

Identification of Methylated Gene Markers in Childhood Atopic Asthma by Integrating Gene Expression and Methylation Profiles Based on Bioinformatic Analysis

Fang Liu, MD,¹ YuHua Zhao, BM,¹ ChunHua Gong, MM,¹ Tong Xu, MM,² YanBin Zhang, MD,¹ Jun Mei, MM,¹ JieMin Lu, BM,¹ XiaoYan Feng, BM,¹ YanMing Rong, BM,¹ and YaHui Wu, BM¹

The study aimed to screen potential key genes related to childhood atopic asthma based on an integrated analysis of gene expression and methylation profiles, as well as provide potential therapeutic targets for the progression of childhood atopic asthma. The gene expression profile of nasal epithelial cells GSE65204 (including 36 atopic asthma patients and 33 healthy controls) and the corresponding methylation profile GSE65163 (including 36 atopic asthma and 36 healthy controls) were obtained from the Gene Expression Omnibus. Following differentially expressed genes (DEGs) and differentially methylated positions (DMPs) were identified, correlation analysis between DEGs and DMPs was conducted. Function and pathway-enrichment analyses were also performed for these candidate genes. Protein–protein interaction (PPI) and transcription factor (TF) regulatory networks were constructed. A total of 199 methylated DEGs were selected in childhood atopic asthma samples. Most were highly correlated with functions in the inflammatory response and the T cell receptor (TCR) signaling pathway. Moreover, *CD8A*, *FYN*, *LCK*, *CXCR6*, *CCL5*, and *POSTN* with differentially methylated sites in their promoter regions were significantly negatively correlated with their gene expression levels, and were found to have high degrees in the PPI network. In addition, TF MAZ may play an important role in the development of childhood atopic asthma by binding its targets, such as *CD8A*, *FYN*, and *LCK*. The methylated genes, *CD8A*, *FYN*, *LCK*, *CXCR6*, *CCL5*, and *POSTN*, may be closely associated with the pathogenesis of childhood atopic asthma owing to their roles in the inflammatory response and in the TCR signaling pathway.

Keywords: childhood atopic asthma, differentially expressed genes, differentially methylated position, protein–protein interaction network, transcription factor regulatory network

Introduction

ASTHMA IS A CHRONIC DISEASE characterized by variable lung function, reversible airflow obstruction, airway inflammation, and hyperresponsiveness; its symptoms include recurrent wheezing, dyspnea, chest distress, and cough.^{1,2} Asthma is a heterogeneous disease with several phenotypes, mainly divided into allergic and nonallergic asthma.³ Symptoms of atopic asthma often begin in early childhood,⁴ and its incidence in children account for a large percentage of total atopic asthma cases.⁵ The prevalence of atopic asthma increases from 5.0% in 1996 to 6.0% in 2006, and further to 7.3% in 2016.⁶ This increase may be attributed to the increased exposure to adverse environmental conditions, such as smok-

ing, cooking, and air particles.^{7,8} Moreover, genetic risk factors also play a significant role in atopic asthma progression.^{9,10}

Asthma is a genetically determined disease and is estimated to be 44%–60% heritable.¹¹ Several genes have been reported to be related to the development of atopic asthma; for example, the downregulation of Forkhead box P3 (*FOXP3*) plays a crucial role in regulating inflammatory processes in atopic asthma through chemokine signaling pathways.¹² DNA methylation has been proved to be an important in regulating gene expression. Various reports have shown that most CpG sites of asthma-associated genes are methylated, such as gasdermin B (*GSDMB*) and *ORMDL* sphingolipid biosynthesis regulator 3 (*ORMDL3*),¹³ gasdermin A (*GSDMA*),¹⁴ leptin (*LEP*),¹⁵ acyl-CoA synthetase long-chain family member

¹Department of Pediatrics, Shanghai East Hospital affiliated to Tongji University, Shanghai, China.

²Department of Pediatrics, Changzheng Hospital Affiliated to the Second Military Medical University, Shanghai, China.

3 (*ACSL3*),¹⁶ interleukin-4 (*IL-4*), and interferon- γ (*IFN- γ*).¹⁷ Most of these genes (eg, *GSDMB*, *ORMDL*, *IL-4*, and *IFN- γ*) are related to the immune system, and changes in their methylation may contribute to the development of asthma either by promoting lymphocyte-driven inflammation or by affecting the differentiation of CD4+ T lymphocytes into T-helper cells.^{13,17} Interleukin-1 receptor type 2 (*IL1R2*) expression is downregulated because of hypermethylation in its promoter region and is associated with allergic asthma by influencing T helper 2 (T_H2) cell imbalance.¹⁸ In addition, AXL receptor tyrosine kinase (*AXL*) acts as a crucial signaling molecule in immune response, and its methylation at the CpG locus (cg00360107) has been reported to be directly correlated with a higher risk for childhood asthma.¹⁹

Since atopic asthma can severely influence quality of life, and the mechanisms underlying the development of atopic asthma are still not fully understood, it is necessary to further explore the molecular basis of this disease. Yang et al. previously analyzed DNA methylation and corresponding gene expression profiles (GEPs) to identify DNA methylation and gene expression changes related to childhood allergic asthma²⁰; however, functional enrichment analysis and prediction of upstream regulators for those genes are yet to be done. Thus, this study aimed to screen important methylated genes in samples from patients with atopic asthma and gain insights into the molecular mechanisms underlying the development of childhood atopic asthma.

Methods and Materials

Microarray data

The GEP of GSE65204 based on the platform of Agilent-028004 SurePrint G3 Human GE 8x60K Microarray was extracted from Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/). This profile consisted of 69 samples, including nasal epithelial cells from 36 African American children with atopic asthma and 33 healthy controls. The corresponding methylation profile (GSE65163) based on Illumina HumanMethylation450 BeadChip was also obtained from the GEO database and was utilized in this study. This profile consisted of 72 samples, including 36 atopic asthma samples and 36 healthy controls. The GSE65204 and GSE65163 were disposed by Yang et al.,²⁰ in which study subjects were supported by the Inner City Asthma Consortium from census tracts. However, we only reanalyzed the microarray data of above 2 datasets, and it was not required for collecting participants or obtaining patients information. Thus, the ethics approval was not needed for the present study.

Data preprocessing and analysis of differentially expressed genes

The raw probe-level data were first converted into an expression value matrix by the Affy package in R (programming language), and then background correction, quartile data normalization, and expression calculation were conducted by the Robust Multi-array Average algorithm.²¹ Afterward, the differentially expressed genes (DEGs) between atopic asthma and normal samples were analyzed by the Linear Model and Empirical Bayes Method based on the Linear Models Microarray Data (limma; www.bioconductor.org/packages/release/bioc/html/limma.html) package in R, and multiple testing adjustment was performed by the Benja-

mini and Hochberg method.²² $P < 0.05$ was used as the cutoff criterion.

