

Genome-wide analysis of DNA methylation and gene expression changes in an ovalbumin-induced asthma mouse model

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Abstract. The aim of the present study was to establish an integrated network of DNA methylation and RNA expression in an ovalbumin (OVA)-induced asthma model, and to investigate the epigenetically-regulated genes involved in asthma development. Genome-wide CpG-DNA methylation profiling was conducted through the use of a methylated DNA immunoprecipitation microarray and RNA sequencing was performed using three lung samples from mice with OVA-induced asthma. A total of 35,401 differentially methylated regions (DMRs) were identified between mice with OVA-induced asthma and control mice. Of these, 3,060 were located in promoter regions and 370 of the genes containing these DMRs demonstrated an inverse correlation between methylation and gene expression. Kyoto Encyclopedia of Genes and Genomes pathway analysis identified that 368 genes were upregulated or downregulated in OVA-induced asthma samples, including genes involved in 'chemokine signalling pathway', 'focal adhesion', 'leukocyte transendothelial migration' and 'vascular smooth muscle contraction signaling' pathways. Integrated network analysis identified four hub genes, consisting of three upregulated genes [forkhead box O1 (*FOXO1*), SP1 transcription factor (*SPI*) and amyloid β precursor protein (*APP*)], and one downregulated gene [RUNX family transcription factor 1 (*RUNX1*)], all of which demonstrated an association between DNA methylation and gene expression. These genes were highly interconnected nodes in the Ingenuity Pathway Analysis module and were functionally significant. A total of four interconnected hub genes, *FOXO1*, *RUNX1*, *SPI* and *APP*, were identified from the integrated DNA methylation and gene expression networks involved in asthma development. These results suggested that

modulating these four genes could effectively control the development of asthma.

Introduction

Asthma is a chronic inflammatory airway disease that is considered an important public health concern. It usually develops in response to the inhalation of allergens, inhalants and air pollutants, and is characterized by pulmonary inflammation, airway hyper-responsiveness and mucus overproduction (1). Asthma and allergic diseases are considered to be complex genetic diseases caused by interactions between various genes and environmental factors, which are very important in the pathogenesis of asthma (2). These gene-by-environment interactions can be explained by the phenomenon of epigenetic regulation, which has provided mechanistic explanations linking molecular events and early-life environmental exposure with subsequent disease development (3). Epigenetic markers have emerged as potential participants in the mechanism of asthma, and have been demonstrated to modulate various immune cell processes involved in the development of disease (4-7). Furthermore, several previous epigenomic and transcriptomic studies have identified DNA methylation as a biomarker involved in the development of asthma (8,9). Numerous asthma-promoting environmental factors can alter the epigenetic profiles of airway cells, suggesting that environmental exposure may cause asthma or contribute to phenotypic heterogeneity changes via DNA methylation (10-12).

Experimental asthma models have been established using a variety of stimuli, such as house dust mites, ovalbumin (OVA), fungi, cockroach extracts, ragweed and latex (13). These animal models have focused on the role of type 2 immune responses driven by allergic reactions in the development of asthma (14). An OVA-induced asthma model was developed to evaluate the efficacy of therapeutic agents prior to clinical trials and to investigate their mechanisms of action; it has been used to identify the signaling pathways involved in the development of asthma (15). Additionally, the OVA-induced asthma model has often been used to identify the epigenetic mechanisms of therapeutic agents and the pathogenesis of asthma (16-18); however, to the best of the authors' knowledge, no studies have explained the association between DNA methylation and gene expression in the development of OVA-induced asthma.

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The present study aimed to establish an integrated network of DNA methylation and RNA expression in an OVA-induced asthma model and to investigate the involvement of epigenetically-regulated genes related to the development of asthma.

Materials and methods

Animals. A total of 6 female BALB/c mice (8 weeks old and weight 20–25 g) were obtained from Daehan BioLink Co., Ltd., and were housed under standard laboratory conditions (22±2°C, 60% relative humidity, 12/12 h light/dark cycle, and food and water *ad libitum*) for at least 1 week before the experiment began. Ethical and scientific management procedures for all animals were approved by The Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine (approval no. 17-073).

