1α and VEGF expression in BHP2-7 cells

In BHP2-7 cells treatment with apigetrin caused inhibition of HIF-1α and VEGF expression in concentration dependent manner (Figure 4). The expression of HIF-1α and VEGF was markedly reduced in BHP2-7 cells at 48 hr of treatment with 150, 200, 250 and 300 μM of apigetrin.

Apigetrin inhibits BHP2-7 cell migration

Exposure of BHP2-7 cells to apigetrin was followed by wound healing assay for analysis of cell migration (Figure 5). The migration of BHP2-7 cells was reduced markedly on treatment 150, 200, 250 and 300 μM of apigetrin in comparison to the untreated cells.

Apigetrin inhibits tumor development *in vivo*

In the mice model of thyroid cancer treatment with apigetrin markedly suppressed growth and development of tumour. The tumour volume was markedly smaller in the apigetrin treated mice in comparison to the untreated group (Figure 6A). Treatment of the thyroid cancer mice with apigetrin at 100, 200 and 300 mg/kg doses markedly reduced tumor weight in comparison to the untreated mice (Figure 6B).

DISCUSSION

Thyroid cancer is one of the very fast growing carcinoma which needs urgent attention of clinicians and researchers. Biocompatibility of natural products and their derivatives together with no or negligible side effects has been a driving force for their investigation against various types of cancers.[13] Around 70% of the compounds possessing anti-cancer activity are either natural products or derivatives of the natural isolates.[14] The present study investigated the effect of apigetrin on BHP2-7 and ATC351 cancer cell proliferation and metastasis potential. The study for the first time demonstrated thyroid cancer growth inhibition potential of apigetrin *in vitro* and *in vivo*.

Apoptosis activation is explored by most of the drug candidates for elimination of the undesired cells from body of multicellular organisms in a programmed manner.[15] Among many factors caspase-3 plays a main role in the activation of molecules which are required for cell apoptosis.[16] The proteolytic breakdown of proteins like PARP, DNA repairing molecule and the protein responsible for integrity of DNA is catalysed by the caspase-3.[16] In the present study initial results showed that apigetrin exhibited cytotoxic effect on BHP2-7 and ATC351 cell viability. Investigation of the mechanism revealed that apigetrin treatment of BHP2-7 cells led to up-regulation of apoptotic pathway. Treatment of BHP2-7 cells with apigetrin promoted expression of caspase-3 which subsequently enhanced cleaved PARP level. The level of Bax was increased and that of Bcl-2 was suppressed in BHP2-7 cells on exposure to apigetrin. These findings proved that apigetrin exhibited cytotoxic effect on BHP2-7 cells by activation of pro-apoptotic molecules. Induction of BHP2-7 cell apoptosis was also confirmed by TUNEL assay which clearly showed apoptotic bodies and chromatin condensation by apigetrin treatment.

Tumor development, metastasis and growth at the site of migration mainly depends on angiogenesis. The tumor angiogenesis is facilitated by the increased expression of VEGF and HIF-1α.[17-19] Since HIF-1α is the signalling molecule for expression of VEGF therefore down-regulation of HIF-1α is has been used for inhibition of tumor angiogenesis in different types of cancers.[19] In the present study apigetrin treatment markedly down-regulated the expression of HIF-1α and VEGF in BHP2-7 cells. Therefore, apigetrin exposure inhibited thyroid cancer cell angiogenesis and migration by targeting HIF-1α and VEGF expression. The *in vitro* cytotoxicity of apigetrin against BHP2-7 cells fascinated for the investigation of its effect on *in vivo* BHP2-7 cell tumor xenograt mice model. The study revealed that apigetrin reduced tumor volume and weight markedly in the mice model of thyroid cancer.

CONCLUSION

In summary, the study demonstrated that apigetrin inhibited thyroid cancer growth *in vitro* as well as *in vivo*, suppressed tumor angiogenesis and activated pro-apoptotic molecules. This suggests that apigetrin is a potent molecule against thyroid cancer and needs to be investigated further for the development of and effective treatment strategy.

CONFLICT OF INTEREST

The authors declare no conflict of interest in the publication of these results.

ABBREVIATIONS

HIF:1α: Hypoxiainducible factor1α; **VEGF:** Vascular endothelial growth factor; **DAP:** Diamidino-2-phenylindole.

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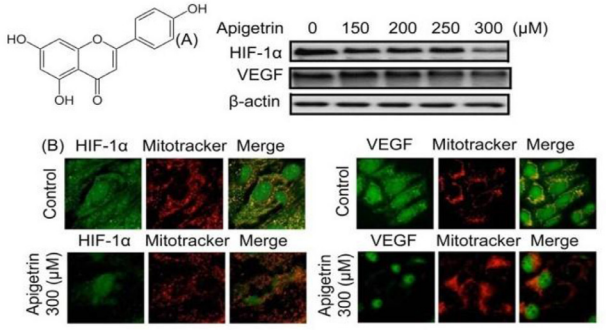
PICTORIAL ABSTRACT



SUMMARY

The present study for the first time demonstrated that apigetrin possesses thyroid cancer growth inhibition potential. Therefore, the study can be of immense importance to the clinicians for the development of thyroid cancer treatment.

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