Regulation of *CBL* and *ESR1* expression by microRNA-22-3p, 513a-5p and 625-5p may impact the pathogenesis of dust mite-induced pediatric asthma

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Abstract. Despite evidence for the involvement of microRNAs (miRNAs or miRs) in pediatric asthma, the mechanism responsible has not yet been fully elucidated. We aimed to identify novel miRNAs and to study their pathogenic role(s) in children with dust mite-induced asthma in order to gain a better understanding of the underlying mechanism responsible for this disease. For this purpose, 62 patients with asthma as well as 62 age- and gender-matched healthy controls were recruited. Twelve pairs of subjects were randomly subjected to microarray-based discovery analysis using a miRCURY LNA™ array. The differential expression of miRNAs and their targeted messenger RNAs were validated using RT-qPCR. Plasma concentrations of cytokines were determined using an enzyme-linked immunosorbent assay (ELISA) kit. The results revealed that three novel miRNAs - miR-22-3p, miR-513a-5p and miR-625-5p - were significantly downregulated in the asthma group compared with the control group ($p<0.01$), whereas the transcript levels of Cbl proto-oncogene, E3 ubiquitin protein ligase (*CBL*), peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*), and estrogen receptor 1 (*ESR1*) that are targeted by these miRNAs were increased ($p<0.01$). There were significant differences in the plasma concentrations of γ-interferon, tumor necrosis factor-α, interleukin (IL)-12 and IL-10 between the two groups ($p<0.05$). Thus, miR-513a-5p, miR-22-3p and miR-625-5p may have an impact on the regulation of the immune response and inflammatory cytokine pathways through the regulation of their target gene(s), *CBL*, *PPARGC1B* and *ESR1*, which may then lead to a dust mite-induced asthma attack. Our findings may provide novel insights into the pathogenesis of pediatric asthma.

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Introduction

Pediatric asthma is one of the most common respiratory diseases in children. A survey of asthma morbidity and mortality conducted by the Centers for Disease Control and Prevention reported that the incidence of this disease was 8.0% during 2006-2010 in the United States, with a prevalence of 9.4% among children (1). A recent study of childhood asthma in urban areas of China described an increasing incidence of asthma, by approximately 10% in one decade, and the overall prevalence of asthma in children <14 years of age in urban areas was 3.02% in 2009-2010 (2). Etiologically, pediatric asthma may result from gene-environment interactions (3). The common environmental factors include air pollution, pollen, fungi and dust mites (4-6). Up to 80% of asthmatics are allergic to house dust mites, although the exact rate depends on the geographic region (7). Currently, the pathogenic mechanism of childhood asthma remains unknown. As a heterogeneous disease with strong genetic factors, the pathogenesis of pediatric asthma is complex (8-10). It is well known that immune regulation is closely associated with the inflammatory response in asthma. Previous studies showed that the abnormal regulation of immune and inflammatory responses occurred in asthma patients, as well as an imbalance in T helper type 1 (Th1)/T helper type 2 (Th2) cells, which increased the inflammatory factors interleukin (IL)-4, IL-6 and IL-13 (mediated by Th2) and decreased IL-12 and γ-interferon (γ-IFN) (mediated by Th1) to cause chronic airway inflammation and hyperresponsiveness (11-13). These inflammatory responses are closely associated with several signaling pathways, such as the phosphoinositide 3-kinase (PI3K)-AKT, Janus kinase (JAK)-signal transducers and activators of transcription (STAT), mitogen-activated protein kinase (MAPK)

and nuclear factor-κB (NF-κB) pathways (14-16), which resulted in alterations in cytokine secretion.

Previous findings have shown that PI3K-AKT and NF-κB are associated with Th1/Th2 differentiation and cytokine secretion (e.g., IL and IFN) (17). It was found that several genes, including Cbl proto-oncogene, E3 ubiquitin protein ligase (*CBL)* and estrogen receptor 1 (*ESR1)*, are involved in the PI3K-AKT and/or NF-κB signal pathway(s) (18,19). These genes may influence downstream genes such as spleen tyrosine kinase (*SYK*) and epidermal growth factor receptor (*EGFR*) in order to regulate the PI3K-AKT and NF-κB pathway(s) (20,21). However, the underlying mechanism of regulation in asthma has not yet been clearly illustrated.

MicroRNAs (miRNAs or miRs), a group of small, non-coding RNAs 21-25 nucleotides in length, have been reported to be transcriptional regulators involved in many complex human disorders, and in biological processes including cell proliferation and apoptosis (22,23). miR-223 was reported to regulate the maturation, function and differentiation of neutrophils (24). Major histocompatibility complex, class I, G (*HLA-G)*, an asthma susceptibility gene, has been found to be a common target of miR-148a, miR-148b, and miR-152 (25). miR-146b, miR-223, miR-29b, miR-29c, miR-483, miR-5745p, miR-672 and miR-690 are differentially expressed in asthma patients (26-28). The miRNA regulation of target genes appears to play an important role in immune and inflammatory responses as well as in the development of asthma (29). As miRNAs are regulators of the inflammatory response in asthma, it is likely that miRNAs may regulate their targeted messenger RNA(s) [mRNA(s)] that are involved in the asthma-associated inflammatory pathway, thereby leading to an asthma attack. In the present study, we conducted a genome-wide investigation of novel miRNA(s) that may have regulatory functions in dust mite-induced asthma.

Subjects and methods

Subjects. Dust mites have been determined to be the predominant allergen in asthma (7). In the present study, we focused on dust mite-induced asthma. The study was approved by the Bioethics Committee of the Shanghai Children's Hospital (Shanghai, China), and written informed consent was obtained from the participants or their guardians.

