Lung cancer is the most common cancer and the leading cause of cancer-related deaths. Panax ginseng has long been used to treat cancer and other diseases worldwide. Most of the pharmacological actions of ginseng are attributed to a variety of ginsenosides, which are often metabolized by intestinal bacteria into more effective forms. In this study, we found that the antiproliferative activity of ginseng was increased after enzymatic processing of ginseng saponin (50% inhibitory concentration, $>$70 $\mu$g/ml).

To elucidate the mechanism by which modified ginseng extract (MGX) induced cell death in human lung cancer cells, the gene expression profiles of A549 cells regulated by MGX were assayed using Agilent PrimeView Human Gene Expression Arrays. The expression of 17 genes involved in the regulation of cell signaling, cell metabolism, transport, and cytoskeleton-regulation was up-regulated, whereas the expression of 16 genes implicated in invasion and metastasis and cellular metabolism was down-regulated in MGX-treated A549 cells. Moreover, nuclear staining with 4′,6-diamidino-2-phenylindole revealed that MGX clearly caused nuclear condensation and fragmentation which are observed in apoptosis cell. These results elucidate crucial anticancer mechanisms of MGX and provide potential new targets for the assessment of anticancer activity of MGX.

**Keywords** ginseng; saponins; cancer; ginsenoside; apoptosis

Received: January 10, 2014    Accepted: February 20, 2014

**Introduction**

Lung cancer is the leading cause of cancer-related deaths worldwide, and $\sim$1.61 million new cases are reported every year [1]. Non-small-cell lung cancer, which is the most common type of lung cancer, accounts for 85%–90% of all lung cancer cases and is strongly associated with smoking [2].

The root of Panax ginseng C.A. Meyer (Korean ginseng) has traditionally been used as a herbal medicine in East Asia for the treatment of various diseases, including cancer [3–7]. The major biologically active components of Panax ginseng are a series of saponin glycosides collectively known as ginsenosides, which are a group of steroidal saponins. To date, over 50 ginsenosides have been isolated and identified from ginseng saponins [8].

Ginsenosides have a steroid-like skeleton consisting of four trans-rings with modifications that depend on the type (e.g. glucose, maltose, and fructose) and number of sugar moieties, as well as the sites of attachment of the hydroxyl group (e.g. C-3, C-6, or C-20). Based on the chemical structural characteristics, ginseng saponins can be divided into two groups, namely protopanaxadiol and protopanaxatriol (PPT), with the exception of ginsenoside Ro, which is derived from an oleanolic group. In the protopanaxadiol group, sugars are attached to the $\beta$-OH at C-3 and another $\alpha$-OH at C-20, as found in Rh1, Rh2, Rr, Rn, Rg3, and Rh2. In the PPT group, sugar residues are attached to the $\alpha$-OH at C-6, with another $\alpha$-OH at C-20, as found in Re, Rg1, Rg2, Rh1, and Rf.

Recent studies have reported the cytotoxic effects of minor saponins such as Rh1 and Rh 2 on the growth of various cancer cells, and the inhibitory effects of human intestinal bacterial saponin metabolites such as Compound K and PPT on the growth, invasion, and migration of tumor cells [9–13]. Human intestinal bacteria have recently been shown to cleave the oligosaccharide connected to the aglycon stepwise from the terminal sugar of ginsenosides to form the metabolite 20S-protopanaxadiol 20-O-$\beta$-D-glucopyranoside (IH-901, Compound K) after oral administration of
ginseng extract [14]. Transformed 20S-protopanaxadiol 20-O-β-d-glucopyranoside from ginsenosides Rb1, Rb2, and Rc induc an antimitotic or antiproliferative effect [15]. Wakabayashi et al. [16] revealed that the antitumor activities of ginsenosides following oral administration are attributable to metabolites formed by colonic bacteria-mediated deglycosylation. In addition, Compound K is known to exhibit cytotoxicity via the induction of apoptosis and cell cycle arrest in G1 phase through a caspase-dependent pathway involving mitochondrial disruption of tumor cells and reversal of multidrug resistance in tumor cells [17–19]. Furthermore, combined treatment with Compound K and radiation enhances human lung cancer cell death [17,20]. Therefore, in this study, we investigated the antiproliferative effects of modified ginseng extract (MGX) after transformation by enzyme treatment. To this end, we used human non-small-cell lung A549 cells and further investigated the possible mechanisms of action.

