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Understanding asthma pathophysiology through gene expression on peripheral blood mononuclear cells

말초혈액 단핵구에서 유전자 발현을 통한 천식 병태생리의 이해

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강민규

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이 논문을 의학박사 학위논문으로 제출함

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강민규의 의학박사 학위논문을 인준함

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Abstract

Understanding asthma pathophysiology through gene expression on peripheral blood mononuclear cells

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Asthma is a chronic inflammatory airway disease characterized by bronchial hyperresponsiveness and reversible airway obstruction. Corticosteroids are known to the most effective treatment for asthma. However, there is substantial variability in response to corticosteroids in asthma patients. Ineffective response to corticosteroids may result in exacerbation of asthma. Although many genetic studies have been conducted, the mechanisms of asthma pathogenesis and steroid

insensitivity in asthma have not been fully elucidated. Gene expression profile represents the complete set of RNA transcripts that are produced by the genome under specific circumstances or in a specific cell. High-throughput methods such as microarray, and recent advances in biostatistics based on network-based approaches provide a quick and effective way of identifying novel genes and pathways related to asthma. This study aimed to understand the pathogenesis and steroid insensitivity in asthma using gene expression profiles of blood cells from asthma patients. To obtain a comprehensive picture of the gene expression in these cells, we used network-based approaches. The study was divided into two separate parts. In the first part of the study, important genetic signatures of acute exacerbation (AE) in asthma were identified using weighted gene co-expression network analysis (WGCNA) in peripheral blood mononuclear cells (PBMCs) from 29 adult asthma patients and lymphoblastoid cell lines (LCLs) from 107 childhood asthma patients. An AE-associated gene module composed of 77 genes was identified from childhood asthma patients and the conservation of this gene module structure was validated in adult asthma patients. The identified module was found to be conserved in terms of the gene expression profile and associated with AE in both childhood and adult asthma patients, and thus it was defined as an AE-associated common gene module. Changes in the expression of genes in the AE-associated common gene module

following *in vitro* dexamethasone (Dex) treatment were examined, to better understand the mechanisms associated with steroid insensitivity. The differential gene expression profiles were classified into two classes according to Dex-induced changes in childhood asthma patients. Thirteen genes showed significant Dex-induced differential expression and were categorized as the A gene set. Sixty-four genes were not significantly altered by Dex were categorized as the B gene set. In the A gene set, the expression of eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*) showed significant Dex-induced differential expression in adult asthma patients as well. In addition, the basal expression of *EIF2AK2* (pre-Dex) were significantly higher in asthma patients with AE compared to those without AE in both childhood and adult asthma. In the B gene set, based on a pathway-based approach, the protein repair pathway was found to be significantly enriched. Among the genes that belong to this pathway, the basal expression of methionine sulfoxide reductase A (*MSRA*) and methionine sulfoxide reductase B2 (*MSRB2*) were significantly lower in asthma patients with AE compared to those without AE in both childhood and adult asthma. These findings suggest that alternate treatment options, apart from corticosteroids, may be needed to prevent AE in asthma. Expression of *EIF2AK2*, *MSRA*, and *MSRB2* in blood cells may help us to identify AE-susceptible asthma patients and adjust treatments to prevent AE events. In the second study,

gene regulatory networks identified gene expression profiles of PBMCs from 23 adult asthma patients were assessed to elucidate the differences in responsiveness to inhaled corticosteroids (ICSs). Among these the top five (top-5) transcriptional factors (TFs; Top-5 TFs: *GATA1*, *JUN*, *NFκB1*, *SPl1*, and *RELA*) showing differential connections between good-responders (GRs) and poor-responders (PRs) were identified. Interestingly, *GATA1* and *JUN* also showed differential connections in the gene regulatory networks identified gene expression profiles of LCLs from 107 childhood asthma patients in a previous study. The top-5 TFs and their connected genes were significantly enriched in distinct biological pathways associated with asthma. Among the genes connected to the top-5 TFs, the expression of *TBX4*, which is regulated by the TF, *NFκB1*, may be helpful in identifying GRs to ICS treatment. In conclusion, the novel genes and biological pathways identified in this study may deepen our understanding of asthma pathophysiology and steroid insensitivity in asthma.

Keywords:

asthma; acute exacerbation; common gene module; peripheral blood mononuclear cell (PBMC); insensitivity to corticosteroids; system biology; gene expression; weighted gene co-expression network analysis (WGCNA); *in vitro* dexamethasone treatment; pathway analysis;

transcription factor; gene regulatory network; passing attributes between networks for data assimilation (PANDA); differential connectivity

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Chapter 1. Introduction

1.1. Asthma - glucocorticoids as important therapeutic drugs

Asthma is a chronic inflammatory disease of the airway characterized by reversible airway obstruction and hyperresponsiveness (1, 2). Chronic inflammation of the airways causes proliferation of smooth muscle cells and fibroblasts that eventually lead to dysplasia of the alveolar epithelial cells. When left untreated, these changes may lead to irreversible airway remodeling that progressively worsens pulmonary function (3). Asthma affected approximately 358 million people globally in 2015 and over 2 million asthma patients were reported in Korea in 2010 (4). The prevalence of asthma is increasing worldwide (5). An additional 100 million asthma cases are expected by 2025 due to the increasing trend in asthma diagnosis (6).

Glucocorticoids are the current mainstay treatment for asthma and are typically administered in an inhaled form. Inhaled corticosteroids (ICSs) effectively inhibit inflammatory airway reactions and prevent asthma exacerbation (7, 8). ICS crosses the epithelial cell membrane in airway, and binds to and activates the glucocorticoid receptors in the cytoplasm (7). Homodimerized glucocorticoid receptors bind to a glucocorticoid response element present in genes to transactivate tgenes encoding anti-inflammatory

proteins (9) and suppress the transcription of pro-inflammatory genes (7). Long-term maintenance treatment with ICSs delays progressive lung function decline and irreversible airway remodeling (10). The global initiative for asthma (*GINA*) guidelines recommend that patients with 'well-controlled' mild persistent asthma maintain ICSs treatment regularly.

1.2. Blood cells as potential candidates for the study of asthma pathogenesis

Airway epithelial cells are optimal candidates to evaluate the pathophysiology or steroid insensitivity in asthma (11). The airway epithelium is at the interface between the host and the environment, and constitutes an epithelial barrier and a place where various immunologic responses to external stimuli occur. As such, airway epithelium orchestrates various innate and adaptive immune responses (12). However, it is not easy to utilize bronchial epithelial cells as bronchoscopy is not only invasive but also challenging to perform in every asthma patient.

Blood sampling is a more practical alternative for clinical sampling in asthma research. Peripheral blood can be easily obtained through relatively non-invasive procedure. Although blood cells may not fully represent the biology of the airway, they have been widely used for asthma genetics studies and provide considerable information regarding the pathophysiology of asthma

(13). Blood cells express approximately 80% of the genes encoded by the human genome (14). In addition, blood contains many of the cells that are involved in immune reactions of asthma. Previous studies showed that immune cells in the blood, such as monocytes or lymphocytes, reflect the pathophysiology of asthma, and genome-wide gene expression profiling perturbated by *in vitro* exposure of corticosteroids were proposed to be a useful tool in understanding *in vitro* drug responses (15–18).

Recently, peripheral blood mononuclear cells (PBMCs) are being increasingly used to analyze the selective responses of the immune system (19). Previous studies have shown that profiling of genes expressed in PBMCs predicts corticosteroid responses in asthma patients (17, 18). A cell line model combined with genome-wide gene expression analysis was proposed to be a useful tool to understand drug response *in vitro* (15, 16). Therefore, *in vitro* changes in genome-wide gene expression or gene regulatory network of PBMCs following dexamethasone (Dex) administration may be useful to evaluate the genetic signatures associated with asthma pathophysiology (20, 21).

Lymphoblastoid cell lines (LCLs) immortalized by Epstein-Barr virus are also good alternatives for such studies (16). Several studies have reported that the corticosteroid-induced gene expression changes in LCLs are like those

observed in PBMCs (22–25). Activated states of LCLs also indicates a suitable system for studying the immunosuppressive effects of corticosteroids. Park et al. reported that Dex-regulated genes significantly overlapped between PBMCs and LCLs (23), indicating that LCLs may good candidates to evaluate steroid response and insensitivity as the main molecular mechanism of acute exacerbation (AE) in asthma. Moreover, some regulatory variants that affect corticosteroid response in LCLs may be shared with other cell types, as observed for baseline expression (26, 27). LCLs may be good candidates to recapitulate the gene expression changes in PBMCs in response to corticosteroids. These studies suggest that LCLs would be suitable for the study of immune-modulatory effects of corticosteroids.

1.3. Role of gene expression studies in evaluating asthma pathogenesis

Asthma is a complex disease, and multiple genetic factors contribute to the disease pathogenesis and susceptibility (28). In twin studies, asthma heritability estimates ranged from 35–70% (29, 30). Overall, genetic variation between individuals can account for approximately 50% of asthma risk (31). Recent studies also reported that genetic susceptibility is important in the progression of asthma and may determine asthma severity (32). Many researchers are attempting to understand the pathophysiology and mechanisms

of asthma by applying genome-wide association studies (GWAS). GWAS is a study designed to identify genetic variations associated with phenotypic variables by scanning single-nucleotide polymorphism (SNP) markers. Rapid advances in high-throughput technologies has allowed affordable profiling of genomes as well as other-omics. Variations in responsiveness to steroids may be due to genetic variations between asthma patients (33). More than 15 loci have been found to be associated with steroid responsiveness from previous GWAS (34–43). Identifying genetic signatures associated with asthma risk and decreased corticosteroid response may provide insight into disease-causing mechanisms and help overcome the ineffective response to corticosteroids through the development of a tailored therapies for patients with severe asthma (31).

Although previous GWAS studies have accelerated our understanding of genetic-based pathophysiology of asthma, these approaches have some limitations (44). Gene expression represents the complete set of RNA transcripts produced by the genome under specific conditions or in a specific cell type. Gene expression is essential for gene function and a sensitive indicator of its biological activity, wherein changes in gene expression pattern are reflected as changes in biological processes. Gene expression profiling goes beyond the static information of the genome sequence into a dynamic functional view of an organism's biology. It is a widely used research approach

in clinical and pharmaceutical settings to better understand individual genes, gene pathways, or gene activity profiles.

1.4. A network-based approach (systems biology)

Previous GWAS studies addressed the association between numerous candidate susceptibility genes and asthma. Although asthma is a complex disease influenced by multiple genetic and non-genetic factors, the interaction of gene-gene or gene-transcription factors (TFs) have not been sufficiently addressed. Genes do not exist or function in isolation. Rather, they interact with other. Recent advances in biostatistics and multi-omics provide an unbiased, systems-based approach to investigate gene-gene interaction or diverse pathways involving multiple genes and other factors such as multi-omic data or TFs (45). Systems biology is an interdisciplinary approach that focuses on complex interactions within biological systems (46). The purpose of the systems biology approach is to create a network that can explain various gene interactions and associations based on existing biological knowledge and mathematical techniques (46). The recent remarkable advances in -omic analysis technologies have enabled the successful discovery of candidate genes through systems biology approaches in predicting various diseases or drug responses (47, 48). A major advantage of a systems biology approach is that it enables the enrichment of existing biological knowledge (such as known

protein interaction networks or pathways), so that genetic data with low P values are obtained even from a small number of samples (which do not reach genome-wide significance). It is that mutations can also be given meaning (49). As mentioned above, asthma has complex pathogenesis with multiple tiers of biological complexity, polygenicity, and gene-environmental interaction. Therefore, a system biology approach may be optimum for the study of steroid insensitivity in AE.

1.5. Weighted gene co-expression network analysis (WGCNA)

WGCNA is an analytic methodology that creates a weighted correlation network based on the co-occurrence of gene expression and identifies gene modules related to the research objective (50). The co-expression network is useful for describing pairwise relationships between gene transcripts (51-53). In co-expression networks, each gene is represented as a node in a graph, and the gene expression profiles were denoted as a node profile. A highly correlated gene module or clustering is created after filtering the fold-changes and p-value adjustment through differential expressed gene analysis of mRNA expression. Eigengene or intramodular hub gene in a specific module is searched through eigengene network methodology (54). Highly connected nodes represent essential genes that may contribute to a disease or phenotype. Functional gene variants underlying disease pathogenesis can also be

identified. Therefore, WGCNA helps identify biomarkers and therapeutic targets related to a specific gene expression or disease phenotype (55).

1.6 Gene regulatory network (GRN) *analysis*

GRN constitutes the principal structural and functional genomic control programs in animals and controls all aspects of development, from cell fate specification to differentiation. By studying GRNs, it is possible to obtain an understanding of the causal mechanisms that underlie the pathogenesis of diseases. Passing attributes between networks for data assimilation (PANDA) has provided insights into the regulatory context of genes and TFs associated with disease and other phenotypes (56, 57). PANDA is an integrative network inference method that explicitly models interactions between TFs and their putative target genes (58). PANDA starts with an initial network model derived from known motif-based TF-target mapping to the genome and uses a message-passing framework to refine that initial model in each phenotype given gene expression and other data (59). Edges in PANDA networks reflect the overall consistency between the regulatory profile of a TF with the co-expression of the target gene. PANDA is then able to compare the network models between experimental groups to explore transcriptional process differences. PANDA has been successfully used to evaluate the regulator

network of specific conditions such as asthma, as well as chronic obstructive pulmonary disease (56), and ovarian cancer (60).

1.7. Purpose of the study

The objective of this study was to understand the pathophysiological mechanisms underlying asthma based on gene expression profiles of PBMCs. As discussed earlier, network-based approaches were used to obtain complete gene expression profiles. This study was divided into two separate parts. In the first study, important genetic signatures of AE in asthma were identified with WGCNA using PBMCs from adult patients and LCLs from childhood asthma patients. In the second study, GRNs were generated using PANDA and the differential connectivity between good-responders (GRs) and poor-responders (PRs) to ICS treatment were compared to infer steroid responses in asthma.

Chapter 2. Part I

2.1. Introduction

Asthma patients occasionally experience a sudden deterioration of the asthmatic symptoms called AE, despite maintenance treatment with ICSs. AE of asthma is defined as the worsening of asthmatic symptoms requiring the use of systemic corticosteroids to prevent serious outcomes (61). Approximately 15% of patients in the GINA step 3 (combination of low-dose ICSs and long-acting β2 agonist) experience AE once a year, and patients with more severe asthma experience AE frequently (10, 62). Various factors such as compliance, genetic susceptibility, viral infection, smoking, air pollution, and a poor response to asthma medications are known risk factor of AE (63–68).

Some asthma patients have recurrent AE despite maintenance on high dose of ICSs or even systemic corticosteroids (69). Response to ICSs seemed to be different between asthma patients. Steroid insensitivity or resistance to corticosteroids is associated with frequent AE and contributes to a high risk of asthma-related morbidity and mortality in asthma patients (70). Approximately 5–10% of asthma patients do not respond well to ICSs. The exact mechanism of steroid insensitivity underlying AE of asthma remains

unclear (71). The healthcare burden of severe steroid-resistant asthma accounts for 50–80% of total asthma-related costs. Therefore, understanding the mechanisms of steroid ineffectiveness is an important unmet need in asthma research.

Understanding the genetic factors of AE is essential to overcome the ineffective response to corticosteroids and tailor therapies for severe asthma (31). In twin studies, asthma heritability estimates ranged from 35–70% (29, 30). Overall, genetic variation among individuals accounts for approximately 50% of asthma risk (31). In addition, recent studies also reported that genetic susceptibility is important in asthma progression and determines the severity of asthma (32). Therefore, it is reasonable to postulate that there exist genetic factors that are specific to AE. Many researchers have been trying to understand the pathophysiology and mechanisms associated with AE by applying GWAS. GWAS is a study designed to find genetic variations associated with phenotypic variables by scanning SNP markers. Rapid advances in high-throughput technologies has allowed affordable profiling of genomes as well as other -omics. Previous GWAS studies identified more than 15 loci associated with corticosteroid responsiveness in asthma (34–43). Gene loci near *APOBEC3B* and *APOBEC3C* on chromosome 22q13.1 were associated with AEs (42). SNP *rs3827907* on chromosome 14q11.2, and SNP

rs1800925 located in the promotor region of IL-13 were also associated with AE (43, 72). SNPs such as r*s1805011*, *rs1801275*, and *rs4950928* associated with chitinase 3-like 1 were associated with severe AE leading to intensive care unit stay (73–75). In a hypothesis-free approach, *GSBMB*, *IL33*, *RAD50*, *IL1RL1*, and rs6967330 in cadherin-related family member 3 (*CDHR3*) were associated with recurrent and severe exacerbation of childhood asthma (76). This analysis hypothesized that ineffective response to corticosteroid may be related to AE. To identify mechanisms underlying this ineffectiveness, the AE-related common gene module was searched using WGCNA in blood cells from children and adult asthma patients. Then, the changes in the expression of genes belonging to the identified module following *in vitro* Dex treatment were measured.

2.2 Methods

All the study protocols were approved by the institutional review boards approved of the affiliated institutions. For the discovery dataset, informed consent was obtained from the children's parents or guardians. The use of the discovery dataset was approved by the Institutional Review Board of the Brigham and Women's Hospital (2002-P-00331/41). The use of the replication dataset was also approved by the Institutional Review Board of the Seoul

National University Hospital (SNUH-1408-051-601). Informed consent was obtained from all the study participants and a parent and/or legal guardian if subjects were under 18 years of age. All procedures were carried out according to the relevant guidelines and regulations. Figure 1 presents the overall scheme of the study.

