ANTICANCER RESEARCH *37*: 1705-1710 (2017)
doi:10.21873/anticanres.11502

Myricetin Induces Apoptosis of Human Anaplastic Thyroid Cancer Cells *via* Mitochondria Dysfunction

SUNHYO JO[1*], TAE KWUN HA[2*], SANG-HUN HAN[3*], MI EUN KIM[1], INAE JUNG[1], HEE-WOO LEE[3], SUNG KWON BAE[4] and JUN SIK LEE[1]

[1]*Department of Life Sciences, BK21-plus Research Team for Bioactive Control Technology, College of Natural Sciences, Chosun University, Gwangju, Republic of Korea;*
[2]*Department of Surgery, Inje University College of Medicine, Busan Paik Hospital, Busan, Republic of Korea;*
[3]*Department of Verterinary Internal Medicine, College of Verterinary Medicine, Seoul National University, Seoul, Republic of Korea;*
[4]*Department of Medical Management, Kosin University, Busan, Republic of Korea*

Abstract. *Aim: Thyroid cancer is the most common endocrine malignancy, with an increasing incidence worldwide. Most thyroid cancers are well differentiated and have a favorable outcome. However, undifferentiated thyroid cancers are one of the most lethal human malignancies. Anaplastic thyroid cancer (ATC) accounts for 2% of all thyroid cancers, and its median survival rate is low. ATC is responsible for more than one-third of thyroid cancer-related deaths. Myricetin is a flavonol compound found in walnuts, herbs, and various berries and is known to induce apoptotic death of various types of cancer cells. However, an anticancer effect of myricetin against human anaplastic thyroid cancer (HATCs) cells has not been demonstrated. Materials and Methods: In the present study, the anticancer effects and mechanism of action of myricetin were examined using SNU-80 HATC cells. SNU-80 HATC cells were treated with various concentrations of myricetin and compared with untreated controls. Results: Myricetin significantly reduced HATC cell proliferation, by approximately 70%. A substantial proportion of dead cells exhibited arrest in the sub-G_1 phase. Myricetin also exhibited cytotoxicity and induced DNA condensation in SNU-80 HATC cells in a dose-dependent manner. The mechanism of myricetin-induced cell death involved an increase in the activation of caspase cascades and the Bax:Bcl-2 ratio at a concentration of 100 μM. Myricetin also induced the release of apoptosis-inducing factor (AIF) from mitochondria into the cytosol and altered the mitochondrial membrane potential. Conclusion: Our results indicate that myricetin is a potent inducer of HATC cell death and may thus prove useful in the development of therapeutic agents for HATC.*

Thyroid cancer is the most common endocrine malignancy, and its worldwide incidence has increased steadily over the past three decades (1). Anaplastic thyroid cancer (ATC) has an incidence of approximately 1 to 2 cases per million persons per year (2). However, patients with ATC have a median survival time of less than 6 months, with fewer than 20% of patients surviving 1 year. Because ATC, although rare, is fatal, accounting for approximately one-third of all thyroid cancer-related deaths, there is a pressing need for new effective therapies (2-4). Generally, thyroid cancer can be treated effectively with surgery or radioactive iodine. The mechanisms driving the progression of ATC are not completely understood, however. ATC is currently treated with radiotherapy, surgery, and chemotherapy (5). Nevertheless, patients with ATC only have a median survival of 5 months, and less than 20% survive for 1 year after diagnosis (1).

Development of new therapies for rare cancers such as ATC has been hindered for several reasons. Traditional approaches to drug development require an often-prohibitive investment of time and money, an issue that is even more significant for developing drugs for treating orphan cancers. A new and inventive approach to cancer therapy development would be to exploit the multitude of already established compounds approved for clinical use for other indications (6). However, this approach requires that these compounds be evaluated for both *in vitro* and *in vivo* anti-cancer activity and exploiting already established compounds would, therefore, assist in the identification of new drugs for clinical trials and therapies to treat thyroid cancers.

*These Authors contributed equally to this study.

Correspondence to: Jun Sik Lee, Department of Life Sciences, BK21-plus Research Team for Bioactive Control Technology, College of Natural Sciences, Chosun University, Gwangju 501-759, Republic of Korea. Tel: +82 622306651, Fax: +82 622306650, e-mail: junsiklee@chosun.ac.kr

Key Words: Myricetin, human anaplastic thyroid cancer cells, cell cycle, Bax/BcL-2 ratio.

