Mol. Cells 2015; 38(9): 765-772
http://dx.doi.org/10.14348/molcells.2015.0062

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The Genome-Wide Expression Profile of *Saussurea lappa* Extract on House Dust Mite-Induced Atopic Dermatitis in Nc/Nga Mice

Hye-Sun Lim[1,3], Hyekyung Ha[1], Hyeun-Kyoo Shin[1], and Soo-Jin Jeong[2,4,*]

***Saussurea lappa* has been reported to possess anti-atopic properties. In this study, we have confirmed the *S. lappa*'s anti-atopic properties in Nc/Nga mice and investigated the candidate gene related with its properties using microarray. We determined the target gene using real time PCR in *in vitro* experiment. *S. lappa* showed the significant reduction in atoptic dermatitis (AD) score and immunoglobulin E compared with the AD induced Nc/Nga mice. In the results of microarray using back skin obtained from animals, we found that *S. lappa*'s properties are closely associated with cytokine-cytokine receptor interaction and the JAK-STAT signaling pathway. Consistent with the microarray data, real-time RT-PCR confirmed these modulation at the mRNA level in skin tissues from *S. lappa*-treated mice. Among these genes, PI3Kca and IL20Rβ were significantly downregulated by *S. lappa* treatment in Nc/Nga mouse model. In *in vitro* experiment using HaCaT cells, we found that the *S. lappa* components, including alantolactone, caryophyllene, costic acid, costunolide and dehydrocostus lactone significantly decreased the expression of PI3Kca but not IL20Rβ *in vitro*. Therefore, our study suggests that PI3Kca-related signaling is closely related with the protective effects of *S. lappa* against the development of atopic-dermatitis.**

INTRODUCTION

Saussurea lappa has traditionally been used in Asian countries as an ingredient in the treatment of abdominal pain and tenesmus, and an analgesic, digestive aid, aphrodisiac, and diuretic. Many authors have reported that *S. lappa* exhibits biological effects such as antiulcer, anticancer, and hepatoprotective effects (Byambaragchaa et al., 2014; Chen et al., 1995; Kim et al., 2014; Yoshikawa et al., 1993). We have recently reported anti-AD effects of *S. lappa* as shown in both *in vitro* and *in vivo* assays (Lim et al., 2014). *S. lappa* decreased the immunoglobulin E, cytokines and chemokines with the reduction in histopatholgical features of atopic dermatitis (AD) lesion. However, there is no study on identifying an active component of *S. lappa* exhibiting curative effects against AD and its mechanism of actions.

AD is a chronic skin disease involving skin barrier dysfunction and cutaneous inflammatory hypersensitivity, and has a strong genetic basis (Cookson, 2001). Various studies have indicated that AD has a complex etiology that involves the activation of multiple immunological and inflammatory mechanisms (Leung and Bieber, 2003; Novak et al., 2003). Currently, many researchers have been investigating target genes that can apply to treat AD using a various experiments (Choi et al., 2014; Choy et al., 2012; Lu et al., 2009; Zhang et al., 2014). Therefore, candidate for treating AD need to identify target molecule exerting its curative effects throughout a gene analysis.

Microarray analysis is a molecular technique that enables the parallel analysis of expression by a very large number of genes encompassing a significant fraction of the human genome. This method is both qualitative and quantitative because it can detect changes in the expression levels in treated cells based on comparisons with control samples (Kim et al., 2006; Wang et al., 2006; Yu et al., 2011). The use of microarray analysis may be helpful in the development of more advanced therapies for the treatment of AD using naturally derived products.

In the present study, we used microarray analysis to evaluate the systemic biological activities of *S. lappa* extract in a house dust mite-induced AD model in Nc/Nga mice. We found that of *S. lappa* extract reduced the expression of genes related to allergy metabolism in Nc/Nga mice. The results of our evaluation and the possible mechanisms to explain these results are discussed herein.