2.2.1 Discovery dataset

The discovery cohort consisted of non-hispanic Caucasian children randomized to budesonide treatment in the Childhood Asthma Management Program trial (77). A total of 1041 children aged between 5 and 12 years were enrolled in eight clinical trial centers between December, 1993 – September, 1995. They had mild to moderate asthma, as defined by the presence of typical respiratory symptoms, or used ICSs at least more than twice a week for the maintenance of asthma control. They were maintained on low-dose ICSs and followed-up for 4.3 years (mean duration). Various factors such as age, sex, race, number of AEs and lung function, and the ICSs dose were assessed. The presence of airway hyperresponsiveness was defined as provoked methacholine

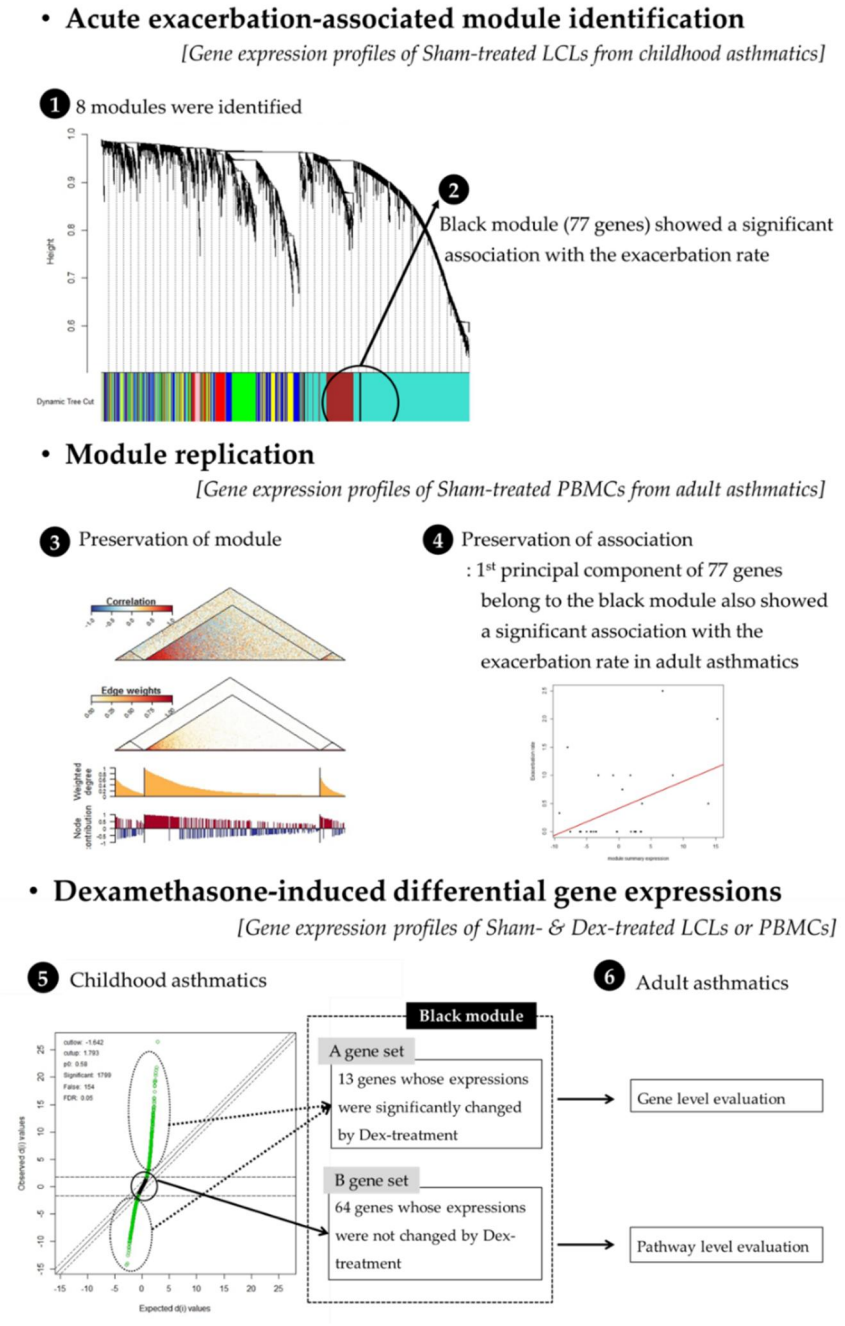


Figure 1. Overall scheme of the study

LCL, lymphoblastoid B cell line; PBMC, peripheral blood mononuclear cell; Dex, dexamethasone.

concentration that caused a 20 percent decrease in forced expiratory volume in 1 second (FEV1) equal to or lesser than 12.5 mg/mL.

2.2.2. Replication dataset

The discovery cohort consisted of adult asthma patients treated at the Seoul National University Hospital (Seoul, Korea). A total of 29 adult asthma patients were studied. They were treated with low, medium, or high dose (based on the GINA guidelines) of ICS plus long-acting β2-agonist combination to achieve asthma control and followed up for one year or longer. Study participants who had smoking history of 10 years or more were excluded. A retrospective review was performed to evaluate the clinical characteristics of the participants. Medical record review included clinical history, demographics (age, gender), pulmonary function test, presence of atopy, ICS dose, and history of AEs.

2.2.3. Definition of AE

The definition of AE differs between studies (62, 78). In the National Institute of Health-sponsored Asthma Outcomes workshop, AE was defined as the worsening of asthma requiring the use of systemic steroids to prevent a serious outcome (78). In this study, the definition of AE included at least one of the following: (a) use of systemic corticosteroids (any tablets, suspension, or

injection), or an increase from a stable maintenance dose for at least three days; (b) hospitalization or emergency room visit because of asthma, requiring systemic corticosteroids. As follow-up periods varied between study participants, the annual number of AE events was counted.

2.2.4. PBMC isolation and RNA extraction

In the discovery data set, Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) from study participants of the childhood asthma management program (CAMP) trial were used (79). PBMCs were isolated from 10 ml of blood from the study participants using IsoPrep (Robbins Scientific, San Diego, California, USA) and transformed with EBV as described in a previous study (80). After six weeks of transfection, viable cells were LCLs.

In the replication data set, PBMCs isolated from the study participants were used. A total of 16 mL of whole blood was drawn into a heparin tube and carefully layered onto 32 mL of Ficoll-Paque containing centrifuge tube. The solution was centrifuged at 1500 rpm for 30 min at room temperature in a swinging-bucker rotor without applying the brake. The upper layer containing plasma and platelets was drawn off, and the undisturbed PBMC interface just above the Ficoll-containing layer was carefully transferred to a sterile centrifuge tube. The PBMCs were washed with an equal volume of phosphate-

buffered saline (PBS, pH 7.4) by centrifugation at 1800 rpm for 10 min. the PBMC pellets were suspended in ammonium chloride (ACK) lysis buffer (Invitrogen, Carlsbad, California, United States) and incubated for 10 min at room temperature with gentle mixing to lyse the contaminating red blood cells. The cells were then washed with PBS-EDTA. PBMCs were cryopreserved and stored in liquid nitrogen in fetal calf serum (FCS; Invitrogen) containing 10% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific). PBMCs from adult asthma patients were incubated in RPMI 1640 medium for 6 h (Sham-treated PBMC). RNA was simultaneously extracted from PBMCs and purified using the AllPrep RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA quantity and quality were determined with an Agilent 2100 Bioanalyzer according to the manufacturer's instructions.

2.2.5 Gene expression analysis

As described previously (79), LCLs derived from childhood asthma patients were cultured in RPMI 1640 medium for 6 h (Sham-treated LCLs). Gene expression levels in Sham-treated LCLs from childhood asthma patients were measured using the Illumina HumanRef8 v2 BeadChip (Illumina, San Diego, CA, USA).

PBMCs from adult asthma patients were also cultured in RPMI 1640 medium for 6 h (Sham-treated PBMCs). Gene expression levels of Sham-treated

PBMCs from adult asthma patients were measured using Affymetrix GeneChip Human Gene 2.0 ST (Affymetrix, Santa Clara, CA, US). Affymetrix GeneChip Human Gene 2.0 ST provides comprehensive coverage of over 30,000 coding transcripts and over 10,000 long intergenic non-coding transcripts. Hybridization was performed at 45 ℃ for 16 h using the Hybridization Oven 640 (Affymetrix). Washing and staining were performed on a Fluidics Station 450, and images were acquired using the Affymetrix 7G GeneChip scanner. Procedures were performed according to the minimum information about a microarray experiment (MIAME) guidelines.

The probes with bad chromosome annotation and those for X or Y chromosome were removed. Then, variance stabilizing transformation and quantile normalization were performed to reduce the effects of technical noises and make the distribution of expression level for each array closer to normal distribution. Sham-treated LCLs from childhood asthma patients or Sham-treated PBMCs from adult asthma patients may represent participant's intrinsic genetic traits, and *in vitro* Dex-mediated perturbation may provide insights into the responses to corticosteroid. Genes or gene modules associated with AE were searched using both the transcriptomic datasets. The relative expression of the genes of interest were compared using raw intensity values and validated using real-time polymerase chain reaction (PCR).

2.2.6. WGCNA

WGCNA is a systems biology method for examining the correlation patterns among genes across microarray samples (50). The significance of gene expression is also denoted as the node significance measure. Modules are clusters of highly interconnected genes. The connectivity is defined as the sum of the connection strengths with the other network genes (81). In the co-expression network, the connectivity measures how correlated a gene is with all other network genes (82). Co-expression modules are defined as branches of a hierarchical clustering tree, and each module is assigned a unique color label. Module eigengene is defined as the first principal component of a given module. Eigengene values are effective and biologically meaningful tools for studying the relationships between modules of a gene expression network and phenotypes. Module eigengene are considered representative of the gene expression profiles in a module. WGCNA can identify cluster modules of highly correlated genes to summarize such clusters using eigengene network methodology and calculate module membership measures.

2.2.7 Identification of AE-associated common gene modules in the discovery data set

To compare data from different microarray platforms, the mean expression value of each gene in the Sham-treated LCLs in the discovery data set after

collapsing probes by genes was calculated. Then, the mean expression levels of genes and the selected top 5,000 genes in common with the highest correlation were compared. Using these 5,000 genes in gene expression profiles of Sham-treated LCLs from childhood asthma patients, WGCNA with R package "WGCNA" ver 1.69 (50) (R Foundation, Vienna, Austria) was performed. Eigengene values of the modules were identified and multivariate linear regression analyses were computed and adjusted by baseline age, gender, atopy, forced expiratory volume in 1 second (FEV1) % predicted value, and FEV1 over forced vital capacity (FVC) ratio to find a module whose eigengene value was significantly associated with the AE rate. The AE-associated common module was defined when its eigengene value was significantly associated with AE, a target phenotype.

2.2.8 Replication of the AE-associated common gene modules in adult asthma patients

The next step was to check whether the modules identified in gene expression profiles of Sham-treated LCLs from childhood asthma patients were replicated in the gene expression profiles of Sham-treated PBMCs from adult asthma patients. Replication was assessed in two ways: preservation of module (the consistency of network module structure across gene expression profiles) and preservation of association (association between corresponding eigengene

values and the AE rate). Module preservation was measured by the R package "NetRep" (81). NetRep quantifies the replication/preservation of a network module's topology across the datasets and produces unbiased p values based on a permutation approach to score module preservation without assuming data are normally distributed. To check the preservation of the association, we calculated the first principal component of the expression of genes belonging to the preserved module in adult asthma patients and performed multivariate linear regression analysis adjusted by age, gender, atopy, FEV1 % predicted value, FEV1/FVC ratio, and the dose of ICSs to check an association between the first principal component calculated and the AE rate. We called the module which satisfied the replication criteria, the AE-associated common gene module.

2.2.9 In vitro Dex-mediated perturbations of the co-expression modules

The effects of *in vitro* perturbation of the AE-associated common gene module gene expression following Dex-treatment was investigated. We evaluated the differential expression pattern of genes belonging to the AE-associated common module using gene expression profiles of Sham- and Dex-treated LCLs and PBMCs. LCLs from childhood asthma patients were cultured in RPMI 1640 medium (sham-treated LCLs) and treated with Dex (10–6 M) for 6 h (Dex-treated LCLs). PBMCs from adult asthma patients were also cultured

in RPMI 1640 medium without (Sham-treated PBMCs) or with Dex (10−6 M) for 6 h (Dex-treated PBMCs). Gene expression levels in Sham- and Dex-treated PBMCs from adult asthma patients were measured using Affymetrix GeneChip Human Gene 2.0 ST (Affymetrix, Santa Clara, CA, US). Hybridization was performed at 45 °C for 16 h using the Hybridization Oven 640 (Affymetrix). Washing and staining were performed on a Fluidics Station 450, and images were acquired using the Affymetrix 7G GeneChip scanner.

2.2.10. Functional interpretation of AE-associated common gene modules

Finally, to assign biological meaning to the interpretability of the gene module, we performed pathway enrichment analyses that is useful to identify key genes in a previously known pathway concerning a particular experiment or pathologic conditions. By examining changes in the expression in a pathway, the biologic activity of gene modules can be examined. We used the web interface of ConsensusPathDB (http://cpdb.molgen.mpg.de), a meta-database that integrates different types of functional interactions from heterogeneous interaction data resources (83).

2.3. Results

2.3.1. Module construction and association

Table 1 summarizes the characteristics of 107 childhood and 29 adult asthma patients that were enrolled in this study. By applying WGCNA to the 5,000 genes in the gene expression profiles of Sham-treated LCLs from childhood asthma patients, we identified eight modules of various sizes ranging from 71 in the pink module to 2,266 genes in the turquoise module (Fig. 1). A total of 16 genes could not be assigned to a module as a membership gene and were grouped into the gray module that was excluded from further analysis. To emphasize the impact of strong correlations over weak ones in the network construction, we chose an empirical soft threshold of 6, representing a strong model fit for scale-free topology ($R2 > 0.97$, Fig. 2). Eigengene values of the black module of 77 genes showed a significant association with the AE rate in multivariate linear regression analysis ($P = 0.04$) (Fig. 3A). Among the eight modules identified in childhood asthma patients, two modules (yellow and black modules) were significantly preserved in the adult asthma patients (Fig. 4). Module preservation statistics and P values are presented in Table 2. In addition, multivariate linear regression analysis showed that the first principal component value of the genes belonging to the black module was also significantly associated with the AE rate in adult asthma patients ($P = 0.03$)

(Fig. 3B). Given that the black module was associated and preserved with AE in both childhood and adult asthma patients, we defined the black module as the AE-associated common gene module.

2.3.2. Corticosteroid-mediated genes within the common module

We then identified the subsets of 1,799 genes that were differentially expressed between Sham- and Dex-treated LCLs and 1,154 genes that were differentially expressed between Sham- and Dex-treated PBMCs (FDR P-value < 0.05, Fig. 5). Among the 77 genes belonging to AE-associated common gene module, 13 were also differentially expressed between Dex and Sham in LCLs and were categorized as the A gene set (Table 3). The other 64 genes in the black module were classified into the B gene set (Table 3). Among the 13 genes belonging to the A gene set, two genes also showed significant differential expression in PBMCs from adult asthma patients (Table 3). As shown in Figure 6, expression of the eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*) in Sham-treated LCLs and PBMCs were significantly higher in asthma patients with AE compared to those without AE. These changes were validated using real-time PCR (Fig. 7). However, the 59 genes belonging to the B gene set showed no significant differential expression changes in PBMCs (Table 3). As shown in Figure 8, the expression of methionine sulfoxide reductase A (*MSRA*) and methionine sulfoxide reductase B2

(*MSRB2*) in Sham-treated LCLs and PBMCs were significantly lower in asthma patients with AE compared to those without AE and Dex-treatment caused no significant changes in the expression of *MSRA* and *MSRB2*. These changes were validated using real-time PCR (Fig 7). The AE-associated common gene module structures based on expression correlations (co-expression networks) showed different connections between Sham-treated and Dex-treated LCLs from childhood asthma patients (Fig 9).

2.3.3. Pathway analysis

Pathway enrichment analyses using complete genes belonging to the AE-associated common gene module identified three reactome biological pathways; protein repair, syndecan interactions, and HATs acetylate histones (Table 4). All three pathways were also identified in enrichment analysis using genes belonging to the B gene set. However, genes belonging to the A gene set provided no enriched biological pathway.

Table 1. Characteristics of the patients with asthma enrolled

Characteristic	Childhood asthmatics*	Adult asthmatics†
Number	107	29
Gender, male	64 (59.9)	10 (34.5)
Age (yr)	8.6 ± 2.2	56.7 ± 11.1
Ethnicity		
Non-Hispanic white	107 (100)	0 (0)
Asian	0 (0)	29 (100)
Atopy, Yes (%)	94 (87.8)	15 (51.7)
Exacerbation, yes	74 (69.2)	11 (37.9)

Values are presented as number (%) or mean ± standard deviation.

CAMP, Childhood Asthma Management Program; ICS, inhaled corticosteroid.

*Characteristics measured at enrollment of the CAMP;

†Characteristics measured at enrollment of the present study;

‡Based on the Global Initiative for Asthma guideline.[2]

Table 2. Module preservation statistics and *P* values in gene expression profiles of PBMCs from adult asthmatics

Characteristic	Module	Avg.weight	Coherence	Cor.cor	Cor.degree	Cor.contrib	Avg.cor	Avg.contrib
Statistics	Turquoise	0.000529033	0.2763263	0.09003795	0.03353621	0.2207718	0.021131235	0.10107644
	Blue	0.000567902	0.288143	0.10124375	−0.03956173	0.2162779	0.025435924	0.10246129
	Brown	0.000465253	0.2922684	0.04617835	−0.03034021	0.1666007	0.0141772	0.08988917
	Yellow	0.002109171	0.2945858	0.88243878	0.77886607	0.958283	0.227128567	0.44573357
	Green	0.000509242	0.2862446	0.06561986	−0.0201276	0.1499842	0.028658762	0.08005171
	Red	0.000513684	0.2316613	0.77535824	0.22444157	0.913583	0.193422996	0.43262193
	Black	0.003983556	0.4620086	0.96671965	0.73920027	0.9919127	0.416523066	0.64944444
	Pink	0.000671975	0.255436	0.02693771	−0.16761045	−0.2164916	0.007584064	−0.09178646
P values	Turquoise	0.00009999	0.00009999	0.00009999	0.06239376	0.00009999	0.00009999	0.00009999
	Blue	0.00269973	0.00009999	0.00009999	0.86371363	0.00009999	0.00009999	0.00009999
	Brown	0.10928907	0.00009999	0.00009999	0.7760224	0.00009999	0.00009999	0.00009999
	Yellow	0.00009999	0.00009999	0.00009999	0.00009999	0.00009999	0.00009999	0.00009999
	Green	0.07279272	0.00009999	0.00009999	0.6530347	0.00059994	0.00009999	0.00009999
	Red	1	0.73732627	0.00009999	0.00249975	0.00009999	0.00009999	0.00009999
	Black	0.00009999	0.00009999	0.00009999	0.00009999	0.00009999	0.00009999	0.00009999
	Pink	0.10838916	0.41445855	0.09519048	0.9250075	0.96620338	0.10588941	0.93210679

Columns correspond to 7 module preservation statistics defined by the “NetRep” package and *P* values are permutation *P* values. ‘Avg.weight’ measures the average magnitude of edge weights in the test dataset, that is, how connected nodes in the module are to each other on average. ‘Coherence’ measures the proportion of variance in the module data explained by the module's summary profile vector in the test dataset. ‘Cor.cor’ measures the concordance of the correlation structure, that is, how similar the correlation heatmaps are between the 2 datasets. ‘Cor.degree’ measures the concordance of the weighted degree of nodes between the 2 datasets, that is, whether the nodes that are most strongly connected in the discovery dataset remain the most strongly connected in the test dataset. ‘Cor.contrib’ measures the concordance of the node contribution between the 2 datasets. This measures whether the module's summary profile summarizes the data in the same way in both datasets. ‘Avg.cor’ measures the average magnitude of the correlation coefficients of the module in the test dataset, that is, how tightly correlated the module is on average in the test dataset. This score is penalized where the correlation coefficients change in sign between the 2 datasets. ‘Avg.contrib’ measures the average magnitude of the node contribution in the test dataset. This is a measure of how coherent the data is in the test dataset. This score is penalized where the node contribution changes in sign between the 2 datasets, for example, where a gene is differentially expressed between the 2 datasets.

PBMC, peripheral blood mononuclear cell.

