SCIENTIFIC REPORTS

OPEN

Let-7a is differentially expressed in bronchial biopsies of patients with severe asthma

SUBJECT AREAS:
RESPIRATORY TRACT DISEASES
TRANSLATIONAL RESEARCH

Received
11 June 2014

Accepted
28 July 2014

Published
18 August 2014

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Asthma is a chronic inflammatory disease. Around 5 to 10% of patients classified as having severe asthma can-not be adequately controlled despite the use of all currently available therapeutic approaches. Previous studies have revealed the potential important role of miRNAs in the regulation of a variety of inflammatory processes, including asthma. Expression of selected miRNAs, specifically let-7a, miR-21 and miR-223, that were shown to have important roles in asthma pathogenesis, were analyzed in bronchial biopsies of 24 patients with asthma, 12 mild and 12 severe, and 10 controls with no chronic disease. We found significantly reduced expression of let-7a in bronchial biopsies from patients with severe asthma in comparison to patients with mild asthma as well as in comparison to the non-asthmatic controls. On the other hand, no significant differences in miR-21 and miR-223 expression were found between the different groups analyzed. Reduced let-7a levels in bronchial biopsies of patients with severe therapy-resistant asthma could not only be used as a potential biomarker to discriminate between different asthma phenotypes, but also might be a target for modulation of treatment at the inflammatory site for a group of patients that are most affected and still lack effective treatment.

Asthma is a chronic inflammatory disease with exaggerated bronchoconstriction after provocation with specific or non-specific stimuli. It is one of the most common chronic diseases, affecting more than 300 million people of all ages worldwide. The incidence continues to increase, and thus represents a serious challenge for public health system[1,2]. Therapy currently primarily consists of inhaled corticosteroids, long- and short-acting beta-agonists, and leukotriene antagonists, which are not directed at the underlying disease, but rather to symptom relief[1–4]. A majority of patients achieve good symptom control and minimal exacerbation using regular controller therapy; however, up to 10% of patients with severe asthma can-not be adequately controlled despite maximal therapy[1,2,5]. The variability in treatment response is based on large asthma clinical heterogeneity, and understanding the pattern of airway inflammation and molecular factors regulating those processes could be helpful in defining asthma endotypes and understanding why therapy is not effective in all patients[6].

MicroRNAs (miRNAs), 19–25 nucleotides long non-coding small RNAs involved in regulation of gene expression through a process known as RNA interference[7–9], may play a role in orchestrating the phenotypic programming of immune and airway epithelial cells to enhance the production of cytokines and other mediators that result in the inflammation that characterizes asthma[3,4,10–25]. Therefore, miRNAs may contribute to asthma pathology. Distinct miRNA expression was found in samples from subjects with asthma, such as epithelial brushings or circulating T cells[12,26], and experiments have also shown altered expression in sensitized and challenged mouse asthma models[27]. Numerous miRNAs have been found to be dysregulated in asthma; however, the replication level is relatively low and data on miRNA expression in human lung tissue are still scarce[3,4,10–25]. MiRNAs have been found to play critical roles in regulating key pathogenic mechanisms in inflammation, including polarization of adaptive immune responses and activation of T cells (e.g., miR-21), regulation of eosinophil development (e.g., miR-21 and miR-223)[4], and regulation of IL-13, a key cytokine in allergic lung inflammation (e.g., let-7a)[11].

Many miRNAs identified as either tumor suppressors or oncogenes in lung cancer were also reported to be involved in the immune response; for example, the let-7 family, the most abundant miRNAs in the lungs[28]. The let-7 family is involved in pro-inflammatory cytokine production[29] because it inhibits IL-13 expression and represents a major regulatory mechanism for modulating IL-13 secretion and thereby T_H2 inflammation, characteristic for T_H2 high asthma[30]. There was reduced expression of let-7a in samples from subjects with asthma[12,15]

and overexpression of let-7a reduced airway inflammation and hyper-responsiveness in the lungs of asthma mouse models[10,11].

Among the top miRNAs expressed in inflamed tissue is miR-21[4]. MiR-21 is thought to play an important role in immunity, in maintaining the effector phase of T cells[31], and in regulation of T_H2 immune responses through IL-12 targeting[32]. In asthma, miR-21 was found to be up-regulated[32] and its expression was associated with eosinophilic T_H2 high asthma[33].

MiR-223 was found to be one of the candidates involved in asthma pathogenesis when asthmatic mouse lung tissue miRNA expression was profiled using a microarray[27]. This miRNA is involved in granulocytopoiesis and granulocyte activation, and is thought to be a regulator for preventing hyper-inflammatory states[34]. It was found to be down-regulated in human asthmatic T cells[26], and in mice elevated pro-inflammatory cytokines IL-6 and IL-1β were found as a result of miR-223 down-regulation upon induction with LPS[35]. In humans, IL-6 levels are known to be elevated in asthma and also correlate with lung function and IL-13 levels[36].