A total of 62 pediatric patients with asthma were recruited, as the case group, from our Asthma Clinic at Shanghai Children's Hospital (Shanghai, China). A diagnosis of asthma was made according to the Global Initiative for Asthma (GINA) guidelines (10). We focused on patients with intermittent-mild asthma in this study, as the pathogenesis of severe asthma is more complex (30). Detailed information regarding patient demographics and enrollment inclusion and/or exclusion criteria is provided in Table I. Forty-one boys and 21 girls (average age, 6.05±2.15 years) were recruited for the asthma case group. During the time that they were enrolled in this study and their blood specimens were being collected, these patients did not have current or recent infections, were not taking any medication, such as inhaled corticosteroid (ICS) (31), and did not experience any acute asthma exacerbations or attacks, in order to avoid some disturbing factors. An independent, unrelated group of 37 boys and 25 girls (average age, 7.83±3.30 years) showing no clincial phenotypes was enrolled as normal controls. No significant difference was observed between the groups of cases and controls, in terms of gender and age (χ^2=0.667, p=0.414).

Immunoglobulin E (IgE) serology. Total IgE and specific serum IgE, which were thresholds of enrollment inclusion (Table I), were assessed using the ImmunoCAP System (Thermo Fisher Scientific/Phadia AB, Uppsala, Sweden). The cut-off of total IgE was 60 kU/l. In the asthma group, total IgE was >60 kU/l, and IgE serology atopic sensitization was indicated if the child had only dust mite allergen-specific serum IgE (≥0.35 kU/l) (data not shown) without food allergens of Fx5 (egg white, milk, fish, wheat, peanut and soy) (32). In the control group, no allergen-specific IgE was >0.35 kU/l, and total IgE was <60 kU/l. The percentage of eosinophils in peripheral blood (EOS) was also counted in order to confirm the patient's atopy. The cut-off value was 4%.

Array hybridization. Twelve pairs of gender- and age-matched children with asthma and normal control individuals were subjected to initial microarray-based discovery analysis. Peripheral blood (5 ml) was collected from each participant using ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. Total RNA was isolated using TRIzol (Invitrogen, Grand Island, NY, USA) and an miRNeasy mini kit (Qiagen, Valencia, CA, USA) kits according to the manufacturer's instructions, which efficiently recovered all RNA species, including miRNAs. The quality and quantity of RNAs collected from all participants were measured using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was determined by gel electrophoresis. After RNA was isolated from the samples, the miRCURY LNA™ microRNA Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's instructions for miRNA labeling. Briefly, 1 μg of each sample was 3'-end-labeled with Hy3 fluorescent dye, using T4 RNA ligase according to the following procedure: RNA in 2.0 μl of water was combined with 1.0 μl CIP buffer and CIP (Exiqon). The mixture was incubated for 30 min at 37˚C and was terminated at 95˚C for 5 min. Then, 3.0 μl labeling buffer, 1.5 μl fluorescent label (Hy3), 2.0 μl dimethyl sulfoxide (DMSO), and 2.0 μl labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16˚C and was terminated by incubation at 65˚C for 15 min. After termination, the Hy3-labeled samples were hybridized on the miRCURY LNA array (v.18.0; Exiqon) according to the manufacturer's instructions. The total 25 μl mixture from Hy3-labeled samples together with 25 μl hybridization buffer were denatured at 95˚C for 2 min, incubated in ice for 2 min, and then hybridized to the microarray at 56˚C for 16-20 h with a 12-Bay Hybridization system (Nimblegen Systems, Inc., Madison, WI, USA). After hybridization, the slides were prepared, washed using a wash buffer kit (Exiqon), and dried by centrifugation at 200 x g for 5 min. The slides were then scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Union City, CA, USA).

miRNA profiling and differential expression analysis. The signal quantification of scanned microarray images was captured using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA). Replicated miRNAs were averaged, and miRNAs whose intensities were ≥30 in all

Table I. Demographics of asthma and control groups.

Group	Gender[a]		Age[a] (years)	IgE (kU/l)	EOS (%)
	Male	Female			
Asthma	41	21	6.05±2.15	453.29±284.28	4.60±2.80
Control	37	25	7.83±3.30	38.16±34.51	3.72±3.40

No significance (χ^2=0.667, p=0.414) observed in gender and age. [a]Enrollment inclusion criteria: i) asthmatic patients aged >4 years old and diagnosed according to the Global Initiative for Asthma (GINA) guidelines were enrolled; ii) patients did not have current or recent infection, were not taking any medication, and did not experience any acute asthma exacerbations or attacks; and iii) patients were only allergic to dust mites. Enrollment exclusion criteria: i) patients were <4 years old; ii) patients with current or recent infection, taking any medication, and who had experienced acute asthma exacerbations or attacks. IgE, total immunoglobulin E; EOS, the percentage of eosinophils in peripheral blood.

Table II. Primers for miRNA sequences.

Name of miRNA	RT primer
U6	5'-CGCTTCACGAATTTGCGTGTCAT-3'
hsa-miR-675-3p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGAGCG-3'
hsa-let-7d-3p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGAAAG-3'
hsa-miR-550b-3p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCAGTGC-3'
hsa-miR-501-5p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCTCAC-3'
hsa-miR-4312	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGGGGA-3'
hsa-miR-151a-5p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACTAGA-3'
hsa-miR-625-5p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGGACTA-3'
hsa-miR-22-3p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAGTT-3'
hsa-miR-126-3p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGCATT-3'
hsa-miR-513a-5p	5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACATGACA-3'

samples were selected for calculation of the normalization factor. Expressed data were normalized using the median normalization. Significant differentially expressed miRNAs were identified through volcano plot filtering. Hierarchical clustering was performed to show distinguishable miRNA expression profiling among samples using MEV software (v4.6, TIGR; www.tm4.org/). A threshold (fold-change ≥2.0 and p-value ≤0.05) was used to determine the significance of differences of up- or downregulated miRNAs. The heat map diagram shows the result of the two-way hierarchical clustering of miRNAs and samples.

