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Gartanin Induces Apoptosis in Oral Squamous Cell Carcinoma Cell Lines through Mitochondrial Signaling Pathway

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INTRODUCTION

Apoptosis is a physiological process of the controlled elimination of unhealthy or damaged cells[1]. When cells were undergoing apoptosis, it would be show nuclear condensation, DNA fragmentation, membrane blebbing through various signaling pathway[2, 3]. Mitochondria signaling pathway is among the apoptosis pathway. Mitochondria play an important role in the regulation of cell apoptosis. Changes in the mitochondria membrane potential (MMP) are considered an early event in apoptosis and many pro-apoptotic proteins can be released from the mitochondria into the cytoplasm when the MMP is damaged[4]. In recent studies, reported that apoptosis might play an important role in various diseases. Especially of cancer is closely associated with apoptosis. So, it is important to study the anti-cancer capacities of natural compounds for the development of anti-cancer drugs without side effects[5].

Oral cancer is the most common type of human cancers in worldwide[6], and oral squamous cell carcinoma (OSCC) arises from the mucosa of oral cavity frequently[7, 8]. The major risk factors of oral cancer are tobacco, alcohol and high risk Human Papilloma Virus (HPV) infection. The overall survival rate has not changed in recent years, despite exte-

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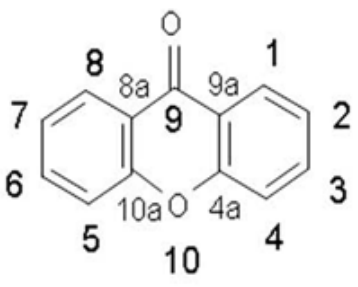
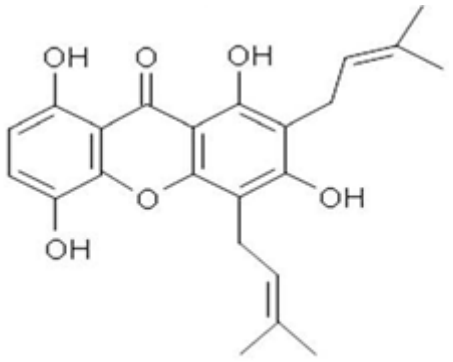
nsive research on the biological and molecular aspects of OSCC[9].

Many tropical fruits have interesting molecular biological activities with potential treatment applications. Garcinia mangostana (mangosteen) has a long history of usage as a medicinal plant in southeast asia[10, 11]. This tropical plant is famous for its nutritious and flavorful fruits. The pericarps of Garcinia mangostana contain numerous xanthones with chemical diversity[12]. Several studies have shown that typical magosteen products, α-mangostin, β-mangostin, γ-mangostin, and gartanin have various effects in diseases such as anti-oxidant, anti-inflammatory, anti-allergic, anti-bacterial, anti-cancer via cell cycle arrest and apoptosis[10, 11, 13, 14]. Although recently mangosteen products, research has been actively promoted but effect in the oral cavity cancer, there is insufficient. In this study, we evaluated the anti-cancer effects of gartanin, one of the major xanthones from the mangosteen fruit in OSCC cell lines.



Xanthone nucleus



Gartanine

Figure 1. Xanthone nucleus with IUPAC numbers of carbons and chemical structure of gartanin[10].

MATERIALS AND METHODS

1. Reagents and cell culture

Gartanin compound was obtained from Chromadex (Irvine, CA, USA). Antibodies against PARP(poly-ADP ribose polymerase) and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Caspase-7, ICAD (Inhibitor of caspase-activated DNase), anti-rabbit IgG antibody, and rabbit anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents were purchased from Sigma unless otherwise specified.

Human OSCC cell lines (HSC-2, -3, -4 cells) that kindly supplied by Prof. Sung-Dae Cho. Chonbuk National University, Jeonju, Korea were cultured in Minimum Essential Medium/Earle's Balanced Salt

Solution (MEM/EBSS) supplemented with 10% FBS and 1% penicillin streptomycin (GIBCO-BRL, Rockville, MD, USA) under a humidified 5% CO_2 atmosphere.

2. Gartanin treatment

The original medium was removed and gartanin (20 mM) stock solution was added to the medium to obtain 0 to 50 μM doses of gartanin. 20 mM stock solution of gartanin was prepared in DMSO and stored frozen at -20°C. The gartanin stock solution was diluted to the indicated concentration with MEM/EBSS when needed.