Flavonoids are natural polyphenols present in a variety of foods, especially fruits and vegetables. The anticancer activities of flavonoids isolated from plants have been intensively studied. Myricetin is a flavonoid that is abundant in fruits and vegetables and has demonstrated anti-cancer activities. Previous studies reported that myricetin induces apoptosis of various types of cancer cells, including those of pancreatic cancer, hepatoma, esophageal cancer, and colon carcinoma. We previously reported that myricetin induces the death of human colon cancer cells *via* Bax- and Bcl-2–dependent pathways (7). However, activity of myricetin against human thyroid cancer cells has not been demonstrated.

In this study, the anticancer activity of myricetin was examined using SNU-80 human anaplastic thyroid cancer (HATC) cells. The mechanism underlying the anticancer activity of myricetin was also investigated. The findings of this investigation suggest that myricetin is a potentially useful chemotherapeutic agent for treating human ATC.

Materials and Methods

Reagents. Myricetin and propidium iodide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazoliumbromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Prolong Gold anti-fade reagent with 4,6-diamidino-2-phenylindole (DAPI) and tetramethylrhodamine methyl ester (TMRM) were obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture. SNU-80 HATC cells were obtained from the Korea Cell Line Bank. Cells were cultured at 37˚C in the presence of 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 IU/ml penicillin, 200 mg/ml streptomycin, 2 mmol L-glutamine, and 1 mmol sodium pyruvate.

Myricetin treatment. Myricetin was dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI 1640 (final DMSO concentration, 0.1% [v/v]). Untreated control cells were treated with an equal volumeof DMSO.

Cell proliferation assay. Cell viability was confirmed using the MTT assay to determine the toxic dose of myricetin. Cells (8×10^3 cells/well) were cultured in 96-well culture plates and then treated with myricetin at various concentrations (25, 50, and 100 μM). After treatment, the cells were incubated at37˚C for 24 h. The treatment medium was removed, and MTT (0.5 mg/ml) dissolved in phosphate-buffered saline (PBS) was added. After incubation for 4h, medium containing the MTT reagent was removed and the formazan precipitate was dissolved using solubilization solution (DMSO: ethanol, 1:1). The absorbance of the formazan solution was measured using an enzyme-linked immunosorbent assay microplate reader at 570 nm.

Nuclear DAPI staining. Chromatin condensation and nuclear fragmentation were confirmed using DAPI staining.SNU-80 HATC cells (8×10^4 cells/well) were seeded in 12-well culture plates containing round cover-slips in the wells. After culture at 37˚C, myricetin was added to each well at various concentrations (25, 50, or 100 μM) and the cells were incubated for 24 h, after which they were washed twice with cold PBS and fixed with cold 100% methanol. Fixed cells on cover-slip were mounted on glass slides using permanent mounting solution with DAPI. Chromatin condensation was observed under a fluorescence microscope.