Materials and Methods

Preparation of enzyme-MGX
The root of regular ginseng (4 years old) was purchased from G&V (Wonju, Korea). A total of 20 g of pulverized ginseng root powder was suspended in 380 ml of distilled water and then sterilized by heating at 121°C for 15 min. To reduce the complexity of the components in the ginseng root, the extract was fractionated via extraction with water, methanol, and butanol. Among the fractions, the ginseng butanol extract (GBX) contained the compounds with specific anticancer activity; therefore, GBX was used as a control for the MGX. In addition, the suspension was treated with aliquots of filter-sterilized commercial enzymes (100 mg laminarinase and 100 mg pectinase) at an equimolar ratio (1 : 1, specific activity unit). For these treatments, the mixture was incubated at 40°C for 2 days and then evaporated to dryness at 60°C. The enzyme-modified ginseng powders were then suspended in 400 ml of 80% (v/v) methanol and 100 mg pectinase) at an equimolar ratio (1 : 1, specific activity unit). For these treatments, the mixture was incubated at 40°C for 2 days and then evaporated to dryness at 60°C. The enzyme-modified ginseng powders were then suspended in 400 ml of 80% (v/v) methanol and subject to ultrasonication for 5 min followed by filtration through Whatman No. 2 filter paper. The wet powder on the filter paper was then collected, suspended, treated in an ultrasound bath, and filtered in the same manner once again, after which the two filtrates were combined and evaporated to dryness at 50°C. The methanol extract was then dissolved in 200 ml of distilled water, washed with 200 ml of ethyl acetate in a separation funnel, and then extracted with 200 ml of butanol. Next, the butanol extract was evaporated to dryness at 50°C and then dissolved to a concentration of 10% (w/v) in 70% ethanol. The final sample was designated as the MGX.

HPLC analysis of ginsenosides
Standard ginsenosides, including Rg1, Re, Rf, Rh1, Rb1, Re, Rb2, f1, Rd, Rg3(S), Rg3(R), PPT, Compound K, Rh2, and PPD, were obtained from ChromaDex, Inc. (Irvine, USA) and analyzed using an Acquity UPLC system (Waters, Milford, USA) with an Acquity BEH C18 HPLC column. The mobile phase consisted of Solution A (CH3CN, HPLC-grade, JT Baker, Phillipsburg, USA) and Solution B (Milli-Q H2O, Millipore, Billerica, USA). The flow rate was set at 0.6 ml/min and the injection volume was fixed at 2 : 1. The separation of ginsenosides was performed using an isocratic gradient of 100% Solution A for 27 min. The column was maintained at room temperature during the separation, and the UV diode array detection was set at 203 nm. To confirm a reliable retention time of sample ginsenosides, the individual ginsenosides were identified and quantified based on comparison with the retention times of standard ginsenosides.

Cell culture
Human non-small-cell cancer cell line A549 was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). A549 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, USA), 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 0.25 µg/ml amphotericin B (Invitrogen) under 5% CO2 at 37°C. Confluent cells were detached with 0.25% trypsin-EDTA for 5 min, after which the aliquots were subcultured.

Cell viability assay
The effects of MGX on cell viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which quantifies the number of viable cells. Cells were seeded (2 × 104 cells/well) in 24-well flat-bottomed plates (Nunc, Thermo Scientific, Waltham, USA). After incubation at 37°C for 24 h, the medium was replaced with MGX at the appropriate concentrations. Control cells were treated with DMSO at a final concentration of <0.2%. After incubation for an additional 24 h, cells were washed three times with Dulbecco’s phosphate-buffered saline, and 500 µl of MTT solution (5 mg/ml in PBS) was added to each well. After incubating for 4 h, the medium was removed and 200 µl of formazan (crystals dissolved in DMSO) was added to the cells. The absorbance of each well was then read at 540 nm using a microplate reader.

RNA extraction and cDNA synthesis
Total RNA was extracted using an Easy-spin RNA Extraction kit (iNtRON, Seoul, Korea) according to the manufacturer’s instructions. The purity of the RNA was assessed by measuring the absorption at 260 and 280 nm (A260/A280 ratios of 1.9–2.1 were considered acceptable) and by ethidium bromide staining of 18S and 28S RNA by gel electrophoresis. RNA concentrations were determined from the A260. Next, 2 µg of total RNA were reverse-transcribed in a 20-µl reaction mixture containing 50 units of
SuperScript II reverse transcriptase (Invitrogen), 5 \( \mu \)M DTT, 40 units of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), 0.5 \( \mu \)M random hexanucleotide primers, and 500 \( \mu \)M of dNTP mixture. The reverse transcription reaction was performed at 50°C for 60 min, after which the reaction was terminated by heating the mixture at 70°C for 15 min. The complementary DNA (cDNA) was then stored at −20°C until use.