Table 3. Genes belonging to the acute exacerbation-associated common gene module and their expression differences between Sham- and Dex-treated blood cells

Gene	LCLs			PBMCs		
	Fold change*	Raw *P* value	Adjusted *P* value	Fold Change*	Raw *P* value	Adjusted *P* value
A gene set						
CALD1	1.060933149	1.4996E-20	**6.46378E-19**	1.0554004	0.759530296	0.909317997
CPOX	0.97263836	8.03415E-20	**3.11401E-18**	0.971923423	0.997767819	0.99919353
EIF2AK2	0.973333718	1.13199E-09	**1.58335E-08**	0.96965243	0.001204344	**0.01062032**
NEXN	0.966314429	1.71367E-08	**1.98112E-07**	0.967349306	0.936257622	0.977318445
NOL11	0.984302863	5.61941E-08	**5.9654E-07**	0.983734232	0.005400716	**0.035069586**
OXR1	0.981110354	1.71286E-07	**1.70603E-06**	0.980770783	0.673246792	0.865133375
DDX5	0.98299549	1.11695E-06	**9.78067E-06**	0.981860055	0.971922243	0.989008159
CEP290	0.984639854	2.44887E-05	**0.000166364**	0.980545301	0.011496491	0.06275377
GLT1D1	1.015204775	0.000255983	**0.001400346**	1.019586373	0.018962813	0.091874096
ZBED2	0.980105373	0.000455575	**0.002331498**	0.980723854	0.143664468	0.375691599
AMPH	1.004154065	0.000587485	**0.002911225**	1.004043207	0.120804068	0.338008025
MAP4K5	0.990445124	0.005850812	**0.02147875**	0.990246636	0.218321105	0.487759396

ST3GAL6	1.020664765	0.011757524	**0.0393755**	1.018811631	0.233623817	0.50699613
B gene set						
GLIS3	1.002342887	0.020643971	0.063637395	1.001197059	0.489962645	0.752795396
VPS72	0.99014686	0.036891865	0.104214308	0.990337803	0.314070853	0.597594631
PROK2	1.003166612	0.062428712	0.15852898	1.004287449	0.284094187	0.5648485
SSTR2	0.995620448	0.212456166	0.39624636	0.998636211	0.865144341	0.952058192
TM7SF2	0.991787778	0.213456324	0.397358444	0.994150099	0.660230898	0.859004551
FARS2	1.00431458	0.220293714	0.406719043	1.009103699	0.292267408	0.572175819
SPINK1	0.998768344	0.251495484	0.445124999	1.00021141	0.900256072	0.966977521
CDKN2B	0.998517994	0.253426808	0.446645767	0.999879523	0.96177239	0.985920656
DMTF1	1.006813917	0.271343402	0.466065617	1.008507968	0.541612206	0.789481043
MTAP	0.995104477	0.300381439	0.501471517	0.993046445	0.434041635	0.711203311
NPC2	1.004223832	0.31879817	0.523536915	1.004515485	0.611714403	0.830907909
TTC14	0.995484106	0.323569317	0.528708035	0.999535927	0.961912344	0.985920656
HIST1H4L	1.001237368	0.325956314	0.531046455	1.000701274	0.806025468	0.932509886
CACHD1	0.999078871	0.349467421	0.557008959	0.998805257	0.560437993	0.800982122
KIAA1524	0.99391181	0.362450994	0.570849805	0.993662723	0.609585907	0.830498512
MSRA	1.002891578	0.376649213	0.584859026	1.004743979	0.471966844	0.740921263
RPL28	1.00408898	0.378247216	0.586248011	1.004284506	0.616847859	0.833101437

RAB8B	0.997950857	0.389600526	0.596814532	0.996016675	0.404928403	0.684108444
NET1	1.002528311	0.399001733	0.605281755	1.001790531	0.758602049	0.909094866
NCOA6	1.002579232	0.441215777	0.641835735	1.003610076	0.629324162	0.838875183
DDX23	0.996413565	0.453040382	0.65298412	0.997614799	0.798379528	0.930573811
MSRB2	1.002896633	0.471116279	0.6690092	1.004005109	0.588816986	0.819166648
SPG21	0.997844152	0.475926594	0.673926075	0.995116221	0.468010928	0.738141808
ADAM23	0.996411727	0.492363414	0.686890923	0.99792013	0.804792845	0.93250926
ANK3	0.99896128	0.497331308	0.690648682	0.998149839	0.624036142	0.835352718
SLAMF8	1.000774252	0.500059469	0.692565882	0.999939814	0.975658576	0.990717482
EPC1	1.003662756	0.505954367	0.697321437	1.004501741	0.000784188	**0.007540268**
AHSA2	1.00585843	0.512405774	0.702888578	1.005613901	3.30E-05	**0.000555592**
INPP4B	0.997864074	0.54511216	0.731497799	1.000276169	0.004916367	**0.032583738**
MARVELD1	1.002603031	0.58430186	0.762398043	1.007447365	0.487930588	0.750893488
DNAH1	0.998768006	0.620516116	0.785463438	0.999086443	0.87040701	0.953766174
ERGIC1	1.002724993	0.632600708	0.793528234	1.00305354	0.816898955	0.937240656
RNASE1	1.001069693	0.63871757	0.798197414	1.001927988	0.657167036	0.857591728
RFX3	1.001661611	0.660370112	0.815575817	1.005170608	0.513437752	0.772424741
NKTR	1.002763795	0.687023254	0.83510105	1.000038788	4.39E-06	**9.62E-05**
PTBP2	1.001724458	0.703431462	0.848939732	0.999546231	0.955500677	0.983326779

OTUD4	1.001451825	0.725614327	0.860299311	0.999887116	0.989306706	0.996481372
OR56B1	0.999210704	0.745311391	0.871709229	1.000304066	0.956188543	0.98368206
PYGL	0.998878481	0.746741007	0.872972886	0.994452447	0.447354347	0.720787851
OR9A4	0.999394631	0.757012646	0.879461511	1.001475646	0.725773418	0.895048917
SERPINA1	1.000814698	0.762283405	0.880726347	1.001109082	0.824383314	0.942152359
NDUFA6	0.999065729	0.768535257	0.885817493	0.999029997	0.892314521	0.964039025
ATP6V1F	1.001440687	0.768775807	0.885890535	1.005957674	0.514667305	0.773278005
XPO1	0.998681558	0.772165242	0.887342268	0.995757109	0.628965426	0.838844259
TAF5L	1.002041816	0.775331753	0.889345897	1.008763354	0.546022324	0.79219529
CCL7	0.999276412	0.81666118	0.913286938	0.999107069	0.892829246	0.964178451
SFTPA2	0.999001319	0.828512118	0.919294688	1.002608616	0.801033129	0.931650534
PDE4DIP	0.999141373	0.82980626	0.91976819	1.000431229	0.961329739	0.985920656
CCDC14	1.000944199	0.831442751	0.920552205	1.000482159	0.957705998	0.984578458
SIPA1L3	0.999499003	0.835294575	0.922603288	0.999017432	0.844384083	0.947833478
MET	0.999554418	0.837011042	0.923647844	0.994889359	0.251389109	0.529359856
SELPLG	1.000835416	0.837194406	0.923647844	1.003529221	0.692652173	0.877600964
FCRL6	0.99915329	0.838797803	0.92440001	1.004774311	0.596940775	0.823623363
ITGB3	0.999627734	0.903512166	0.956151883	0.99603341	0.557545291	0.798677856
PPP3R1	0.998967761	0.913756019	0.962335991	1.002598969	0.902742594	0.96798197

NRIP3	1.000305095	0.938212507	0.974216306	1.000677275	0.000936725	**0.008689469**
SCFD2	1.000312055	0.93725572	0.974216306	1.002921629	0.747597418	0.904951305
CFH	1.000211059	0.943238885	0.976033615	1.002317965	0.703939041	0.884432298
CBX5	0.999762656	0.945996598	0.977064002	1.00211238	0.770255015	0.914140773
SIRPB1	0.999926251	0.957501043	0.982316839	1.001755353	0.523813854	0.778357992
SDC2	1.000136335	0.961597461	0.983832066	1.002481329	0.706725057	0.886207735
RAD17	1.000211856	0.967764869	0.985256299	0.99988785	0.992496141	0.99695365
PPBP	0.999898874	0.978076571	0.98935522	1.00380721	0.639730598	0.845757004
UTS2	1.000023252	0.994875001	0.996691549	1.001393036	0.847260891	0.948765239

Bold denotes adjusted *P* value less than 0.05.

A set, genes showing significant differential expressions between Sham- and Dex-treated LCLs; B set, genes showing insignificant differential expressions between Sham- and Dex-treated LCLs; Dex, dexamethasone; LCL, lymphoblastoid B cell line; PBMC, peripheral blood mononuclear cell.

*Log2 fold changes.

Table 4. Biological pathways enriched in the AE-associated common gene module (black module)

Pathway	Reactome_ID*	Raw *P* value	FDR *P* value‡	Overlapped genes
Whole genes				
Protein repair	R-HSA-5676934	0.000285721	0.025429145	*MSRB2*; *MSRA*
Syndecan interactions	R-HSA-3000170	0.003477859	0.105783533	*ITGB3*; *SDC2*
HATs acetylate histones	R-HSA-3214847	0.003565737	0.105783533	*HIST1H4L*; *TAF5L*; *EPC1*; *VPS72*
Genes belong to B set				
Protein repair	R-HSA-5676934	0.000202316	0.015780612	*MSRB2*; *MSRA*
HATs acetylate histones	R-HSA-3214847	0.001895864	0.064446115	*HIST1H4L*; *TAF5L*; *EPC1*; *VPS72*
Syndecan interactions	R-HSA-3000170	0.002478697	0.064446115	*ITGB3*; *SDC2*
Peptide ligand-binding receptors	R-HSA-375276	0.00580197	0.113138409	*UTS2*; *PPBP*; *SSTR2*; *PROK2*
Transcriptional regulation by E2F6	R-HSA-8953750	0.007491007	0.116859712	*CBX5*; *EPC1*

AE, acute exacerbation; FDR, false discovery rate; HAT, histone acetyltransferase.

*Gene ontology biological pathway; †Only depths 1-3 were presented; ‡Benjamini-Hochberg FDR *P* value.

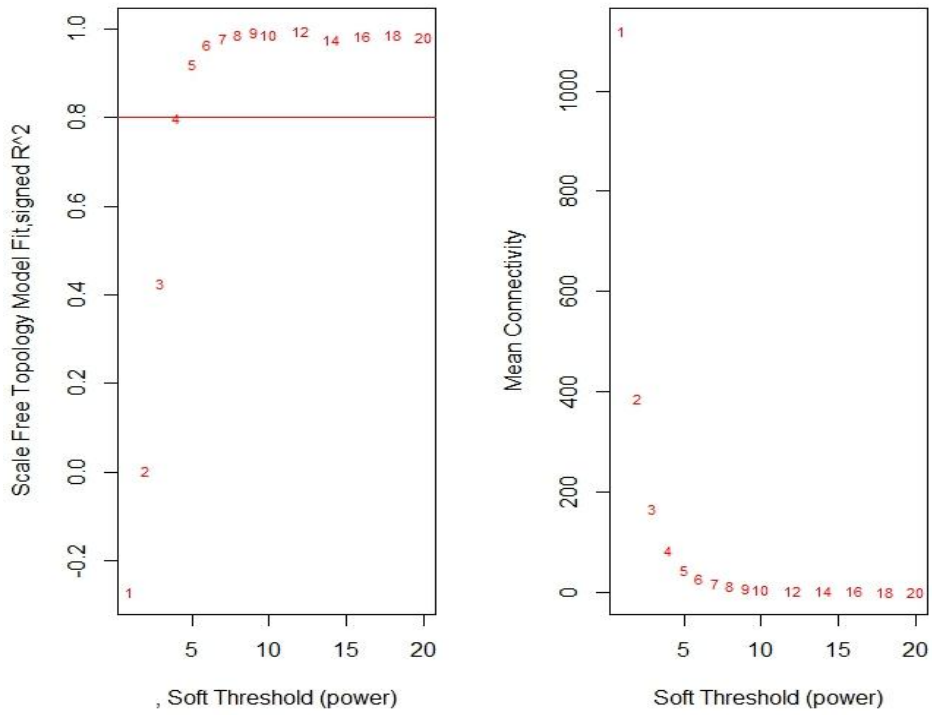


Fig. 2. Assessing scale-free model fitting in gene expression profiles of Sham-treated lymphoblastoid B cell lines from childhood asthmatics. The left panel shows a scale-free topology plotted by a soft threshold. The red horizontal line represents the cutoff for identifying a strong model fit. The right panel shows mean gene connectivity plotted by a soft threshold.

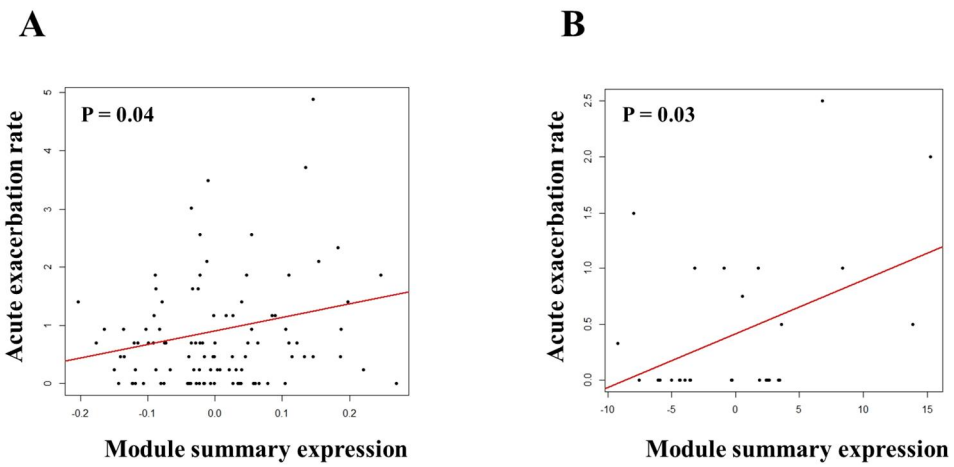


Fig. 3. Correlations between the eigengene value of the preserved gene module (black module) and the AE rate. (A) Childhood asthmatics. (B) Adult asthmatics. Both *P* values were adjusted ones.

AE, acute exacerbation.

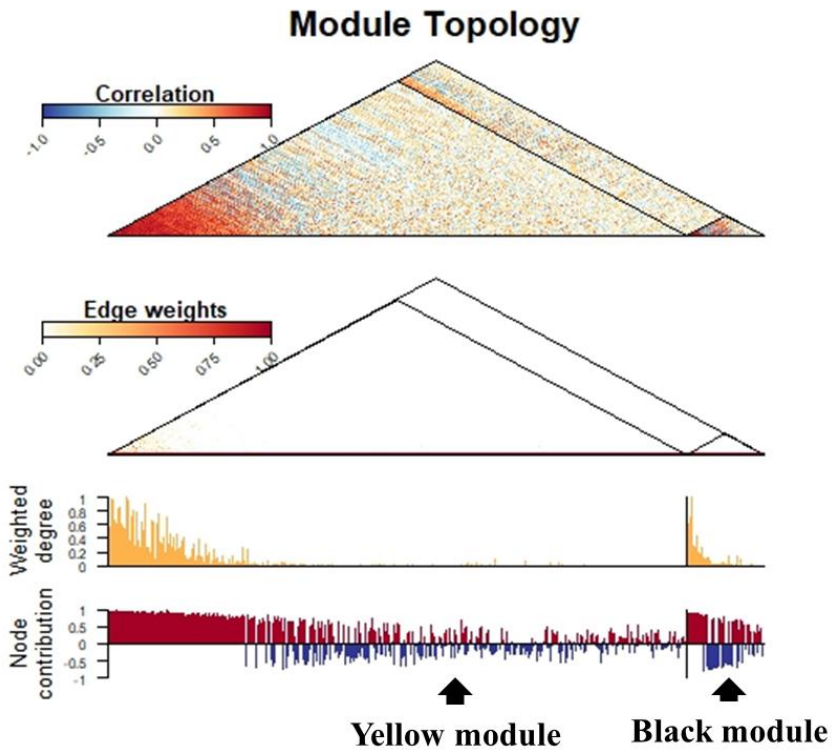
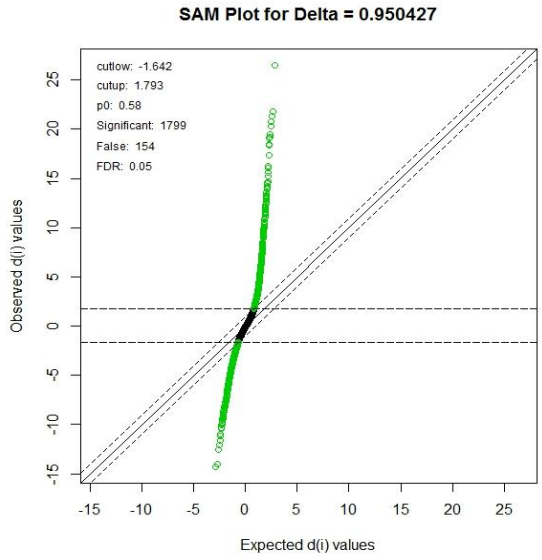


Fig. 4. Preservation of gene modules identified in LCLs from childhood asthmatics in PBMCs from adult asthmatics. The first (top) panel shows a heatmap of pairwise correlations among the genes comprising the turquoise, magenta, and purple modules. The second panel shows a heatmap of the edge weights (connections) among the genes comprising the 3 modules. The third panel shows the distribution of scaled weight degrees (relative connectedness) among the genes comprising the 3 modules. The fourth panel shows the distribution of node contributions (correlation to module eigengene) among the genes comprising the 3 modules. Genes are ordered from left to right based on their weighted degree in the discovery cohort to highlight the consistency of the network properties in the replication cohort.

LCL, lymphoblastoid B cell line; PBMC, peripheral blood mononuclear cell.

(a)



(b)

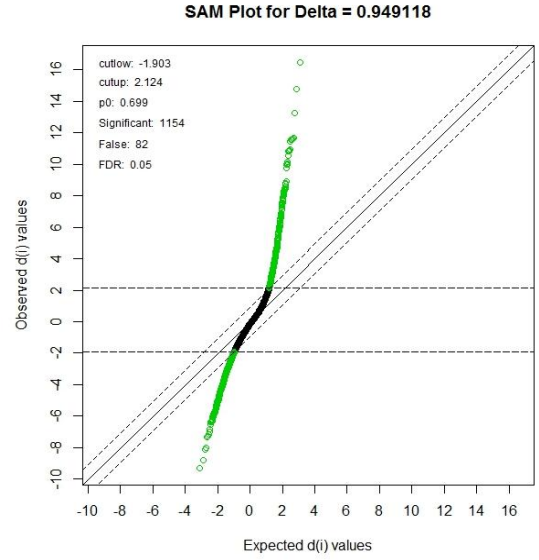


Fig. 5. Differentially gene expressions between Sham- and Dex-treated blood cells (SAM plot). (a) Lymphoblastoid cell lines from childhood asthmatics. (b) Peripheral blood mononuclear cells from childhood asthmatics.
SAM, significance analysis of microarrays; Dex, dexamethasone.

