

Mitigating effect of fermented Korean red ginseng extract with yeast and probiotics in 1-chloro-2,4-dinitrobenzene-induced skin allergic inflammation

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Abstract

Backgrounds: Atopic dermatitis (AD) frequently affects both infants and adults, impacting on the quality of life of the individual and imposing a significant psychosocial burden. The efficacy against AD-like skin lesions of the fermented form of Korea red ginseng (FRG), that contains ginsenoside metabolites, has not been investigated.

Methods: FRG was prepared after fermentation of Korean red ginseng (KRG) extract in the presence of yeast and probiotics. The effects of FRG on skin allergic inflammation were then investigated by examining the frequency of scratching, and assessing the levels of representative allergic markers in 1-chloro-2,4-dinitrobenzene (DNCB)-challenged Balb/c mice. After two weeks of DNCB challenge, vehicle, dexamethasone (positive control), and FRG (10 and 50 µg/mL) were applied top-

ically on skin lesions for 2 weeks. At the end of the experiment, scratching behavior, immunoglobulin E (IgE), and interleukin 4 (IL-4) levels were measured. The status of mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB) were determined by immunoblot analysis in DNCB-treated primary splenocytes.

Results: Topical administration of FRG significantly suppressed scratching behavior, serum IgE, and IL-4 levels ($P < 0.05$, $n = 5/\text{group}$). In addition, FRG treatment significantly decreased the mRNA expression levels of IL-4 and IL-10 in DNCB-exposed splenocytes ($P < 0.05$, $n = 6/\text{group}$). Moreover, FRG suppressed DNCB-induced phosphorylation of p38 MAPK and activation of NF-κB.

Conclusion: FRG could ameliorate skin allergic inflammation via suppression of p38 MAPK and NF-κB. Therefore, FRG may be a potential therapeutic agent for the prevention and/or treatment of atopic-like dermatitis.

Keywords: Atopic-like dermatitis, Fermentation, Immunoglobulin E, Interleukin, Mitogen-activated protein kinase, Nuclear factor kappa-B

Introduction

Atopic dermatitis (AD) is one of the most common chronic allergic and inflammatory skin conditions, manifested by intense itching and recurrent eczematous lesions^{1,2}. It is characterized by pruritus, xerosis, lichenification, and mechanical skin injury, and these manifes-

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tations are related to various inflammatory cells including eosinophils, mast cells, and lymphocytes that infiltrate the AD skin lesions^{3–6}. Atopic dermatitis frequently affects both infants and adults, impacting on the quality of life of the individual and imposing a significant psychosocial burden. The direct and indirect economic burdens of AD treatment are increasing annually⁷.

The pathogenesis of AD involves multifaceted etiologies, including genetic predisposition, impairment of the normal barrier function of the skin, and immunological aberrations¹. The most widely accepted hypothesis that explains the involvement of immune dysfunction in AD is the elevation of immunoglobulin E (IgE) levels and immune dysregulation between type-1 (Th₁) and type-2 helper T (Th₂) cells⁸. Aberrant immune stimulation mediated by interleukin (IL)-4, IL-5, IL-10, IL-13, or IgE, which are regulated by Th₂ cells, may contribute to the pathogenesis of allergic diseases such as AD^{4,8–14}. Although there has been substantial progress in understanding the pathogenesis of AD, there is still no effective therapy for this condition^{1,14}. Topical corticosteroids like dexamethasone (Dex) constitute one of the oldest and most effective treatments for dermatological conditions, including contact dermatitis and AD^{15,16}. However, there is some evidence that they have severe side effects¹⁷. A recent study suggested that cutaneous diseases, including AD, can contribute to cardiovascular morbidity due to dysregulation of atherosclerosis mediators and inflammatory markers¹⁸. Therefore, development of therapeutic alternatives for AD that can ameliorate the clinical symptoms is urgently needed, both to reduce the public health burden as well as improve the quality of life of the patients.

Korean red ginseng (KRG) extract can be obtained by subsequent heat and steam treatment (98–100°C for 2–3 h) of the roots of *Panax ginseng* C.A. Meyer (Araliaceae). This process induces a change in its chemical composition, including formation of ginseng saponins (ginsenosides) that have been shown to exhibit antioxidant, immunostimulatory, anti-aging, and several additional biological activities^{19,20}. Moreover, KRG also possesses anticancer, antidiabetic, and antiallergic properties^{21–24}. 1-Chloro-2,4-dinitrobenzene (DNCB) is a potent contact allergen, which has been used as an inducer of AD *via* elevation of the levels of IgE and cytokines (IL-4 and IL-10) produced by Th₂ lymphocytes^{25,26}. In a previous study, we showed that topical application of KRG could improve skin lesions and reduce IL-4, IL-10, and IgE levels in a DNCB-induced AD-like mouse model²⁷. However, the effectiveness of oral administration of KRG may differ depending on individual differences in gut microflora^{28,29}. Artificial processing of natural products, such as drying, fermentation, and roasting, has been used to increase their health benefits and

bioavailability^{30,31}. It was shown that the contents of compound K and several ginsenosides that exhibit biological activities (Rh₁, Rh₂, Rg₅, and Rk₁ in KRG) could be increased by fermentation¹⁹. However, the efficacy against AD-like skin lesions of the fermented form of KRG (FRG), that contains ginsenoside metabolites, has not been investigated.

In the present study, we examined the effects of FRG on AD-like skin allergic inflammation *in vivo* by examining the frequency of scratching and assessing the levels of representative allergic markers, like serum IgE and splenocyte IL-4, in DNCB-exposed mice. In addition, we aimed to elucidate the underlying mechanism by investigating the potential effects of FRG on the mRNA expression levels of IL-4 and IL-10; expression of mitogen-activated protein kinase (MAPK) family members like extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK; and nuclear translocation of nuclear factor kappa B (NF-κB, p65) in DNCB-stimulated primary splenocytes.