Data preprocessing of the methylation profile and screening of differentially methylated positions

RnBeads is a package in R for the large-scale analysis and interpretation of DNA methylation data (<http://rnbeads.mpi-inf.mpg.de/>).²³ The RnBeads software was used to handle the raw methylation data, including quality control, background correction, data normalization, and probe filtration. In addition, the β -value, which is the ratio between methylated probe intensity and the overall intensity, was converted into M value and was used to show methylation level. After data preprocessing, the Bayes method in limma package was used to identify differentially methylated positions (DMPs) between atopic asthma and healthy samples. To account for the potential confounders, such as ethnicity, age, and gender differences between the samples, the known or predicted unknown concomitant variables were used to adjust the raw P values as the false discovery rate (FDR) using the SVA package in R.²⁴ FDR < 0.05 was chosen as the cutoff criterion to screen DMPs.

Correlation analysis between DEGs and DMPs

We obtained 4 gene sets, including upregulated and downregulated DEGs, and hypomethylated and hypermethylated genes. A Venn diagram was used to select the overlapped genes between the 4 gene sets. Upregulated DEGs that were simultaneously hypomethylated/hypermethylated, and the downregulated DEGs that were hypermethylated/hypomethylated were chosen. To narrow the possible range, the hypomethylated, upregulated DEGs and the hypermethylated, downregulated DEGs were considered as candidate genes and used for subsequent analyses.

Next, above 69 samples that performed both the methylation and GEPs analyses were screened, and correlation analysis was performed by calculating the Spearman correlation coefficient between the β -values and expression values. To explore the genes whose expression levels may be influenced by negative regulation of their methylation sites, the threshold values of $P < 0.05$ and $r < -0.4$ were defined as significant negative correlation between the DEGs and methylation levels. In addition, the DEGs with methylated sites in the promoter region were screened.

Function and pathway-enrichment analysis of candidate genes

The Database for Annotation, Visualization, and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/>) is a gene functional classification tool for grouping functionally related genes and terms into a number of biological modules for efficient interpretation of gene function.²⁵ Gene Ontology (GO; www.geneontology.org/) function and Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) pathway-enrichment analyses were performed on the upregulated DEGs with hypomethylated levels and downregulated DEGs with hypermethylated levels using the online DAVID tool.²⁶ $P < 0.05$ was chosen as the cutoff criterion for the selection of significant functional and pathway terms.

Protein–protein interaction network construction of candidate genes

The Search Tool for the Retrieval of Interacting Genes (STRING; <http://string-db.org/>) is a database for the prediction and analysis of functional associations between proteins encoded by DEGs.²⁷ The STRING database was used to explore the protein–protein interaction (PPI) pairs between DEGs with combined score >0.4 set as the threshold. After the PPI pairs were obtained, the Cytoscape software (www.cytoscape.org/) was used for network visualization.²⁸ Then, the hub proteins were selected based on the scale-free property of the network.

Prediction of transcription factors of candidate genes and regulatory network construction

The WEB-based GENE SeT Analysis Toolkit (WebGestalt; www.webgestalt.org/option.php) is a popular software tool for functional genomic, proteomic, and large-scale genetic studies for large sets of genes.^{29,30} It was used to predict the transcription factors (TF) that bind to the candidate genes. Then, count ≥ 2 and adj. $P < 0.01$ were chosen as the cutoff criteria. Moreover, the top 10 TFs (ranked by adj. P value) and their related genes were selected to conduct a TF-target regulatory network using Cytoscape software.

Results

Data preprocessing and DEGs screening

Based on the data preprocessing, the average expression values of each sample almost fell on the same straight line (Supplementary Fig. S1; Supplementary materials are available online at www.liebertpub.com/ped), which demonstrated that the normalized data could be used for further analysis. With the cutoff criterion, a total of 1,757 DEGs across atopic asthma and healthy samples were selected, including 1,106 upregulated genes and 651 downregulated genes.

Data preprocessing of methylation profile and DMP screening

According to the criterion, a total of 3,847 DMPs were obtained between atopic asthma and healthy samples, including 1,244 hypermethylated positions corresponding to 976 genes and 2,603 hypomethylated positions corresponding to 1,958 genes in atopic asthma samples.

The associations between DEGs and DMPs

A total of 147 upregulated DEGs corresponding to 226 hypomethylated DMPs, and 45 upregulated DEGs corresponding to 62 hypermethylated DMPs, whereas 52 downregulated DEGs corresponding to 83 hypermethylated DMPs and 34 downregulated DEGs corresponding to 39 hypomethylated DMPs were selected (Fig. 1). The top 5 genes (ranked according to P value of DEGs) in the above 4 gene sets are shown in Table 1.

Further correlation analysis indicated that among the 147 upregulated DEGs, 61 DEGs (eg, *ALOX15*, *POSTN*, and *CTSC*) were significantly negatively correlated with their hypomethylated DMPs. Meanwhile, among the 52 downregulated DEGs, 44 DEGs (eg, *HLA-DPA1*, *CXCR6*, and

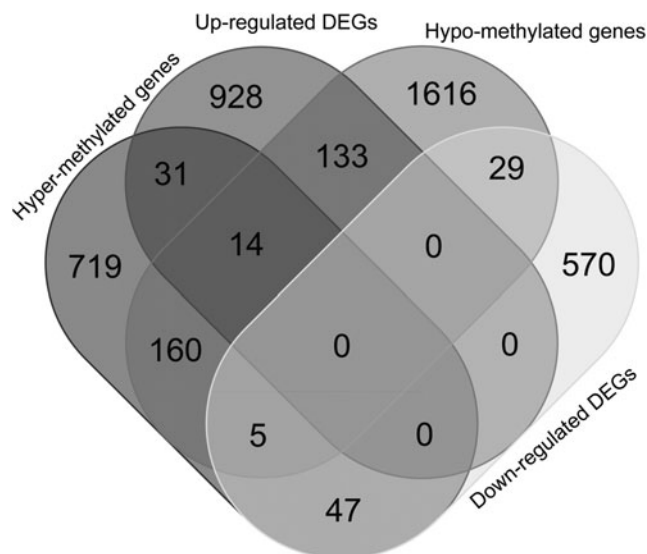


FIG. 1. Venn diagram of up-regulated DEGs, down-regulated DEGs, hypomethylated genes and hypermethylated genes.

CCL5) were significantly negatively correlated with their hypermethylated DMPs. Genes, such as *ALOX15*, *POSTN*, *CTSC*, *HLA-DPA1*, *CXCR6*, and *CCL5*, were also identified as DEGs that had significant relationships between their methylation status and gene expression level by Yang et al.²⁰ In addition, we have focused on the DEGs with methylation sites in promoter regions. There were 22 upregulated DEGs with methylation sites in the promoter region, and among these, the expression of *ARNTL2* (cg17367616), *TM4SF1* (cg16810293), *TMEM71* (cg05598678), and *POSTN* (cg13634560) were significantly negatively correlated with their methylation levels. On the other hand, there were 19 downregulated DEGs with methylation sites in the promoter region, and expressions of *CD8A* (cg03318654), *FYN* (cg04657000), *LCK* (cg11683242), *CXCR6* (cg05705212), *CCL5* (cg19411729), and *CD2* (cg16719404) were significantly negatively correlated with their methylation levels (Fig. 2).

