

Apoptotic effects of 7,8-dihydroxyflavone in human oral squamous cancer cells through suppression of Sp1

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Abstract. 7,8-Dihydroxyflavone (7,8-DHF) is a member of the flavonoid family and has recently been identified as a brain-derived neurotrophic factor mimetic that selectively activates tropomyosin-receptor kinase B with high affinity. The antioxidant and anticancer effects of 7,8-DHF have been reported. However, the pharmacological mechanisms of 7,8-DHF in oral cancer are unclear. Thus, we investigated the mechanisms of the antiproliferative action of 7,8-DHF on HN22 and HSC4 oral squamous cell carcinoma cell lines. We demonstrated that 7,8-DHF decreased cell growth and induced apoptosis in the HN22 and HSC4 cells through regulation of specificity protein 1 (Sp1) using the MTS assay, DAPI staining, Annexin V, propidium iodide staining, reverse transcription-polymerase chain reaction, immunocytochemistry, pull-down assay and western blot analysis. The results showed that the Sp1 protein bound with 7,8-DHF in the HN22 and HSC4 cells. Taken together, the results suggest that 7,8-DHF could modulate Sp1 transactivation and induce apoptotic cell death by regulating the cell cycle and suppressing antiapoptotic proteins. Furthermore, 7,8-DHF may be valuable for cancer prevention and better clinical outcomes.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common types of malignant tumors in the world. Approximately 275,000 new cases of oral cancer occur each year, and OSCC accounts for more than 90% of the diagnosed cases of oral cancer (1-4). Oral cancer is the eighth leading cause of cancer-related mortality in men. The causes of oral cancer are tobacco, alcohol and ultraviolet light (5). Although conservative treatments for oral cancer, including surgery, radiation, and chemotherapy are well advanced, the 5-year survival rate remains at <50% (6). Surgical resection, radiotherapy and combination therapy with chemotherapy are typical OSCC therapeutic methods (7). Failure of treatment is often due to local and regional recurrence. However, due to improvements in local disease control, treatment failure of oral cancer occurs most frequently as metastasis (8). Therefore, new anticancer agents are urgently required to improve the therapeutic effect.

7,8-Dihydroxyflavone (7,8-DHF) is a flavonoid that exerts beneficial pharmacological and biochemical activities. 7,8-DHF has high selectivity and binding affinity to the tropomyosin-receptor kinase B (TrkB) receptor and activates downstream signaling (9,10). 7,8-DHF is a powerful synthetic analog to brain-derived neurotrophic factor leading to robust activation of TrkB in the mouse brain (10). Flavonoids offer neuronal protection against oxidative stress due to glutamate toxicity (11) and show a spectrum of biological activities, including antiinflammatory, antioxidant, antimutagenic and anticarcinogenic effects (12-16). Therefore, the development of anticancer agents from flavonoids and other natural products is an important topic. However, little is known concerning the other biological effects of 7,8-DHF. In the present study, we investigated whether 7,8-DHF could modulate cell cycle progression and specificity protein (Sp) repression; thus, leading to the apoptotic death of OSCC.

Sp is a transcription factor that is generally expressed in all mammalian cells (17), and protein expression levels of Sp1 are often greater in cancer cells than those in normal cells (18).

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Sp1 is a recently defined transcription factor (19) including Sp/Krüppel. These factors are involved in controlling the cell cycle, apoptosis, and angiogenesis and play an important role in other physiological processes (20-23). For example, Sp1 levels are higher in lung, breast, gastric, thyroid and colorectal cancers (17,21,24). Moreover, Sp1 plays important roles in the carcinogenesis and metastasis of human tumors by regulating growth-related signal transduction, apoptosis, tumor-suppressor genes, cell cycle control molecules, oncogenes and angiogenesis-related factors (25,26). Therefore, inhibiting Sp1 is an effective therapeutic strategy for preventing cancer.

We specifically examined the anticancer effect of 7,8-DHF on cell viability against the OSCC cell lines HN22 and HSC4 and identified proteins regulated by 7,8-DHF in the cells. We investigated whether expression of Sp1 and its downstream proteins and other important apoptotic proteins were altered by 7,8-DHF treatment. Our results provide evidence for the chemotherapeutic efficacy of 7,8-DHF in oral squamous cells. Our results suggest that 7,8-DHF has chemotherapeutic efficacy.

Materials and methods

Cell culture. The HN22 and HSC4 human OSCC lines were obtained from Dankook University (Cheonan, Korea) and Hokkaido University (Hokkaido, Japan), respectively, and were cultured in Hyclone Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum, and 100 U/ml each of penicillin and streptomycin (Thermo Fisher Scientific) at 37°C with 5% CO₂ in humidified air.