**Quantitative real-time reverse transcriptase polymerase chain reaction**

Overexpression of the target genes was confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). All real-time PCR analyses were performed using an ABI Step One Real-time PCR system (Applied Biosystems, Foster City, USA). Each reaction contained 0.1 \( \mu \)M of each primer, 10 \( \mu \)l of the 2× SYBR Green PCR master mix (including AmpliTaq Gold DNA polymerase with buffer, dNTPs mix, SYBR Green I dye, ROX dye, and 10 mM MgCl₂), and 1 \( \mu \)l of the template cDNA in a 20-\( \mu \)l total reaction volume. The typical amplification program included activation of the enzyme at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, then annealing and extension at 60°C for 1 min. The \( C_T \) (cycle threshold) value for each gene was determined using the automated threshold analysis function of the ABI instrument and normalizing to \( C_T(GAPDH) \) to obtain \( dC_T \) (\( = C_{T(GAPDH)} - C_{T(test)} \)). The difference in \( n \) between the 2 \( C_T \) or \( dC_T \) values indicates a 2\(^n\)-fold difference in the amount of the target sequence between the two cDNA samples being compared. The primers used for quantitative PCR are shown in **Table 1**.

**Microarray assay**

Gene expression was analyzed using the Agilent PrimeView Human Gene Expression Arrays containing 36,000 gene transcripts and variants from the UniGene resource (Affymetrix, Santa Clara, USA). This array is composed of over 45,000 probe sets representing \( \approx \)38,500 well-characterized human genes. For each gene, 11 pairs of oligonucleotide probes were synthesized *in situ* on the arrays. Biotinylated cRNA was then prepared according to the standard Affymetrix protocol from 100 ng of total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 12 \( \mu \)g of RNA was hybridized for 16 h at 45°C on a GeneChip Human Genome Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, after which they were scanned using an Affymetrix GeneChip Scanner 3000 7G. The data were then analyzed using the Microarray Suite version 5.0 and the Affymetrix default analysis settings, and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized and log-transformed intensity values were then analyzed using GeneSpring GX 11.5.1 (Agilent Technologies, CA). Fold-change filters included the requirement that the genes be present in at least 200% of the controls for genes showing up-regulated expression and <50% of controls for genes showing down-regulated expression. Hierarchical cluster analysis using GeneSpring GX 11.5.1 (Agilent technologies) identified clustered groups as those that behaved in a similar manner in all experiments. The clustering algorithm was Euclidean distance, with average linkage.

**DAPI staining**

To detect the apoptotic cells, the cells were first washed twice with cold PBS and then fixed with 4% paraformaldehyde (Sigma, St Louis, USA) for 30 min. The fixed cells were then washed again with PBS and stained with 1 \( \mu \)g/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma) solution for 30 min. Finally, the cells were washed two more times with PBS and analyzed by fluorescence microscopy (Axiovert 200 inverted microscope; Zeiss, Oberkochen, Germany).

**Results**

**Effects of MGX on cell growth and viability in human non-small lung cancer A549 cells**

Ginsenosides have been shown to inhibit tumor cell proliferation and tumor growth, as well as to induce differentiation
and apoptosis [21]. To determine the antiproliferative effects of MGX on human non-small lung cancer A549 cells, we conducted an MTT assay. MGX inhibited A549 cell growth in a dose-dependent manner at 14.8 22.2, 33.3, 50, and 100 μg/ml at 24 h. The concentration at which 50% growth inhibition occurred was 70 μg/ml (Fig. 1). However, exposure of A549 cells to 100 μg/ml non-treated ginseng saponin extract showed less growth inhibition (~10%). Therefore, these results suggest that enzymatic processing of ginseng saponin increased its antiproliferative effects on A549 cells.

**Identification of differentially expressed genes in A549 human lung adenocarcinoma cells treated with MGX**

Although ginseng has been long used as a traditional medicine for the treatment of cancer, the underlying mechanisms for the varying activities observed following exposure to ginseng are largely unclear. To identify relevant alterations in gene expression associated with MGX treatment, we analyzed the gene expression profiles of A549 cells by using Affymetrix Gene Chip arrays containing more than 38,500 genes. Of a total of 38,500 genes, we identified known 33 genes whose expression was significantly up- or down-regulated with magnitude exceeding a cutoff of 2-fold change following treatment with MGX. Among these, 17 had significantly up-regulated expression, and 16 had significantly down-regulated expression when compared with the control treatment (Tables 2 and 3).

