**Title:** microRNA in children with asthma: findings from bronchoalveolar lavage fluid

**Running title:** microRNA and asthma

**Authors:** LIU Min [1]: Doctor. 6518115@ zju.edu.cn

LV Jian-Hai [1,2]: Doctor.

WU Lei [1]: Doctor

TANG Lan-Fang [1]: PhD, MD, Professor. 6195007@zju.edu.cn

LIU Min and LV Jian-Hai contributed equally to this article

1. Department of Pulmonology, Children's Hospital of Zhejiang University School of Medicine

2. Department of Pediatric, Shangyu People Hospital

**Corresponding author:** TANG Lan-Fang

3333 Binsheng Road, Hangzhou 310051, China

Tel: +86-13868138022

E-mail: 6195007@zju.edu.cn

Fax: +86-571-87033296

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**Abstract**

Objective: To investigate the pathogenesis of miRNA in asthma by measuring the microRNAs (miRNAs) levels in the bronchoalveolar lavage fluid (BALF) cells of children with asthma.

Methods: A total of 15 children with asthma were enrolled as the asthma group and another 13 children who were subjected to airway foreign removal within 24 hours were enrolled as the control group. The levels of miRNAs were measured by miRNA microarray and verified by qRT-PCR. The expression of target genes E-cadherin was assessed as well.

Results: A total of 65 miRNAs in the BALF cells of the asthma group were different from those in the control group. Six miRNAs of miR-34/449 family and seven miRNAs of miR-200 family were decreased. For 13 differentially expressed miRNAs, qRT-PCR confirmed that 10 miRNAs were consistent with the results of miRNAs microarray, including miR-34b-3p, miR-34b-5p, miR-34c-3p, miR-34c-5p, miR-200a-3p, miR-200a-5p, miR-200b-3p, miR-200b-5p, miR-200c-3p, miR-141-3p. E-cadherin, the predicted target of miR-34/449 family and miR-200 family, was down-regulated in children with asthma as well.

Conclusions: These data suggested that miRNAs, especially the miR-34/449 and miR-200 families, were involved in the mechanism of childhood asthma by regulating E-cadherin expression. Further studies on the mechanism of miRNAs in asthma are required.

**Key words：** Asthma; Bronchoalveolar lavage fluid; microRNA; microRNA-34; microRNA-200; *E-cadherin*.

## Introduction

Asthma is a common chronic inflammatory disease of the airway contributing to significant morbidity and mortality worldwide [1, 2]. It is characterized by reversible airflow obstruction, bronchial hyper-responsiveness and chronic inflammation. Asthma is caused by a combination of complex environmental and genetic interactions. Expression changes of some genes (e.g. cytokines, IgE, MMP-9, TIMP-1 and sonic hedgehog) is an important mechanism of asthma [3-5], which may induce the infiltration of inflammatory cells, proliferation of smooth muscle and mucus hypersecretion; however, the inner mechanism by which these genes change still needs investigation.

microRNAs (miRNAs) are a group of 19-22 nucleotide single-stranded noncoding RNAs. Increased evidences have shown that miRNA and other non-coding RNA (e.g. lncRNA) play an important role on regulating gene expression. Every miRNA regulates hundreds of genes (both coding and non-coding) by post-transcriptional (and possibly also translational) silencing target mRNA. Recently, some miRNAs have been reported to participate in the mechanism of lung diseases, including asthma, chronic obstructive pulmonary diseases and lung cancer [6, 7]. One study showed that the expressions of 227 miRNAs in the airway biopsies were not different between the mild asthma patients and controls [8]. However, many studies indicated that some miRNAs were abnormally expressed in the animal asthmatic models, including let-7, miR-146, miR-181, miR-223, miR-29b, miR-672 and miR-690; studies have also demonstrated that miRNAs may regulate a variety of cell signaling pathways, immune cell development, and inflammatory response, for example IL-13, IL-6, STAT6, TGF-β and MAPK signal pathways [9-11]. Differences in miRNA expression (miR-16, miR-125b, miR-146a and miR-155) were also found in the blood and sputum of asthmatic patients [12, 13]. However, the samples of these studies were from the animal asthmatic models or lung in adults but not in children.