Materials & Methods

Chemicals and reagents

1-Chloro-2,4-dinitrobenzene, dithiothreitol (DTT), Dex, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NonidetTM P-40 (NP-40), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ginsenosides Rb₁ (C₅₄H₉₂O₂₃, MW = 1109.307), R_d (C₄₈H₈₂O₁₈, MW = 947.15), and Rg₃ (C₄₂H₇₂O₁₃, MW = 785.01), the main active compounds found in KRG, were obtained from the Korea Ginseng and Tobacco Central Research Institute (KGTORI, Daejeon, Korea) and were used as standards for high-performance liquid chromatography (HPLC) analysis. All other chemicals were purchased from Sigma-Aldrich. All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Experimental animals

Six-week-old male Balb/c mice were obtained from Dae-Han Biolink (Eumsung, Korea) and housed with *ad libitum* access to food and water. The mice were maintained in the experimental animal facility, and the protocols were approved by the Animal Care Committee at Sungkyunkwan University. Animal experiments were performed in accordance with the National Institutes of Health (NIH) guidelines. During the experiment, all animals were maintained at a temperature of 23 ± 1°C and humidity of 55 ± 5% with 10–18 circulations/h under 12 h light/dark cycles. The animals were randomly

divided into five groups ($n = 5/\text{group}$), as follows: 1) normal control group; 2) DNCB-treated disease group; 3) DNCB-treated group receiving 10 $\mu\text{g/mL}$ FRG; 4) DNCB-treated group receiving 50 $\mu\text{g/mL}$ FRG; and 5) DNCB-treated group receiving Dex.

Preparation of FRG

Korean red ginseng extract was obtained from KGC (Korea Ginseng Corporation, Seoul, Korea) and its ginsenoside contents have been previously addressed³². Korean red ginseng extract was processed once with 70% ethanol (Korea Ethanol Supplies, Seoul, Korea) for 8 h and then twice with 15% ethanol. After concentrating (60–64 cm Hg, 65 brix) to remove the ethanol, a 5-fold diluted product was prepared. Korean red ginseng was obtained by filtration with centrifugation (Continuous Centrifuge, Kansai, Japan), followed by vacuum evaporation (60–64 cm Hg, 67 brix) at 60°C. Fermented red ginseng was prepared as previously described³³. Briefly, KRG (10 g) was suspended in water (600 mL) and then fermented in yeast extract broth for 3 d at 26°C. The yeast extract broth was incubated with *Saccharomyces cerevisiae* (IFO 0309) and *Kluyveromyces lactis* (ATCC 8585) for 5 d at 26°C. The cultured KRG was then further fermented by *Lactobacillus plantarum* (299V Probi AB, Sweden), *Lactobacillus casei* (Shirota, Yakult, Japan), and *Bifidobacterium longum* (BB536, Morinaga Milk Industry Co., Ltd. Japan) for 5 d at 36°C. The endotoxin level in FRG (10 mg/mL) was determined using a QCL-1000 chromogenic limulus amebocyte lysate end-point assay (Lonza, Walkersville, MD, USA), as previously described³⁴. It was less than 0.1 EU/mL.

HPLC analysis

High-performance liquid chromatography analysis was performed using an SIL-20A equipped with an SPD-M20A PDA and CTO-20A column oven (SHIMADZU Corp., Kyoto, Japan). Fractions were separated at 40°C on an ACE-121-2546 column (Advanced Chromatography Technologies Corp., Aberdeen, Scotland) using one-step linear gradient elution. The eluent consisted of 2% acetic acid in water (A) and 0.5% acetic acid in acetonitrile (B). The gradient program was as follows: 0–70 min, linear change from 0% to 55% A in B; 70–80 min, linear change from 55% to 0% A in B. Flow rate, ultraviolet (UV) absorption wavelength, and injection volume were set at 0.8 mL/min, 280 nm, and 10 μL , respectively.

Splenocyte preparation and culture

Under anesthesia with diethyl ether, spleens were collected from the mice, and splenocytes were processed

as previously described³⁵. Briefly, the spleen was placed in ice-cold phosphate-buffered saline (PBS) and passed through a 100- μm nylon mesh (BD Biosciences, CA, USA) to obtain a single-cell suspension. After centrifugation ($300 \times g$, 15 min), the pellets were resuspended in PBS. Red blood cells were lysed by a brief hypotonic shock using ice-cold water for 15 s, and the remaining cells were washed with PBS by centrifugation ($300 \times g$, 15 min). Splenocytes (3×10^7 cells/mL) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, NY, USA) supplemented with 10% fetal bovine serum (HyClone, UT, USA), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin, and incubated at 37°C in a humidified incubator with 5% CO_2 .

Measurement of IL-4 and IL-10 mRNA expression levels by RT-PCR

The mRNA expression levels of IL-4 and IL-10 were determined using reverse transcription polymerase chain reaction (RT-PCR). Splenocytes (3×10^7 cells/mL) were treated with DNCB (1 $\mu\text{g/mL}$) in the presence or absence of FRG (50 $\mu\text{g/mL}$) for 30 min. Then, total RNA was extracted using TRIzol2[®] reagent (Invitrogen) and processed to cDNA using RevertAid[™] (Fermentas, Vilnius, Lithuania). The Solgent[™] EF-Taq DNA polymerase kit was used for PCR, and cDNA was amplified using primers specific for IL-4 (forward, 5'-ACAGGAGAA GGGACGCCA T-3'; reverse, 5'-GAAGCCCTACAGA CGAGCTCA-3'), IL-10 (forward, 5'-GGTTGCCAAG CCTTATCGGA-3'; reverse, 5'-ACCTGCTCCACTGC CTTGCT-3'), and GAPDH (forward, 5'-TTCACCAC CATGGAGAAGGC-3'; reverse, 5'-GGCATGGACTG TGGTCATGA-3')³⁶.

