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Genome Wide Expression Profile of *Agrimonia pilosa* in LPS-stimulated BV-2 Microglial Cells

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Abstract

Microglial cells constitute the first line of defense against infection and injury in the brain. This study was conducted to evaluate the protective mechanisms of *Agrimonia pilosa* (AP) on LPS-induced activation of BV-2 microglial cells. The effects of AP on gene expression profiles in activated BV-2 microglial cells were evaluated using microarray analysis. BV-2 microglial cells were cultured in a 100 mm dish (1 × 10^7/mL) for 24 hr and then pretreated with 1 g/mL AP or left untreated for 30 min. Next, 1 g/mL LPS was added to the samples and the cells were reincubated at 37°C for 30 min, 3 hr and 6 hr. The gene expression profiles of the BV-2 microglial cells varied depending on the AP. The microarray analysis revealed that MAPK signaling pathway-related genes were down-regulated and IL10 gene was up-regulated in AP-treated BV-2 microglial cells. AP can affect the inflammatory response and MAPK pathway in BV-2 microglial cells.

Keywords: LPS, *Agrimonia pilosa*, Gene expression profile, Microarray

Microglia plays a role in host defense and tissue repair in the central nervous system[1,2]. Activated microglia release neurotoxic factors such as nitric oxide (NO), as well as cytokines and chemokines, such as IL-1β, IL6, TNF-α, and MIP-1α[3-5]. NO is known to be an important mediator of acute and chronic inflammation[6]. In addition, NO plays an important role in diverse physiological processes, including immune responses, platelet inhibition, neurotransmission, inflammation, and smooth muscle relaxation. Inhibition of microglial activation, therefore, would be an effective therapeutic approach to alleviate the progression of neuroinflammation disease including Alzheimer's and Parkinson's disease[3,7-9].

During a search for new agents from medicinal plants for use in the treatment of neuroinflammation disease, the spray-dried extracts of 270 herbal medicines in a phytolibrary™ kit were tested for their ability to inhibit LPS-induced NO production in BV-2 microglial cells. Of these medicinal plants, *Agrimonia pilosa* (AP) was selected for this study based on its higher inhibitory activity. AP is a well-known traditional Chinese medicine that is used as inflammation-related diseases, and recent studies have reported that their extracts possess anti-oxidant, anti-inflammatory, anti-virus, anti-bacteria, anti-tumor, and anti-hyperglycemic effects[10-12]. However, little is known of the inhibitory mechanisms of AP in microglial activation.

Therefore, this study was conducted to determine the protective mechanisms of AP on LPS-induced activation in BV-2 microglial cells. Specifically, AP was evaluated to determine if it could prevent LPS induced activation of microglial cells by using microarray analysis. The anti-neuroinflammation strategies and their possible mechanisms are also discussed herein.

Gene Expression Profiles in BV-2 Microglial Cells

Gene expression profiles were significantly up- or down-regulated in the experimental groups (LPS or LPS plus AP-treated BV-2 microglial cells) when compared with the control (non-treated BV-2 microglial cells). When the microglial cells were treated with LPS and AP were evaluated, 748 up-regulated probe sets and 1,252 down-regulated probe sets were selected from the experimental group using approximately 45,100 oligonucloetide probes. Genes showing highly altered expression levels were aligned according to the magnitude of the altered expression. The most differ-

entially expressed genes (1 up-regulated, 250 down-regulated) are listed in Table 1, which shows a comparison of the expression levels of a variety of genes between the experimental group and the control. All genes were grouped into functional categories and metabolic pathways based on the KEGG database.

Discussion

Natural producs have long been used in traditional medicine to treat inflammatory and other allergic diseases. The raw materials are also used to develop new drugs[13]. Microarray technique is qualitative as well as quantitative because it possesses the sensitivity to detect changes in the levels of gene expression in the investigated cells when compared with the control samples[14,15]. An understanding of these molecular processes can then be used in the development of more advanced therapies for the herbal treatment of neuroinflammation disease. Our objective was to determine how AP effect gene expression profiles in BV-2 microglial cells. In this study, we used that BV-2 microglial cells were stimulated with LPS with or without AP. The early signaling events involved in LPS-induced microglial activation are not completely understood; therefore the effects of AP on the gene expression profiles of BV-2 microglial cells that were treated for different lengths of time (30 min, 3 hr, and 6 hr) were evaluated. Specific and significant alterations of the expression profile of AP-treated BV-2 microglial cells were observed (Table 1). The genes found to be differentially expressed were responsible for inflammatory and immune response processes.