MTS cell viability assay. Cell viability was measured using the CellTiter 96™ AQueous assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. HN22 and HSC4 cells were seeded in a 96-well plate for 24 h and then treated with 7,8-DHF (5, 10, 20 and 40 μM) for 24 and 48 h. Cell viability was measured by adding the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dehydrogenase enzyme substrate and the electron coupling reagent phenazine methosulfate. The plates were incubated at 37°C in 5% CO₂ for 2 h after a 24 h or 48 h post-treatment with 7,8-DHF. Absorbance at 490 nm was recorded using a GloMax-Multi Microplate Multimode reader (Promega).

DAPI staining. The levels of nuclear condensation and fragmentation were observed by nucleic acid staining with DAPI. HN22 and HSC4 cells treated with 7,8-DHF were harvested by trypsinization, and fixed in 100% methanol at room temperature for 20 min. The cells were seeded on slides, stained with DAPI (2 μg/ml), and monitored by FluoView confocal laser microscopy (Fluoview FV10i; Olympus Corp., Tokyo, Japan).

Propidium iodide (PI) staining. After 48 h of 7,8-DHF treatment, the HN22 and HSC4 cells were collected by centrifugation and combined with adherent cells. The cells were washed with cold phosphate-buffered saline (PBS), pooled, and centrifuged before being fixed in 70% ice-cold ethanol overnight at -20°C, and then treated with 150 μg/ml RNase A and 20 μg/ml PI (Sigma-Aldrich, St. Louis, MO,

USA). The stained cells were analyzed, and the distribution of the cells in different phases of the cell cycle was calculated using flow cytometry with a MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Annexin V/7-AAD assay. The cells were seeded on a 100-mm dish containing 5.2x10⁵ cells/well for HN22 cells and 8.8x10⁵ cells/well for HSC4 cells and treated with various concentrations of 7,8-DHF (10, 20 and 40 μM) for 48 h. Both adherent and floating cells were harvested and washed once with PBS. The cells were incubated with Annexin V/7-AAD for 20 min at room temperature in the dark to detect apoptosis, followed by a 6 h incubation at 37°C. Apoptotic and necrotic cells were analyzed by flow cytometry (Muse Cell Analyzer; Merck Millipore, Billerica, MA, USA) using the Muse Annexin V/7-AAD & Dead Cell kit (MCH100105; Merck Millipore). The experiment was performed in triplicate.

Reverse transcription-polymerase chain (RT-PCR) reaction. Total RNA was extracted from the cells using the TRIzol® reagent (Life Technologies, Carlsbad, CA, USA), and 2.5 μg of RNA was used to synthesize cDNA using the HelixCript™ first-strand cDNA synthesis kit (NanoHelix, Seoul, Korea). cDNA was obtained by PCR using β-actin-specific and Sp1-specific primers as described below under the following PCR conditions (35 cycles: 1 min at 95°C, 1 min at 56°C and 1 min at 72°C). The β-actin primers were: forward, 5'-GTG GGG CGC CCC AGG CAC CA-3' and reverse, 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'; and the Sp1 primers were: forward, 5'-ATG CCT AAT ATT CAG TAT CAA GTA-3' and reverse, 5'-CCC TGA GGT GAC AGG CTG TGA-3'. PCR products were analyzed by 1% agarose gel electrophoresis.

Immunocytochemistry. HN22 and HSC4 cells were seeded onto sterilized glass coverslips on 6-well tissue culture plates for 24 h and incubated with 7,8-DHF for 48 h. The cells were fixed and permeabilized with Cytifix/Cytoperm solution for 30 min. The cells were blocked with 1% bovine serum albumin and then incubated with monoclonal Sp1 and cleaved caspase-3 antibody at 4°C overnight to express Sp1 and cleaved caspase-3. After washing with PBS containing 0.05% Tween-20 (PBST), Sp1 and cleaved caspase-3 antibodies were reacted with a Jackson 488-conjugated anti-mouse and Jackson 647-conjugated anti-rabbit secondary antibody at room temperature for 1 h and mounted with Vectashield mounting medium to assess DAPI fluorescence (Vector Laboratories, Inc., Burlingame, CA, USA). The cells were visualized using a FluoView confocal laser microscope.