Preparation of whole cell lysates and nuclear extracts

Whole cell lysates and nuclear extracts were obtained from DNCB-treated (1 $\mu\text{g/mL}$) splenocytes in the presence or absence of FRG (50 $\mu\text{g/mL}$) for 30 min. To obtain whole cell lysates, the cells were collected and lysed in a lysis buffer containing 20 mM Tris, 150 mM NaCl, and 1% Triton X-100, pH 7.6, with a protease inhibitor cocktail. Nuclear extracts were obtained after cellular fractionation. Briefly, cells were suspended in 0.2 mL of buffer A (10 mM HEPES at pH 7.5, 1.5 mM MgCl_2 , 10 mM KCl, 1 mM DTT, 0.1% NP-40, and 0.2 mM PMSF) and then lysed on ice for 15 min. After centrifugation ($5,000 \times g$, 5 min, 4°C), the obtained nucleic pellets were washed with buffer A without NP-40, and resuspended in 0.025 mL of buffer C (20 mM HEPES at pH 7.5, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl_2 , 1 mM DTT, and 0.2 mM PMSF) for 30 min on ice. After centrifugation at $13,000 \times g$ for 10 min

at 4°C, the supernatant was collected as the nuclear extract.

Induction of atopic-like dermatitis and treatment of mice

1-Chloro-2,4-dinitrobenzene was used to induce atopic-like dermatitis, as previously described²⁷. Balb/c mice were shaved with an electric clipper and depilatory cream. Briefly, 1% DNCB was freshly dissolved in acetone : olive oil (4 : 1) and applied topically on the shaved area of the dorsal surface, twice for one week. Then, 0.2% DNCB was applied in the same way (four times in total), as previously described²⁷. After induction of atopic-like dermatitis, the lesions were topically treated via skin smear with PBS (vehicle), dexamethasone (positive control), or FRG (10 and 50 µg/mL) every other day (four times in total).

Measurement of scratching behavior frequency

Scratching behavior, defined as movement with the hind limb on the side of the body, was observed after all treatments. Briefly, mice were placed in cages, and the number of scratching events was counted for 10 min. This was repeated five times (50 min in total) for different mice.

Measurement of serum IgE levels

At the end of the experiment, blood was collected from the abdominal *vena cava* under anesthesia with diethyl ether. Blood was centrifuged at $3,000 \times g$ for 10 min at 4°C to obtain the serum. Serum IgE concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit purchased from BioLegend (San Diego, CA, USA).

Measurement of splenocyte IL-4 levels

At the end of the experiment, the spleen was aseptically removed, and the splenocytes were processed. The splenocytes (5×10^5 cells/well) were placed in 96-well plates and incubated for 24 h. The supernatants were then collected, and IL-4 secretion from the splenocytes was assayed using an ELISA kit (BioLegend, San Diego, CA, USA).

Immunoblot analysis

The protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad). The samples were diluted with $1 \times$ lysis buffer containing 1% β -mercaptoethanol. Equal amounts of cellular protein (50 µg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred

to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skimmed milk, the membranes were incubated with the target primary antibodies and then with horseradish peroxidase-conjugated secondary anti-IgG antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence (ECL) western blot detection system. Protein expression was compared to that of a loading control, such as lamin A or the respective non-phosphorylated protein.

Statistical analysis

Data are expressed as mean $\pm$ SEM (standard error of the mean; in *in vitro* study) or mean $\pm$ SD (standard deviation; in animal study). Significance was determined by one-way analysis of variance (ANOVA) using Sigma-Plot (Systat Software, Inc., San Jose, CA, USA), where $P < 0.05$ was considered significant ($^*P < 0.05$, compared to the vehicle control, and $^{\#}P < 0.05$, compared to the DNCB-treated group).

Results

FRG exhibits higher R_d and R_{g3} contents

In this study, fermentation was performed in two steps. Korean red ginseng extract was fermented using cellulose-degrading *S. cerevisiae* and polysaccharide-degrading *K. lactis*. It was then further fermented to increase the production of monosaccharides from disaccharides using *L. plantarum*, *L. casei*, and *B. longum*. All the used microorganisms are edible bacteria and are widely used as probiotics. To verify the effect of KRG fermentation, the contents of R_{b1}, R_d, and R_{g3} in FRG were compared to those in unfermented KRG using HPLC. As shown in Figure 1, the relative content of R_{b1} in FRG decreased (1.1 vs. 4.5 mg/mL); however, both R_d (13.6 vs. 6.7 mg/mL) and R_{g3} (4.4 vs. 1.5 mg/mL) contents increased compared to unfermented KRG. This finding indicates that fermentation successfully increased the ginsenoside content.

FRG suppresses IL-4 and IL-10 mRNA expression in DNCB-exposed splenocytes

It has been suggested that IL-4 stimulates Th₂ cell production and plays a key role in AD progression in the early stages². Splenocytes were treated with DNCB (1 µg/mL) in the presence or absence of FRG (10 and 50 µg/mL) for 30 min. The effects of FRG on the mRNA expression levels of IL-4 and IL-10 were then determined by RT-PCR. As shown in Figure 2, the levels of IL-4 and IL-10 mRNA decreased significantly in the presence of FRG, at both 10 and 50 µg/mL ($n = 5$, $P < 0.05$).