MiRNAs let-7a, miR-21, and miR-223 were shown to either directly or indirectly repress the translation of several crucial factors with well-defined roles in asthma pathogenesis, such as signal transducer and activator of transcription 3 (STAT3), interleukins (IL-6, IL-1β, IL-13), interferon gamma (IFN-γ), transforming growth factor, beta receptor (TGF-β receptor), toll-like receptor 4 (TLR4), and vascular endothelial growth factor (VEGF)[3,4,10–25]. Dysregulated miRNAs could have a role in the pathogenesis of asthma and could be used as potential biomarkers to discriminate between different asthma phenotypes. Furthermore, miRNAs are promising targets for a novel and effective therapeutic strategy for modulation of inflammatory response for a group of patients that still lack effective treatment.

Let-7a, miR-21, and miR-223 seem to be involved in modulation of inflammation in the lungs, and therefore we hypothesized that they are differentially expressed in bronchial biopsies of patients with severe asthma.

Results

Patient characteristics. The characteristics of the study groups from which the lung biopsies were derived are shown in Table 1. Patients with severe asthma had significantly reduced FEV_1% predicted and Tiffeneau index (TI) in comparison to patients with mild asthma and non-asthmatic controls ($P < .01$ and $P < .001$, respectively). Vital capacity was lower in the severe asthma group than in the control group ($P < .05$). In addition, patients with severe asthma experienced more asthma exacerbations per year ($P < .001$) and also required oral corticosteroids more often ($P = .003$, OR 33.0, 95%CI 2.9−374.5) in comparison to those with mild asthma. Other clinical characteristics did not differ between the study groups.

miRNA expression. Expression of selected miRNAs was determined in bronchial biopsy tissue samples and normalized to a sample from the control group. When we stratified asthma patients according to severity, we found significantly reduced expression of let-7a in bronchial biopsies from patients with severe asthma in comparison to bronchial tissues from patients with mild asthma as well as to bronchial tissues from the control group (Figure 1). When analyzing the entire group of asthma patients (24), we did not observe any difference in expression of let-7a between patients and controls. Moreover, no significant differences in miR-21 and miR-223 expression were found between the different groups analyzed (Figure 2).

Discussion

Asthma is a complex disorder of the immune system characterized by chronic inflammation, airflow obstruction, and bronchial hyper-responsiveness. Tightly balanced pathways, networks of activators, and suppressors are needed for proper regulation of immune responses. MiRNAs have been shown to be important regulators of variety of immunologically driven processes[3,4,21–23] and are emerging as important biomarkers in the pathogenesis of asthma[3,4,10–25]. However, the data on miRNA expression in the lungs of human subjects with asthma are still scarce. Several miRNAs, such as let-7a, miR-21, and miR-223, have been shown to have a role in airway inflammation, T_H1/T_H2 polarization, and eosinophil development either directly or indirectly repress the translation of several crucial factors, and were shown to be differentially expressed in asthma[3,4,10–25].

In the present study we analyzed the expression of three miRNAs (let-7a, miR-21, and miR-223) in bronchial biopsy samples from patients with mild and severe asthma and non-asthmatic controls. The expression of let-7a was significantly reduced in severe asthma

Table 1 | Characteristics of the study groups

Study group	Control group	Mild asthma	Severe asthma
	$n = 10$	$n = 12$	$n = 12$
Male/female	4/6	5/7	6/6
Age (years), median (IQR)	53.5 (29.5)	44.0 (24.0)	54.5 (17.8)
BMI (kg/m^2), median (IQR)	26 (6.5)	28 (8.6)	30 (6.2)
Vital capacity (%),median (IQR)	104 (6.2)	106 (31.8)	86 (27.8)^{a,*}
FEV_1% predicted, median (IQR)	104 (23.1)	95 (27.6)	67 (24.0)^{b,**}
TI (%), median (IQR)	80 (11.5)	73 (5.5)	52 (18.5)^{b,***}
TLCO (%), median (IQR)	88 (23.0)	91 (22.5)	78 (37.0)
Histology of eosinophil bronchitis	0	4	4
Atopy	3	4	6
Smoking status; never, current, ex	4, 4, 2	10, 2, 0	5, 0, 7
No. of M-SAE/year, median (IQR)	/	0 (0.0)	1 (2.5)^{b,***}
No. of MAE/year, median (IQR)	/	1 (0.8)	3 (3.5)^{b,**}
Asthma therapy			
LABA/ICS, ICS alone, ALT	/	9, 3, 3	10, 2, 6
OCS continuously, ≥3×, 1−2× per year	/	0, 0, 1	2, 4, 3^{a,**}

[a]Statistically different between patients with severe and mild asthma.
[b]Statistically different between patients with severe asthma and both those with mild asthma and non-asthmatic controls.
$^*P < .05$, $^{**}P < .01$, $^{***}P < .001$
BMI = body mass index; TI = Tiffeneau index; TLCO = transfer factor of the lung for carbon monoxide; M-SAE = moderate-severe asthma exacerbations; MAE = mild asthma exacerbations; LABA = long-acting beta-agonist; ICS = inhaled corticosteroid; ALT = antileukotriene; OCS = oral corticosteroid.