Pull-down assay. This method was previously described (27). Briefly, HN22 and HSC4 cell lysate (500 μg) was reacted with Sepharose 4B or 7,8-DHF-Sepharose 4B (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) matrix beads (0.2 g) in reaction buffer [50 mM Tris, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM phenylmethylsulfonyl fluoride (PMSF) and 1X proteinase inhibitor cocktail]. After overnight incubation with gentle rocking at 4°C, the beads were washed five times with washing buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 150 mM

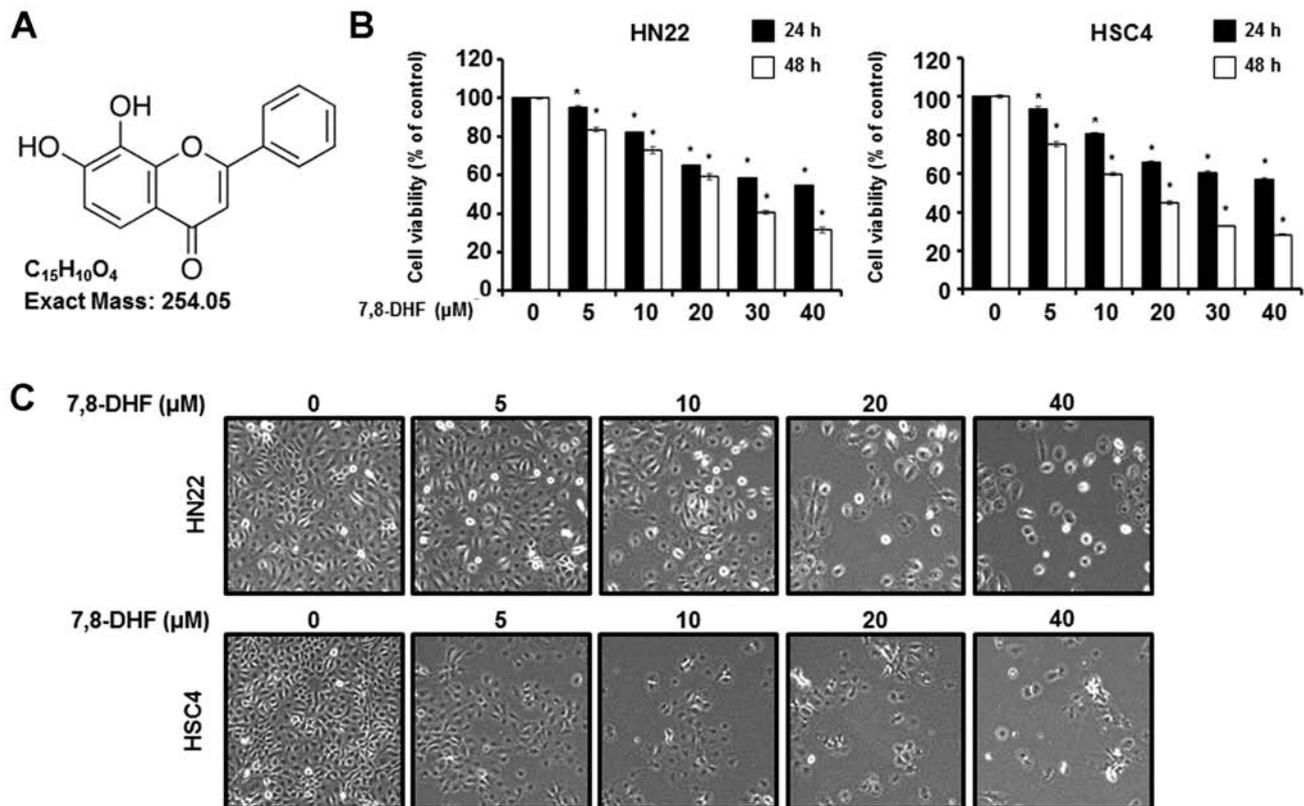


Figure 1. Effect of 7,8-DHF on the viability of oral squamous carcinoma cells. (A) Chemical structure of 7,8-DHF. (B) Cell viability in 7,8-DHF (5, 10, 20, 30, and 40 μM)-treated HN22 and HSC4 cells was detected using an MTS assay kit. Data represent mean percentage levels \pm standard deviations. Significantly different compared with DMSO-treated control by the paired t-test ($n=3$; $^*P<0.05$). (C) Morphological changes observed in the 7,8-DHF treated (5, 10, 20, 30, and 40 μM) or untreated HN22 and HSC4 cells 48 h post-treatment.

NaCl, 1 mM DTT, 0.01% Nonidet P-40 and 0.02 mM PMSF) and proteins bound to the beads were analyzed by western blot analysis.

Western blot analysis. HN22 and HSC4 cells were treated with 7,8-DHF (10, 20 and 40 μM) for 48 h, washed with PBS, and harvested in ice-cold PRO-PREPTM protein extraction solution (Intron Biotechnology, Inc., Daejeon, Korea) containing a protease inhibitor. The extracted proteins were measured using a Pierce[®] BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein were separated via 10% or 15% (v/v) SDS-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. After blocking for 2 h at room temperature with 5% non-fat dried milk in PBST containing 0.1% Tween-20, the membrane was incubated overnight at 4°C with the specific antibodies. Enhanced chemiluminescence western blotting was performed according to the manufacturer's instructions (Thermo Fisher Scientific).

