




Anticancer effects of 6-shogaol via the AKT signaling pathway in oral squamous cell carcinoma

Abstract

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Objective: Oral squamous cell carcinoma (OSCC) is one of the common type of cancer that leads to death; and is becoming a global concern. Due to the lack of efficient chemotherapeutic agents for patients with oral cancer, the prognosis remains poor. 6-shogaol, a bioactive compound of ginger, has a broad spectrum of bioactivities and has been widely used to relieve many diseases. However, its effects on human oral cancer have not yet been fully evaluated. In our study, we investigated the anticancer effects of 6-shogaol on the proliferation, migration, invasion, apoptosis, and underlying mechanisms within human OSCC cell lines. **Methodology:** We investigated the effect of 6-shogaol on the growth of OSCC cells by cell viability and soft agar colony formation assay. Migration and invasion assays were conducted to confirm the effect 6-shogaol on OSCC cell metastasis. Apoptosis was detected by flow cytometry and the underlying mechanism on the antigrowth effect of 6-shogaol in OSCC cells was assessed using western blotting. **Results:** In our results, 6-shogaol not only suppressed proliferation and anchorage-independent cell growth in OSCC cells, but also induced apoptosis by regulating the apoptosis-associated factors such as p53, Bax, Bcl-2, and cleaved caspase-3. Migration and invasion of OSCC cells were inhibited following the regulation of E-cadherin and N-cadherin by 6-shogaol. Additionally, 6-shogaol treatment significantly inhibited the PI3K/AKT signaling pathway. **Conclusion:** Therefore, our results may provide critical evidence that 6-shogaol can be a potential new therapeutic candidate for oral cancer.

Keywords: 6-shogaol. Oral squamous cell carcinoma. PI3K/AKT signaling pathway

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Introduction

Cancer within the oral cavity and pharynx is one of the most common types worldwide, occurring more often in men than women.¹ Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers and is caused by various factors, including tobacco, alcohol, and heredity.² Since most oral cancers are diagnosed at a late stage, the 5-year survival rate for OSCC patients was reported to be approximately of 55%.³ Surgical resection is the primary treatment for most oral cancers, followed by either radiation or chemotherapy, or a combination thereof.⁴ Despite advances in anticancer treatment techniques, patients with oral cancer develop esthetic and functional impairments due to the surgical defect at the head and neck region.⁵ As a result, the patient's quality of life deteriorates. Therefore, it is important to develop nontoxic and effective new drugs for the prevention and treatment of oral cancer in patients.

Compared to traditional cytotoxic agents, recent oncology has focused on molecules regulating signal transduction pathways, which are key factors in cancer development. Of these pathways, AKT signaling is a central mechanism for carcinogenic activation. The AKT-modified expression has been implicated in various cancers, including esophageal and colon cancer, and is also associated with cell growth, apoptosis or epithelial–mesenchymal transition (EMT) during the process of carcinogenesis.^{6,7} EGFRvIII and phosphorylated AKT are considered as predictive value for patient survival outcome.⁸ Several studies have reported that the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway is involved in oral cancer progression and the AKT signaling pathway may be an important therapeutic target of oral cancer.^{8–11}

6-shogaol is one of the major physiologically active compounds in dried ginger and has various pharmacological activities, including anti-inflammatory, antioxidant, and antitumor.¹² In particular, the antitumor activity of 6-shogaol is displayed by regulating signaling molecules such as AKT, mitogen-activated protein kinases, signal transducer and activator of transcription-3 (STAT3), cyclin D1, Bcl-2 and caspases; all of these are associated with cell proliferation, cell cycle arrest, anti-apoptosis, and tumor progression.^{11,13,14} However, few studies have reported on the effect of 6-shogaol in oral cancer.

In our study, we explored the anticancer effects of 6-shogaol on cell proliferation and the mechanisms involved in regulating survival in human OSCC cells.