Induction of asthma using OVA. Mice were randomly divided into two groups: Vehicle-treated (n=3) and OVA-treated (n=3). Asthma was induced using OVA according to the previously described experimental procedures (19). To boost immune responses, mice were injected intraperitoneally with OVA (50 µg) emulsified with 200 µl aluminum sulfate (InvivoGen) twice, 7 days apart (on day 0 and 7). The mice were administered intranasally with 50 µl OVA (25 µg) from day 12 to 15 under anesthesia with 2% isoflurane (Piramal Critical Care, Ltd.).

Evidence of OVA-induced asthma. Pathophysiological evidence of OVA-induced asthma was indicated by airway inflammation, eosinophil and interleukin (IL)-4 production (Fig. S1). The right lungs of the mice were fixed with 10% neutral buffered formalin at 25°C for 24 h, embedded in paraffin, sectioned into 4-µm thick slices, and stained with hematoxylin for 5 min and eosin 3 min (Sigma-Aldrich; Merck KGaA) at 25°C to evaluate inflammatory responses in lung tissues, as well as with periodic acid-Schiff (IMEB Inc.) to evaluate mucus production. A tracheostomy was performed in mice anesthetized with 85 mg/kg alfaxan (Alfaxalone®; Jurox Pty Ltd.) to obtain bronchoalveolar lavage fluid (BALF). The lungs were lavaged twice with cold PBS (total volume 1.4 ml) and the obtained BALF was centrifuged at 380 x g for 5 min at 4°C. The supernatant was used for an ELISA assay (cat. no. M400B; R&D Systems, Inc.) in order to analyze level of IL-4, according to the manufacturer's protocol. The pellets of BALF were used to count inflammatory cells in the BALF. The eosinophil count was determined using a cell counting machine (Countess II™ Automated Cell Counter, Thermo Fisher Scientific, Inc.).

Statistical analysis. Statistical analysis was performed using Student's t-test with Prism 8 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Microarray data. A schematic diagram for identifying target genes involved in asthma development is presented in Fig. 1. Gene expression profile data was used from a previous study by Baek *et al.* (18), which were deposited in Gene Expression Omnibus (GSE114587; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114587>). A total of six chips were

available for further analysis, including samples from three vehicle- and three OVA-treated animals. Detailed information is available from the GEO database (18).

RNA-sequencing (RNA-seq) and data analysis. Total RNA was extracted from the lung tissues of saline-treated (normal) and OVA-induced mice. RNA isolation was performed using the PureLink™ RNA Mini kit (cat. no. 12183018A; Thermo Fisher Scientific, Inc.). RNA-seq libraries were prepared using the TruSeq RNA Sample Prep kit (Illumina, Inc.) and sequenced with a NextSeq® 500 System Whole-Genome Sequencing Solution (Illumina, Inc.) using the 76-bp paired-end reads. The raw FASTQ reads were trimmed to remove adapters and low-quality reads (per-base quality <20) using cutadapt (v.1.10, <https://cutadapt.readthedocs.io/en/stable/>) (20), then the high-quality sequence reads were aligned to the *Mus musculus* genome (mm10) using HISAT2 (v.2.1.0) and StringTie (v.1.3.4, <https://ccb.jhu.edu/software/stringtie/>) (21). Gene expression was quantified using the ballgown package in R (v.2.6.0) (22). Differentially expressed genes (DEGs) between the normal and OVA-induced mice groups were identified using the edgeR package (v.3.16.5) (23) based on negative binomial models of RNA-seq count data. Candidate DEGs were filtered using log₂(FC) ≥11 and P<0.05 as thresholds, where FC indicates the fold change. DAVID was used to functionally annotate the genes (Tables SI and SII) (24,25).

Methyl-sequencing and data analysis. DNA methylation profile data from the previous study by Baek *et al.* (18) was used. DNA libraries were prepared according to the SureSelectXT Methyl-Seq Target Enrichment System protocol (Agilent Technologies, Inc.) and sequenced using an Illumina HiSeq 2500 platform (Illumina, Inc.) to generate 101-bp paired-end reads. After sequencing, the raw FASTQ reads were trimmed to remove adapters and low-quality reads (per-base quality <20) using cutadapt (v.1.10) and mapped onto the bisulfite converted *Mus musculus* genome (mm10) using Bismark v.0.19.0 (<https://www.bioinformatics.babraham.ac.uk/projects/bismark/>). The methylKit R package (v.1.6.0) (26) was used to analyze DNA methylation. Differentially methylated regions (DMRs) are presented in Fig. 2. The base-pair methylation profile was summarized over a tiling window (window size: 1,000 bp and step-size: 500 bp). Statistically significant DMRs were selected based on their Q-value and the percentage of methylation difference (CpGs, mean methylation difference in groups >10%; Q<0.01). The genomation R package (27) was used to annotate the genomic features of DMRs, which were classified into six types (intergenic, 5'UTR, 3'UTR, promoter, CDS and intron) based on University of California Santa Cruz Genome Browser (28).