As the lung tissue is difficult to obtain from asthmatic patients, bronchoalveolar lavage fluid (BALF) cells were used as a best marker reflecting the lung tissue. BALF cells were mainly from alveolar tissue. Hence, to investigate the pathogenesis of miRNA in asthma, we analyzed the miRNAs levels in the BALF of asthmatic adolescents by miRNA microarray. Then, the

differentially expressed miRNAs were verified by qRT-PCR and the possible target gene *E-cadherin* was assessed.

## Subjects and Methods

### Subjects

This study was approved by the ethical committee of Children Hospital of Zhejiang University School of Medicine, and informed consents were obtained from parents or guardians of all children, and confirmed that all experiments were performed in accordance with relevant guidelines and regulations.

A case-control study was designed. A total of 15 children with asthma were enrolled as the asthma group. The diagnosis of asthma was according to the guidelines of Global Initiative for Asthma [GINA] [14]. All these patients had received the therapy of glucocorticoid over without improvement or progressed recently. Fibrobronchoscopy was performed to exclude some other diseases. Another 13 children with airway foreign body that were removed by fibrobronchoscopy within 24 hours were enrolled as the control group. Children with other lung, immunologic or other diseases were excluded.

### Fibrobronchoscopy and BALF collection

Midazolam maleate (0.3 mg/kg) was injected 15-30 minutes before fibrobronchoscopy. Topical anesthesia with 2% of lidocaine was utilized. BALF with saline were conducted in the right middle lobe (1 $mL/kg^{-1}$ each time × 3 times, max 20 ml each time) with -25 to -100 mmHg (-3.3 to -13.3kPa) suction. The sucked first-time samples were used for pathogen analysis. The second- and the third-time samples were centrifuged at 300×g for 10 min at 4ºC. Then, cells and supernatants of BALF samples were used for further studies.

The BALF cells from five boys of the asthma group and five boys of the control group (one sample excluded because of the RNA degradation) were used for miRNAs microarray. For PCR measurements, BALF cells of five samples in the asthma group and three in the control group were used to verify miR-34/449 family as the first batch of samples. Other BALF cells of five samples in the asthma group and five in the control group were used to verify miR-200a/b/c and

*E-cadherin* as the second batch of samples. E-cadherin protein levels of the BALF supernatant of two batches including ten of the asthma group and eight of the control group were measured by ELISA. Characteristics of the two groups for different measurements are summarized in Table 1.

**miRNA microarray**

Total RNA was isolated from BALF cells using QIAGEN RNeasy Mini Kit (Qiagen N.V, Germany) for assessment of miRNA. Microarray profiling for miRNA was performed using AffymetrixGeneChip miRNA 4.0 arrays (Affymetrix Inc. USA) according to manufacturer's recommended protocol. Briefly, 1 mg of total RNA from the cells was labeled by polyA polymerase using the FlashTag® Biotin HSR RNA Labeling Kit following the manufacturer's recommendations (Affymetrix Inc, USA). RNA was hybridized to the Affymetrix miRNA array as recommended by thevendor. Standard Affymetrix array cassette staining, washing and scanning was performed using the GeneChip2 Hybridization, Wash, and Stain Kit (Affymetrix Inc, USA) and GeneChip2 Scanner 3000 7G. Feature extraction was performed using Affymetrix Command Console software. The raw data were processed in the following sequence: background detection was followed by RMA global background correlation, quantile normalization, median estimation and log2-transformation using the miRNA QC software tool (Affymetrix Inc, USA). Each cell line performed miRNA microarray in triplicates.

**Analysis of differentially expressed miRNAs**

The random variance model *t*-test was used to identify differentially expressed genes between the control and asthma groups. After the significant analysis and false discovery rate analysis, we selected the differentially expressed genes according to at least 2-fold change expression levels and predefined P-value thresholds (0.05). The results of differentially expressed genes were subjected to unsupervised hierarchical clustering (Cluster 3.0) and TreeView analysis (Stanford University, Stanford, CA, USA).