3. Cell viability assay

In order to measure of the cell viability, we used the MTT assay. Firstly, we were seeded OSCC cell lines on a 96-well microtiter plate (1×10^4cells/well) and then treated with different concentration of gartanin (0 to 50 μM). Secondly, the cells were then incubated for different time periods in the presence of same concentrations of gartanin (25μM). Next, removed existing medium and added 100 μl of MTT solution (500 mg/mL) to each well. The cells were incubated for 4 h at 37°C.There action was stopped by the addition of DMSO (150 μl/well) and constantly shook for 10 min. The cell viability was monitored on an ELISA reader (Tecan, Mänedorf, Switzerland) at 620 nm excitatory emission wavelength. The MTT data were obtained from at least three independent experiments.

4. Hoechst staining for morphological aspects of apoptosis.

The number of cells undergoing apoptosis following treatment with gartanin was quantified by Hoechst staining. 24 h post-treatment with different concentration of gartanin (0 to 50 μM), HSC-2 cells were harvested and cytocentrifuged onto clean, fat-free glass slide with a cytocentrifuge. The cells stained with Hoechst 33342 (1 μg/ml) for 10 min at 37°C. After being washed with phosphate-buffered saline (PBS) and mounted with glycerol, they were observed using an inverted fluorescence microscope (Carl Zeiss, Goettingen, Germany). Untreated cell nuclei showed dispersion and uniform fluorescence, while apoptotic cells were characterized by nuclear shrinkage, condensation, and fragmentation.

5. Flow cytometry analysis and measurements of mitochondrial transmembrane potential (MMP)

Regulation of HSC-2 cell cycle profile and apoptosis was analyzed by FACS. First, 70-80% confluent cells cultured in 100 mm culture dishes (2×10^6 cells/dishes) for 24 h. After treatments, the cells were harvested and centrifugeat

3000 rpm for 5 min. Next, fixed in 95% ice-cold ethanol with 0.5% Tween 20 overnight, washed in 1% bovine serum albumin PBS solution and resuspended in PBS containing 50 μg/mL RNase A, incubated at 4°C for 30 min. The cells were stained with Propidium iodide (50 μg/ml) and Stained cells were measured using a CYTOMICS FC500 flow cytometer system. The data was analyzed using Multi Cycle software that allowed a simultaneous estimation of cell-cycle parameters and apoptosis.

In order to measure of MMP, HSC-2 cells were seeded onto 60mm dishes and incubate for one day. After treatment with gartanin for 24 h, collected cells and $DIOC_6$ stained directly to the cell culture medium (final concentrationis 1 μM) and incubation for 30 min. MMP was analyzed with a flowcytometry system (Beckman Coulter, CA, USA).

6. Western blot analysis

HSC-2 cells were treated with gartanin in the indicated concentrations (0 to 50 μM) for 24 h. After then, the cells were washed twice in ice-cold PBS, resuspended in 200 μl ice cold solubilizing buffer (300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 μl/ml aprotinin and 2 μl/ml leupeptin). The lysates were centrifuged at 13,200 rpm for 30 min at 4°C. Protein concentrations of cell lysates were determined using a Bradford protein assay (Bio-Rad, Richmond, CA, USA). The lysates resolved on sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred, and the membrane was blocked with 5% skim milk for 2 h at room temperature. After blocking, the membranes were incubated overnight at 4°C with the respective primary antibodies. The membranes were washed 6th and conjugated secondary antibodies for 2 h at room temperature. After six washes, the membranes were detected using Super Signal West Femto (Pierce, Rockford, IL, USA) enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, CA USA).

RESULTS

Effect of Gartanin on the cell viability OSCC cell lines and assessed apoptosis of OSCC cells.

Measurement of gartanin on the proliferation and viability of OSCC cell lines (HSC-2, HSC-3, and HSC-4) firstly, we investigated the effects of gartanin, OSCC cell lines (HSC-2, HSC-3, and HSC-4). Thereafter we were cultured with various concentrations of gartanin (0 to 50 μM) for 24 h. As shown in Fig. 2A, gartanin significantly reduced the cell viabilities of OSCC cells dose-dependently.

Next in Fig. 2B, the viability of OSCC cells was decreased in a time-dependent manner by gartanin treatment (25 μM) for 24 h, 48 h, 72 h. The results of the analysis of the MTT assay of OSCC, we observed HSC-2 cells were most reactive for gartanin. For that reason, we conducted an experiment using the HSC-2 cells.