Methodology

Reagents and antibodies

6-shogaol (purity \geq 97% from HPLC and NMR analysis) was obtained from Weikeyi Biological Technology Co. Ltd (Wuhan, Hubei, China) (Figure 1A). Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), antibiotic–antimycotic mixture containing 100 U/ml of penicillin and 100 ug/ml of streptomycin, and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL. (Grand Island, NY, USA). Anti-p-PI3K, anti-PI3K, anti-p-AKT, anti-AKT, anti-p-mTOR, anti-mTOR, anti-Bax, anti-p53, anti-E-cadherin, and anti-N-cadherin antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-actin, anti-Bax, and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines and cell culture

Human OSCC cell lines (YD-10B and Ca9-22) were obtained from the Department of Oral Biology, College of Dentistry, Yonsei University (Seoul, Korea). Human oral mucosal fibroblasts (hOMFs), as a normal oral cells, were purchased from Aiyuan Biotech Co., Ltd (Shanghai, China). OSCC cells and hOMFs were cultured in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic mixture, and incubated in a humidified atmosphere with 5% CO₂ at 37°C.

Cell viability assay

Cells (1×10^4 cells/well) were seeded in 96-well plates and cultured in a complete medium overnight. The attached cells were incubated in serum-free media with varying concentrations of 6-shogaol for 24, 48, and 72 h. MTT solution (5 mg/ml) was added to each well, and incubated at 37°C for 4 h. The medium was subsequently discarded, and DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader

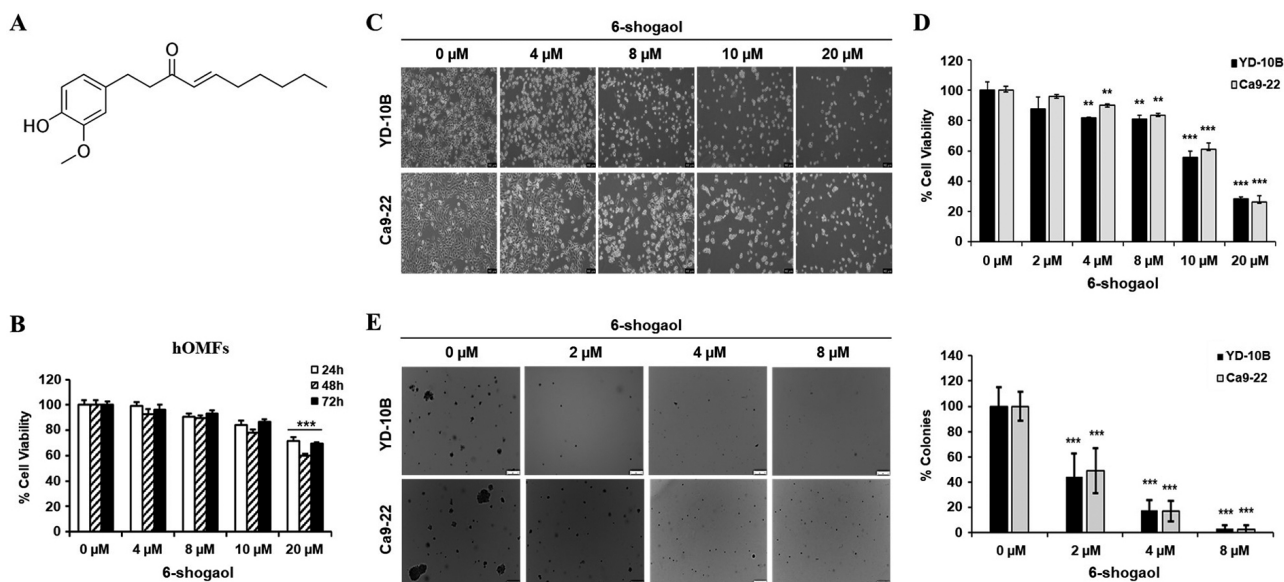


Figure 1- Effects of 6-shogaol on the growth of OSCC cells. (A) The chemical structure of 6-shogaol. (B) The toxicity effect of 6-shogaol on human oral mucosal fibroblasts at different concentrations. (C) The morphological analysis of YD-10B and Ca9-22 cells treated with various concentrations of 6-shogaol for 24 h using MTT assay. (E) In colony formation assay, representative photographs and quantitative graphs of colonies formed on soft agar from YD-10B and Ca9-22 cells treated with 0, 2, 4, and 8 μM 6-shogaol. Data are expressed as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control (0 μM 6-shogaol)

(Thermo Fisher Scientific, Waltham, MA, USA).

