MicroRNAs in Asthma and COPD; from biomarkers to regulators of disease pathogenesis

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A novel role of bronchial microRNAs and long non-coding RNAs in asthma remission

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To the editor,

Complete asthma remission in adulthood occurs in a minority of patients, but knowledge about its fundamental mechanisms is lacking. We explored underlying mechanisms by investigating bronchial microRNA expression, because microRNAs are increasingly recognized as important regulators of lung development and growth, as well as disease pathogenesis, including T-helper cell type 2 (Th2)-driven airway inflammation [1]. We investigated differential microRNA expression among subjects with complete asthma remission, subjects with persistent asthma, and healthy control subjects. In addition, we integrated these findings with protein-coding RNA and long noncoding RNA (lncRNA) expression.

All subjects with persistent asthma or complete remission had a doctor’s diagnosis of asthma and documented airway hyperresponsiveness (AHR) to histamine in the past. Complete asthma remission was further defined as absence of wheeze or asthma attack in the last 3 years, no use of inhaled corticosteroids (ICS) or β-agonists, and absence of AHR and airway obstruction (FEV₁ > 80%). Persistent asthma was defined by doctor-diagnosed asthma and documented AHR in the past, and current respiratory symptoms and/or asthma medication use.

Subjects with asthma either stopped their ICS 6–8 weeks before inclusion or did not use ICS at all. They had no exacerbation within 2 months before the study. Healthy subjects had no respiratory symptoms and normal pulmonary function. Bronchoscopy was performed 3–6 weeks after inclusion. Total RNA was isolated from bronchial biopsies. During RNA extraction, library preparation, and sequencing, all samples were equally distributed across all batches to minimize technical variation. Library preparation was done for microRNAs, protein-coding RNAs, and lncRNAs separately, and RNA sequencing was performed. Genes with appreciable expression (for microRNAs: one fragment per million in at least one of the clinical groups; for long RNAs: five normalized counts in half of the samples) were used for analysis. The differential expression of microRNAs was analyzed with DESeq2 (v1.14.1) [2], adjusting for age, gender, smoking status, and library preparation batch, and correcting for multiple testing using the Benjamini-Hochberg method. Next, we integrated microRNA, protein-coding RNA, and lncRNA expression in association with complete remission by performing Bayesian network modeling using the CGBayesnets package in MATLAB (The MathWorks) [3]. We assessed the predictive performance of the network by calculating the area under the receiver operator characteristic curve (AUC) by means of permutation testing (10,000 iterations). Finally, we performed in vitro validation of a top ten microRNA by microRNA transfection of tracheobronchial and 16HBE cells.
The study included 14 subjects with complete remission (7 females (50%), 4 current smokers (29%), mean FEV$_1$ 103% predicted (standard deviation [SD] ±13), median age 48 years (interquartile range [IQR] 36-53) and median time since first asthma attack 45 years (IQR, 40–50). It also included 46 patients with persistent asthma (24 females (52%), 16 current smokers (35%), mean FEV$_1$ 84% predicted (SD ±16%), median age 52 years (IQR, 35–57), and median time since first asthma attack 41 years([IQR 23–49). Among the subjects with asthma, 28 (61%) did not use ICS at all, and 18 (39%) had stopped their ICS 6–8 weeks before inclusion in the study. The study included 82 healthy controls, 36 females (44%), 40 current smokers (49%), mean FEV$_1$ 101% predicted (SD ±12%) and median age, 42 years (IQR, 23–56). All of the subjects were white individuals.

