

# Biochanin-A induces apoptosis and suppresses migration in FaDu human pharynx squamous carcinoma cells

IN-A CHO<sup>1</sup>, SANG-JOUN YOU<sup>2</sup>, KYEONG-ROK KANG<sup>1</sup>, SU-GWAN KIM<sup>1,6</sup>,  
JI-SU OH<sup>1</sup>, JAE-SEEK YOU<sup>1</sup>, GYEONG-JE LEE<sup>3</sup>, YO-SEOB SEO<sup>4</sup>, DO KYUNG KIM<sup>5</sup>,  
CHUN SUNG KIM<sup>5</sup>, SOOK-YOUNG LEE<sup>6</sup> and JAE-SUNG KIM<sup>5</sup>

Departments of <sup>1</sup>Oral and Maxillofacial Surgery, <sup>2</sup>Periodontology, <sup>3</sup>Prosthodontics, <sup>4</sup>Oral and Maxillofacial Radiology, <sup>5</sup>Oral Biology Research Institute, <sup>6</sup>Regional Innovation Center for Dental Science and Engineering, School of Dentistry, Chosun University, Gwangju 61452, Republic of Korea

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**Abstract.** The aim of the present study was to investigate biochanin-A-induced anticancer effects and their cellular signaling pathway in FaDu pharyngeal squamous carcinoma cells. Biochanin-A induced cell death through increased cytotoxicity of FaDu cells in a dose- and time-dependent manner. The number of cells with nucleus condensation and the apoptotic population were increased in the FaDu cells stimulated with biochanin-A for 24 h. Furthermore, extrinsic apoptotic factors such as FasL and their downstream target caspase-8 were increased and activated in the FaDu cells treated with biochanin-A in a dose-dependent manner. Moreover, biochanin-A decreased the expression of intrinsic anti-apoptotic factors such as Bcl-2 and Bcl-xL, and increased the level and activation of intrinsic apoptotic factors such as Bad and caspase-9. Finally, biochanin-A induced the activation of caspase-3 and Poly(ADP ribose) polymerase (PARP) in FaDu cells. Our results suggest that biochanin-A-induced apoptosis was mediated by death receptor mediated-extrinsic and mitochondria-dependent intrinsic apoptotic signaling pathways. Biochanin-A also inhibited wound healing migration and proliferation of FaDu cells via the downregulation and inactivation of matrix metalloproteinase-2 and -9 that are mediated by the suppression of p38, mitogen activated protein kinase (MAPK), NF- $\kappa$ B and Akt cellular signaling pathways. Therefore, these data suggest that the biochanin-A may act as a potential chemotherapeutic compound to treat head and neck cancer.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer with over 640,000 cases diagnosed annually and high mortality worldwide (1). Lifestyles such as smoking, alcohol consumption, and betel nut chewing are primary etiological factors of HNSCC and are linked to the increased prevalence of HNSCC worldwide (2). Although clinical interventions such as surgery, radiotherapy, chemotherapy, and chemo-radiotherapy have rapidly advanced, 5-year survival and morbidity rates of patients with HNSCC have not improved significantly in the last 30 years (2,3). Furthermore, despite the advancement of diagnostic techniques and therapeutics, clinical interventions frequently result in irreversible loss of function and disfigurement (4). Therefore, there is an urgent need to develop chemotherapeutic agents that effectively treat the cancer with fewer adverse effects.

Recently, natural compounds purified from herbal plants used traditionally in folk and oriental medicine have received considerable interest as a potential preventive or clinical drugs due to their various pharmacological effects combined with potential biological safety (5). Many recent studies have tested the anticancer effects of natural compounds to develop chemotherapeutic agents with fewer side-effects. Due to these studies, several natural compounds with effective anticancer effects and strong safety data have been used as clinical chemotherapeutic agents for cancer patients, after approval by the United States Food and Drug Administration (5).

Apoptosis is defined as programmed cell death characterized by morphological shrinkage (6), nucleus condensation (7) and DNA degradation (8). Cancer cell-specific apoptosis is one of current strategies for developing chemotherapeutic agents from natural compounds (9).

Biochanin-A (5,7-dihydroxy-4-methoxy-isoflavone) is a phytoestrogen, which is a natural estrogen analogue, derived from edible and herbal plants such as peanuts, alfalfa sprouts, soy and red clover (10). Recent studies have reported that biochanin-A alleviates climacteric vasomotor symptoms and reduces low-density lipoprotein levels in menopausal women (11). Furthermore, biochanin-A has been reported to

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*Correspondence to:* Professor Jae-Sung Kim, Department of Pre-Dentistry, School of Dentistry, Chosun University, 309 Philmun-daero, Dong-gu, Gwangju 61452, Republic of Korea  
E-mail: js\_kim@chosun.ac.kr

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possess various pharmacological effects including antioxidant activity (12), anti-inflammation (13), neuroprotection (14) and the prevention of articular cartilage degeneration (10). Biochanin-A has also been reported to show anticancer activity such as increased radiotoxicity in colon cancer (15), the increased survival in pancreatic cancer, and induction of apoptosis in prostate cancer (16) and hepatoma (17).

