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25-Hydroxycholesterol Induces Death Receptor-mediated Extrinsic and Mitochondria-dependent Intrinsic Apoptosis in Head and Neck Squamous Cell Carcinoma Cells

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Abstract. *Background/Aim: Oxysterol plays important physiological roles in diverse biological processes including apoptosis. However, the mechanisms underlying oxysterol-induced apoptosis remain unknown. 25-hydroxycholesterol (25-HC) is an oxysterol synthesized by cholesterol 25-hydroxylase from cholesterol during sterol metabolism. The aim of present study was to investigate 25-HC-induced apoptosis and associated signalling pathways in FaDu cells, which is originated form human head and neck squamous cell carcinoma cells. Materials and Methods: 25-HC-induced apoptosis was investigated by cell cytotoxicity assay using MTT, cell viability assay using cell LIVE/DEAD cell viability assay, haematoxylin & eosin staining, nuclear staining, fluorescence-activated cell sorting, western blotting using specific antibodies associated with extrinsic and intrinsic apoptosis pathways, and caspase-3/-7 activity assay in FaDu cells. Results: 25-HC dose-dependently decreased the viability of FaDu cells and up-regulated apoptotic events, such as alteration in morphology, and nuclear condensation. Flow cytometric analysis showed an increase in apoptotic population upon 25-HC treatment, suggesting that 25-HC induces apoptosis in FaDu cells. Moreover, 25-HC-induced apoptosis in FaDu cells was dependent on the activation of caspases by Fas antigen ligand-triggered death receptor-mediated extrinsic pathway and mitochondria-dependent intrinsic pathway via mitogen activated protein kinases. Conclusion: Cholesterol-derived oxysterol, 25-HC has potential anti-cancer function in FaDu cells and may have potential properties for the discovery of anti-cancer agents.*

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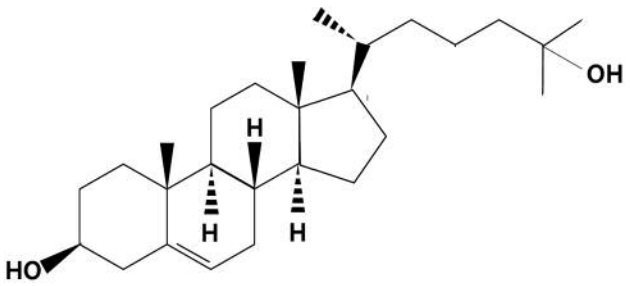
Key Words: Apoptosis, cholesterol, head and neck squamous cell carcinoma, oxysterol, 25-hydroxycholesterol.

Cholesterol, a type of lipid sterol is biosynthesized by all animal cells and is a crucial structural component that modulates the fluidity of cell membranes. Additionally, it is an essential precursor of steroid hormones and bile acid which maintain physiological homeostasis (1). Oxysterols are 27-carbon derivatives are derived from cholesterol *via* endogenous auto-oxidation or enzymatic processes (2). Oxidation promotes the addition of hydroxyl, keto, hyperoxy, carbonyl, or epoxy group at C4-7 or C24, C25, and C27 positions in the cholesterol backbone (3). The derivatives synthesized from oxidation of cholesterol are crucial physiological factors that are associated with the maintenance of diverse biological processes such as cholesterol homeostasis, lipid metabolism, protein prenylation, cell proliferation, and cell differentiation (4). A recent study on the physiological role of oxysterol reported that short-term exposure of cholesterol derivatives such as 7-ketocholesterol, cholestane-3β-5α-6β-triol, and 5α-cholestane-3β,6β-diol selectively triggers apoptosis in tumour cell lines (5). Furthermore, these cholesterol derivatives have showed varying effects depending on the cell type and oxysterol concentration (5). Thus, many recent studies have suggested the potential pharmacological efficacy of cytotoxic oxysterols in chemotherapy using oxysterol synthesized by auto-oxidation of cholesterol (6).

As shown in Figure 1, 25-hydroxycholesterol (25-HC, CAS No. 2140-46-7, $C_{27}H_{46}O_2$) is an oxygenated metabolite

of cholesterol that is catalysed by cholesterol-25-hydroxylase (CH25H) (7). Many studies have reported that 25-HC is associated with cell death in different types of cells such as macrophages, human keratinocytes, oligodendrocytes, and others (8-10). Furthermore, the anti-tumour activity of 25-HC has been reported in hepatocellular carcinoma (11, 12) and human leukemic cell line, CEM (13).

Consequently, the aim of this study was to verify the anti-tumour activity of 25-HC and its underlying cellular signalling pathways associated with cell death in human head and neck squamous cell carcinoma (HNSCC), which is diagnosed in the oral cavity, oropharynx, larynx, and hypopharynx (14).



- 25-Hydroxycholesterol
- CAS 2140-46-7
- Molecular formula : $C_{27}H_{46}O_2$
- Molecular weight : 402.663 g/mol

Figure 1. *Chemical structure of 25-hydroxycholesterol (25-HC).*

Materials and Methods

Cell culture. An HNSCC cell line, FaDu was obtained from the American Type Culture Collection (ATCC). FaDu cells were cultured in minimum essential medium (Life Technologies, Grand Island, NY, USA) containing 10% fatal bovine serum (FBS) in a humidified incubator at 37˚C with 5% CO_2.