Combined analysis of DNA methylation and RNA expression. DNA methylation and gene expression are known to be closely related. To identify the genes whose RNA expression levels were affected by DNA methylation (i.e. inverse correlation between RNA expression and DNA methylation) in asthmatic mice, the expression level was adjusted to between 0 and 1 (as methylation β values range between 0 and 1). Spearman's rank correlation coefficient was used to evaluate the correlation between CpG methylation and gene expression. To understand

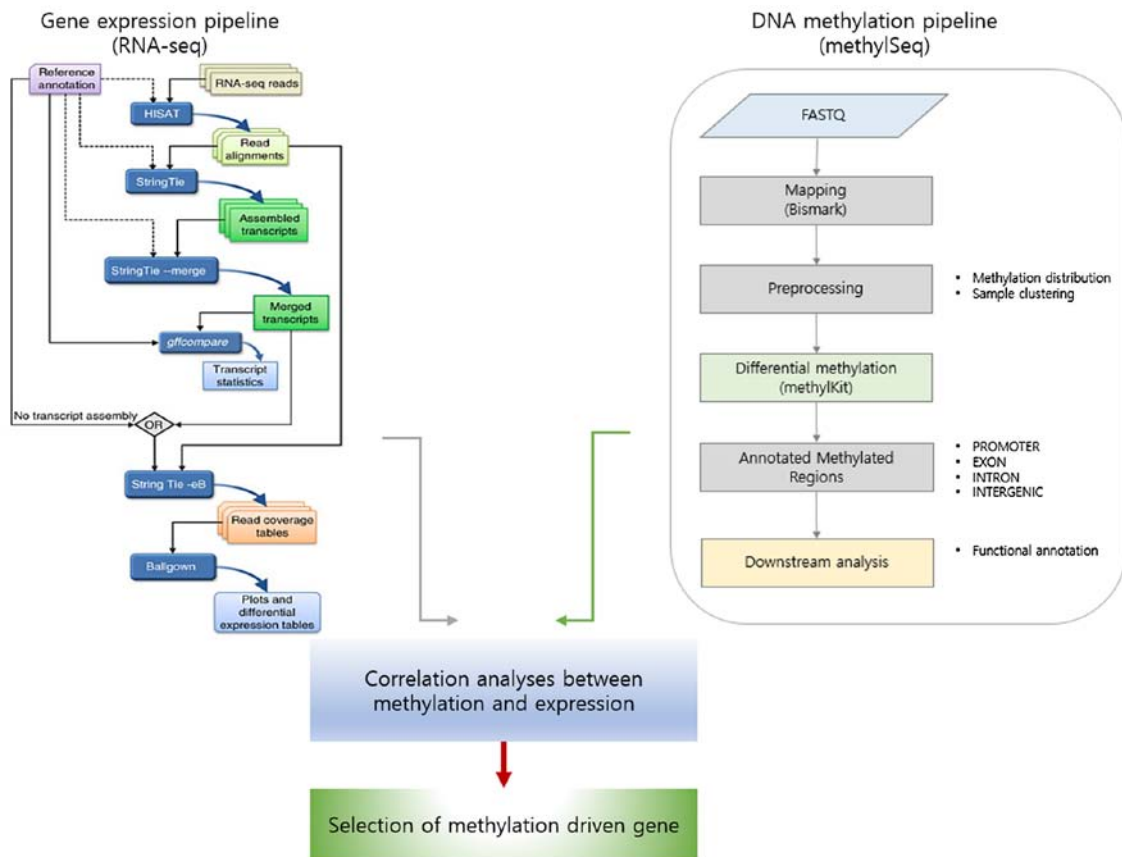


Figure 1. Schematic diagram for identifying target genes involved in asthma development. Gene Expression Omnibus (GSE114587; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114587>). RNA-seq, RNA-sequencing.

