Immunotherapeutic effects of recombinant colorectal cancer antigen produced in tomato fruits

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The production of pharmacological vaccines in plants has been an important goal in the field of plant biotechnology. GA733-2, the protein that is also known as colorectal carcinoma (CRC)-associated antigen, is a strong candidate to produce a colorectal cancer vaccine. Tomato is one of the major targets for production of an edible vaccine, as tomato is a fruit consumed in fresh form. It also contains high content of vitamins that aid activation of immune response. In order to develop an edible colorectal cancer vaccine, the transgene rGA733-Fc that encodes a fusion protein of GA733-2, the fragment crystallizable (Fc) domain, and the ER retention motif (rGA733-Fc) was introduced into tomato plants (Solanum lycopersicum cv. Micro-Tom). The transgenic plants producing rGA733-Fc (rGA733-FcOX) protein were screened based on stable integration of transgene expression cassette and expression level of rGA733-Fc protein. Further glycosylation pattern analysis revealed that plant derived rGA733-Fc protein contains an oligomannose glycan structure, which is a typical glycosylation pattern found on ER-processing proteins. The red fruits of rGA733-FcOX transgenic tomato plants containing approximately 270 ng/g FW of rGA733-Fc protein were orally administered to C57BL/6 mice. Oral administration of tomato fruits of the rGA733-Fc expressing transgenic plants delayed colorectal cancer growth and stimulated immune responses compared to oral administration of tomato fruits of the h-Fc expressing transgenic plants in the C57BL/6J mice. This is the first study showing the possibility of producing an edible colorectal cancer vaccine using tomato plants. This research would be helpful for development of plant-derived cancer edible vaccines.

The recent vigorous isolation and characterization of tumor antigens has opened up the possibility for development of cancer vaccines. In the case of metastatic cancer, the therapeutic effect of immunotherapy with cancer vaccines is expected to be more effective than radiation treatment and chemotherapy1–6. The immune responses mediated by T cells and B cells lead to the removal of cancer cells, without affecting the surrounding normal cells in humans and animals5–7. In addition, the consistent maintenance of the immune response decreases the rate of cancer recurrence8. These findings provide strong evidences that cancer can be successfully treated with a recombinant antigen vaccine either alone or in conjunction with hormone therapy, chemotherapy or monoclonal antibody therapy9–15.

As a tool for pharmacological recombinant protein production, plants have several advantages compared with other production system, such as low production cost, appropriate post-translation modification, and lack of human pathogen contamination16–23. Another outstanding advantage of plant system for vaccine development is that plants producing pharmacological recombinant proteins can be used as edible vaccines17. Among the plants, tobacco system has been extensively used for production of vaccine candidates in plants. Despite of several benefits of tobacco system, such as easy transformation, robust expression of recombinant protein18,23, tobacco system is not the best target for development of edible vaccine. Thus, production of vaccine candidates in the edible plants will be an alternative for development of edible vaccines. Tomato is the most producing fruits

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Results
Stable expression of rGA733-Fc and human Fc in transgenic tomato plants. To generate transgenic tomato plants that stably express rGA733-Fc, a plant expression vector that expresses rGA733-Fc fused with ER-targeting signals and ER retention motif under the control of the 35S promoter (pBINPLUS-GA733-Fc) were transformed into Solanum lycopersicum cv. Micro-Toms plants using Agrobacterium-mediated genetic transformation (Fig. 1a). In addition, the pBINPLUS-Fc vector that expresses human-Fc (h-Fc) protein driven by 35S promoter was also transformed into Micro-Toms plants (Fig. 1a). Integration of genetic cassette into tomato plants were validated by PCR analysis with specific primers for the expression cassettes. Through the process, we finally obtained total 16 transgenic plants containing rGA733-Fc expression cassette (rGA733-FcOX) and 13 transgenic plants containing h-Fc expression cassette (h-FcOX) (Fig. 1b and Fig. S1). Expression level of rGA733-Fc protein in the leaves of selected transgenic plants was determined by ELISA and western blot analysis (Fig. 1c,d, and Fig. S2). Among 16 rGA733-FcOX transgenic plants, rGA733-FcOX #16 showed the highest expression of rGA733-Fc (43.21 μg of rGA733-Fc/g fresh weight) (Fig. 1c and Table 1). In case of the h-FcOX transgenic plants, h-FcOX #1 produced 5.23 μg of h-Fc protein per gram of fresh weight (Fig. 1c and Table 1). Four independent transgenic plants that express corresponding recombinant proteins were chosen to monitor rGA733-Fc protein level in tomato fruits. Among the rGA733-FcOX transgenic plants, rGA733-FcOX #16 showed the highest expression of rGA733-Fc in both pink and red tomato fruits (Fig. 2a and Table 1). Similarly, h-FcOX #1 produced the highest level of h-Fc protein in both leaves and pink tomato fruits. However, h-FcOX #12 that produced relatively low h-Fc protein in leaves showed the highest expression level of h-Fc protein in red tomato fruits (Fig. 2b and Table 1).