Signaling from the TGFβ stimulates the MAPK pathways[16]. The MAPK pathways are deeply involved in signaling for various immune responses including apoptosis. In our experiments, we detected the down-regulation of apoptosis related genes, such as *Casp3, CASP8* and FADD-like (*Cflar*), and lymphotoxin A (*Lta*) in AP-treated BV-2 microglial cells (Table 1). MAPKs are serine/threonine kinases, which include the extracellular signal-related kinases (ERKs), p38 kinases, and c-Jun N-terminal kinases (JNKs). Activation of the MAPK pathway often occurs in response to growth factor stimulation of receptor tyrosine kinases, which are coupled to the activation of Ras G-proteins through Src homology 2 domain-containing proteins, such as Shc and Grb2, and quinine nucleotide exchange factors such as SOS[17,18]. In this study, we detected the down-regulation of Src family associated phosphoprotein 1 (*Skap1*), RAS p21 protein activator 4 (*Rasa 4*), RAS protein activator like 2 (*Rasal2*), Ras association (*RalGDS/AF-6*) domain family 1 (*Rassf1*), G protein-coupled receptor 15 (*Gpr15*), *Gpr125*, *Gpr158*, prostaglandin F receptor (*Ptgfr*), chemokine (C-X-C motif) receptor 7 (*Cxcr7*), chemokine (C-C motif) receptor 9 (*Ccr9*), histamine receptor H 3 (*Hrh3*), MAD homolog 9 (Drosophila) (*Smad9*), noggin (*Nog*), fibroblast growth factor receptor 3 (*Fgfr3*), *Fgf4*, calcium channel, voltage-dependent, alpha 2/delta subunit 2 (*Cacna2d2*), interleukin 1 receptor, type I (*Il1r1*), Mitogen activated protein kinase 1 (*Mapk1*), and nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 (*Nfatc2*) in AP-treated BV-2 microglial cells (Table 1). Each of the MAPKs has also been implicated in neuroinflammatory events, including mediation of many of the physiological responses to NO. For example, NO regulation of matrix metalloproteinases proteins, including MMP1, during inflammatory and angiogenic responses may require MAP kinase proteins[24,25]. In our experiments, we detected the down-regulation of *MMP2*, and *MMP15* in AP-treated BV-2 microglial cells (Table 1). NO is a signaling molecule, neurotransmitter, and immune effector[17,19,20]. Also NO is produced by the activity of the family of enzymes nitrix-oxide synthases (NOSs). This gene encodes a nitric oxide synthase which is expressed in liver and is inducible by a combination of lipopolysaccharide and certain cytokines[17,19-21]. In this study, we found that *Nos1ap* was down regulated in AP-treated BV-2 microglial cells (Table 1).

TGFβ signaling is often coupled with alteration in cell adhesion and motility[16]. In many of these inflammatory situations, the expression of adhesion molecules is induced by cytokines. Adhesion molecules are shown to play important roles in the induction of inflammation. The interaction of the adhesion molecules (e.g., ICAM1, VCAM1 and E selectin) with their counter-receptors on circulating leukocytes (e.g., β1 and β2 integrins) results in the capture, rolling, and firm adhesion of the leukocytes to the vascular endothelium. The arrested leukocytes then transmigrate the vascular wall and move toward the lesion along the chemotactic gradient[22-25]. In our experiments, we detected the down-regulation of integrin binding sialoprotein (*Ibsp*), immunoglobulin kappa chain variable 1 (V1) (*Igk-v1*), immunoglobulin superfamily, member 4B (*Igsf4b*), immunoglobulin superfamily containing leucine-rich repeat (*Islr*), integrin alpha 8 (*Itga8*), *Itgb-8*, *Itgav*, *Icam1*, and *Vcam1* in AP-treated BV-2 microglial cells (Table 1). Also in this study, we found that immune and inflammatory response-related genes, such as *Il24*, kininogen 1 (*Kng1*), tachykinin 1 (*Tac1*), IL2-inducible T-cell kinase (*Itk*), myelin basic protein (*Mbp*), colony stimulating factor 2 (granulocyte-macrophage) (*Csf2*), DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (*Ddx58*), *Il3*, *Il9*, T-cell specific

Table 1. Up- and down-regulation of genes based on comparison of gene expression between experimental (*Agrimonia pilosa* (AP)-treated) and control (non-treated or LPS-treated) BV-2 cells.