Bioinformatics analysis. Web-based programs, namely miRBase (http://mirbase.org/), PicTar (http://pictar.mdc-berlin.de/), miRDB (http://mirdb.org/miRDB/), miRanda (http://www.microrna.org/microrna/) and TargetScan (http://www.targetscan.org/), were employed to predict miRNA target(s). The miRNA-targeted transcripts, predicted by a minimum of three programs as the miRNA-targeted candidates, were selected for further analysis. The functional enrichment was analyzed using the DAVID program (http://david.abcc.ncifcrf.gov/), in which gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were provided for analysis. Venny software (http://bioinfogp.cnb.csic.es/tools/venny/) was used to show the predicted target genes that are co-regulated by multi-miRNA.

Validation of miRNAs and mRNA using reverse transcription-quantitative polymerase chain reaction. To validate the microarray results, miRNA expression was quantified in all 124 samples using RT-qPCR. The primers used for reverse transcription are presented in Table II, and the PCR conditions are shown in Table III. miRNA-U6 (33) was applied as an internal control. Briefly, reverse transcription was performed using MMLV Reverse Transcriptase and RNase inhibitor (both from Epicentre, Madison, WI, USA), 10X buffer (250 mM Tris-HCl, pH 8.3; 200 mM KCl; 40 mM $MgCl_2$; 5 mM DTT), 2.5 mM dNTP with RT primers at 16˚C for 30 min, followed by 42˚C for 40 min and 85˚C for 5 min. Twenty microliters of RNA (final concentration 50 ng/μl) was used as a template. The cDNA products from reverse transcription reactions were quantified using a real-time PCR System 7500 (Applied Biosystems, Foster City, CA, USA). The resulting amplicon was quantified by RT-qPCR with end-point SYBR-Green fluorescence. Each assay was performed in triplicate. RT-qPCR was also performed to determine the expression of the target genes regulated by the miRNAs that were discovered in our microarray analysis. The reverse transcription reaction was performed as descibed above except using SuperScript™ III reverse transcriptase (Invitrogen). RT primers were added into the reaction mixture at 37˚C for 1 min, followed by 50˚C for 60 min and 75˚C for 15 min. Twenty microliters of RNA (final concentration 75 ng/μl)

Table III. Primers used for RT-qPCR of miRNAs and PCR conditions.

Name of miRNA	Bidirectional primer sequence	Annealing temperature (˚C)	Amplicom length (bp)
U6	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'		
	R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'	60	89
hsa-miR-675-3p	GSP: 5'-GAAGGCTGTATGCCCTCACC-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	63
hsa-let-7d-3p	GSP: 5'-GGGCTATACGACCTGCTGC-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	63
hsa-miR-550b-3p	GSP: 5'-GGGGGGTCTTACTCCCTCAG-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	64
hsa-miR-501-5p	GSP: 5'-GGAGAATCCTTTGTCCCTGG-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	64
hsa-miR-4312	GSP: 5'-GGGGTAAGCCTTGTTCCTGT-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	63
hsa-miR-151a-5p	GSP: 5'-GGGGTCGAGGAGCTCACAG-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	63
hsa-miR-625-5p	GSP: 5'-GGGGAGGGGGAAAGTTCTA-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	63
hsa-miR-22-3p	GSP: 5'-GGGAAGCTGCCAGTTGAAG-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	63
hsa-miR-126-3p	GSP: 5'-GGGGTCGTACCGTGAGTAAT-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	64
hsa-miR-513a-5p	GSP: 5'-GGAGGGTTCACAGGGAGGT-3'		
	R: 5'-GTGCGTGTCGTGGAGTCG-3'	60	62

GSP, gene-specific primer, which is the corresponding miRNA-specific primer and different from the RT primer. R, a primer that matches the RT primer.

Table IV. Primers used for RT-qPCR of target genes regulated by miRNAs.

Gene	Primer sequence	Annealing temperature (˚C)	Amplicom length (bp)
β-actin	F: 5'-CCTGTACGCCAACACAGTGC-3'		
	R: 5'-ATACTCCTGCTTGCTGATCC-3'	60	211
PPARGC1B	F: 5'-GGCGGTAGACGAATGACAGAC-3'		
	R: 5'-CTTAGTGGAGGCAGCAGAAAGA-3'	60	199
ESR1	F: 5'-CCTTGCTATGTTACTAAGCGTGAG-3'		
	R: 5'-GGGTGCTATAATAAACCCTTGAC-3'	60	247
CBL	F: 5'-GGACAAGAAGATGGTGGAGAAGT-3'		
	R: 5'-CTTGACAAGATAGTACGGAGATGC-3'	60	150

PPARGC1B, peroxisome proliferator-activated receptor gamma, coactivator 1 beta; *ESR1*, estrogen receptor 1; *CBL*, Cbl proto-oncogene, E3 ubiquitin protein ligase.

was used as a template. The cDNA products from the reverse transcription reactions were quantified using a real time PCR system 7500 (Applied Biosystems). The resulting amplicon was quantified by RT detection of end-point SYBR-Green fluorescence. Each assay was performed in triplicate. The primers and reaction conditions are presented in Table IV. The relevant quantification was obtained for each sample with normalization to the internal control β-actin.