Function and pathway-enrichment analyses of candidate genes

Based on the DAVID database, the hypomethylated, up-regulated DEGs were significantly related to functions such as “positive regulation of cell proliferation” and pathways such as “PI3K-Akt signaling pathway” (Table 2A), whereas the hypermethylated, downregulated DEGs were significantly associated with functions such as “inflammatory response” and pathways such as the “T cell receptor (TCR) signaling pathway” (eg, *CD8A*, *FYN*, and *LCK*) (Table 2B).

PPI network analysis

By integrating information from the STRING database with the identified DEGs, a total of 191 PPIs involving 83 upregulated DEGs with hypomethylation and 35 downregulated DEGs with hypermethylation, were included in the network (Fig. 3). The top 10 nodes with high degrees in the PPI network were selected, such as *CD8A*, *TNF*, *FYN*, *LCK*, *CXCR6*, *CCL5*, and *DPP4* (Table 3).

TABLE 1. TOP 5 METHYLATED DIFFERENTIALLY EXPRESSED GENES LISTED BY THE EXPR.P VALUE IN EACH GENE SETS

CpG site	Gene symbol	Feature	Meth.diff	Meth.p.fdr	Exprs.log ₂ FC	Expr.p
Hypermethylated DEGs with downregulation						
cg05705212	CXCR6	TSS1500	0.023	0.035	-0.906	3.41E-05
cg14117297	TMIGD2	Body	0.036	0.029	-1.001	7.63E-05
cg11927233	NPM1	Body	0.022	0.038	-0.177	2.25E-04
cg03318654	CD8A	5'UTR	0.033	0.020	-0.729	2.27 E-04
cg05875421	GPR68	5'UTR	0.071	0.001	-0.603	6.85 E-04
Hypomethylated DEGs with upregulation						
cg16118839	CTSC	Body	-0.077	2.78 E-04	0.830	2.73E-06
cg22112035	ANO1	Body	-0.096	3.41E-05	1.327	1.08E-05
cg13213755	NPTN	TSS1500	-0.084	5.34 E-04	0.277	1.31E-05
cg11609940	ALOX15	Body	-0.075	1.53E-09	1.322	1.85E-05
cg05715138	ALOX15	3'UTR	-0.067	4.81E-06	1.322	1.85E-05
Hypomethylated DEGs with downregulation						
cg26175846	HLA-DOA	3'UTR	-0.032	0.016	-0.914	1.68E-04
cg12193731	ACVRL1	TSS1500	-0.026	0.046	-0.528	2.67E-04
cg03113916	AIG1	3'UTR	-0.092	0.019	-0.126	3.10E-03
cg03503642	COL12A1	5'UTR	-0.087	0.009	-0.660	5.69E-03
cg10070788	BIRC5	3'UTR	-0.013	0.026	-0.494	6.65E-03
Hypermethylated DEGs with upregulation						
cg06648455	ANO1	Body	0.030	0.040	1.327	1.08E-05
cg25723217	ANO1	Body	0.018	0.021	1.327	1.08E-05
cg13965707	BDKRB1	TSS1500	0.028	0.006	0.417	8.26E-05
cg00335735	EPHA4	Body	0.016	0.037	0.706	9.48E-05
cg24355048	CTSG	Body	0.052	0.050	2.585	3.69 E-04

CpG site stands for the position of the methylation microarray. Feature represents the corresponding gene location of the CpG site. Meth.p.fdr indicates the significance methylation level of adjusted fdr. Exprs.log₂FC stands for the log₂-fold change of expression values, and Expr.p indicates the significance level of *t*-test.

DEGs, differentially expressed genes; fdr, false discovery rate.

Predicted TFs of candidate genes and the regulatory network

Based on the WebGestalt software, the upstream TFs related to the candidate genes were screened. As a result, a total of 223 TFs that targeted 159 candidate genes were identified. Then, the regulatory network containing the top 10 TFs (eg, *MAZ*, *NFAT* and *SPI1*) that corresponded to 129 target genes was constructed, and the network consisted of 338 regulation interactions (Fig. 4). Interestingly, in this regulatory network, the hub genes such as *CD8A*, *TNF*, *FYN*, and *LCK* were found to have relationships with the aforementioned TFs.

Discussion

In the present study, a total of 199 candidate genes were identified between atopic asthma children and healthy samples through association analysis of gene expression and methylation profiles. The correlation analysis showed the expression of *CD8A*, *FYN*, *LCK*, *CXCR6*, *CCL5*, and *POSTN*, whose methylation sites in the promoter regions, were significantly negatively correlated with their methylation levels. Functional enrichment analysis revealed that DEGs in the PPI network were significantly associated with the functions of "TCR signaling pathway" (eg, *FYN* and *LCK*) and "inflammatory response" (eg, *CXCR6* and *CCL5*). Additionally, *MAZ* was shown to be a key TF in the regulation of the expression of the aforementioned DEGs.

Allergic airway inflammation with eosinophilic and/or neutrophilic infiltration is a key symptom of asthma,³¹ and

the chronic inflammation induced by increased IgE is considered as a pathology of allergic asthma.³² It is well known that the increased IgE is mainly dependent on the activation and differentiation of T helper 2 (T_H2) cells, which release cytokines [eg, IL-4, IL-5, and IL-13].³³ In addition, it has been proven that different T cell subsets can also cause recurrent infections and airway inflammation, which contribute to the development of allergic asthma, more than just T_H2 cells.³⁴ Similarly, our functional enrichment analysis results showed that the hub genes, *CD8A*, *LCK*, *FYN*, *CXCR6*, and *CCL5* in the PPI network, were relevant to T cell development, activation, immune function, and inflammatory response.

In our study, *CD8A*, *LCK*, and *FYN* were all down-regulated DEGs with hypermethylation promoter regions, and were associated with "TCR signaling pathway." Notably, those genes were regulated by the TF MYC-associated zinc finger protein (*MAZ*). CD8 alpha (*CD8A*), which encodes the α chain of the CD8 protein, acts as a coreceptor with the cytotoxic T cells. Previous studies have proved that *CD8A* plays essential roles in cell-mediated immune defense and T cell development, and it can regulate antigen-specific effector T cells to suppress autoimmune thyroiditis.^{35,36} Additionally, the TF *MAZ*-related factor (*MAZR*) has been shown to regulate *CD8A* expression in CD8⁺ T cells based on TCR signaling strength, and the potential for cytotoxic lineage differentiation of CD8⁺ effector T cells is impaired in *MAZR*-deficient thymocytes.³⁷ Moreover, *CD8A* mutation can lead to CD8 deficiency, which increases susceptibility to recurrent respiratory infections.³⁸ Furthermore, the silencing of *CD8* expression is due to CpG methylation of several regions of the

