

ORIGINAL ARTICLE

Differential gene expression profiles of peripheral blood mononuclear cells in childhood asthmaQian Kong, PhD^{1,2}, Wen-Jing Li, MM², Hua-Rong Huang, MM^{1,2}, Ying-Qiang Zhong, PhD^{1,3}, and Jian-Pei Fang, PhD^{1,2}¹Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, P.R. China, ²Department of Pediatrics, and ³Department of Gastroenterology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, P.R. China**Abstract**

Objective: Asthma is a common childhood disease with strong genetic components. This study compared whole-genome expression differences between asthmatic young children and healthy controls to identify gene signatures of childhood asthma. **Methods:** Total RNA extracted from peripheral blood mononuclear cells (PBMC) was subjected to microarray analysis. QRT-PCR was performed to verify the microarray results. Classification and functional characterization of differential genes were illustrated by hierarchical clustering and gene ontology analysis. Multiple logistic regression (MLR) analysis, receiver operating characteristic (ROC) curve analysis, and discriminate power were used to scan asthma-specific diagnostic markers. **Results:** For fold-change ≥ 2 and $p < 0.05$, there were 758 named differential genes. The results of QRT-PCR confirmed successfully the array data. Hierarchical clustering divided 29 highly possible genes into seven categories and the genes in the same cluster were likely to possess similar expression patterns or functions. Gene ontology analysis presented that differential genes primarily enriched in *immune response*, *response to stress or stimulus*, and *regulation of apoptosis* in biological process. MLR and ROC curve analysis revealed that the combination of ADAM33, Smad7, and LIGHT possessed excellent discriminating power. **Conclusions:** The combination of ADAM33, Smad7, and LIGHT would be a reliable and useful childhood asthma model for prediction and diagnosis.

Keywords

ADAM33, allergic asthma, childhood asthma, differential gene expression, LIGHT, microarray, Smad7

HistoryReceived 4 July 2014
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Asthma is a common respiratory disorder characterized by recurrent and reversible symptoms of coughing, wheezing, and dyspnea. Over the past 20 years, the prevalence of asthma was increasing at an alarming rate in China. The national epidemiological surveys by Collaborative Group of Childhood Asthma indicated that prevalence of asthma in children aged 0–14 years was 0.77%, 2.60%, and 1.39% in 1990, and it reached 2.69%, 4.63%, and 1.33% in 2000 in Beijing, Chongqing, and Guangzhou, respectively [1]. A similar cross-sectional survey showed that the prevalence was up to 3.15%, 7.45%, and 2.09% from October 2008 to March 2009 in the above three cities, respectively [2]. Researchers attributed higher prevalence to air pollution, climate transition, life style change, and increasing rate of diagnosis.

Various inducing factors cause excessive Th2-driven inflammation in genetically susceptible individuals, and subsequently result in attack of asthma. Numerous studies,

including our previous study, suggested that genetic susceptibility to asthma was polygenic [3,4]. Since multiple drawbacks of glucocorticoid therapy occurs in children, gene therapy attracts increasing attention in recent years. The scientists have found a series of asthma biomarkers by detecting the differential biomolecules which have undergone cellular, biochemical, or molecular alterations in sputum, nasal lavage fluid, bronchoalveolar lavage fluid, or lung tissues [5–13]. But most previous data were collected from adults of other nations, which might not be universal for kids in China. Besides, collecting samples by biopsy was invasive, expensive, and not suitable for children. Thus, it is imperative to obtain data of childhood asthma in China, and the peripheral blood cell may be an ideal surrogate used to analyze gene profile [14,15].

The present study compared whole-genome expression differences between asthmatic young children and healthy controls, and identified gene signatures which could reflect asthma or be used in the prevention and treatment of asthma.

Materials and methods**Ethics statement**

This study was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. Written

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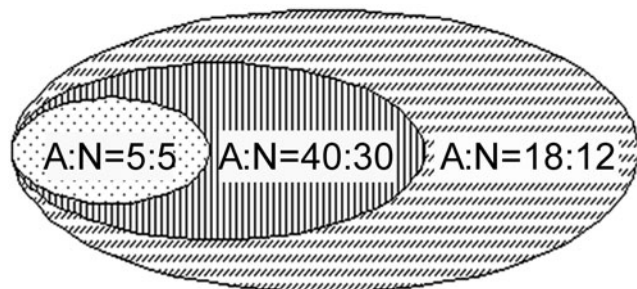


Figure 1. The strategy of gene expression profiling. (a) Microarray was performed on five asthma children and five control subjects. Hierarchical clustering and GO analysis were processed based on these array data. (b) Forty asthma children and 30 control subjects were added in. QRT-PCR was carried out on total 80 samples aiming at eight genes. Using the QRT-PCR outcomes, MLR analysis and ROC curve analysis were performed to identify the best gene combination. (c) Eighteen asthma children and 12 control subjects were recruited to evaluate the discriminating power of the best gene combination.

informed consents were obtained from all participants or their parents.

The overall process and strategy

As shown in Figure 1, we performed gene microarray analysis on samples of five asthma children and five control subjects, and completed hierarchical clustering and gene ontology analysis on these array data with the criteria of $p < 0.05$ and a fold-change > 2 . Then, 40 asthma children and 30 control subjects were recruited. QRT-PCR was carried out on total 80 samples aiming at eight genes which fulfilled more stringent criteria of $p < 0.0001$ and a fold-change > 2.5 . Using the QRT-PCR outcomes, MLR analysis and ROC curve analysis were performed to identify the best gene combination for asthma. Then, 18 asthma children and 12 control subjects were recruited and used to verify the excellent discriminating power of the gene combination.

Subjects

A total of 110 young children who enrolled in the present case-control study were long-term residents in the Pearl River Delta region of southern China. They were hospitalized during November 2012–April 2014 in pediatric ward of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China.