MATERIALS AND METHODS

Reagents and chemicals

Biostir-AD®, which is an ointment that contains house dust mite (*Dermatophagoides farinae*) extract, was purchased from Biostir

[1]K-herb Research Center, [2]Korean Medicine Convergence Research Division, Korea Institute of Oriental Medicine, Daejeon 305-811, Korea, [3]Division of Allergy and Chronic Respiratory Diseases, Center for Biomedical Sciences, Korea National Institute of Health, Cheongju 361-951, Korea, [4]Korea Medicine Life Science, University of Science & Technology, Daejeon 305-350, Korea
*Correspondence: sjijeong@kiom.re.kr

Received 9 March, 2015; revised 31 July, 2015; accepted 5 August, 2015; published online 21 August, 2015

Keywords: atopic dermatitis, microarray, Nc/Nga mouse model, PI3Kca, *Saussurea lappa*

eISSN: 0219-1032

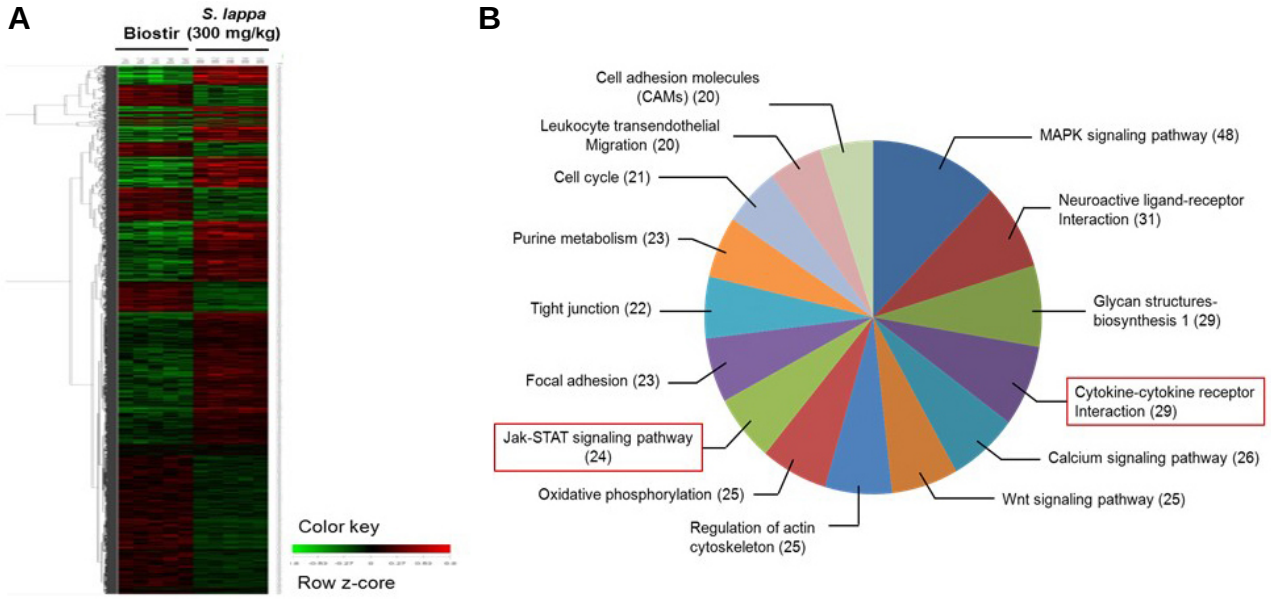

Fig. 1. Gene ontology assignment of differentially expressed genes. Clustergram of up- and down-regulated genes in skin tissue from Nc/Nga mice. Microarray data from control (untreated cells) and experimental groups (300 mg/kg *S. lappa*-treated group) were combined and clustered. (A) Each gene is represented by a single row of clustered boxes and each group is represented by a single column. (B) Gene ontology classification based on biological processes.

Inc. (Japan). Reference compounds, costunolide, dehydrocostus lactone, alantolactone, costic acid, and trans- caryophyllene, were purchased from ChemFaces (China). The purities of the five components were ≥ 98.0% by HPLC analysis.

Plant materials and preparations 70% methanol extract

The *S. lappa* material used in this experiment was purchased from HMAX (Korea) in 2009. A specimen (2009-KIOM62) has been deposited in the K-herb Research Center, Korea Institute of Oriental Medicine. Dried *S. lappa* (100 g) was extracted three times with 70% methanol (1 L) by refluxing for 90 min. The extract was filtered, evaporated to dryness, and freeze-dried using a freeze dryer (PVTFD100R, Ilshin Lab Co., Korea). The yield was 28.6%.

Experimental animals

Specific pathogen-free male Nc/Nga mice (eight weeks old) were purchased from Central Laboratory Animal Inc. (Korea). The animals were maintained in an air-conditioned room and maintained at 24 ± 2°C with 55 ± 15% humidity. The animals were housed one per cage and were allowed sterilized tap water and standard rodent chow (Samyang Feed Co, Korea) ad libitum. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee (Approval number: #12-047).