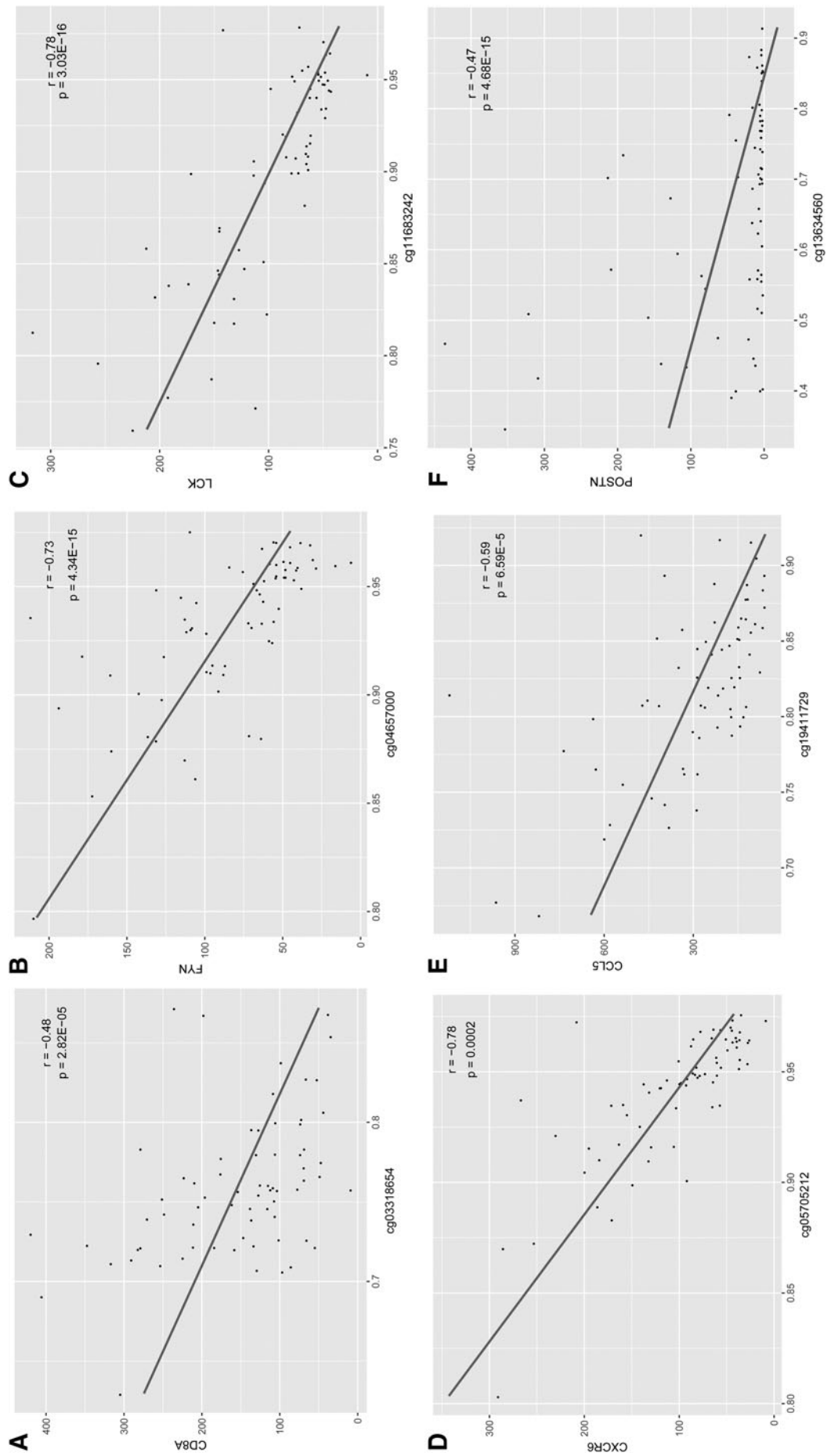


FIG. 2. Scatter plots show the significantly negative correlations between the gene expressions and methylation levels. **(A)** The Scatter plot for *CD8A*; **(B)** The scatter plot for *FYN*; **(C)** The scatter plot for *LCK*; **(D)** The scatter plot for *CXCR6*; **(E)** The scatter plot for *CCL5*; **(F)** The scatter plot for *POSTN*. Horizontal axis indicates the methylation levels (beta value) at each site, and the vertical axis indicates the relative gene expression value corresponding with the each methylated site.

TABLE 2. THE RESULTS OF FUNCTIONAL ENRICHMENT ANALYSIS THAT SHOWS TOP 10 BIOLOGICAL PROCESS AND TOP 5 PATHWAYS OF CANDIDATE GENES BASED ON THE *P* VALUE

Category	Term	Count	Ratio	<i>P</i>	Genes
A					
GO:0042490	Mechanoreceptor differentiation	3	0.020	9.86E-04	BDNF, NTRK1, NTRK2
GO:0007169	Transmembrane receptor protein Tyrosine kinase signaling pathway	6	0.041	0.001018	NTRK1, NTRK2, NPTN, TNK1, MST1R, IL31RA
GO:0030855	Epithelial cell differentiation	5	0.034	0.002639	PTHLH, LGALS3, SIX1, EHF, CPT1A
GO:0030335	Positive regulation of cell migration	7	0.048	0.003451	GCNT2, ADAM10, RHOD, THBS1, MMP14, CCL26, CYR61
GO:0010811	Positive regulation of cell–substrate adhesion	4	0.027	0.003721	ALOX15, THBS1, AGR2, CYR61
GO:0030198	Extracellular matrix organization	9	0.061	0.004136	LAMA4, LAMB2, ADAM10, LGALS3, HSPG2, POSTN, THBS1, MMP14, CYR61
GO:0003151	Outflow tract morphogenesis	4	0.027	0.004306	DHRS3, SIX1, ZFPM1, THBS1
GO:0046777	Protein autophosphorylation	6	0.041	0.012345	NTRK1, NTRK2, MINK1, TNK1, DAPK2, SIK2
GO:0034976	Response to endoplasmic reticulum stress	4	0.027	0.021497	ALOX15, THBS1, AGR2, OS9
GO:0008284	Positive regulation of cell proliferation	9	0.061	0.036769	PTHLH, NACC1, GCNT2, ADAM10, CHRM1, NTRK2, MST1R, DPP4, IL31RA
hsa04512	Extracellular matrix-receptor interaction	5	0.034	0.012119	LAMA4, TNXB, LAMB2, HSPG2, THBS1
hsa01212	Fatty acid metabolism	4	0.027	0.013017	ACOX1, ELOVL5, SCD5, CPT1A
hsa01040	Biosynthesis of unsaturated fatty acids	3		0.023032	ACOX1, ELOVL5, SCD5
hsa04151	PI3K-Akt signaling pathway	9	0.061	0.024713	LAMA4, TNXB, LAMB2, CHRM1, FGF11, KITLG, ANGPT1, CREB3 L4, THBS1
hsa05230	Central carbon metabolism in cancer	4	0.027	0.027914	NTRK1, SLC2A1, HK2, HK1
B					
GO:0050852	T cell receptor signaling pathway	9	0.173	9.54E-09	CARD11, FYN, PIK3CD, LCK, HLA-DPA1, HLA-DPB1, PSMB8, PAG1, PSMB9
GO:0031295	T cell costimulation	6	0.115	2.91E-06	CARD11, FYN, LCK, TMIGD2, HLA-DPA1, HLA-DPB1
GO:0042102	Positive regulation of T cell proliferation	5	0.010	2.27E-05	CARD11, ITGAL, HLA-DPA1, HLA-DPB1, CCL5
GO:0032729	Positive regulation of interferon-gamma production	4	0.077	2.67E-04	TNF, IL12RB1, HLA-DPA1, HLA-DPB1
GO:0042110	T cell activation	4	0.077	3.06E-04	CD8A, FYN, PIK3CD, CD2
GO:0071346	Cellular response to interferon-gamma	4	0.077	4.69E-04	CCL25, IL12RB1, HLA-DPA1, CCL5
GO:0006954	Inflammatory response	7	0.134	6.03E-04	CCL25, ITGAL, TNF, PIK3CD, CXCR6, GPR68, CCL5
GO:0050870	Positive regulation of T cell activation	3	0.058	0.001108	LCK, HLA-DPA1, HLA-DPB1
GO:0001819	Positive regulation of cytokine production	3	0.058	0.002038	CARD11, TNF, TMIGD2
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	3	0.058	0.030269	CD8A, FYN, LCK
hsa05323	Rheumatoid arthritis	6	0.115	1.18E-05	ITGAL, TNF, HLA-DPA1, HLA-DPB1, CCL5, HLA-DMA
hsa04660	T cell receptor signaling pathway	6	0.115	2.70E-05	CARD11, TNF, CD8A, FYN, PIK3CD, LCK
hsa05416	Viral myocarditis	5	0.010	4.68E-05	ITGAL, FYN, HLA-DPA1, HLA-DPB1, HLA-DMA
hsa05321	Inflammatory bowel disease	5	0.010	7.39E-05	TNF, IL12RB1, HLA-DPA1, HLA-DPB1, HLA-DMA
hsa04514	Cell adhesion molecules	6	0.115	1.32E-04	ITGAL, CD8A, CD2, HLA-DPA1, HLA-DPB1, HLA-DMA