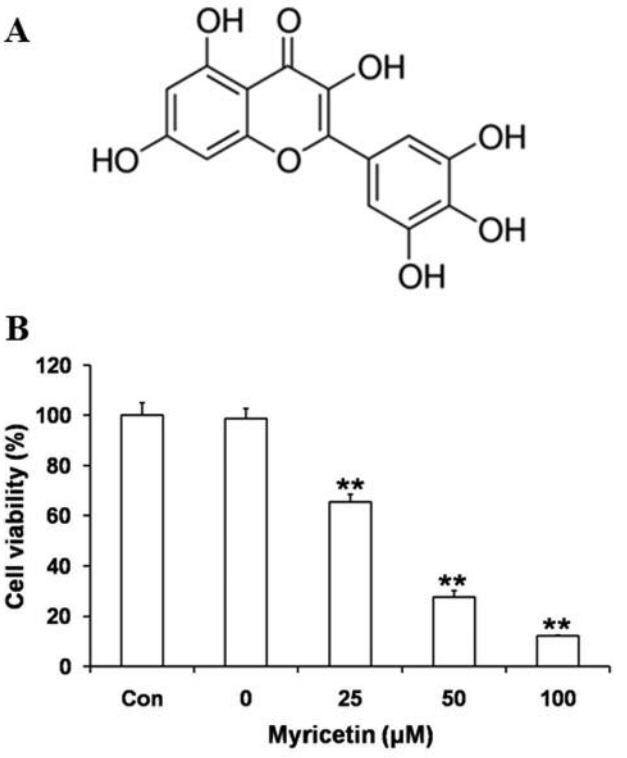


Figure 1. *Myricetin treatment induces the cytotoxicity of SNU-80 HATC cells. Structure of myricetin (A). SNU-80 HATC cells were plated in 96-well microplates and experimental medium containing various concentrations of myricetin was added. Cell survival was assessed using the MTT assay after 24 h of incubation, as described in the Materials and Methods section. Data are reported as the number of viable cells, expressed as a percentage of control cells exposed only to 0.1% DMSO. The data represent the average (±SD) from four replicate wells and are representative of three independent experiments. Differences were considered statistically significant at **p<0.01.*

Cell-cycle analysis. SNU-80 HATC cells (1×10^6 cells/well) were seeded in 100-mm culture plates and treated with myricetin (100 μM) for 24 h. Treated cells were then harvested using trypsin-EDTA and washed twice with cold PBS.Cells were fixed overnight at −20˚C using cold 70% ethanol. After centrifugation, the ethanol-fixed cells were washedtwice with cold PBS to remove the ethanol and then stained with 50 μg/ml of propidium iodide (PI) for 15 min at 4˚C in the dark. Cell-cycle stage was assessed using flow cytometry (Beckman, FC 500), and the resulting data were analyzed using Kaluza software.

Western blot analysis. Myricetin-treated (25, 50, or 100 μM) SNU-790 HPTC cells were lysed with cold whole-lysate buffer (RIPA) containing 150 mM sodium chloride, 0.1% sodium dodecyl sulfate (SDS), 50 mMTris (pH 8.0), 0.5% sodium deoxycholate, 1% NP-40, protease inhibitor cocktail, and phosphatase inhibitor

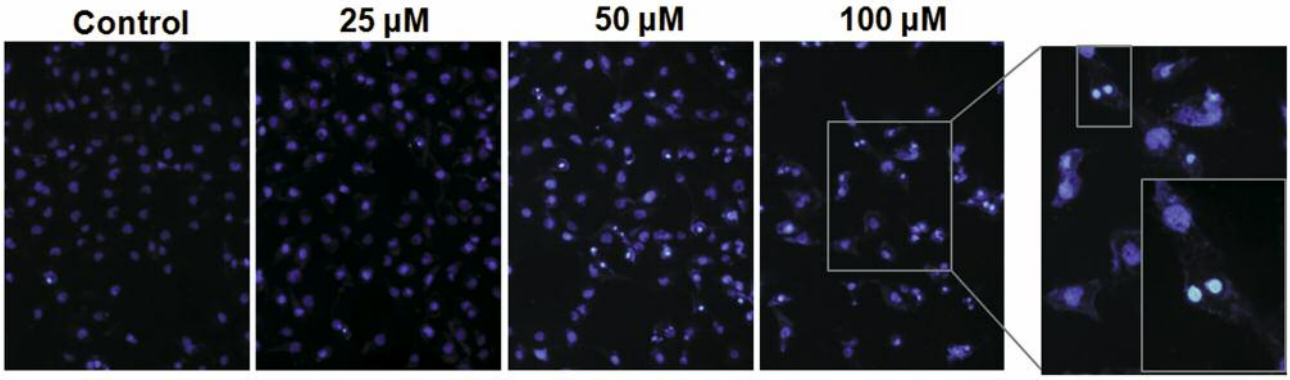


Figure 2. *Myricetin induces nuclear condensation in SNU-80 HATC cells. SNU-80 HATC cells were treated with various concentrations of myricetin (from 25 to 100 μM) for 24 h, after which the cells were stained with DAPI, as described in the "Materials and Methods" section. The cells werethen examined under a fluorescence microscope. Original magnification was 200× and 400×. Data are representative of three independent experiments.*

cocktail and harvested using a cell scraper. Cell debris was removed by centrifugation at 14,000 rpm at 4˚C for 15 min. Protein concentration was determined using a bicinchoninic acid assay kit (Pierce, Rockford, IL, USA). Protein samples (20 μg) were loaded on 10% SDS–polyacrylamide gel electrophoresis (PAGE) gels and separated. After SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with 5% non-fat milk at room temperature for 1h. The membrane was then blotted using anti-caspase 3, 8, 9, anti-apoptosis inducing factor(AIF), anti–pro-apoptotic factor Bax, anti-Bcl-2, and anti-β-actin (Santa Cruz Biotechnology) primary antibodies. The blots were developed and using anti-mouse and anti-rabbit secondary antibodies and visualized using chemiluminescence (ECL).