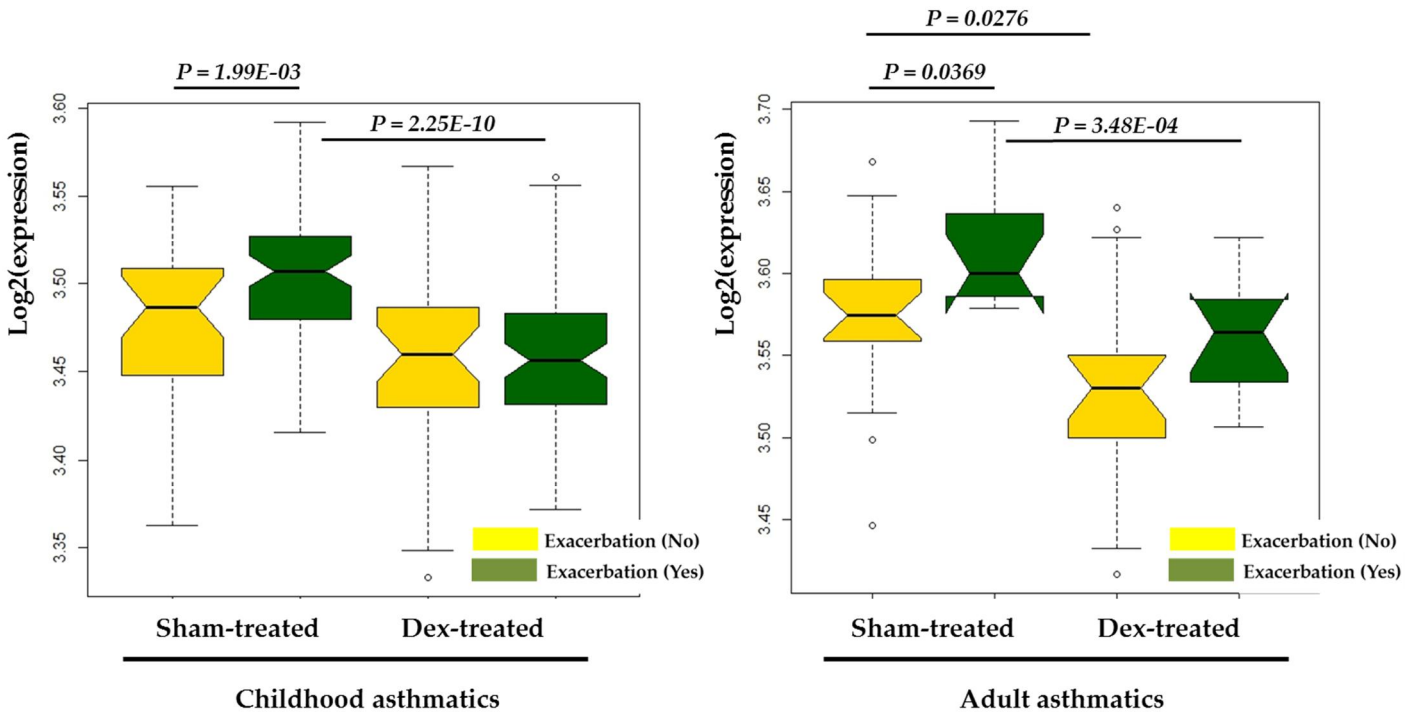


Fig. 6. Eukaryotic translation initiation factor 2-alpha kinase (*EIF2AK2*) expressions in lymphoblastoid cell lines form childhood asthmatics and peripheral blood mononuclear cells from adult asthmatics.

Dex, dexamethasone.

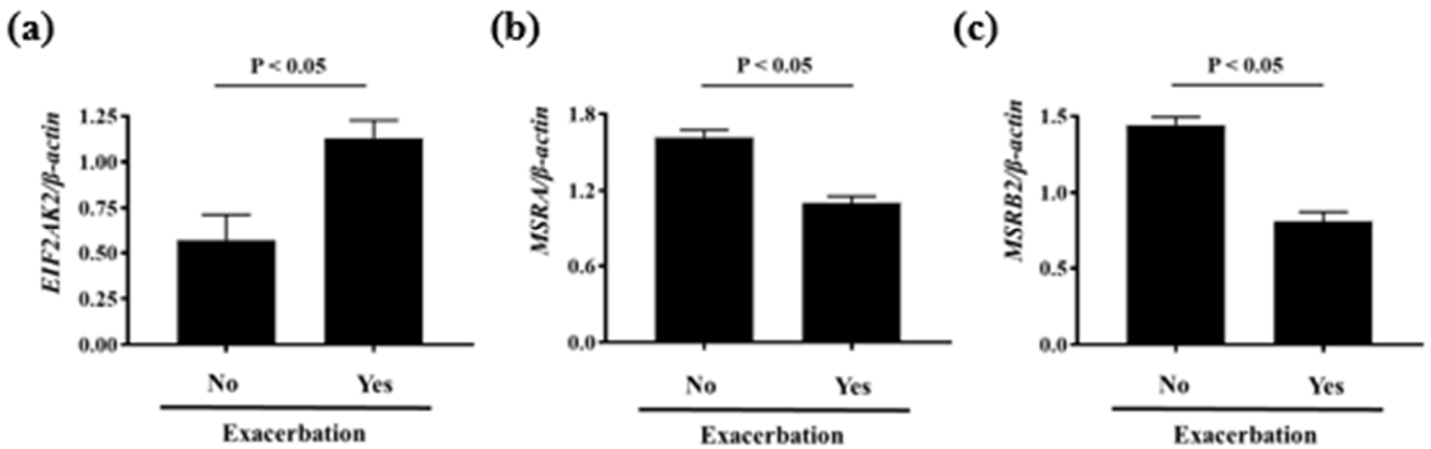


Fig. 7. Validation using real-time PCR (Sham-treated PBMCs from adult asthmatics). (a) *EIF2AK2*. (b) *MSRA*. (c) *MSRB2*.

PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell.

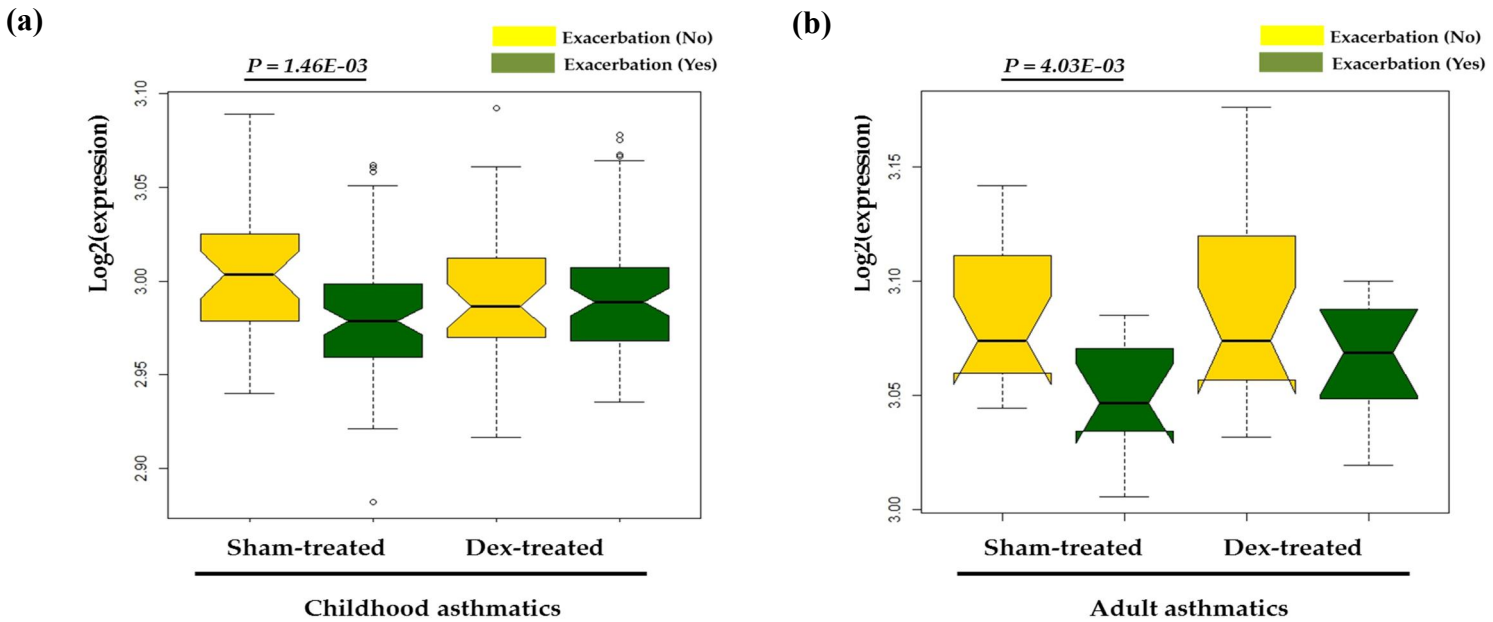


Fig. 8 Methionine sulfoxide reductase A (*MSRA*) and methionine sulfoxide reductase B2 (*MSRB2*) expressions in lymphoblastoid cell lines form childhood asthmatics and peripheral blood mononuclear cells from adult asthmatics. (A) *MSRA* gene expression. (B) *MSRB2* gene expression.

Dex, dexamethasone.

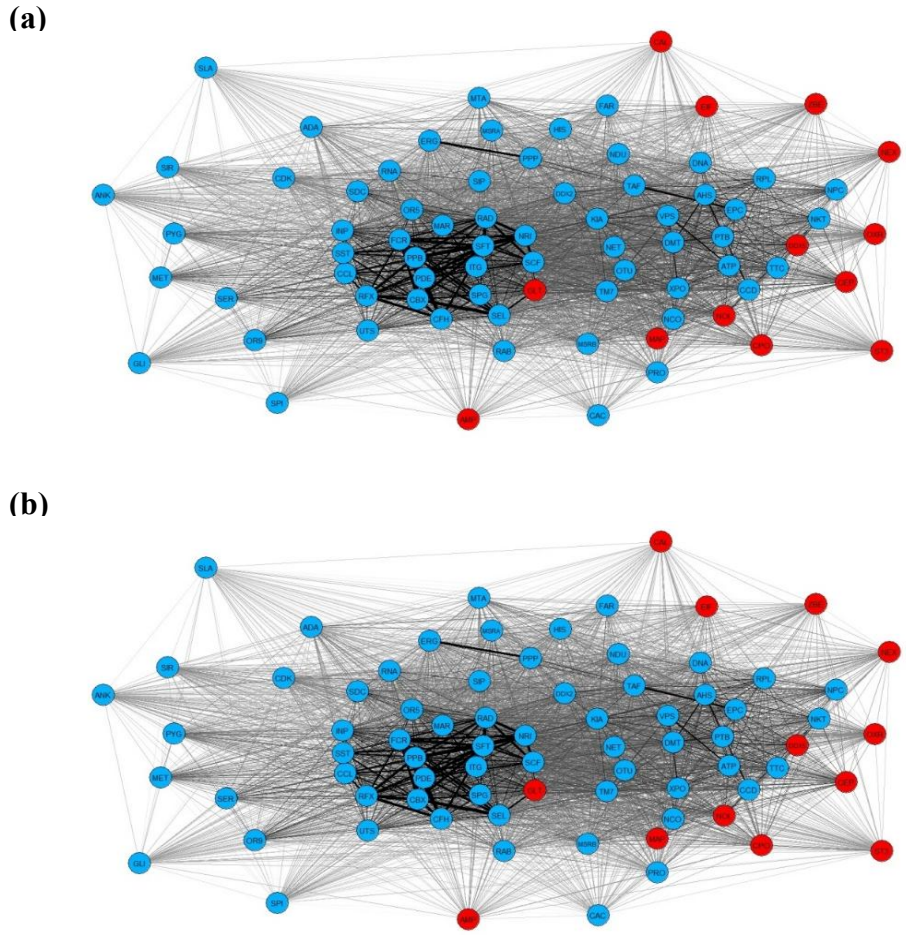


Fig. 9. Structures of the acute exacerbation-associated common gene module (co-expression networks). (a) Sham-treated lymphoblastoid cell lines from childhood asthmatics. (b) Dex-treated lymphoblastoid cell lines from childhood asthmatics. For clarity, only the edges corresponding to the Pearson correlation coefficient > 0.8 were shown. The edge width is proportional to the Pearson correlation coefficient between 2 nodes. Reds nodes represented genes belonging to the A gene set. The network was visualized using qgraph R package.

Dex, dexamethasone.

2.4. Discussion

In this analysis, co-expressed gene modules associated with AE rate were evaluated using LCLs from childhood patients and PBMCs from adult asthma patients. AE is a distinct domain of asthma management (10) and, thus, an important target phenotype in asthma pharmacogenomics studies (64). Our analysis led to the identification of a gene module consisting of 77 genes that showed significant association with AE rate in Sham-treated LCLs from childhood asthma patients, and this gene module structure was significantly preserved in the gene expression profiles of Sham-treated PBMCs from adult asthma patients. In addition, this gene module also showed significant association with AE rate in adult asthma patients. This gene module can thus be regarded as an 'AE-associated common gene module.'

Among the 77 genes belonging to the AE-associated common gene module, 13 genes showed significant changes in expression between Sham- and Dex-treated LCLs derived from the pediatric cohort. Gene expression profiling of *in vitro* drug perturbations is useful for many biomedical discovery applications, including drug repurposing. We presumed that the differential connections between Sham-treated and Dex-treated LCLs in the AE-associated common gene module may help us to acquire the whole genomic picture of acute AE related to ineffective response to corticosteroid (84).

As no biological pathway was enriched in this gene set, we focused on the individual genes for further analysis and found that two genes *EIF2AK2* and nucleolar protein 11 (*NOL11*) showed significant decrease in expression after Dex-treatment in both childhood and adult asthma patients. *EIF2AK2,* also known as protein kinase R, is an interferon-inducible double-stranded RNA protein kinase with multiple effects in cells (85, 86). *EIF2AK2* actively contributes to cellular response to various types of stress and plays a critical role in the antiviral defense mechanisms of the host induced by interferons (86, 87). Expression of *EIF2AK2* in Sham-treated LCLs and PBMCs were significantly higher in asthma patients with AE compared to those without AE (Fig. 4). As *EIF2AK2* expression is activated by virus infection as a part of the host viral defense mechanism (88) and found to be increased in respiratory virus-infected airway epithelium even from subjects without respiratory illness (89), our observation did not seem to be the etiology but likely a consequence of AE. Interestingly, corticosteroid-treatment significantly decreased *EIF2AK2* expression in both childhood and adult asthma patients with AE (Fig. 4). A previous report showed that mice that were given intranasal treatment with corticosteroids prior to influenza developed a more severe disease associated with amplified virus replication (90). Based on these findings, increased *EIF2AK2* expression in blood cells from asthma patients

may reflect previous AE. Although a confirmatory study is required, physicians should be cautious while prescribing corticosteroids when a viral infection is suspected to be a cause of AE in patients with genetic variations affecting *EIF2AK2* expression.

Among the 77 genes belonging to the AE-associated common gene module, 64 showed no changes in gene expression between Sham- and Dex-treated LCLs, and these genes were categorized as the B gene set. Three biological pathways, protein repair, syndecan interactions, and HATs acetylate histones were significantly enriched in this gene set. The protein repair pathway maintains overall protein integrity by reduction or methyl group transfer (91). It contains genes encoding methionine sulfoxide reductases that can reduce methionine sulfoxide to methionine and restore the scavenging function of methionine (92, 93). Methionine sulfoxide reductases have wide tissue distribution and protect cells from oxidative-stress-induced cell injury (93–95). However, its role in the airway has not yet been fully understood, although it is well known that AE of asthma is associated with increased oxidative stress (96, 97). *MSRA* and *MSRB2* expression in Sham-treated LCLs and PBMCs were significantly lower in asthma patients with AE compared to those without AE and Dex-treatment caused no significant changes in *MRSA* and *MRSB2* expression (Fig. 5). Taken together, it is possible that asthma patients with

decreased expression of *MSRA* and *MSRB2* in blood cells are susceptible to AE. Thus, we may need a new treatment, in addition to corticosteroids, for the effective prevention of AE in these patients. Antioxidant supplementation based on genetic susceptibility may be an option worth considering (98).

Oxidative stress due to mitochondrial dysfunction is associated with airway remodeling in asthma patients (99), and also with smooth muscle remodeling in patients with chronic obstructive pulmonary disease (100). Interestingly, syndecans, the transmembrane heparan sulfate proteoglycans, play key roles in development, tumorigenesis and inflammation, and there is growing evidence for their involvement in tissue regeneration (101). Syndecan-1 promotes lung fibrosis by regulating epithelial reprogramming through extracellular vesicles (102). Taken together, genes belonging to the protein repair and syndecan interaction pathway may collectively contribute to airway remodeling found in asthma patients and relative insensitivity to corticosteroid treatment.

EBV-transformed LCLs are widely used for human genomics study. However, EBV transformation itself alters gene expression, and therefore whether the gene regulation observed in these LCLs recapitulates that of untransformed primary cells remains controversial. Recently, it has been reported that genes involved in cholesterol metabolism are similarly regulated by statin between

LCLs and primary B cells from the same donors (103). Similarly, we observed that the Dex-regulated genes significantly overlapped in LCLs and primary B cells, and the expression of these genes showed significant correlations between treatment-naive LCLs and primary B cells (23). Based on these findings, we used gene expression in PBMCs to recapitulate changes in gene expression in LCLs. However, PBMCs harbored various cell types, including B cells, although LCL gene expression showed little association with the differential blood count (104). We did not adjust PBMC gene expression by the differential blood count in this study, and this point needs to be considered before generalizing our observations.

A weakness of this study was that we utilized gene expression profiles of peripheral blood cells. Although previous reports showed that tissue-specific genes may be expressed in a non-tissue-specific manner (105, 106) and peripheral blood cells express approximately over 80% of the genes encoded by the human genome (14), it is difficult to fully ascertain whether peripheral blood cell can be a surrogate for airway cell biology. Given that peripheral blood is an easily accessible tissue, further studies are warranted to test the utility of gene expression in blood cells to predict the AE of asthma. The small number of participants, belonging to different ethnicities, and a relative low rate of AE (especially in adult asthma patients) are other issues to keep in mind

before generalizing the results of the present study. As most of the asthma patients enrolled in this study were treated with low- to medium-dose ICSs, replicative studies performed in asthma patients treated with high-dose ICSs are warranted.

Chapter 3. Part 2

3.1 Introduction

Corticosteroids are the mainstay in asthma treatment. Corticosteroids reduce inflammation through the activation of suppressors and inhibition of inducers of inflammation. The detailed molecular mechanisms of corticosteroids have been studied extensively (107). Corticosteroids exert their action from the cell membrane to their target genes inside the nucleus via multiple processes involving various genes and TFs (107). The dysregulation of any of the interactions could potentially cause the non-responsiveness or insensitivity to the corticosteroids (108). However, to date, the precise molecular mechanism of non-responsiveness to steroids, including that associated with severe asthma or frequent exacerbation, remains unclear.

Using differential gene expression, SNPs, and eQTL (expression quantitative trait loci), previous studies have shown that multiple genes are associated with drug responses in asthma. (109–111). These studies mostly focused on one gene or a single genetic trait and explored the association of specific genes with asthma or a given phenotype of asthma. The expression of a specific gene is regulated by a TF (TF). TFs can regulate the expression of multiple genes. TFs exert their deferential action as activators or repressors depending on the specific condition. TFs also usually work together to coregulate gene

expression. Genes with similar functions tend to be co-expressed. The interaction of genes and their regulators are complex and are influenced by multiple factors. However, systematic approaches for addressing how genetic factors and their regulators determine variations in drug response in asthma treatment are lacking.

GRN attempts to evaluate beyond the co-expression of genes and understand the patterns of TFs that influence gene expressions (112). GRN can describe the hierarchical relationship between TFs and a set of target genes. The activation or repression of different TFs and their regulatory roles in gene expression may be one of the critical mechanisms underlying diverse asthma phenotypes and determining the responsiveness to anti-inflammatory drugs (113).

