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# Genkwadaphnin induces reactive oxygen species (ROS)-mediated apoptosis of squamous cell carcinoma (SCC) cells

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ABSTRACT

Genkwadaphnin is a daphnane diterpene ester molecule isolated from the flower buds of *Daphne genkwa*. In the present study, we investigated the apoptosis-inducing effect of genkwadaphnin in squamous cell carcinoma (SCC) cells. Apoptosis was triggered in SCC12 cells following genkwadaphnin treatment in a time- and concentration-dependent manner. Genkwadaphnin treatment increased phosphorylation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). Knockdown of JNK and p38 MAPK by recombinant adenovirus expressing microRNA (miR) resulted in significant inhibition of genkwadaphnin-induced apoptosis in SCC12 cells. Finally, pretreatment with the reactive oxygen species (ROS) scavenger N-acetylcysteine (NAC) markedly reduced SCC12 cell apoptosis, concomitant with significant inhibition of MAPK activation. These results indicate that genkwadaphnin has the potential to induce apoptosis in SCC cells, providing information on which to base further research with the aim of developing a cure for SCC.



## 1. Introduction

Cutaneous squamous cell carcinoma (SCC) originates from the upper layers of skin epidermis. SCC incidence is relatively high, ranking as the second most frequent among the non-melanoma skin cancers [1]. The primary cure for SCC is surgical excision; topical treatment (e.g., 5% imiquimod cream) is also used to successfully manage SCC [2–4]. Imiquimod stimulates tumor destruction by recruiting cutaneous effector T cells from blood and by inhibiting tonic anti-inflammatory signals within the tumor [5]. In addition, imiquimod directly induces apoptosis of SCC cells via an A20-dependent mode of action [6]. This fact supports the idea that drug development for SCC can be based the ability of a compound to induce apoptosis in SCC cells. In fact, various chemotherapeutic drugs that have been used to treat many different cancers appear to exert their effects via apoptosis [7,8].

Medicinal plants are an important source of chemotherapeutic drugs. Diverse chemotherapeutic materials have been isolated from plant extracts, with anti-cancer effects based on their apoptosis-inducing potential [9–11]. In a preliminary study, we attempted to identify the apoptosis-inducing constituent of a medicinal plant, and found that genkwadaphnin had such an effect. Genkwadaphnin is the daphnane diterpene ester molecule isolated from the flower buds of *Daphne genkwa* [12]. Genkwadaphnin has been reported to exert anti-neoplastic activity against a leukemic cell line [13,14]. However, the effect of genkwadaphnin on cutaneous SCC cells remains unclear. In this study, we demonstrated that genkwadaphnin induces apoptosis in SCC cells, a process in which reactive oxygen species (ROS)-mediated mitogen-activated protein kinase (MAPK) activation is involved.

## 2. Materials and methods

### 2.1. Cell culture

SCC12 cells, a human squamous cell carcinoma line, were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies Corporation, Grand Island, NY). Genkwadaphnin was dissolved in dimethyl sulfoxide (DMSO). For viability testing, SCC12 cells were seeded into six-well plates at a density of $2 \times 10^5$ cells/well,

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treated with genkwadaphnin for 24 h, and then the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed.

### 2.2. Detection of apoptosis

Apoptotic cells were identified using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany). After treatment with genkwadaphnin for 24 h, cells were incubated with a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) reaction mixture for 2 h at 37 °C, rinsed, counterstained with 2% Evans blue solution (Sigma–Aldrich, St. Louis, MO), and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

For flow cytometry analysis, a Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit was used (BD Biosciences, San Jose, CA). Cells were washed twice with cold phosphate-buffered saline (PBS) and stained with FITC annexin V and propidium iodide (PI). Cells were then analyzed using FACScanto (BD Biosciences).

### 2.3. Western blotting

Cells were lysed in Proprep solution (Intron, Daejeon, Korea). Total protein was measured using Bicinchoninic Acid (BCA) Protein Assay Reagent (Pierce Biotechnology, Rockford, IL). Samples were run on sodium dodecyl sulfate (SDS)–polyacrylamide gels, transferred onto nitrocellulose membranes and incubated with the appropriate antibodies. Blots were then incubated with peroxidase-conjugated secondary antibodies and visualized using enhanced chemiluminescence (ECL; Intron). The following primary antibodies were used in this study: poly ADP ribose polymerase (PARP; BD Biosciences, San Jose, CA), caspase-3, JNK, phospho-JNK, p38 MAPK, phospho-p38 MAPK (Cell Signaling Technology, Beverly, MA), and actin (Sigma–Aldrich, St. Louis, MO).