Among them, 63 asthmatic children were diagnosed by pediatricians. The inclusion criteria were as follow: (1) ages were less than 5 years; (2) meeting the definition of asthma given in the Global Initiative for Asthma (GINA) guidelines; (3) total serum IgE (TIgE) levels were beyond the general population mean for their ages (0–100 IU/mL); (4) positive results of skin prick tests. Since the young ages of enrolled children, most of them failed to complete the pulmonary function test or the methacholine challenge test. Among the 63 subjects, 12 children were out of symptoms and had stopped corticosteroid therapy before this recurrence, and the others experienced intermittent attacks of asthma but did not take any corticosteroid within the last 30 d before hospitalization. All patients with mild-to-moderate asthma were then treated according to guidelines of the GINA.

Forty-seven children hospitalized for umbilical hernia or phimosis operation were enrolled in the control group. They must meet all the following criteria: (1) ages were less than 5 years; (2) healthiness, no acute infections, and major chronic illnesses; (3) no symptoms or history of allergic diseases (including wheezing, allergic rhinitis, and atopic dermatitis), and no family history of asthma; (4) total serum IgE levels were within the normal range.

Exclusion criteria of asthmatic children were as follows: (1) ages were more than 5 years; (2) fever ($> 37.4^{\circ}\text{C}$); (3) C-reactive protein (CRP) > 5.0 mg/mL or procalcitonin (PCT) (+); (4) etiological tests suggested current infection of mycoplasma (MP), chlamydia pneumonia (CP), respiratory syncytial virus (RSV), or adenovirus (ADV); (5) chest X-ray showed other cardiopulmonary diseases; (6) treatment with systemic corticosteroid within the last 30 d before hospitalization. All laboratory values were obtained during hospitalization and before any treatment.

Peripheral blood mononuclear cells isolation and total RNA extraction

Four to five milliliters of venous blood was collected before treatment and the peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Paque plus (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) according to the manufacturer's recommendations. Total RNA was extracted from PBMC by TRIZOL (Invitrogen Corp, San Diego, CA) according to the manufacturer's recommendations. Total RNA was quantified by the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE) and RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

RNA labeling and array hybridization

A human gene expression array (Roche NimbleGen, Basel, Switzerland) which contained massive probes represented 45 033 human genes and gene transcripts were used for gene expression analysis. Total RNA of each sample was conducted according to the following steps: (1) reverse transcription; (2) ds-cDNA labeling; (3) array hybridization and washing; (4) array scanning with the Axon GenePix 4000B microarray scanner (Molecular Devices Corporation, Sunnyvale, CA).

Quantitative real-time PCR

Real-time quantitative PCR (QRT-PCR) was performed to analyze eight genes, which were CD69, LIGHT, Smad7, IRF1, Id2, TNFSF13, ADAM33, and CNTF. Primer sequences used for QRT-PCR are shown in Supplementary Table 1. Reactions were carried out in a Roche Light Cycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. With the $2^{-\Delta\Delta C_t}$ method, the data were presented as the fold-change of gene expression compared with the control after normalization by GAPDH.

General statistical analysis

Data of normal distribution were presented as mean $\pm$ SD, while abnormal distribution data were described with median

(interquartile range). If the quantitative data distribution showed normality and equality in the variances in each group, a classic two-sample *t*-test was used. If the quantitative data distribution did not show normality in each group, then the Mann–Whitney *U* test was applied. Qualitative data were analyzed using the Chi-square tests or Fisher's exact probabilities. Bilateral tests were used for all analyses. $p < 0.05$ was considered to be statistically significant.

Further data processing

For microarray analysis, background-corrected values of each probe on microarray were extracted by the GenePix Pro software (Molecular Devices, Sunnyvale, CA). Scanned images were imported into the NimbleScan software (NimbleGen Systems, Inc., Madison, WI) and expression data were normalized. Detection of *p* values and normalization were performed for the extracted values which were then exported to the GeneSpring software (Agilent Technologies, Inc., Santa Clara, CA) and further normalized to the median. The statistical significance of the microarray data was calculated by *t*-tests using 'R' (ver. 3.0.0; <http://www.r-project.org/>). If the distribution of the mRNA expression showed normality and equality in the variances in each group, a classic two-sample *t*-test was used. If the distribution of mRNA expression levels showed normality and inequality in the variances for each group, the Welch two-sample *t*-test was used. If the distribution of mRNA expression levels did not show normality in each group, then the Mann–Whitney *U*-test was applied. The criteria of differentially expressed genes between the two groups were the following: (1) more than 2-fold changed and (2) the extent of difference of gene expression was significant ($p < 0.05$).

Unsupervised hierarchical clustering was performed in MultiExperiment Viewer (MeV, version 4.8.1, Dionex Corporation, Sunnyvale, CA), using an average linkage and the Pearson correlation as the default distance metric. Gene ontology analysis was performed by the DAVID Bioinformatics Resources 6.7 (National Cancer Institute at Frederick, Frederick, MD) (<http://david.abcc.ncifcrf.gov/>). The significance was defined as $p < 0.05$.

Through literature retrieval by joining the search terms of asthma, remodeling, inflammation, immune, respiratory hypersensitivity, fibrosis, allergy with the Boolean operator OR on PubMed, we narrowed down our focus on 29 highly possible genes. Then we selected eight candidate genes that satisfied the criteria of $p < 0.0001$ and a fold-change > 2.5 from these 29 genes. A power set was made with the eight candidate genes and the multiple logistic regression (MLR) analysis was performed on the data from QRT-PCR. Receiver operating characteristic (ROC) curves for all models were obtained, and areas under the curve (AUCs) were calculated. Contingency tables were used to evaluate the discriminating power.

Results

Characteristics of subjects

As shown in Table 1, five asthma children and five control subjects were recruited for the microarray. Moreover, we expanded the sample size with 58 asthma children and 42 control subjects for further data analysis. There were no differences in the distribution of gender and age between the groups ($p > 0.05$). With respect to laboratory examinations, the counts of leucocytes, neutrophils, lymphocytes, and eosinophils showed no statistical difference ($p > 0.05$).