Therefore, the aim of the present study was to determine whether biochanin-A has the potential to function as a chemotherapeutic agent to treat HNSCC. We evaluated the potential apoptotic effects of biochanin-A on HNSCC and elucidated the induced apoptotic signaling pathway.

## Materials and methods

**Cell culture.** FaDu cells originating from human pharynx squamous carcinoma were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured by following the instruction provided by ATCC. Briefly, FaDu cells were maintained in minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100  $\mu\text{g/ml}$  streptomycin and 100 Unit/ml penicillin (Gibco) in a humidified, 5%  $\text{CO}_2$  and 37°C incubator.

**Cell cytotoxicity assay.** To determine the cytotoxicity of biochanin-A in FaDu cells, the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described (2). Briefly, FaDu cells were cultured at a density of  $0.2 \times 10^5$  cells/ml in 96-well plates and allowed to attach to the well overnight. After incubation, cultured FaDu cells were treated with 25, 50 and 100  $\mu\text{M}$  biochanin-A (Santa Cruz Biotechnology, Dallas, TX, USA) for 24 and 48 h. Thereafter, 200  $\mu\text{l}$  of 5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO, USA) was added into cultured FaDu cells and incubated for another 4 h. Sequentially, supernatant was removed, and MTT crystals were dissolved in 200  $\mu\text{l/well}$  dimethyl sulfoxide (DMSO; Sigma-Aldrich). Finally, optical density was measured at 570 nm by a spectrophotometer (Epoch; BioTek Instruments, Inc., Winooski, VT, USA).

**Live and dead cell assay.** To visualize the live and dead FaDu cells following treatment with biochanin-A, the cell live and dead assay was performed with green calcein AM and ethidium homodimer-1 as previously described (4). Briefly, FaDu cells were cultured at a density of  $0.2 \times 10^5$  cells/ml in an 8-well chamber slide (Electron Microscopy Sciences, Hatfield, PA, USA) and allowed to attach to the well overnight. After incubation, cultured FaDu cells were treated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. Thereafter, to visualize either live or dead FaDu cells, live and dead cell assay was performed by live and dead cell assay kit (Thermo Fisher Scientific, Rockford, IL, USA), which is composed of green calcein AM to stain the live cells with green fluorescence and ethidium homodimer-1 to stain the dead cells with red fluorescence, following the instructions provided by the manufacturer. Cells were imaged using fluorescence microscopy (Eclipse TE2000; Nikon Instruments, Inc., Melville, NY, USA).

**DAPI staining.** To determine the nucleus condensation in FaDu cells treated with biochanin-A, DAPI (4',6-diamidino-2-phenylindole) staining was performed by the protocol previously described (18). Briefly, FaDu cells were cultured at a density of  $0.2 \times 10^5$  cells/ml in an 8-well chamber slide (Electron Microscopy Sciences) and allowed to attach to the well overnight. After incubation, cultured FaDu cells were treated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. Thereafter, FaDu cells were fixed with 4% paraformaldehyde after washing with phosphate-buffered solution (PBS) and stained with 1 mg/ml DAPI for 20 min. The nucleus of FaDu cells were imaged using fluorescence microscopy (Eclipse TE2000; Nikon Instruments).

**Flow cytometric analysis.** Flow cytometric analysis (FACS) was performed to determine the extent of apoptosis and necrosis using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), respectively. The FaDu cells were cultured at a density of  $1 \times 10^5$  cells/ml and allowed to attach to the well overnight. After incubation, cultured FaDu cells were treated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. Thereafter, FaDu cells were washed twice in PBS and re-suspended in a binding buffer (BD Biosciences, San Diego, CA, USA). Annexin V-FITC and PI (BD Biosciences) were added to the cells and incubated in the dark for 15 min. The cells were analyzed using a fluorescence-activated cell sorting Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). Data analysis was performed using standard CellQuest software (Becton-Dickinson) and WinMDI version 2.9 software (The Scripps Research Institute, San Diego, CA, USA).

**Immunoblotting.** The FaDu cells were cultured at a density of  $1 \times 10^5$  cells/ml and allowed to attach to the well overnight. After incubation, cultured FaDu cells were treated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. Thereafter, FaDu cells were harvested, lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor cocktails (Sigma-Aldrich) according to the manufacturer's protocol. Total protein concentrations of cell lysates were determined by bicinchoninic acid (BCA) protein assays (Pierce, Rockford, IL, USA). In addition, conditioned media were harvested to detect the proteins secreted from FaDu cells. Equal amounts of protein and conditioned media were mixed with 5X loading buffer, boiled at 90°C for 10 min, separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (BD Biosciences). After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich) in TBS-T (Tris-buffered saline with 0.1% Tween-20 (Sigma-Aldrich) at room temperature for 1 h, membranes were reacted with TBS-T containing 5% BSA and primary antibodies of the corresponding proteins at 4°C for 12 h and then incubated with horseradish peroxidase-conjugated secondary antibody. The following antibodies were used: antibodies against FasL, caspases (-3, -7, -8 and -9), Bcl-2, Bcl-xL, Bad, Poly(ADP ribose) polymerase (PARP), phospho-NF- $\kappa\text{B}$  and total NF- $\kappa\text{B}$  were purchased from Cell Signaling Technology.  $\beta$ -actin, phospho-ERK, total ERK, phospho-p38, total p-38, phospho-Akt, total Akt, matrix metalloproteinase (MMP)-2