Statistical analysis. Statistical significance was assessed using the Student's t-test. $P<0.05$ relative to the control was considered significant.

Results

7,8-DHF inhibits cell viability and increases apoptosis in OSCC cells. The aim of the present study was to investigate the efficiency of 7,8-DHF to inhibit the growth of OSCC cells. The

chemical structure of 7,8-DHF is shown in Fig. 1A. To confirm the growth inhibitory effect of 7,8-DHF on OSCC, HN22 and HSC4 cells were treated with 7,8-DHF, and cell viability was determined by the MTS assay. As shown in Fig. 1B, the MTS assay was performed after treatment with 7,8-DHF at various concentrations (5, 10, 20, 30 and 40 μM) for 24 and 48 h. Fig. 1B shows that 7,8-DHF inhibited the viability of OSCC cells in a dose-dependent manner. The changes in the appearance of OSCC cells were observed with an optical microscope after 48 h (Fig. 1C). The apoptotic phenotype was a rounded cell, with cytoplasmic blebbing and an abnormal shape. These results indicate that 7,8-DHF inhibited the growth of human OSCC.

7,8-DHF causes G_1 phase cell cycle arrest of OSCC cells. Cancer cell proliferation can be suppressed by apoptosis, inducing cell cycle arrest or both. DAPI staining was performed to confirm the induction of apoptosis by 7,8-DHF in HN22 and HSC4 cells, as DAPI specifically stains nuclei. The results showed fragmented and condensed nuclei in cells treated with 7,8-DHF (10, 20 and 40 μM) for 48 h compared to that in the control (Fig. 2A). The cell cycle distribution was analyzed by FACS analysis. As shown in Fig. 2C, a significant increase in the number of sub- G_1 phase HN22 cells was observed (11.2 \pm 1.4% in the presence of 10 μM 7,8-DHF, 31.0 \pm 2.3% in the presence of 20 μM 7,8-DHF, and 44.0 \pm 2.6% in the presence of 40 μM 7,8-DHF) compared with that of untreated control cells. An increase in the number of sub- G_1 phase HSC4 cells was