Several genes involved in the regulation of cell signaling (FHL1, IGF-2, BPTF2, LUZP1, and MYEF2) were expressed at higher levels following treatment with MGX. Furthermore, several genes implicated in cell metabolism (ACAT2, RDH10, and NAPEPLD), transport (AQP3 and SLC11A2), and cytoskeleton regulation (DST and KIF21A) showed increased expression in A549 human non-small-cell lung cancer cells that were treated with MGX (Table 2).

In contrast, 16 genes showed reduced expression in A549 human non-small-cell lung cancer cells treated with MGX. There was an over-representation of members of genes involved in invasion and metastasis (CXCL1, CXCL3, Rhotekin 2, TPPI2, NPTX1, EFNA1, and MMP7) and cellular metabolism (MT1, ARSI, and YWHAE). Table 3 lists the names of genes exhibiting reduced expression where the difference was greater than 2-folds. This differently expressed group of genes may serve as an important target in MGX-mediated anticancer activity in human non-small-cell lung cancer cells.

**Real-time RT-PCR validation of microarray data**

To confirm the results obtained using microarrays, we investigated the expression of six target genes in MGX treated A549 cells by qRT-PCR analysis. These genes included four that showed highly up-regulated expression, namely FHL1, CYP1B1, AQP3, and ACAT2 and two that was highly down-regulated, namely, CXCL3 and MMP7. To accurately quantify the expression of these six genes, the data were

![Figure 1. Antiproliferative effects of MGX on human A549 lung cancer cells](image-url)

- (A) Cell viability at the indicated concentrations of MGX at 24 h was assessed by the MTT assay.
- (B) Microscopic images of A549 cells treated with MGX (0, 50, and 100 μg/ml) (~100-folds).
normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

The expression levels of FHL1, CYP1B1, AQP3, and ACAT2 increased by 3-folds, 18-folds, 2.7-folds, and 2-folds, respectively, upon treatment with MGX (Fig. 2). Conversely, the expressions of CXCL1 and MMP7 were decreased by 4-folds and 2-folds, respectively. These results indicate that the qRT-PCR results are consistent with the microarray data.

MGX-mediated cell death in A549 non-small-cell lung cancer cells

To determine whether MGX has an effect on lung cancer cells, A549 cells treated with DMSO or MGX for 24 h were subjected to fluorescence microscopic analysis after DAPI staining. In the control cells, the nuclei showed uniform staining, thereby indicating that cells were healthy and the nuclei were intact. However, after treatment with 50–100 μg/ml MGX for 24 h, some nuclei exhibited typical apoptotic characteristics such as nuclear condensation and fragmentation. Some floating dead cells were also observed at this time (Fig. 3). These results suggest that MGX induces cell death in A549 cells.

Table 2. Genes with highly up-regulated expression due to MGX in A549

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<td>DST</td>
<td>Dystonin</td>
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<td>AQP3</td>
<td>Aquaporin 3</td>
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<td>BPTF2</td>
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<td>Solute carrier family 6</td>
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<td>Anoactinin 6</td>
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<td>Lipin 2</td>
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<td>MKLN1</td>
<td>Muskelin 1</td>
<td>2.1</td>
<td>NM001145354</td>
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Table 3. Genes with highly down-regulated expression due to MGX in A549

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<td>Interleukin 15</td>
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Some recent studies including ours have reported the cytotoxic effects of minor saponins such as Rh1 and Rh 2 on the growth of various cancer cells, and the inhibitory effects of human intestinal bacterial saponin metabolites such as Compound K and PPT on the growth, invasion, and migration of tumor cells [9–13,22]. In this study, we used cDNA microarray analysis to identify novel target genes involved in the anticancer activity of MGX (Fig. 4). We found that several genes involved in the regulation of cell signaling (FHL1, IGF-2, BPTF2, LUZP1, and MYEF2), cell metabolism (ACAT2, RDH10, and NAPEPLD), transport (AQP3 and SLC11A2), and cytoskeleton regulation (DST and KIF21A) were expressed at higher levels following treatment with MGX.

