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), SPI 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 Bio Link 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.). Intranasal OVA administration began. Ethical and scientific management procedures were affected by DNA methylation (i.e. inverse correlation 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

RNA-seq (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 TrueSeq 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 log2FC ≥1 and P<0.05 as thresholds, where FC indicates the fold change. DAVID was used to functionally annotate the genes (Tables S1 and SII) (24,25).

Methyl-seq 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
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).
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 $\beta$ 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).
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>
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<th>Hit</th>
<th>Total</th>
<th>P-value</th>
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<td>389</td>
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<tr>
<td>Neuroactive ligand receptor interaction</td>
<td>55</td>
<td>272</td>
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<td>14</td>
<td>140</td>
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<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>72</td>
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<td>113</td>
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4,866 genes; Gene Ontology gene set (min. size=3; max. size=1,000); 170 significant KEGG pathways with P<0.01. KEGG, Kyoto Encyclopaedia of Genes and Genomes.
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] 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.

References


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