Cell cytotoxicity assay. FaDu cells (1×10^5 cells/ml) were cultured in 96-well plates and treated with 0.1, 1, 10, and 20 μM of 25-HC for 24 h at 37˚C. Subsequently, FaDu cells were incubated for additional 4 h with 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Life Technologies, Grand Island, NY, USA). Supernatant was aspirated and dimethyl sulfoxide (200 μl/well) was added in each sample well to dissolve the MTT crystals. Optical density (OD) of each well was measured at 570 nm using Epoch microplate spectrophotometer (BioTek instruments, Winooski, VT, USA).

Cell viability assay. Cell viability assay was performed using LIVE/DEAD cell viability assay kit (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instruction. Briefly, FaDu cells (1×10^5 cells/ml) were cultured in an 8-well chamber slide (Sigma-Aldrich, St. Louis, MO, USA) and treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Further, FaDu cells were stained with calcein green, AM to stain the live cells (green fluorescence) and ethidium homodimer-1 to stain the dead cells (red fluorescence). Subsequently, cells were imaged using fluorescence microscope (Eclipse TE2000; Nikon Instruments, Melville, NY, USA).

Haematoxylin & eosin (H&E) staining. FaDu cells (1×10^5 cells/ml) were cultured in an 8-well chamber slide (Sigma-Aldrich, St. Louis, MO, USA) and treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Subsequently, FaDu cells were fixed with 4% paraformaldehyde for 30 min at 4˚C. H&E staining was performed to evaluate the morphological alterations in FaDu cells. Cells were observed and imaged using Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland).

Nuclear staining. FaDu cells (1×10^5 cells/ml) were cultured in an 8-well chamber slide (Sigma-Aldrich, St. Louis, MO, USA) and treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Further, cells were stained with 1 mg/ml DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Nuclear condensation was spotted and imaged using fluorescence microscope (Eclipse TE200; Nikon Instruments, Melville, NY, USA).

Caspase-3/-7 activity assay. Caspase-3/-7 activity was assayed using cell-permeable fluorogenic substrate, PhiPhiLux®-G_1D_2 (OncoImmunin Inc.; Gaithersburg, MD, USA). Briefly, FaDu cells (1×10^5 cells/ml) were cultured in an 8-well chamber slide (Sigma-Aldrich, St. Louis, MO, USA) and treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Subsequently, cells were stained using cell-permeable fluorogenic substrate PhiPhiLux®-G_1D_2 according to manufacturer's instructions. Following, cells were imaged using fluorescence microscopy (Eclipse TE200; Nikon Instruments, Melville, NY, USA).

Fluorescence-activated cell sorting (FACS). FaDu cells (5×10^5 cells/ml) were cultured in a 6-well plate for 24 h and treated with 10 and 20 μM of 25-HC for 24 h. Further, the collected FaDu cells were stained with annexin V-FITC and propidium iodide (PI) (Cell Signaling Technology, Danvers, MA, USA), and incubated for 15 min at 37˚C. Apoptotic populations were analysed using BD Cell Quest® version 3.3 (Becton Dickinson, San José, CA, USA).

Western blotting. FaDu cells (5×10^5 cells/ml) cultured in a 6-well plate were treated with 10 and 20 μM of 25-HC for 24 h. Subsequently, cell lysates were prepared using cell lysis buffer (Cell Signaling Technology) according to the manufacturer's instructions. Protein concentrations were measured by performing bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of the cell lysates were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting using specific antibodies: Fas antigen Ligand (FasL, 40 kDa; Cell Signaling Technology), caspase-8 (18 kDa and 43 kDa; Cell Signaling Technology), BH3-interacting domain death agonist (Bid, 20 kDa, Santa Cruz Biotechnology Inc., Dallas, TX, USA), B-cell lymphoma-2 (Bcl-2, 26 kDa; Santa Cruz Biotechnology Inc.), B-cell lymphoma-extra large (Bcl-xL, 30 kDa;