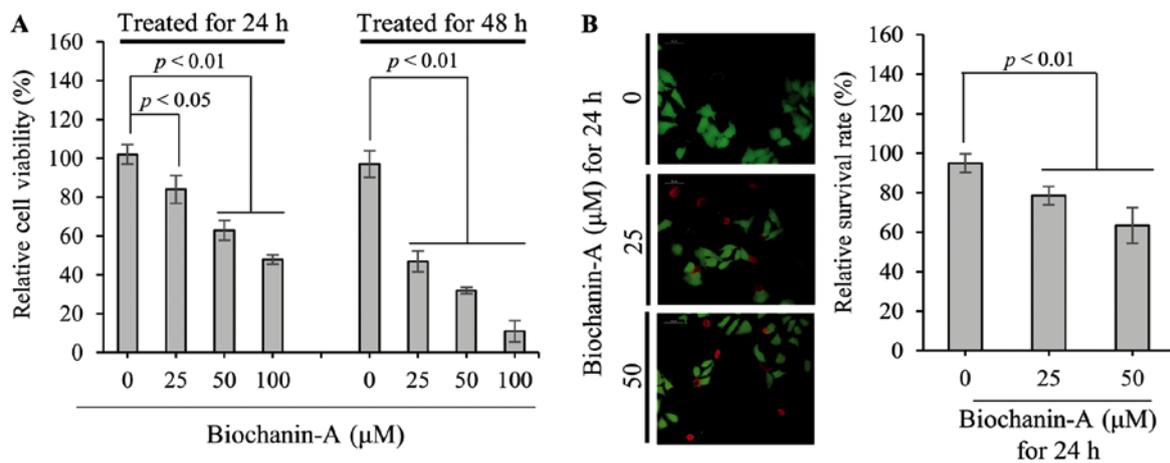


Figure 1. Biochanin-A induces cell death through the increase of cytotoxicity in FaDu cells. (A) Biochanin-A decreases the viability of FaDu cells in a dose- and time-dependent manner. FaDu cells were treated with different doses of Biochanin-A (0, 25, 50 and 100  $\mu\text{M}$ ) for 24 and 48 h. Under the indicated treatment conditions, cell viability was measured using an MTT assay. The data represent the results of three independent experiments and are expressed as the mean  $\pm$  standard deviation (SD); \* $P < 0.05$  and \*\* $P < 0.01$  compared to the control). (B) Biochanin-A induces the death of FaDu cells. FaDu cells cultured at a density of  $0.2 \times 10^5$  cells/ml in 8-well chamber slide were stimulated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. Live cells were stained with green calcein-AM and dead cells were stained with ethidium homodimer-1 (red) and then examined and photographed using fluorescence microscopy (Eclipse TE200; Nikon Instruments).

and MMP-9 were purchased from Santa Cruz Biotechnology. The immunoreactive bands were visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and were exposed on radiographic film.

**In vitro migration assay.** To perform the migration assay, FaDu cells were cultured onto  $2 \times 0.22 \text{ cm}^2$  culture inserts (Ibidi, Regensburg, Germany) at a density of  $1 \times 10^4$  cells/well. Wounds were introduced by removing the culture inserts after 24 h of incubation. Thereafter, cultured FaDu cells were treated with 25  $\mu\text{M}$  biochanin-A for 48 and 72 h. Wound widths were imaged using an inverted microscope (Eclipse TE2000; Nikon Instruments).

**Colony formation assay.** A colony formation assay was performed according to the previously described protocol (19). Briefly, FaDu cells were cultured at a density of 200 cells/well in a 6-well culture plate and allowed to attach to the well overnight. After incubation, cultured FaDu cells were treated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h and were then incubated in culture medium without biochanin-A for 5 days. Thereafter, the medium was removed and the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at  $4^\circ\text{C}$ . Sequentially, the colonies were stained with 2% crystal violet for 10 min. Finally, the colonies were washed with PBS and dried at room temperature, before being imaged by a digital camera (Nikon Instruments).

**Gelatin zymography.** Gelatin zymography was performed to assess the activity of matrix metalloproteinases (MMPs) secreted from FaDu cells treated with biochanin-A. An equal volume of conditioned media was mixed with non-reducing sample buffer [4% SDS, 0.15 M Tris (pH 6.8) and 20% (v/v) glycerol containing 0.05% (w/v) bromophenol blue] and resolved on a 10% polyacrylamide gel containing copolymerized 0.2% (1 mg/ml) swine skin gelatin. After electrophoresis of the conditioned media samples, gels were washed with

cold PBS containing 2.5% (v/v) Triton X-100 for 30 min and washed twice with cold PBS for 15 min. After washing, gels were incubated in the zymogram renaturing buffer [50 mM Tris-HCl (pH 7.6), 10 mM  $\text{CaCl}_2$ , 50 mM NaCl and 0.05% Brij-35] at  $37^\circ\text{C}$  for 72 h. After renaturation of MMPs, gels were stained with 0.1% Coomassie brilliant blue R250. The gelatinolytic activity was revealed as a clear band on a background of uniform light blue staining.