N-glycosylation pattern analysis of purified rGA733-Fc protein from transgenic tomato plants. Purification of plant-derived protein is important for its application as vaccine candidate. Since rGA733-2 protein was fused with h-Fc domain, we used protein A column to purify rGA733-Fc proteins produced from transgenic tomato plants. Coomassie brilliant staining of the eluted fractions showed that most of the purified proteins were detected in 5th elution fraction (Fig. 3a and Fig. S3). The purified rGA733-Fc protein was confirmed by western blot analysis using a rGA733 specific antibody (Fig. 3b and Fig. S3). Final concentration of the purified rGA733-Fc was 8.69 μg/g from rGA733-FcOX #16 transgenic tomato plants. We next determine glycan structure of plant-derived rGA733-Fc protein through MALDI-TOF MS analysis. The observed masses (1989 and 2914 Da) were corresponded to Hex7HexNAc2 and Hex8HexNAc2, which were identified as oligomannose glycan structure. Thus, rGA733-Fc proteins obtained from rGA733-FcOX transgenic tomato plants contained oligomannose glycan structure, which are typically found from ER-associated proteins (Fig. 3c).

Inhibitory effect of the plant-derived rGA733-Fc protein for colorectal cancer growth in tumor-bearing mice model. To test effect of rGA733-FcOX transgenic plants for colorectal cancer development, ground red fruits of rGA733-FcOX transgenic plants were orally administered to C57BL/6 mice every other day for two weeks. Fruits of transgenic plants expressing h-Fc protein were used as a negative control. After two weeks of the treatments, MC38 colon cancer cells were injected subcutaneously (s.c.) in the right flank of pre-administrated mice. Colorectal tumor volumes were measured to determine whether the plant-derived rGA733-Fc inhibits colorectal cancer growth. The C57BL/6 mice pre-administered with fruits of rGA733-FcOX transgenic plants showed delayed tumor growth compared to the mice pre-treated with fruits of the h-FcOX transgenic plants (Fig. 4a).

To evaluate whether the plant-derived rGA733-Fc protein activates the immune responses in tumor-bearing mice model, a production of IgA and IgG was analyzed from the fecal extracts and serum samples, respectively.
Pre-administration of rGA733-FcOX fruits significantly increased cellular IgA production compared to pre-administration of h-FcOX fruits (Fig. 4b). On the other hand, cellular IgG level was not significantly changed by rGA733-FcOX fruit treatments (Fig. 4b).

To understand the anti-cancer effect of the rGA733-Fc transgenic tomato fruits, we next examined expression level of the genes related to the cell cycle and apoptosis in the tumors of tumor-bearing mice (Fig. 4c). Expression of tumor suppressor gene p53 and p21 was increased in the tumor tissue of the mice pre-administered with plant-derived rGA733-Fc. Among two pro-apoptotic genes, expression of bax was significantly induced by oral administration of the plant-derived rGA733-Fc, while no significant change was detected on the expression of bak gene by the treatment. In addition, expression of chemokine mcp1 was significantly up-regulated by rGA733-Fc treatment. These data suggest that the plant-derived rGA733-Fc inhibited the growth of colorectal carcinoma potentially through activation of tumor suppressor and pro-apoptotic molecules (Fig. 4c).

Table 1. Quantification of the rGA733-Fc and h-Fc protein in transgenic tomato plants. rGA733-FcOX, rGA733-Fc overexpressing plants; h-FcOX, human-Fc overexpressing transgenic plants.
Discussion