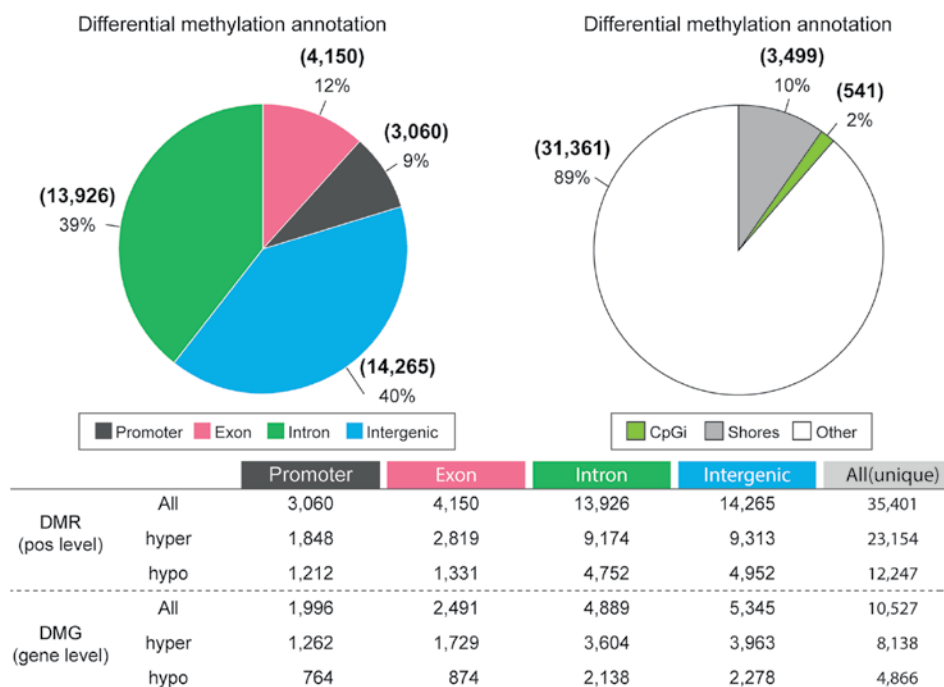


Figure 2. Genome-wide methylation profile of DMRs. DNA methylation profile data was used from the previous study by Baek *et al* (18). DNA libraries were prepared according to the SureSelectXT Methyl-Seq Target Enrichment System Protocol. CpGi, CpG islands; DMR, differentially methylated region; DMG, differentially methylated gene.

the biological mechanisms and pathways related to these genes in asthma, Gene Ontology (GO) enrichment analysis (29,30)

and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (31) mapping were conducted using DAVID (24).

Table I. Hyper-KEGG pathway functional annotation of DNA methylation.

Term	Hit	Total	P-value
Olfactory transduction	13	389	3.80×10^{-141}
Cytokine-cytokine receptor interaction	89	267	1.22×10^{-43}
Neuroactive ligand receptor interaction	98	272	1.08×10^{-41}
Pathways in cancer	170	328	4.18×10^{-34}
Systemic lupus erythematosus	25	140	8.18×10^{-34}
Huntington's disease	60	185	4.45×10^{-31}
MAPK signalling pathway	129	267	2.51×10^{-30}
Oxidative phosphorylation	30	135	2.68×10^{-29}
Chemokine signalling pathway	70	190	1.13×10^{-28}
Alzheimer's disease	55	169	1.66×10^{-28}
Natural killer cell mediated cytotoxicity	36	137	5.31×10^{-27}
Parkinson's disease	34	133	1.01×10^{-26}
Antigen processing and presentation	10	89	5.96×10^{-26}
Spliceosome	33	128	1.28×10^{-25}
Purine metabolism	55	159	2.00×10^{-25}
Regulation of action cytoskeleton	102	216	2.19×10^{-25}
Focal adhesion	100	201	3.38×10^{-22}
Cell cycle	41	128	4.23×10^{-22}
Lysosome	36	121	5.19×10^{-22}
Endocytosis	87	183	1.66×10^{-21}

8,138 genes; Gene Ontology gene set (min. size=3; max. size=1,000); 1,235 significant KEGG pathways with $P < 0.01$. KEGG, Kyoto Encyclopaedia of Genes and Genomes.