**Quantitative RT-PCR**

Quantitative RT-PCR (qRT-PCR) was used to verify differential expression of miRNAs that were detected by the miRNA microarray and predicted target gene. Total RNA was extracted with

Trizol reagent (Invitrogen Co, USA). RNA concentration was measured with a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA). The qRT-PCR of miRNA was using miDETECT A TrackTM miRNA qRT-PCR Starter Kit (RiboBio Co.Ltd, China). According to miRNA concentration, the 3 μL total RNA was used in the poly(A) tailing. For the expression of target gene *E-cadherin*, cDNA was obtained with GoScriptTM Reverse Transcription System and assessment of target gene used GoTaq® qPCR Master Mix kit to verify (Promega Co, USA) according to the manufacturer's instructions. The relative quantities of miRNA and target genes were calculated using $2^{-\Delta\Delta CT}$ method and ΔCT (CT value of target gene - CT value of U6/ GAPDH) was used for statistical analyses. U6 and GAPDH were used for internal reference of the expressions of miRNAs and *E-cadherin*, respectively (Table 2).

**Target genes analyzed and validated**

miRBase database (http://www.mirbase.org/) was used to predict targets genes. Also, relevant literatures were reviewed. We selected the target gene and signal pathways related to target miRNAs. We selected *E-cadherin* because it was the target genes of miRNA-34/449 and miRNA-200 family, which was down-regulated in the BALF cells of asthmatic children.

The mRNA levels of *E-cadherin* were detected by qRT-PCR as above-mentioned. Total protein levels in supernatants of BALF were measured with the BCA Protein Assay Kit (Beyotime Lit Co, China). Protein levels of E-cadherin were measured with Human E-cadherin Quantikine ELISA Kit (R&D systems, USA) following the manufacturer's instructions. The ratio of E-cadherin protein to total protein was used to estimate the E-cadherin protein levels.

**Statistical analysis**

Statistical analyses were performed using SPSS 20.0 software (SPSS: Chicago, IL, USA) and GraphPad Prism 5.01 (GraphPad Software Inc, USA). The numerical data were expressed as mean ± SEM. Differences expressions between the asthma and control groups were analyzed using independent *t* test. The difference of age and sex between the asthma and control groups was analyzed using independent *t* test and the Fisher's exact test, respectively. Significant difference was regarded if a *P* value of $< 0.05$.

## Results

### Differential miRNAs found by miRNA array

Compared the miRNA levels between the control and asthma groups, 65 differentially expressed miRNAs were found (Figure 1). Among these differential miRNAs, 22 miRNAs in the asthma group exhibited 2-fold or greater increase than those in the control group (Table 3). The dramatic up-regulated miRNAs were miR-143-3p, miR-424-3p, miR-199b-3p and miR-199a-3p, with 3.71, 3.58, 3.46 and 3.46 folds increased. Other up-regulated miRNA included miR-3197, miR-4484, miR-503-5p, miR-1290, miR-1304-5p, miR-6126, miR-212-3p, miR-7515, miR-3135b, miR-6780b-5p, miR-4430, miR-6772-5p, miR-4417, miR-4485, miR-204-3p, miR-4533, miR-1183 and miR-6758-3p.

Forty-three miRNAs were down-regulated in asthma group (Table 4). The dramatic down-regulated were the three miRNAs of miR-34/449 family, including miR-34b-5p, miR-34b-3p and miR-34c-5p, which were only 0.068, 0.08 and 0.087 folds of these in the control group. Other down-regulated miRNA included other members of miR-34/449 family (e.g. miR-34c-3p, miR-449), miR-200 family (miR-200a/b/c, miR-429 and miR-141), miR-23b/27b cluster (miR-23b, miR-27b), miR-92b, miR-100, and so on. Six miRNAs of miR-34/449 family (miR-34b-3p, miR-34b-5p, miR-34c-3p, miR-34c-5p, miR-449a and miR-449b-5p) and seven miRNAs of miR-200 family (miR-200a-3p, miR-200a-5p, miR-200b-3p, miR-200b-5p, miR-200c-3p, miR-429 and miR-141-3p) decreased, which account for 30.2% (13/43) down-regulated miRNAs in the BALF cells from asthmatic children.