Gene description	Affimetrix probe set ID	Gene symbol	Regulation profile and ratio					
			LPS			AP		
			30 min	3 hr	6 hr	30 min	3 hr	6 hr
interleukin 10	1450330_at	Il10	−2.7	−0.5	−0.4	3.5	0.42	0.49
aldo-keto reductase family 1, member C20	1425387_at	Akr1c20	0.8	2.4	2.4	−1.9	−1.9	−3.0
amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13 (human)	1444629_at	Als2cr13	2.5	4.5	1.1	−1.8	−2.7	−1.8
ankyrin repeat and SOCS box-containing protein 15	1439836_at	Asb15	2.7	6.3	6.8	−1.6	−3.2	−4.9
biphenyl hydrolase-like	1430531_at	Bphl	3.4	3.4	4.9	−2.3	−3.7	−5.8
brain protein 44	1430071_at	Brp44	0.7	2.2	1.1	−2.2	−2.5	−1.5
cadherin 10	1425092_at	Cdh10	5.4	3.7	3.8	−1.6	−2.2	−1.8
casein beta	1420369_a_at	Csn2	2.5	3.1	3.8	−3.3	−2.8	−3.0
chemokine (C-C motif) receptor 9	1421919_a_at	Ccr9	1.9	1.7	1.2	−3.9	−2.5	−3.2
coagulation factor XIII, A1 subunit	1448929_at	F13a1	4.0	3.1	3.5	−3.7	−1.9	−2.4
cytochrome P450, family 11, subfamily b, polypeptide 1	1456362_at	Cyp11b1	1.9	2.0	2.6	−3.1	−1.6	−3.0
cytochrome P450, family 2, subfamily a, polypeptide 4	1422230_s_at	Cyp2a4	4.2	4.5	3.4	−3.8	−2.4	−2.2
cytochrome P450, family 2, subfamily j, polypeptide 13	1426102_at	Cyp2j13	1.6	1.9	2.3	−3.0	−1.8	−2.9
Dedicator of cytokinesis 4	1459279_at	Dock4	3.8	2.7	2.0	−2.9	−2.7	−1.6
deleted in azoospermia-like	1419542_at	Dazl	1.9	3.9	2.0	−2.1	−4.8	−2.3
enolase 2, gamma neuronal	1418829_a_at	Eno2	5.0	5.1	2.3	−3.2	−1.9	−2.2
Erythrocyte protein band 4.1-like 1	1443806_x_at	Epb4.1l1	5.0	3.5	4.0	−4.8	−2.4	−3.9
fibroblast growth factor 4	1420086_x_at	Fgf4	2.7	4.7	2.9	−1.8	−2.3	−1.9
G protein-coupled receptor 158	1438526_at	Gpr158	1.4	2.6	2.4	−2.8	−2.4	−4.7
galactosidase, beta 1 like 3	1453143_at	Glb1l3	2.9	2.5	3.0	−3.2	−2.6	−1.7
killer cell lectin-like receptor, subfamily A, member 19	1426140_x_at	Klra19	4.6	4.8	4.2	−4.9	−3.1	−2.8
kininogen 1	1426045_at	Kng1	2.1	2.9	1.5	−2.4	−3.4	−3.5
Mannosidase, alpha, class 1A, member 2	1456534_at	Man1a2	2.4	2.1	2.8	−1.9	−2.1	−3.2
phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	1421704_a_at	Pik3c2g	3.2	2.9	3.7	−3.4	−4.1	−3.5