Table 1. Characteristics of study subjects.

	Array samples		Expanded samples	
	Asthma	Normal control	Asthma	Normal control
Subjects (n)	5	5	58	42
Gender (male/female)	3/2	3/2	31/27	20/22
Age (years) ^a	3.17 (3.96–1.96)	2.80 (3.52–1.90)	3.65 (4.02–3.10)	3.82 (4.21–2.82)
Age-onset (years) ^a	3.00 (3.20–1.75)	–	3.10 (3.50–2.60)	–
Newly diagnosed ^b	3/5	–	34/58	–
Severity (mild/moderate)	0/5	–	12/46	–
Allergic history ^c	3/5 positive*	Negative*	31/58 positive**	Negative**
Family history ^d	4/5 positive*	Negative*	38/58 positive**	Negative**
Passive smoking ^e	3/5 positive	2/5 positive	21/58 positive	18/42 positive
Leucocytes ($\times 10^9/L$) ^a	7.83 (9.65–5.67)	7.39 (8.78–6.06)	8.42 (8.79–6.91)	7.68 (8.31–6.74)
Neutrophils ($\times 10^9/L$) ^a	2.83 (3.80–2.52)	3.62 (4.71–2.97)	3.11 (3.76–2.92)	3.44 (4.29–2.98)
Lymphocytes ($\times 10^9/L$) ^a	4.25 (4.84–2.48)	2.73 (3.44–2.31)	3.79 (4.17–3.50)	4.12 (4.44–3.22)
Eosinophils ($\times 10^9/L$) ^a	0.41 (0.62–0.12)	0.13 (0.18–0.08)	0.61 (1.17–0.23)	0.42 (0.56–0.21)
Serum TlgE (IU/mL) ^a	187.00 (384.50–138.00)*	21.00 (28.00–16.50)*	178.00 (230.50–142.50)**	42.0 (59.50–28.80)**
Etiological tests ^f	2/5 mildly abnormal	Negative	7/58 mildly abnormal	Negative

* and ** represents $p < 0.05$. All *p* values were calculated using Chi-square tests or Mann–Whitney *U* tests or Fisher's exact probabilities depending on different data types.

^aData of abnormal distribution were described with median (interquartile range).

^bNewly diagnosed: all asthmatic subjects were diagnosed by pediatricians according to the GINA. It was noteworthy that just one instance of wheezing was not enough to diagnose asthma.

^cPositive allergic history: the child has or had eczema, rhinitis, hay fever, etc. diagnosed by pediatrician.

^dPositive family history: first-degree relatives have or had allergic diseases diagnosed by physician.

^eThe smokers specifically refer to the close contacts.

^fIncluding the tests of MP-IgM, CP-IgM, RSV-IgM, ADV-IgM. Mildly abnormal in this study refer to MP-IgM (1:40 or 1:80). MP, mycoplasma; CP, chlamydia pneumonia; RSV, respiratory syncytial virus; ADV, adenovirus.

Table 2. Potential functions of 14 up-regulated genes on asthma.

	Name	Potential functions
Cluster 1	GFRA4	Extended linkage disequilibrium around ADAM33
	PDGFB	Changing the airway vascular angiogenesis and remodeling in asthma murine model
	HSPB2	Inducing T cell regulation of chronic inflammation; protecting bronchial epithelial cells against oxidative stress-mediated apoptosis
	PDE7B	Inducing T cell activation; its inhibitors can ease asthma and COPD
Cluster 2	SCIN	Involving in secretion of goblet cell
	LIGHT*	Involving in airway remodeling, lymphocyte activation and T cell proliferation
	PRTN3	Involving in neutrophils releasing and migration
	ENG	The ancillary TGF- β receptor
Cluster 3	TPSB2	A mediator in several allergic disorders; expressed in mast cells
	ADAM33*	Involving in cell–cell and cell–matrix interactions, FEV1 decline, bronchial hyper-responsiveness and airway remodeling
	EPX	A member of peroxidase gene family; expressed in eosinophils
	CNTF*	Enhancing spontaneous IgE production by B cells
	HP	Involving in iron metabolic pathway related to asthma and COPD.
	MADCAM1	Supporting eosinophil firm adhesion under conditions of flow

Abbreviations are listed in Supplementary Table 2.

*These highly possible genes fulfilled criteria of $p < 0.0001$ and a fold-change > 2.5 .

Table 3. Potential functions of 15 down-regulated genes on asthma.

	Name	Potential functions
Cluster 4	IL7R	An inhibitor of allergic inflammation
Cluster 5	DNMT1	A negative regulator of allergic skin inflammation
Cluster 6	CD69*	Involving in allergic airway inflammation and activation of T cells and eosinophils
	TNFSF13*	Activation of B cells; suppression of Th2 and allergic lung inflammation
	LILRB4	Inhibition of Th2 immune responses and pulmonary inflammation
	ID2*	An indispensable regulator of the Th1/Th2 balance and IgE class switching
	CEBPD	A regulator of IgG-induced lung inflammation and has anti-inflammatory and anti-apoptotic roles in pancreatic beta cells
Cluster 7	PXN	Th2 cytokines down-regulate PXN, thereby disrupting the cell-matrix interactions
	RELB	Lack of RELB can induce T cell-dependent skin lesions
	FPR1	A neutrophil formyl peptide receptor involving in pro-inflammatory and anti-inflammatory effects and may be used to reduce injuries in asthma
	IRF1*	Promoting T cells to differentiate into Th1
	SMAD7*	An intracellular mediator of airway inflammation and remodeling.
	CD55	Reduced in asthma mice and suggested abnormalities in inositol signaling pathway
	FTL	Toluene diisocyanate inhibits FTL and then induces occupational asthma
	HDAC6	Regulating of cell migration, cell–cell interaction, Treg or effector T cell function

Abbreviations are listed in Supplementary Table 2.

* These highly possible genes fulfilled criteria of $p < 0.0001$ and a fold-change > 2.5 .