Induction of AD in Nc/Nga mice

AD-like skin lesions were induced in 10-week-old male Nc/Nga mice using Biostir-AD, as described by the manufacturer (Lee et al., 2010). The animals were housed in conventional conditions. Briefly, the upper back was shaved, and 200 μl of 4% (w/v) sodium dodecyl sulfate was applied to the shaved dorsal skin and both surfaces of each ear to induce barrier disruption. Two hours after shaving, 50 mg of Biostir-AD was applied topically twice per week for six weeks. Prednisolone and *S. lappa* were dissolved in distilled water and administrated by oral gavage 6 h after Biostir-AD application for six weeks. The mice (n = 30) were allocated to five groups (n = 6/group): (A) normal control group that received distilled water (DW, p.o.); (B) Biostir group that received Biostir-AD (topical application) and DW (p.o); (C) prednisolone group that received Biostir-AD (topical application) and prednisolone (3 mg/kg, p.o.); *S. lappa* groups that received Biostir-AD (topical application) and *S. lappa* (100 or 300 mg/kg, p.o.). Prednisolone was used as a positive control and was a recommended medication to patient with AD. The mice were sacrificed under anesthesia via intraperitoneal injection of pentobarbital sodium (Entobar Inj., Hanlim Pharm. Co., Ltd., Korea) on the day of the experiment. The incidence of atopic dermatitis was determined by gross and histopathological examination for back skin and ear and the level of IgE in plasma (Supplementary Fig.1).

RNA preparation

RNA was isolated from dorsal skin tissue using TRIzol reagent (Invitrogen, USA) using the following procedure. Initially, 1 ml of TRIzol reagent was added to cells grown in culture dishes. After 5 min at room temperature, 0.2 ml of chloroform was added for each sample of reagent, and the tubes were shaken vigorously by hand for 15 s and then incubated at room temperature for 3 min. Next, the mixture was centrifuged at 14,000 rpm for 15 min at 4°C, after which the resulting upper aqueous phase (400 μl) was transferred to a fresh tube into which 0.5 ml of 2-propanol was also added. After incubation for 10 min at 4°C, the mixture was centrifuged again at 14,000 rpm for 10 min at 4°C. After separation, the supernatant was removed, washed with 1 ml of 75% ethanol and centrifuged again at 10,000 rpm for 5 min at