Mitochondrial membrane potential (MMP, ΨΔm) measurement. SNU-80 HATC cells were seeded in 100-mm culture plates and treated with 100 μM myricetin. Floating and adherent cells were harvested with trypsin-EDTA at 0, 15, 30, 60, 120 and 180 min and then washed with cold PBS. The collected cells were stained with the fluorescent dye TMRM ester (100 nM). Stained cells were separated using flow cytometry (Beckman, FC 500), and the resulting data were analyzed using Kaluza software.

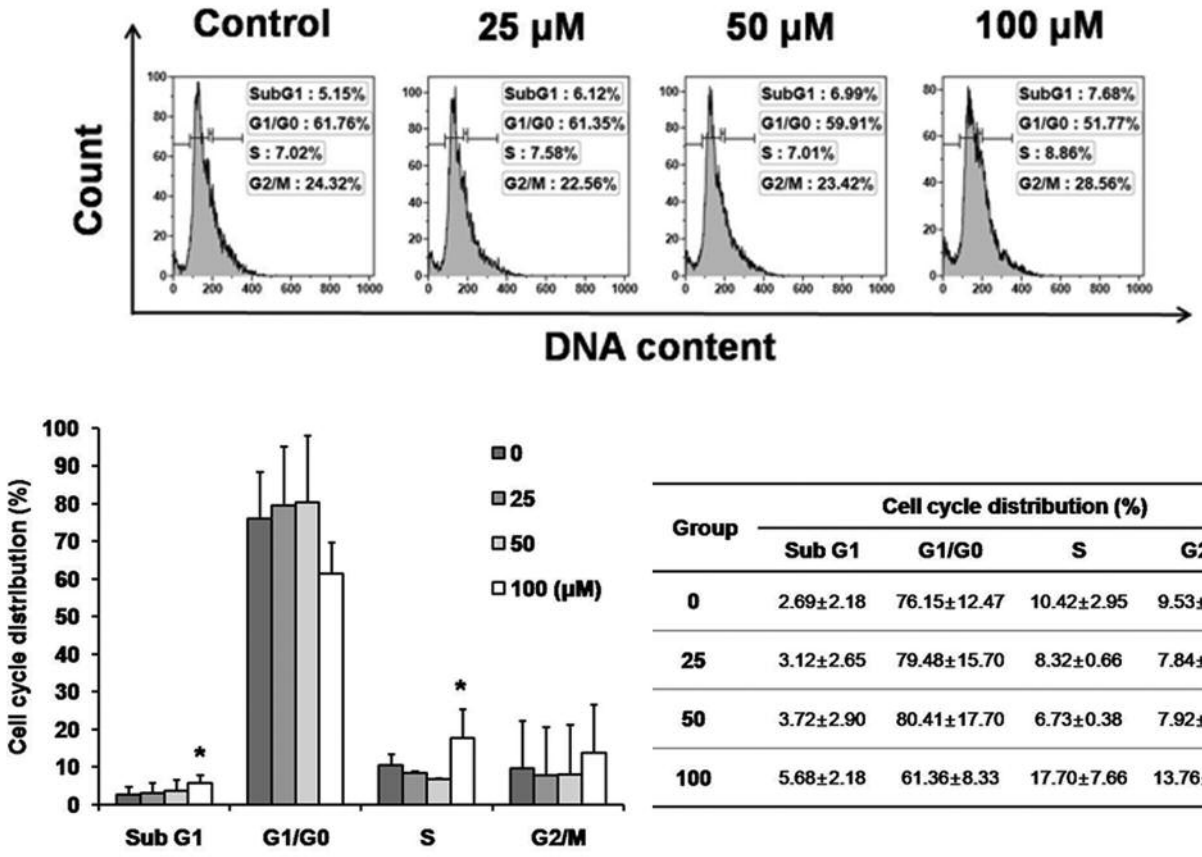
Statistical analysis. Results are presented as mean±standard deviation (SD). All data were evaluated using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) using SPSS (IBM, New York, NY, USA). A p-value<0.05 was considered indicative of statistical significance.