A: The functional enrichment analysis of upregulated hypomethylated genes. B: The functional enrichment analysis of downregulated hypermethylated genes. Count represents the number of DEGs enriched in the corresponding GO and KEGG categories, and ratio is a relationship between gene count in the corresponding term and numbers of the gene set.

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

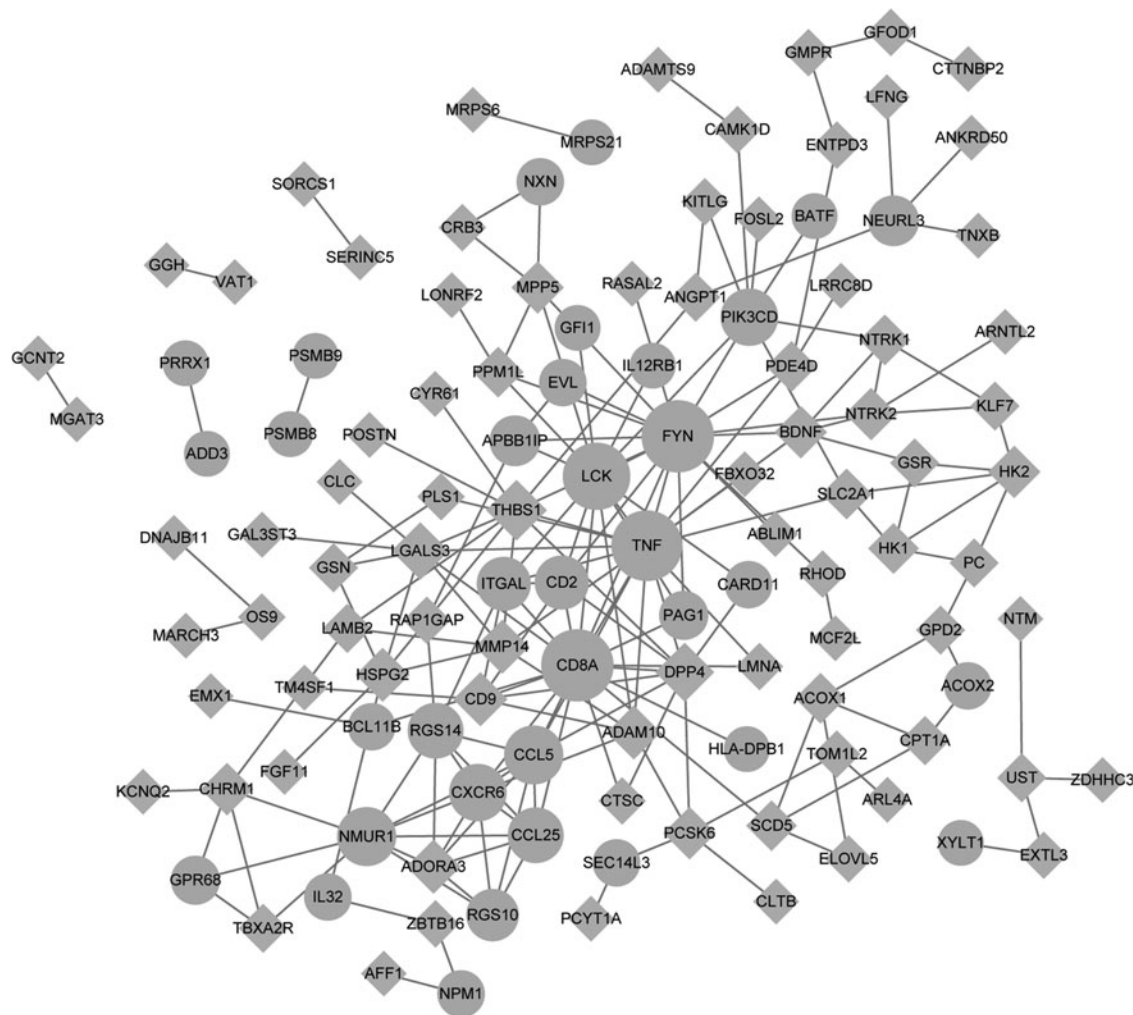


FIG. 3. The protein–protein interaction network of candidate genes. The *nodes* stand for the genes and the *lines* represent the corresponding interaction relationships. The *circular-shaped nodes* represent downregulated hypermethylated DEGs, and the *diamond-shaped nodes* represent upregulated hypomethylated DEGs.

CD8A locus.³⁹ Similarly, our results showed that *CD8A* was downregulated in atopic asthma children samples compared with its expression in the healthy controls, which was also shown to be significantly negatively correlated with its hypermethylation at cg03318654 site of the promoter region.

TABLE 3. THE TOP 10 NODES WITH HIGH DEGREE IN PROTEIN–PROTEIN INTERACTION NETWORK BASED ON THE VALUES OF DEGREE CENTRALITY

Gene	DC	BC	CC
<i>FYN</i>	17	2745.2800	0.044965412
<i>CD8A</i>	17	2389.5051	0.044879172
<i>TNF</i>	16	2259.3620	0.045051984
<i>LCK</i>	14	1210.6234	0.044605415
<i>THBS1</i>	10	2042.7175	0.044690605
<i>NMUR1</i>	9	434.82626	0.04293578
<i>DPP4</i>	9	904.0701	0.044452887
<i>CCL5</i>	9	613.2962	0.04418429
<i>LGALS3</i>	8	820.4850	0.044234406
<i>CXCR6</i>	8	241.1321	0.043771043

BC, betweenness centrality; CC, closeness centrality; DC, degree centrality.