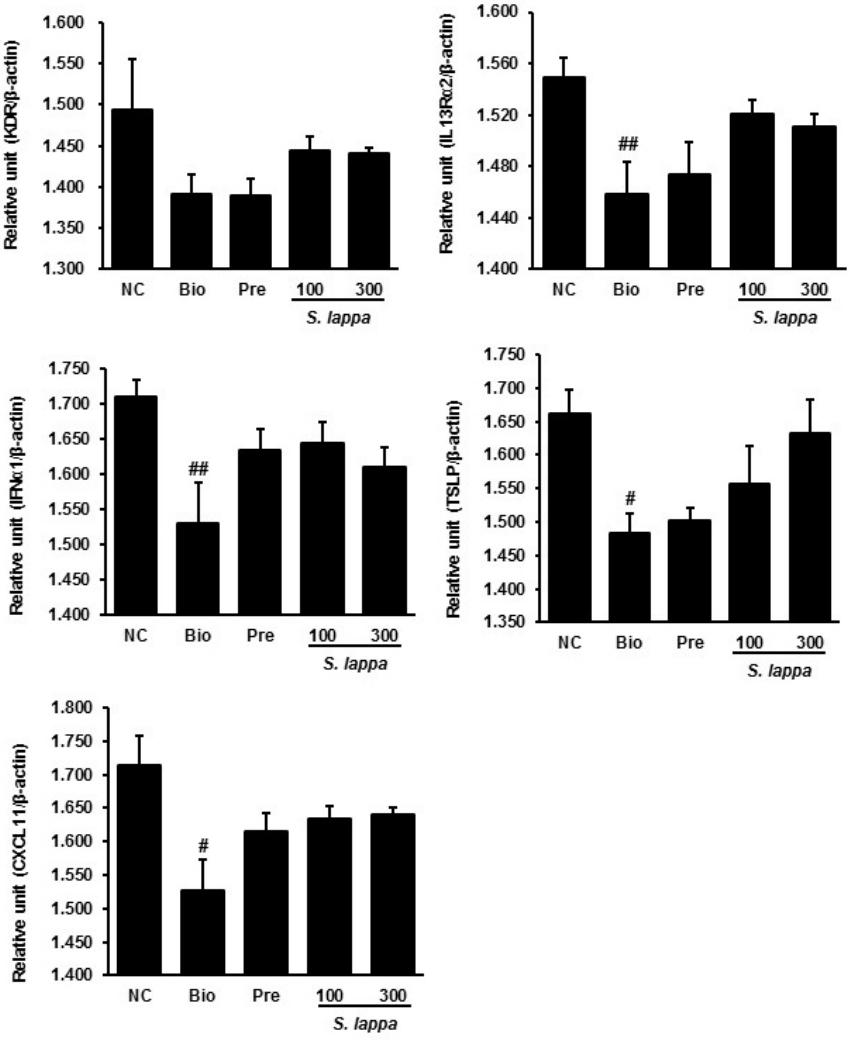

Fig. 2. Validation of selected up-regulated genes using real-time qPCR in skin tissue from Nc/Nga mice. The results are normalized as a ratio of each specific mRNA signal (KDR, IL13Rα2, IFNα1, TSLP, and CXCL11) to the β-actin gene signal within the same sample and the values expressed. Data are presented as mean ± SD (n = 3). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. normal control.

4°C. The resulting RNA pellet was then dried briefly, and the purified RNA was dissolved in diethyl pyrocarbonate (DEPC)-distilled water. The RNA was cleaned up using the Rneasy Mini kit (Qiangen, Germany) in accordance with the manufacturer's instructions. The quality and quantity of total RNA were measured with an Experion™ system (Bio-Rad, USA).

Labeling and purification

Total RNA was amplified and purified using and Ambion Illumina RNA amplification kit (Ambion, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, transcribed *in vitro*, and labeled with biotin-NTP. After purification, the cRNA was quantified using an ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA).

Hybridization and data export

Illumina Mouse Ref-8 expression BeadChip (P/N BD-25-203, Illumina Inc., Ambion) arrays were used in this study. Seven hundred fifty nanograms of labeled cRNA samples were hybridized to each mouse Ref-8 expression bead array for 16-18 h at 58°C according to the manufacturer's instructions. The array signal was detected using Amersham Fluorolink streptavidin- Cy3 (GE Healthcare Bio-Sciences, USA) following the bead array reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis were performed using Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4).

Data analysis

The BeadStudio (version 3.0) was used to evaluate the expression signals generated by the Illumina Mouse Ref-8 expression BeadChip array. Global scaling normalization was then performed, and the normalized data were log-transformed using base 2. Next, the fold change and Welch's t-test were applied to select the differentially expressed genes (DEGs) using a fold change threshold of 2-fold and a $p < 0.05$ to indicate significance. The 2-fold DEGs were clustered using GenPlex™ v3.0 software (ISTECH Inc., Korea) using hierarchical clustering with Pearson correlation as a similarity measure and complete linkage as the linkage method. Gene ontology classification was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Real-time RT-PCR analysis

To verify the microarray results, real-time RT-PCR analysis was performed for selected genes using an Applied Biosystems