**Statistical analysis.** Data are reported as the mean  $\pm$  SD of three individual experiments performed. Statistical analysis was carried out using the Student's t-test and a P-value  $< 0.05$  was considered to indicate a statistically significant result.

## Results

**Biochanin-A induces death of FaDu cells via increased cytotoxicity.** To measure the viability of FaDu cells treated with 25, 50 and 100  $\mu\text{M}$  biochanin-A for 24 and 48 h, MTT assays were performed. As shown in Fig. 1A, relative viabilities of FaDu cells treated with 25, 50 and 100  $\mu\text{M}$  biochanin-A for 24 h are  $84 \pm 7.2$ ,  $63 \pm 5.15$  and  $48 \pm 2.4\%$ , respectively, compared to non-treated control ( $102 \pm 5.1\%$ ). Furthermore, relative viabilities of FaDu cells treated with the same concentrations of biochanin-A for 48 h are  $47 \pm 5.3$ ,  $32 \pm 1.6$  and  $11 \pm 5.5\%$ , respectively. Live and dead cell assays were performed to visualize the live and dead cells after treatment with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. In the non-treated control, as shown in Fig. 1B, almost all cells are stained fluorescent green by membrane permeable calcein AM, which is cleaved by cytosolic esterase in living cells. In contrast, the number of dead cells stained fluorescent red by ethidium homodimer-1 increases with biochanin-A treatment in a dose-dependent manner. The relative rate of live FaDu cells after treatment with 25 and 50  $\mu\text{M}$  biochanin-A were measured as  $78.5 \pm 4.7$  and  $63.5 \pm 9\%$ , respectively. These results consistently demonstrate that biochanin-A suppresses

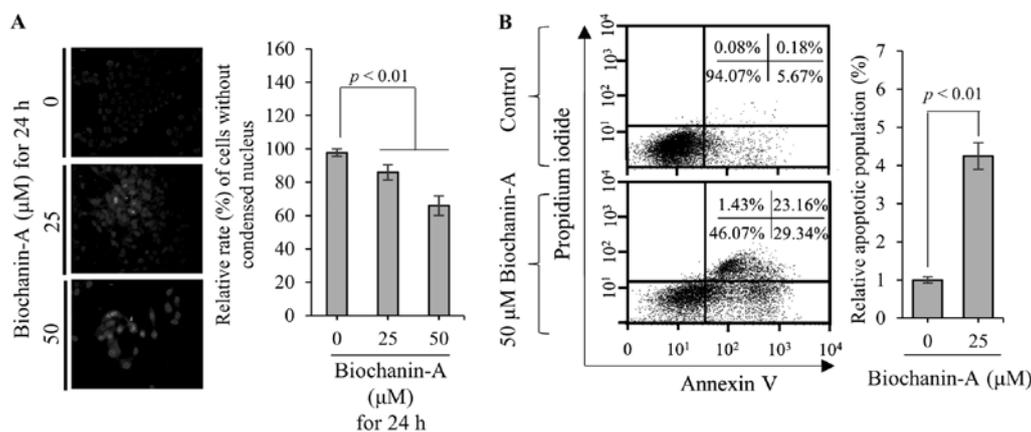


Figure 2. Biochanin-A-induced FaDu cell death is mediated by apoptosis. (A) Biochanin-A increases FaDu cells with condensed nucleus in a dose-dependent manner. Detection of apoptotic cells was accomplished by fluorescently staining DNA to examine chromosomal condensation. FaDu cells cultured at a density of  $1 \times 10^5$  cells/ml were treated with 0, 25 and 50  $\mu\text{M}$  biochanin-A and incubated for 24 h. Cells were stained with 4'-6-diamidino-2-phenylindole (DAPI) and then examined and photographed using fluorescence microscopy. (B) Increased apoptotic population in FaDu cells treated with biochanin-A. Flow cytometric analysis was performed on cells co-stained with Annexin V-FITC and propidium iodide (PI) (Cell Signaling Technology) to detect apoptosis. After  $5 \times 10^5$  cells/ml of FaDu cells were plated into a 6-well plate. After 24 h, the cells were treated with 50  $\mu\text{M}$  biochanin-A. Both floating and attached cells were then collected, washed twice with ice-cold phosphate-buffered saline, and resuspended in 500  $\mu\text{l}$  of 1X binding buffer. Annexin V-FITC and PI were added to the cells for 15 min at 37°C in the dark. The population of Annexin V-positive cells and the cell cycle phase were analyzed using a BD CellQuest<sup>®</sup> version 3.3 instrument (Becton-Dickinson) and WinMDI version 2.9 software (The Scripps Research Institute).