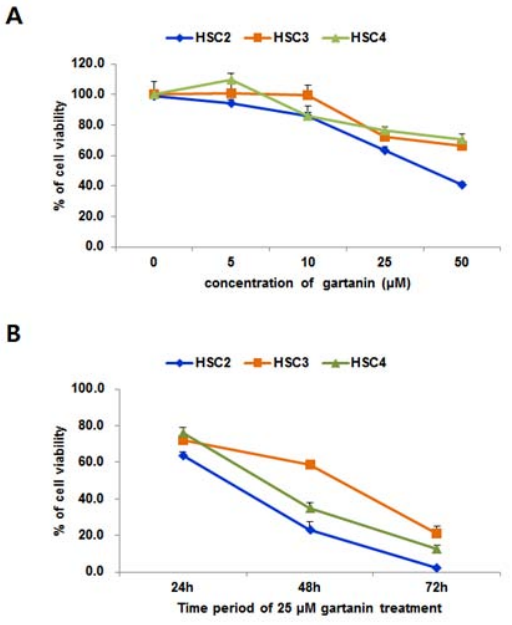


Figure 2. Measurement of cell viability in OSCC cells (HSC-2, HSC-3, and HSC-4) after gartanin treatment. (A) Gartanin (0 to 50 μM) was to treat in OSCC cells for 24 h. (B) OSCC cells were treated with 25 μM of gartanin for 24 h, 48 h 72 h. Values are the mean ±SE from three independent experiments.
* P < 0.05 versus untreated samples.

To further investigate the mechanism of gartanin-induced apoptosis was detected using hoechst staining and flow cytometry. Hoechst staining proved that gartanin induced a change in nuclear morphology. Untreated control cells no significant changes were seen in cell nuclei or cell membrane integrity. However, the HSC-2 cells treated with 0 to 50 μM gartanin for 24 h observed fragmented and cleavage nuclei (Fig. 3).

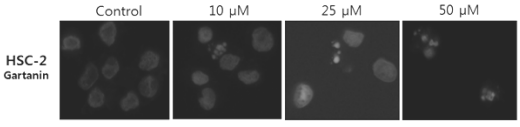


Figure 3. Gartanin induced apoptosis in HSC-2 cells. HSC-2 cells treated with (0 to 50 μM) of gartanin for 24 h. After HSC-2 cells were stained with hoechst and observed a fluorescence microscope. We showed various extent of cell shrinkage, volume reduction, apoptotic body formation and cell blebbing.

DNA condensation and fragmentation are biochemical hallmark of apoptosis. In this experiment, we observed that gartanin-treated cells showed nuclear condensation and fragmentation, and gartanin was significantly effective in HSC-2 cells dose-dependently. To further investigate the apoptotic activity of gartanin, PI staining-based cell cycle analysis was performed. The percentages of sub-G1 fraction in HSC-2 cells were stained with PI solution and analyzed by flowcytometry at 24 h of treatment with 0 to 50 μM gartanin. Following 24 h of incubation with gartanin the sub-G1 population was higher than non-treated HSC-2 cells. Gartanin treated HSC-2

cells showed significant increases in PI positive apoptosis (Fig. 4).

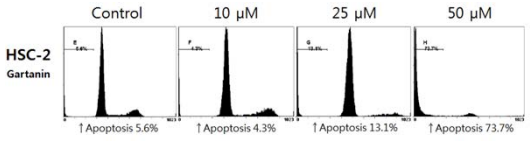


Figure 4. Effects of gartanin treatment HSC-2 cells on the induction of apoptosis. A flow cytometry analyses of HSC-2 cells using PI staining. In order to analysis of sub-G1 cells, we were treated gartanin (0 to 50) for 24 h.

Measurements of mitochondrial membrane potential (MMP) and caspase-mediated apoptosis induced by gartanin.

The result of mitochondrial membrane potential (MMP) measurement, we verified the change of MMP in grtanin-induced apoptosis using $DIOC_6$ staining.The mitochondria membrane potential was measured by flowcytometry. Gartanin treated HSC-2 cells showed a loss of mitochondrial membrane potential in dose-dependently (Fig. 5).

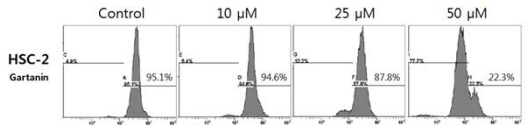


Figure 5. Measure the mitochondrial membrane potential in gartanin-iduced apoptosis. Cells were treated with 0 to 50 μM concentration of gartianin for 24 h and incubation with 1 μM $DIOC_6$ and analyzed using flowcytometry.