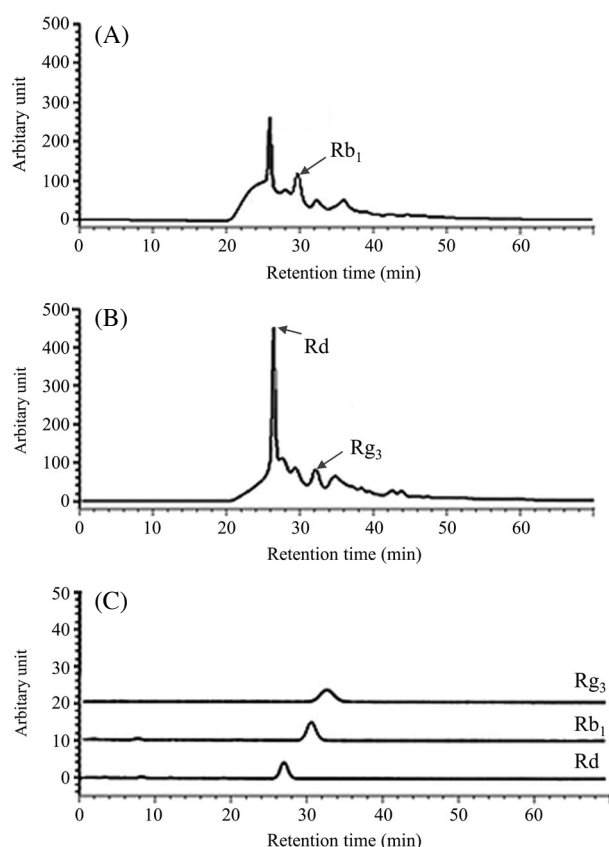


Figure 1. The contents of Rb_1 , R_d , and Rg_3 in KRG and FRG. HPLC analysis was performed to determine Rb_1 , R_d , and Rg_3 contents in KRG (A) and FRG (B), as described in the Materials and methods. Standard ginsenosides, including Rb_1 , R_d , and Rg_3 , were used as specific standards (C). KRG, Korean red ginseng extract; FRG, the fermented form of KRG.

In addition, FRG significantly suppressed the levels of IL-10 mRNA in the DNCB-exposed splenocytes. In splenocytes, Dex was more potent at suppressing IL-4 expression, but less potent at suppressing IL-10 expression, compared to FRG (Figure 2B).

FRG suppresses NF- κ B nuclear translocation in DNCB-treated mouse splenocytes

Nuclear factor kappa B has been shown to play an important role in allergic responses and IL-4 production³⁷. The transcriptional activity of NF- κ B can be indirectly assayed by measuring the nuclear translocation of its p65 subunit. As shown in Figure 3, DNCB exposure significantly increased the nuclear translocation of NF- κ B (p65); however, this effect was significantly suppressed by FRG treatment at 50 μ g/mL ($n = 5$, $P < 0.05$). Fermented red ginseng alone did not alter the level of p65 subunit in the nucleus. This finding suggested that the downregulation of IL-4 expression might be attribut-

able, at least in part, to the suppression of the NF- κ B signaling pathway by FRG.

Effects of FRG on MAPK activation in DNCB-treated mouse splenocytes

Activation of MAPKs is involved, either directly or indirectly, in NF- κ B transcriptional activity^{38,39}. Treatment of splenocytes with DNCB significantly activated ERK, JNK and p38 MAPK, as evidenced by the increase in the relative ratio of their phosphorylated forms (Figure 4). Although FRG treatment alone resulted in activation of p38 MAPK, DNCB-mediated activation of p38 MAPK was significantly suppressed by FRG. These suppressive effects of FRG against DNCB-mediated p38 MAPK activation might be, at least in part, associated with immune suppression and reduction of NF- κ B activity.

Topical FRG treatment suppresses DNCB-induced atopic-like dermatitis lesions

All the animals survived until the end of the study. A comprehensive clinical examination of the back of the mice was undertaken. Topical application of DNCB induced severe atopic-like dermatitis lesions in Balb/c mice, as evidenced by the development of erythema and eczema with keratosis compared to the untreated group (Figure 5). Both Dex and FRG (10 and 50 μ g/mL) visibly alleviated the skin lesions.

Effects of FRG on the frequency of scratching behavior

Itch-induced scratching is associated with accumulated protein allergens in the epidermis of AD patients. In this study, scratching behavior was used as a typical clinical symptom of AD. In accordance with the atopic-like dermatitis lesions shown in Figure 5, the scratching frequency increased significantly in DNCB-treated mice ($n = 5$, $P < 0.05$). Dex treatment almost completely suppressed scratching (Figure 6), whereas FRG (10 and 50 μ g/mL) significantly suppressed the scratching frequency ($n = 5$, $P < 0.05$). These results imply that topical application of FRG was beneficial in reducing dermatitis lesions and AD-like symptoms.

FRG suppresses serum IgE levels in DNCB-treated mice

Next, we investigated whether FRG treatment could reduce the serum levels of IgE, an obvious indicator of AD that is well correlated with its severity. Insult with DNCB on the back of Balb/c mice induced a significant increase in serum IgE levels compared to the vehicle-only control ($n = 5$, $P < 0.05$). Fermented red ginseng at