### 2.4. Knockdown of gene expression

For JNK- and p38 MAPK-specific microRNA (miR), target sequences were designed using Invitrogen's RNAi Designer. The double-stranded DNA oligonucleotides were synthesized and cloned into the parental vector pcDNA6.2-GW/EmGFP-miR (Invitrogen, Carlsbad, CA). In this vector system, the target gene miR sequence is located downstream of the emerald green fluorescent protein (EmGFP) coding sequence, allowing the identification of miR expressing cells through fluorescence microscope observation of GFP. The miR expression cassette was moved into the pENT/CMV vector, and then adenovirus was generated as reported previously [15]. The miR sequences are as follows: JNK1, top strand 5′-TGCTGATGAGTCTGATTCTGAAATGGGTTTTGGCCACTGACTGACCCATTTCAATCAGACTCAT, bottom strand 5′-CCTGATGAGTCTGATTGAAATGGGTCAGTCAGTGGCCAAAACCCATTTCAGAATCAGACTCATC; JNK2, top strand 5′-TGCTGCTGACTGTCACATTTACTGTCGTTTTGGCCACTGACTGACGACAGTAAGTGACAGTCAG, bottom strand 5′-CCTGCTGACTGTCACTTACTGTCGTCAGTCAGTGGCCAAAACGACAGTAAATGTGACAGTCAGC; p38 MAPK, top strand 5′-TGCTGATGAATGATGGACTGAAATGGGTTTTGGCCACTGACTGACCCATTTC ACCATCATTCAT, bottom strand 5′-CCTGATGAATGATGGTGAAATGGGTCAGTCAGTGGCCAAAACCCATTTCAGTCC ATCATTCATC.

For JNK knockdown, SCC12 cells were co-transduced with 10 multiplicity of infection (MOI) of adenovirus expressing miR-JNK1 and miR-JNK2 for 6 h. Cells were supplied with fresh medium, and incubated for a further 2 d. Then, cells were treated with genkwadaphnin (200 ng/mL) for 24 h. For p38 MAPK knockdown, cells were transduced with 10 MOI of adenovirus expressing miR-p38 and then processed in the same way.

### 2.5. Statistical analysis

Data were evaluated statistically using one-way analysis of variance (ANOVA) with the SPSS software (v 22.0; IBM, Seoul, Korea). Statistical significance was set at $p < 0.01$.

## 3. Results

To investigate its effect, we treated SCC12 cells with genkwadaphnin and evaluated viability using an MTT assay. Genkwadaphnin induced cell death in a concentration-dependent manner (Fig. 1A). To verify that genkwadaphnin induced apoptosis in SCC12 cells, we performed a TUNEL assay. Compared with the control, the number of TUNEL-positive cells increased significantly in the genkwadaphnin-treated group (Fig. 1B). Flow cytometry analysis showed that treatment with genkwadaphnin resulted in a marked increase in the proportion of apoptotic cells (Fig. 2A). Consistent with these data, genkwadaphnin induced the cleavage of PARP and caspase-3 in a concentration- and time-dependent manner (Fig. 2B). To verify that the effect of genkwadaphnin was not specific to SCC12 cells alone, we treated another SCC cell line, SCC13; genkwadaphnin also induced apoptosis in SCC13 cells (Suppl. Fig. 1). Together, these results indicate that genkwadaphnin has apoptosis-inducing potential in SCC cells.

We attempted to examine the putative mechanism of genkwadaphnin-induced apoptosis in SCC12 cells. We first investigated whether genkwadaphnin influenced intracellular signaling molecules. After treatment with genkwadaphnin, JNK phosphorylation was markedly increased for up to 24 h. Although the effect was not as marked as that on JNK, p38 MAPK phosphorylation was also