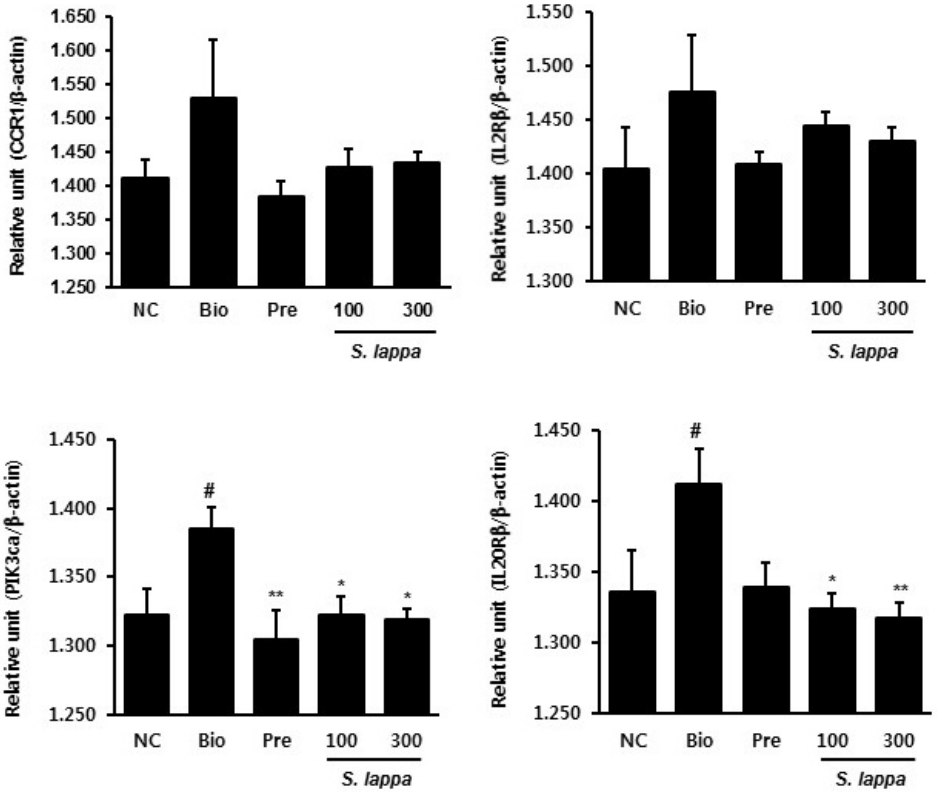

Fig. 3. Validation of selected down-regulated genes using real-time qPCR in skin tissue from Nc/Nga mice. The results are normalized as a ratio of each specific mRNA signal (CCR1, IL2Rβ, PI3Kca, and IL20Rβ) to the β-actin gene signal within the same sample and the values expressed. Data are presented as mean ± SD (n = 3). #p < 0.05 vs. normal control. *p < 0.05 and **p < 0.01 *vs.* Biostir-treated group.

7300 Real-time PCR system and the SYBR green fluorescence quantification system (Applied Biosystems, USA) to quantify the amplicons. cDNA was synthesized using 100 ng of RNA in a reverse transcription reaction. The PCR conditions were 50 cycles of 95°C (30 s), 53°C (30 s), and a standard denaturation curve. The primer sequences are listed in the 5′ to 3′ orientation in Supplementary Table 1. The PCR conditions for each target were optimized according to the primer concentration, the absence of primer dimer formation, and the efficiency of amplification of both the target genes and the housekeeping control gene. PCR reactions mixture comprised 1 μl of cDNA and 9.5 μl of PCR master mix, which contained 2× SYBR Green, 10 pmole each of the forward and reverse primer, and 4.5 μl of DEPC-distilled water in a final volume of 15 μl. To normalize the cDNA content of the samples, we used the comparative threshold (C_T) cycle method, which includes normalization of the number of target gene copies vs. the endogenous reference gene, β-actin. The C_T is defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe passes a fixed threshold baseline when amplification of the PCR products is first detected.

Cell culture

Human keratinocyte HaCaT cells were obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco Inc., USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco Inc.), penicillin (100 U/ml), and streptomycin (100 μg/ml), in a 5% CO_2 incubator at 37°C.

Statistical analyses

The data are expressed as the mean ± SEM. The data were analyzed by one-way analysis of variance and Dunnett's multiple-comparisons test. Results with a P value < 0.05 were considered significant.

RESULTS

Gene expression profile of *S. lappa* extract in a house dust mite-induced AD Nc/Nga mouse model

Gene expression profiles were significantly up or downregulated in the *S. lappa* extract group compared with the Biostir group. A total of 4255 genes that were differentially expressed from about 24,000 genes detected in the experimental group. A hierarchical clustering algorithm was used to group the genes based on their similar expression patterns. Gene ontology annotation was achieved using the KEGG database and the genes were placed into 173 biological pathways (Supplementary Table 2): MAPK signaling pathway (48 genes), neuroactive ligand-receptor interaction (31 genes), glycan structures biosynthesis 1 (29 genes), cytokine-cytokine receptor interaction (29 genes), calcium signaling pathway (26 genes), Wnt signaling pathway (25 genes), regulation of actin cytoskeleton (25 genes), oxidative phosphorylation (25 genes), JAK-STAT signaling pathway (24 genes), focal adhesion (23 genes), tight junction (22 genes), purine metabolism (23 genes), cell cycle (21 genes), leukocyte transendothelial migration (20 genes), and cell adhesion molecules (20 genes) (Fig. 1). Among the 173 biological pathways that were up or downregulated, we selected five upregulated and six downregulated allergy-related pathways. The functionally annotated upregulated (40) and downregulated (45) genes are listed in Supplementary Tables 3 and 4, which show comparisons of the expression levels for a variety of genes between the Biostir and *S. lappa* group.