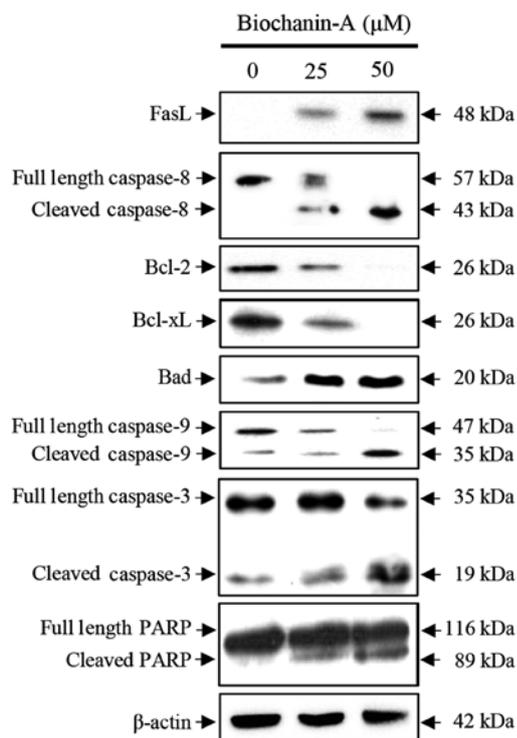


Figure 3. Biochanin-A-induced apoptosis is mediated by extrinsic and intrinsic apoptotic pathways. FaDu cells cultured at a density of  $1 \times 10^5$  cells/ml in 6-well plates were stimulated with 25 and 50  $\mu\text{M}$  biochanin-A for 24 h. The activation of caspase-3 and PARP via biochanin-A induced extrinsic death receptor-dependent and intrinsic mitochondrial-dependent apoptosis signaling pathways. Biochanin-A-induced apoptosis of FaDu cells is dependent on caspase activation.

the viability of FaDu cells through an increase in cell cytotoxicity, in a dose- and time-dependent manner.

*Biochanin-A induced FaDu cell death is mediated by apoptosis.* Nucleus condensation is a typical phenomenon

of apoptosis (7). Therefore, DAPI staining was performed to observe the nucleus morphology of FaDu cells treated with biochanin-A. As shown in Fig. 2A, the relative rate of FaDu cells without condensed nucleus is  $\sim 85.9 \pm 4.7$  and  $66.0 \pm 5.9\%$  at 25 and 50  $\mu\text{M}$  biochanin-A compared with non-treated control, respectively. These data show that biochanin-A increased the number of FaDu cells with condensed nuclei in a dose-dependent manner. Therefore, to verify the biochanin-A-induced apoptosis of FaDu cells, FACS analysis was performed using Annexin V-FITC and PI. As shown in Fig. 2B, the apoptotic population of FaDu cells treated with 50  $\mu\text{M}$  biochanin-A increases  $\sim 4.2$ -fold compared with than non-treated control. Taken together, these data consistently indicate that biochanin-A-induced FaDu cell death is mediated by apoptosis.

*Biochanin-A induces the apoptosis of FaDu cell through extrinsic and intrinsic apoptotic pathways.* Next, to verify biochanin-A-induced apoptosis in FaDu cells, the alteration in expression of pro- and anti-apoptotic factors were observed by western blot analysis. As shown in Fig. 3, the expression of death ligand FasL (48 kDa) increases in FaDu cells treated with 25 and 50  $\mu\text{M}$  biochanin-A in a dose-dependent manner. Sequentially, the expression of full length caspase-8 (57 kDa), which is a down-stream target molecule of FasL and is part of the death receptor mediated extrinsic apoptotic signaling pathway, gradually decreased in the FaDu cells treated with biochanin-A. In contrast, the expression of cleaved caspase-8 (43 kDa) increased due to the cleavage of full length caspase-8 by FasL in the FaDu cells treated with biochanin-A. Furthermore, the expression of cleaved caspase-3 increased due to the cleavage of full length caspase-3 (35 kDa) by the cleaved caspase-8. Sequentially, the expression of cleaved PARP (89 kDa; full length PARP: 116 kDa) increased by cleaved caspase-3 and then induced apoptosis. Therefore, these data indicate that the biochanin-A induces cells death through the FasL-caspase-8-caspase-3-