Ten microRNAs were differentially expressed between subjects with complete remission and those with persistent asthma: 9 upregulated (miR-320a, miR-193a-5p, miR-320c, miR-4532, miR-320d, miR-320b, miR-423-3p, miR-133b, and miR-3960) and 1 downregulated (miR-126-3p) (Figure 1). Seventy-seven microRNAs were differentially expressed between subjects with complete remission and healthy controls (62 upregulated and 15 downregulated) (Figure 1). For subjects in complete remission and persistent asthma, we integrated microRNA, protein-coding RNA, and IncRNA expression. For this purpose, we used the expression levels of 518 microRNAs and 22,729 protein-coding RNAs, IncRNAs, and pseudogenes as input in a Bayesian network analysis. The gene network that associated with the binary phenotype complete remission (using participants with persistent asthma as control subjects) consisted of 24 microRNAs, 20 protein-coding RNAs, 35 IncRNAs, and 14 pseudogenes (Figure 2, area under the curve (AEC) 0.99, $p = 0.0027$). Of interest, 6 of the 24 microRNAs in the network were also identified in our differential expression analysis: miR-126a-3p, miR-320a, miR-320b, miR-320c, miR-193a-5p, and miR-133b. MicroRNAs and IncRNAs, but not protein-coding RNAs, were directly connected to complete remission. A permutation analysis (100 iterations in which we swapped the phenotype values) showed that our network consistently contained a lower proportion of protein-coding RNAs than what could be expected by chance ($p < 0.01$).
Next, we performed *in vitro* validation of miR-320d, one of the top-10 microRNAs that were upregulated in complete remission compared with persistent asthma. Of interest, in a previous report of our group, miR-320d was also found to have anti-inflammatory effects in primary bronchial epithelial cells [4]. In tracheobronchial cells and 16HBE cells stimulated with the viral mimic poly-(I:C), miR-320d transfection significantly decreased the production of GM-CSF (granulocyte-macrophage colony-stimulating factor) compared with mimic controls ($p < 0.05$).

We observed a clear signal that the expression levels of 10 microRNAs differentiated complete remission from persistent asthma. Moreover, expression changes in microRNAs and lncRNAs, which are abundantly present in a Bayesian network, were strongly associated with complete remission of asthma. This suggests that noncoding RNAs may play an important role in complete remission of asthma.

**Figure 1: Heatmap of 83 microRNAs (miRNAs) differentially expressed between complete remission and persistent asthma, and between complete remission and healthy.**
Figure 2: Bayesian network of microRNAs, protein-coding RNAs, long noncoding RNAs, and pseudogenes predicting complete remission. The predictive performance of the network was assessed by calculating the area under the receiver operator characteristic curve (AUC). We determined the statistical significance of the AUC by permutation testing (10,000 iterations) in which we compared the AUC of our network with the AUCs of the permuted networks. The AUC of the network was 0.99 (P=0.0027). Distinct gene types are shown in different colors. Black-lined nodes represent nodes that are directly connected to complete remission. Diamond-shaped nodes represent microRNAs that were also identified in the differential expression analysis.

One other group studied microRNA expression in remission of asthma by performing Bayesian network modeling [5]. McGeachie and colleagues identified a network based on serum-RNA profiles of children with asthma between the ages of 5 and 12 years that was associated with asthma remission at age 14. Although their study differed in many aspects from ours, the importance of microRNAs exhibiting their function by acting in a network is supported by both studies.

Ten microRNAs were differentially expressed between subjects in complete remission and those with persistent asthma. Also, we observed that subjects in complete remission had a distinct bronchial microRNA profile (77 differentially expressed microRNAs) compared with healthy controls. This suggests that subjects in complete remission do not resemble healthy
subjects but represent a third, separate molecular state in addition to asthma and healthy. Exploring this phenomenon further could aid in the development of new asthma therapies.

Among the differentially expressed microRNAs, miR-126-3p has been found to be an agonist of Th2 inflammation, an important mechanism underlying asthma [6]. We found that miR-126-3p was downregulated in complete remission compared with persistent asthma, suggesting that this microRNA might play a role in achieving asthma remission. Four members of the miR-320 family, namely, miR-320a, miR-320b, miR-320c, and miR-320d, were upregulated in complete remission compared with persistent asthma. It has been suggested that miR-320 has anti-inflammatory effects [4,7], and this was confirmed by our in vitro experiments showing decreased GM-CSF production upon poly-(I:C) stimulation in miR-320d transfected cells compared with mimic controls. This upregulation of miR-320 in complete remission may potentially act to suppress inflammatory processes in asthma.

A strength of this study is the careful characterization of the subjects at baseline and at follow-up with standardized questionnaires and extensive pulmonary function tests. A limitation is the lack of a replication cohort for our findings. Also, the presence of only white individuals in our study population limits generalizability to other groups.

In conclusion, we show that subjects in complete remission of asthma, subjects with persistent asthma, and healthy control subjects differed with regard to their bronchial microRNA expression profiles. Of interest, when we integrated microRNA, protein-coding RNA, and lncRNA expression by performing Bayesian network modeling, we identified a network characteristic of complete remission of asthma in which microRNAs and lncRNAs are abundantly present. Hence, future research should focus on the functional characterization of these noncoding RNAs in asthma remission.
References