phytanoyl-CoA hydroxylase interacting protein-like	1427023_at	Phyhipl	4.3	4.5	3.5	−2.1	−2.9	−3.4
potassium large conductance calcium-activated channel, subfamily M, beta member 1	1421400_at	Kcnmb1	1.8	2.3	2.6	−2.9	−3.2	−2.1
Potassium voltage-gated channel, subfamily H- (eag related), member 8	1459015_at	Kcnh8	3.8	3.3	2.9	−4.2	−2.0	−2.5
Pre B-cell leukemia transcription factor 3	1440154_at	Pbx3	4.6	4.7	3.7	−2.4	−4.7	−4.2
prostaglandin F receptor	1420349_at	Ptgfr	2.1	2.8	3.2	−3.6	−2.6	−2.6
proteasome 26S subunit, non-ATPase, 11	1432726_at	Psmd11	3.0	2.5	2.6	−3.0	−3.7	−3.1
Protein kinase C, mu	1446596_at	Prkcm	3.2	2.7	4.5	−2.1	−1.9	−2.2
solute carrier family 39 (zinc transporter), member 14	1457770_at	Slc39a14	1.1	1.9	1.0	−2.3	−5.0	−2.1
sulfotransferase family 1A, phenol-preferring, member 1	1427345_a_at	Sult1a1	1.7	3.6	3.6	−1.9	−3.8	−3.2
tachykinin 1	1431883_at	Tac1	4.2	3.5	3.5	−3.6	−2.2	−1.7
TBC1 domain family, member 21	1429408_at	Tbc1d21	4.5	3.9	3.3	−2.7	−2.5	−2.0
transmembrane protein 116	1453710_at	Tmem116	2.2	2.2	2.3	−2.4	−2.7	−2.4
TRIO and F-actin binding protein	1442383_at	Triobp	3.0	4.6	2.7	−3.1	−2.6	−2.8
vacuolar protein sorting 24 (yeast)	1459584_at	Vps24	2.4	3.1	2.7	−1.8	−1.8	−3.2
leucine-rich repeat LGI family, member 1	1435851_at	Lgi1	5.2	2.7	5.3	−4.3	−1.3	−4.3
interleukin 24	1426181_a_at	Il24	0.8	1.8	1.2	−2.0	−2.7	−3.9
kallikrein 1-related peptidase b5	1449313_at	Klk1b5	3.3	1.4	3.3	−3.2	−2.3	−3.0
serine/threonine kinase 11	1448062_at	Stk11	2.8	3.7	4.1	−1.4	−2.8	−3.3
serine/threonine kinase 32B	1431236_at	Stk32b	3.3	3.2	3.3	−1.3	−2.0	−2.1
SH3-domain GRB2-like interacting protein 1	1431300_at	Sgip1	2.5	2.6	3.0	−0.2	−1.6	−2.0
solute carrier family 23, member 3	1460042_at	Slc23a3	4.7	3.2	5.0	−0.1	−3.2	−2.1
solute carrier family 35, member A4	1420199_at	Slc35a4	2.2	3.4	3.1	0.1	−3.0	−2.3
solute carrier family 4 (anion exchanger), member 4	1450169_at	Slc4a4	2.7	2.5	2.4	−0.1	−3.5	−2.4
Transferrin receptor 2	1459061_at	Trfr2	0.7	3.8	3.6	−1.1	−2.8	−2.2
transmembrane protease, serine 11f	1441981_at	Tmprss11f	1.8	2.3	3.7	−1.2	−1.7	−4.6
Utrophin	1459009_at	Utrn	1.5	2.4	1.4	−0.7	−1.7	−2.7