Recently, Qiu et al. reported the differential connectivity of GRN according to corticosteroid response in childhood asthma patients. They applied PANDA to a set of LCLs of ICS-treated childhood asthma patients from the CAMP trial (114). Differential connectivity between the GRN was assessed according to the response to ICSs (GRs versus PRs). By applying GRN, they found that TFs differentially affected gene expression in LCLs from children with asthma, including good and PRs to ICS treatment (115). The purpose of this analysis was to assess GRN of adult asthma patients who showed good or poor

improvement in lung function in response to ICSs. To do this, genome-wide gene expression levels in PBMCs from adult asthma patients were analyzed. Following this, GR or PR-specific regulatory patterns of GRN were assessed using PANDA algorithm.

3.2. Methods

3.2.1. Study population

GRs and PRs to ICSs were defined as follows: GRs were patients who had less or more than 12% improvement in FEV1 compared to baseline values at 4 weeks after initiation of treatment, respectively. PRs eventually achieved more than 12% improvement in FEV1 response to ICSs, but it took longer than 4 weeks.

3.2.2. Gene expression array

Data quality was separately checked for the two treatment types of arrays. Paired samples were pooled together and $\log_2$ transformation and quantile normalization were performed. The $\log_2$ difference in expression level between Dex- and sham-treated cell lines was used to measure the effect of drug treatment on gene expression.

3.2.3. PANDA algorithm

PANDA is a message-passing model to construct directed networks between TFs and genes using genomic information sources to predict regulatory relationships (116). The nodes in a PANDA network are TFs or genes. The directed edges extend from TFs to genes. Each edge has a weight value indicating the probability that a TF regulates a gene.

3.2.4. Network analysis

Network analysis was performed using R version 4.0.2 (www.r-project.org). We performed PANDA analysis on gene expression profiles from GRs and PRs using the R package "pandaR" Network (ver 1.22.0) (117). To seed the PANDA algorithm, the transcriptional regulatory relationships identified from the TRRUST database was used to map between TF motifs and target genes (118). This mapping file consisted of 8444 regulatory interactions corresponding to 800 TFs and 2521 target genes. There were 796 TFs in both our gene expression data and the mapping file. These TFs corresponded to 8392 pairs (TF and gene) and 2490 genes in our expression data. To minimize the effect of outliers in our networks built from a smaller sample size, two-thirds of the participants were chosen from each GR and PR group at random (without replacement) to form subsamples. These 50 subsamples were used to

construct 50 GRNs in GRs and PRs. PANDA reports the probability that a connection (edge) exists between a TF and gene in an estimated network as a Z-score. A single aggregate GRN was generated by averaging the Z-scores of edges across the 50 networks identified from the subsamples, as described elsewhere (58). We then selected high-confidence edges that had an average edge Z-score greater than 0 in the aggregate GR or PR networks. These edges can be interpreted as edges that are most likely to exist in each aggregate network. To quantify differences in high-confidence edges, we calculated an edge-enrichment score (EES): $EESi = \log_2[(kgi/kpi)/(Ng/Np)]$ where kgi and kpi are the (out-degree) numbers of high-confidence edges for TF i in the aggregate GR and PR networks, respectively, and Ng and Np are the total number of high-confidence edges in each network (58). Note that the EES is positive for edge-enrichment from a particular TF in the aggregate GRN, and negative for edge-enrichment from a particular TF in the aggregate PR network.

3.2.5. Gene set enrichment analysis

Based on EES, we selected the top-5 TFs from the aggregate networks. We then identified genes connected to these five TFs differentially in the aggregate GR and PR networks by selecting genes whose differences in high-confidence

edge Z-scores were greater than 0.75. This means that these genes have at least a 75% chance of existing and being different in each aggregate network. As we assumed that these five TFs and their differentially-connected genes were the main drivers in each aggregate network, we used them to construct GR and PR subnetworks. To assign biological meaning to the interpretability of each subnetwork, we performed pathway enrichment analyses using the web interface of ConsensusPathDB (http://cpdb.molgen.mpg.de), a meta-database that integrates different types of functional interactions from heterogeneous interaction data resources (83).

3.3. Results

A total of 23 adult asthma patients (13 GRs and 10 PRs) were enrolled in this study and Table 5 summarizes their baseline characteristics. There were no significant differences between the GRs and PRs with respect to age, sex, atopy, blood eosinophil counts, or pulmonary functions at baseline (before initiation of treatment). GRs showed significant improvement in FEV1 compared to PRs at 4 weeks after initiation of treatment (534.3 ± 310.9 mL in GRs vs. 78.4 ± 172.5 mL in PRs, $P = 6.82 \times 10^{-5}$), as expected from the definition of the two groups.

We next evaluated the differential connectivity between the GR and PR

networks. Using PANDA, we created aggregate GR and PR network and identified the top-5 TFs with the largest absolute differences of edge weights between the two networks (Table 6). *GATA1* showed the highest ratio (*n*Edge(g)/*n*Edge(p) = 1.36; P < 0.05 by permutation analysis) whereas *RELA* showed the lowest ratio (*n*Edge(g)/*n*Edge(p) = -1.219; P < 0.05 by permutation analysis). We performed a 2-sample t-test to test whether the TF was differentially expressed between Dex- and Sham-treated PBMCs. Table 7 shows the top-5 TFs and their differentially-connected genes in each aggregate GR and PR network.

It was challenging to visualize the differential connectivity of TFs and connected genes in the aggregate GR and PR networks when all TF-gene connections were considered. Hence, we illustrated subnetworks using the top-5 TFs identified and their differentially-connected genes in each aggregate GRs and PRs network. Distinct differences in connectivity between the GRs and PRs are shown in Figure 2-2. The red edges are from the network of GRs, and the blue edges are from the network of PRs. Figure 10 demonstrates the vast difference of connectivity between GRs and PRs.

Table 8 shows the gene ontology (GO) pathways in which the top-5 TFs and their differentially-connected genes were significantly enriched in each GR and PR subnetwork (adjusted P < 0.01). The identified pathways helped us

understand the differences in regulatory control driven by the top-5 TFs between GRs and PRs.

T-box transcription factor (*TBX4*) gene was differentially connected to *NFκB1*. As shown in Figure 11, expression of *TBX4* was not different in Sham-treated PBMCs in both GRs and PRs. However, in GRs, the expression of *TBX4* was significantly decreased in Dex-treated PBMCs.

Table 5. Baseline characteristics of the study participants according to response to inhaled corticosteroid

	Good-Responder n=13	Non-responder n=10	P value
Age, yr	49.1 (11.8)	50.2 (12.7)	0.83
Male, n	7 (53.8%)	4 (40.0%)	0.51
Atopy, n	8 (61.5%)	4 (40.0%)	0.31
Blood eosinophil, n	341.2 (260.3)	405.3 (372.3)	0.28
FEV1, ml	2145.1 (401.2)	2423.1 (887.6)	0.19
FEV1 pred., %	65.5 (15.2)	73.9 (19.2)	0.21
FVC, ml	3177.7 (664.6)	3412.4 (815.1)	0.12
FVC pred., %	79.1 (13.5)	86.2 (15.8)	0.52
FEV1/FVC ratio, %	67.5 (11.5)	70.9 (10.2)	0.36
FEV1 inc., ml†	534.3 (310.9)	78.4 (172.5)	6.82×10^{-5}
FEV1 inc., %	25.5 (42.7)	3.2 (8.1)	4.24×10^{-3}

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; %pred, % predicted value.

†The change in FEV1% between baseline and 1-month follow-up after inhaled corticosteroid use

Table 6. The characteristics of the top-5 unique TFs in both good-responders and poor- responders.

TF	*n*Edge(GR)	*n*Edge(PR)	*n*Diff	Log_2(EES)
GATA1	193	77	116	1.364
JUN	103	228	-125	-1.108
NFKB1	44	102	-58	-1.174
SPI1	71	169	-98	-1.212
RELA	46	110	-64	-1.219

Top-5 TFs (*GATA1, JUN, NFKB1, SP1, RELA*) have permutation p-value <0.05

*n*Diff, *n*Edge(GR) - *n*Edge(PR),

EES, ednge-enrichment score = log2[(kgi/kpi)/(Ng/Np)],

kgi and kpi - (out-degree) number of high-confidence edges for TF i in the aggregate GRs and PRs networks, respectively

Ng and Np are the total number of high-confidence edges in each network.

Table 7. Differentially-connected transcription factor and genes according to the response to inhaled corticosteroids in adult patients with asthma.

Transcription factor	Connected genes
GATA1	Good responder] *BAG1;BARD1;DR1;GP9;HBA1;HBA2;HBD;HESX1;IL17RB* Poor responder] *ATF7;FGFR4;HOXD9;LTBP4;NFATC1;RUNX1*
JUN	Good responder] *FGFR4;NGF;OXTR* Poor responder] *CNP;CYP11A1;IL3;MSR1;PANK2;SPRR1B*
NFKB1	Good responder] *ABCG2;CRMP1;CYP19A1;E2F1;FGF9;GATA3;HDAC7;IL21;MYC;NLRP2;PROX1;SFTPC; SIN3A;SMYD1;TBP;TBX4* Poor responder] *ABCB1;ACBD3;ARF1;BCAS2;CHRNE;CNP;COL2A1;CXCL12;CYP2E1;ELL; GCGR;GTF2B;KMT5A;MMP1;PANK2;PTGER2;RPL3;SET;SNF8;TNFRSF10B*
RELA	Good responder] *ABCG2;CYP19A1;E2F1;FGF9;FGFR4;GATA3;RELA;HOXC8;KDM2A;KLK3;MAT2A; MYC;NLRP2;OXTR;PROX1;RPRM;SFTPC;SIN3A;SMYD1;TBP;TBX4;TP53;UMOD;WT1*

	Poor responder] *ABCB1;ACBD3;ARF1;BCAS2;CASP9;CHRNE;CNP;COL2A1;CXCL12;CXCR4;CYP2E1;* *ELL;GCGR;GCLC;GTF2B;HOXB1;HPSE;IL22;IL3;KMT5A;LHCGR;MADCAM1;MMP1;* *MUC17;PANK2;PTGER2;RIPK2;RPL3;SET;SNF8;SPRR1B*
SP1	Good responder] *BRCA1;CCL5;CERS2;CREB1;DEFB1;ERBB2;IL2RG;KLK3;LCAT;LTBP4;OXTR;RASSF1;* *SOX3* Poor responder] *CDKN1B;COL1A1;CTGF;DNMT3B;EGR1;GSTA1;HOXB1;IL2;IL3;IRS2;LTC4S;* *MMP2;ODC1;PDE6B;PHGDH;POU4F1;SCT;SOD1;TFF1;TGF*

Table 8. The differentially enriched gene ontology and biologic processes according to the response to inhaled corticosteroids in adult patients with asthma.

Pathway name	GO:ID	adj P-value	Overlapped genes
Good responders			
angiogenesis	GO:0001525	0.000302085	*BRCA1; ERBB2; TBX4; HDAC7; SP1; JUN; KLK3; FGF9*
blood vessel development	GO:0001568	8.53E-05	*BRCA1; ERBB2; TBX4; HDAC7; WT1; SP1; JUN; KLK3; FGF9; PROX1*
response to hypoxia	GO:0001666	0.018494289	*MYC; TP53; E2F1; CREB1*
cell fate determination	GO:0001709	0.012314626	*GATA3; PROX1*
endothelial cell proliferation	GO:0001935	0.013986802	*SP1; JUN; PROX1*
regulation of immune system process	GO:0002682	0.002857509	*ERBB2; RELA; CCL5; CYP19A1; JUN; CREB1; IL21; NFKB1; MYC; SIN3A; GATA3; GATA1*
nucleobase-containing compound metabolic process	GO:0006139	0.000223767	*ERBB2; HDAC7; HOXC8; HESX1; TBX4; SOX3; NLRP2; CCL5; JUN; GATA3; CRMP1; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; KDM2A; SMYD1; FGF9; FGFR4; PROX1; GATA1; RELA; TP53; E2F1; DR1; TBP*
chromatin organization	GO:0006325	8.10E-05	*BRCA1; TP53; HDAC7; DR1; GATA3; SMYD1; KDM2A; MYC; SIN3A; RELA; GATA1*
lipid metabolic process	GO:0006629	0.001358255	*BRCA1; ERBB2; CERS2; SP1; CYP19A1; LCAT; CREB1; FGF9; NFKB1; FGFR4; PROX1; SIN3A*
cellular aromatic compound metabolic process	GO:0006725	0.000154293	*ERBB2; HDAC7; HOXC8; HESX1; TBX4; SOX3; NLRP2; CCL5; JUN; GATA3; CRMP1; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; KDM2A; SMYD1; FGF9; FGFR4; PROX1; GATA1; ABCG2; RELA; TP53; E2F1; DR1; TBP*

phosphorus metabolic process	GO:0006793	0.009073295	*ERBB2; NGF; TP53; BARD1; LCAT; IL2RG; JUN; CREB1; PROX1; IL21; CCL5; FGF9; OXTR; NFKB1; FGFR4; MYC; GATA1*
chemotaxis	GO:0006935	0.004214006	*ERBB2; DEFB1; CCL5; CYP19A1; CREB1; CRMP1; GATA3*
defense response	GO:0006952	0.001716866	*IL17RB; CYP19A1; NLRP2; DEFB1; JUN; UMOD; CCL5; IL21; KLK3; NFKB1; RELA; GATA3; SIN3A*
response to oxidative stress	GO:0006979	0.00078078	*TP53; SP1; JUN; HBA1; RELA; HBA2; SIN3A*
cell cycle arrest	GO:0007050	4.50E-05	*BRCA1; TP53; E2F1; BARD1; RASSF1; RPRM; MYC*
mitotic cell cycle checkpoint	GO:0007093	0.018855792	*BRCA1; TP53; E2F1*
cell surface receptor signaling pathway	GO:0007166	0.000324485	*BRCA1; ERBB2; NGF; BAG1; TP53; MYC; IL2RG; CCL5; JUN; CREB1; IL17RB; IL21; RELA; LTBP4; FGF9; OXTR; NFKB1; FGFR4; GATA3; GATA1*
sex determination	GO:0007530	0.000206859	*SOX3; FGF9; WT1*
sex differentiation	GO:0007548	0.002556648	*WT1; CYP19A1; GATA3; FGF9; GATA1*
memory	GO:0007613	0.009282368	*NGF; CREB1; OXTR*
response to radiation	GO:0009314	0.000939213	*BRCA1; TP53; JUN; CREB1; GATA3; MYC; RELA*
response to wounding	GO:0009611	0.005051223	*ERBB2; CERS2; HBD; JUN; GP9; GATA3; GATA1*
response to toxic substance	GO:0009636	1.67E-05	*CCL5; HBD; JUN; CREB1; OXTR; HBA1; NFKB1; RELA; HBA2; GATA3*
response to hormone	GO:0009725	0.003526954	*BRCA1; WT1; SP1; JUN; CREB1; OXTR; NFKB1; RELA; GATA1*
embryo development	GO:0009790	0.010497319	*BRCA1; WT1; TBX4; GATA3; FGF9; SIN3A; PROX1; GATA1*
animal organ morphogenesis	GO:0009887	0.003453128	*HOXC8; RELA; TBX4; WT1; JUN; PROX1; FGF9; MYC; GATA3*
tissue development	GO:0009888	0.011129319	*WT1; RELA; TBX4; UMOD; JUN; CREB1; PROX1; SMYD1; FGF9; MYC; GATA3; GATA1*

response to organic substance	GO:0010033	1.09E-05	*ERBB2; NGF; CCL5; IL21; JUN; GATA3; NFKB1; MYC; SIN3A; BRCA1; WT1; LTBP4; MAT2A; SP1; CREB1; FGF9; FGFR4; RELA; GATA1; TP53; E2F1; IL2RG; OXTR; IL17RB*
regulation of hormone levels	GO:0010817	0.000435789	*NGF; LTBP4; CCL5; CYP19A1; CREB1; NFKB1; GATA3; SIN3A*
programmed cell death	GO:0012501	0.000145176	*BRCA1; WT1; NGF; BAG1; TP53; E2F1; BARD1; NLRP2; JUN; CREB1; CCL5; RELA; NFKB1; MYC; SIN3A; GATA3; GATA1*
regulation of metabolic process	GO:0019222	0.000153725	*ERBB2; NGF; HDAC7; CCL5; HESX1; IL21; TBX4; SOX3; NLRP2; JUN; GATA3; KDM2A; NFKB1; MYC; SIN3A; BRCA1; WT1; LTBP4; BARD1; SP1; CREB1; SMYD1; FGF9; HOXC8; FGFR4; PROX1; GATA1; RELA; TP53; E2F1; DR1; TBP; OXTR*
protein metabolic process	GO:0019538	0.000287899	*ERBB2; NGF; HDAC7; CCL5; LCAT; IL21; NLRP2; SFTPC; JUN; GATA3; GP9; KDM2A; NFKB1; MYC; SIN3A; BRCA1; LTBP4; BARD1; CREB1; KLK3; SMYD1; FGF9; FGFR4; PROX1; GATA1; RELA; TP53; IL2RG; DR1; OXTR*
diencephalon development	GO:0021536	0.003588481	*HESX1; SOX3; CREB1*
cellular component assembly	GO:0022607	0.011856049	*BRCA1; MAT2A; HDAC7; CCL5; DR1; HBD; LCAT; TBP; JUN; CREB1; OXTR; HBA1; TP53; MYC; HBA2; PROX1*
signal transduction by protein phosphorylation	GO:0023014	0.000711583	*ERBB2; NGF; IL2RG; CCL5; JUN; FGF9; OXTR; NFKB1; FGFR4; MYC*
regulation of signaling	GO:0023051	0.000822167	*BRCA1; ERBB2; NGF; MYC; TP53; HDAC7; E2F1; CCL5; TBP; CYP19A1; JUN; CREB1; IL21; RELA; LTBP4; FGF9; OXTR; NFKB1; FGFR4; GATA3; GATA1*
signal release	GO:0023061	0.015601378	*LTBP4; CYP19A1; GATA3; CREB1; CCL5*
cell differentiation	GO:0030154	0.000317248	*HOXC8; NGF; MYC; HDAC7; DEFB1; IL21; SOX3; CERS2; JUN; GATA3; CRMP1; NFKB1; RELA; SIN3A; ERBB2; WT1; LTBP4; CREB1; SMYD1; FGF9; PROX1; GATA1; TP53; E2F1*
forebrain development	GO:0030900	0.001853872	*E2F1; HESX1; CREB1; OXTR; SOX3; PROX1*