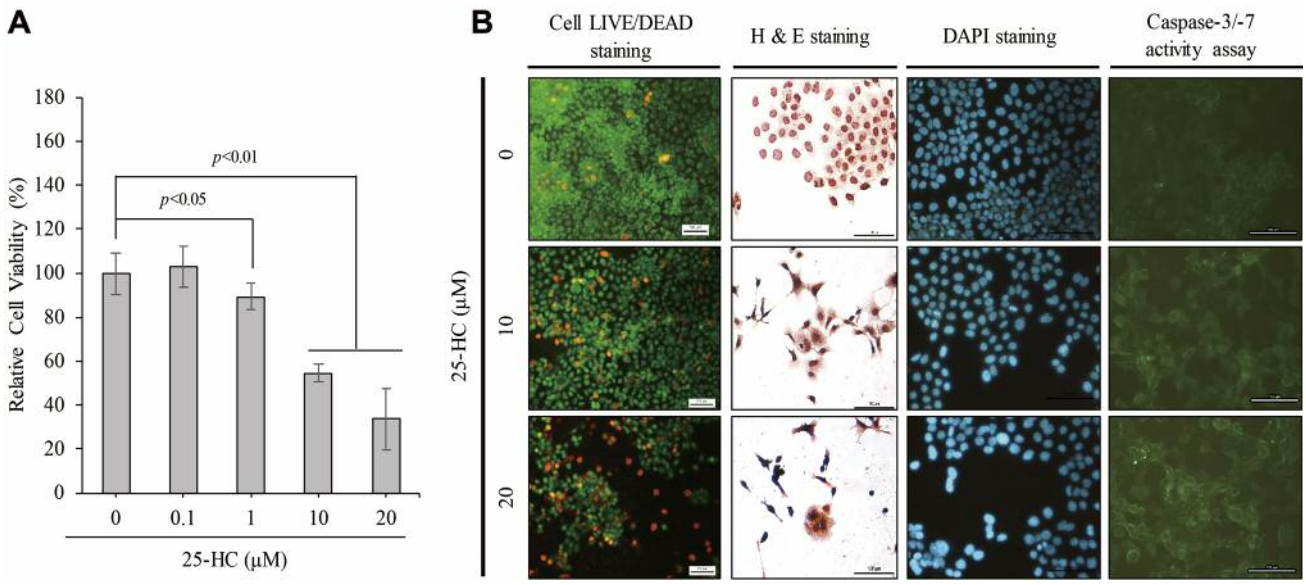


Figure 2. *25-HC induced cell death is involved with apoptosis in FaDu cells. (A) 25-HC decreases viability of FaDu cells. FaDu cells were cultured in 96-well plates and treated with 0.1, 1, 10, and 20 μM of 25-HC for 24 h at 37˚C. Subsequently, an MTT assay was performed to measure the viability of FaDu cells. (B) Apoptotic cell death was increased in FaDu cells when treated with 25-HC. FaDu cells were cultured in an 8-well chamber slide and treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Thereafter, Cell LIVE/DEAD assay, H&E staining, DAPI staining and caspase-3/-7 activity assay were performed to investigate the cell survival, morphological alteration, nucleus condensation and the caspase-3 activation, respectively. Cells were imaged using fluorescence microscope (Eclipse TE2000; Nikon Instruments, Melville, NY, USA).*

Cell Signaling Technology), Bcl-2-associated death promoter (Bad, 23 kDa; Cell Signaling Technology), Bcl-2-associated X protein (Bax, 20 kDa; Cell Signaling Technology), cleaved caspase-9 (35 kDa; Cell Signaling Technology), cleaved caspase-3 (17 kDa and 19 kDa; Cell Signaling Technology), poly(ADP-ribose) polymerase (PARP, 89 kDa and 116 kDa; Cell Signaling Technology), β-actin (45 kDa; Santa Cruz Biotechnology Inc.), phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2, 42 kDa and 44 kDa; Santa Cruz Biotechnology Inc.), total ERK (42 kDa and 44 kDa; Santa Cruz Biotechnology Inc.), phospho-p38 (38 kDa; Santa Cruz Biotechnology Inc.), total p38 (38 kDa; Santa Cruz Biotechnology Inc.), phospho-JNK (46 kDa and 54 kDa; Cell Signaling Technology), total JNK (46 kDa and 54 kDa; Cell Signaling Technology), phospho-protein kinase B (p-Akt, 60 kDa; Cell Signaling Technology), total-Akt (60 kDa; Cell Signaling Technology), phospho-nuclear factor kappa B (p-NFκB, 65 kDa; Cell Signaling Technology), and total NFκB (65 kDa; Cell Signaling Technology). The immunoreactive bands were visualized using ECL System (Amersham Biosciences, Piscataway, NJ) and exposed on radiographic film or MicroChemi 4.2 (Dong-Il SHIMADZU Crop., Seoul, Republic of Korea).

Statistical analysis. The experimental data are presented as the mean±standard deviation and were compared using analysis of variance, followed by post-hoc multiple comparison (Tukey's test) using SPSS software version 25 (SPSS, Inc., Chicago, IL, USA). $p<0.05$ was considered to indicate statistically significant differences. All the data were obtained from three independent experiments except animal study.

Results

The MTT assay was performed to assess the cytotoxicity of 25-HC in FaDu cells. As shown in Figure 2A, FaDu cells were treated with 1, 10, and 20 μM of 25-HC for 24 h and cell viability was measured as 89.3±7%, 54.4±6%, and 33.8±9%, respectively in comparison to untreated control cells (100±9%). Thus, the data revealed that the percentage of viable FaDu cells decreased on 25-HC treatment in a dose-dependent manner. To further verify cell survival, cell viability assay was performed using calcein green, AM (to stain live cells for green fluorescence) and ethidium homodimer-1 (to stain dead cells for red fluorescence) on FaDu cells treated with 10 and 20 μM of 25-HC for 24 h. The result of Cell LIVE/DEAD staining in Figure 2B showed that 25-HC decreased the total number of FaDu cells similar to that observed in Figure 2A. Furthermore, we observed that number of dead cells stained for red fluorescence increased on 25-HC treatment in a dose-dependent manner. Overall, the data suggested that 25-HC induces cell death by increasing cytotoxicity in FaDu cells.
Next, to investigate whether 25-HC-induced cell death was associated with apoptosis, we performed H&E staining and nucleus staining using DAPI to verify apoptotic events such as alteration in morphology and nucleus condensation,