Colony formation assay

OSCC cells (8×10^3 cells/well), suspended in a complete medium, were added to 0.3% agar with 6-shogaol in the top layer over a base layer of 0.6% agar with 6-shogaol. The cultures were maintained under 5% CO_2 at 37°C for two weeks, as previously described.¹⁵ Colonies were subsequently visualized under a microscope and counted using Image-Pro Plus software.

Cell migration and invasion assay

Transwell chambers (Corning Costar, Lowell, MA, USA) with 8.0 μm pore polycarbonate membrane insert were used for the migration and invasion assay according to the manufacturer's procedure and published methods.¹⁶ OSCC cells (1×10^5 cells/well) were seeded onto the upper surface of 1 mg/ml Matrigel-coated membranes (for invasion assay) or uncoated membranes (for migration assay), and the medium containing 6-shogaol was added to the upper and lower chambers. The non-invaded/migrated cells from the upper surface were removed after 24 h of incubation at 37°C. The invaded/migrated cells on the lower surface were fixed with cold 4% formaldehyde, permeabilized with 100% methanol, and stained with 0.5% crystal violet. The membrane with migrated or invaded cells was imaged using a Leica SP2 confocal

microscope (Leica Microsystems, Wetzlar, Germany) and quantified using the Image-Pro Plus software.

TUNEL assay

Apoptotic cell death was conducted using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. OSCC cells (1×10^5 cells/well) were seeded on chamber slides and incubated in DMEM with 6-shogaol for 24 h. Apoptotic cells were detected with the TUNEL assay using the *in situ* apoptosis detection kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Cells were stained with DAPI, mounted, and examined using fluorescence microscopy (Leica Microsystems). The percentage of apoptotic cells were estimated using annexin V/propidium iodide (PI) staining as described previously.¹⁷

Binding assay

Cell lysates were incubated with 6-shogaol-Sepharose 4B beads (Sepharose 4B only beads served as a negative control) in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.01% NP-40, and 2 mg/ml bovine serum albumin at 4°C rotator overnight. The beads were washed three times with a washing buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and 0.01% NP-40. The binding was visualized by western blot analysis

with an anti-AKT antibody.

Western blot analysis

OSCC cells were lysed using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM PMSF, protease/phosphatase inhibitor cocktail) for the collection of total protein. The protein concentrations of samples were quantified using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Equal amounts of protein were loaded onto a sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) and subsequently incubated with primary antibodies overnight at 4°C. After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The detected proteins were visualized using the Amersham enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, UK) and analyzed using Da Vinci software.

Statistical analysis

All quantitative results are expressed as the mean \pm standard error of three independent experiments. Significant differences were compared using a two-tailed independent sample *t*-test between the different groups, and *p*-values less than 0.05 were considered statistically significant.

Results

6-shogaol inhibits the growth of OSCC cells

To investigate the effect of 6-shogaol on the growth of oral cancer cells, we conducted assays on cell proliferation and colony formation. To first confirm the toxicity of 6-shogaol in normal human oral cells, we measured the viability of hOMFs by treating with different concentrations of 6-shogaol. Figure 1B shows that no toxicity was exhibited in normal oral cells at 0–10 μ M 6-shogaol. According to the previously indicated 6-shogaol concentrations, the MTT assay determined the viability of OSCC cells. Figures 1C and 1D show that 6-shogaol dose-dependently suppressed the proliferation of both YD-10B and Ca9-22 cells. A 20 μ M concentration of 6-Shogaol inhibited both types of cells by more than 70%. In subsequent

experiments, we used the concentration of 6-shogaol with a survival rate of 80% or more. For anchorage-independent cell growth assessment, we conducted the colony formation assay for two weeks; 6-shogaol dose-dependently decreased both the size and the number of colonies in the two cell lines (Figure 1E).