These collectively imply that the downregulation of *CD8A* might be caused by the CpG hypermethylation, and this might influence the TCR signaling pathway, thereby regulating atopic asthma progression in children.

Lymphocyte cell-specific protein tyrosine kinase (*LCK*) and nonreceptor tyrosine protein kinase (*FYN*) belong to the members of the protein tyrosine kinase oncogene family, which are crucial in T cell development and activation mediated by TCR signaling, and they are both cancer-related oncogenes regulated by *MAZ*.^{40,41} The *LCK* gene deficiency can cause T cell immunodeficiency by impairing TCR signaling in T cells.⁴² Similarly, it has been reported that *FYN* plays a role in downstream signaling pathways related to T cell proliferation and differentiation following TCR stimulation, and several polymorphisms in *FYN* are closely connected with childhood asthma.⁴³ Moreover, the altered methylation level of *LCK* acts as an immune response factor correlated with T lymphocyte infiltration in breast cancers.⁴⁴ The high DNA methyltransferase activity is likely to downregulate *FYN* in T lymphoma cell.⁴⁵

In the PPI network, another 2 hub-downregulated genes, *CXCR6* and *CCL5*, differentially hypermethylated in their promoter regions at cg05705212 and cg19411729 CpG sites,

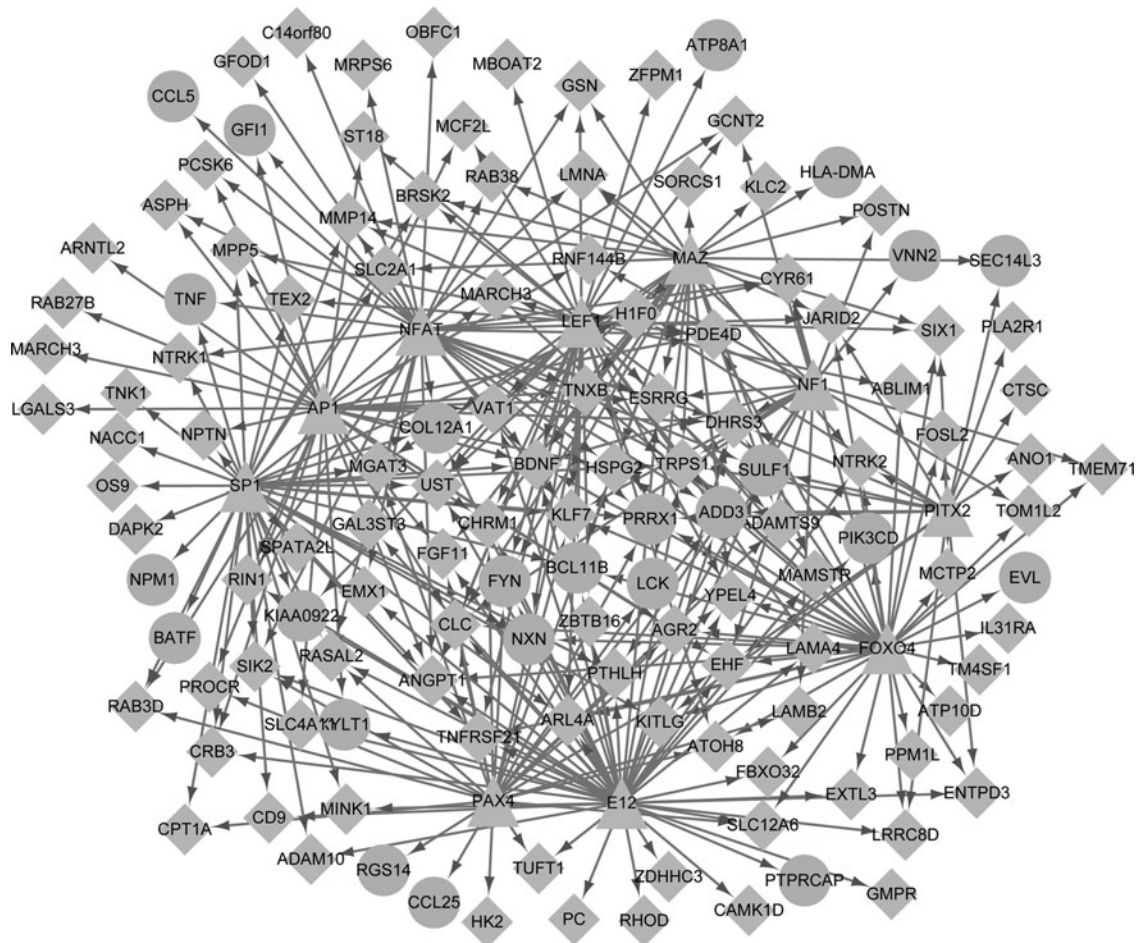


FIG. 4. The regulatory network of TFs and their target genes. The *triangle-shaped nodes* represent the top 10 predicted TFs, the *diamond-shaped nodes* represent downregulated hypermethylated DEGs and *circular-shaped nodes* represent upregulated hypomethylated DEGs. TFs, transcription factors.

respectively, were involved in the function of “inflammatory response.” C-X-C Motif Chemokine Receptor 6 (*CXCR6*) is not only closely related to T cell activation, but also its downregulated expression may inhibit a downstream process of continued inflammation in the animal model with allergen challenged asthma.⁴⁶ Chemokine ligand 5 (*CCL5*) is a chemotactic cytokine for airway recruitment of eosinophils to induce airway inflammation and hyperresponsiveness in asthma.⁴⁷ An enhanced level of *CCL5* is detected in adult atopic asthmatics,⁴⁸ however, Hamsten et al. have suggested that *CCL5* is downregulated in childhood asthma,⁴⁹ which is consistent with our result. Although we speculated that *CXCR6* hypermethylated at cg05705212 and *CCL5* hypermethylated at cg19411729 were closely associated with the inflammatory response in children with atopic asthma, no related studies have reported about the expression of these genes being associated with the alteration of their methylation levels in asthmatics. Therefore, future studies are required to confirm this hypothesis.

According to our result, the hypomethylated periostin (*POSTN*) at cg13634560 was upregulated in atopic asthma children samples, and its expression level was significantly negatively correlated with its methylation level. Periostin is an extracellular matrix protein involved in allergic inflammation.⁵⁰ Similarly, Jia et al. have indicated that periostin levels in serum

are remarkably enhanced in asthmatics, and its increase is a marker for eosinophilic airway inflammation.⁵¹ However, this was firstly suggested that the hypomethylation of *POSTN* at cg13634560 may be associated with childhood atopic asthma.

It was worth mentioning that, although we used the same dataset as the study by Yang et al.,²⁰ different analyses were performed in the present study. In the study by Yang et al., only 186 DMPs or differentially methylated probes, 53 DEGs, and 32 DEGs that showed significant relationships between DNA methylation and gene expression within 5 kb were screened, which could have limited the subsequent bioinformatics analysis. In the present study, a less restrictive threshold value, as well as different methods were employed to identify more DEGs and DMPs. Notably, the DEGs with an associated change in DNA methylation mentioned in their article (eg, *CXCR6*, *CCL5*, and *POSTN*) were also identified in our study. However, novel DEGs that had significant relationships between their DNA methylation status were identified in our study and should be validated in future studies.