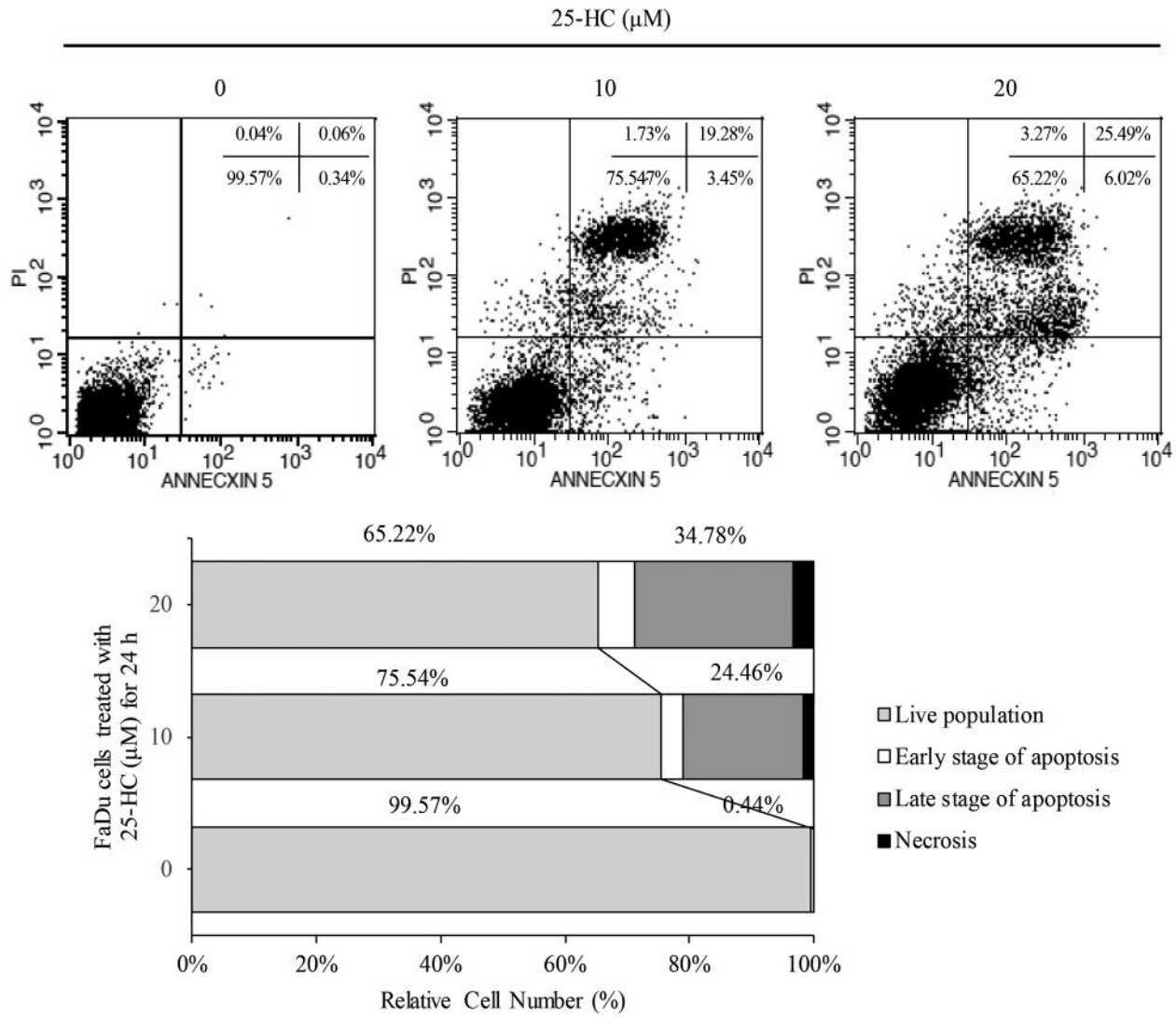


Figure 3. *Apoptotic population was increased in FaDu cells when treated with 25-HC. FaDu cells were treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. FACS analysis was then performed to measure the apoptotic population.*

respectively. The results of H&E staining in Figure 2B show that the number of FaDu cells with altered morphology was observed to increase on 25-HC treatment in a dose-dependent manner. Furthermore, the results of DAPI staining in Figure 2B show that FaDu cells with condensed nucleus were increased in a dose-dependent manner on 25-HC treatment. Overall, in FaDu cells, 25-HC-induced cell death was found to be associated with apoptotic events such as alteration in morphology and nucleus condensation. Hence, a caspase-3/-7 activity assay was performed to determine the activation of caspase-3 that is activated in the execution-phase of cell apoptosis, as shown in the Figure 2. The results of caspase-3/-7 activity assay showed that the activity of cleaved caspase-3 was increased in FaDu cells on treatment with 25-HC. Consequently, the data suggested that 25-HC-induced cell death is significantly associated with apoptosis in FaDu cells.