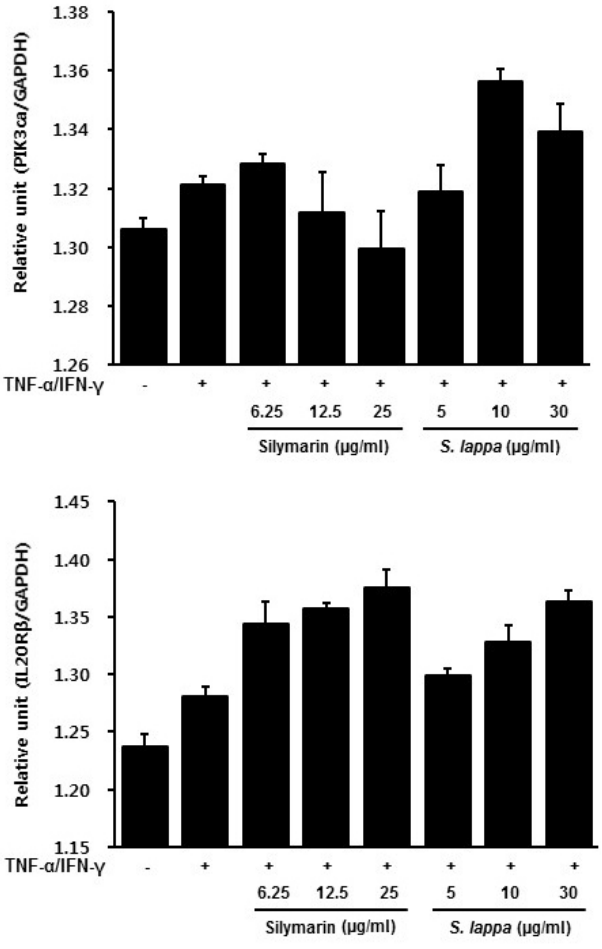

Fig. 4. Effects of *S. lappa* on gene expression determined using real-time RT-PCR in TNF-α and IFN-γ-treated HaCaT cells. The results are normalized for each specific mRNA signal to the GAPDH gene signal within the same sample and the values expressed. (A) PI3Kca and (B) IL20Rβ.

Validation of selected genes using real-time RT-PCR

To confirm the effects of *S. lappa* extract on AD metabolism genes at the mRNA level, real-time RT-PCR was conducted for 10 selected genes involved in cytokine-cytokine receptor interaction and the JAK-STAT signaling pathway. Consistent with the results of the microarray analysis, *S. lappa* extract increased the expression of kinase insert domain protein receptor (KDR), interleukin 13 receptor alpha 2 (IL13Rα2), interferon alpha 1 (IFNα1), thymic stromal lymphopoietin (TSLP), and chemokine (C-X-C motif) ligand 11 (CXCL11) in the Biostir group (Fig. 2). In addition, *S. lappa* extract suppressed the expression of chemokine (C-C motif) receptor 1 (CCR1), interleukin 2 receptor beta (IL2Rβ), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3Kca), and interleukin 20 receptor beta (IL20Rβ) in the Biostir group (Fig. 3). Among the downregulated genes, *S. lappa* significantly suppressed PI3Kca and IL20Rβ. The relative expression levels of each gene were normalized relative to the expression of β-actin, a well-known housekeeping gene.

Effects of *S. lappa* and its components on validation of gene expression

To examine the downregulatory effects of PI3Kca, and IL20Rβ in *S. lappa*-treated in HaCaT cells, the cells were treated with tumor necrosis factor alpha (TNF-α) and IFN-γ in the absence or presence of *S. lappa* extract. As shown in Figure 3, *S. lappa* had no significant effect on PI3Kca or IL20Rβ expression in TNF-α/IFN-γ stimulated HaCaT cells. However, five components of *S. lappa* suppressed PI3Kca mRNA expression in TNF-α/IFN-γ-stimulated HaCaT cells. Among the five components of *S. lappa*, costic acid and dehydrocostus lactone suppressed PI3Kca mRNA expression in a dose-dependent manner (Fig. 4). By contrast, no inhibitory effect of these components on IL20Rβ expression was observed in TNF-α/IFN-γ-stimulated HaCaT cells (Fig. 5).