Results

Myricetin induces death of SNU-80 HATC cells. In the present study, we examined whether myricetin exhibits anticancer activity against SNU-80 HATC cells. The cytotoxic effect of myricetin on SNU-80 HATC cells was measured by MTT assay. The treatment of myricetin reduced cell viability of SNU-80 HATC cells in a dose-dependent manner. In comparison to the control (treated with only 0.1% of DMSO), cell treated with 100 μM of myricetin induced about 85% reduction in cell viability on SNU-80 HATC cells (Figure 1). This result suggests that myricetin significantly induces cell death of SNU-80 HATC cells.

Myricetin induces chormatin condensation in SNU-80 HATC cells. As described in the previous result, myricetin significantly reduced cell viability of SNU-80 HATC cells. Therefore, we explored the mechanism of the cytotoxicity of myricetin using SNU-80 HATC cells treated with myricetin at concentration of 25, 50 and 100 μM. To investigate chromatin condensation of SNU-80 HATC cells, cells were treated with myricetin at various concentrations from 25 to 100 μM for 24h. As shown in Figure 2, DAPI staining showed that myricetin-treated SNU-80 HATC cells induced significant unclear shrinkage and rounding compared with 0.1% of DMSO (control) treated cells at 50 and 100 μM concentration. Therefore, myricetin plays a major role in initiating cell death in cancer cells through the DNA damage.

Myricetin induces sub-G_1 cell-cycle arrest in SNU-80 HATC cells. Considering the fact that myricetin inhibited cell proliferation, flow cytometric analysis on cell-cycle progression was performed to determine the mechanism of the anti-proliferative effect exhibited by the tested myricetin against SNU-80 HATC cells. As shown in Figure 3, it appears that myricetin induced a dose-dependent growth arrest in the sub-G_1 phase of SNU-80 HATC cells. The percentage of cell in the sub-G_1 phase was lower in untreated control cells compared with cells treated with 100 μM myricetin (2.69±2.1% *vs*. 5.68±2.18%). The percentage of cells in the S phase was 10.42±2.95% in the untreated control cells, compared with 17.70±7.66% in cells treated with 100 μM of myricetin. This result suggests that myricetin induces the death of SNU-80 HATC cells in part by arresting cells in the sub-G_1 phase.



Group	Cell cycle distribution (%)			
	Sub G1	G1/G0	S	G2/M
0	2.69±2.18	76.15±12.47	10.42±2.95	9.53±12.81
25	3.12±2.65	79.48±15.70	8.32±0.66	7.84±12.75
50	3.72±2.90	80.41±17.70	6.73±0.38	7.92±13.42
100	5.68±2.18	61.36±8.33	17.70±7.66	13.76±12.82

Figure 3. *Cell-cycle analysis of SNU-80 HATC cells treated with myricetin. SNU-80 HATC cells were treated with medium only (0.1% DMSO) or 25, 50, and 100 μM of myricetin. The cell-cycle assay was performed as described in the Materials and Methods section. Data are representative of three independent experiments. Differences were considered statistically significant at *p<0.05.*

Myricetin induces the death of SNU-80 HATC cells via up-regulation of Bax/Bcl-2 ratio and activation of caspase cascades. The activation of the caspase cascade and an increase in the Bax/Bcl-2 ratio are important for induction and processing of apoptosis. To this address, the downstream events of myricetin-induced apoptosis in SNU-80 HATC cells were characterized by activation of caspase-3, -8, -9 and Bax/Bcl-2 ratio using western blot analysis. As shown in figure 4, myricetin increased the activation of caspase-3, -8and -9. Moreover, 100 μM of myricetin treatment significantly induces the Bax/Bcl-2 ratio. These data indicate that myricetin might induce SNU-80 HATC cells death *via* increasing the activation of caspase and Bax/Bcl-2 ratio.

Myricetin induces AIF release and disruption of mitochondrial function. Dysfunction of mitochondria can result in the release of pro-apoptotic molecules including AIF (apoptosis-inducing factors). The Bax expression resulted in release of AIF from mitochondria, and activation of pro-caspase-3/-9. Moreover, mitochondrial dysfunction appears all morphologic and biochemical markers of apoptosis. Therefore, mitochondrial dysfunction can result in the release of pro-apoptotic molecules such as AIF and activation of caspase cascades. As shown in Figure 4, treatment with myricetin induced the release of AIF in SNU-80 HATC cells at 100 μM.