Furthermore, FACS analysis using PI and Annexin-V-FITC was performed to verify 25-HC-induced cell death *via* apoptosis. As shown in Figure 3, from total dead FaDu cells (24.46%) on 10 μM 25-HC treatment; 3.45%, 19.28%, and 1.73% were found to be in early apoptotic phase, late apoptotic phase, and necrosis, respectively. Furthermore, from total dead cells (34.78%), percent of cells in early apoptotic, late apoptotic, and necrosis were found to be 6.02%, 25.49%, and 3.27%, respectively on treating FaDu cells with 20 μM

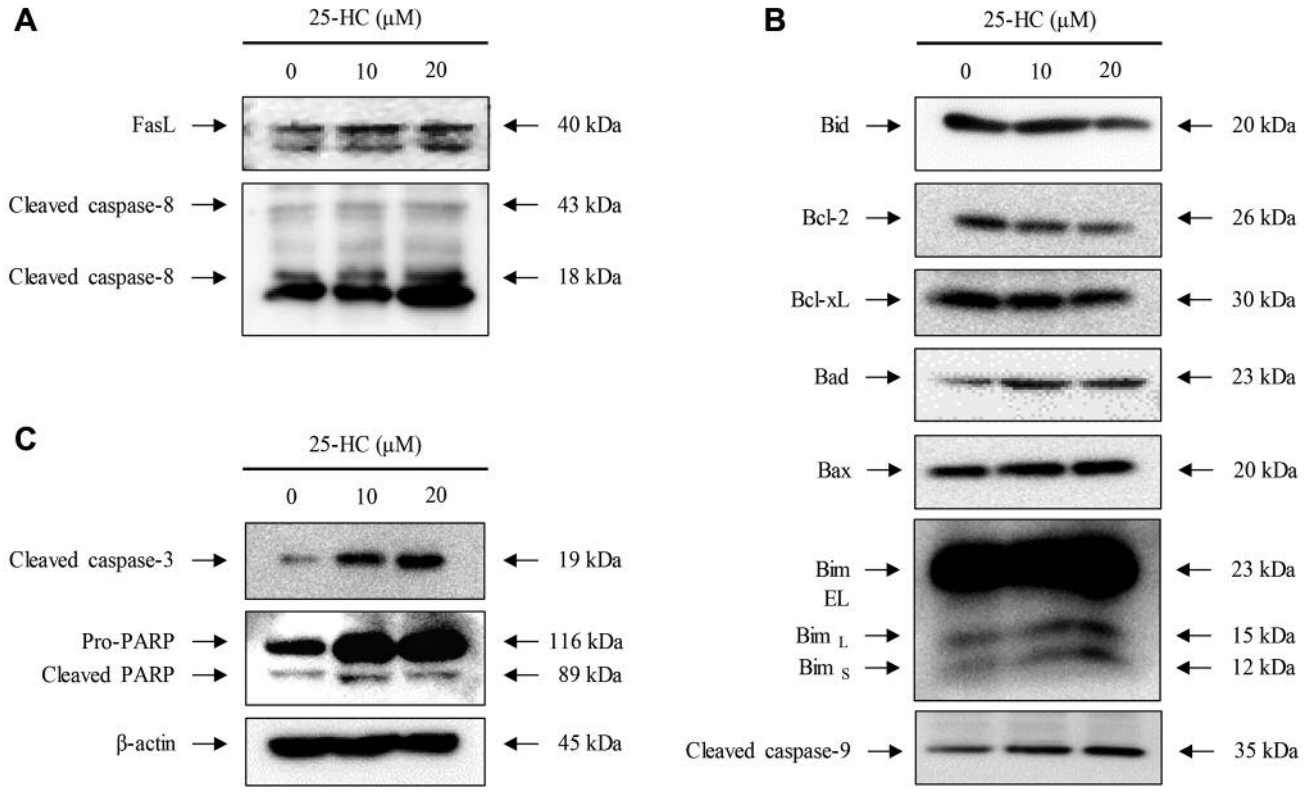


Figure 4. *25-HC-induced apoptosis is mediated by caspase-dependent signalling through FasL-induced death receptor-mediated extrinsic pathway and mitochondria-dependent intrinsic apoptosis in FaDu cells. Apoptotic population was increased in FaDu cells when treated with 25-HC. FaDu cells were treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Further, total proteins were extracted and electrophorized to perform western blotting. Data show that (A) 25-HC induced death receptor-mediated extrinsic apoptosis in FaDu cells that was regulated by cleavage of caspase-8 and (B) 25-HC induced mitochondria-dependent intrinsic apoptosis through activation of caspase-9 in FaDu cells. (C) Caspase-3 was cleaved by both, cleaved caspase-8 and cleaved caspase-9, in FaDu cells.*

25-HC. Thus, the results confirmed that 25-HC-induced cell death is mediated by apoptosis in FaDu cells.