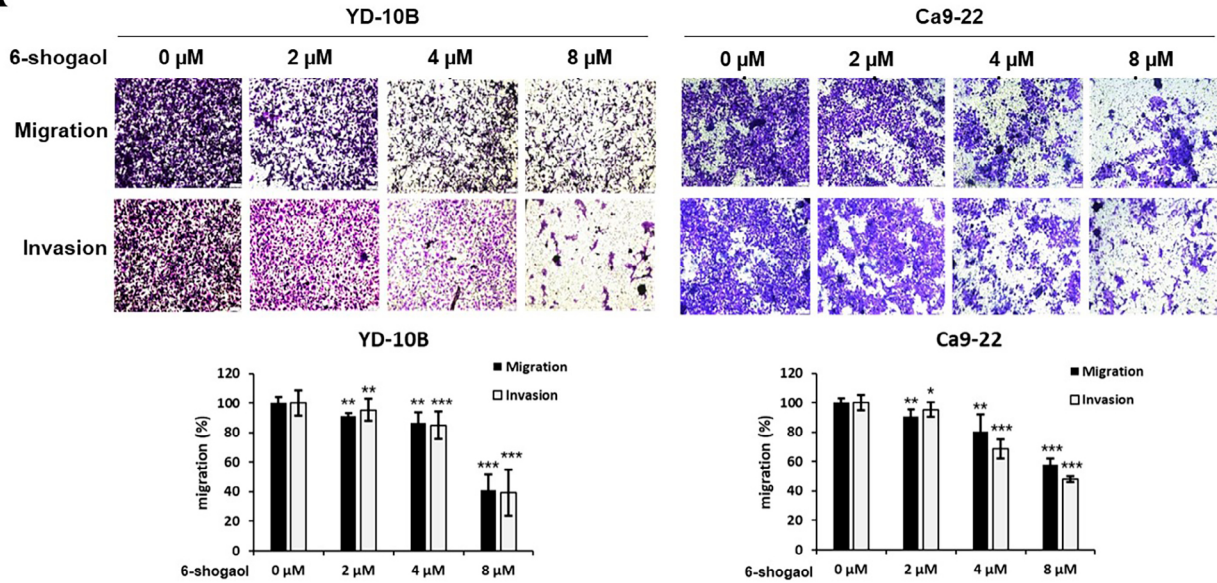
6-shogaol suppresses the migration and invasion in OSCC cells

To confirm the effects of 6-shogaol on oral cancer cell metastasis, we investigated the migration and invasive abilities of OSCC cells and the expression EMT-related proteins, which play an essential role in local recurrence and lymph node metastasis of cancer. The results indicated that the ability of OSCC cells to migrate and invade was dose-dependently reduced by 6-shogaol as compared to the control (0 μ M of 6-shogaol) (Figure 2A). Quantitative analysis showed that the migration and invasion were reduced by approximately 60%, using 8 μ M concentration of 6-shogaol in both OSCC cell lines. Treatment with 6-shogaol dose-dependently induced an increase in E-cadherin and a decrease in N-cadherin. These results inhibited the migration and invasion of OSCC cells (Figure 2B). Thus, 6-shogaol may suppress the EMT process of human OSCC cells.