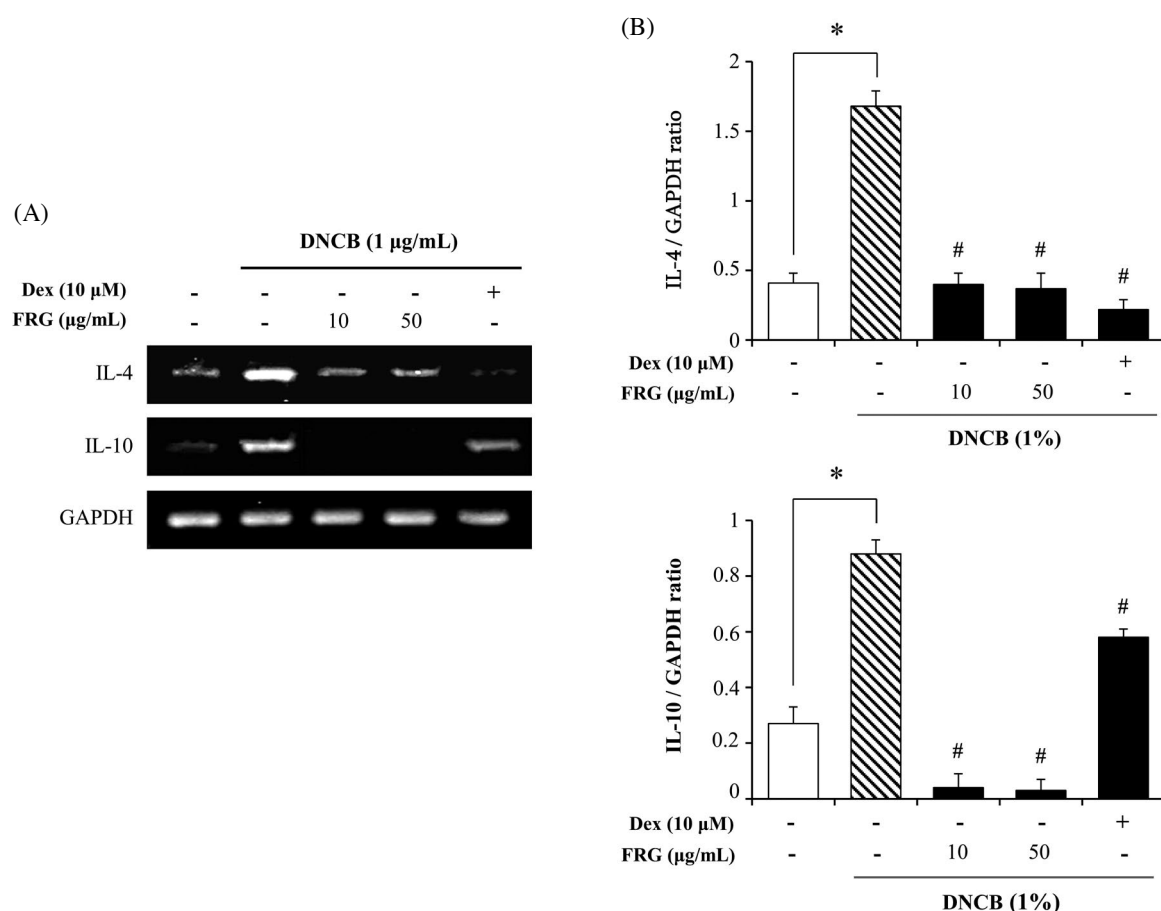


Figure 2. Suppressive effects of FRG against DNCB-induced mRNA expression of IL-4 and IL-10 *in vitro*. Splenocytes (3×10^7 cells/mL) were treated with DNCB (1 $\mu\text{g/mL}$) in the presence or absence of FRG (10 and 50 $\mu\text{g/mL}$) for 24 h. The mRNA levels of IL-4 and IL-10 were measured by RT-PCR. Data are expressed as the means $\pm$ SEM ($n=6$). (A) A representative image of RT-PCR, and (B) densitometric analysis for IL-4 and IL-10 expression. GAPDH mRNA expression was used as an internal control. Dex, dexamethasone; DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract; IL, interleukin. * $P < 0.05$ vs. the control group, # $P < 0.05$ vs. the DNCB-treated disease group.

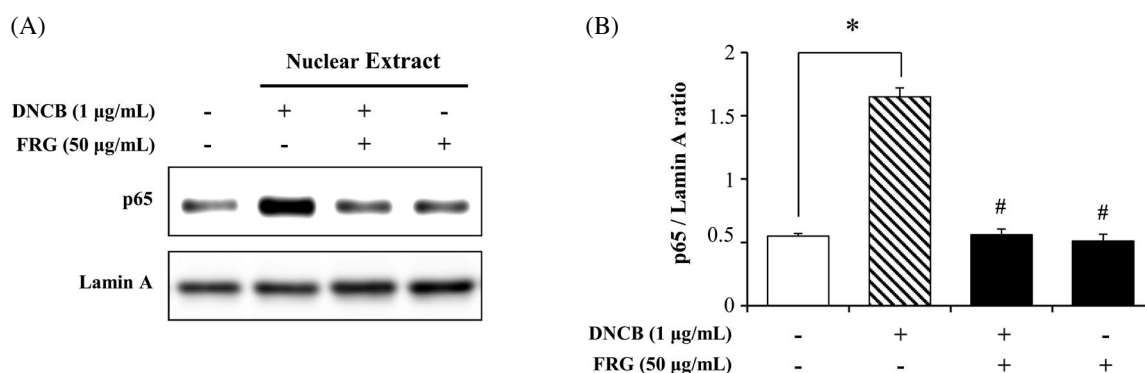


Figure 3. Suppressive effects of FRG against DNCB-induced nuclear translocation of the p65 subunit of NF- κB . Splenocytes were treated with DNCB (1 $\mu\text{g/mL}$) in the presence or absence of FRG (50 $\mu\text{g/mL}$) for 30 min. After cellular fractionation, the nuclear translocation of the NF- κB p65 subunit was assessed by immunoblot assay. Data are expressed as the means $\pm$ SEM ($n=6$). (A) A representative image from the immunoblot analysis, and (B) densitometric analysis. Lamin A was used as a loading control for nuclear protein fractions. DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract. * $P < 0.05$ vs. the control group, # $P < 0.05$ vs. the DNCB-treated disease group.