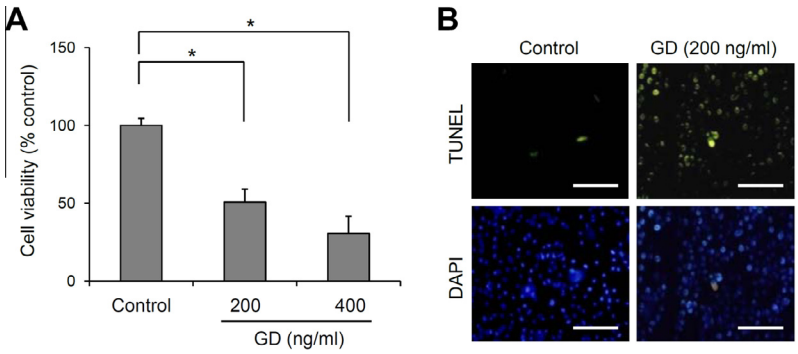


**Fig. 1.** Genkwadaphnin-induced apoptosis of SCC12 cells. (A) Cells were treated with genkwadaphnin (GD) at the indicated concentrations for 24 h. Cell viability was determined by MTT assay. Data are expressed as percentage of control. The mean values ± SD are averages of triplicate measurements. (B) Cell apoptosis was analyzed by TUNEL assay. The number of TUNEL-positive cells was highly increased by genkwadaphnin (GD) treatment. Bar = 100 μm.

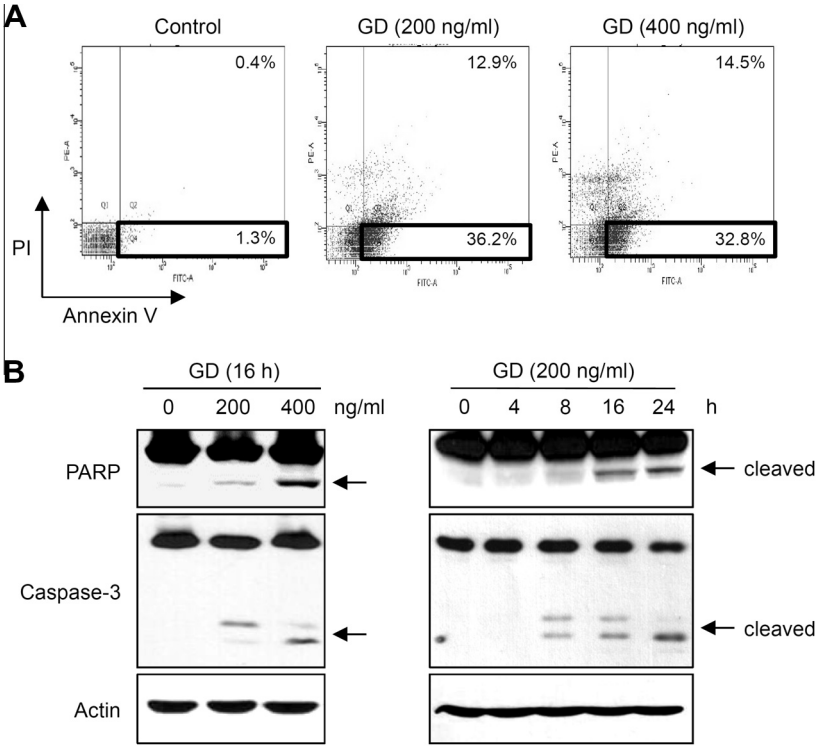


**Fig. 2.** Genkwadaphnin-induced apoptosis of SCC12 cells. (A) Cells were treated with genkwadaphnin (GD) at the indicated concentrations for 24 h. Apoptosis was determined by flow cytometry. Annexin V high and propidium iodide (PI) dim cells (bold box) represent apoptotic cells. (B) Cleavages of PARP and caspase-3 were detected by Western blot. Genkwadaphnin induced the cleavages of PARP and caspase-3 in the dose- and time-dependent manner.

increased by genkwadaphnin (Fig. 3A). As these signaling molecules are implicated in the onset of apoptosis, these findings suggest that genkwadaphnin-induced activation of JNK and p38 MAPK might be functionally involved in SCC12 cell apoptosis. To further demonstrate this, we performed knockdown experiments using recombinant adenovirus expressing miR specific for JNK1/2 and p38 MAPK [16]. Fluorescence microscopy observation revealed that almost all cells were transduced with the recombinant adeno-