DNA integrity checkpoint	GO:0031570	0.017852092	*BRCA1; TP53; E2F1*
interleukin-12 production	GO:0032615	0.017880409	*NFKB1; RELA*
interleukin-13 production	GO:0032616	0.005051223	*GATA3; IL17RB*
interleukin-5 production	GO:0032634	0.004758763	*GATA3; IL17RB*
regulation of localization	GO:0032879	2.90E-05	*ERBB2; IL17RB; CYP19A1; TP53; HDAC7; E2F1; BARD1; CCL5; SP1; DEFB1; JUN; CREB1; PROX1; NLRP2; IL2RG; FGF9; OXTR; NFKB1; CERS2; GATA3; SIN3A*
cellular response to stress	GO:0033554	0.010497319	*BRCA1; BAG1; TP53; E2F1; BARD1; JUN; KDM2A; NFKB1; MYC; CERS2; RELA; SIN3A*
cellular nitrogen compound metabolic process	GO:0034641	9.37E-05	*ERBB2; NGF; HDAC7; HOXC8; HESX1; TBX4; SOX3; CERS2; NLRP2; CCL5; JUN; GATA3; CRMP1; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; KDM2A; SMYD1; FGF9; FGFR4; PROX1; GATA1; ABCG2; RELA; TP53; E2F1; DR1; TBP*
tube morphogenesis	GO:0035239	1.97E-05	*BRCA1; ERBB2; TBX4; HDAC7; WT1; SP1; JUN; PROX1; KLK3; FGF9; MYC; GATA3*
tube development	GO:0035295	2.61E-07	*BRCA1; ERBB2; TBX4; HDAC7; WT1; SP1; UMOD; JUN; CREB1; PROX1; KLK3; FGF9; OXTR; MYC; GATA3; GATA1*
intracellular signal transduction	GO:0035556	0.000151338	*BRCA1; ERBB2; NGF; MYC; TP53; HDAC7; E2F1; IL2RG; RASSF1; TBP; DEFB1; JUN; CCL5; IL21; RELA; FGF9; OXTR; NFKB1; FGFR4; GATA3*
modification of morphology or physiology of other organism	GO:0035821	0.018437536	*SP1; JUN; CCL5*
regulation of growth	GO:0040008	0.000393428	*ERBB2; WT1; NGF; LTBP4; TP53; CREB1; IL17RB; FGF9; PROX1*
regulation of locomotion	GO:0040012	0.00027674	*ERBB2; CYP19A1; CERS2; HDAC7; CCL5; SP1; DEFB1; JUN; PROX1; FGF9; GATA3*
cellular ketone metabolic process	GO:0042180	0.004565514	*BRCA1; FGFR4; CYP19A1; PROX1*

response to drug	GO:0042493	7.99E-06	*BRCA1; ABCG2; RELA; TP53; HBD; JUN; CREB1; OXTR; HBA1; NFKB1; MYC; HBA2; GATA3; SIN3A*
homeostatic process	GO:0042592	0.01852947	*ABCG2; UMOD; BARD1; CCL5; LCAT; GATA3; OXTR; MYC; SIN3A; FGFR4; GATA1*
regulation of circadian rhythm	GO:0042752	0.000822167	*TP53; PROX1; CREB1; SIN3A*
macromolecule metabolic process	GO:0043170	0.001358255	*ERBB2; NGF; HDAC7; HOXC8; HESX1; LCAT; IL21; TBX4; SOX3; NLRP2; SFTPC; CCL5; JUN; GATA3; GP9; KDM2A; NFKB1; MYC; SIN3A; BRCA1; WT1; LTBP4; BARD1; SP1; CREB1; KLK3; SMYD1; FGF9; FGFR4; PROX1; GATA1; RELA; TP53; E2F1; IL2RG; DR1; TBP; OXTR*
response to external biotic stimulus	GO:0043207	0.012050253	*CCL5; DEFB1; JUN; KLK3; NFKB1; RELA; GATA3; SIN3A*
macromolecule methylation	GO:0043414	0.003588481	*BRCA1; WT1; MYC; GATA3; SMYD1*
regulation of multi-organism process	GO:0043900	0.001853872	*CCL5; SP1; JUN; OXTR; PROX1; SIN3A*
regulation of cellular component biogenesis	GO:0044087	0.009282368	*BRCA1; TP53; DR1; JUN; LCAT; CREB1; OXTR; PROX1*
negative regulation of molecular function	GO:0044092	0.001931578	*NGF; TP53; E2F1; NLRP2; JUN; CRMP1; NFKB1; SIN3A; PROX1; GATA1*
positive regulation of molecular function	GO:0044093	0.013173804	*ERBB2; NGF; RELA; PROX1; NLRP2; CCL5; JUN; GATA3; NFKB1; FGFR4; MYC*
cellular biosynthetic process	GO:0044249	1.04E-05	*ERBB2; HDAC7; CCL5; HESX1; LCAT; IL21; TBX4; SOX3; CERS2; NLRP2; JUN; GATA3; PROX1; KDM2A; NFKB1; MYC; SIN3A; BRCA1; WT1; MAT2A; BARD1; SP1; CREB1; SMYD1; FGF9; HOXC8; FGFR4; RELA; GATA1; TP53; E2F1; DR1; TBP; CYP19A1*
cellular macromolecule metabolic process	GO:0044260	0.000548924	*ERBB2; NGF; HDAC7; HOXC8; HESX1; LCAT; IL21; TBX4; SOX3; NLRP2; SFTPC; CCL5; JUN; GATA3; KDM2A; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; KLK3; SMYD1; FGF9; FGFR4; PROX1; GATA1; RELA; TP53; E2F1; IL2RG; DR1; TBP; OXTR*

symbiont process	GO:0044403	0.001022433	*TP53; CCL5; SP1; IL2RG; JUN; CREB1; TBP; RELA; PROX1*
G0 to G1 transition	GO:0045023	0.013988137	*BRCA1; E2F1*
innate immune response	GO:0045087	0.009258114	*CCL5; DEFB1; IL21; NLRP2; NFKB1; RELA; GATA3; SIN3A*
development of primary sexual characteristics	GO:0045137	0.001358255	*WT1; CYP19A1; GATA3; FGF9; GATA1*
cell fate commitment	GO:0045165	0.010497319	*WT1; GATA3; PROX1; GATA1*
heterocycle metabolic process	GO:0046483	0.000145176	*ERBB2; HDAC7; HOXC8; HESX1; TBX4; SOX3; NLRP2; CCL5; JUN; GATA3; CRMP1; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; KDM2A; SMYD1; FGF9; FGFR4; PROX1; GATA1; ABCG2; RELA; TP53; E2F1; DR1; TBP*
response to antibiotic	GO:0046677	0.000939213	*TP53; JUN; HBA1; RELA; HBA2; GATA3*
cell development	GO:0048468	0.001358255	*ERBB2; WT1; NGF; CERS2; E2F1; DEFB1; JUN; CREB1; PROX1; IL21; CRMP1; SOX3; RELA; GATA3; GATA1*
animal organ development	GO:0048513	0.001453756	*ERBB2; HOXC8; TBX4; RELA; UMOD; E2F1; WT1; HESX1; CYP19A1; JUN; CREB1; PROX1; SMYD1; FGF9; OXTR; SOX3; MYC; SIN3A; GATA3; GATA1*
positive regulation of biological process	GO:0048518	2.73E-05	*ERBB2; NGF; HDAC7; CCL5; DEFB1; IL21; NLRP2; JUN; GATA3; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; SMYD1; FGF9; HBA1; FGFR4; HBA2; PROX1; GATA1; RELA; TP53; E2F1; IL2RG; DR1; TBP; CYP19A1; OXTR; IL17RB*
negative regulation of biological process	GO:0048519	1.40E-06	*ERBB2; NGF; BAG1; MYC; HDAC7; HOXC8; RASSF1; SOX3; CERS2; NLRP2; RPRM; CCL5; JUN; GATA3; CRMP1; NFKB1; RELA; SIN3A; BRCA1; WT1; BARD1; UMOD; CREB1; KLK3; SMYD1; FGF9; PROX1; GATA1; TP53; E2F1; DR1; CYP19A1; OXTR*
regulation of response to stimulus	GO:0048583	0.001205929	*ERBB2; NGF; BAG1; HDAC7; CCL5; IL21; CERS2; JUN; GATA3; NFKB1; MYC; SIN3A; BRCA1; LTBP4; FGF9; FGFR4; RELA; GATA1; TP53; E2F1; TBP; CYP19A1; IL17RB*

reproductive structure development	GO:0048608	0.012389807	*WT1; CYP19A1; GATA3; FGF9; GATA1*
smooth muscle cell proliferation	GO:0048659	0.011912636	*JUN; FGF9; CCL5*
system development	GO:0048731	5.28E-05	*ERBB2; NGF; HDAC7; HOXC8; HESX1; TBX4; SOX3; CERS2; JUN; GATA3; CRMP1; MYC; SIN3A; BRCA1; WT1; SP1; UMOD; CREB1; KLK3; SMYD1; FGF9; FGFR4; PROX1; GATA1; RELA; E2F1; CYP19A1; OXTR*
regulation of developmental process	GO:0050793	2.61E-07	*ERBB2; NGF; MYC; HDAC7; SOX3; CERS2; JUN; GATA3; CRMP1; NFKB1; RELA; SIN3A; BRCA1; WT1; LTBP4; SP1; CREB1; KLK3; SMYD1; FGF9; PROX1; GATA1; E2F1; OXTR*
regulation of cellular process	GO:0050794	1.09E-05	*ERBB2; NGF; BAG1; HDAC7; HOXC8; RASSF1; DEFB1; LCAT; IL21; TBX4; SOX3; CERS2; NLRP2; RPRM; CCL5; JUN; PROX1; HESX1; CRMP1; NFKB1; MYC; SIN3A; BRCA1; WT1; LTBP4; BARD1; SP1; UMOD; CREB1; KDM2A; SMYD1; FGF9; HBA1; FGFR4; HBA2; GATA3; GATA1; RELA; TP53; E2F1; IL2RG; DR1; TBP; CYP19A1; OXTR; IL17RB*
regulation of body fluid levels	GO:0050878	0.005820894	*HBD; CREB1; GP9; OXTR; GATA3; GATA1*
regulation of DNA-binding transcription factor activity	GO:0051090	0.011129319	*NFKB1; RELA; JUN; PROX1; NLRP2*
regulation of binding	GO:0051098	0.000354349	*NGF; E2F1; JUN; CRMP1; SIN3A; GATA3; GATA1*
regulation of multicellular organismal process	GO:0051239	2.61E-07	*ERBB2; NGF; MYC; HDAC7; LCAT; IL21; SOX3; CERS2; NLRP2; JUN; GATA3; CRMP1; NFKB1; RELA; SIN3A; BRCA1; WT1; SP1; CREB1; KLK3; FGF9; PROX1; GATA1; E2F1; OXTR; IL17RB*
interaction with symbiont	GO:0051702	0.003655541	*SP1; JUN; CCL5*
head development	GO:0060322	0.010330604	*E2F1; HESX1; CREB1; FGF9; OXTR; SOX3; PROX1*
heart growth	GO:0060419	0.004393732	*WT1; PROX1; FGF9*
response to oxygen levels	GO:0070482	0.005837025	*MYC; TP53; E2F1; CREB1; OXTR*

cellular response to chemical stimulus	GO:0070887	1.11E-07	*ERBB2; NGF; CCL5; HBD; IL21; JUN; GATA3; NFKB1; MYC; SIN3A; BRCA1; WT1; LTBP4; MAT2A; SP1; CREB1; FGF9; HBA1; FGFR4; HBA2; RELA; GATA1; TP53; E2F1; IL2RG; CYP19A1; OXTR; IL17RB*
neuron death	GO:0070997	0.005192509	*NGF; JUN; GATA3; CREB1; CCL5*
cellular response to biotic stimulus	GO:0071216	0.008173356	*NFKB1; RELA; TP53; CCL5*
cellular response to endogenous stimulus	GO:0071495	1.11E-07	*BRCA1; ERBB2; NGF; RELA; TP53; E2F1; CCL5; SP1; JUN; CREB1; LTBP4; FGF9; OXTR; NFKB1; FGFR4; SIN3A; WT1; GATA3; GATA1*
response to fibroblast growth factor	GO:0071774	0.001974602	*FGFR4; GATA3; FGF9; CCL5*
nephron development	GO:0072006	0.001793883	*WT1; MYC; UMOD; GATA3*
nephron morphogenesis	GO:0072028	0.003588481	*WT1; MYC; GATA3*
cell proliferation involved in metanephros development	GO:0072203	0.001358255	*WT1; MYC*
reactive oxygen species metabolic process	GO:0072593	0.002306645	*BRCA1; HBD; HBA2; TP53; HBA1*
epithelium migration	GO:0090132	0.002873277	*SP1; JUN; GATA3; HDAC7; PROX1*
apoptotic signaling pathway	GO:0097190	0.003009306	*BRCA1; NGF; TP53; E2F1; JUN; RELA; GATA1*
cell-cell adhesion	GO:0098609	0.012921229	*ERBB2; CCL5; UMOD; IL21; RELA; GATA3; GATA1*
cellular oxidant detoxification	GO:0098869	0.00631764	*HBA1; HBD; HBA2*
organic cyclic compound metabolic process	GO:1901360	4.29E-05	*ERBB2; HDAC7; HOXC8; HESX1; LCAT; TBX4; SOX3; NLRP2; CCL5; JUN; GATA3; PROX1; CRMP1; NFKB1; MYC; SIN3A; BRCA1; WT1; BARD1; SP1; CREB1; KDM2A; SMYD1; FGF9; FGFR4; RELA; GATA1; ABCG2; TP53; E2F1; DR1; TBP; CYP19A1*

organonitrogen compound metabolic process	GO:1901564	0.000767995	*ERBB2; NGF; HDAC7; CCL5; LCAT; IL21; CERS2; NLRP2; SFTPC; JUN; GATA3; GP9; KDM2A; NFKB1; MYC; SIN3A; BRCA1; LTBP4; BARD1; CREB1; KLK3; SMYD1; FGF9; FGFR4; PROX1; GATA1; ABCG2; RELA; TP53; IL2RG; DR1; OXTR*
organic substance biosynthetic process	GO:1901576	1.09E-05	*ERBB2; HDAC7; CCL5; HESX1; LCAT; IL21; TBX4; SOX3; CERS2; NLRP2; JUN; GATA3; PROX1; KDM2A; NFKB1; MYC; SIN3A; BRCA1; WT1; MAT2A; BARD1; SP1; CREB1; SMYD1; FGF9; HOXC8; FGFR4; RELA; GATA1; TP53; E2F1; DR1; TBP; CYP19A1*
organic hydroxy compound metabolic process	GO:1901615	0.001681597	*SP1; CYP19A1; LCAT; PROX1; NFKB1; FGFR4; GATA3*
response to nitrogen compound	GO:1901698	0.001618548	*BRCA1; WT1; TP53; SP1; JUN; CREB1; OXTR; NFKB1; RELA; SIN3A*
response to oxygen-containing compound	GO:1901700	3.27E-05	*BRCA1; WT1; RELA; TP53; E2F1; CCL5; SP1; JUN; CREB1; OXTR; HBA1; NFKB1; FGFR4; HBA2; GATA3; SIN3A*
response to nerve growth factor	GO:1990089	0.001453756	*NGF; E2F1; CREB1*
Poor responder			
cellular response to chemical stimulus	GO:0070887	7.89E-13	*CDKN1B; GSTA1; RIPK2; IRS2; COL2A1; MSR1; LHCGR; CYP11A1; ARF1; EGR1; JUN; GCLC; PTGER2; LTC4S; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; SOD1; SP1; RPL3; POU4F1; PHGDH; GCGR; FGFR4; CXCL12; GATA1; IL22; CYP2E1; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; MMP2; MMP1*
response to organic substance	GO:0010033	3.90E-12	*CDKN1B; RPL3; RIPK2; IRS2; COL2A1; MSR1; LHCGR; CYP11A1; ARF1; EGR1; JUN; GCLC; PTGER2; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; CNP; SOD1; SP1; TFF1; POU4F1; GCGR; FGFR4; CXCL12; GATA1; IL22; CYP2E1; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; MMP2; MMP1*

response to hormone	GO:0009725	1.71E-11	*CDKN1B; TFF1; IL22; LHCGR; CYP11A1; EGR1; JUN; GCLC; PTGER2; NFKB1; RELA; DNMT3B; SP1; POU4F1; GCGR; CXCL12; GATA1; IRS2; CASP9; CTGF; RUNX1; COL1A1*
response to oxygen-containing compound	GO:1901700	5.64E-11	*CDKN1B; TFF1; RIPK2; IRS2; LHCGR; CYP11A1; EGR1; JUN; GCLC; PTGER2; NFKB1; RELA; DNMT3B; CNP; SOD1; SP1; POU4F1; GCGR; FGFR4; CXCL12; CYP2E1; CASP9; CTGF; IL2; COL1A1; MMP2*
cellular response to endogenous stimulus	GO:0071495	1.12E-10	*RIPK2; COL2A1; LHCGR; CYP11A1; EGR1; JUN; GCLC; PTGER2; NFKB1; RELA; DNMT3B; LTBP4; SOD1; SP1; POU4F1; GCGR; FGFR4; GATA1; IRS2; CASP9; CTGF; RUNX1; COL1A1; MMP2*
response to nitrogen compound	GO:1901698	2.42E-09	*DNMT3B; LHCGR; CYP11A1; SOD1; CDKN1B; EGR1; JUN; GCLC; CYP2E1; TFF1; CTGF; NFKB1; IRS2; SP1; COL1A1; GCGR; RELA; CXCL12; MMP2; RIPK2*
cell surface receptor signaling pathway	GO:0007166	2.53E-09	*NFATC1; CDKN1B; TNFRSF10B; RIPK2; COL2A1; MADCAM1; TGFA; ARF1; EGR1; JUN; GCLC; CXCR4; NFKB1; RELA; LTBP4; SOD1; CHRNE; MUC17; GCGR; FGFR4; CXCL12; GATA1; IL22; IRS2; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; MMP2; MMP1*
regulation of response to stimulus	GO:0048583	1.82E-07	*NFATC1; CDKN1B; KMT5A; TFF1; TNFRSF10B; RIPK2; COL2A1; TGFA; EGR1; JUN; GCLC; ABCB1; CXCR4; SCT; RELA; LTBP4; SOD1; MADCAM1; POU4F1; SNF8; NFKB1; MUC17; FGFR4; CXCL12; GATA1; HPSE; IL22; IRS2; CASP9; PDE6B; CTGF; RUNX1; IL2; IL3; COL1A1*
positive regulation of biological process	GO:0048518	2.14E-07	*NFATC1; CDKN1B; TNFRSF10B; RIPK2; ODC1; MSR1; TGFA; LHCGR; ARF1; EGR1; JUN; GCLC; PTGER2; GTF2B; ABCB1; CXCR4; NFKB1; RELA; DNMT3B; HOXD9; ELL; SOD1; SP1; HOXB1; POU4F1; SNF8; SCT; MUC17; MADCAM1; FGFR4; CXCL12; GATA1; HPSE; IRS2; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; MMP2; MMP1*

response to toxic substance	GO:0009636	2.14E-07	*DNMT3B; CNP; CHRNE; SOD1; EGR1; JUN; GSTA1; CYP2E1; LTC4S; IL2; COL1A1; NFKB1; RELA*
response to drug	GO:0042493	2.91E-07	*DNMT3B; EGR1; SOD1; CDKN1B; COL1A1; JUN; GCLC; CYP2E1; CASP9; ABCB1; RIPK2; IL2; PTGER2; CHRNE; NFKB1; RELA; CXCL12*
regulation of signaling	GO:0023051	4.67E-07	*NFATC1; KMT5A; TFF1; TNFRSF10B; RIPK2; COL2A1; TGFA; ARF1; EGR1; JUN; GCLC; CXCR4; SCT; RELA; LTBP4; SOD1; POU4F1; SNF8; NFKB1; FGFR4; CXCL12; GATA1; HPSE; IL22; IRS2; PDE6B; CTGF; RUNX1; IL2; IL3; COL1A1*
organic substance biosynthetic process	GO:1901576	8.81E-07	*NFATC1; CDKN1B; KMT5A; GSTA1; RIPK2; IRS2; ODC1; TGFA; LHCGR; CYP11A1; ELL; ARF1; EGR1; JUN; GCLC; GTF2B; LTC4S; NFKB1; RELA; DNMT3B; HOXD9; CNP; SOD1; SP1; HOXB1; RPL3; POU4F1; SET; SNF8; PHGDH; MUC17; FGFR4; GATA1; PANK2; CYP2E1; ACBD3; CTGF; RUNX1; IL2; COL1A1; ATF7*
cellular biosynthetic process	GO:0044249	2.11E-06	*NFATC1; CDKN1B; KMT5A; GSTA1; RIPK2; IRS2; ODC1; TGFA; LHCGR; CYP11A1; ELL; ARF1; EGR1; JUN; GCLC; GTF2B; LTC4S; NFKB1; RELA; DNMT3B; HOXD9; CNP; SOD1; SP1; HOXB1; RPL3; POU4F1; SET; SNF8; PHGDH; MUC17; FGFR4; GATA1; PANK2; CYP2E1; CTGF; RUNX1; IL2; COL1A1; ATF7*
response to acid chemical	GO:0001101	2.14E-06	*CDKN1B; EGR1; GCLC; CYP2E1; CTGF; PTGER2; COL1A1; FGFR4; MMP2; RELA*
regulation of metabolic process	GO:0019222	2.83E-06	*NFATC1; CDKN1B; KMT5A; RPL3; TNFRSF10B; RIPK2; COL2A1; ODC1; MSR1; TGFA; LHCGR; SET; ARF1; EGR1; JUN; GCLC; GTF2B; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; HOXD9; ELL; SOD1; SP1; HOXB1; POU4F1; PANK2; SNF8; PHGDH; GCGR; FGFR4; GATA1; IRS2; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; ATF7*