Here, we proposed the potential application of the tomato expressing rGA733-Fc protein as an oral vaccine for prevention of colorectal carcinoma. To successfully produce GA733-2 colorectal antigen in tomato, we introduced pBINPLUS-GA733-Fc plant expression vector that expresses GA733-2 fused with a Fc domain and ER retention motifs (rGA733-Fc) under the control of 35S promoter into tomato plants. Four selected transgenic tomato plants accumulated rGA733-2 protein (Fig. 1 and Table 1). Among them, rGA733-FcOX #16 transgenic tomato plants accumulated significantly higher rGA733-Fc protein in leaves, and the rGA733-Fc protein level in leaves was even higher than that in transgenic tobacco plants transformed with same vector. Moreover, the rGA733-FcOX #16 transgenic tomato plants accumulated fivefold more rGA733-Fc protein in fruits. The level of rGA733-Fc in fruits was maintained at a similar level at the end of maturity (Fig. 2 and Table 1). This finding suggests that a more powerful edible vaccine can be developed to treat colorectal carcinoma using tomato. The rGA733-Fc protein level was significantly high in leaves compared to fruits in the rGA733-FcOX transgenic plants (Table 1). Even though the 35S promoter has been regarded as a constitutive promoter, its activity decreased during fruit ripening in tomato plants. The use of a promoter showing higher activity during fruit ripening will be helpful to further improve rGA733-Fc protein production in tomato fruits.

Figure 2. The rGA733-Fc and h-Fc protein expression levels in the transgenic tomato fruits. ELISA analysis was performed to quantify the protein expression level in the pink and red stage of transgenic fruits. (a) rGA733-Fc protein levels in fruits of rGA733-FcOX transgenic plants. (b) h-Fc protein levels in fruits of h-FcOX transgenic plants. Data represent mean ± standard deviation (N = 3).

Figure 3. The rGA733-Fc protein purification and glycosylation pattern analysis. rGA733-Fc protein was purified from the transgenic tomato plants using a protein A column. (a) The eluted fractions were separated by SDS-PAGE and visualized by Coomassie brilliant blue staining. M, protein size marker; T, total soluble protein; E, purified rGA733-Fc protein. (b) rGA733-Fc protein in 5th eluted fraction was detected with the rGA733-2 antibody by western blot analysis. 5th E, 5th eluted fraction. (c) Glycosylation patterns of purified rGA733-Fc protein were analyzed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Blue square, N-acetylglucosamine (GlcNAc); green circle, mannose.
For successful development of plant-derived vaccine, it is crucial to reduce the potential for safety issue. Plant specific glycosylation pattern, such as β-1,2-xylose and α-1,3-fucose, often act as allergen for mammalian system. The N-glycosylation patterns are known to be incorporated into plant protein in Golgi. Directing recombinant protein into ER might be effective to avoid unnecessary complex glycosylation on recombinant protein. Moreover, oligomannose glycan patterns processed in ER improve antigen presentation and enhance the immune response through perception by mannose receptor on macrophages and dendritic cells. Ishii and Kojima reported that oligomannose-coated liposomes showed enhanced immunization effects compared to uncoated liposomes. Thus, oligomannose glycan patterns detected from rGA733-Fc would be also helpful for effective delivery of orally treated rGA733-Fc to immune system. It has been reported that fusion with KDEL ER retention motif is sufficient to target rGA733-Fc to ER. Fc fragment fusion has several advantage for expression of recombinant protein, such as improvement of stability and yield of recombinant protein. In addition, Fc fragment fusion can be used for one step purification of recombinant protein with protein A column. SDS-PAGE analysis confirmed that rGA733-Fc protein was successfully purified with protein A column (Fig. 3a). In addition to rGA733-Fc protein, additional protein band was also detected from protein A column purified sample. It has been reported that Fc fusion with recombinant protein results in production of degradation products. ER targeting of recombinant protein fused with Fc fragment generally reduces production of degradation products.
rGA733-Fc protein greatly reduced the formation of degradation products in tobacco plants. Different with tobacco system, ER targeting of rGA733-Fc still produced significant amount of degradation products in tomato system (Fig. 3a). Additional process, such as size exclusion chromatography, during purification for excluding the degraded products will be helpful for large scale production of rGA733-Fc protein.

To confirm the anti-cancer effects of tomato fruits producing rGA733-Fc protein, colorectal cancer was induced in the rGA733-Fc-immunized C57BL/6J mice. Pre-administration of the rGA733-FcOX transgenic tomato fruits (0.1 g per mice) significantly delayed development of colon cancer in the colorectal cancer mouse model (Fig. 4a). Similar with oral administration of rGA733-Fc through transgenic tomato fruits, injection of purified GA733-Fc produced in either mammalian and plant system successfully induced immune responses in mice. The sera of mice immunized with plant-derived GA733-Fc showed similar immunogeniciy compare with the sera of mice immunized with mammalian-derived GA733-Fc. In addition, the sera of mice immunized either insect cell-derived or plant-derived GA733-Fc showed similar binding affinity to colorectal cancer cell. These results indicate that plant-derived GA733-Fc is as efficient as mammalian or insect cell-derived GA733-Fc for inducing immune response in mice.