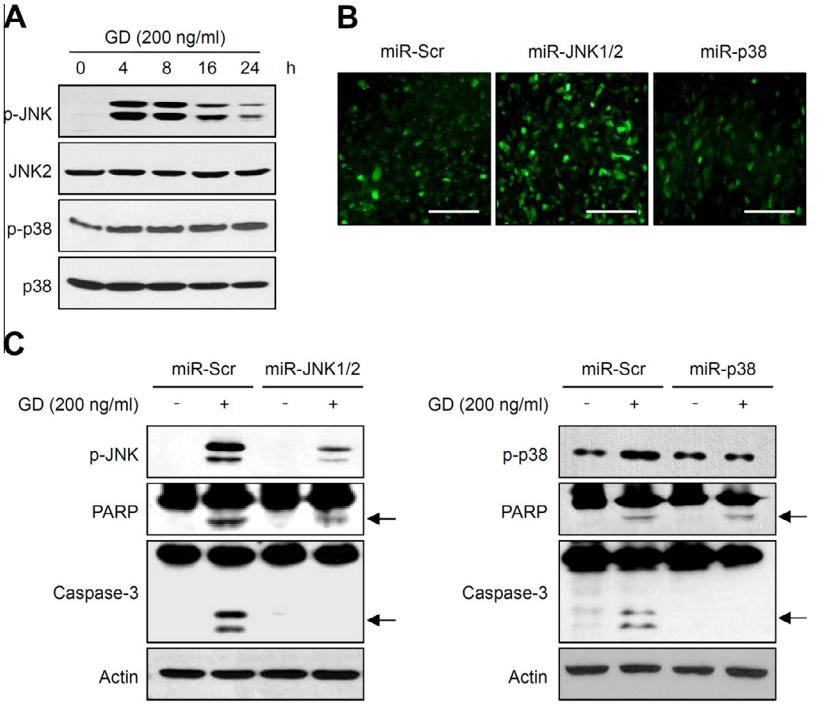


**Fig. 3.** Involvement of MAPK pathway activation in genkwadaphnin-induced apoptosis of SCC12 cells. (A) Cells were treated with genkwadaphnin (GD) for the indicated time points. The phosphorylation of JNK and p38 MAPK was determined by Western blotting. (B) For knockdown experiment, cells were transduced with adenoviruses expressing miR-scrambled control (miR-Scr), miR-JNK1/2 and miR-p38 MAPK. The miR-expressing cells were verified by EmGFP expression. (C) Cells were transduced with the indicated adenovirus expressing miR, then treated with genkwadaphnin (GD). Cleavages of PARP and caspase-3 were detected by Western blot. Bar = 100 μm.

virus expressing miR (Fig. 3B). Western blotting showed that JNK knockdown significantly inhibited the genkwadaphnin-induced cleavage of PARP and caspase-3, supporting the notion that genkwadaphnin triggered SCC12 cell apoptosis in a JNK-dependent manner. When p38 MAPK was knocked down by miR, PARP cleavage was not markedly prevented, but caspase-3 activation was inhibited (Fig. 3C). Similarly, JNK knockdown resulted in marked inhibition of genkwadaphnin-induced cleavage of PARP and caspase-3 in SCC13 cells (Suppl. Fig. 2). Therefore, genkwadaphnin-induced activation of JNK might be an important step in triggering SCC cell apoptosis.

Since ROS reportedly mediate apoptosis in several cell types [17–20], we investigated the involvement of ROS in genkwadaphnin-induced apoptosis in SCC12 cells. We pretreated SCC12 cells with N-acetylcysteine (NAC), a well-known ROS scavenger, followed by treated with genkwadaphnin. As shown in Fig. 4A, pretreatment with NAC significantly blocked genkwadaphnin-induced cell death. Flow cytometry also showed that the apoptotic cell population induced by genkwadaphnin was markedly diminished by pretreatment with NAC (Fig. 4B). Furthermore, genkwadaphnin-induced phosphorylation of JNK and p38 MAPK was inhibited by pretreatment with NAC, as were PARP cleavage and caspase-3 activation (Fig. 4C). These results suggest that genkwadaphnin induces apoptosis in SCC12 cells via ROS-mediated MAPK activation.

## 4. Discussion

Cutaneous SCC is a common malignancy, and surgical excision is the first choice for SCC treatment. Since surgical procedures can have unsatisfactory cosmetic results, development of novel anti-cancer agents for SCC is in demand. In this study, we showed that genkwadaphnin induced apoptosis in SCC12 cells, suggesting that it may be a promising agent for cutaneous SCC therapy.

In Oriental medicine, *D. genkwa* flower buds are used to treat edema, cancer and asthma [21]. The main ingredients of the genus *Daphne* include flavonoids, lignans, and daphnane-type diterpenes. Among those, daphnane-type diterpene reportedly possesses a wide variety of bioactivities, including melanogenesis inhibition, anti-HIV-1 activity, and anti-cancer effects [22–24]. Genkwadaphnin is a daphnane-type diterpene that exerts apoptosis-inducing potential in SCC cells. We demonstrated that genkwadaphnin induced activation of MAPKs, including JNK and p38 MAPK. Specifically, JNK phosphorylation was markedly and persistently increased. Since persistent JNK activation contributes to tumor necrosis factor (TNF)-induced apoptosis [25], genkwadaphnin likely regulates JNK activity as a key process in SCC12 cell apoptosis. This notion is supported in part by our knockdown experiment in which the miR-induced decrease in JNK1/2 resulted in a significant reduction in PARP cleavage and caspase-3 activation.