Measurement of inflammatory cytokines. The plasma concentrations of IL-4, -6, -10, -12 and -13; γ-IFN; and tumor necrosis factor-α (TNF-α) were determined using

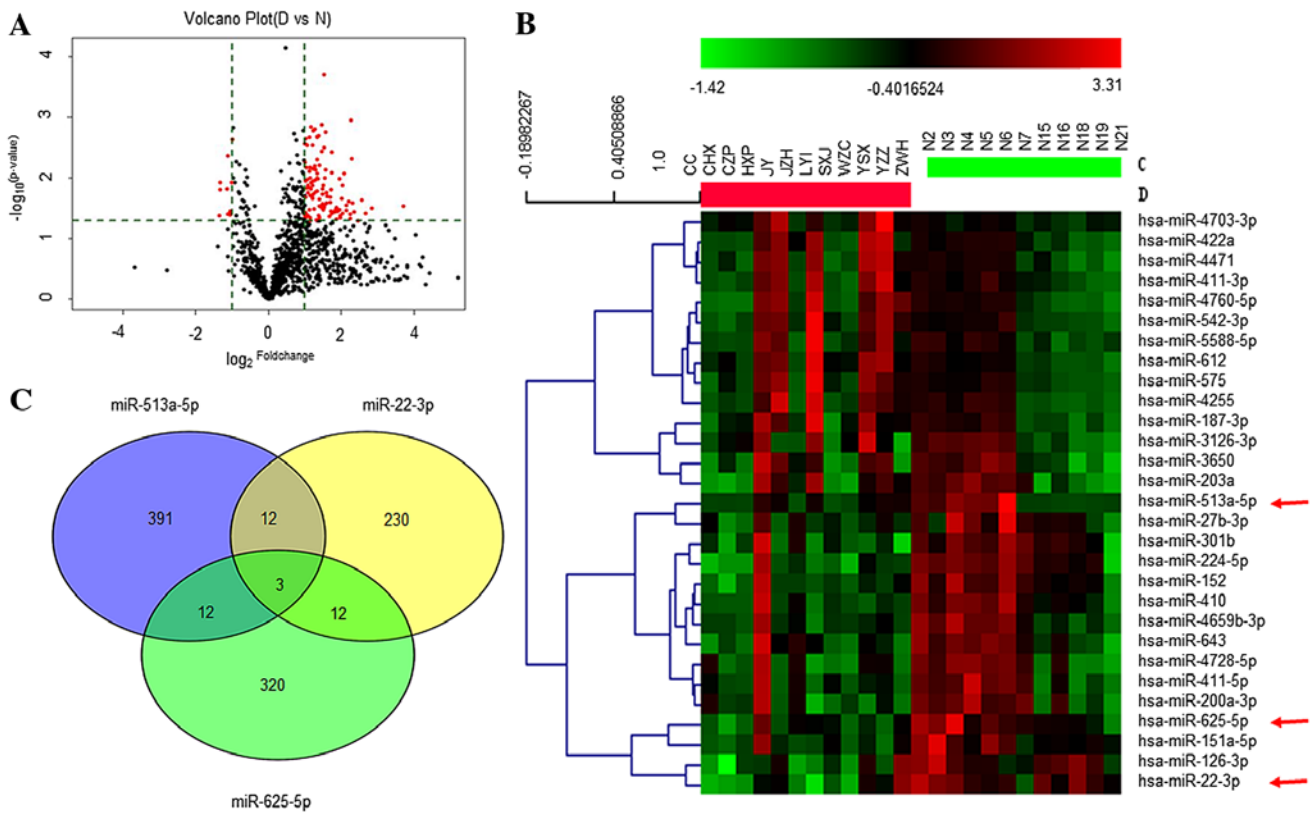

Figure 1. Identification of microRNAs (miRNAs or miRs) differentially expressed in childhood asthma. (A) Volcano plot presents differential expression of miRNAs. The vertical lines corresponds to fold changes of -2 (downregulation) and +2 (upregulation). The horizontal line represents a p-value of 0.05. The red points in the plot represent the differentially expressed miRNAs with statistical significance. There were 112 upregulated and 10 downregulated miRNAs identified. (B) A heat map shows a main part of the clustering of miRNAs. Upregulated miRNAs are colored red, and downregulated are green. The expression of miR-513a-5p, miR-22-3p and miR-625-5p was downregulated in the asthma group compared with the control group ($p<0.05$). miR-625-5p was changed 2.17-fold, miR-513a-5p was changed 2.07-fold and miR-22-3p was changed 2.21-fold in the asthma group. (C) miRNA-target genes are co-regulated by multi-miRNAs. Three target genes were co-regulated by miR-513a-5p, miR-22-3p and miR-625-5p, and three pairs of any two miRNAs combined may have 12 target genes co-regulated individually.

an enzyme-linked immunosorbent assay (ELISA) kit (Multiscience Biotech Co. Ltd., Shanghai, China).

Statistical analysis. The differences between the groups were analyzed using the Student's t-test when two groups were compared or by one-way ANOVA when > two groups were compared. Analyses were performed using SPSS 19.0 software (IBM, Armonk, NY, USA). A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Differential expression of miRNAs in children with asthma. A total of 122 differentially expressed miRNAs were identified from 12 children with asthma in a discovery study with a microarray that detects 3,100 genome miRNAs. Among differentially expressed miRNAs, 112 were identified as upregulated, and 10 were downregulated (Fig. 1A and B). To confirm the microarray results, five upregulated (miR-let-7d-3p, miR-550b-3p, miR-501-5p, miR-4312 and miR-675-3p) and five downregulated (miR-126-3p, miR-22-3p, miR-513a-5p, miR-151a-5p and miR-625-5p) miRNAs were randomly subjected to RT-qPCR with the 12 pairs of RNA samples that were subjected to the original array-based discovery study. The differential expression of four upregulated (miR-let-7d-3p, miR-550b-3p, miR-501-5p and miR-675-3p) and all five downregulated miRNAs were validated to be significant ($p<0.05$) (Table V). Three downregulated miRNAs (miR-625-5p with 2.17-fold decrease, miR-513a-5p with 2.07-fold-change, and miR-22-3p with 2.21-fold-change) (Table VI), which were statistically significant differences ($p<0.05$) and functionally linked to inflammation and apoptosis by bioinformatics analysis (Fig. 2), were applied for prediction of targeting transcripts.