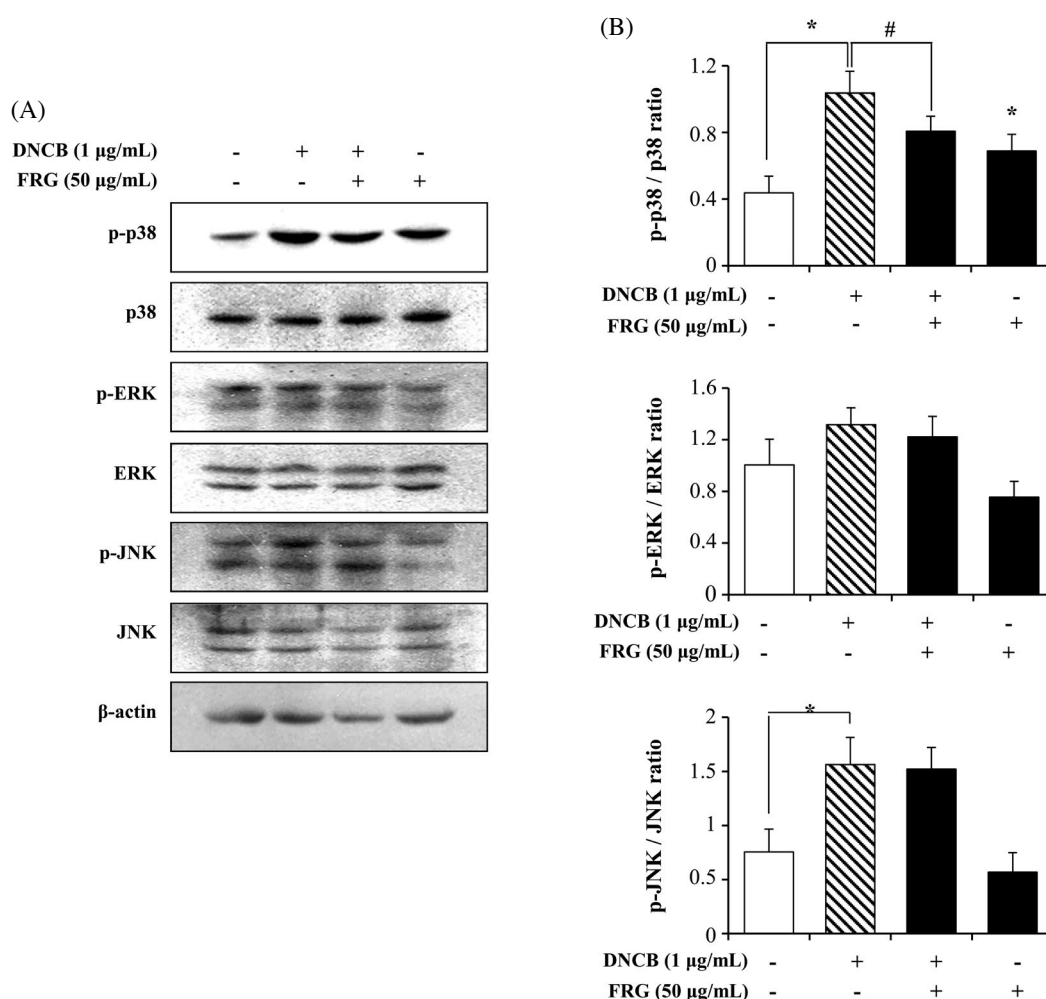


Figure 4. Suppressive effects of FRG against DNCB-induced activation of MAPKs. Splenocytes were treated with FRG (50 $\mu\text{g/mL}$) in the presence or absence of DNCB (1 $\mu\text{g/mL}$) for 30 min. Then, whole cell lysates were subjected to immunoblot analysis. Data are expressed as the means $\pm$ SEM ($n=6$). (A) A representative image from the immunoblot analysis, and (B) densitometric analysis. The phosphorylation of ERK 1/2, JNK, and p38 MAPK is expressed as the relative ratio of their total level. DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract; MAPK, mitogen-activated protein kinase. * $P<0.05$ vs. the control group, # $P<0.05$ vs. the DNCB-treated disease group.

10 $\mu\text{g/mL}$ did not significantly reduce IgE levels (Figure 7). However, FRG at 50 $\mu\text{g/mL}$ significantly suppressed the serum levels of IgE in DNCB-treated mice to a level comparable to that of Dex (10 μM).

FRG suppresses IL-4 secretion from DNCB-treated mouse splenocytes

Interleukin 4 is a key Th₂ cytokine that stimulates immune responses in allergic reactions, and serves as a therapeutic target for AD¹⁴. As shown in Figure 8, IL-4 secretion from DNCB-exposed splenocytes was significantly suppressed by FRG treatment at both 10 and 50 $\mu\text{g/mL}$ ($n=5$, $P<0.05$). This finding indicates that the observed FRG-induced reduction in IL-4 secretion may

be attributable to its inhibitory effects on the expression of IL-4 (Figure 2).

Discussion

In the present study, FRG treatment significantly suppressed DNCB-induced activation of NF- κ B and p38 MAPK in splenocytes. These suppressive effects of FRG might be associated with the reduced levels of IgE, IL-4, and IL-10, that in turn resulted in the attenuation of atopic-like skin lesions and scratching behavior frequency in Balb/c mice.

Korean red ginseng is a heat-processed product derived from the root of Korean white ginseng. Korean