To investigate the pathways involved in 25-HC-induced apoptosis in FaDu cells, western blotting was performed as shown in Figure 4. When FaDu cells were treated with 10 and 20 μM of 25-HC, FasL (40 kDa), a ligand that induces apoptosis on binding with its receptor, expression was found to be up-regulated in a dose-dependent manner (Figure 4A). Furthermore, the expression of cleaved caspase-8 (18 and 43 kDa), a downstream target molecule of FasL, was found to be up-regulated in FaDu cells on treatment with 10 and 20 μM of 25-HC (Figure 4A). Subsequently, data showed that expression of cleaved caspase-3 (pro-caspase-3: 35 kDa; cleaved caspase-3: 19 kDa) and cleaved PARP (Pro-PARP: 116 kDa; cleaved PARP: 89 kDa) was sequentially increased to induce cell death in FaDu cells on 25-HC treatment (Figure 4C). Our data suggested that 25-HC-induced FaDu cell death is mediated by death receptor-mediated extrinsic apoptosis through up-regulation of FasL. Furthermore, expression of Bid (20 kDa), a downstream target of cleaved caspase-8, was observed to be decreased due to its cleavage into tBid on treating FaDu cells with 25-HC (Figure 4B). Expression of Bcl-2 and Bcl-xL, anti-apoptotic factors associated with the maintenance of mitochondrial membrane potential, was also found to be decreased in dose-dependent manner in 25-HC-treated FaDu cells. On the other hand, expressions of pro-apoptotic factors such as Bad (23 kDa), Bax (20 kDa), and Bim (BimEL: 23 kDa, BimL: 15 kDa, BimS: 12 kDa) were found to be up-regulated in 25-HC-treated FaDu cells (Figure 4B). Finally, the expression of cleaved caspase-9, cleaved caspase-3, and cleaved PARP was observed to be up-regulated sequentially in 25-HC-treated FaDu cells (Figure 4C). Thus, our data proved that 25-HC-induced cell death is mediated by mitochondria-dependent intrinsic pathway in FaDu cells. Furthermore, to verify whether 25-HC-induced apoptosis is dependent on the activation of caspases in FaDu cells, cell viability and caspase-3 expression were assessed in the presence or

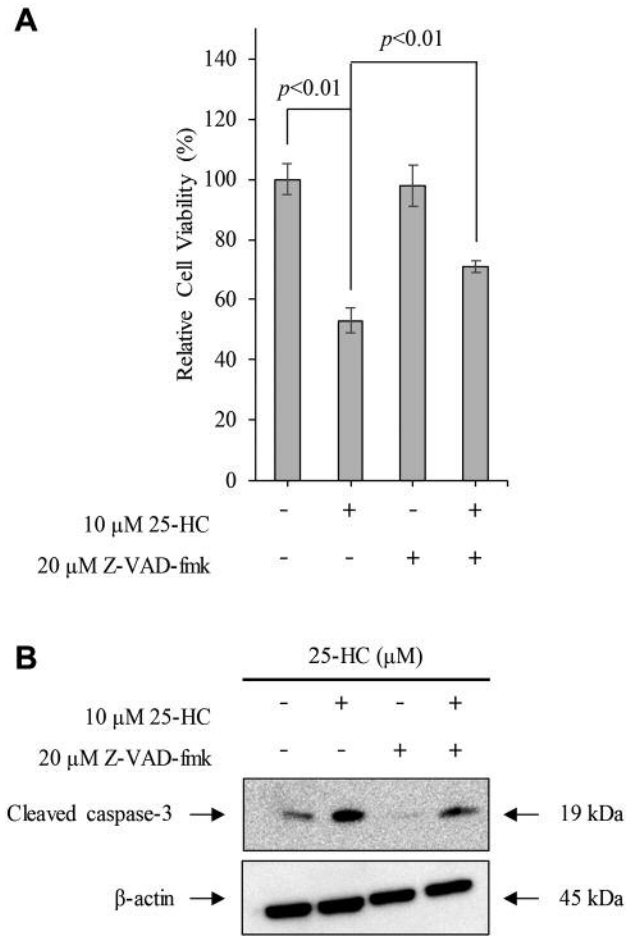


Figure 5. *25-HC-induced apoptosis is dependent on the activation of caspases. FaDu cells were treated with 10 μM 25-HC in the presence or absence of a cell-permeant pan-caspase inhibitor, 20 μM Z-VAD-FMK, for 24 h at 37˚C. Subsequently, MTT assay (A) and western blotting (B) were performed using cleaved caspase-3 antibody to measure cell viability and investigate the altered activity of caspase-3 in FaDu cells when treated with 25-HC, respectively. Data revealed that (A) Z-VAD-FMK neutralized the effect of 25-HC-induced apoptosis in FaDu cells and (B) activation of caspase-3 was significantly suppressed by Z-VAD-FMK in FaDu cells when treated with 25-HC.*