To elucidate the molecular mechanisms of gartanin-induced apoptosis, we further examined the expression levels of the apoptosis-associated proteins, such as caspase-7, caspase-3, and PARP using western blot analysis. The expression levels of caspase-7, caspase-3, ICAD and PARP proteins decreased dose- dependently and, in particular, cleaved PARP significantly increased in HSC-2 cells (Fig. 6). The level of GAPDH was used as an internal standard for caspase-7, caspase-3, ICAD and PARP expression.

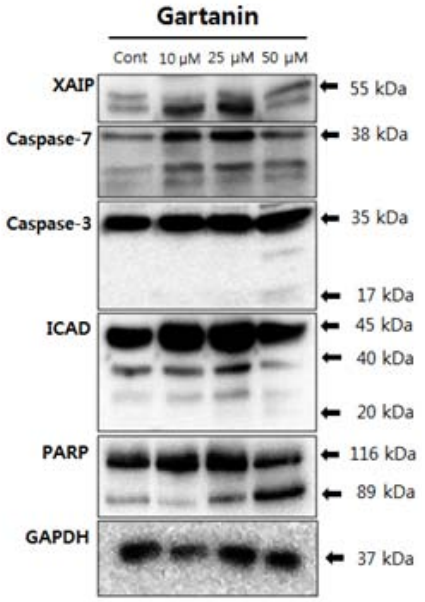


Figure 6. Gartanin affects the expression levels of apoptosis associated molecules (caspase-7, caspase-3, ICAD and PARP) in HSC-2 cells using western blot analysis. The HSC-2 cells were treated with gartanin (0 to 5 μM) for 24 h and caspase-7, caspase-3, ICAD proteins were decreased and, in particular, cleavage PARP significantly increased in HSC-2 cells by Western blot analysis.

DISCUSSION

Garcinia mangostana has a long history of usage as a medicinal plant in southeast asia[11]. In many studies, mangosteen has been reported to have potent biological advantages against various types of cancer cells, including canine osteosarcoma cells, human colorectal cancer cells and pancreatic cancer cells[15, 16]. Gartanin has received significant attention because of its molecular biological advantages, such as anti-oxidant, anti-inflammatory, anti-allergic, anti-bacterial and anti-cancer properties[11, 17, 18]. However, the biological mechanism of gartanin-induced cell death against OSCC cell lines has not been well studied. OSCC is a malignant tumor common diagnosed oral cancer in the world. The age of OSCC onset tends to be younger than that of other tumors at approximately 30~50 years of age[19]. In this study, we provided emerging proof that gartanin has anti-tumor effects against HSC-2 cells. Gartanin was accompanied by the inhibition of cell proliferation, the acceleration of mitochondria-controlled apoptosis. Based on preliminary experiments, we found that the cell viability and proliferation effect of gartanin to OSCC cell lines. Firstly we treated with various doses (0 to 50 μM) of each cells (HSC-2, HSC-3, HSC-4), and cell viability was determined by MTT assay. There was significant difference of cell viability between control and gartanin treated cells (Fig. 2A). Secondary we were treated 25 μM gartanin on HSC-2 cells for 24 h, 48 h, 72 h. As a result, HSC-2 cells were more sensitive to gartanin than HSC-3 and HSC-4 cells. Therefore, we conducted an experiment using the HSC-2 cells. Next, we observed that gartanin induced apoptosis in OSCC cell lines using hoechst staining and flow cytometry with PI staining (Fig. 3 and 4). During the apoptosis, the cell was become shrinkage, plasma membrane "blebbing", DNA fragmentation and chromatin condensation. These dynamic morphological changes are biochemical hallmark of apoptosis[20]. In order to hoechst staining, cells were treated with 0 to 50 μM gartanin for 24 h, then stained with DAPI and analyzed under a fluorescence microscope (Fig. 3). In this study, we investigated the possible mechanisms of gartanin-induced apoptosis in HSC-2 cells. Additionally we performed the flow cytometry with PI staining. In this experiment, we assessed sub-G1 phase proportion in HSC-2 cells. Few researchers demonstrated that the proportion of sub-G1 phase was increased by gartanin in prostate, breast, and colon cancer cells[21, 22]. So we observed from flow cytometric assay using PI staining revealed that treatment with 0 to 50 μM gartanin resulted in increased accumulation of sub-G1 phase in HSC-2 cells dose-dependently (Fig. 4). As a result when compared

with the controls, HSC-2 cells were consistent with the enhanced effect of gartanin on the induction of apoptosis.