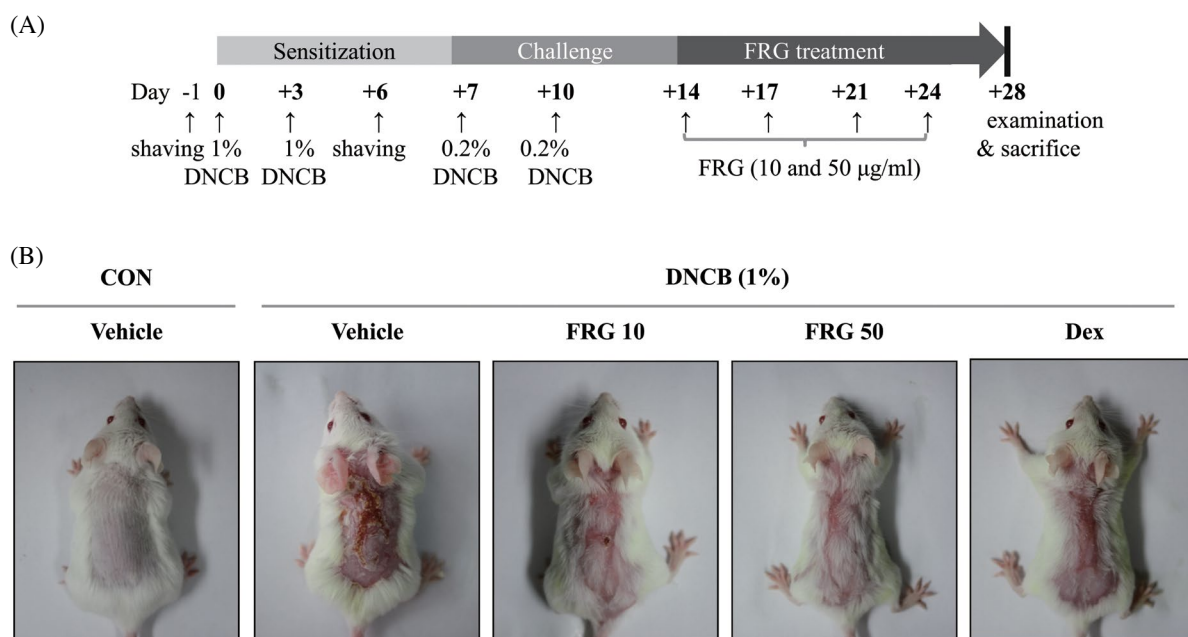


Figure 5. Effects of FRG on DNCB-induced atopic-like dermatitis in mice. Balb/c mice were challenged with DNCB to induce atopic-like dermatitis lesions. Then, Dex and FRG were applied topically for 2 weeks. (A) Animal study design, and (B) a representative image ($n = 5$). Dex, dexamethasone; DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract.

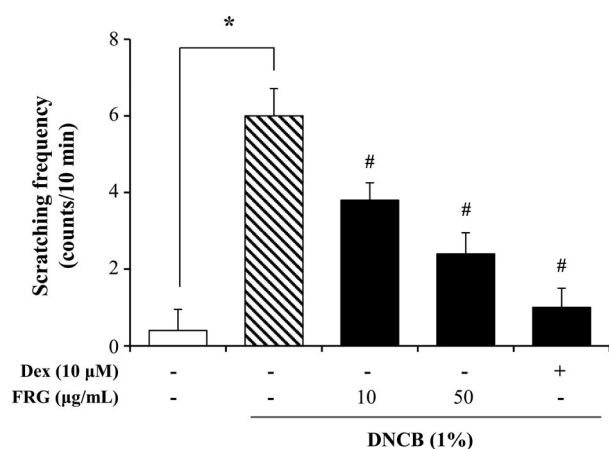


Figure 6. Inhibitory effects of FRG against DNCB-induced scratching behavior in mice. The scratching behavior of each mouse was counted five times, 10 min each time (50 min in total). Data are expressed as the means $\pm$ SEM ($n = 5$). Dex, dexamethasone; DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract. * $P < 0.05$ vs. the control group, # $P < 0.05$ vs. the DNCB-treated disease group.

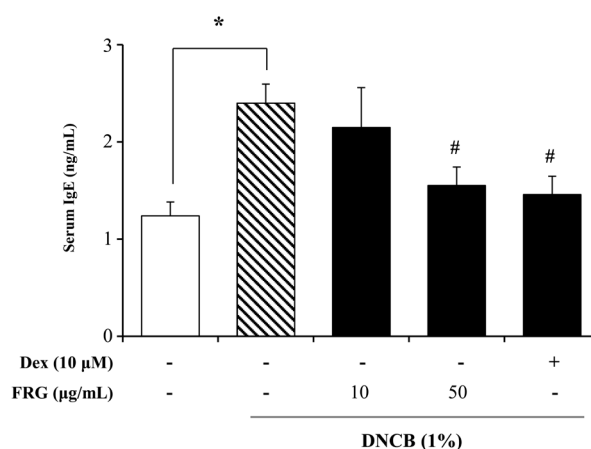


Figure 7. Effects of FRG on the serum level of IgE in mice. Serum IgE levels were measured by ELISA. Data are expressed as the means $\pm$ SEM ($n = 5$). Dex, dexamethasone; DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract. * $P < 0.05$ vs. the control group, # $P < 0.05$ vs. the DNCB-treated disease group.

red ginseng is enriched with ginsenosides like Rh₂, RS₄, and Rg₃ compared to the unprocessed counterpart⁴⁰. Besides exhibiting improved ginsenoside bioavailability and physiological activities, FRG has been shown to be a practically non-irritating material, based on its

primary irritation index, making it superior to KRG⁴¹. Therefore, FRG is considered a relatively safe and effective aid for use in topical preparations. Although the biological effects of ginsenosides have not been fully elucidated, Rg₃ and Rh₂ were shown to be effective in suppressing histamine release from mast cells *in vitro*^{24,42}.

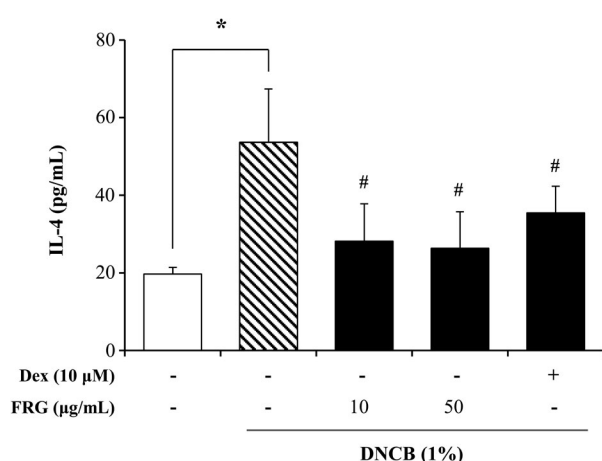


Figure 8. Inhibitory effects of FRG against IL-4 production in DNCB-treated mouse splenocytes. Spleens were removed, and the splenocytes were isolated. Splenocytes (5×10^5 cells/well) were cultured for 24 h, and IL-4 secretion in the culture medium was assayed using ELISA. Data are expressed as the means $\pm$ SEM ($n = 5$). Dex, dexamethasone; DNCB, 1-chloro-2,4-dinitrobenzene; FRG, fermented Korean red ginseng extract; IL, interleukin. * $P < 0.05$ vs. the control group, # $P < 0.05$ vs. the DNCB-treated disease group.