Pathway analysis. Ingenuity Pathway Analysis (IPA; v.1.13, Qiagen, Inc.) software was used to predict putative sub-networks containing the four candidate genes, whose expression altered due to changes in DNA methylation in asthma. Briefly, the Molecule Activity Predictor tool in IPA was used to interrogate sub-networks using the methylation-expression candidate genes, then integrated the sub-network by overlaying the genes involved in asthma. To examine how each gene affected the sub-network, the fold change of gene expression and DNA methylation β values were included.

Data access. The data from the present study were deposited in the GEO under accession number GSE114587 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114587>; secure token for reviewer: itytgkuoptclnej) (18).

Results

Methyl-sequencing exhibits DNA methylation alterations between vehicle-treated mice and mice with OVA-induced asthma. The DNA methylation of samples from the same animals were profiled in order to identify DMRs between the vehicle and the OVA-induced asthma samples (Fig. 2). After filtering the methylation profile, a total of 35,401 DMRs (base pair-level) were identified between the OVA-induced asthma and the saline-treated mice. Of these, 3,060 were located in promoter regions and 370 of the genes containing these DMRs demonstrated an inverse correlation between methylation and gene expression. Additionally, the number of hyper-methylated

regions was twice as high as the number of hypo-methylated regions. Nevertheless, the proportion of functional regions, such as promoters and exons, was similar between the hyper- and hypo-methylated regions (hyper-methylated: 20.1%; hypo-methylated: 20.7%). The regional distribution of DMRs was assessed based on their distance from the nearest CpG island (CpGi); shores were defined as the regions flanking the CpGi (0-2 kb range). The majority of DMRs identified in mice with OVA-induced asthma were located in other regions (89% non-CpGi and non-shore); this was followed by shores (10%) and CpGi (2%). The basepair-level DMRs were combined with the differentially methylated genes to perform an integrated analysis of methylation and gene expression. The number of hyper-methylated genes was also higher than the number of hypo-methylated genes. These DMRs, which were verified as important genes, were used to infer the mechanisms underlying the development of asthma. DAVID was used to functionally annotate the genes (Tables I and II). KEGG pathway analysis identified that 1,235 genes were more highly expressed in the OVA-induced asthma samples, than in the vehicle samples, and were mainly involved in 'chemokine signalling pathway', 'cytokine-cytokine receptor interaction', 'MAPK signalling pathway', 'neuroactive ligand receptor interaction', 'olfactory transduction' and 'oxidative phosphorylation' pathways (Table I). Conversely, 170 genes were expressed at lower levels in the OVA-induced asthma samples than in the vehicle samples, and were enriched in the 'cytokine-cytokine receptor interaction', 'MAPK signalling pathway', 'neuroactive ligand receptor interaction' and 'olfactory transduction' pathways (Table II).

Table II. Hypo-KEGG pathway functional annotation of DNA methylation.

Term	Hit	Total	P-value
Olfactory transduction	7	389	5.65×10^{-102}
Neuroactive ligand receptor interaction	55	272	5.59×10^{-33}
Systemic lupus erythematosus	14	140	1.07×10^{-25}
Cytokine-cytokine receptor interaction	72	267	5.55×10^{-25}
Pathways in cancer	113	328	9.82×10^{-23}
Huntington's disease	38	185	1.69×10^{-22}
Alzheimer's disease	31	169	2.02×10^{-22}
Oxidative phosphorylation	20	135	1.04×10^{-20}
MAPK signalling pathway	88	267	1.00×10^{-19}
Parkinson's disease	22	133	5.98×10^{-19}
Spliceosome	22	128	4.89×10^{-18}
Drug metabolism cytochrome P450	3	72	2.35×10^{-17}
Regulation of action cytoskeleton	69	216	7.53×10^{-17}
Antigen processing and presentation	9	89	1.05×10^{-16}
Ubiquitin mediated proteolysis	30	138	2.40×10^{-16}
Purine metabolism	41	159	6.54×10^{-16}
Calcium signalling pathway	52	178	1.37×10^{-15}
Cell cycle	27	128	1.63×10^{-15}
Endocytosis	57	183	6.20×10^{-15}
Steroid hormone biosynthesis	1	55	7.97×10^{-15}

4,866 genes; Gene Ontology gene set (min. size=3; max. size=1,000); 170 significant KEGG pathways with $P < 0.01$. KEGG, Kyoto Encyclopedia of Genes and Genomes.