The concentration of serum TIgE was higher in asthma children than in control subjects ($p < 0.01$). Pathogenic tests showed that all subjects did not have current infections of mycoplasma (MP), chlamydia pneumonia (CP), respiratory syncytial virus (RSV), and adenovirus (ADV).

Overview of differential gene expression

A whole-genome microarray comprising 45 033 human gene transcripts was used to perform gene microarray analysis on asthma and normal subjects. All values of the microarray were normalized in order to make the results comparable.

Based on the criteria of fold change larger than 2 and $p < 0.05$, the present study has obtained 795 (758 named, 345/413 = up/down-regulated) differential transcripts in the asthma group relative to the healthy controls. After literature retrieval by using asthma-related searching terms on PubMed, we focused on 29 highly possible genes concerning asthma, remodeling, inflammation, or immune reaction (Tables 2 and 3). Out of 29 highly possible genes, only eight genes that

fulfilled more stringent criteria of $p < 0.0001$ and a fold-change > 2.5 (three were up-regulated and five were down-regulated).

There were three up-regulated genes which fulfilled more stringent criteria but were not included in 29 highly possible genes. Among them, FGA was related to clot formation and fibrinolysis. Very little was known about the other two genes, C1orf91 and LOC644830. We hope to unearth more substantial evidence to verify their relationship with asthma in future studies.

Identification of gene clusters differentially regulated in childhood asthma

Based on a total of 795 differential transcripts, hierarchical clustering divided 10 samples into two groups which were exactly consistent with the grouping of subjects (five asthmatic patients versus five control subjects). Furthermore, hierarchical clustering classified 29 differential expressed genes (14 up-regulated and 15 down-regulated) into seven

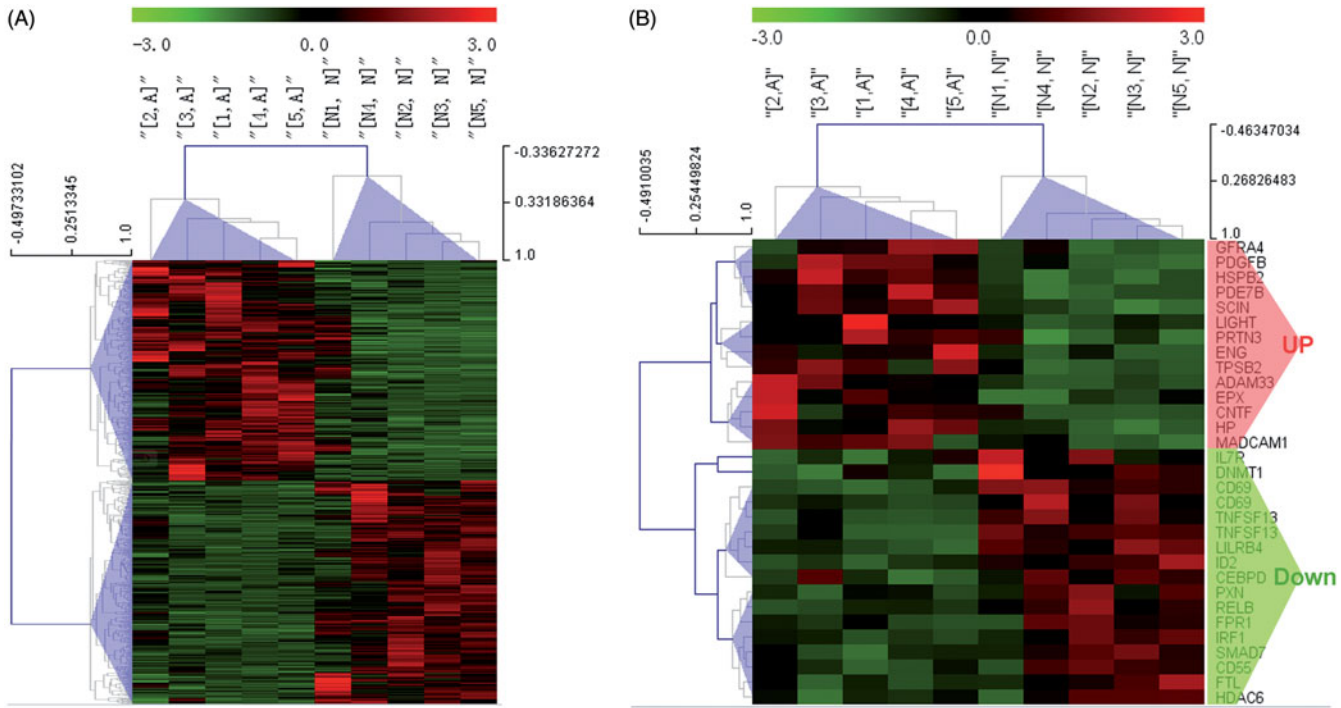


Figure 2. Hierarchical cluster analysis. (A) Hierarchical cluster analysis based on 795 differential expressed genes. Ten samples were divided into two groups which were exactly consistent with the grouping of subjects. (B) Hierarchical clustering classed 29 highly possible genes (covering 31 transcripts) into seven categories. Genes in the same cluster were likely to possess similar expression patterns or functions.

categories (shown in Tables 2 and 3, Supplementary Table 2, and Figure 2).

Hierarchical clustering belongs to an exploratory data analysis and it can divide a limited number of objects into several categories. With the help of Genbank (<http://www.ncbi.nlm.nih.gov/Genbank>), we acquired potential functions of these 29 highly possible genes. The functions mainly related to activation, proliferation, differentiation, and migration of various immune cells. Airway remodeling and some signaling pathways were also involved. Genes with coherent expression patterns were gathered in the same cluster, and they probably possessed similar functions or belonged to the same pathways.

Functional characterization of differential genes in childhood asthma

Using DAVID Bioinformatics Resources 6.7 (National Cancer Institute at Frederick, Frederick, MD), we got 604 gene annotations from 758 named genes, and other 144 genes were undefined or different transcripts of the same gene. Aiming at 578 DAVID IDs which mapped from 604 annotations, we performed gene ontology analysis. The gene ontology covers three subcategories including biological process (BP), cellular component (CC), and molecular function (MF). In each subcategory, the GO terms with minimum p values were picked out which represented the functions enriched by differential genes.