In a previous study, we found that unfermented KRG was effective at reducing AD-like skin lesions²⁷. To enhance its efficacy and reduce adverse effects, FRG was prepared by fermentation using edible bacteria like *S. cerevisiae*, *K. lactis*, *L. plantarum*, *L. casei*, and *B. longum*, resulting in an increase in the contents of Rg₃ and R_d (Figure 1). We subsequently examined the suppressive effects of FRG on AD-like skin lesions based on the previous experimental setting²⁷. Mitogen-activated protein kinases are involved in the transduction of external signals from the membrane to the nucleus⁴³. In DNCB-insulted splenocytes, there was a significant increase in JNK and p38 MAPK phosphorylation; ERK phosphorylation levels also increased, although not significantly (Figure 4). Fermented red ginseng significantly suppressed the DNCB-induced phosphorylation of p38 MAPK, which plays a central role in the pathogenesis of dermal inflammatory responses⁴⁴, but not the phosphorylation of JNK and ERK. This was in contrast to that previously observed for KRG, which significantly suppressed the phosphorylation of JNK, ERK, and p38 MAPK in DNCB-insulted splenocytes²⁷. It is not clear why FRG, unlike KRG, did not inhibit the phosphorylation of all three MAPKs in similar models of DNCB insult. However, we speculate that changes in the active components of KRG, in addition to fermentation, may have contributed to the differences in inhibitory potential towards MAPKs in DNCB-stimulated splenocytes.

Production of cytokines such as IL-4 and IL-10 is regulated either by the NF- κ B or p38 MAPK signaling pathway. In this study, we found that FRG was more potent than KRG at suppressing IL-4 and IL-10 mRNA expression (Figure 3), where the inhibitory effects of FRG were evident at 10 μ g/mL, whereas KRG was effective only at 100 μ g/mL²⁷. In addition, both the increase in serum IgE levels (Figure 7) and secretion of IL-4 from splenocytes (Figure 8) were almost completely inhibited by FRG at 50 μ g/mL, in contrast to KRG where no significant differences were observed, even at the concentration of 100 μ g/mL. In addition, FRG administration at 50 μ g/mL resulted in a reduction of scratching behavior frequency of approximately 50%, whereas with KRG this effect could only be achieved at a higher concentration (100 μ g/mL). Interestingly, FRG treatment alone activated p38 MAPK in splenocytes (Figure 4B), but FRG did not evoke any cumulative effect in the presence of DNCB. In contrast, FRG suppressed the DNCB-induced activation of p38 MAPK, suggesting that FRG-induced p38 MAPK activation at the baseline might mediate the beneficial effects of p38 MAPK in the skin and/or in immune signaling pathways since there were no significant changes either in IL-4 and IL-10 production or NF- κ B activation in the same experimental setting (Figures 2 and 3). The conversion of KRG components into more biologically active components *via* fermentation might vary depending on the microorganisms used and on the fermentation process. The fermentation process used in this study may not be optimal for the preparation of FRG-based anti-AD formulations, and an improved and standardized fermentation process for producing FRG with high efficacy and reduced safety concerns should be developed in the future.

Similar to Dex (10 μ M), FRG at 50 μ g/mL was highly effective in reducing the AD-like skin lesions and scratching behavior in Balb/c mice by suppressing the production of IL-4 and IL-10 that are involved in allergic skin reactions. Despite the potent anti-inflammatory activity of glucocorticoids (GCs), there is consensus that even topical GCs may induce local and systemic adverse effects, depending on their potency and treatment duration⁴⁵. The anti-inflammatory actions of GCs are largely attributed to GC receptor (GR)-mediated repression of inflammatory gene expression¹⁶. Hu *et al.* (2017) showed that ginsenosides were less effective and showed weaker binding to the GR than Dex. Therefore, FRG likely acts *via* a GR-independent mechanism to alleviate atopic-like dermatitis. Additionally, the reduced activation of GR by FRG suggests that topical FRG might be safer, and have fewer adverse effects, than GCs. Of note, FRG was administered topically, and it will be important to determine the concentration of FRG

that penetrated the skin barrier. However, this aspect was beyond the scope of the current study, and should be considered in future investigations.

Conclusion

Fermented red ginseng exhibited significant anti-AD activity via the suppression of IL-4 and IL-10 production, p38 MAPK activation, and NF- κ B nuclear translocation. The beneficial effects of FRG in the treatment of atopic-like dermatitis may potentially result in the development of a new therapy for AD, or at least in reduced doses of GCs if administered in combination.

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Conflict of Interest Se Chan Kang, Sung Ryul Lee, Mediana Hadiwidjaja, Manorma Negi, Hyun Jung Koo, Seon-A Jang, Jeong-Eun Kwon, Hyejung Hong, Jung Dae Lim, Seung Namkoong, Kyungho Kim & Eun-Hwa Sohn declare that they have no conflict of interest.

Human and animal rights All experimental procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and National Animal Welfare Law in Korea. The experimental animal facility and protocols were approved by the Institutional Animal Care and Use Committee of Radiation Health Research Institute (Seoul, Korea).

Author's contributions Se Chan Kang, Sung Ryul Lee and Eun-Hwa Sohn designed the research study. Mediana Hadiwidjaja, Manorma Negi, Seon-A Jang, Hyun Jung Koo, Jeong-Eun Kwon, Kyungho Kim, and Hyejung Hong performed the experiments and analyzed data. Jung Dae Lim, Seung Namkoong, Se Chan Kang, Sung Ryul Lee and Eun-Hwa Sohn interpreted data and wrote manuscript.

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