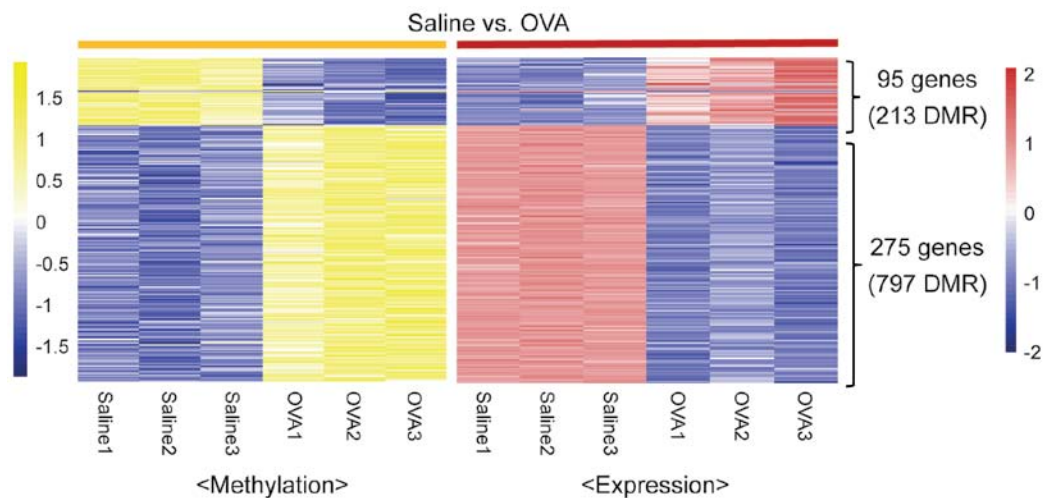


Figure 3. Correlation analysis between DNA methylation and gene expression. Gene Ontology enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway mapping using DAVID. OVA, ovalbumin; DMR, differentially methylated region. Yellow indicates hyper-methylation and blue indicates hypo-methylation in the methylation heatmap. Red indicates upregulation and blue indicates downregulation in the expression heatmap.

Identification of epigenetically-regulated genes in the development of asthma. To verify the genes modulated by DNA methylation in the development of asthma, the RNA-seq data were analyzed for correlations between DNA methylation and gene expression (Fig. 3). A total of 95 genes were upregulated, whilst 275 genes were downregulated in the OVA-induced asthma samples. DAVID was used to functionally annotate the genes. KEGG pathway analysis identified that 368 genes were

upregulated or downregulated in the OVA-induced asthma samples, and were mainly involved in 'acute myeloid leukemia', 'chemokine signalling pathway', 'focal adhesion', 'leukocyte transendothelial migration', 'vascular smooth muscle contraction' and 'neurotrophin signalling pathway' (Table III).

Identification of four genes associated with asthma development using integrated network analysis. An integrated

Table III. Kyoto Encyclopaedia of Genes and Genomes pathway functional annotation of DNA methylation and gene expression.

Term	Count	%	P-value
mmu05221: Acute myeloid leukemia	7	2.07715134	0.00156622
mmu04062: Chemokine signalling pathway	12	3.56083086	0.00232787
mmu04510: Focal adhesion	12	3.56083086	0.00446246
mmu04670: Leukocyte transendothelial migration	9	2.67062315	0.00486667
mmu04270: Vascular smooth muscle contraction	9	2.67062315	0.00511869
mmu04070: Phosphatidylinositol signalling system	7	2.07715134	0.00627478
mmu04916: Melanogenesis	8	2.37388724	0.00669627
mmu04722: Neurotrophin signalling pathway	9	2.67062315	0.00822449
mmu05200: Pathways in cancer	15	4.45103858	0.01234863
mmu04666: Fc γ R-mediated phagocytosis	7	2.07715134	0.02170314
mmu05212: Pancreatic cancer	6	1.7841543	0.02172617
mmu05213: Endometrial cancer	5	1.48367953	0.02805151
mmu00562: Inositol phosphate metabolism	5	1.48367953	0.03168093
mmu04512: ECM-receptor interaction	6	1.78041543	0.03721468

Program: DAVID Functional Annotation tool; Input: 368 genes (correlation <0 and P<0.05).