As alterations of the MMP on mitochondria may occur during cell death, we determined whether myricetin treatment affects the MMP in SNU-80 HATC cells using TMRM fluorescence analysis. As shown in Figure 5, the MMP in SNU-80 HATC cells treated with 100 μM myricetin was altered in a time-dependent manner. Synthetically, these results suggest that myricetin induces the death of SNU-80 HATC cells *via* a mitochondrial dysfunction-mediated mechanism.

Discussion

Cancer represents a serious threat to human health and life, especially thyroid cancer is a kind of most common endocrine gland carcinomas. Surgery, chemotherapy, drugs, and radiation are the most commonly utilized

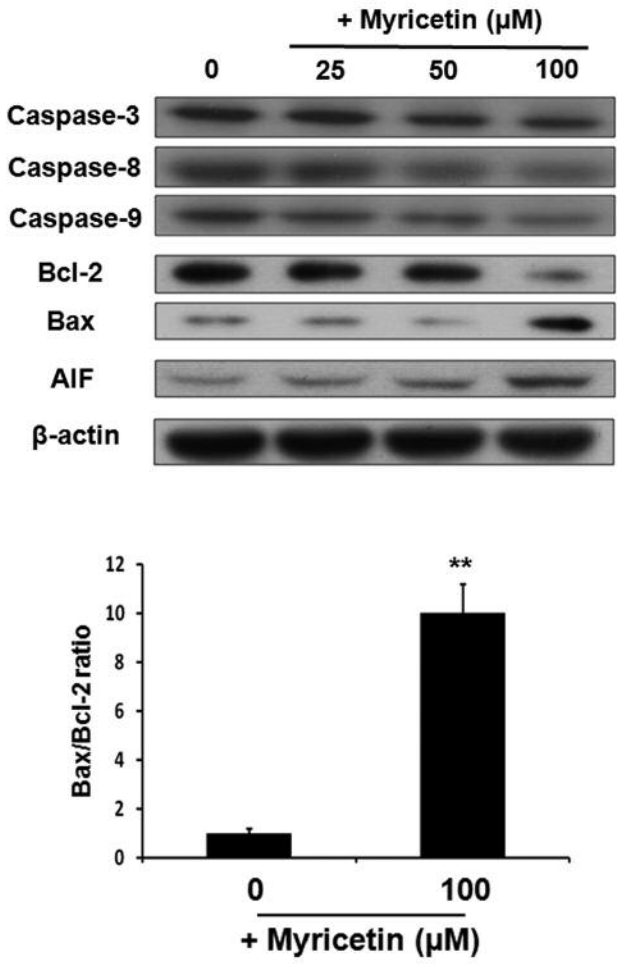


Figure 4. *Myricetin activates caspase cascades and induces apoptotic proteins. SNU-80 HATC cells were incubated with different concentrations of myricetin (from 25 to 100 μM) for 24 h and subsequently lysed. Equal amounts of lysate were then separated on 10 or 12% of SDS-PAGE gels and transferred to PVDF membranes. The membranes were probed with antibodies to the indicated factors (AIF, caspase-3, caspase-8, caspase-9, Bax, Bcl-2, and β-actin) and detected by the addition of ECL solution. β-actin was used as the internal control. The results shown are from one experiment representative of four performed that showed similar patterns.*

methods for treating most cancers. It's associated with unacceptable rates of cytotoxicity toward normal cells and many side-effects, such as nephrotoxicity, neurotoxicity, anemia, and nausea (8). Therefore, novel therapies and more selective drugs are needed for the treatment of human cancers.

In previous studies, myricetin has been reported to induce the apoptotic cell death of human hepatocarcinoma and human ovarian cancer (9, 10). The present study, we report that myricetin induces the death of human anaplastic thyroid cancer cells. We found significant induction of apoptotic biomarkers in SNU-80 HATC cells. The effect of myricetin on the proliferation of SNU-80 HATC cells was evaluated by treating cells with the compound at various concentrations. As shown in Figure 1, myricetin induces cytotoxicity of SNU-80 HATC cells in a dose-dependent manner. Interestingly, when cell treated with 100 μM of myricetin induced about 85% reduction in cell viability on SNU-80 HATC cells.