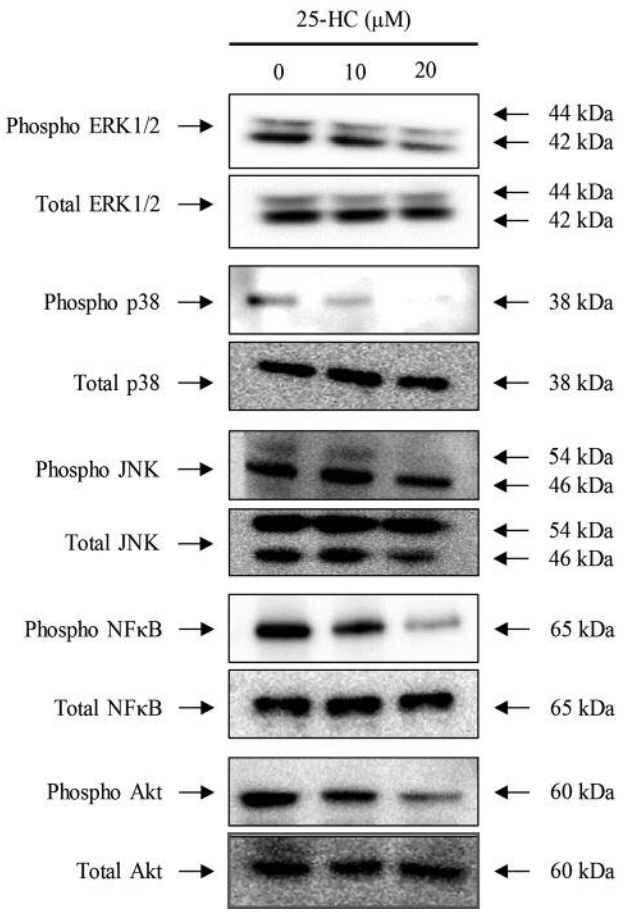


Figure 6. *25-HC-induced apoptosis is mediated by alteration of MAPK, NFκB, and Akt cellular signalling pathways in FaDu cells. FaDu cells were treated with 10 and 20 μM of 25-HC for 24 h at 37˚C. Subsequently, total proteins were extracted and electrophorized to perform western blotting. (A) Phosphorylation of MAPK markers including ERK1/2, p38, and JNK was suppressed in dose-dependent manner in FaDu cells when treated with 25-HC. (B) Phosphorylation of NFκB and Akt was suppressed by 25-HC in FaDu cells.*

absence of a cell-permeant pan-caspase inhibitor, Z-VAD-FMK. As shown in Figure 5A, percentage of viable FaDu cells on 25-HC treatment was found to be decreased by 56.2±2% compared to the percent viability of untreated control cells (101±3%). Furthermore, no statistical significance was observed in cell viability on treating FaDu cells with 20 μM Z-VAD-FMK for 24 h compared to viability of untreated control cells. However, viability of FaDu cells on 25-HC treatment was enhanced by 73±4% in the presence of Z-VAD-FMK. Furthermore, although cleaved caspase-3 expression was significantly up-regulated in 25-HC-treated FaDu cells, it was found to be decreased in the presence of Z-VAD-FMK (Figure 5B). Overall, our data consistently suggested that 25-HC-induced apoptosis is dependent on the cascade activation of caspases in FaDu cells.

Next, to investigate the potential cellular signalling associated with 25-HC-induced apoptosis, FaDu cells were treated with 10 and 20 μM of 25-HC for 24 h. Subsequently, total proteins were extracted and electrophoresed to perform western blotting using specific antibodies associated with MAPK signalling such as ERK1/2, p38, JNK, NFκB, and Akt. As shown in Figure 6, phosphorylation of ERK1/2, p38, JNK, and NFκB was observed to be down-regulated in a

dose-dependent manner on treating FaDu cells with 25-HC. Similarly, phosphorylation of Akt was found to be decreased in 25-HC-treated FaDu cells in a dose-dependent manner. Thus, the data revealed that modulations in MAPK, NFκB, and Akt signalling are significantly associated with 25-HC-induced apoptosis either directly or indirectly in FaDu cells.

Discussion

HNSCC is the seventh most common malignant cancer that is formed in the oral cavity, oropharynx, hypopharynx, and larynx with survival rate of less than 60% (15-19). Although clinical interventions such as surgery, radiation therapy, and chemotherapy are performed to treat patients with HNSCC (20), the strategy is not effective due to several hindrances such as rapid tumour growth, tumour progression, and malnutrition caused by the alteration of mechanical oral functions and dysfunction in swallowing (15, 21). As a result, clinical interventions with a therapeutic goal of achieving effective chemotherapy with less side-effects and minimum toxicity are urgently required for treating patients with HNSCC (15).

Fundamentally, apoptosis is programmed cell death that is tightly regulated for the development and maintenance of homeostasis in healthy tissues (22). Thus, tumour development and metastasis are closely associated with impaired apoptotic signalling (22). Subsequently, recent chemotherapeutic strategies are considering cancer cell specific-induction of apoptosis using natural compounds with less side-effects and minimum toxicity.

Cholesterol is an essential component of cell membrane that regulates the stability and fluidity of cell membrane (5). Furthermore, cholesterol is a well-known precursor of steroid hormones, vitamin D, oxysterols, and bile acids which are associated with the regulation of several cell physiological functions such as growth, proliferation, differentiation, and death (5, 23). Recent studies have reported that many oxysterols are involved in diverse pathophysiological processes including atherosclerosis and cancer (5, 24, 25). Moreover, it has been shown that many oxysterols promote cell death by increasing cytotoxicity in different types of cells including smooth muscle cells (26), human alveolar epithelial cells (27), and vascular endothelial cells (28). Additionally, they have been reported to induce apoptosis in several tumour cell lines (5). Recently, Levy *et al*. revealed that oxysterols such as 7-ketocholesterol, cholestane-3α-5β-6α-triol, and (3α-5β-6α)-cholestane-3,6-diol induce apoptosis in *Mus musculus* skin melanoma cells, B16-F10 and human breast cancer cells, MDA-MB-231(5). Furthermore, oxysterols have shown different effects and IC_{50} values of each has been observed to be dependent on the cell type and its concentration. Thus, a recent study suggested use of oxysterols as a potential pharmacological candidate with cytotoxic effects on cancer cells.