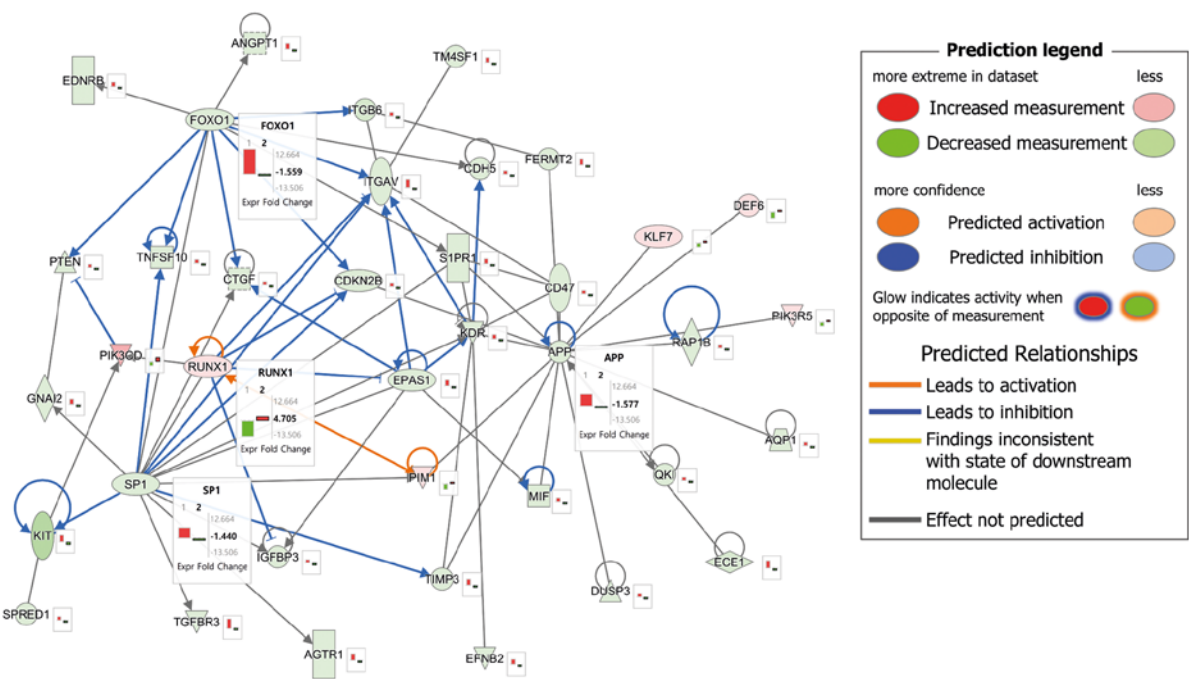


Figure 4. Gene networks involved in asthma development. Ingenuity Pathway Analysis software was used to predict putative sub-networks containing the four candidate genes. FOXO1, forkhead box O1; APP, amyloid β precursor protein; RUNX1, RUNX family transcription factor 1; SP1, SP1 transcription factor.

network analysis of DNA methylation and gene expression was conducted to understand the interactions between the genes related to asthma development. The predicted pathway is presented in Fig. 4. There were four hub genes, consisting of three upregulated genes [forkhead box O1 (*FOXO1*), SP1 transcription factor (*SP1*) and amyloid β precursor protein (*APP*)] and one downregulated gene [RUNX family transcription factor 1 (*RUNX1*)], which demonstrated the association between DNA methylation and gene expression. These genes were closely interconnected nodes in the IPA module, and were functionally significant. Therefore, *FOXO1*, *SP1*, *APP*

and *RUNX1* were identified as key integrated network regulators in the development of asthma.

Discussion

The purpose of the present study was to conduct an in-depth analysis of DNA methylation and gene expression in the lung tissues of an OVA-induced asthma mouse model. The important features of DNA methylation in asthma development and how they differ from those of gene expression were investigated. The present results identified important genes

underlying asthma development, and provided an incorporated network of DNA methylation and gene expression in mice with OVA-induced asthma.