The pre-administration of rGA733-FcOX transgenic tomato fruits also activated immune responses (Fig. 4b) and induced expression of cell cycle arrest and pro-apoptotic genes in the colorectal cancer-induced mice (Fig. 4c). Generally, IgA is mainly produced and secreted by B cells in mucosal area including intestine and respiratory tracts, and it is well known to play important roles in mucosal immune responses. On the other hand, IgG is involved in immune responses of blood and body tissue. In this study, we showed that the production of IgA was increased by the oral-administration of rGA733-FcOX fruits in the mucosal area, while no difference was observed in the IgG production of blood serum. Thus, these data indicate that the oral-administration of rGA733-FcOX fruits lead to enhance the immune responses in the mucosal area, not in the whole-body area. Recent studies report that not only IgG but also IgA have therapeutic potentials in antibody-based cancer immunotherapy using tumor antigen-targeting monoclonal antibodies. The antigen-targeting monoclonal antibodies enhance cancer cytotoxicity through inducing complement activation, antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis. Although, further studies are necessary to clarify the direct mechanisms of IgA antibody-mediated anti-cancer effect, we suggest that the oral-administration of rGA733-Fc increases the production of tumor antigen-targeting antibodies and may have a therapeutic potential for colon cancer patients. Collectively, we suggest that tomato fruits producing rGA733-Fc protein have a potential to be used as an edible vaccine to suppress the development of colorectal cancer. In addition, rGA733-FcOX transgenic tomato may function as a research tool that will help to further elucidate the anti-cancer mechanism of rGA733-Fc as an oral vaccine against various types of cancer.

Methods

Plant expression vector. A truncated form of human colorectal carcinoma antigen GA733-2 (aa 17–266) and Fc fragment of human IgG1 (Val97-Gly328, GenBank accession No.AY172957) were used in this study. The GA733-2 fused with human Fc-and ER signal peptide (rGA733-Fc) were amplified using the primers GA733-Fsas (5′-ACGTCCAGATGATTATGCCCG-3′), Ig-Fsal (5′-ACGTCCAGGTTAGCCCCCTTCTG-3′) and KDEL-Rsnab (5′-TGATGCTGTTAGGGTTATCTT-3′). The PCR products were introduced into a plant expression vector pBINPLUS through SalI and SacI restriction enzyme sites. The final constructs named as pBINPLUS-GA733-Fc and pBINPLUS-h-Fc were used for tomato transformation.

Agrobacterium-mediated transformation of Solanum lycopersicum cv. Micro-Tom. Wild tomato seeds (Solanum lycopersicum cv. Micro-Tom) were provided by Sanghyeob Lee of Sejong University in Republic of Korea (ROK). WT tomato plants and transgenic lines were grown under controlled growth room condition with 16 h light/8 h dark cycle (22–24 °C). Collection of plant material, must comply with relevant institutional, national, and international guidelines and legislation. This experiment was conducted with permission at Chonnam National University (35° 10′ N, 126° 53′ E) in Gwangju, Republic of Korea. The plant materials and residues were discarded according to the biosafety guidelines. Transgenic tomato plants expressing rGA733-Fc or h-Fc were produced using the Agrobacterium-mediated transformation method. Cotyledons from a sterile cultured three-week-old tomato plants were cut into two pieces and inoculated with Agrobacterium tumefaciens bacteria solution. After the inoculation, the explants were placed on co-culture medium [3% sucrose (Duchefa, RV Haarlem, Netherlands), 4.4 g/L MS salt (Duchefa, RV Haarlem, Netherlands), 0.1 mg/L IAA (Duchefa, RV Haarlem, Netherlands), 1 mg/L zeatin (Duchefa, RV Haarlem, Netherlands), and 200 μM acetosyringone (MBcell, Seoul, Korea), pH 5.2]. After 2 days of co-cultivation, the coyledon pieces were transferred to shoot induction medium [3% sucrose, 4.4 g/L MS salt, 50 mg/L kanamycin (Biosesang, Sungnam, Korea), 0.1 mg/L IAA, 1 mg/L zeatin, and 250 mg/L cefotaxime (Biosesang, Sungnam, Korea), pH 6.0], and the explants were transferred to fresh shoot induction medium every 2–3 weeks until regenerated shoot appeared. The regenerated shoots were then transferred into shoot elongation medium (1.5% sucrose, 4.4 g/L MS salt, 50 mg/L kanamycin, and 250 mg/L cefotaxime, pH 6.0) for further development. The callus with well-developed shoots were then transferred into root induction medium [1.5% sucrose, 4.4 g/L MS salt, 50 mg/L kanamycin, 2 mg/L IBA (Duchefa, RV Haarlem, Netherlands), and 50 mg/L cefotaxime, pH 6.0]; the regenerated plantlets with roots were transferred to soil and grown in a growth room (16 h light/8 h dark cycle, 22–24 °C).