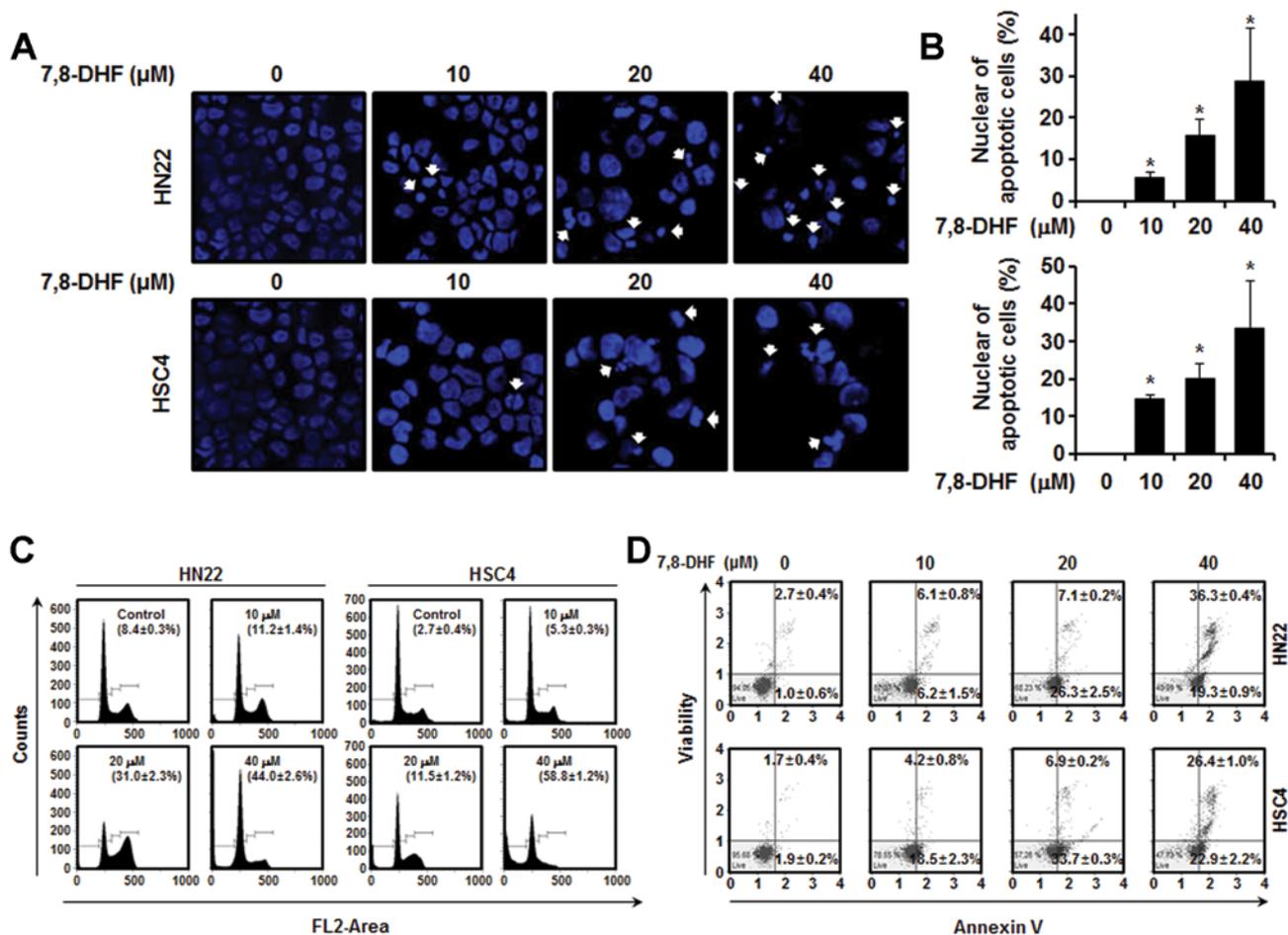


Figure 2. Apoptotic effect induced by 7,8-DHF in oral squamous cell carcinoma. HN22 and HSC4 cells were cultured without 7,8-DHF (control) or with 7,8-DHF (10, 20 and 40 μ M) for 48 h. (A) Fluorescence microscopic (magnification, x200) images of DAPI-stained cells. White arrows indicate DNA fragmentation and nuclear condensation. (B) DNA fragmentation and nuclear condensation were quantified, and data represent the mean percentage levels \pm standard deviations (n=3; *P<0.05). (C) HN22 and HSC4 cells were treated with 10, 20 and 40 μ M 7,8-DHF or DMSO (vehicle), and the cells were washed, fixed, stained with propidium iodide (PI) and analyzed for DNA content by flow cytometry 48 h after treatment. The ratio of apoptotic cells was measured by flow cytometry after PI staining. (D) Quantitative detection of Annexin V/7-AAD positive cells was performed with the Muse Cell Analyzer. Cells stained with Annexin V only were defined as early apoptotic (lower right); Annexin V and 7-AAD double-stained cells were defined as late apoptotic (upper right).

also observed (5.3 \pm 0.3% in the presence of 10 μ M 7,8-DHF, 11.5 \pm 1.2% in the presence of 20 μ M 7,8-DHF and 58.8 \pm 1.2% in the presence of 40 μ M 7,8-DHF) compared with that in the untreated control cells. Cells stained with Annexin V only were defined as early apoptotic and Annexin V (lower right) and 7-AAD double-stained cells were defined as late apoptotic (upper right). As shown in Fig. 2D, 7,8-DHF displayed marked effects to induce apoptosis of HN22 and HSC4 cells in a dose-dependent manner. Treatment of the HN22 cells with 10, 20 and 40 μ M of 7,8-DHF for 48 h resulted in 6.2 \pm 1.5, 26.3 \pm 2.5 and 19.3 \pm 0.9% of early apoptotic cells and 6.1 \pm 0.8, 7.1 \pm 0.2 and 36.3 \pm 0.4% of late apoptotic cells, respectively. Similarly, treatment of HSC4 cells with 7,8-DHF also led to 18.5 \pm 2.3, 33.7 \pm 0.3 and 22.9 \pm 2.2% of early apoptotic cells and 4.2 \pm 0.8, 6.9 \pm 0.2 and 26.4 \pm 1.0% of late apoptotic cells at the same three concentrations as above, respectively. Apparently, 7,8-DHF-mediated apoptosis of HN22 and HSC4 cells, at least in part, contributed to its antiproliferative effects.

7,8-DHF suppresses Sp1 expression and binds with Sp1 in OSCC cells. As the Sp1 protein plays an important role in oncogenesis, a therapeutic agent that can effectively modulate the

Sp1 protein may be a suitable anticancer drug to suppress tumor progression (28). Both HN22 and HSC4 cells were treated with various concentrations of 7,8-DHF (10, 20 and 40 μ M) for 48 h to observe Sp1 protein expression levels. Fig. 3A and B shows a significant decrease in Sp1 protein expression levels in the HN22 and HSC4 cells in a dose-dependent manner. We also observed downregulation of the Sp1 protein in HN22 and HSC4 cells at 40 μ M 7,8-DHF after different periods of time (0, 12, 24, 36 and 48 h). Additionally, Sp1 mRNA was suppressed by 7,8-DHF in both HN22 and HSC4 cells (Fig. 3C). Consistent with these observations, the immunocytochemistry results revealed decreased Sp1 and increased cleaved caspase-3 levels in a dose-dependent manner in both the HN22 and HSC4 cell lines (Fig. 3D). We conclude that suppression of Sp1 by 7,8-DHF treatment leads to apoptotic cell death. Next, a pull down assay was performed to determine whether 7,8-DHF directly binds to Sp1. Fig. 4A and B showed that 7,8-DHF strongly suppressed Sp1 activity by directly binding to Sp1.