Table 1. Continued

Gene description	Affimetrix probe set ID	Gene symbol	Regulation profile and ratio					
			LPS			AP		
			30 min	3 hr	6 hr	30 min	3 hr	6 hr
vascular cell adhesion molecule 1	1448162_at	Vcam1	2.4	3.3	5.6	0.4	−1.7	−2.9
caspase 3	1430192_at	Casp3	1.8	0.8	2.0	−1.7	−1.6	−1.0
chemokine (C-X-C motif) receptor 7	1417625_s_at	Cxcr7	4.7	4.4	0.5	−1.6	−3.5	0.2
dentin matrix protein 1	1443745_s_at	Dmp1	2.9	4.3	1.1	−3.4	−3.1	−0.1
glutamate receptor, ionotropic, kainate 2 (beta 2)	1457683_at	Grik2	4.0	1.5	4.0	−3.9	−2.1	−0.5
integrin beta 8	1436223_at	Itgb8	5.3	6.5	5.3	−4.2	−4.0	0.1
Rho guanine nucleotide exchange factor (GEF) 11	1446904_at	Arhgef11	3.3	3.7	2.1	−1.8	−1.9	−1.3
Src family associated phosphoprotein 1	1441418_at	Skap1	4.1	3.3	1.4	−1.8	−1.6	−0.9
synapsin II	1428460_at	Syn2	2.7	2.9	0.5	−1.8	−2.7	−1.4
T-cell receptor beta, variable 13	1427752_a_at	Tcrb-V13	4.0	3.5	4.2	−3.2	−2.5	−0.2
ATPase, Na^+/K^+ transporting, alpha 3 polypeptide	1424856_at	Atp1a3	3.3	3.0	3.3	−2.9	−0.9	−1.0
fibroblast growth factor receptor 3	1425796_a_at	Fgfr3	3.3	3.7	3.1	−3.4	−0.6	−0.5
immunoglobulin kappa chain variable 1 (V1)	1452536_s_at	Igk-V1	3.4	3.4	2.2	−2.5	−0.6	−0.4
integrin binding sialoprotein	1417484_at	Ibsp	4.5	1.6	3.1	−3.6	−0.6	−1.4
mast cell protease 4	1425967_a_at	Mcpt4	4.0	4.6	3.3	−3.5	−1.4	−0.5
Ras association (RalGDS/AF-6) domain family 1	1456994_at	Rassf1	4.3	3.5	3.9	−3.8	−0.9	−0.3
T-cell specific GTPase	1449009_at	Tgtp	2.2	1.0	4.1	−3.5	−1.5	−0.9
ubiquitin carboxyl-terminal esterase L4	1421766_at	Uchl4	5.7	4.9	4.3	−4.1	−1.2	−1.5
aspartic peptidase, retroviral-like 1	1452732_at	Asprv1	2.7	2.1	2.3	−0.7	−3.5	−1.0
CASP8 and FADD-like apoptosis regulator	1425687_at	Cflar	0.2	2.1	2.2	0.5	−1.7	−1.4
histamine receptor H 3	1448807_at	Hrh3	2.0	2.2	2.7	−0.5	−0.6	−0.8
immunoglobulin superfamily containing leucine-rich repeat	1418450_at	Islr	4.9	4.6	5.0	−1.3	−0.8	−1.2
intercellular adhesion molecule	1424067_at	Icam1	−0.1	2.1	1.9	0.6	0.1	−0.1
interleukin 7	1422080_at	Il7	2.1	5.7	2.5	−1.4	−4.3	−0.7
lymphotoxin A	1420353_at	Lta	2.2	4.4	6.0	−0.7	−1.6	−0.7
macrophage activation 2 like	1447927_at	Mpa2l	−0.4	2.2	4.0	0.6	−0.4	−0.5
MAD homolog 9 (Drosophila)	1450265_at	Smad9	1.5	3.0	2.4	−1.5	−2.6	0.0
matrix metallopeptidase 15	1437462_x_at	Mmp15	2.2	2.9	2.9	−0.2	−0.9	0.1
matrix metallopeptidase 2	1416136_at	Mmp2	1.6	3.0	3.8	−0.7	0.1	−0.4
nitric oxide synthase 1 (neuronal) adaptor protein	1437485_at	Nos1ap	2.8	3.6	1.6	−1.3	0.1	−0.1
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	1426031_a_at	Nfatc2	1.2	0.8	2.2	−1.3	−0.4	−0.6
RAS p21 protein activator 4	1417333_at	Rasa4	0.4	2.1	3.1	−0.7	−0.6	−1.4
reproductive homeobox 9	1449540_at	Rhox9	3.8	2.3	2.5	−4.6	−1.8	−1.8
protocadherin beta 4	1440632_at	Pcdhb4	3.0	4.5	4.6	−3.5	−3.7	−5.5

GTPase (*Tgtp*), *Il7*, and macrophage activation 2 like (*Mpa2l*) were down regulated in AP-treated BV-2 microglial cells (Table 1). In addition, *Il10*, a potent anti-inflammatory cytokine[26] was significantly increased in this study (Table 1).

Taken together, these results indicate that AP may have potential efficacy for the treatment of inflammation disease and other neurodegenerative diseases through anti-neuroinflammation by inhibiting TGFβ signaling and MAPK pathway. The microarray-based genomic survey has been of interest in the study of herbal-medicines because it can quickly identify herbs with the potential for use for treatment of specific diseases based on their characteristic expression profiles and the generated profiles can also be used to identify putative mechanisms of action.