The top five of the minimum p values of each subcategory are listed in Table 4, the differential genes were enriched in GO terms of immune response, response to stress or stimulus, and regulation of apoptosis in BP. Most of them were down-regulated genes. In CC, differential genes were mainly related

to platelet alpha granule, vesicle lumen, cell surface, and extracellular space. With respect to MF, there was significant enrichment of enzyme activity, protein binding, and signal transducer activity.

Data validation by QRT-PCR analysis on expanding samples

QRT-PCR analysis was applied to confirm the microarray outcomes and the PCR data were also used to perform multiple logistic regression analysis later. To this end, it was essential to enlarge the sample size. Apart from 10 cases used for microarray, we recruited another 40 asthmatics and 30 normal controls. After literature retrieval and using more stringent criteria of $p < 0.0001$ and a fold-change > 2.5 , we picked eight genes. In the case of ADAM33, LIGHT and CNTF, gene expressions were dramatically increased in the asthma group than those in the controls. Reversely, gene expressions of Smad7, TNFSF13, CD69, IRF1, and ID2 were evidently decreased in asthmatic children. The results showed that even in a larger sample size, the variation tendency of these genes were completely consistent with those in array data (Figure 3). But the expression levels of eight candidate genes detected by QRT-PCR showed no difference between mild and moderate asthma (data not shown).

Selection of the best gene model by MLR analysis and ROC curve analysis

Based on the QRT-PCR data, we performed a MLR analysis on eight candidate genes and measured the AUCs of ROC curves to select asthma-specific diagnostic gene markers.

We built 255 ($2^8 - 1$) models with different combinations of eight candidate genes, and computed p values of genes in each

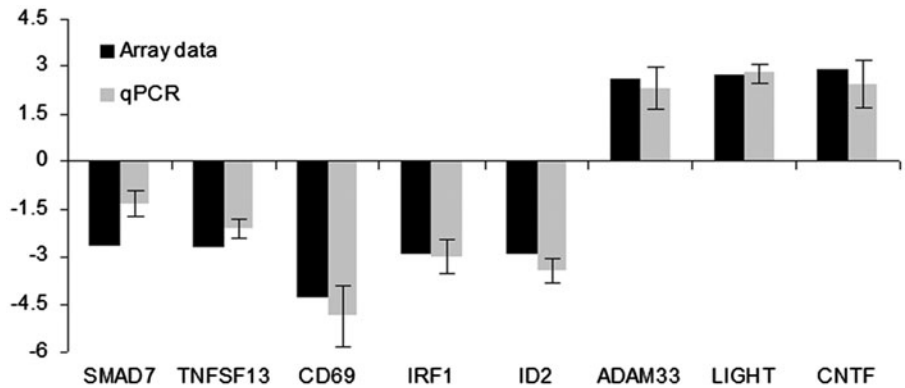
Table 4. The GO analysis of differential genes.

GO ID	Term	Genes (up/down-regulated)	Proportion ^a (%)	<i>p</i> Value*
GO:0006955	BP: immune response	46 (7/39)	7.96	3.56E–06
GO:0002376	BP: immune system process	58 (11/47)	10.03	1.03E–05
GO:0006950	BP: response to stress	78 (26/52)	13.49	5.68E–04
GO:0050896	BP: response to stimulus	140 (45/95)	24.22	1.45E–03
GO:0042981	BP: regulation of apoptosis	42 (5/37)	7.27	1.83E–03
GO:0031091	CC: platelet alpha granule	8 (1/7)	1.38	2.98E–03
GO:0060205	CC: cytoplasmic membrane-bounded vesicle lumen	7 (2/5)	1.21	3.77E–03
GO:0031983	CC: vesicle lumen	7 (2/5)	1.21	4.72E–03
GO:0009986	CC: cell surface	23 (7/16)	3.98	4.73E–03
GO:0005615	CC: extracellular space	37 (19/18)	6.40	7.98E–03
GO:0016301	MF: kinase activity	46 (9/37)	7.96	2.29E–03
GO:0005515	MF: protein binding	317 (92/225)	54.84	2.31E–03
GO:0043028	MF: caspase regulator activity	6 (1/5)	1.04	2.98E–03
GO:0004871	MF: signal transducer activity	11 (3/8)	1.90	3.58E–03
GO:0016504	MF: peptidase activator activity	22 (3/19)	3.81	3.59E–03

**p* < 0.05 only the top five of the minimum *p* values in each category are listed in this table. BP, biological process; CC, cellular component; MF, molecular function.

^aThe proportion is the percentage of differential genes in 578 DAVID IDs.

Figure 3. Expression of eight differential expressed genes was verified by QRT-PCR. QRT-PCR was performed on 80 subjects for eight candidate genes. Compared with array data, the results of QRT-PCR displayed the same change tendency.



model. We divided 255 models into eight groups (groups 1–8). Group *n* indicated the model which was made of *n* genes. Only groups 1–4 contained combinations with significant *p* values. Among them, only 51 models met the requirement that all *p* values for each gene are less than 0.05. The MLR analysis data for total 51 models are presented in Supplementary Table 3.

Then AUC and ROC were calculated for these 51 models (Supplementary Table 3). Higher AUC value indicated better discrimination between two groups. The top five AUC values of groups 1–4 are presented in Figure 4. The best AUC value (0.952) was observed in a combination of three genes, which were ADAM33, Smad7, and LIGHT. Their asymptotic *p* value was 6.34×10^{-15} , and the asymptotic 95% confidence interval was 0.915–0.990 (Figure 5).

Discriminating power of the best combination model

According to the outcomes of MLR and ROC curve analysis, we evaluated discriminating ability of the best combination of ADAM33, Smad7, and LIGHT with a contingency table on total 80 subjects (45 asthmatics and 35 controls) which was used in QRT-PCR before. The sensitivity, specificity, and accuracy were 84.44%, 85.71%, and 85.00%, respectively (shown in Table 5).