animal organ development	GO:0048513	3.36E-06	*CDKN1B; RIPK2; SPRR1B; COL2A1; ODC1; LHCGR; EGR1; JUN; CXCR4; SCT; RELA; HOXD9; CNP; SOD1; HOXB1; POU4F1; PHGDH; CXCL12; GATA1; HPSE; IRS2; CASP9; PDE6B; CTGF; RUNX1; IL2; IL3; COL1A1; MMP2*
positive regulation of molecular function	GO:0044093	5.30E-06	*TGFA; LHCGR; SOD1; POU4F1; CDKN1B; ARF1; EGR1; JUN; ABCB1; TNFRSF10B; CASP9; GTF2B; LTC4S; CTGF; RIPK2; IL2; CXCR4; NFKB1; FGFR4; RELA*
system development	GO:0048731	5.43E-06	*NFATC1; CDKN1B; RIPK2; SPRR1B; COL2A1; ODC1; LHCGR; ARF1; EGR1; JUN; CXCR4; SCT; RELA; DNMT3B; HOXD9; CNP; SOD1; SP1; HOXB1; POU4F1; PHGDH; FGFR4; CXCL12; GATA1; HPSE; IRS2; CASP9; PDE6B; CTGF; RUNX1; IL2; IL3; COL1A1; MMP2*
cell differentiation	GO:0030154	8.77E-06	*NFATC1; CDKN1B; GSTA1; RIPK2; SPRR1B; COL2A1; MSR1; PANK2; ARF1; EGR1; JUN; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; HOXD9; CNP; SOD1; TFF1; POU4F1; PHGDH; CXCL12; GATA1; IRS2; CASP9; CTGF; RUNX1; IL2; COL1A1; MMP2*
response to antibiotic	GO:0046677	9.05E-06	*SOD1; CDKN1B; EGR1; JUN; CYP2E1; CASP9; IL2; COL1A1; RELA*
intracellular signal transduction	GO:0035556	9.05E-06	*NFATC1; CDKN1B; KMT5A; TNFRSF10B; RIPK2; TGFA; LHCGR; JUN; PTGER2; CXCR4; NFKB1; RELA; SOD1; POU4F1; SNF8; SCT; GCGR; FGFR4; CXCL12; HPSE; IRS2; CASP9; CTGF; IL2; IL3*
apoptotic signaling pathway	GO:0097190	2.88E-05	*SOD1; TNFRSF10B; JUN; GCLC; CASP9; POU4F1; IL2; RELA; CXCL12; COL2A1; GATA1*
response to xenobiotic stimulus	GO:0009410	4.13E-05	*DNMT3B; SOD1; EGR1; GCLC; CYP2E1; CASP9; PHGDH; RELA*
response to mechanical stimulus	GO:0009612	4.57E-05	*JUN; GCLC; TNFRSF10B; COL1A1; NFKB1; RELA; CXCL12*
programmed cell death	GO:0012501	4.57E-05	*SET; TNFRSF10B; SOD1; CDKN1B; EGR1; JUN; GCLC; IRS2; CASP9; POU4F1; CTGF; RIPK2; IL2; CXCR4; NFKB1; RELA; CXCL12; COL2A1; SPRR1B; GATA1*

regulation of developmental process	GO:0050793	6.10E-05	*NFATC1; CDKN1B; RIPK2; MSR1; ARF1; EGR1; JUN; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; SOD1; SP1; POU4F1; CXCL12; GATA1; HPSE; CTGF; RUNX1; IL2; COL1A1*
regulation of cellular process	GO:0050794	7.16E-05	*NFATC1; CHRNE; CDKN1B; KMT5A; TFF1; TNFRSF10B; RIPK2; MADCAM1; COL2A1; ODC1; MSR1; TGFA; LHCGR; SET; ARF1; EGR1; JUN; GCLC; PTGER2; GTF2B; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; HOXD9; ELL; SOD1; SP1; HOXB1; POU4F1; PANK2; SNF8; SCT; MUC17; GCGR; FGFR4; CXCL12; GATA1; HPSE; IL22; IRS2; CASP9; PDE6B; CTGF; RUNX1; IL2; IL3; COL1A1; ATF7; MMP2; MMP1*
response to oxygen levels	GO:0070482	0.000125408	*DNMT3B; CXCR4; CDKN1B; EGR1; CTGF; COL1A1; CXCL12; MMP2*
regulation of catalytic activity	GO:0050790	0.000193556	*TGFA; LHCGR; SET; TNFRSF10B; ELL; SOD1; CDKN1B; ARF1; EGR1; JUN; IRS2; CASP9; LTC4S; CTGF; RIPK2; IL2; SNF8; CXCR4; NFKB1; FGFR4*
signal transduction by protein phosphorylation	GO:0023014	0.000241753	*TGFA; SOD1; JUN; IRS2; CTGF; RIPK2; IL2; IL3; CXCR4; NFKB1; FGFR4; SNF8*
cellular ketone metabolic process	GO:0042180	0.000250127	*PANK2; EGR1; IRS2; CYP11A1; FGFR4; ODC1*
head development	GO:0060322	0.000250127	*CXCR4; CNP; COL2A1; HOXB1; IRS2; POU4F1; PHGDH; COL1A1; SCT; CXCL12; MMP2*
regulation of immune system process	GO:0002682	0.000262934	*NFATC1; SOD1; MADCAM1; JUN; IRS2; POU4F1; RIPK2; RUNX1; IL2; COL1A1; MUC17; NFKB1; RELA; CXCL12; COL2A1; GATA1*
symbiont process	GO:0044403	0.000284545	*SET; SP1; ARF1; JUN; GTF2B; SNF8; CXCR4; RELA; CXCL12; ATF7; MMP1*
extracellular structure organization	GO:0043062	0.000324258	*MMP2; ARF1; MADCAM1; CTGF; COL1A1; FGFR4; COL2A1; MMP1*
response to inorganic substance	GO:0010035	0.000421004	*SOD1; TFF1; CDKN1B; JUN; GCLC; CYP2E1; CASP9; COL1A1; RELA*

homeostatic process	GO:0042592	0.000421004	*SOD1; ARF1; EGR1; GCLC; PTGER2; TFF1; PDE6B; CTGF; IL2; IRS2; CXCR4; MUC17; GCGR; FGFR4; CXCL12; COL2A1; GATA1*
response to nutrient	GO:0007584	0.000448338	*DNMT3B; SOD1; GCLC; COL1A1; GCGR; RELA*
macromolecule metabolic process	GO:0043170	0.00048474	*NFATC1; CDKN1B; KMT5A; RPL3; TNFRSF10B; RIPK2; SPRR1B; COL2A1; ODC1; MSR1; TGFA; SET; BCAS2; ARF1; EGR1; JUN; GCLC; GTF2B; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; HOXD9; ELL; SOD1; SP1; HOXB1; POU4F1; SNF8; PHGDH; MUC17; GCGR; FGFR4; GATA1; HPSE; IRS2; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; ATF7; MMP2; MMP1*
cellular response to abiotic stimulus	GO:0071214	0.000505507	*EGR1; GCLC; CASP9; PDE6B; TNFRSF10B; COL1A1; NFKB1*
cellular response to environmental stimulus	GO:0104004	0.000505507	*EGR1; GCLC; CASP9; PDE6B; TNFRSF10B; COL1A1; NFKB1*
signal transduction in absence of ligand	GO:0038034	0.000514581	*IL2; GATA1; COL2A1; CASP9*
response to external biotic stimulus	GO:0043207	0.000514581	*CNP; ARF1; JUN; CYP2E1; CASP9; RIPK2; PTGER2; CXCR4; NFKB1; RELA; CXCL12; ODC1*
neuron death	GO:0070997	0.000538759	*SET; SOD1; EGR1; JUN; GCLC; CASP9; POU4F1*
regulation of multicellular organismal process	GO:0051239	0.000589056	*NFATC1; CDKN1B; RIPK2; ARF1; EGR1; JUN; PTGER2; CXCR4; SCT; RELA; DNMT3B; SOD1; SP1; POU4F1; NFKB1; CXCL12; GATA1; HPSE; CTGF; RUNX1; IL2; COL1A1*
tissue development	GO:0009888	0.000628601	*HPSE; HOXD9; SOD1; CDKN1B; EGR1; JUN; GSTA1; MMP2; POU4F1; CTGF; RUNX1; PHGDH; COL1A1; RELA; COL2A1; SPRR1B; GATA1*
small molecule biosynthetic process	GO:0044283	0.000656811	*LHCGR; CNP; SOD1; SP1; EGR1; CYP2E1; LTC4S; PHGDH; NFKB1; FGFR4*
direct ossification	GO:0036072	0.000754594	*MMP2; COL1A1*

organic cyclic compound metabolic process	GO:1901360	0.001296543	*NFATC1; CDKN1B; KMT5A; RPL3; RIPK2; BCAS2; TGFA; CYP11A1; ELL; EGR1; JUN; GCLC; GTF2B; NFKB1; RELA; DNMT3B; HOXD9; CNP; SOD1; SP1; HOXB1; POU4F1; SET; SNF8; FGFR4; GATA1; PANK2; CYP2E1; ACBD3; CTGF; RUNX1; IL2; COL1A1; ATF7*
cellular macromolecule metabolic process	GO:0044260	0.001296543	*NFATC1; CDKN1B; KMT5A; RPL3; TNFRSF10B; RIPK2; SPRR1B; COL2A1; TGFA; SET; ARF1; EGR1; JUN; GCLC; GTF2B; CXCR4; NFKB1; RELA; DNMT3B; HOXD9; ELL; SOD1; SP1; HOXB1; POU4F1; SNF8; MUC17; GCGR; FGFR4; GATA1; HPSE; IRS2; CASP9; CTGF; RUNX1; IL2; IL3; COL1A1; ATF7; MMP2; MMP1*
embryo development	GO:0009790	0.001404932	*HOXD9; ELL; SOD1; MMP2; HOXB1; IL3; PHGDH; COL1A1; SCT; COL2A1; GATA1*
cell development	GO:0048468	0.001404932	*DNMT3B; PANK2; HOXD9; CNP; SOD1; ARF1; JUN; IRS2; CASP9; POU4F1; RUNX1; IL2; PHGDH; CXCR4; RELA; CXCL12; GATA1*
phosphorus metabolic process	GO:0006793	0.001608131	*CDKN1B; TNFRSF10B; RIPK2; TGFA; LHCGR; SET; CNP; ARF1; EGR1; JUN; CXCR4; NFKB1; ELL; SOD1; PANK2; SNF8; FGFR4; GATA1; IRS2; CTGF; IL2; IL3*
regulation of signaling receptor activity	GO:0010469	0.002112828	*TGFA; TFF1; CTGF; IL22; IL2; IL3; SCT; CXCL12*
response to hypoxia	GO:0001666	0.002112828	*DNMT3B; CDKN1B; EGR1; CXCR4; CXCL12; MMP2*
response to oxidative stress	GO:0006979	0.002112828	*SOD1; SP1; JUN; GCLC; CYP2E1; COL1A1; RELA*
protein metabolic process	GO:0019538	0.002397648	*CDKN1B; KMT5A; RPL3; TNFRSF10B; RIPK2; SPRR1B; COL2A1; ODC1; TGFA; SET; EGR1; JUN; GCLC; GTF2B; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; SOD1; SNF8; MUC17; FGFR4; GATA1; HPSE; IRS2; CASP9; CTGF; IL2; IL3; MMP2; MMP1*
lipid metabolic process	GO:0006629	0.002461168	*CYP11A1; SOD1; SP1; ARF1; EGR1; GSTA1; CYP2E1; ACBD3; LTC4S; PANK2; IRS2; NFKB1; FGFR4*
response to radiation	GO:0009314	0.002624078	*DNMT3B; EGR1; JUN; CASP9; PDE6B; RELA; CXCL12*

interleukin-12 production	GO:0032615	0.002691403	*NFKB1; RELA; RIPK2*
cellular response to external stimulus	GO:0071496	0.002885885	*SOD1; JUN; GCLC; TNFRSF10B; COL1A1; NFKB1*
negative regulation of biological process	GO:0048519	0.003388318	*NFATC1; CDKN1B; KMT5A; RPL3; TNFRSF10B; RIPK2; COL2A1; MSR1; SET; ARF1; EGR1; JUN; GCLC; SCT; RELA; DNMT3B; HOXD9; ELL; SOD1; TFF1; POU4F1; NFKB1; CXCL12; GATA1; IRS2; CTGF; RUNX1; IL2; COL1A1*
vitamin metabolic process	GO:0006766	0.003388318	*PANK2; NFKB1; CYP11A1; GCLC*
cellular chemical homeostasis	GO:0055082	0.00348114	*SOD1; ARF1; GCLC; PTGER2; PDE6B; IL2; IRS2; CXCR4; CXCL12*
circulatory system process	GO:0003013	0.003778958	*SOD1; GCLC; PTGER2; CTGF; IL2; GCGR; CXCL12*
organonitrogen compound metabolic process	GO:1901564	0.003931079	*CDKN1B; KMT5A; RPL3; TNFRSF10B; RIPK2; SPRR1B; COL2A1; ODC1; TGFA; SET; EGR1; JUN; GCLC; GTF2B; CXCR4; NFKB1; RELA; DNMT3B; LTBP4; SOD1; GSTA1; PANK2; SNF8; PHGDH; MUC17; FGFR4; GATA1; HPSE; IRS2; CASP9; CTGF; IL2; IL3; MMP2; MMP1*
response to fibroblast growth factor	GO:0071774	0.004137885	*GCLC; CTGF; FGFR4; COL1A1*
animal organ morphogenesis	GO:0009887	0.004137885	*HOXD9; SOD1; MMP2; HOXB1; JUN; POU4F1; CTGF; COL1A1; RELA; COL2A1*
blood vessel development	GO:0001568	0.004347224	*HPSE; SP1; EGR1; JUN; CTGF; RUNX1; COL1A1; MMP2*
tube development	GO:0035295	0.004577073	*HPSE; MMP2; JUN; CTGF; RUNX1; PHGDH; SP1; SCT; COL2A1; GATA1*
organic hydroxy compound metabolic process	GO:1901615	0.004721522	*LHCGR; CYP11A1; SOD1; SP1; PANK2; NFKB1; FGFR4*
response to extracellular stimulus	GO:0009991	0.004920936	*DNMT3B; SOD1; JUN; GCLC; COL1A1; GCGR; RELA*

cellular nitrogen compound metabolic process	GO:0034641	0.005029533	*NFATC1; CDKN1B; KMT5A; GSTA1; RIPK2; BCAS2; ODC1; TGFA; SET; ELL; EGR1; JUN; GCLC; GTF2B; NFKB1; RELA; DNMT3B; HOXD9; CNP; SOD1; SP1; HOXB1; RPL3; POU4F1; PANK2; SNF8; FGFR4; GATA1; CTGF; RUNX1; IL2; COL1A1; ATF7*
heterocycle metabolic process	GO:0046483	0.005442942	*NFATC1; CDKN1B; KMT5A; RPL3; RIPK2; BCAS2; TGFA; SET; ELL; EGR1; JUN; GCLC; GTF2B; NFKB1; RELA; DNMT3B; HOXD9; CNP; SP1; HOXB1; POU4F1; PANK2; SNF8; FGFR4; GATA1; CYP2E1; CTGF; RUNX1; IL2; COL1A1; ATF7*
platelet formation	GO:0030220	0.005983203	*GATA1; CASP9*
cellular aromatic compound metabolic process	GO:0006725	0.006114334	*NFATC1; CDKN1B; KMT5A; RPL3; RIPK2; BCAS2; TGFA; SET; ELL; EGR1; JUN; GCLC; GTF2B; NFKB1; RELA; DNMT3B; HOXD9; CNP; SP1; HOXB1; POU4F1; PANK2; SNF8; FGFR4; GATA1; CYP2E1; CTGF; RUNX1; IL2; COL1A1; ATF7*
adult locomotory behavior	GO:0008344	0.006173293	*CXCL12; HOXD9; CNP*
response to bacterium	GO:0009617	0.006173293	*CNP; JUN; PTGER2; CASP9; RIPK2; CYP2E1; NFKB1; RELA*
response to hyperoxia	GO:0055093	0.006269149	*DNMT3B; COL1A1*
interaction with symbiont	GO:0051702	0.006441104	*GTF2B; SP1; JUN*
nucleobase-containing compound metabolic process	GO:0006139	0.007037656	*NFATC1; CDKN1B; KMT5A; RPL3; RIPK2; BCAS2; TGFA; SET; ELL; EGR1; JUN; GCLC; GTF2B; NFKB1; RELA; DNMT3B; HOXD9; CNP; SP1; HOXB1; POU4F1; PANK2; SNF8; FGFR4; GATA1; CTGF; RUNX1; IL2; COL1A1; ATF7*
hormone biosynthetic process	GO:0042446	0.007674617	*NFKB1; CYP11A1; EGR1*
organic acid metabolic process	GO:0006082	0.007674617	*PANK2; GCLC; CYP2E1; GSTA1; LTC4S; IRS2; PHGDH; NFKB1; FGFR4; ODC1*
trabecula formation	GO:0060343	0.009035967	*MMP2; COL1A1*
replacement ossification	GO:0036075	0.00962511	*COL2A1; COL1A1*

cellular modified amino acid metabolic process	GO:0006575	0.011113111	*PANK2; SOD1; GCLC; GSTA1*
response to virus	GO:0009615	0.011248126	*CXCL12; ARF1; RELA; ODC1; CXCR4*
cellular oxidant detoxification	GO:0098869	0.011417819	*LTC4S; GSTA1; SOD1*
immune response-regulating signaling pathway	GO:0002764	0.011612694	*NFATC1; JUN; RUNX1; MUC17; NFKB1; RELA; RIPK2*
face morphogenesis	GO:0060325	0.012719632	*MMP2; COL1A1*
angiogenesis	GO:0001525	0.01334341	*HPSE; SP1; JUN; CTGF; RUNX1; MMP2*
response to wounding	GO:0009611	0.014318564	*HPSE; SOD1; CDKN1B; JUN; CTGF; COL1A1; GATA1*
alcohol metabolic process	GO:0006066	0.014366699	*LHCGR; SOD1; CYP11A1; SP1; NFKB1*
type I interferon production	GO:0032606	0.015776743	*NFKB1; RELA; RIPK2*
sulfur compound metabolic process	GO:0006790	0.016549133	*SP1; SOD1; GCLC; PHGDH; GSTA1*
head morphogenesis	GO:0060323	0.016701272	*MMP2; COL1A1*
regulation of binding	GO:0051098	0.01753358	*GTF2B; POU4F1; RIPK2; JUN; GATA1*
regulation of hormone levels	GO:0010817	0.017536441	*CYP11A1; EGR1; IRS2; LTBP4; SCT; NFKB1*
regulation of multi-organism process	GO:0043900	0.018409769	*GTF2B; SP1; RIPK2; JUN; SNF8*
response to ischemia	GO:0002931	0.018409769	*EGR1; CASP9*
collagen catabolic process	GO:0030574	0.018409769	*MMP2; MMP1*
defense response	GO:0006952	0.019481226	*IL22; SOD1; EGR1; JUN; PTGER2; RIPK2; IL2; CXCR4; MUC17; NFKB1; RELA; CXCL12*
cellular response to stress	GO:0033554	0.019817894	*DNMT3B; KMT5A; SOD1; CDKN1B; EGR1; JUN; CASP9; TNFRSF10B; CTGF; RIPK2; NFKB1; RELA; CXCL12*
collagen biosynthetic process	GO:0032964	0.020502514	*CTGF; COL1A1*

smooth muscle cell proliferation	GO:0048659	0.020630926	*CDKN1B; JUN; MMP2*
regulation of lipid storage	GO:0010883	0.021011029	*NFKB1; MSR1*

GO, gene ontology.