Materials & Methods

Preparation of *Agrimonia pilosa* (AP)

AP that was purchased from Sun Ten Pharmaceutical (Taipei, Taiwan), powdered to 0.1 g and then extracted by stirring in 10 mL of DW (distilled water) overnight at room temperature. The sample was then centrifuged for 10 min at 3,000 rpm, after which the supernatant was removed and sterilized by passing it through a 0.22 μm syringe filter and then used for the experiments.

Cell Culture

The immortalized murine BV-2 microglial cell line, which exhibits both the phenotypic and functional properties of reactive microglia cells, was grown and maintained in 100% humidity and 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin, and penicillin (Invitrogen Life Technologies, Rockville, USA). BV-2 microglial cells were then plated onto 100 mm dish (1×10^7/mL) for 24 hr, and then pretreated with 1 μg/mL AP or left untreated and incubated for 30 min. Next, 1 μg/mL LPS was added to the samples, and the cells were then reincubated at 37°C for 30 min, 3 hr and 6 hr.

RNA Preparation

BV-2 microglial cells were initially cultured in a 100 mm dish (1×10^7/mL) for 24 hr, and then pretreated with 1 μg/mL AP or left un-treated. The cells were then incubated for 30 min, after which 1 μg/mL LPS was added. Next, the cells were re-incubated at 37°C for 30 min and 1 hr. The RNA was then isolated from the BV-2 microglial cells using an Rneasy® mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, after which the RNA was quantified using NanoDrop (NanoDrop Technologies, Inc ND-1000; Wilmington, DE, U.S.A).

Oligonucleotide Chip Microarray

Oligonucleotide chip microarray was performed using single round RNA amplification protocols, following the Affimetrix specifications (Affimetrix GeneChip Expression Analysis Technical Manual). Briefly, 3 micrograms of total RNA were used to synthesize first-strand complementary DNA (cDNA) using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primers (Proligo LLC, Boulder, CO) and the Superscript Choice System (Life Technologies, Invitrogen, Milan, Italy). After double-stranded cDNA synthesis, the products were purified by phenol-chloroform extraction, and then biotinylated antisense complimentary RNA (cRNA) was generated through *in vitro* transcription using a BioArray RNA High-Yield Transcript Labeling Kit (ENZO Life Sciences Inc., Farmingdale, NY). The biotinylated labeled cRNA was then fragmented, and 10 μg of the total fragmented cRNA was hybridized to the Affymetrix Mouse 430 2.0 GeneChip array (P/N900470, Affymetrix Inc., USA). The Affimetrix Fluidics Station 400 was then used to wash and stain the chips, after which the nonhybridized target was removed. Next, the samples were incubated with a streptavidin-phycoerythrin conjugate to stain the biotinylated cRNA. The staining was then amplified using goat IgG as blocking reagent and biotinylated antistreptavidin antibody (goat), followed by a second staining step using a streptavidin-phycoerythrin conjugate. The fluorescence was detected using the Genechip System Confocal Scanner (Hewlett-Packard), and analysis of the data contained on each GeneChip was conducted using the GeneChip 3.1 software produced by Affymetrix, using the standard default settings. To compare different chips, global scaling was used, with all probe sets being scaled to a user-defined target intensity of 150.

Data Analysis

The MAS5 algorithm was used to evaluate the expression signals generated by the Affymetrix Mouse 430 2.0 array. Global scaling normalization was then performed and the normalized data were log-transformed with base 2. Next, Fold change was applied to select the differentially expressed genes (DEGs) using a fold change threshold of 1.5-fold and a $P < 0.05$ to indicate significance. Each probe set used in the Affymetrix GeneChip produces a detection call, with P (present call) indicating good quality, M (marginal call) indicating intermediate quality and A (absent call) indicating relatively low reliability. Therefore, probe sets that resulted in A calls in the compared groups were removed to filter false positives. The 1.5-fold DEGs were clustered using the GenPlex™ v2.3 software (ISTECH Inc., Korea using hierarchical clustering with Pearson correlation as a similarity measure and complete linkage as the linkage method). In addition, gene ontology significance analysis was conducted to investigate the functional relationships among the 1.5-fold DEGs using high-throughput GoMiner. The 1.5-fold DEGs were then mapped to relevant pathways using GenPlex™ v2.4 software (ISTECH Inc., Korea). The pathway resources were provided by the KEGG database.

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