### miRNA levels verified by qRT-PCR

Because six miRNAs of miR-34/449 family and seven miRNAs of miR-200 family accounted for a large proportion of all down-regulated miRNA, we selected 13 down-regulated miRNAs to verify the results of miRNA array by qRT-PCR. Consistent with the results of miRNA microarray, 10 of 13 (76.9%) miRNAs, including miR-34b-3p, miR-34b-5p, miR-34c-3p, miR-34c-5p, miR-200a-3p, miR-200a-5p, miR-200b-3p, miR-200b-5p, miR-200c-3p and miR-141-3p, were significantly lower in the asthma group than those in the control group ($P<0.05$,

respectively) (Figure 2). However, the levels of miR-449a, miR-449b-5p and miR-429 had no significant differences between the two groups (P>0.05, respectively).

**Levels of target gene**

The mRNA of *E-cadherin* gene was significant different between the asthma and control groups (Figure 3A). The ratios of E-cadherin to total protein (E-cadherin/protein ratio) in the asthma and control groups (0.0016 ± 0.0004% vs. 0.0035 ± 0.0007%) were significantly different (P=0.031). E-cadherin protein in the asthma group was significantly lower than those in the control group (Figure 3B).

## Discussion

Our study found that 65 miRNAs in the BALF cells of the asthma group were different from these in the control group, and then qRT-PCR confirmed that 10 miRNAs were consistent with the results of miRNAs microarray, including six miRNAs of miR-34/449 family and seven miRNAs of miR-200 family. More and more animal evidences have shown that miRNAs play an important role on the regulation of gene expression, which involved in lung development and lung diseases [6, 7]. Although some studies about the miRNA in blood of asthma patients or animal model have been reported [8-11], to our knowledge. BALF cells may provide more direct insight about the lung pathology of asthma.

Our previous study reported that most common BALF cells are inflammatory cells (macrophages, neutrophils, lymphocytes and eosinophils) accounting for 90-95% of the total cells, although airway epithelium and fibroblasts were also involved. Among the inflammatory cells, macrophages were predominant, which account for 70%-90% the inflammatory cells. Also found that there was no significant difference in total and macrophage numbers in bronchoalveolar lavage fluid between asthmatic children and controls. [3, 4]. We found that 65 miRNAs levels of BALF cells were different between the controls and asthma children. This suggested that miRNAs were involved in pathogenesis of asthma. These differences of 65 miRNAs may be associated with the proportion and function changes of inflammatory cells [4, 15, 16], even airway epithelium or fibroblasts as well [17-19]. Differential miRNAs found in our study were a

little different from some previous reports [8, 11], which can be explained by different species (animal vs. human), samples (BALF cell vs. lung tissue, blood cell), and phases (acute vs. catabasis) or severity (sever vs. mild). Further researches on the cellular origin, specific mechanism of expression and actual function of these differentially expressed miRNAs are required.

Although 22 kinds of up-regulated miRNA and miR-143-3p in the asthmatic children were 3.71 folds higher than that in the controls, we noted that the overall trend about miRNAs change was decreased. Two-thirds (43/65) of miRNAs were down-regulated in the BALF cells from asthmatic children, three miRNAs (miR-34b-5p, miR-34b-3p and miR-34c-5p) were less than one-tenth of those in the controls; and the other seven miRNAs (miR-361-5p, miR-34c-3p, miR-141-3p, miR-1180-3p, miR-23b-5p and miR-100-5p) were less than one-fifth of those in the controls.

MiRNAs in one miRNA family usually have similar miRNA sequences, and may target similar genes and play similar role [20]. In this study, we found that six miRNAs levels of miR-34 family and seven of miR-200 family decreased. Moreover, four miRNAs of miR-34/449 family and six of miR-200 family were verified by qRT-PCR. E-cadherin is an important adhesion molecular in maintaining epithelial integrity and mediating immunological function of airway epithelium through the release of growth factors and proinflammatory factor. The loss and redistribution of E-cadherin may promote the inflammatory activity of epithelium and play an important role in the pathogenesis of asthma [21]. miR-200 family was reciprocally inhibited with zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2 [22-26] that are important signal proteins in TGF-β signaling pathway, and regulated cell cycle and remodeling thought the effect of E-cadherin [27]. Several studies revealed that miR-34/449 family and miR-200 family form complex feedback loops with TGF-β, including SNAI1/miR-34 double-negative feedback loop and ZEB/miR-200 double-negative feedback loop. SNAI1 and ZEB have negative consequences on the expression of *E-cadherin* [24, 28, 29]. Many studies have confirmed that miR-34/449 and miR-200 families can participate in the progression of diseases by regulating cell proliferation, differentiation and apoptosis in a variety of ways. In our study, we confirmed low E-cadherin in