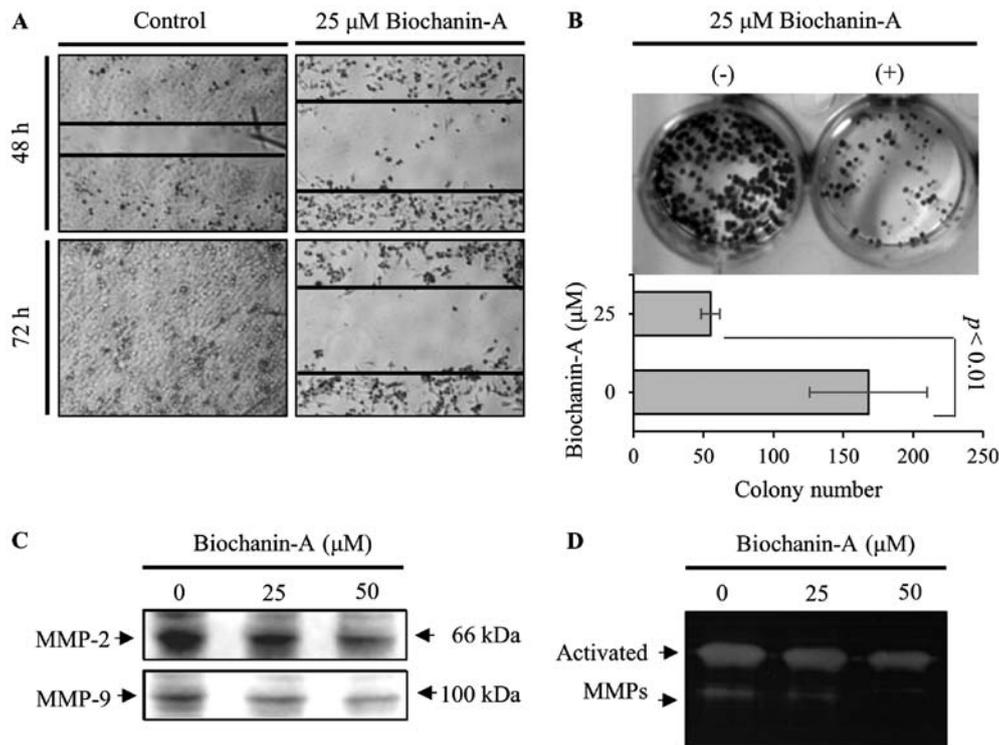


Figure 4. Biochanin-A suppresses the migration and proliferation of FaDu cells through downregulation and inactivation of matrix metalloproteinase (MMP)-2 and -9. (A) Wound healing migration is attenuated in FaDu cells treated with biochanin-A. FaDu cells were treated with 25  $\mu$ M biochanin-A for 48 and 72 h. Wound widths were imaged using reverse microscopy (Eclipse TE2000; Nikon Instruments). (B) Biochanin-A suppresses the proliferation of FaDu cells. To perform a colony formation assay, FaDu cells were treated with 25  $\mu$ M biochanin-A for 24 h and then were incubated in the culture media without biochanin-A for 5 days. Thereafter, formed colonies were stained by crystal violet and were counted. (C and D) The expressions and activities of MMP-2 and MMP-9 decreased in FaDu cells treated with biochanin-A in a dose-dependent manner. FaDu cells were stimulated with 25 and 50  $\mu$ M biochanin-A for 24 h. Thereafter, (C) immunoblotting and (D) gelatin zymography were performed to verify the expression and activity of MMP-2 and MMP-9 in the conditioned media of FaDu cells treated with biochanin-A, respectively.

PARP axis mediated extrinsic apoptosis signaling pathway in FaDu cells.

Furthermore, the expressions of Bcl-2 (26 kDa) and Bcl-xL (26 kDa), anti-apoptotic factors associated with the mitochondria-dependent apoptotic signaling pathway, decreased in the FaDu cells treated with 25 and 50  $\mu$ M biochanin-A in a dose-dependent manner. Whereas, biochanin-A increased the expression of Bad (20 kDa) and cleaved caspase-9 (35 kDa; full length caspase-9: 47 kDa), pro-apoptotic factors associated with the mitochondria-dependent apoptotic signaling pathway. The cleaved caspase-9 increased the amount of cleaved caspase-3 and cleaved PARP and then induced the apoptosis of FaDu cells treated with biochanin-A. Therefore, these data indicate that biochanin-A induces cell death via the mitochondria-dependent intrinsic apoptosis signaling pathway in FaDu cells.

*Biochanin-A suppresses the migration and proliferation of FaDu cells through the downregulation and inactivation of MMP-2 and -9.* FaDu cells were treated with 25  $\mu$ M biochanin-A for 48 and 72 h to verify whether biochanin-A can suppress the migration and invasion. As shown in Fig. 4A, biochanin-A effectively suppressed migration compared with non-treated control. In addition, to verify whether biochanin-A suppress the proliferation of FaDu cells, a colony formation assay was performed as shown in Fig. 4B. The number of colonies in the untreated control was  $168 \pm 42$ . In contrast, the number of

colonies was  $55 \pm 6.6$  in the FaDu cells treated with 25  $\mu$ M biochanin-A for 5 days. These data indicate that biochanin-A effectively suppressed the migration and proliferation of FaDu cells. In order to verify the expression alteration of implicating factors associated with the migration and proliferation of cancer cells, western blot analysis and gelatin zymography were performed as shown as Fig. 4C and D, respectively. The expressions of MMP-2 and MMP-9 gradually decreased in the FaDu cells treated with 25 and 50  $\mu$ M biochanin-A in a dose-dependent manner. Clear bands formed on the gelatin zymogram gels by active MMPs gradually decreased in the FaDu cells treated with 25 and 50  $\mu$ M biochanin-A in a dose-dependent manner. These data consistently indicate that biochanin-A suppresses the migration and invasion of FaDu cells through the downregulation expression and activation of MMPs. Taken together, these data suggest that the biochanin-A may act as a potential anti-metastatic reagent in HNSCC.