Mitochondrial permeabilization and release of intermembrane space proteins are important features of both models of cell death[23, 24]. A number of studies have demonstrated that mangosteen product induces apoptosis through the stimulation of mitochondrial signaling pathway[25]. So, in this study, we observed that gartanin also induces apoptosis in OSCC cell lines via measurement of MMP. As a result of gartanin treated HSC-2 cells showed a loss of mitochondrial membrane potential in dosedependently (Fig. 5).

It has been acknowledged that apoptosis is divided into two principal pathways. Among them intrinsic pathway is activated on caspase cascade. Caspases are a family of genes important for maintaining homeostasis through regulating apoptosis [26]. Apoptotic caspases are activated with the initiator caspases cleavage and activate the executioner caspases-3 or -7[27]. Pro-caspase-3 becomes activation when it is cleaved to two monomers. This has potent activity, including an ability to auto-catalytical activation that contributes to the observed cascade effect of increasing caspase-3 activity as apoptosis progresses[27, 28]. Caspase-7 can also play an important role in the outcome of the intrinsic pathways. Mature caspases-7 cleaves a large set of substrates, ultimately resulting in the characteristic morphological and biochemical hallmarks of apoptosis[29]. Caspase-activated DNase (CAD), which causes a genome fragmentation at the final stage of apoptosis exists as a complex form with the ICAD [30]. When apoptotic stimuli activates, the caspase cascade, caspase 3 cleaves ICAD. In this course CAD released from ICAD, degrades chromosomal DNA in nuclei[30], [31]. Poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme, has a particularly well known role in base excision repair[32]. PARP is part of the caspase-dependent pathway of apoptosis. Cleaved PARP is increased during apoptosis and it is important of apoptotic marker[33]. So, we concluded that DNA fragmentation factor, which comprises a caspase-3-activated DNase (CAD) and its inhibitor (ICAD), may influence the rate of cell death by generating PARP-activating DNA fragmentation[31].

In order to confirm the expression levels of Caspase-7, caspase-3, ICAD and PARP we explored this process in more detail by western blot analysis (Fig. 6). We clarified activation of caspase-7, caspase-3, ICAD and PARP, which are associated with apoptosis. The expression levels of caspase-7, caspase-3, ICAD and PARP proteins decreased and, in particular, cleaved PARP significantly increased in HSC-2 cells. These results clearly suggest that gartanin-induced apoptosis is involved in

the intrinsic pathway and caspase cascades in HSC-2 cell lines. Gartanin help cells to survive from cancer and could be a potential treatment application for OSCC and provide valuable data for the development of a novel anti-cancer strategy.

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국문초록

구강편평상피암종에서 가르타닌이 유도하는 세포자멸사의 기전연구

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가르타닌은 동남아시아에서 주로 재배되는 망고스틴에 많이 존재하는 Xantone 구조를 가진 화합물의 하나로 항산화, 항염증, 항바이러스, 항암효과 등 여러 가지 생리학적 활성을 갖는 것으로 알려져 있다. 현재까지 보고된 논문에 의하면 가르타닌은 항암, 항염증, 항산화 기전등의 연구가 진행되어왔지만 치과영역과 관련하여 구강편평상피세포암종세포에서의 효과와 기전에 대한 규명은 아직 미미하다. 본 연구에서는 구강편평상피세포암종세포에서의 가르타닌의 항암 효과와 분자생물학적 기전을 밝히기 위하여 실험을 수행하였다. 가르타닌 처리된 HSC-2 세포의 생존률 감소는 MTT기법과 Flow cytometry를 이용한 Sub-G1기 비율의 변화를 통하여 확인하였고, 세포자멸사 유도를 확인 하기위하여 Hoechst 염색과 미토콘드리아 막전위의 변화를 확인 하였다. 그 결과 가르타닌을 처리한 HSC-2 세포에서 세포자멸이 유도되었으며, 가르타닌이 유도하는 세포자멸사는 미토콘드리아 관련 기전으로 이루어지는 것을 알 수 있었다. 이러한 이유로 가르타닌은 구강암 치료와 관련하여 훌륭한 항암제가 될 수 있으며 지속적인 연구와 개발의 가치가 있는 것으로 생각된다.

주제어 : 가르타닌, 세포자멸사, 구강편평상피세포암