Apoptosis is programmed cell death Chromatin condensation is a morphological hall marker of apoptotic cell death and DNA damage can induce cell death which leads to a cell cycle arrest (11). The cell cycle involves a series of events that take place within a cell that lead to its division and duplication, resulting in the production of two daughter cells. The cell cycle can be divided into three periods: interphase (G_1, S, and G_2 phases), mitosis (M phase), and cytokinesis. Dysregulation of the cell cycle canis critical characteristic, and the development of investigational anti-cancer drugs hasrecently focused on the molecular targets involved in cell cycle control mechanisms in chemotherapy of cancer (12, 13). In addition, it has been previously reported that the sub-G_1 phase arrest was observed in cancer cells including melanoma cell (14). Our data showed that myricetin induced SNU-80 cell accumulation in sub-G_1 phase and chromatin condensation, suggesting that myricetin treatment induce the cell cycle arrest followed by apoptosis.

The mechanism of apoptotic cell death is divided into two major pathways, extrinsic and intrinsic pathway. Extrinsic pathway is mediated by death receptors, which can induce the cleavage of caspase-8. Cleaved caspase-8 directly activates caspase-3 and regulates pro-apoptotic (Bax)/anti-apoptotic (Bcl-2) protein ratio (15). In intrinsic pathway, various apoptotic stimuli trigger the increase the Bax/Bcl-2 ratio and alteration of mitochondria permeability. Increased Bax protein induces cytochrome *c* and AIF (apoptosis-inducing factor) are release from mitochodnria, sequentially activates caspase-dependent and -independent pathways. Caspase-9, -8, and -3 are activated in caspase-dependent pathway, and AIF directly lead the apoptotic cell death following DNA degradation and damages (16, 17). In this study, myricetin treatment induced caspase-3, -8, -9 cleavage, Bax/Bcl-2 ratio, and AIF release. In addition, myricetin also led the alteration of MMP. These results indicated that myricetin can induce apoptotic cell death through intrinsic pathway in SNU-80 HATC cells.

In conclusion, our data demonstrated that myricetin treatment reduced cell viability and induced apoptotic cell death of SNU-80 HATC through an increase of AIF release and mitochondrial dysfunctions. Thus, the present study suggests the therapeutic potential of myricetin for the treatment of human anaplastic thryroid cancer.

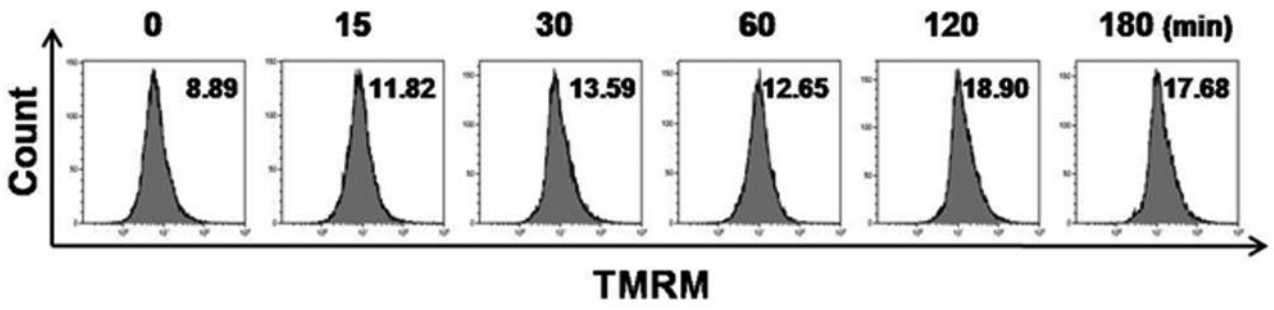


Figure 5. *Myricetin-induced MMP alteration in SNU-80 HATC cells. SNU-80 HATC cells were treated with 100 μM myricetin for various time points (from 0 to 180 min) and then stained with 20 nM TMRM. Fluorescence intensity was measured by flow cytometry and is presented as mean fluorescence intensity (MFI). The results are representative of three identical experiments.*

Disclosures

The Authors have no financial conflict of interest.

Acknowledgements

This study was supported by research fund from Chosun University 2014 (K206212007).

References

1 Kim SM, Park KC, Jeon JY, Kim BW, Kim HK, Chang HJ, Choi SH, Park CS and Chang HS: Potential anti-cancer effect of N-hydroxy-7-(2-naphthylthio) heptanomide (HNHA), a novel histone deacetylase inhibitor, for the treatment of thyroid cancer. BMC Cancer *15*: 1003, 2015.