To further confirm the microarray results, an additional independent 100 RNA samples, including 50 from individuals with asthma and 50 from control subjects, were subjected to RT-qPCR in order to validate the expression of miR-625-5p, miR-22-3p and miR-513a-5p. These three miRNAs were selected on the basis of predicting their targeted mRNAs: *CBL*, peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*) and *ESR1*, which is involved in the PI3K-AKT and NF-κB pathways (18,19). Our results showed that the average levels of miR-625-5p, miR-22-3p and miR-513a-5p in the asthma group were significantly lower than those in the control group (1.13±0.22 vs. 2.21±0.17, 0.84±0.17 vs. 1.58±0.27 and 1.03±0.14 vs. 2.29±0.26, respectively; $p<0.01$) (Fig. 3A). No statistical significance was observed, regarding the expression levels of these three miRNAs, among individual participants within either the asthma group or the control group ($p>0.05$).

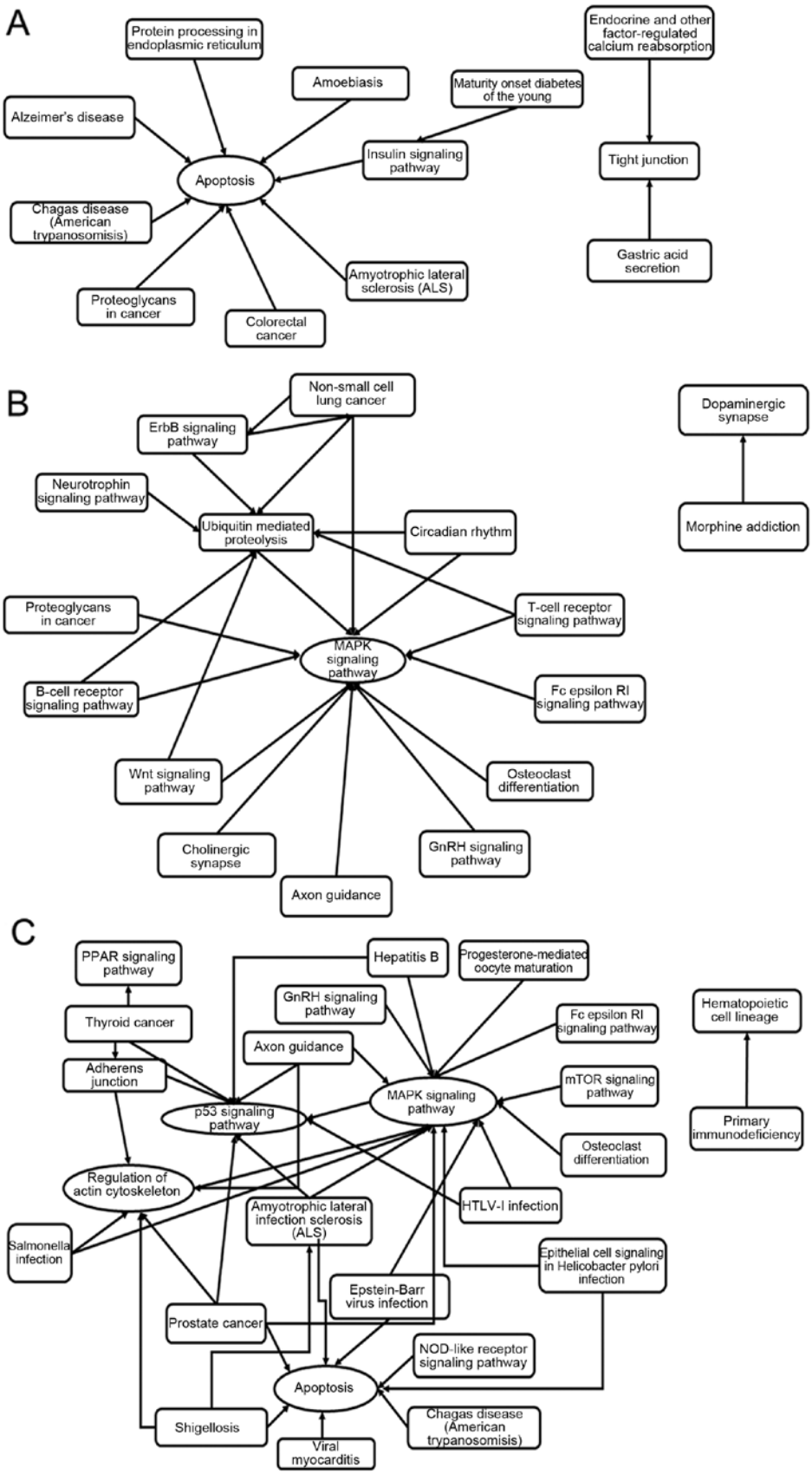

Figure 2. Molecular networks of miRNAs and their associated pathways. (A) miR-513a-5p, (B) miR-625-5p and (C) miR-22-3p.

Predicted target gene mRNAs. A total of 418 genes were predicted as the targets of miRNA-513a-5p, 257 targeted by miRNA-22-3p, and 347 targeted by miRNA-625-5p, respectively, in three web-based programs (miRDB, miRBase and miRanda). These listed target genes were subjected to further analysis with Venny software, through which miRNA targets were found to be over-

Table V. Up- and downregulated miRNAs determined using RT-qPCR ($2^{-\Delta\Delta CT}$).

miRNA	Asthma (n=12)	Control (n=12)	t-value	p-value	95% confidence interval of difference
Upregulated					
let-7d-3p	0.943±0.120	0.434±0.089	8.650	0.000	0.385 to 0.632
miR-550b-3p	0.937±0.130	0.472±0.056	7.632	0.000	0.337 to 0.593
miR-501-5p	1.252±0.244	0.786±0.183	3.893	0.001	0.215 to 0.717
miR-675-3p	1.063±0.172	0.660±0.044	5.092	0.000	0.237 to 0.569
miR-4312	1.268±0.282	1.130±0.207	0.999	0.331	-0.152 to 0.428
Downregulated					
miR-151a-5p	1.006±0.193	2.024±0.374	-8.028	0.000	-1.284 to -0.751
miR-625-5p	1.130±0.222	2.206±0.168	-9.858	0.000	-1.305 to -0.847
miR-126-3p	0.992±0.142	1.790±0.161	-24.619	0.000	-0.957 to -0.639
miR-513a-5p	1.031±0.140	2.288 ±.02565	-14.089	0.000	-1.445 to -1.070
miR-22-3p	0.836±0.175	1.580±0.269	-7.215	0.000	-0.961 to -0.527

Table VI. Differential expression of miR-22-3p, miR-513a-5p and miR-625-5p.

miRNA	Fold-change (A vs. C)	p-value	CV-value	
			A	C
miR-22-3p	0.452	0.015	0.422	0.693
miR-513a-5p	0.484	0.040	0.426	0.792
miR-625-5p	0.460	0.004	0.646	0.506

A, asthma group (n=12); C, normal control group (n=12); CV, coefficient of variation.