7,8-DHF modulates the factors concerned with cell cycle arrest or apoptosis of OSCC cells. In many previous studies, the downregulation of Sp1 was found to induce apoptosis, and Sp1

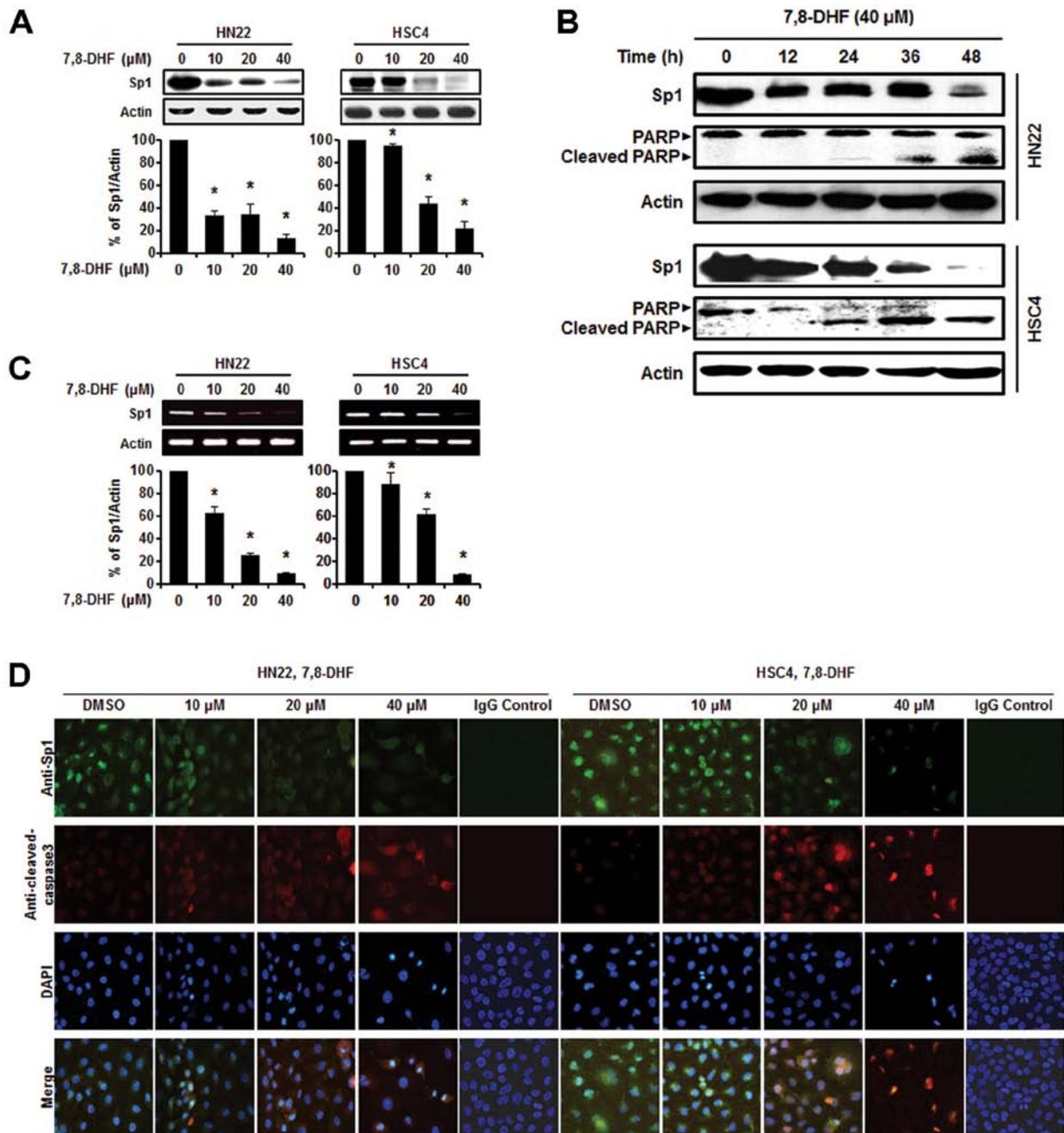


Figure 3. 7,8-DHF suppresses the Sp1 protein via apoptosis in oral squamous cell carcinoma. (A) HN22 and HSC4 cells were treated with 10, 20 and 40 μ M 7,8-DHF for 48 h, whole-cell extracts were prepared, separated by SDS-PAGE gel electrophoresis, and subjected to western blotting with the Sp1 antibody. Actin was employed as the loading control. The histograms indicate the ratio of Sp1 to actin expression. (B) Experiments to assess time-dependent effects of 7,8-DHF on Sp1 and PARP were performed using HN22 and HSC4 cells treated with 40 μ M 7,8-DHF for 0, 12, 24, 36 and 48 h. (C) The effect of 7,8-DHF (10, 20 and 40 μ M) on Sp1 mRNA after 48 h. (D) Immunofluorescence microscopy was performed in 7,8-DHF-treated HN22 and HSC4 cells. HN22 and HSC4 cells were treated with different concentrations of 7,8-DHF (10, 20 and 40 μ M) for 48 h and the cells were immunostained with anti-Sp1 and anti-cleaved caspase-3. The signals were detected with Jackson 488-conjugated anti-mouse and Jackson 647-conjugated anti-rabbit secondary antibody. DAPI was used for nuclear staining.