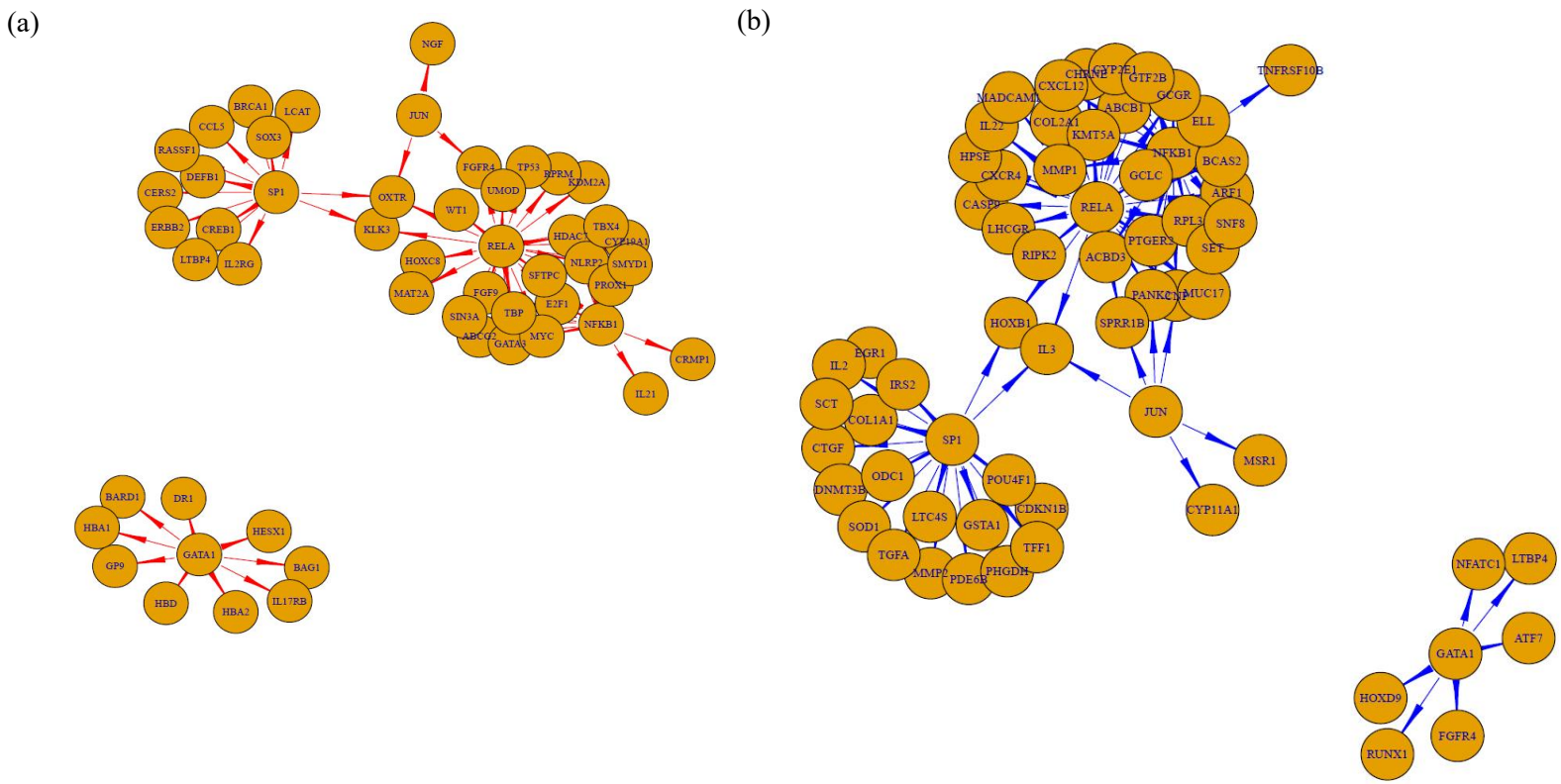


Figure 10. Differential connectivity by using the networks of top-5 transcription factor-gene pairs in good-responders and poor-responders. (a) good-responders, (b) poor-responders. The red edges are for the networks for good-responders; the blue edges are for the network of poor-responders.

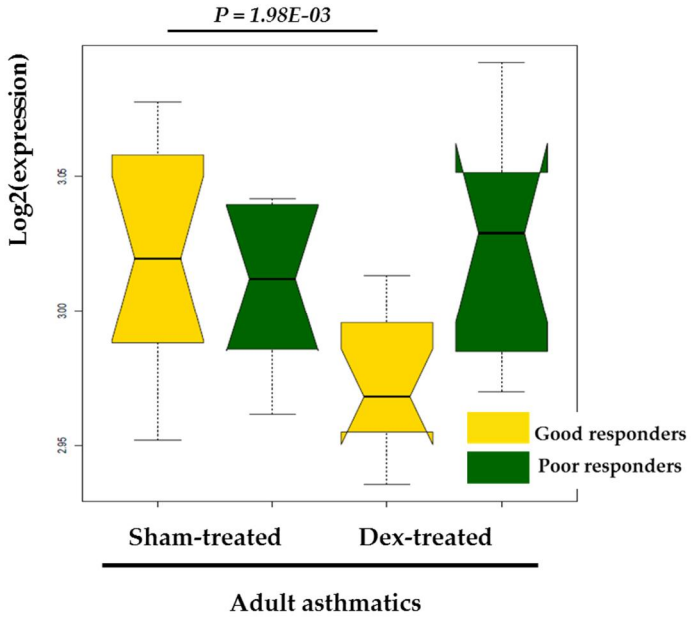


Figure 11. T-box transcription factor Tbx4 (TBX4) expression in PBMCs from adult patients with asthma.

Dex, dexamethasone.

3.4. Discussion

In this study, we used PANDA to investigate the transcriptional regulatory networks in PBMCs from adult asthma patients according to their response to ICSs. A total of 796 TFs and 2490 genes were used to construct specific 8,392 (TF, gene) edges in our data. The top-5 TF hubs obtained from TF-gene networks characterizing the effect of ICS treatment on gene expression were also identified in TF-gene networks characterizing differential ICS responses. The GRs and PRs networks showed differential connectivity and distinct enriched gene ontology. *TBX4* gene of *NFκB1* was differentially expressed between GRs and PRs following Dex treatment.

As we can see from the network in Figure 10 and Table 7, the top 5-TFs were significantly differentially connected to their target genes. The top 5-TFs that were the key 'hub" TFs, possessed 15 differentially connected edges on average between Dex- and Sham-treated PBMCs. This suggests that the top 5-TFs may be transcriptional regulatory "hot spots". The differential connectivity of these TFs-genes may help to explain why some adult asthma patients were non-responsive to ICS. *GATA1* is a critical TF for eosinophil development. Eosinophil has a clinically relevant role in severe asthma and steroid responsiveness. *GATA1* is also known to be a potent activator of IL-9 expression of mast cells. IL-9 leads to airway inflammation in airway

epithelial cells (119). Th9 cells are recognized as a novel subset of T cell subsets associated with asthma (120). JUN encoded c-Jun, and other TF families, including c-Fos, ATF, and JDP proteins constitute the activator protein-1 (AP-1) family that regulates various cellular processes such as differentiation, proliferation, and apoptosis. AP-1 regulates many inflammatory genes overexpressed in asthma (113). A recent study reported that JUN was involved in glucocorticoid receptor DNA accessibility by regulating chromatin structure (121). *RELA* encodes the TF p65, which is also known as nuclear factor NF-κB p65 subunit. *RELA* is involved in NF-κB heterodimer formation, translocation, and activation in the nucleus. Tian et al. reported that *RELA* triggered critical epithelial-mesenchymal transition pathway, including the Wnt morphogen pathway, and the JUN TF, SNAl1. *RELA* is a key transcriptional regulator of the epithelial-mesenchymal transition in airway epithelial cells, which is regarded as important pathophysiology of airway remodeling in asthma (122). SP1 is a zinc finger TF that binds to GC-boxes and related motifs of many promoters (123). In airway smooth muscle of asthma patients. SP1 is involved in prostaglandin E2 induced vascular endothelial growth factor (VEGF) production that accelerates bronchial vascular remodeling and chronic inflammation (124). *NFκB1* is one of the top-5 differentially-connected TFs (125). *NFκB1* is a TF

encoded by the *NFκB1* gene. Various stressful stimuli such as cytokines, oxidant-free radicals, and microorganisms such as bacteria or viruses promote the transcription of *NFκB1*. Overactivation of *NFκB1* is associated with airway inflammation in asthma (126). Activation of *NFκB* gene induces the expression of cytokines, chemokines, and cell adhesion molecules (CAMs) (127-130) that activate inflammatory cell infiltration in the airway. *In vivo* studies showed that intranasal challenge with an allergen (131), endotoxin (132), or microbial infection (133) increased the level of NFκB followed by inflammatory cells infiltration in airway tissue. Hence, *NFκB1* is the main target of glucocorticoid therapy in asthma management.

T-box transcription factor 4 (*TBX4*) is one of the genes that is differentially connected to *NFκB1* in GRs. *TBX4*, a member of the T-box gene family, is known mainly for its role in the development of the hind limb. However, it has recently been reported to play an important role in lung development during embryogenesis. *TBX4* is highly expressed in the visceral mesoderm of the lung primordium and is involved in multiple processes during the development of the respiratory tract. Recently, *TBX4* variants were reported to be associated with pulmonary artery hypertension (PAH) in neonates. The effect of *TBX4* variant is not clear in pulmonary disease in adults. *TBX4* variants were observed in approximately 1.4% of adult patients with PAH. Interestingly, the

clinical presentation of *TBX4* variants seemed to be milder than other PAH-associated gene mutations. To date, there has been no report on the association between *TBX4* and asthma. *TBX4*, together with TBX5, is known to regulate the growth of lung buds and airway branching by activating fibroblast growth factor 10 (FGF10) expression (134). FGF10 signaling pathway functions as a gatekeeper for airway epithelial quiescence and controls sustained airway remodeling (135). *FGF10* haploinsufficiency is associated with chronic obstructive airway disease (136). Airway remodeling is one of the main pathophysiology of severe asthma or steroid-resistant asthma. In this study, gene expression of *TBX4* did not significantly differ between GRs and PRs in the baseline Sham-treated PBMCs. However, in Dex-treated PBMCs, *TBX4* expression (*ENSG00000121075*) was significantly decreased in the GRs compared to that in PRs. These findings was not observed in the gene expression omnibus (GEO) dataset *GDS3864* (*https://www.ncbi.nlm.nih.gov/sites/GDSbrow-ser?acc=GDS3864*), which contains gene expression data of mononuclear cells from non-leukemic individuals up to 24 h after glucocorticoid injection (137). GEO dataset *GSE52778* contains the mRNA expression profiling changes of human airway smooth muscle in response to Dex. *In vitro* Dex-treatment did not change *TBX4* expression in airway smooth muscles. This suggests that the

corticosteroid-induced repression of *TBX4* was a specific finding confined to adult asthma patients who showed good response to ICSs. Although the exact biological mechanisms and network dynamics of *NFκB1* and *TBX4* remain unclear, the interaction and modulation of the network including *NFκB1-TBX1*, may be of therapeutic benefit and warrant further investigation.

The weakness of our study was the relatively small sample size. This limitation may affect the stability of the gene regulatory networks. Several previous small-sized studies have applied PANDA to construct a glucorticoid-responsing elements. In this study, to overcome the outliers in the GRN, the study participants were randomly chosen without replacement to form subsamples. These 50 subsamples were used to construct the 50 gene regulatory networks according to ICS responses. Despite the relatively small sample size, the GRE was clearly differentially connected between the GRs and the PRs. Another limitation is that functional validation studies were not conducted in this study. Qiu et al. experimentally validated the differentially-connected GRNs. They performed functional validation analysis by silencing the *NFκB* with siRNA and demonstrated the differential downstream expression based on the ICS responses. Further functional studies are needed to validate the novel differentially-connected TFs in adult asthma patients and will provide new biological and translational insights into steroid

responsiveness in adult asthma patients.

Chapter 4. Conclusions

Unlike the variations in genomic DNA, gene expression is cell-specific and changes over time in response to environmental changes. Gene expression studies may thus allow us to accurately predict the individual-level risk for asthma and response to treatments. Moreover, network-based approaches using gene expression data may provide novel insights into the pathogenesis of asthma.

In this study, we identified a common gene module associated with AE rate in both childhood and adult asthma patients using gene expression profiles of blood cells. Most genes belonging to the AE-associated common gene module showed no change in expression after *in vitro* Dex-treatment, which suggested that we need a new treatment, other than corticosteroids, to prevent AE of asthma. In addition, we have identified the GRN to elucidate the differences between GRs and PRs to ICSs in asthma patients. We identified the top-5 TFs showing differential connections between GRs and PRs and found that these top-5 TFs and their differentially-connected genes were significantly enriched in distinct biological pathways. Focusing on the expression of the individual genes, *EIF2AK2*, *MSRA,* and *MSRB2* in blood cells may help us to identify asthma patients who are susceptible to AE and adjust treatments to prevent

AE. Gene expression of *TBX4*, which is regulated by the TF, *NFκB1*, may enable us to identify GRs to ICS treatments.

Genes and biological pathways identified in this study potentially open new avenues to understand asthma pathogenesis and to overcome corticosteroid insensitivity in asthma patients. Further large-scale and prospective studies are needed to confirm our results.

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국문초록

말초혈액 단핵구에서 유전자 발현을 통한 천식 병태생리의 이해

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코르티코스테로이드는 천식 치료의 중요한 약제이다. 하지만 코르티코스테로이드에 대한 치료 효과는 환자마다 상당한 차이가 있는 것으로 알려져 있다. 특히 코르티코스테로이드에 대한 낮은 반응성은 중증 천식 또는 잦은 급성 악화와 관련이 있을 수 있다. 비록 많은 유전체 연구들이 진행되었지만, 스테로이드 저반응성과 관련된 천식의 병태 생리에 대해서는 아직까지 충분히 연구되지 않았다. 따라서 생체외 덱사메타손 처리에 따른 유전자 발현 양상의 변화를 분석하는 것은 스테로이드 저반응성과 관련된 천식 급성악화의 기전을 연구하는데 도움이 될 수 있다. 본 연구는 천식 환자의 말초 혈액 단핵구 세포의 유전자 발현 양상 및 생체외 덱사

메타손 처리에 따른 변화를 분석함으로써 스테로이드 저반응성과 관련된 병태생리 기전 및 생물학적 경로를 탐색해보고자 한다.

본 연구는 두 파트로 나누어 진행되었다. 첫번째 파트는 Weighted Gene Co-expression Network Analysis (WGCNA) 방법론을 통해, 소아천식 환자와 성인천식 환자에서 공통적으로 관찰되는 급성악화와 관련된 유전체 모듈이 존재하는지 그리고 해당 모듈에 생체외 덱사메타손 처리를 유전자 발현 양상의 변화를 탐색하였다. 소아 천식 환자 107명의 불멸화된 림프모세포 세포주와 성인천식 환자 29명의 말초혈액 단핵구에서 유전자 발현 양상을 분석하였다. 천식 급성악화는 전신스테로이드를 3일이상 복용하거나 천식으로 인해 응급 방문 또는 입원으로 정의하였다. 소아천식 환자군과 성인천식 환자군에서 공통적으로 관찰되는 총 77개의 유전자로 구성된 급성악화과 관련된 유전체 모듈을 찾았다. 해당 모듈의 *EIF2AK2* 유전체와 NOL11 유전체는 덱사메타손 처리시 소아 천식환자군과 성인천식 환자군 모두에서 유전체 발현양이 유의하게 감소하였다. 해당 모듈 중 64개의 유전체는 덱사메타손 처리시 유전자 발현양이 유의하게 변하지 않았는데, 이들 유전자들은 단백질 수리 경로 (protein repair pathway) 등과 관련성이 있었다. 단백질 수리 경로와 관련된 유전자 중에서 *MSRA*와 *MSRB2*의 중요한 역할은 산화 스트레스를 조절하는 것으로 알려져 있다. 본 연구의 두번째 파트는 유전자 조절 네트워크를 통해 성인 천식환자에

서 흡입용 스테로이드에 대한 반응성에 영향을 미치는 요인들을 탐색하였다. 흡입용 스테로이드에 대한 치료 효과가 있었던 환자군과 치료 효과가 없었던 환자군에서 생체외 덱사메타손 처리시 전사인자 차별발현을 보였던 상위 5개의 전사인자효과는 *GATA1*, *JUN*, *NFKB1*, *SPI1* 그리고 *RELA*였다. 이들 전사인자는 흡입용 스테로이드에 반응이 있었던 환자군과 없었던 환자군에서 서로 다른 유전자들과 다양한 생물학적 경로에서 연결되어 있었다. *TBX4* 유전자는 흡입용 스테로이드에 좋은 치료효과를 보였던 환자군에서 염증반응과 관련된 *NFKB1* 전사인자와 연결되어 있었다.

본 연구를 통해 규명된 새로운 유전자 및 생물학적 경로 탐색을 통해 스테로이드 저반응성과 관련된 유전적 특질을 이해하는데 도움이 되었고, 이는 천식의 다양한 병태생리에 기반한 새로운 치료제 또는 생물지표를 개발하는데 이바지할 수 있을 것이다.

주요어: 천식; 급성악화; 유전체 공통 모듈; 말초혈액단핵구; 스테로이드 저반응성; 시스템 생물학; 유전자 발현; Weighted gene co-expression network analysis; in vitro dexamethasone treatment; 생체외 덱사메타손 처리; 유전자 경로 분석; 전사인자; 유전자 조절 네트워크; Passing Attributes between Networks for Data Assimilation; 차별적 연결상

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