Genomic DNA extraction and PCR analysis of transgenic plants. Genomic DNA was isolated from 300 mg transgenic tomato leaves to confirm the insertion of the transgene cassette. Transgenic tomato leaves were ground to fine powder with liquid nitrogen, and mixed well with 700 µl of DNA extraction buffer (0.05 M Tris–HCl, pH 7.6 (Biosesang, Sungnam, Korea), 0.5% SDS (Sigma-Aldrich, St. Louis, MO, USA), 0.1 M
NaCl (Sigma-Aldrich, St. Louis, MO, USA), 0.05 M EDTA (Sigma-Aldrich, St. Louis, MO, USA), and 0.1 M β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). The mixtures were incubated at 50 °C for 10 min with inversion every 2 min. The extracts were thoroughly mixed with 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1, Biooneang, Sungnam, Korea). The samples were centrifuged at 13,000 rpm for 15 min, and then upper aqueous phase was transferred to fresh micro centrifuge tubes. 2 volumes of 100% ethanol were added to the tubes and the samples were mixed by gently inversion. The tubes were centrifuged at 13,000 rpm for 10 min at 4 °C. The pellet was dissolved in distilled water after washing with 75% ethanol. The presence of the rGA733-Fc or human Fc expression cassette in plants was determined by PCR analysis using 35S-F (5′-GGATGAGCGACA ATCCCCACTATCC-3′), Fc-R (5′-AGGGAGAGGCTCCTTCGCGT-3′) primers. PCR reactions were carried out following conditions; preheating at 95 °C for 5 min, 34 amplification cycles (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min), final incubation at 72 °C for 10 min.

rGA733-Fc protein purification. The transgenic plants harboring rGA733-Fc expression cassette were ground in 0.5 x PBS buffer (8.17 mM Na₂HPO₄, 1.34 mM KCl, 0.47 mM K₃PO₄, 68.45 mM NaCl, pH 7.4). The soluble fraction was dialyzed in binding buffer (10 mM NaH₂PO₄, 150 mM NaCl, pH 8.2). After dialysis, total soluble proteins were purified via Affi-Gel Protein A Gel column (Bio-Rad Laboratories, Hercules, CA, USA) at a flow rate of 0.5 ml/min. The glycosylation patterns of purified rGA733-Fc proteins were analyzed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis.

Western blot analysis and quantification of rGA733-Fc and human Fc proteins in transgenic plants. Total soluble proteins were isolated from 200 mg of tomato leaves. The harvested tomato leaves were homogenized using a TissueLyser II (Qiagen, CA, USA) with one volume of Brady buffer at 30 Hz for 5 min. 50 μg of total soluble proteins were separated on a 10% or 12% SDS polyacrylamide gel, and transferred to a polyvinylidene fluoride membrane (PVDF; GE Healthcare, USA). The PVDF membrane was incubated with a blocking solution [1% bovine serum albumin (BSA) and 5% skim milk] at 25 °C for 3 h and then washed with TBS-T (150 mM NaCl, 2.5 mM KCl, 25 mM Tris base, and 0.1% Tween-20, pH 7.4). The rabbit rGA733-2 specific antibody was used as a primary antibody (1:5000 dilution) and the HRP-conjugated goat anti-human IgG was used as a secondary antibody (1:5000 dilution, Thermo Scientific, Waltham, USA). The rGA733-Fc proteins were visualized using an Immobilon western chemiluminescent HRP system (Millipore, Billerica, MA, USA) and a LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

Quantification of rGA733-Fc protein was performed as described previously. 10 μg/ml of total soluble proteins from transgenic tomato leaf and fruits were plated on a 96-well immunoassay plate. The individual wells were blocked with 200 μl of 5% skim milk in PBS buffer (16.34 mM Na₂HPO₄, 2.68 mM KCl, 0.94 mM K₃PO₄, 136.9 mM NaCl, pH 7.4) for 2 h. The immunoplate was then incubated with 1:2000-diluted rabbit rGA733-2 specific antibody, and then incubated with 1:2000-diluted AP-conjugated goat anti-human IgG (Thermo Scientific, Waltham, USA). The concentration of immunoblotted proteins was determined with p-nitrophenyl phosphate (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) as a substrate by measuring an absorbance at 450 nm using a VERSA max microplate reader (Molecular Devices, California, USA).