To verify the cellular signaling associated with migration and proliferation of FaDu cells, alterations in ERK1/2, p38 and NF- $\kappa$ B expression were observed by immunoblotting in FaDu cells treated with 50  $\mu$ M biochanin-A for 15, 30 and 60 min as shown in Fig. 5. Biochanin-A did not induce the phosphorylation of ERK1/2 in FaDu cells at the defined treatment time, whereas, the phosphorylation of p38 and NF- $\kappa$ B decreased in the FaDu cells treated with biochanin-A in a time-dependent manner compared with non-treated control. Furthermore, the phosphorylation of Akt, which is closely associated with

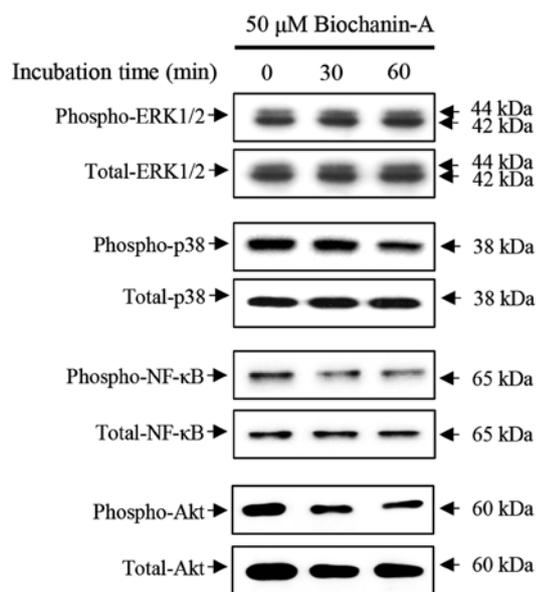


Figure 5. Biochanin-A suppresses the migration and proliferation of FaDu cells through the inhibition of p38 MAPK, NF- $\kappa$ B and Akt phosphorylation. FaDu cells cultured at a density of  $1 \times 10^5$  cells/ml in 6-well plates were stimulated with  $50 \mu\text{M}$  biochanin-A for 30 and 60 min. Thereafter, FaDu cells were harvested and lysed using cell lysis buffer containing the phosphatase inhibitors. Immunoblotting was performed to verify the alterations of ERK1/2, p38, NF- $\kappa$ B and Akt. The immunoreactive bands were visualized using the ECL system and were exposed on radiographic film.

cellular signaling of cell proliferation, significantly decreased in FaDu cells treated with biochanin-A, in a time-dependent manner compared with non-treated control. Therefore, these data suggest that biochanin-A induced suppression of migration and proliferation is associated with the alteration of p38 MAPK, NF- $\kappa$ B and PI3K/Akt cellular signaling pathways in FaDu cells.

## Discussion

Oral cancer is the most common type of head and neck cancer and head and neck squamous cell carcinomas worldwide (20). Despite therapeutic and technological advances, the 5-year survival rate of oral cancer has not increased (21). Furthermore, the clinical interventions for patients with oral cancer have multiple serious side-effects such as the loss of function of speech, breathing, mastication and swallowing (22). Therefore, to reduce the side-effects caused by clinical interventions to treat oral cancer, the development of chemotherapy using natural compounds that possess anticancer properties and excellent biological safety is critical.

Phytoestrogens are plant-derived compounds that are structurally and physiologically similar to estrogen. Recent studies have reported that the phytoestrogens such as coumestrol, kaempferol and genistein induce apoptosis in various cancers including breast, prostate and hepatocellular carcinoma (23-25).

Biochanin-A is a phytoestrogen derived from red clover and used as a folk medicine worldwide. In the present study, we demonstrated that biochanin-A induced anticancer activities including the suppression of cellular proliferation and migration, and the increase in apoptosis through the induction

of caspase activation in FaDu head and neck squamous cell carcinoma.

In our previous study, we reported that biochanin-A did not affect the viability of primary chondrocytes isolated from the articular cartilage of rats for 21 days (10). However, the present study showed that biochanin-A increased cell death through the increase of cytotoxicity in FaDu cells, as shown in Fig. 1. Furthermore, recent studies have reported that biochanin-A increased the cytotoxicity of pancreatic (26) and prostate cancer (16). Therefore, these studies suggest that biochanin-A is a phytoestrogen that induce specific cancer cell death.