Evidence suggests that many anti-cancer drugs destroy tumor cells via ROS generation. ROS regulates intracellular signaling events, thereby influencing cell death processes [26,27]. We demonstrated that the apoptosis-inducing potential of genkwadaphnin was related to ROS production. When ROS was scavenged by the antioxidant NAC, genkwadaphnin-induced apoptosis was suppressed markedly. Furthermore, NAC pretreatment resulted in reduced JNK phosphorylation. Thus, ROS production is likely the primary event induced by genkwadaphnin in SCC12 cells; JNK

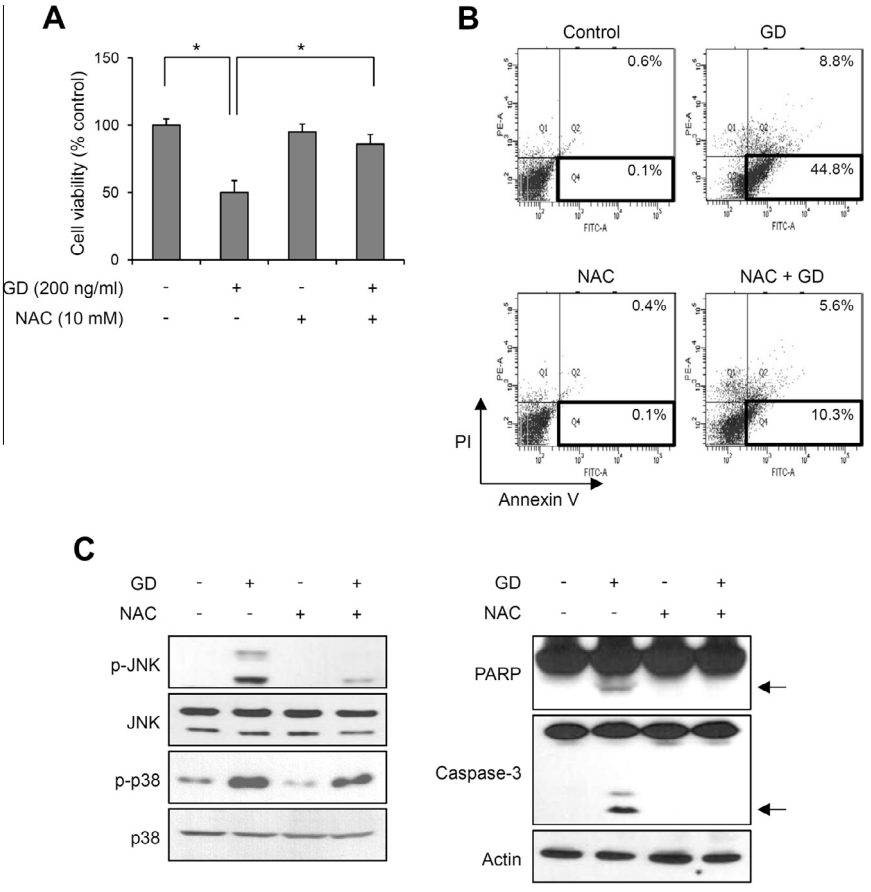


**Fig. 4.** Involvement of ROS production in genkwadaphnin-induced apoptosis of SCC12 cells. (A) Cells were pretreatment with ROS scavenger NAC. After 1 h incubation, cells were further treated with genkwadaphnin (GD) for 24 h. Cell viability was determined by MTT assay. Data are expressed as percentage of control. The mean values ± SD are averages of triplicate measurements. (B) Apoptosis was determined by flow cytometry. Annexin V high and propidium iodide (PI) dim cells (bold box) represent apoptotic cells. (C) Cleavages of PARP and caspase-3 were detected by Western blot.

activation may be a downstream event following ROS production. The precise mechanism of action underlying the genkwadaphnin-induced ROS production remains to be determined.

In summary, we demonstrated that genkwadaphnin induced apoptosis in SCC12 cells via ROS production and MAPK activation. Our data provide information on which to base further research with the aim of developing a cure for SCC.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.118.

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