DISCUSSION

Genome-wide association studies are a hypothesis-free way to discover disease-associated microarray (Barnes, 2010). This avoids the limitations of using candidate genes, while retaining the advantages of the case-control approach. Genome sequencing has identified large numbers of genetic variants that can be read on microarrays (Lindblad-Toh et al., 2005). This allows identification of microarrays that are expressed more frequently in affected individuals than in control subjects. The disease-associated microarray marker regions of the genome identified in this study may be involved in the pathogenesis of AD. In the present study, we used microarray analysis to elucidate the underlying biological effects in *S. lappa* extract-treated Nc/Nga mice. The genes regulated by *S. lappa* extract in Nc/Nga mice were classified using the KEGG database. Interestingly, we detected genes related to allergy-related molecular mechanisms including cytokine-cytokine receptor interaction, JAK-STAT signaling, T-cell receptor signaling, Toll-like receptor signaling, and the mTOR signaling pathways. To confirm these findings, we selected genes related to cytokine-cytokine receptor interactions (CCR1, IL2Rβ, TSLP, KDR, and CXCL11) and JAK-STAT signaling (PI3Kca, IL20Rβ, IFNα1, TSLP, and IL13Rα2).

Several researchers have demonstrated the role of cytokine-cytokine receptor interactions and JAK-STAT signaling in development of AD. TSLP, an interleukin 7-like cytokine, is known to trigger dendritic cell-mediated Th2 inflammatory responses and highly expresses in activated mast cells and skin lesion of AD, which is triggers allergic inflammation (Isaksen et al., 2002; Liu et al., 2006; Sebastian et al., 2008). KDR is a protein-coding gene that is also known as vascular endothelial growth factor receptor 2 (VEGFR-2), the gene that encodes the VEGFR. Activation of the VEGFR leads to activation of endothelial nitric oxide synthase, which also depends on the activation of KDR (Holmes et al., 2007). KDR significantly up-regulated by activated protein C (Tanimoto et al., 2002). CXCR3 is activated by the three IFN-γ-inducible chemokines of the CXC family: CXCL9, CXCL10, and CXCL11 (Lu et al., 1999). The three ligands for CXCR3 are responsible for the recruitment of immune cells at sites of infection and inflammation (Lasagni et al., 2003; Lu et al., 1999). By contrast, interferon alpha1 (INF-α1) has immunoregulatory functions in autoimmune inflammatory diseases and is an important component of the innate immune system in a number of autoimmune or inflammatory diseases including AD (Borden et al., 2007; Theofilopoulos et al., 2005). IL-13 is associated with multiple diseases such as asthma and allergy (Heinzmann et al., 2000). Two IL-13 receptors have