DNA fragmentation caused by nucleus condensation is a typical phenomenon of apoptosis, which is a programmed cell death. As shown in Fig. 2A, the number of FaDu cells with condensed nucleus was increased by biochanin-A in a dose-dependent manner. Therefore, these data indicate that biochanin-A-induced FaDu cell death may involve apoptosis. In order to verify this, FACS analysis using Annexin V and PI staining was performed, as shown in Fig. 2B. At the early stage of apoptosis, membrane phosphatidylserine is translocated to the cell surface from the inner plasma membrane. Therefore, Annexin V, which is a  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein with binding activity to phosphatidylserine, was used as a marker to detecting the early stage of apoptosis (21). Furthermore, translocated membrane phosphatidylserine precedes the loss of membrane integration. Subsequently, PI without membrane permeability binds to DNA by intercalating between the bases through the membranes of dead and damaged cells in the late stage of apoptosis (21). In this study, the results of FACS analysis showed that the biochanin-A increased the apoptotic population of FaDu cells in both early and late stage compared with non-treated control. Taken together, these data consistently indicate that biochanin-A induces apoptosis in FaDu cells.

Apoptosis is generally divided into the death receptor mediated extrinsic pathway and mitochondrial-dependent intrinsic pathway (27,28). The death receptor mediated extrinsic pathways are triggered by death ligands such as FasL and TNF-related apoptosis-inducing ligand (TRAIL), and are sequentially mediated by the activation of caspase-8, caspase-3, and PARP to induce DNA fragmentation (27,28). However, the mitochondria-dependent intrinsic pathway associated with apoptosis is triggered by growth hormone withdrawal, DNA damage by UV- or  $\gamma$ -radiation, chemotherapeutic drugs, and the activation of caspase-8 associated death receptor mediated extrinsic apoptosis through the upregulation or activation of pro-apoptotic factors such as Bad, Bid, Bax and caspase-9 and the downregulation of anti-apoptotic factors such as Bcl-2 and Bcl-xL (27,28). Finally, activated caspase-9 induces the activation of caspase-3 and PARP to induce DNA fragmentation (27,28). In the present study, biochanin-A increased the expression of FasL and induced the activation of its downstream pro-apoptotic factor caspase-8 as shown in Fig. 3. Furthermore, the expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL dose-dependently decreased in the FaDu cells treated with biochanin-A. Whereas, biochanin-A increased the expression of Bad and activated caspase-9 in FaDu cells. Finally, both activated caspase-8 and caspase-9 increased the activation of caspase-3 and PARP to induce the apoptosis of FaDu cells treated with biochanin-A. Therefore,

these data indicate that biochanin-A-induced FaDu cell death is mediated by both death receptor mediated extrinsic and mitochondria dependent intrinsic apoptosis pathways.

Next, in the present study, we demonstrated that the migration and proliferation of FaDu cells were significantly decelerated by biochanin-A as shown in Fig. 4A and B. Cancer cells metastasize to other tissues through bloodstream and lymphatic system in the terminal stage of cancer (29). Hence, the deceleration of cancer cell growth and the suppression of metastasis are crucial aims of clinical treatment for metastatic cancer (30). Furthermore, the expressional upregulation and activation of matrix metalloproteinase such as MMP-2 and MMP-9 are crucial factors associated with the migration and proliferation of cancer cells (29,31). We demonstrated that the expression and activation of MMP-2 and MMP-9 significantly decreased in the FaDu cells treated with biochanin-A, as shown in Fig. 4C and D. Taken together, biochanin-A decelerates the migration and proliferation of FaDu cells through the expression downregulation and inactivation of MMP-2 and MMP-9.

In the present study, biochanin-A suppressed the phosphorylation of p38 MAPK and NF- $\kappa$ B in FaDu cells as shown in Fig. 5. As well as this study, Kole *et al* (32), reported that biochanin-A-induced anti-proliferation through the inhibition of p38 MAPK and the blocking of NF- $\kappa$ B nuclear translocation. Recent studies have also reported that the expression of MMP-2 and MMP-9, associated with cell growth, proliferation and migration, are closely regulated by MAPK cellular signaling in various cancer cells including colon cancer (33) and osteosarcoma (34). Furthermore, Bindhu *et al* (35) and Qin *et al* (36) reported that the activation of MMP-2 is closely regulated by NF- $\kappa$ B cellular signaling in oral squamous cell carcinoma and breast cancer. These consistently indicate that biochanin-A-induced anti-proliferation and anti-migration are mediated by the expressional downregulation and inactivation of MMP-2 and MMP-9 through the suppression of p38 MAPK and NF- $\kappa$ B cellular signaling pathways in FaDu cells. In addition, PI3K/Akt signaling is closely associated with cell proliferation, survival, growth and metastasis (37,38). Recent studies have reported that the suppression of PI3K/Akt signaling enhances cytotoxicity through the inhibition of the mTOR signaling pathway in various cancers such as breast and colorectal cancer (39-41). In the present study, we demonstrated that biochanin-A suppressed the phosphorylation of Akt in FaDu cells. Taken together, these studies indicate that biochanin-A-induced anticancer activities including apoptosis, anti-proliferation, and anti-migration may be mediated by the suppression of PI3K/Akt cellular signaling pathway in FaDu cells.

In conclusion, we demonstrated that biochanin-A-induced anti-apoptotic effects such as death receptor mediated extrinsic and mitochondria-dependent intrinsic apoptosis, anti-metastasis and anti-proliferation in FaDu cells. These findings suggest that biochanin-A may be a promising natural chemotherapy candidate for HNSCC treatment.

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