Table VII. Plasma concentrations of inflammatory factors in dust mite–induced asthma.

Inflammatory factor	Asthma (pg/ml) (n=62)	Control (pg/ml) (n=62)	t-value	p-value
γ-IFN	3.44±2.87	7.08±4.30	8.178	0.000
TNF-α	93.80±83.41	221.06±150.33	6.767	0.045
IL-13	6.24±3.58	7.59±5.04	1.528	0.109
IL-12	1.50±0.66	3.43±1.37	2.898	0.007
IL-4	0.83±0.64	0.91±0.78	6.920	0.623
IL-6	19.83±18.79	18.79±12.64	10.717	0.918
IL-10	2.39±2.47	3.74±1.98	2.598	0.006

γ-IFN, γ-interferon; TNF-α, tumor necrosis factor-α; IL, interleukin.

lapped (Fig. 1C). Three mRNAs (PLCXD3, MSL2 and BRWD3) are the overlapped targets of miRNA-513a-5p, miRNA-22-3p and miRNA-625-5p. Three pairs of any two miRNAs combined may have 12 target genes co-regulated. However, with bioinformatics functional analysis using the GO and KEGG pathways, only three transcripts of mRNAs, *CBL*, *PPARGC1B* and *ESR1,* were predicted as the targets of miR-513a-5p, miR-22-3p and miR-625-5p, respectively (34). Transcripts of *CBL* and *PPARGC1B* were dually targeted by miR-513a-5p and miR-22-3p, and that of *ESR1* was targeted by miR-625-5p and miR-22-3p. mRNAs of *CBL*, *PPARGC1B* and *ESR1* were differentially elevated in the asthma group (*CBL*, 2.61E-02 vs. 1.66E-02; *PPARGC1B*, 3.42E-02 vs. 1.94E-02; *ESR1*, 6.28E-03 vs. 3.67E-03; p<0.01), as compared with the control group (Fig. 3B) and have been found to be associated with the PI3K-AKT and NF-κB signaling pathways (Fig. 4) (35,36). It was also confirmed by GO and KEGG pathway analysis in DAVID software that the CBL was significantly associated with T- and B-cell differentiation and inflammatory factor signaling pathways (GO enrichment value >1.9, p<0.05).

SYK and EGFR are two molecules downstream from CBL and may be mediated by the activation of CBL through the PI3K-AKT and NF-κB pathways. Our assessment showed that in the asthma group, levels of *EGFR* declined (1.26E-03 vs. 2.71E-03; p<0.01), whereas levels of the mRNA of *SYK* involved in this pathway were elevated (4.30E-02 vs. 3.05E-02; p<0.01) (Fig. 3B).

ELISA analysis of the plasma concentrations of associated inflammatory cytokines. We further examined the concentrations of inflammatory cytokine factors (γ-IFN, TNF-α, IL-13, IL-12, IL-4, IL-6 and IL-10) which may be associated with the Th1, Th2 and inflammation pathways PI3K-1KT and NF-κB that are mediated by the miRNA-targeted genes *CBL*, *PPARGC1B* and *ESR1* (18,19,37) (Table VII). Decreased concentrations of γ-IFN, TNF-α, IL-12 and IL-10 in the plasma were found in the asthma group, compared with those in the control group (3.44±2.87 vs. 7.08±4.30 pg/ml, 93.80±83.41 vs. 221.06±150.33 pg/ml, 1.50±0.66 vs. 3.43±1.37 pg/ml, and 2.39±2.47 vs. 3.74±1.98 pg/ml, respectively, p<0.05). These decreased concentrations may be associated with the different expression of upstream genes with a regulatory function. The cytokine concentrations of IL-4, IL-6 and IL-13 in the asthma