Conclusions

In summary, genes such as *CD8A*, *FYN*, *LCK*, *CXCR6*, *CCL5*, and *POSTN* with changes in the methylation levels of their promoter region might play an important role in the

development of childhood atopic asthma by influencing the “inflammatory response” and “TCR signaling pathway.” However, future experiments are necessary to validate these findings.

Author Disclosure statement

No competing financial interests exist.

References

- Drews AC, Pizzichini MMM, Pizzichini E, Pereira MU, Pitrez PM, Jones MH, et al. Neutrophilic airway inflammation is a main feature of induced sputum in nonatopic asthmatic children. *Allergy* 2009; 64:1597–1601.
- Kroegel C. Global Initiative for Asthma (GINA) guidelines: 15 years of application. *Expert Rev Clin Immunol* 2009; 5: 239–249.
- Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012; 18:716.
- Van-Den-Toorn L, Overbeek S, De-Jongste J, Leman K, Hoogsteden H, Prins J. Airway inflammation is present during clinical remission of atopic asthma. *Am J Respir Crit Care Med* 2001; 164:2107.
- Ellwood P, Asher MI, Beasley R, Clayton TO, Stewart AW. The International Study of Asthma and Allergies in Childhood (ISAAC): phase three rationale and methods. *Int J Tuberc Lung Dis* 2005; 9:10–16.
- Backman H, Räisänen P, Hedman L, Stridsman C, Andersson M, Lindberg A, et al. Increased prevalence of allergic asthma from 1996 to 2006 and further to 2016—results from three population surveys. *Clin Exp Allergy* 2017; 47:1426–1435.
- Wallace LA, Mitchell H, O'Connor GT, Neas L, Lippmann M, Kattan M, et al. Particle concentrations in inner-city homes of children with asthma: the effect of smoking, cooking, and outdoor pollution. *Environ Health Perspect* 2003; 111:1265–1272.
- Wagner JG, Morishita M, Keeler GJ, Harkema JR. Divergent effects of urban particulate air pollution on allergic airway responses in experimental asthma: a comparison of field exposure studies. *Env Health* 2012; 11:1–13.
- Mukherjee AB, Zhang Z. Allergic asthma: influence of genetic and environmental factors. *J Biol Chem* 2011; 286: 32883–32889.
- Zhang YN, Li YJ, Li H, Zhou H, Shao XJ. Association of CD14 C159T polymorphism with atopic asthma susceptibility in children from Southeastern China: a case-control study. *Genet Mol Res* 2015; 14:4311–4317.
- Mathias RA, Freidhoff LR, Blumenthal MN, Meyers DA, Lester L, King R, et al. Genome-wide linkage analyses of total serum IgE using variance components analysis in asthmatic families. *Genet Epidemiol* 2001; 20:340–355.
- Krogulska A, Polakowska E, Wąsowska-Królikowska K, Mafachowska B, Młynarski W, Borowiec M. Decreased FOXP3 mRNA expression in children with atopic asthma and IgE-mediated food allergy. *Ann Allergy Asthma Immunol* 2015; 115:415–421.
- Acevedo N, Reinius LE, Greco D, Gref A, Orsmarkpietras C, Persson H, et al. Risk of childhood asthma is associated with CpG site polymorphisms, regional DNA methylation and mRNA levels at the GSDMB/ORMDL3 locus. *Hum Mol Genet* 2015; 24:875.
- Al TA, Gagné-Ouellet V, Madore AM, Laprise C, Naumova AK. Local genotype influences DNA methylation at two asthma-associated regions, 5q31 and 17q21, in a founder effect population. *Group.bmj.com* 2016; 53:232–241.
- Mukherjee N, Lockett GA, Merid SK, Melén E, Pershagen G, Holloway JW, et al. DNA methylation and genetic polymorphisms of the Leptin gene interact to influence lung function outcomes and asthma at 18 years of age. *Int J Mol Epidemiol Genet* 2016; 7:1–17.
- Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, et al. Relation of DNA methylation of 5f 5tic polymorphisms of the leptin gene interact to influence lung function outcomes and asns and childhood asthma. *PLoS One* 2009; 4: 3138–3144.
- Kwon NH, Kim JS, Lee JY, Oh MJ, Choi DC. DNA methylation and the expression of IL-4 and IFN- γ promoter genes in patients with bronchial asthma. *J Clin Immunol* 2008; 28:139–146.
- Gagné-Ouellet V, Guay SP, Boucher-Lafleur AM, Bouchard L, Laprise C. DNA methylation signature of interleukin 1 receptor type II in asthma. *Clin Epigenetics* 2015; 7:80.
- Gao L, Millstein J, Siegmund K, Dubeau L, Avol E, Gilliland F, et al. Association between DNA methylation in the AXL gene at birth and childhood respiratory health. *Am J Respir Crit Care Med* 2017; 195:A7221.
- Yang IV, Pedersen BS, Liu AH, O'Connor GT, Pillai D, Kattan M, et al. The nasal methylome and childhood atopic asthma. *J Allergy Clin Immunol* 2016; 139:1478–1488.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 2003; 4:249–264.
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Methodol* 1995; 57:289–300.
- Assenov Y, Müller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA methylation data with RnBeads. *Nat Methods* 2014; 11:1138–1140.
- Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 2012; 28:882–883.
- Da WH, Sherman BT, Tan Q, Collins JR, Alvord WG, Roayaei J, et al. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. *Genome Biol* 2007; 8: 90–105.
- Kanehisa M, Goto S. KEGG: Kyoto Encyclopaedia of Genes and Genomes. *Nucleic Acids Res* 2000; 28:27–30.
- Von MC, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B. STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res* 2003; 31:258–261.
- Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; 13:2498–2504.
- Wang J, Duncan D, Shi Z, Zhang B. WEB-based GENE SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res* 2013; 41:77–83.
- Zhang B, Kirov S, Snoddy J. WebGestalt: an integrated system for exploring gene sets in various biological contexts. *Nucleic Acids Res* 2005; 33:741–748.
- Hiroshi N, Koichi H. Role of IL-23 and Th17 cells in airway inflammation in asthma. *Immune Netw* 2010; 10:1–4.
- Yalcin AD, Bisgin A, Górczynski RM. IL-8, IL-10, TGF- β , and GCSF levels were increased in severe persistent allergic asthma patients with the anti-IgE treatment. *Mediators of Inflamm* 2012; 2012:720976.