6-shogaol induces the apoptosis in OSCC cells

To investigate whether apoptosis affected the antigrowth effect of 6-shogaol, we conducted Annexin V/PI detection and TUNEL assay in two OSCC cell lines. 6-shogaol-treated OSCC cells were double-stained with annexin V-FITC and PI, and analyzed using a flow cytometry. 6-shogaol remarkably induced both early and late stages of apoptosis in OSCC cells in a dose-dependent manner compared to the control (Figure 3A–B). The apoptotic populations treated by concentrations of 2, 4, and 8 μ M of 6-shogaol were measured to be approximately 10%, 20%, and 30% in both YD-10B and Ca9-22 cells. From the results of the TUNEL assay, it was clear that treatment with 6-shogaol dose-dependently raised the apoptosis rate of OSCC cells compared to the control group (Figure 3C). To confirm the mechanism of apoptosis which was induced by 6-shogaol, the level of apoptosis-related proteins was also investigated using western blotting. 6-shogaol treatment significantly increased the expression of p53, Bax, and cleaved caspase-3 in a dose-dependent manner, but decreased the anti-apoptotic protein Bcl-2 (Figure 3C). These results

A



B

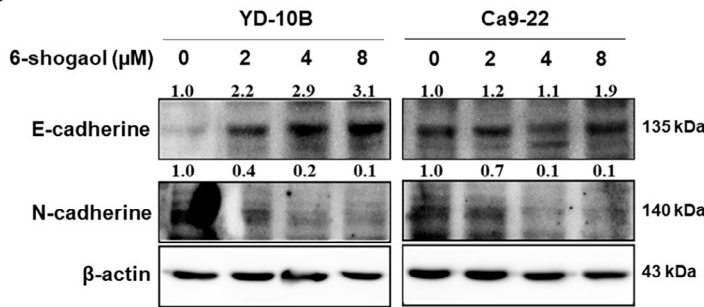
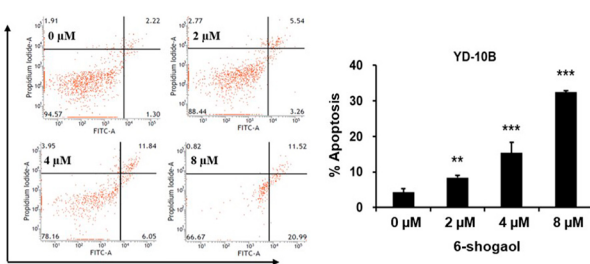
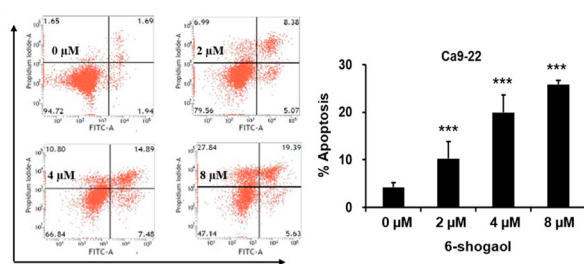


Figure 2- Effects of 6-shogaol on the migration and invasion of OSCC cells. (A) Representative photographs and quantitative graphs of the migrated and invaded YD-10B and Ca9-22 cells treated with 0, 2, 4, and 8 μM 6-shogaol using transwell. Data are expressed as the mean ± SEM. * p<0.05, ** p<0.01, and *** p<0.001 vs control (0 μM 6-shogaol). (B) Western blot analysis of E-cadherin and N-cadherin expression by 6-shogaol treatment in YD-10B and Ca9-22 cells