Discussion

Recent studies including ours have reported the cytotoxic effects of minor saponins such as Rh1 and Rh 2 on the growth of various cancer cells, and the inhibitory effects of human intestinal bacterial saponin metabolites such as Compound K and PPT on the growth, invasion, and migration of tumor cells [9–13,22]. In this study, we used cDNA microarray analysis to identify novel target genes involved in the anticancer activity of MGX (Fig. 4). We found that several genes involved in the regulation of cell signaling (FHL1, IGF-2, BPTF2, LUZP1, and MYEF2), cell metabolism (ACAT2, RDH10, and NAPEPLD), transport (AQP3 and SLC11A2), and cytoskeleton regulation (DST and KIF21A) were expressed at higher levels following treatment with MGX.
In this study, FHL1 proteins, which are a family of LIM domain-only proteins implicated in transcriptional regulation and suppression of tumor growth, were significantly up-regulated by MGX. The FHL1 protein has been shown to interact with Smad proteins, increase the expression of growth inhibitor genes such as the CDK inhibitor p21, and decrease the expression of the growth-promoting gene c-myc [23]. Furthermore, FHL1 inhibits hepatocellular carcinoma cell growth, both in vitro and in nude mice.

Cytochrome P450 is a member of the cytochrome P450 monoxygenase superfamily that plays an important role in xenobiotic metabolism as well as in carcinogenesis [24]. In this study, the CYP1B1 levels were significantly increased after treatment with MGX. CYP1B1 enzymes mediate the bioactivation of carcinogens found in both the human diet and the environment, including compounds such as polycyclic aromatic hydrocarbons, which include 3-methylcholanthrene, benzo(a)pyrene, polyhalogenated aromatic hydrocarbons, and certain congeners of polyhalogenated biphenyls [25]. Our finding that MGX increased the expression of CYP1B1 is consistent with the results of previous studies that showed the induction of CYP1B1 expression by ginsenosides Rg1 and Rb1 [26]. A similar result was shown in our previous study with MGX in HepG2 hepatocarcinoma cell [22].

However, several genes implicated in invasion and metastasis (CXCL1, CXCL3, Rhotekin 2, TFPI2, NPTX1, EFNA1, and MMP7) and cellular metabolism (MT1, ARSI, and YWHAE) exhibited decreased expression in A549 human lung adenocarcinoma cells treated with MGX. Chemokine (C–X–C motif) ligand (CXCL) is a chemokine that enhances cancer cell survival and chemoresistance. Furthermore, CXCL1 blocker increases the effectiveness of chemotherapy against breast cancer, particularly, against metastasis [27]. Rhotekin is a scaffold protein that interacts with S100A4 to facilitate invasion and metastasis [28]. Rhotekin is over-expressed in metastatic colon cancer cells [29] and gastric adenocarcinoma cells, where it confers resistance to apoptosis through activation of nuclear factor-κB [30].

Matrix metalloproteinase 7 (MMP7), also known as matrilysin, is a member of a family of zinc-dependent
endopeptidases [31]. MMP7 is over-expressed in pancreatic, gastric, and colorectal cancers [32,33], and MMP levels correlate with clinical stages of malignancy, including lymph node involvement and distant metastasis [34–36]. Our results showing the decreased expression of several genes involved in invasion and metastasis in A549 cells treated with MGX may facilitate elucidation of the anticancer mechanisms of MGX.

Figure 3. MGX induces cell death in A549 cells  A549 cells were exposed to MGX (50 and 100 μg/ml) for 24 h, after which the DAPI-stained nuclei were analyzed for apoptotic morphology by fluorescence microscopy. A549 cells were also photographed by inverted phase-contrast microscopy (upper panel, original magnification ×400).

Figure 4. A schematic diagram of microarray data analysis  Microarray data analysis used the Agilent PrimeView human Gene expression array containing 38,500 gene transcripts and variants via UniGene annotations. Gene expression profiles of the A549 cells regulated by MGX were assayed. Scatter plot was shown for global comparison of gene expression. Only mRNAs with a cutoff of 2-fold change in expression were reported.
Moreover, nuclear staining with DAPI revealed that MGX clearly caused nuclear condensation and fragmentation observed in apoptosis in A549 human lung adenocarcinoma. Taken together, these results elucidate crucial anticancer mechanisms of MGX and provide potential new targets for the assessment of anticancer activity of MGX.

Funding

This work was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MOE) (2010–0022628, 2013R1A1A2011375) and an Inha University Grant.

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