Histopathology was conducted, and the eosinophil count and IL-4 level were determined, as indicators for establishment of asthma in animals. In the development of asthma, IL-4 leads to eosinophil-rich airway inflammation, elevated immunoglobulin E production and mucus hypersecretion by goblet cells (15). In addition, eosinophilia is considered as an important feature in allergic asthma. Activated eosinophils, as a result of various allergens, can lead to changes in levels of reactive oxygen species, cationic granule proteins, growth factors and cytokines (32). Therefore, resulting in the aggravation of allergic responses, such as airway inflammation and mucus hypersecretion (13). In the present study, the experimental model demonstrated that the eosinophil count and IL-4 were increased with inflammatory cell infiltration into lung tissue in comparison with the normal controls. This suggested that allergic asthma was successfully established in the animal model.

The present study primarily profiled the genome-wide gene and methylation expression patterns of lung tissue from OVA-induced asthmatic animals described by Baek *et al* (18) in order to examine the effect of methylation on asthma development. GO analysis demonstrated that the candidate genes were associated with cytokines/chemokines, MAPKs and oxidative stress signaling pathways. Asthma is characterized by airway inflammation, hyper-responsiveness and mucus overproduction (32); airway inflammation is induced by various factors, including cytokines, chemokines, reactive oxygen species and MAPK signaling pathways, all of which accelerate the development of asthma (33). Cytokines and chemokines induce asthmatic pathophysiological alterations, such as eosinophilia, smooth muscle contraction and mucus hyperproduction (34). MAPK signaling also plays a crucial role in the development of asthma, and MAPK phosphorylation induces airway inflammation and mucus production by activating epidermal growth factor signaling (35).

GO analysis identified a strong enrichment of terms associated with acute myeloid leukemia, chemokines, focal adhesion and vascular smooth muscle contraction signaling pathways. Furthermore, *FOXO1*, *RUNX1*, *SPI* and *APP* were identified as closely connected hub genes in the integrated networks of DNA methylation and gene expression affecting the development of asthma. *FOXO1* promotes inflammation by increasing the expression of several pro-inflammatory genes, including *IL-1B*, *IL-6* and *IL-13*, and exacerbates type 2 immune allergic airway inflammation in response to allergen challenge (36). In contrast, *RUNX1* mediates the normal maturation of immune system functions in various organisms; for example, *RUNX1* promotes T helper type 1 cell development by activating *IL-4* silencers, reducing *IL-4* expression, and elevating interferon- γ expression (37); therefore, *RUNX1* dysfunction activates T helper type 2 cell immune responses and induces asthmatic phenotypes (37). *SPI* is the transcription factor of *WNT-5A* expression in airway smooth muscle cells, and its activity and expression are regulated by the phosphorylation of MAPKs, such as p38 and JNK (38).

Recently, it was identified that *APP* was associated with childhood-onset asthma in a network-assisted analysis of

genome-wide association studies of asthma (39). Of the four genes, previous studies have demonstrated that *FOXO1* and *RUNX1* are correlated with the development of asthma (37,38). However, to the best of the authors' knowledge, *SPI* and *APP* have not been studied extensively and therefore there are insufficient data to correlate these genes with the development of asthma. In particular, in the case of *APP*, only clinical values were provided and to the best of the authors' knowledge, no studies have been performed on the association with the development of asthma. The results of the present study may differ from those of previous studies. However, these differences are considered to be due to various experimental conditions, such as transgenic, knockout mice and stimulants. For example, in the case of aquaporin-3, the elevation of its expression induced the development of asthma in a previous study (40). However, in another previous study, aquaporin-3 expression was not altered compared with normal controls in the OVA-induced asthma model and was only upregulated in an IL-13-induced asthma model (41). As such, small differences may be identified depending on the respective experimental conditions. Therefore, further studies are needed to elucidate the association between these two genes and the development of asthma.

In summary, the present study focused on RNA-DNA methylation, which is one of the factors that can affect RNA expression in the development of asthma. In the present study, the genes that were highly associated with both RNA expression and DNA methylation were analyzed in the OVA-induced asthma model. *FOXO1*, *RUNX1*, *SPI* and *APP* were identified as closely connected hub genes in an integrated network of DNA methylation and gene expression affecting the development of asthma. The results of the present study suggested that the modulation of these four genes could contribute to the development of asthma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JSK and ISS drafted the manuscript. ISS, JSK and CK conceived and designed the study. NRS, JYN, JSK and ISS analyzed the data. JSK and CK reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by The Institutional Animal Care and Use Committee of Korea Institute of Oriental

Medicine (approval no. 17-073) and the animals were cared for in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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