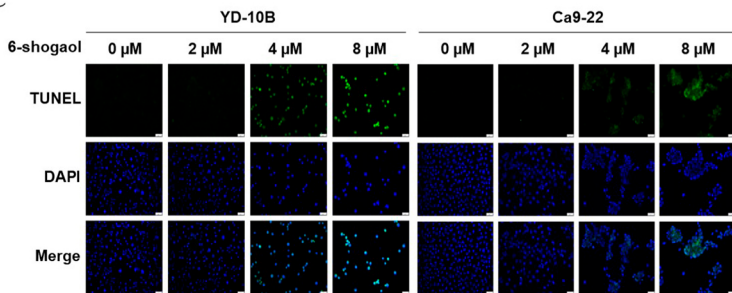
A



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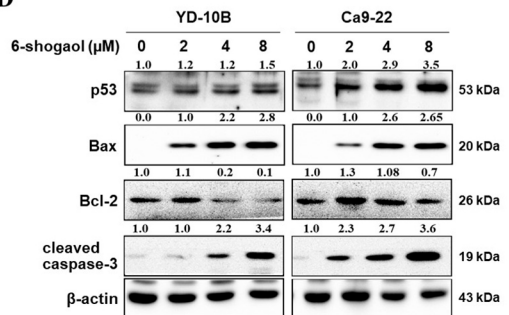
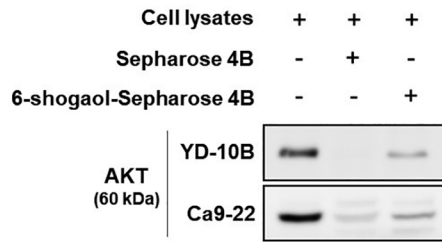


Figure 3- Effects of 6-shogaol on the apoptosis of OSCC cells. (A and B) Annexin V staining and quantification of apoptosis rates in YD-10B and Ca9-22 cells by 6-shogaol treatment. Data are expressed as the mean ± SEM. * p<0.05, ** p<0.01, and *** p<0.001 vs control (0 μM 6-shogaol). (C) The apoptosis phenomenon of YD-10B and Ca9-22 cells treated with 6-shogaol using the TUNEL assay. (D) The expression of cell apoptosis markers, including p53, Bax, Bcl-2, and cleaved caspase-3 in 6-shogaol-treated OSCC cells by western blotting

A



B

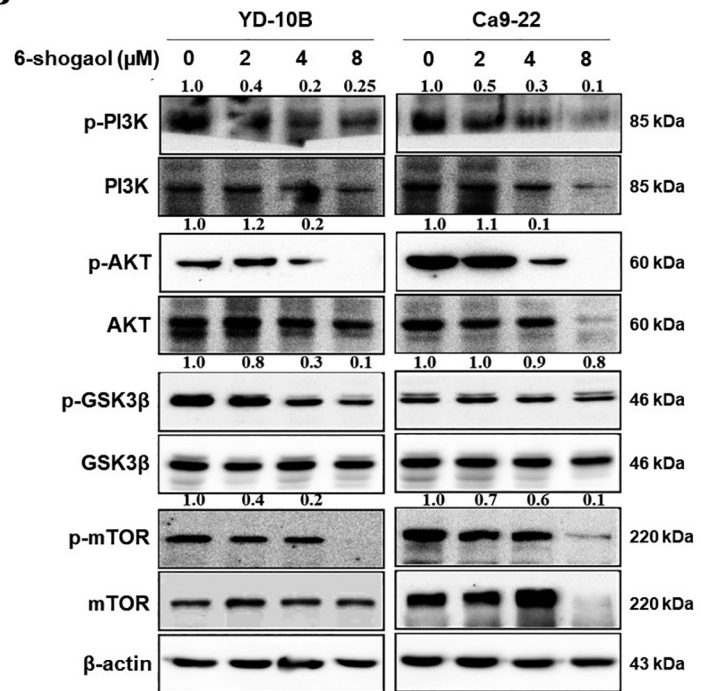


Figure 4- Effects of 6-shogaol on the PI3K/AKT signaling pathway in OSCC cells. (A) Pull-down assay showing 6-shogaol bound to AKT. (B) Western blot analysis of the expressions of proteins related to the PI3K/AKT/mTOR signaling pathway in OSCC cells treated by 6-shogaol

suggest that 6-shogaol significantly induces the apoptosis of OSCC cells.