N-glycan analysis. Samples were digested into glycopeptides by pepsin, followed by peptide N-glycosidase (PNGase) A (Roche, Basel, Switzerland) treatment, as previously described. The released N-glycans were purified using graphitized carbon resin from Carbograph (Alltech, Lexington, MA). Purified glycans were dried and then re-dissolved in a mixture of 90 μl dimethyl sulfoxide (DMSO), 2.7 μl water, and 35 μl iodomethane (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) as a substrate by measuring an absorbance at 450 nm using a VERSA max microplate reader (Molecular Devices, California, USA).

Mouse immunization and induction of colorectal cancer. Potential anti-cancer effects of rGA733-Fc was tested in a colorectal cancer mouse model with transgenic tomato fruits. Seven-week-old male C57BL/6 mice were purchased from the Damool Animal Breeding Company (Daejeon, Korea). Three C57BL/6 mice per each condition were immunized every other day for 4 weeks with 100 mg of transgenic tomato fruit extract of each condition were immunized every other day for 4 weeks with 100 mg of transgenic tomato fruit extract (rGA733-FcOX #16 and h-FcOX #12) in 100 μl PBS by oral injection. Fourteen days later, the mice were hypodermically injected with 1 x 10⁶ MC38 cells. Beginning from 5 days after MC38 cell injection, tumor volume was measured for 13 days. At the end of the experiment (D28), the mice were euthanized. Total IgA and IgG were extracted from fecal and serum, respectively, and quantified by ELISA. The experiment using mice was carried out in compliance with the ARRIVE guidelines.
Real-time PCR analysis. Total RNAs were extracted from cancer tissues using TRIzol reagent (MRC, Cincinnati, OH, USA), according to the manufacturer’s instruction. 1 μg of total RNAs was used for cDNA synthesis using the QuantiTect® Reverse Transcription Kit (Qiagen, CA, USA) and quantitative real-time PCR (qRT-PCR) analysis was performed with the QuantiTect® SYBR® Green PCR Kit (Qiagen, CA, USA) and the Rotor-Gene 6000 real-time amplification operator (Qiagen, CA, USA). The primers used in this study were purchased from Cosmo Genetech (Seoul, Korea) and the sequence of the primers is as follows: β-actin (forward, 5′-GAC GGC GAC  GAG CTG GTATA-3′ and reverse, 5′-CAC CTG CTA CTC ATT CA-3′) and reverse, 5′-CAC TGA CAT CTA CTG TTG TGC GGT GTG TTG GAG CTG GTATA-3′; bax (forward, 5′-ACC AGC TCT GGA CAT CAT G-3′ and reverse, 5′-ACT TTGA TGC CAC AGG GCC TGT G-3′), bak (forward, 5′-AGA CAA TCT TCA CCA AGAT CGC CT-3′ and reverse, 5′-TCA AAC AGC GTG TAG CTG TCA-3′) mcp1 (forward, 5′-CAC TGT CAC ACT GTG TCC AGA CAT TCA-3′ and reverse, 5′-TGT CCT CGT TGT CAG TGC C-3′), ccl17 (forward, 5′-CAG ATC ATG-3′ and reverse, 5′-CCA AGA TCG CCT -3′), p53 (forward, 5′-CAC TGT CAC ACT GTG TCC AGA CAT TCA-3′ and reverse, 5′-TTG TGT CCG TGT AGT GCA TTA-3′), ccl21 (forward, 5′-AGACT CAG AGC CCA AGC GTA-3′ and reverse, 5′-GTT GAG CAG GGA GAG GGT-3′). The β-actin was used as internal control for normalization and relative expression was calculated by ΔΔCt method.

Statistical analysis. Experimental results were presented as mean ± standard error of the mean (SEM) and analyzed with unpaired Student’s t test by GraphPad Prism (Version 8.4.2, GraphPad Prism Software Inc., La Jolla, CA, USA). The data were considered to be statistically significant *p < 0.05, **p < 0.01, and ***p < 0.001.

Data availability
The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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Competing interests

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