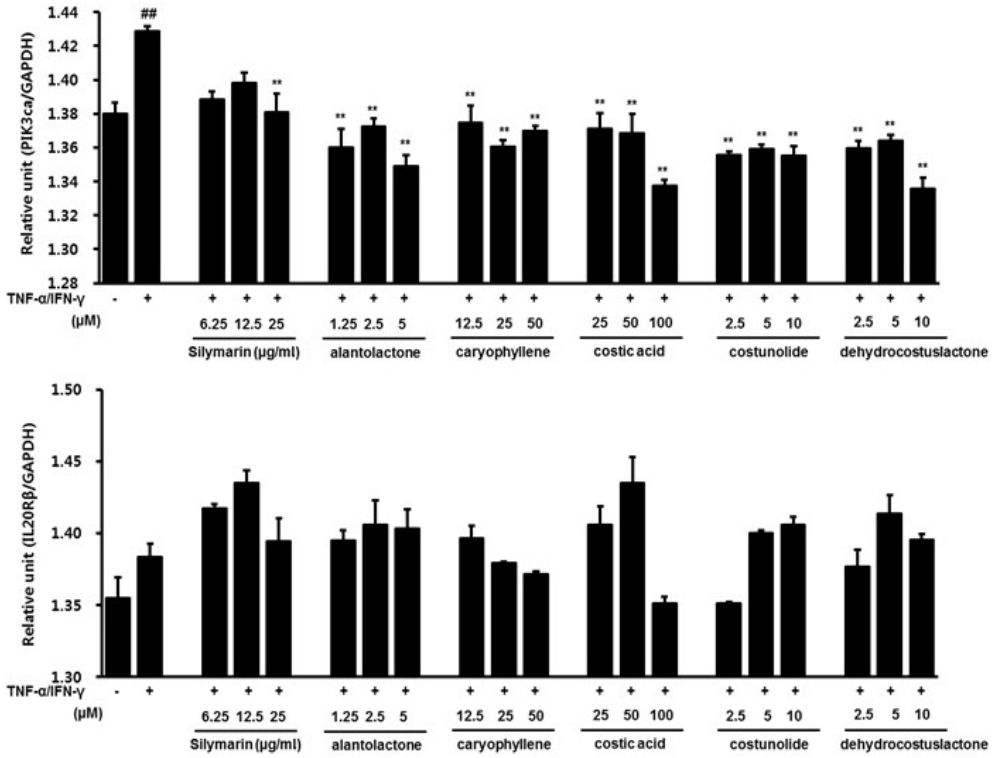

Fig. 5. Effects of five components of *S. lappa* on the validation of gene expression using real-time RT-PCR in HaCaT cells. The results are normalized as a ratio of the (A) PI3Kca and (B) IL20Rβ mRNA signal to the GAPDH gene signal within the same sample, and the values expressed. Values are expressed as mean ± SEM of three independent experiments. ##P < 0.01 vs. vehicle control cells; **P < 0.01 *vs.* TI-treated cells.

been identified, IL-13Rα1 and IL-13Rv2. IL-13Rα2 has been implicated in allergy, bronchial asthma, atopy, and esophageal diseases (Heinzmann et al., 2000; Zuo et al., 2010). In addition, IL2Rβ is associated with a greater predisposition to type I diabetes, rheumatoid arthritis, and multiple sclerosis (Burchill et al., 2007). The association between the IL2Rβ genes and multiple autoimmune diseases suggests a common mechanism in their pathogenesis (Burchill et al., 2007; Matesanz et al., 2001). In this study, we found that *S. lappa* extract upregulated the genes related to AD metabolism in skin samples from Nc/Nga mice. *S. lappa* extract caused overexpression of TSLP, KDR, CXCL11, IFNα1, and IL13Rα2 genes in house dust mite-treated mice. These results suggest that *S. lappa* extract has protective effects on AD metabolism.

PI3K, the most important member of the PI3K complex, comprises a heterodimer with a p85 regulatory subunit and a p110 catalytic subunit (PI3Kca). PI3Kca is one of the most important downstream regulators of multiple receptor kinase families, which are involved in many fundamental cellular processes, including proliferation, cell survival, motility, and cell growth (Bader et al., 2005; Engelman et al., 2006). The importance of PI3K p110 in various functions of leukocytes such as B cells, T cells, NK cells, myeloid cells, macrophages, keratinocytes, and mast cells is well documented (Fung-Leung, 2011). Previous studies have demonstrated that blockade of p110 activity significantly inhibits allergic inflammation, which suggests an important role for PI3K p110 in allergy (Nashed et al., 2007). The IL-20 cytokine subunit, IL20Rβ is highly expressed in skin lesions (Sa et al., 2007) and induces STAT3 phosphorylation (Dumoutier et al., 2001). Previous studies have shown that STAT3 phosphorylation in epidermal keratinocytes is implicated in the development of psoriasis (Sano et al., 2005). In the present study, the effects of *S. lappa* extract on genes involved in cytokine-cytokine receptor interaction and JAK-STAT signaling were confirmed by real-time RT-PCR. We found that *S. lappa* extract significantly decreased the mRNA levels of PI3Kca and IL20Rβ in a dose-dependent manner in skin tissue from Nc/Nga mice. Although *S. lappa* extract did not affect PI3Kca expression, some of its components significantly reduced PI3Kca mRNA expression in TNF-α/IFN-γ-treated HaCaT cells. Difference between *S. lappa* extract and its components was considered to be due to biological condition of *in vivo* and *in vitro*. These results indicate that *S. lappa* include another components exerting elevation of PI3Kca expression. The drugs may change its character by biological conditions including gastric acid and digestive enzymes in the body. Therefore, we considered that PI3Kca mRNA expression *in vivo* significantly decreased because components of *S. lappa* exhibiting PI3Kca expression eliminate or change by biological system in the body.

Overall, our genomic analysis data demonstrate that *S. lappa* can regulate the expression of genes related to cytokine-cytokine receptor interactions and JAK-STAT signaling in a house dust mite-induced Nc/Nga mouse model of AD. Real-time PCR analysis confirmed that PI3Kca and IL20Rβ expression were significantly downregulated at the mRNA level by *S. lappa* treatment in Nc/Nga mouse model. We also found that the *S. lappa* components alantolactone, caryophyllene, costic acid, costunolide and dehydrocostus lactone significantly decreased the expression of PI3Kca but not IL20Rβ *in vitro*. Our study suggests that *S. lappa* extract may be a potential candidate for the treatment of AD by targeting PI3Kca-related signaling.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by a grant from the Korea Institute of Oriental Medicine (No. K14030).

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