33. Grunstein MM, Hakonarson H, Leiter J, Chen M, Whelan R, Grunstein JS, et al. IL-13-dependent autocrine signaling mediates altered responsiveness of IgE-sensitized airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2002; 282:520–528.
34. Lloyd CM, Hessel EM. Functions of T cells in asthma: more than just TH2 cells. *Nat Rev Immunol* 2010; 10:838–848.
35. Ganesh BB, Cheatem DM, Jian RS, Vasu C, Prabhakar BS. GM-CSF-induced CD11c+CD8a—dendritic cells facilitate Foxp3+ and IL-10+ regulatory T cell expansion resulting in suppression of autoimmune thyroiditis. *Int Immunol* 2009; 21:269–282.
36. Staff TPO. Correction: DNA methylation and regulation of the CD8A after duck hepatitis virus type 1 infection. *PLoS One* 2014; 9:e88023.
37. Sakaguchi S, Hombauer M, Bilic I, Naoe Y, Schebesta A, Taniuchi I, Ellmeier W. The zinc finger protein MAZR is part of the transcription factor network controlling CD4/CD8 cell fate decision of DP thymocytes. *Nat Immunol* 2010; 11: 442–448.
38. Dumontet E, Osman J, Guillemont-Lambert N, Cros G, Moshous D, Picard C. Recurrent respiratory infections revealing CD8 α deficiency. *J Clin Immunol* 2015; 35:692–695.
39. Harland KL, Day EB, Apte SH, Russ BE, Doherty PC, Turner SJ, et al. Epigenetic plasticity of Cd8a locus during CD8+ T-cell development and effector differentiation and reprogramming. *Nat Commun* 2011; 5:3547.
40. Laird RM, Hayes SM. Roles of the Src tyrosine kinases Lck and Fyn in regulating $\gamma\delta$ TCR signal strength. *PLoS One* 2010; 5:e8899.
41. Hu H. An efficient algorithm to identify coordinately activated transcription factors. *Genomics* 2010; 95:143–150.
42. Hauck F, Randriamampita C, Martin E, Gerart S, Lambert N, Lim A, et al. Primary T-cell immunodeficiency with immunodysregulation caused by autosomal recessive LCK deficiency. *J Allergy Clin Immunol* 2012; 130:1144–1152.
43. Szczepankiewicz A, Breborowicz A, Skibińska M, Wiłkość M, Tomaszewska M, Hauser J. Association analysis of tyrosine kinase FYN gene polymorphisms in asthmatic children. *Int Arch Allergy Immunol* 2008; 145:43–47.
44. Dedeurwaerder S, Desmedt C, Calonne E, Singhal SK, Haibe-Kains B, Defrance M, et al. DNA methylation profiling reveals a predominant immune component in breast cancers. *EMBO Mol Med* 2011; 3:726–741.
45. Kozłowski A. Inhibition of DNA methyltransferase activity upregulates Fyn tyrosine kinase expression in Hut-78 T-lymphoma cells. *Biomed Pharmacother* 2008; 62:672–676.
46. Kallinich T, Schmidt S, Hamelmann E, Fischer A, Qin S, Luttmann W, et al. Chemokine-receptor expression on T cells in lung compartments of challenged asthmatic patients. *Clin Exp Allergy* 2005; 35:26–33.
47. Isgro M, Bianchetti L, Marini MA, Bellini A, Schmidt M, Mattoli S. The C-C motif chemokine ligands CCL5, CCL11, and CCL24 induce the migration of circulating fibrocytes from patients with severe asthma. *Mucosal Immunol* 2013; 6:718.
48. Powell N, Humbert M, Durham SR, Assoufi B, Kay AB, Corrigan CJ. Increased expression of mRNA encoding RANTES and MCP-3 in the bronchial mucosa in atopic asthma. *Eur Respir J* 1996; 9:2454.
49. Hamsten C, Häggmark A, Grundström J, Mikus M, Lindskog C, Konradsen JR, et al. Protein profiles of CCL5, HPGDS, and NPSR1 in plasma reveal association with childhood asthma. *Allergy* 2016; 71:1357.
50. Izuhara K, Arima K, Ohta S, Suzuki S, Inamitsu M, Yamamoto K. Periostin in allergic inflammation. *Allergol Int* 2014; 63:143–151.
51. Jia G, Erickson RW, Choy DF, Mosesova S, Wu LC, Solberg OD, et al. Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J Allergy Clin Immunol* 2012; 130:647–654.

Address correspondence to:

YaHui Wu, BM

Department of Pediatrics

Shanghai East Hospital Affiliated to Tongji University

No.1800, Yuntai Road, Pudong New Area

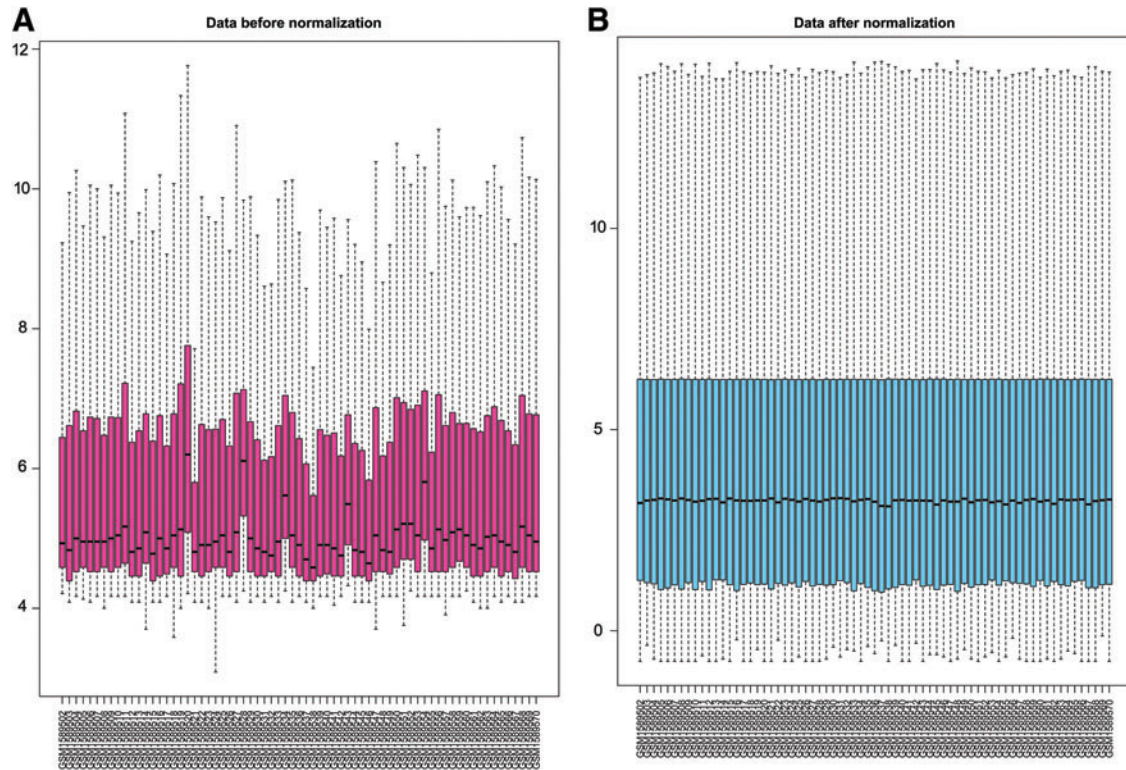
Shanghai 200003

China

E-mail: wuyahui0813@outlook.com

Received for publication July 23, 2017; accepted after revision May 5, 2018.

Supplementary Data



SUPPLEMENTARY FIG. S1. The box plots of gene expression profile. **(A)** Before data normalization; **(B)** after data standardization. The black line in the cassette stands for the median of gene expression and the y-axis represents the average log₂ expression levels across all genes in each samples