the BLAF cells in asthma children. These suggested that miR-34/449 and miR-200 families were involved in pathogenesis of childhood asthma by regulating E-cadherin. According to our results and relevant literature, we speculated that the main relation signaling pathways were ERK-MAPK and TGF-β signaling pathways [30-38] (Figure 4). Further study about the accurate mechanism of these two miRNA families in asthma may provide new targets for childhood asthma treatment.

There were several limitations of our study. First, the controls were not actually normal children. They were patients under airway foreign aspiration. Although we selected patients whose foreign removal within 24 hour, the BALF cells maybe still a little different from that of normal children. Second, as the cells in BALF were limited, the samples used for miRNA array, qRT-PCR and ELISA were from different patients, but not one-for-one correspondence. Third, the sample is too small to find the difference among the miRNAs. Fourth, the relationship between miRNAs and target genes was not verified by gene knockout or transfection. Further study is required to investigate the association between these miRNA and the mechanism of asthma.

In conclusion, our study suggests that miRNAs were involved in the mechanism of childhood asthma, especially the miR-34/449 family and miR-200 family. miR-34/449 family and miR-200 family may regulate the pathogenesis of asthma by regulating expression *E-cadherin*. Further studies are required to increase the understanding of the regulation of miRNAs in asthmatic children.

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**Conflict of interest**

There are no competing interests.

## Data availability

All data generated or analyzed during this study are included in this published article.

## Author Contributions

TANG Lan-Fang and LIU Min conceived and designed the experiment. LIU Min and LV Jian-Hai wrote the paper and analyzed the data. WU Lei collected the data.

## Ethics approval and consent to participate

This study was approved by the ethical committee of Children Hospital of Zhejiang University School of Medicine(2015- HP-037), and informed consents were obtained from parents or guardians of all children, and confirmed that all experiments were performed in accordance with relevant guidelines and regulations**.**

## Consent for publication

Not applicable.

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**Table Legends**

Table 1. The characteristics of asthma and control groups for different measurement.

qRT-PCR 1, for miR-34/449 family; qRT-PCR 2, for miR-200a/b/c and E-cadherin measurement.

Table 2. The primers of qRT-PCR for miRNA and E-cadherin.

Table 3**.** 22 up-regulated miRNAs in the BALF cells from asthmatic children

miRNA, microRNA; miR, microRNA; FDR, false discovery rate.

Table 4. 43 down-regulated miRNAs in the BALF cell from asthmatic children

**Figure Legends**

Figure 1. The different expression of miRNA between asthma and control groups. Heat maps show differentiated miRNAs from BALF. Each sample was assayed in triplicate. Both down-regulated (green) and up-regulated (red) miRNAs were identified.

Figure 2. Differences of miR-34/449 and miR-200 families confirmed by qRT-PCR. (A) miR-34b-3p; (B) miR-34b-5p; (C) miR-34c-3p; (D) miR-34c-5p; (E) miR-449a; (F) miR-449b-5p; (G) miR-200a-3p; (H) miR-200a-5p; (I) miR-200b-3p; (J) miR-200b-5p; (K) miR-200c-3p; (L) miR-141-3p; (M) and miR-429. Levels were significantly down-regulated in the asthmatic children than those in the controls (*$P<0.05$).

Figure 3. The expression levels of E-cadherin by qPCR and ELISA. (A) E-cadherin expression was measured by qPCR. (B) Quantification of E-cadherin protein was assessed from ELISA. For relative expression of protein were expressed as the ratio of target protein to total protein. (*$P<0.05$).

Figure 4. The possible miRNA-mRNA network involved ERK-MAPK and TGF-β signaling pathways (Sirt1, Sirtuin type 1; KLF5, Krueppel-like factor 5; ZEB, zinc finger E-box-binding homeobox; CBP, CREB-binding protein; Wnt , wingless-type).