25-HC is an oxysterol synthesized from cholesterol by CH25H and is expressed in various organs to contribute in diverse physiological processes such as lipid metabolism, inflammation, and innate immune response. Furthermore, 25-HC-induced apoptosis has been reported in different types of cells including macrophages (8), oligodendrocytes (10), rat pheochromocytoma cell line PC12 (29), rat Leydig cells (30), human aortic smooth muscle cells (31), thymocytes (32), and human leukemic cell line CEM-C7 (13). Additionally, oxysterol-binding protein-related protein (ORP) is known as a sterol sensor to regulate a sterol and neutral lipid metabolism. Recently, ORP8, a member of the ORP family that is comprised a 12-member gene family, induced Fas-mediated apoptosis in hepatocellular carcinoma and gastric cancer (33, 34). Especially, Li *et al*. have reported that 25-HC-increased cytotoxicity is mediated by the up-regulation of ORP8 through endoplasmic reticulum stress response pathway in hepatoma cell lines such as HepG2 and Huh77 (35).

However, 25-HC shows varying characteristics depending on cell type. Recently, Olivier *et al*. reported that 25-HC not only induces caspase-dependent apoptosis in human keratinocytes, but also stimulates P2X purinoceptor 7 (P2X7) receptor-dependent pyroptosis through activation of caspase-1 and release of proinflammatory cytokines (9). Pyroptosis is a newly discovered form of inflammatory programmed cell death that is mediated on cleavage of gasdermin D *via* activation of proinflammatory caspases including caspase-1, caspase-4, and caspase-5 in human; and caspase-1 and caspase-11 in mice (36, 37). Furthermore, cleaved gasdermin D induces collapse of cell membrane *via* pore formation in the cell membrane. The process is shown to initiate cell death which is accompanied by the release of inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 (37). In addition, recent studies have shown that 25-HC not only contributes in cerebral inflammation of X-linked adrenoleukodystrophy *via* activation of NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome (38), but also induces proinflammatory response through activation of integrin-focal adhesion kinase signalling after binding to integrins, α5β1 and αvβ3 in macrophages (39). On the other hand, some studies have reported that 25-HC reduces inflammation in ZIKA-infected U-87 MG glioma cell line (40) and even suppresses IL-1-derived inflammation (41). In the present study, inflammatory responses were not observed in 25-HC-treated FaDu cells. Furthermore, although some studies have shown that 25-HC induces apoptosis by increasing cytotoxicity in many types of cells; however, many studies have reported conflicting results. Chen *et al*. have reported that 25-HC promotes migration and invasion without proliferation by up-regulating liver X receptor (LXR) and Snail in lung adenocarcinoma (42). Furthermore, Lappano *et al*. have shown that 25-HC together with the estrogen receptor α-dependent ability like 17β-estradiol might be

considered as an additional factor associated with the progression of breast and ovarian tumours (43).

However, 25-HC-induced apoptosis and cellular signalling pathways have not been reported in human HNSCC. In our current study, we showed that 25-HC decreases cell viability by increasing cytotoxicity in FaDu cells as shown in Figure 2. Furthermore, we revealed that 25-HC-induced cell death is associated with apoptotic events such as morphological alteration and nucleus condensation in FaDu cells. FACS analysis showed that the apoptotic population increased dose-dependently in FaDu cells when treated with 25-HC (Figure 3). Moreover, 25-HC-induced apoptosis was mediated by FasL-induced extrinsic and caspase-8-triggered mitochondria-dependent intrinsic apoptosis pathways in FaDu cells that were dependent on the cascade activation of caspases such as caspase-8, caspase-9, and caspase-3 (Figures 5 and 6). Additionally, we showed that cellular signalling pathways associated with 25-HC-induced apoptosis might be mediated by alteration of MAPKs, NFκB, and Akt in FaDu cells. Overall, our results proved that 25-HC significantly induces apoptosis in FaDu cells. However, as in current our study, we did not investigate the cellular linkage between oxysterol binding protein-related protein and cytotoxicity associated with apoptosis of FaDu cells, we would suggest to further verify cellular signalling pathways associated with 25-HC-induced apoptosis. Although 25-HC-induced apoptosis has been reviewed for its consistency in different cell types and its cellular signalling mechanism associated with cell death needs to be verified, our study suggests that cholesterol-derived oxysterol, 25-HC has potential anti-cancer function in FaDu cells and may have potential properties for the discovery of anti-cancer agents.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

J.S.Y., H.L., T.H.K. and J.S.K. contributed to the experimental work; All Authors including J.S.O., G.J.L., Y.S.S., S.K.Y., D.K.K., H.J.K., C.S.K., and J.S.K. participated in data analysis and interpretations; J.S.Y. and J.S.K drafted the article. All Authors gave final approval for publication.

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