was confirmed to be associated with cell apoptosis (28-30). We used western blot analysis to clarify the association between 7,8-DHF and Sp1-mediated apoptosis. We investigated the cell-cycle arrest proteins such as p27 and p21 and found increased levels following 7,8-DHF treatment. We also investigated cell proliferation and survival-related proteins such as cyclin D1, Mcl-1 and survivin, which were decreased in a dose-dependent manner following 7,8-DHF treatment (Fig. 5). We found a decrease in BID and Bcl_{xl} and an increase in Bax expression. These proteins were associated with the apoptotic

cell death induced by 7,8-DHF. Finally, cleaved caspase-3 and cleaved PARP were induced by 7,8-DHF in a dose-dependent manner (Fig. 6). These results revealed that 7,8-DHF treatment of OSCC decreases Sp1, resulting in growth arrest and apoptotic cell death.

Discussion

Flavonoids demonstrate antiallergic, antiinflammatory, antioxidant, and anticancer effects. They also regulate enzyme

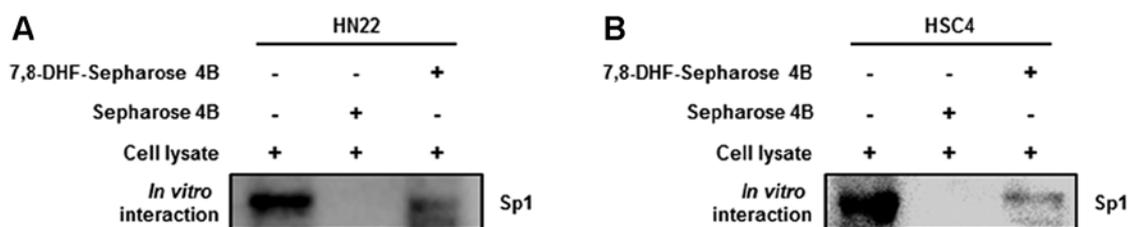


Figure 4. 7,8-DHF binds with Sp1. HN22 or HSC4 cell lysates were mixed with 7,8-DHF-conjugated Sepharose 4B beads or with Sepharose 4B beads alone, and the pulled-down proteins were analyzed by western blotting.

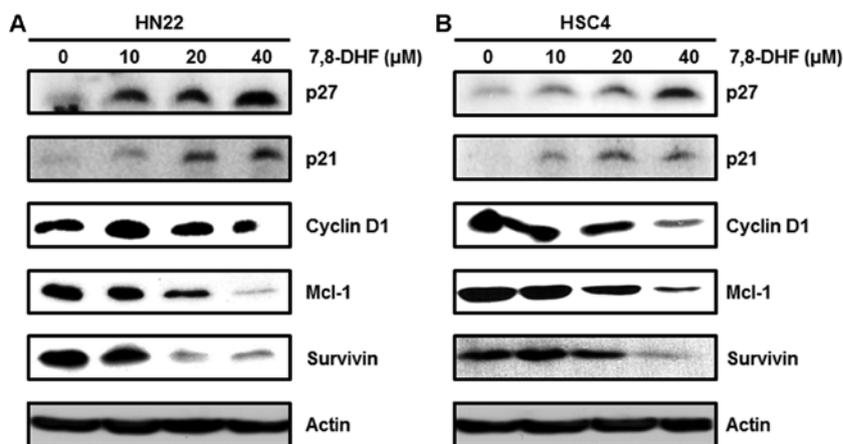


Figure 5. Effect of 7,8-DHF on Sp1 downstream target proteins. (A) HN22 and (B) HSC4 cells were treated with 10, 20 and 40 μM 7,8-DHF for 48 h, and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to western blotting using p27, p21, cyclin D1, Mcl-1 and survivin antibodies. Actin was employed as a loading control. Results shown are representative of three independent experiments.