To further verify this gene combination model, we recruited other 30 samples (18 asthmatics and 12 controls) and repeated the calculation. The sensitivity and the specificity were 88.89% and 83.33%, respectively, and the accuracy reached 86.67% (shown in Table 6).

Data of total 110 samples showed pretty good discriminating power which could distinguish asthmatic children from normal controls.

Discussion

Asthma is a common chronic disease of children. To our knowledge, some studies have investigated PBMC from adults or other tissue samples, but few researchers focused on gene profiling of PBMC from the asthmatic children by microarray, especially in China. A child is not an adult in miniature, and the data from adults are not universally suitable for kids. The nature of asthma varies considerably with age. Scientists have observed phenotypic heterogeneity of asthma between children and adults, suggesting that different pathways operate at different ages [16]. And several large-scale genome-wide association studies (GAWs) have found that some genomic regions were unique to childhood and adult-onset asthma [17–19]. Multiple markers were strongly associated with childhood-onset asthma rather than adult onset [20–22].

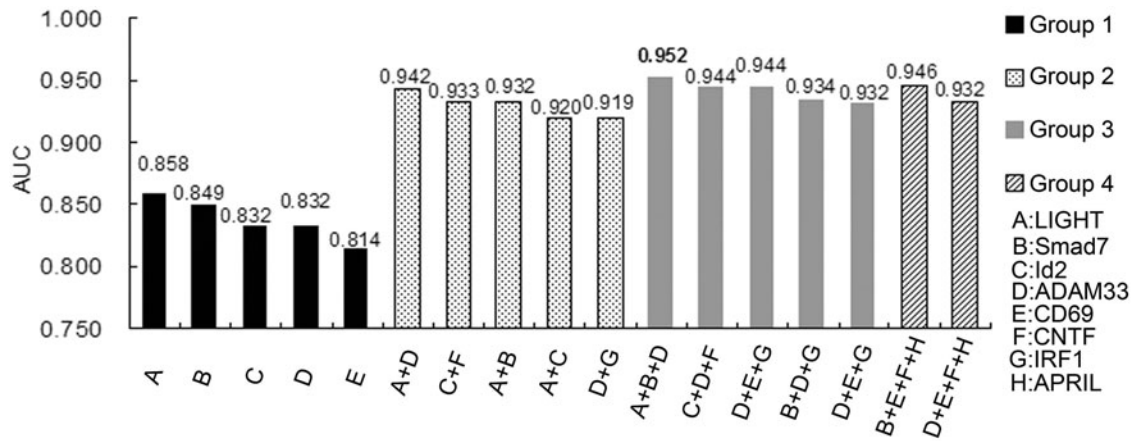


Figure 4. Top AUC values in groups 1, 2, 3, and 4. Among 51 models of which all *p* values <0.05 in MLR analysis, only the maximal five AUC values of each group were presented in this figure. The combination of ADAM33, Smad7, and LIGHT possessed the best AUC value (0.952). Group N: a group which consists of *N* genes.

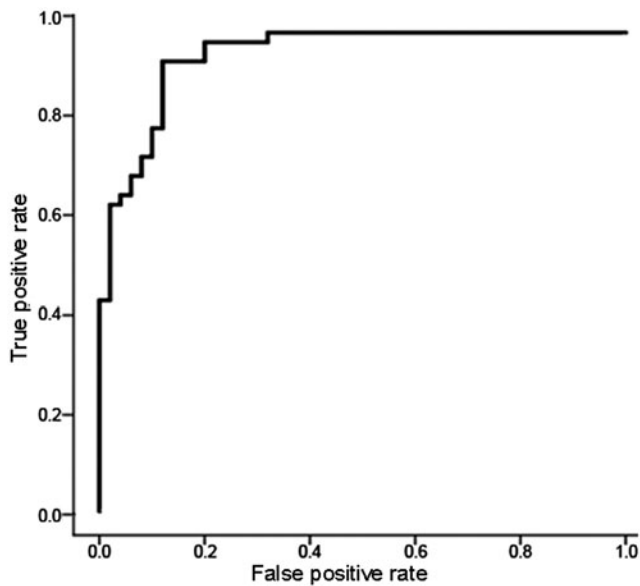


Figure 5. ROC curve of the best combination of ADAM33, Smad7, and LIGHT. The combination of ADAM33, Smad7, and LIGHT possessed the best AUC value (0.952). Their asymptotic *p* value was 6.34×10^{-15} , and the 95% confidence interval was 0.915–0.990.

Table 5. Contingency table of the best model combination for 80 samples.

Prediction outcome	Actual value		Sensitivity (%)	Specificity (%)	Accuracy (%)
	Asthma	Control			
Asthma	38	5	84.44	85.71	85.00
Control	7	30			

Genetic risk was higher in children with early-onset asthma, while adult-onset asthma was less obviously associated with allergy and was thought to have a minor atopic component [17].

PBMC is an ideal material for comparative studies in asthma. Bjornsdottir et al. [23] demonstrated that both the innate immunity and adaptive immunity were activated in

Table 6. Contingency table of the best model combination for 30 expanded samples.

Prediction outcome	Actual value		Sensitivity (%)	Specificity (%)	Accuracy (%)
	Asthma	Control			
Asthma	16	2	88.89	83.33	86.67
Control	2	10			

adult asthma patients. Their oligonucleotide array results showed that analysis of PBMCs could reveal systemic changes accompanying asthma exacerbation. Another research in childhood asthma using microarray and flow cytometry suggested that respiratory viral infection could amplify and sustain airway inflammation via atopy-associated mechanisms [24]. In our study, microarray analysis was performed to explore differential genes which related to childhood asthma. The validated data revealed that the combination of three genes, namely ADAM33, Smad7, and LIGHT, showed the best power for predicting asthma.