2 Mehta A, Zhang L, Boufraqech M, Liu-Chittenden Y, Zhang Y, Patel D, Davis S, Rosenberg A, Ylaya K, Aufforth R, Li Z, Shen M and Kebebew E: Inhibition of Survivin with YM155 Induces Durable Tumor Response in Anaplastic Thyroid Cancer. Clin Cancer Res *21*: 4123-4132, 2015.

3 Kebebew E, Greenspan FS, Clark OH, Woeber KA and McMillan A: Anaplastic thyroid carcinoma. Treatment outcome and prognostic factors. Cancer *103*: 1330-1335, 2005.

4 Nilubol N and Kebebew E: Should small papillary thyroid cancer be observed? A population-based study. Cancer *121*: 1017-1024, 2015.

5 Smallridge RC: Approach to the patient with anaplastic thyroid carcinoma. J Clin Endocrinol Metab *97*: 2566-2572, 2012.

6 Mehta A, Zhang L, Boufraqech M, Zhang Y, Patel D, Shen M and Kebebew E: Carfilzomib is an effective anticancer agent in anaplastic thyroid cancer. Endocr Relat Cancer *22*: 319-329, 2015.

7 Kim ME, Ha TK, Yoon JH and Lee JS: Myricetin induces cell death of human colon cancer cells *via* BAX/BCL2-dependent pathway. Anticancer Res *34*: 701-706, 2014.

8 Garrastazu Pereira G, Lawson AJ, Buttini F and Sonvico F: Loco-regional administration of nanomedicines for the treatment of lung cancer. Drug Deliv *23*: 2881-2896, 2016.

9 Xu Y, Xie Q, Wu S, Yi D, Yu Y, Liu S, Li S and Li Z: Myricetin induces apoptosis *via* endoplasmic reticulum stress and DNA double-strand breaks in human ovarian cancer cells. Mol Med Rep *13*: 2094-2100, 2016.

10 Zhang XH, Chen SY, Tang L, Shen YZ, Luo L, Xu CW, Liu Q and Li D: Myricetin induces apoptosis in HepG2 cells through Akt/p70S6K/bad signaling and mitochondrial apoptotic pathway. Anticancer Agents Med Chem *13*: 1575-1581, 2013.

11 Liu CY, Hsieh CH, Kim SH, Wang JP, Ni YL, Su CL, Yao CF and Fang K: An indolylquinoline derivative activates DNA damage response and apoptosis in human hepatocellular carcinoma cells. Int J Oncol *49*: 2431-2441, 2016.

12 Brough R, Bajrami I, Vatcheva R, Natrajan R, Reis-Filho JS, Lord CJ and Ashworth A: APRIN is a cell cycle specific BRCA2-interacting protein required for genome integrity and a predictor of outcome after chemotherapy in breast cancer. EMBO J *31*: 1160-1176, 2012.

13 Filipits M, Pirker R, Dunant A, Lantuejoul S, Schmid K, Huynh A, Haddad V, Andre F, Stahel R, Pignon JP, Soria JC, Popper HH, Le Chevalier T and Brambilla E: Cell cycle regulators and outcome of adjuvant cisplatin-based chemotherapy in completely resected non-small-cell lung cancer: the International Adjuvant Lung Cancer Trial Biologic Program. J Clin Oncol *25*: 2735-2740, 2007.

14 Wang JJ, Wu CC, Lee CL, Hsieh SL, Chen JB and Lee CI: Antimelanogenic, Antioxidant and Antiproliferative Effects of Antrodia camphorata Fruiting Bodies on B16-F0 Melanoma Cells. PLoS One *12*: e0170924, 2017.

15 Kim KH, Seo HS, Choi HS, Choi I, Shin YC and Ko SG: Induction of apoptotic cell death by ursolic acid through mitochondrial death pathway and extrinsic death receptor pathway in MDA-MB-231 cells. Arch Pharm Res *34*: 1363-1372, 2011.

16 Fulda S and Debatin KM: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene *25*: 4798-4811, 2006.

17 Jin C and Reed JC: Yeast and apoptosis. Nat Rev Mol Cell Biol *3*: 453-459, 2002.

Received January 21, 2017
Revised March 7, 2017
Accepted March 8, 2017