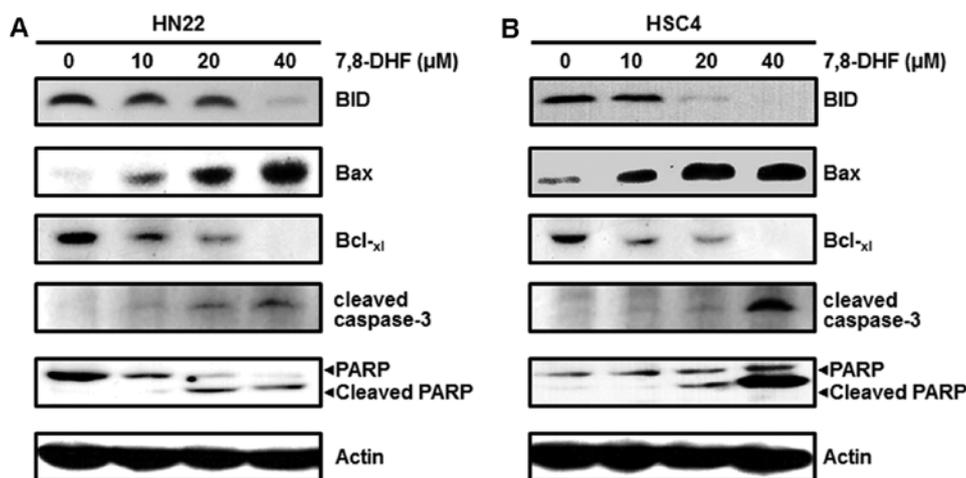


Figure 6. Effect of 7,8-DHF on apoptosis of oral squamous cell carcinoma. (A) HN22 and (B) HSC4 cells were treated 7,8-DHF (10, 20 and 40 μM) for 48 h. The cell lysates were evaluated by western blot analysis with BID, Bax, Bcl- $_{xL}$, cleaved caspase-3, PARP and cleaved PARP antibodies. Equal loading of proteins was confirmed by western blotting of actin antibody. The results shown are representative of three independent experiments.

activities and the immune system (12,31-34). Among them, 7,8-DHF is a novel compound isolated from the flavone family that appears to inhibit the proliferation of cancer cells (11), but its mechanism of action has not yet been investigated in detail.

In the present study, we examined the apoptotic effect of 7,8-DHF in OSCC. The effect of 7,8-DHF treatment on initiating apoptosis and cell cycle arrest in HN22 and

HSC4 cells was determined by flow cytometry and DAPI staining. The percentage of sub-G₁ phase cells increased in 7,8-DHF-treated cells compared with that in untreated control cells. Furthermore, the Annexin V assay revealed that 7,8-DHF induced early apoptosis (Fig. 2C). To determine the level of protein Sp1 expression due to 7,8-DHF treatment, HN22 and HSC4 cells were treated with various concentrations (10, 20 and 40 μM) of 7,8-DHF for 48 h and different periods of time

(12, 24, 36 and 48 h). As shown in Fig. 3A and B, treatment with 7,8-DHF induced a significant decrease in Sp1 protein expression levels in HN22 and HSC4 cells in a dose- and time-dependent manner. Furthermore, Sp1 mRNA was suppressed by 7,8-DHF in both HN22 and HSC4 cells (Fig. 3C). The immunocytochemistry results revealed decreased Sp1 levels and increased levels of cleaved caspase-3 in a dose-dependent manner in the HN22 and HSC4 cell lines (Fig. 3D). These results suggest that 7,8-DHF plays an important role as an anti-tumor agent by downregulation of Sp1, leading to apoptotic cell death in OSCC.

We examined a 7,8-DHF pull-down assay to identify 7,8-DHF molecular targets in tumorigenesis and confirmed that 7,8-DHF specifically binds with Sp1 (Fig. 4). To further characterize the effects of 7,8-DHF on Sp1, we analyzed the effect of p27, p21, cyclin D1, Mcl-1, and the survivin protein on Sp1 protein levels by western blot analysis (35-37). The results showed that levels of the Sp1 target proteins such as p27, p21, cyclin D1, Mcl-1, and survivin were inhibited by 7,8-DHF in a dose-dependent manner (Fig. 5). 7,8-DHF reduced Bcl-2, increased Bax, and cleaved caspase-3 and PARP, suggesting that 7,8-DHF regulates Sp1, ultimately leading to apoptotic cell death (Fig. 6). Our results indicate that 7,8-DHF may be capable of effectively treating cancer. Sp1 expression increases during cancer transformation and plays an important role in the maintenance and development of tumors. Downregulation of Sp1 is useful for treating tumor cells, and Sp1 overexpression induces the proliferation of cancer or transformed cells (22).

7,8-DHF clinical studies are necessary to describe the clinical applications and potential unexpected toxicities.

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