The validated microarray data were reliable

Hierarchical clustering outcomes showed that the samples were divided into two bundles which were exactly consistent with the grouping of subjects (Figure 2A), and the differential genes were also classified as two categories which corresponded to tendency (Figure 2B). These results certified that the data came from PBMC were subtle and reliable.

Given that asthma might be related to pathogens and PBMC are susceptible to many variations, we tried to rule out bacterial infection with rigorous exclusive criteria. Besides, the small sample size is a common inadequacy in this kind of research, and our present study was not exception. Therefore, we further expanded sample size and verified the array data by QRT-PCR (Figure 3). The validated outcomes announced that the microarray data were credible for further research. But the expression levels of eight candidate genes detected by QRT-PCR showed no difference between 12 mild asthma cases and 51 moderate asthmatic children (data not shown).

This result should be validated in a larger sample size with approximately equal numbers of each asthma severity in following research.

Through literature retrieval by using the search terms of asthma, remodeling, inflammation, immune, respiratory hypersensitivity, fibrosis, or allergy, we narrowed down our focus to 29 highly possible genes. This search strategy could fully cover the pathogenesis and pathological changes of allergic asthma. Using the Boolean operator OR to join those search terms could help to screen the genes that have been found to work in other diseases which shared a similar etiology and pathogenesis with asthma. Literature retrieval could help to enhance the validity of data and avoid unavailable information.

Differential genes which clustered in same division possessed similar functions

In this study, hierarchical clustering classified 29 highly possible genes into seven categories (Tables 2 and 3 and Supplementary Table 2). Although diverse functions were scattered in each cluster, we deemed that the genes clustered in the same division owned a similar genetic expression pattern. If different genes possessed a similar genetic expression pattern, they were inclined to execute in the same way or participate in the same biological process. Hierarchical clustering provided a cue from functionally known genes, and made the mysterious biomarkers explicit in asthma.

Since the classical microarray data analysis could generate a very large number of differential genes, we applied GO analysis to gain a comprehensive insight into the functions of the asthmatic responses, especially in biological processes (Table 4). GO analysis clearly revealed that there was significant enrichment of immune response, response to stress or stimulus, and regulation of apoptosis. The outcomes were in accord with complicated pathophysiological changes of asthma as we have known, and the immune mechanisms were still the core of study.

The combination of ADAM33, Smad7, and LIGHT could be an asthma prediction and diagnostic model

There was rarely a unique gene maker in most diseases, and many researchers adopted the parallel test or the serial test to demonstrate that multiple indexes were superior to a single one. We usually got higher sensitivity and lower specificity in the parallel test, but lower sensitivity and higher specificity in the serial test. The AUC–ROC curve analysis, as an authentic approach in selecting candidate genes, can strike a balance between sensitivity and specificity [25]. However, the complex dependencies among genes could generate unpredictable variation in discriminative performance of the biomarker model which contained diverse gene combinations. MLR analysis might be a better tool [26–29], but data from asthmatic children have never been described before.

In this research, after performing the MLR and AUC–ROC curve analysis, the model consisted of LIGHT, Smad7, and ADAM33 genes which showed the highest AUC value (Supplementary Table 3 and Figures 4 and 5). Based on 80 samples and an expanded sample of 30 subjects, we evaluated

the best combination, and the sensitivity, specificity, and accuracy showed that the combination owned quite a good discriminating power (Tables 5 and 6). Thus, we regarded the combination of ADAM33, Smad7, and LIGHT as an excellent asthma prediction and diagnostic model. In other words, combined detection of ADAM33, Smad7, and LIGHT in peripheral blood of children will provide clues for early diagnosis. In future, further studies and larger sample size are required to set appropriate cut-off points.

ADAM33

ADAM33 (A disintegrin and metalloprotease 33) was identified as an asthma susceptibility gene [30–32]. Some clinical studies of asthmatic adults revealed that expression of ADAM33 has a negative correlation with lung function [33,34]. Th2 cytokines could elevate the expression of ADAM33 in human fibroblasts, while IFN- γ could reduce ADAM33 in airway smooth muscle cells [35,36].

Whereas Wang et al [37]. believed that ADAM33 gene had no association with asthma in Chinese population. Tripathi et al [38]. considered that increased expression of ADAM33 protein had no relationship with the severity of asthma.

Our study data were consistent with most previous information and showed that ADAM33 was up-regulated in asthmatic children. On account of this discrepancy, more proofs were required to uncover the truth and filled up vacancy of the data in children.

SMAD7

TGF- β 1 has intertwined functions including inflammation promotion, anti-inflammation effect, and airway remodeling. Smad7 can be rapidly induced by TGF- β family and inversely antagonize TGF- β signaling [39,40]. Studies *in vivo* showed that blocking the TGF- β signal by over-expression of Smad7 could significantly relieve allergic asthma [41,42]. In asthmatic patients, the expression of Smad7 in bronchial epithelial cells was inversely correlated to basement membrane thickness and airway hyper-responsiveness [43].

In our study, the expression of Smad7 decreased remarkably in asthmatic children. Children with low Smad7 expression are prone to suffer from asthma and more likely turn to be the severe or drug-resistant cases. Targeting this pathway may be a novel therapeutic strategy in future. However, we cannot ignore that disruption of TGF- β signaling by Smad7 might aggravate lung carcinogenesis in mouse [44]. Therefore, further credible scientific evidences are demanded to clarify the double nature of Smad7.

LIGHT

LIGHT, also called the TNFSF14, is expressed in immune cells and binding to herpesvirus entry mediator (HVEM) and lymphotoxin β receptor (LT β R). Hastie et al. [45] found that higher level of LIGHT was associated with lower baseline forced expiratory volumes or poor lung function. Blockage of LIGHT-LT β R or LIGHT-HVEM interaction would lessen fibrosis, repair airway remodeling, alter smooth muscle mass, and reduce inflammation *in vitro* and *in vivo* [46–48].