6-shogaol blocks the PI3K/AKT signaling pathway in OSCC cells

To elucidate the regulatory mechanism underlying the antigrowth effect of 6-shogaol in OSCC cells, we assessed the expression of critical PI3K/AKT pathway proteins. We conducted an *ex vivo* pull-down assay with OSCC cell lysates to establish whether AKT is a target of 6-shogaol. We identified that 6-shogaol did indeed bind with AKT directly (Figure 4A). Significant proteins involved in the PI3K/AKT/mTOR signaling pathway were subsequently analyzed using western blotting. Figure 4B shows that 6-shogaol significantly decreased the expression of p-PI3K, p-AKT, and p-mTOR in a dose-dependent manner in both YD-10B and Ca9-22 cells. However, no significant change in the total protein expression of PI3K, AKT, and mTOR was observed. Additionally, glycogen synthase kinase 3beta (GSK3 β) was also inhibited following 6-shogaol treatment. These results indicate that 6-shogaol may increase apoptosis of OSCC cells through the PI3K/AKT/mTOR signaling pathway.

Oral Squamous Cell Carcinoma Cell

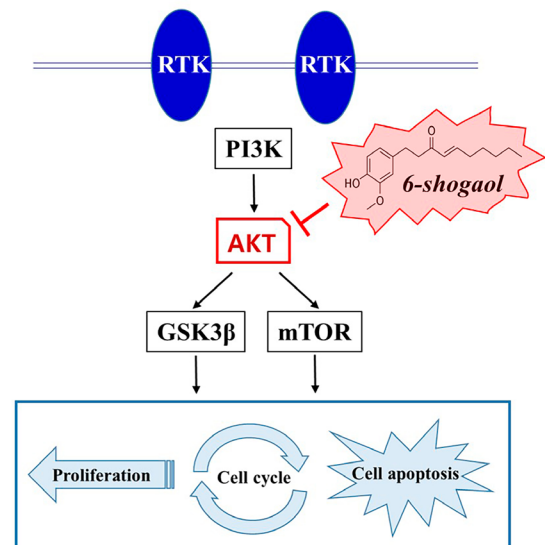


Figure 5- A proposed schematic diagram illustrating the roles of AKT signaling pathway by 6-shogaol in OSCC cells. 6-shogaol exerts the anticancer effects by regulating cell proliferation, cell cycle arrest, and apoptosis via PI3K/AKT signaling pathway by targeting AKT

Discussion

Oral cancer is a common fatal cancer and remains a major threat to global public health. Local recurrence and lymph node metastasis are considered the mainly responsible for the low survival rate in patients with OSCC.^{18,19} Therefore, although current therapeutic methods, such as surgical resection and radiotherapy,

are necessary, it is difficult to obtain satisfactory results because of local recurrence and metastasis of cervical lymph nodes after surgery.²⁰ Furthermore, despite the advances in drugs for various cancers, adjuvant chemotherapy for oral cancer patients has not yet been established.²¹ Recently, compounds isolated from plants or fruits have garnered interest due to their comparatively lower toxic effects and multiple targets.²² Therefore, many researchers are investigating natural products to develop effective prevention and therapeutic agents for various cancers. However, there are few studies on the efficacy of natural products for oral cancer. Here, we introduced 6-shogaol, one of the main compounds in dried ginger, which was reported to possess activities in cancer prevention.^{13,23,24} The anticancer ability of 6-shogaol has been studied in various cancers;²⁴⁻²⁶ however, the mechanism of 6-shogaol in oral cancer has not yet been reported.

Previous reports showed that 6-shogaol plays a crucial role in kidney cancer osteoclastogenic activity and metastatic potential by suppressing 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP).²⁷ Bawadood et al. reported that 6-shogaol induces apoptosis by targeting notch signaling pathway, which therefore suppresses breast cancer cells proliferation and autophagy.²⁸ It has been shown that targeting the TLR4 signaling pathway with 6-shogaol plays an important role in cancer therapy.²⁹ Back to our concern, our findings suggest that 6-shogaol may affect oral cancer cell growth by inhibiting AKT pathway. Notably, 6-shogaol is a multi-targeted drug.