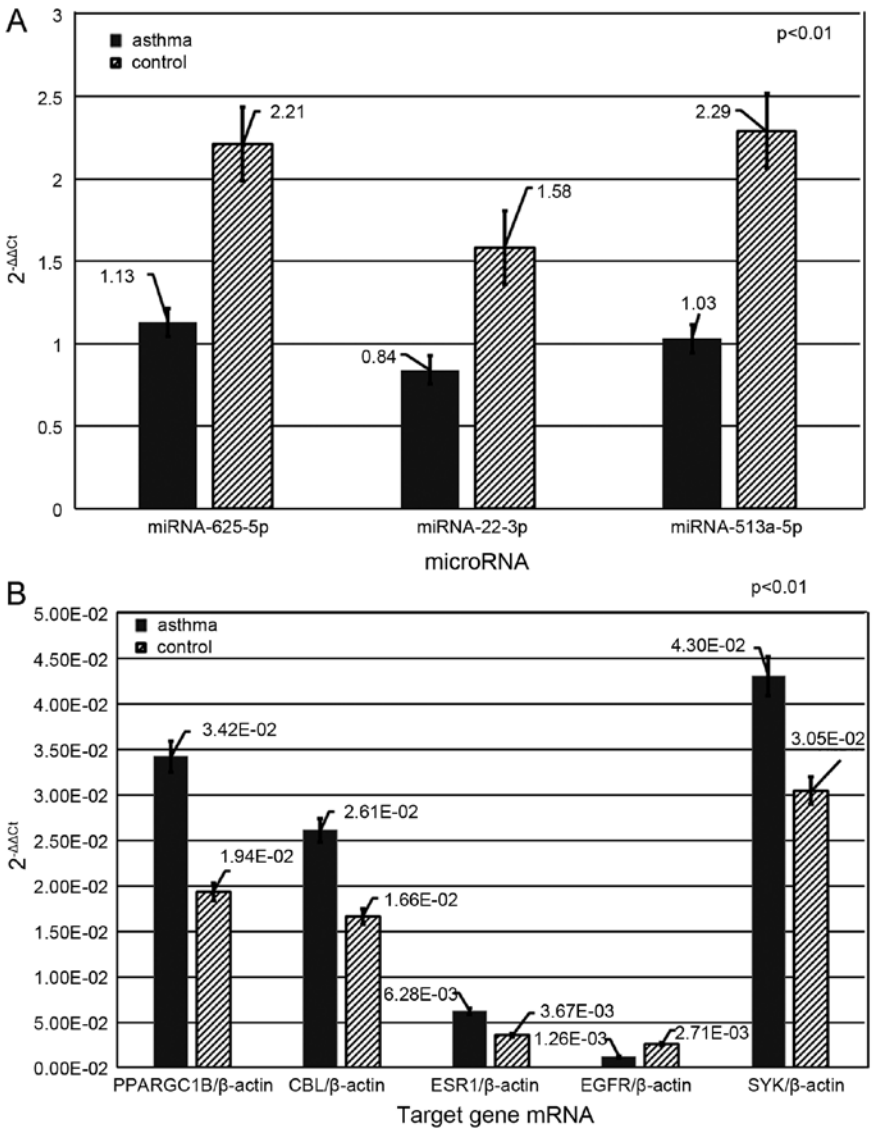

Figure 3. Quantitative measurement of miRNAs and mRNAs. RT-qPCR of downregulated transcribed miRNAs and mRNAs. The x-axis is (A) the miRNAs or (B) the mRNAs. The y-axis is the relevant expression of (A) miRNAs or (B) mRNAs represented by $2^{-\Delta\Delta CT}$. The results showed that the average levels of (A) miR-625-5p, miR-22-3p and miR-513a-5p were significantly lower than those of the control group (p<0.01) and (B) mRNAs in the asthma group were significantly higher than those of the control group (p<0.01). (Asthma, n=62; control, n=62).

group were neither changed nor showing statistically significant differences compared with those in the control group (p>0.05).

Discussion

Previous studies demonstrated that the abnormal regulation of immune and inflammatory responses occur in asthma patients (11-13). House dust mite allergen (Der f 2) may activate the PI3K-AKT pathway (14), which subsequently activates the NF-κB pathway and induces IL-13 expression in human airway epithelial cells according to *in vitro* research. PI3K-AKT was also involved in the induction of IL-6 in Der-p1-stimulated nasal epithelial cells (NECs) from patients with allergic rhinitis (AR), and may have potential implications for the prevention and treatment of AR and asthma (38). There was an interaction between the PI3K-AKT and NF-κB pathways (38). NF-κB activation is the crucial step between contact with an allergen and the downstream manifestations of asthma (16). In the present study, we found that these pathways may be regulated by *CBL*, *PPARGC1B* and *ESR1*, which may be mediated by miRNA, and alter the concentrations of downstream cytokines.

As an immune regulator, miRNA appears to play an important role in the regulation of immune responses and the inflammatory signaling pathway in pediatric asthma (39). However, the mechanism responsible for the involvement of miRNAs in the development of asthma has not yet been fully elucidated. In the present study, children with asthma were enrolled in a genome-wide investigation of novel miRNA(s) that may have a regulatory function in pediatric asthma. The results showed that the expression of three miRNAs (miR-513a-5p, miR-22-3p and miR-625-5p) was downregulated in samples from the asthma group and they were closely associated with the immune response and cytokine secretion. GO and KEGG pathway analysis using DAVID software demonstrated that the target gene *CBL*, regulated by miR-513a-5p and miR-22-3p, was significantly associated with