Recently, researchers scanned genes after allergen challenge on people by genome-wide expression analysis, and

LIGHT was found as a novel mediator in human eosinophils [12]. Our results also exposed that LIGHT was increased among asthmatic children. Accordingly, this gene marker may be used as a potential strategy targeting asthma, and other diseases concerning fibrosis or immunity.

Clinical implications of ADAM33, Smad7, and LIGHT in treating asthma

All three potential biomarkers are closely associated with the pathogenesis of asthma. In Th2-dominant allergic asthma, IL-4 and IL-13 can elevate the expression of ADAM33. The rising ADAM33 always along with prominent sub-epithelial collagen deposition increased contractile elements and airway smooth muscle cells dysfunction [35,49]. Smad7, a key negative regulator of TGF- β signaling, can block synthesis of ECM and inhibit EMT in lung, and involves in immune cell maturation, proliferation, and activation. The decreased expression of Smad7 will reduce the anti-fibrosis and anti-inflammation effect, which are likely to increase susceptibility to asthma [50]. LIGHT can affect the subepithelial fibrosis, smooth muscle hypertrophy and hyperplasia, and airway hyper-responsiveness after allergen challenge. TGF- β and IL-13 are also important in LIGHT-induced airway remodeling [46]. Therefore, those three biomarkers may also be effective targets in asthma treatment. Reducing the expression of ADAM33 and LIGHT or rising Smad7 could prevent fibrosis, reverse airway remodeling, and play a positive role in anti-inflammation.

The relationship among ADAM33, Smad7, and LIGHT has not been reported. But they are all involved in TGF- β signaling, which is known to stimulate extracellular matrix production by fibroblasts [46,50,51]. Due to this, they also take part in the pathogenesis of chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) [52–54]. Fortunately, both COPD and IPF are not hard to make a differential diagnosis with asthma in children. Thus, the value of these three biomarkers in asthma cannot be denied.

Conclusions

The present study on genome-wide mRNA expression of PBMC in asthmatic children manifested differential expression levels of 758 named genes according to the preset criteria. Among them, 29 highly possible genes were related to asthma, remodeling, inflammation, or immune reaction. Then eight genes were selected as candidate biomarkers of asthma using more stringent criteria. The genetic marker combination of ADAM33, Smad7, and LIGHT showed superior discriminative power and which might be a reliable model of diagnostic in childhood asthma. In the future, those gene markers require more validation in a larger population, especially in children with different stages and severity of asthma, and further data mining is demanded to trace the target genes or pathways of asthma.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online
Supplementary Tables 1–3.

Supplementary Table 1. Primer sequences used for QRT-PCR

Gene name	Primer sequence (homo sapiens)		Product length
SMAD7	Forward	5'-GGACAGCTCAATTCGGACAAC-3'	105 bp
	Reverse	5'-GTACACCCACACACCATCCAC-3'	
TNFSF13	Forward	5'-TTGGAAGACCAGGGTGGGTA-3'	199 bp
	Reverse	5'-CACACGCAAGAATTCGGAGC-3'	
ADAM33	Forward	5'-CCAACCACACGGATCATTGC-3'	131 bp
	Reverse	5'-ACGCAGATAATAGCTGGCATTTC-3'	
LIGHT	Forward	5'-ATACAAGAGCGAAGGTCTCACG-3'	102 bp
	Reverse	5'-CTGAGTCTCCCATAACAGCGG-3'	
CD69	Forward	5'-GAAGCATCACAGGGTGACCA-3'	172 bp
	Reverse	5'-GTGCAAATGCATGAAGGGCT-3'	
IRF1	Forward	5'-GGTGAAGGAAATGACACGCC-3'	169 bp
	Reverse	5'-CCCTACTTAACTCTCGCGCA-3'	
Id2	Forward	5'- ATGAAAGCCTTCAGTCCCGT-3'	139 bp
	Reverse	5'- TGAGCTTGGAGTAGCAGTCG-3'	
CNTF	Forward	5'-TGATTAGGCCCGCCAAACTT-3'	120 bp
	Reverse	5'-AACCTGGTATAAGCCGTGCC-3'	
GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'	129 bp
	Reverse	5'-TGGTGAAGACGCCAGTGGA-3'	

Abbreviations: ADAM33: a disintegrin and metallopeptidase domain 33; CD69: CD69 molecule; CNTF: ciliary neurotrophic factor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Id2: inhibitor of DNA binding 2, dominant negative helix-loop-helix protein; IRF1: interferon regulatory factor 1; LIGHT: tumor necrosis factor (ligand) superfamily, member 14; SMAD7: SMAD family member 7; TNFSF13: tumor necrosis factor (ligand) superfamily, member 13.

Supplementary Table 2. Abbreviations of Table 2 and Table 3

Abbreviations	Full name
ADAM33	a disintegrin and metallopeptidase domain 33
CD55	CD55 molecule, decay accelerating factor for complement
CD69	CD69 molecule
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
CNTF	ciliary neurotrophic factor
DNMT1	DNA (cytosine-5-)-methyltransferase 1
ENG	endoglin
EPX	eosinophil peroxidase
FPR1:	formyl peptide receptor 1
FTL	ferritin, light polypeptide
GFRA4	GDNF family receptor alpha 4
HDAC6	histone deacetylase 6
HP	haptoglobin
HSPB2	heat shock 27kDa protein 2
ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
IL7R	interleukin 7 receptor
IRF1	interferon regulatory factor 1
LIGHT	tumor necrosis factor (ligand) superfamily, member 14
LILRB4	leukocyte immunoglobulin-like receptor, subfamily B, member 4
MADCAM1	mucosal vascular addressin cell adhesion molecule 1
PDE7B	phosphodiesterase 7B
PDGFB	platelet-derived growth factor beta polypeptide
PRTN3	proteinase 3
PXN	paxillin
RELB	v-rel avian reticuloendotheliosis viral oncogene homolog B
SCIN	scinderin
SMAD7	SMAD family member 7
TNFSF13	tumor necrosis factor (ligand) superfamily, member 13
TPSB2	tryptase beta 2