Our study showed that 6-shogaol inhibits cell proliferation by inducing apoptotic cell death in two different human OSCC cell lines, YD-10B and Ca9-22. The regulation of apoptotic gene expression, such as the increase in p53, cleaved caspase-3, and Bax along with the decrease in Bcl-2, supports the antitumor effect of 6-shogaol on OSCC cells. Moreover, the EMT event is a pivotal process in tumorigenesis and metastasis.^{30,31} To further understand EMT mechanisms in OSCC cells, we conducted a migration and invasion assay using transwell with and without Matrigel in YD-10B and Ca9-22 cells and confirmed the expression of EMT-related proteins. In the two OSCC cell lines, 6-shogaol treatment significantly suppressed the ability of migration and invasion of the cells; resulting in an alteration in the expression of EMT markers, including promotion of E-cadherin expression and

inhibition of N-cadherin expression compared to the control group. These results indicate the inhibitory effect of 6-shogaol on the EMT process in oral cancer and also suggest a potential therapeutic value of 6-shogaol for treating oral cancer.

The mechanism of action assays for 6-shogaol in various cancer cells showed that it is involved in multiple signaling pathways during initiation, progression, and metastasis.^{26-29,32} As one of the important pathways in tumorigenesis, once the PI3K/AKT/mTOR signaling pathway is activated, various oncogenes that can lead to cancer progression are upregulated.³³ Yang and Wang investigated the role of AKT in oral cancer using different compounds.^{9,34} Kim, et al.¹¹ (2014) reported that 6-shogaol could inhibit cell proliferation in lung cancer by directly targeting AKT. Studies have also reported that 6-shogaol reduces the survival of fibrosarcoma cells by inducing an increase in ROS production and activation of AKT/mTOR.³⁵ Furthermore, Matsuo, et al.³⁶ (2018) reported the pathologic significance of AKT, mTOR, and GSK3 β proteins in OSCC patients, and confirmed that GSK3 β drove the cervical lymph node metastatic spread of OSCC cells. Therefore, AKT, mTOR and GSK3 β at the process of carcinogenesis were appreciated as target molecules for the prevention and treatment of oral cancer.³⁷

In our study, we confirmed that 6-shogaol could directly bind with AKT in oral cancer cells and inhibit the downstream effect of AKT. 6-shogaol significantly reduced the expressions of p-PI3K, p-AKT, and p-mTOR in OSCC cells; and also inhibited GSK3 β downstream of AKT, which plays an important role in the proliferation and apoptosis of cancer cells. As in the aforementioned anticancer studies of 6-shogaol in various cancer cells, in our study, 6-shogaol also inhibited the PI3K/AKT pathway, thus inhibiting cell proliferation and inducing apoptosis (Figure 5). Undoubtedly, targeting AKT with 6-shogaol will help treat oral cancer. Therefore, AKT could be a novel biomarker for diagnosis and prognosis and could serve as a therapeutic target for patients with oral cancer. Taken together, 6-shogaol inhibited cell proliferation, migration, and invasion as well as promoted the apoptosis of OSCC cells. These results indicate that 6-shogaol may be a potential new therapeutic agent for patients with oral cancer.

Conclusion

Our study shows that 6-shogaol suppresses the proliferation of two OSCC cell lines, YD-10B and Ca9-22, in a dose-dependent manner by targeting AKT. The results also indicate that 6-shogaol inhibits the EMT process and triggered apoptosis by activating the PI3K/AKT/mTOR pathway in OSCC cells. Our findings support the development of natural product-derived compound as a new therapeutic candidate for patients with oral cancer. However, since these are cellular-level results, *in vivo* studies should be further evaluated for the potential application of 6-shogaol in the prevention and treatment of oral cancer.

Conflicts of interest

The authors declare no conflicts of interest.

Author's contributions

Huang, Hai: Investigation (Equal); Methodology (Equal); Writing-original draft (Equal). **Kim, Myoung-Ok:** Conceptualization (Equal); Methodology (Equal); Data curation (Equal); Supervision (Equal); Validation (Equal); Writing-review & editing (Equal). **Kim, Ki-Rim:** Conceptualization (Equal); Investigation (Equal); Data curation (Equal); Supervision (Equal); Validation (Equal); Writing-review & editing (Equal)

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