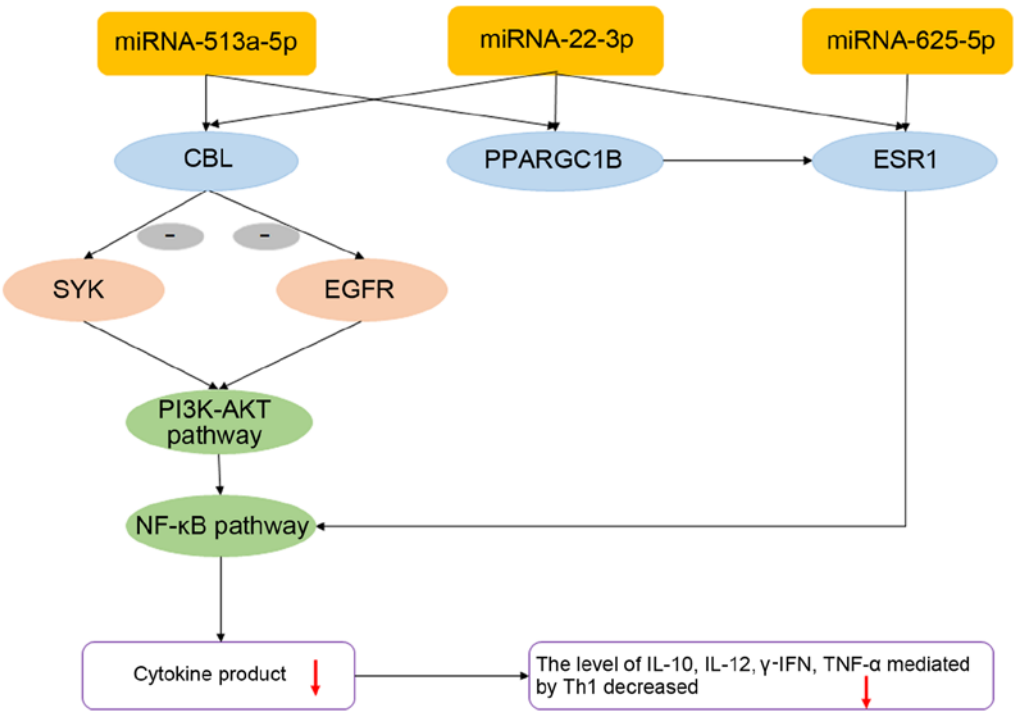

Figure 4. miRNAs regulate pathways. miR-513a-5p and miR-22-3p co-regulated *CBL* (Cbl proto-oncogene, E3 ubiquitin protein ligase), negatively impacting the phosphoinositide 3-kinase (PI3K)-AKT and nuclear factor-κB (NF-κB) pathways through its downstream genes spleen tyrosine kinase (*SYK*) and epidermal growth factor receptor (*EGFR*). The NF-κB pathway is regulated by estrogen receptor 1 (*ESR1*), which is upregulated by miR-625-5p and miRNA-22-3p. In addition, *ESR1* may be regulated by peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*PPARGC1B*) that is upregulated by miR-513a-5p and miR-22-3p. The decreased secretion of cytokine factors interleukin (IL)-10, γ-interferon (γ-IFN), tumor necrosis factor-α (TNF-α), and IL-12 mediated through the differential expression of the NF-κB pathway.

T- and B-cell differentiation and inflammatory factor signaling pathways (GO enrichment value above 1.9, $p<0.05$) (data not shown). The signaling pathways that were mainly involved included the PI3K-AKT and NF-κB as well as the T-cell receptor pathway (14-16,18). The PI3K-AKT pathway, in particular, may affect NF-κB in the T- and B-cell receptor signal pathways in the KEGG pathway maps [map04660 (http://www.genome.jp/kegg-bin/show_pathway?map04660), map04062 (http://www.genome.jp/kegg-bin/show_pathway?map04062) and map04668 (http://www.genome.jp/kegg-bin/show_pathway?map04668)]. However, the mechanism through which miRNAs regulate their targeting genes to affect the inflammatory pathway as well as identifying which pathway is principally involved needs to be further elucidated.

CBL is an adapter protein that functions as a negative regulator of many signaling pathways that are triggered by the activation of cell surface receptors (34). *CBL* alters the inflammatory signaling pathways in pediatric asthma by inhibiting the expression of *SYK* and *EGFR*, which mediate the PI3K-AKT, MAPK and NF-κB signaling pathways (21,40), resulting in the onset of asthma (35,36). Data from our present study provide evidence that miR-513a-5p and miR-22-3p are involved in dust mite-induced pediatric asthma through regulating *CBL*, which possibly alters the gene expression of *EGFR* and *SYK* and further decreases levels of the cytokines γ-IFN, TNF-α, IL-12 and IL-10.

Additionally, *PPARGC1B*, predicted to be regulated by miR-513a-5p and miR-22-3p, and *ESR1*, regulated by miR-625-5p and miR-22-3p, were found by GO and KEGG analysis to be significantly associated with inflammatory factor signaling pathways. *PPARGC1B* is a protein that stimulates the activity of transcription factors and nuclear receptors, and ESR1 is a nuclear hormone receptor. There is an interaction between PPARGC1B and ESR1 (34). ESR1, a negative regulator of several inflammatory signaling pathways, directly affects the transcription factor signaling pathway by inhibiting NF-κB (18), thereby mediating changes in the secretion of several inflammatory cytokines such as IL-10, TNF, IL-6, γ-IFN and others in the NF-κB signaling pathway. Some studies reported that *ESR1* gene expression affected the airway resistance of asthmatic patients (18,41). In this study, RT-qPCR determined that the expression of the target genes *PPARGC1B* and *ESR1* regulated by miRNAs was significantly upregulated in the samples from asthma patients.

The present study showed that levels of the inflammatory cytokines TNF-α, IL-12, γ-IFN and IL-10 were lower in the asthma group than in the control group. We assumed that the decreased secretion of these cytokines was a result of the upregulated expression of the associated target genes, which may be the negative regulators of several inflammatory signaling pathways such as PI3K-ATK, MAPK and NF-κB, and subsequently impacted on the pathways mediating inflammatory cytokine secretion that lead to pediatric asthma attacks, although this assumption requires further investigation. In this study, no statistically significant differences were observed in the concentrations of IL-4, IL-6 and IL-13 between the asthma group and the control group, which does not concur with the findings of previously mentioned *in vitro* research (14). This study demonstrated that miRNAs and their target genes principally affected the secretion of the inflammatory cytokines TNF-α, IL-12, γ-IFN, IL-10,

and not the secretion of IL-4, IL-6 and IL-13. On the other hand, we wondered whether our results may be associated with the fact that the enrolled patients were not receiving any medication or experiencing any acute asthma attacks. This is due to the fact that different cytokine levels have been associated with different phenotypes of asthma and medication therapy (30,31). Therefore, in future studies, the enrollment of additional patients, including those receiving medication or experiencing acute asthma attacks, would further validate these findings.

In conclusion, we have identified three significant miRNAs - miR-513a-5p, miR-22-3p and miR-625-5p - that were differentially expressed in pediatric asthma. These miRNAs regulated their target genes (*CBL*, *PPARGC1B* and *ESR1*) and are likely have an impact on the regulation of immune reactions and inflammatory cytokine pathways. We believe that these three miRNAs, particularly miR-513a-5p and miR-22-3p, and their target gene *CBL*, as reported in this study, may play important roles in the epigenetic mechanisms underlying pediatric asthma.

Acknowledgements

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