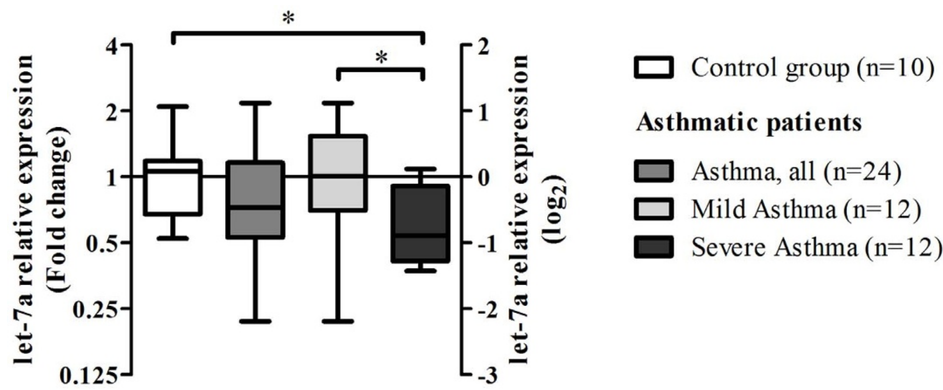

Figure 1 | Analysis of let-7a expression in bronchial biopsies from control individuals and patients with varying asthma severity. Data are presented as medians with ranges and interquartile ranges. * $P < .05$.

compared to mild asthma and controls, whereas the expressions of miR-21 and miR-223 were not altered.

Altered expression of let-7a has been found in many diseases; for example, in various cancers, Alzheimer's disease, and immune system diseases, such as asthma, allergies, allergic rhinitis, and atopic dermatitis[37]. Let-7a is highly expressed in human lungs[19] and it is hypothesized to have an anti-inflammatory role[38]. In mouse asthma models with overexpressed let-7a, reduction of airway inflammation and hyper-responsiveness was observed[11] and it has numerous targets related to asthma pathogenesis, such as IL-13, ADRB2, TLR4, and TGF-ß receptor[15,38,39]. It is hypothesized that the main contribution of let-7a in asthma is targeting of IL-13 and IL-6, and thus modulating T_H2 responses in lung inflammatory processes, which was shown on mouse models and cell cultures[3,10,11,16]. Increased T_H2 response and airway inflammation in asthma is often associated with severe therapy-resistant asthma, characterized by frequent and severe exacerbations, poor asthma control, and severe airflow obstruction[30,40]. Moreover, a correlation was found between IL-6 and lung function[5,36], which further supports the potential role of down-regulated let-7a in severe asthma, for which poor lung function is typical[1,5,6].

Regarding the let-7a expression in asthma, inconsistent results have been found because a few studies have indicated no difference between healthy subjects and asthma patients[19,25]. Williams et al. found no differences in miRNA expression in patients with mild asthma in comparison to healthy controls[19], whereas a study by Jardim et al. found differentially expressed miRNAs in patients with mild asthma, but let-7a expression did not differ between patients with mild asthma and controls[25]. On the other hand, other studies have demonstrated reduced let-7a expression in asthma patients. Pinkerton et al. examined miRNA expression in exhaled breath condensate in asthma patients, and found let-7a levels to be lower than in COPD and in healthy controls[14]. A microarray analysis performed on bronchoalveolar lavage fluid exosomes of asthma patients revealed 8 qPCR confirmed altered miRNAs, including down-regulated let-7a and miR-21. Pathway analysis of differentially expressed miRNAs belonged to JAK-STAT signaling, cytokine network, and cytokine and inflammatory response[13]. Let-7a was also found to be reduced in the airway epithelial cells[12] and serum[15] of asthma patients. Thus, reduced expression of let-7a in bronchial biopsies from patients with severe asthma is consistent with the findings of other studies[3,10–15,19,25].

Similar to let-7a, inconsistent results on miR-21 and miR-223 expression in asthma have also been reported[12,15,19,26,27]. MiR-21 was significantly up-regulated in asthma regardless of treatment[33] and its expression was also altered in various asthma mouse models[32]; however, as in ours, no changes were found in other studies[19]. MiR-223 was previously shown to be down-regulated in asthma[26]; however, we found no alternation in its expression. MiR-21 and miR-223 are thought to play a role in eosinophil development[4] and they were both up-regulated in eosinophilic esophagitis patients[41]. Various complex immunological processes that simultaneously occur in the lungs of asthma patients could be the reason for inconsistent results between studies[3]. Some miRNA expressions are constitutively altered in disease, whereas others need an induction in order to be expressed differentially[42].

In addition to the miRNAs analyzed in our study, several others were shown to be involved in asthma. For example, miR-146a is involved in TLR and cytokine signaling[4,23], and miR-29, miR-126, and miR-155 play important roles in airway remodeling[4,20]. In animal models, modulation of several miRNAs, specifically let-7a, miR-21, miR-145, miR-126, and miR-106a, resulted in decreased asthma

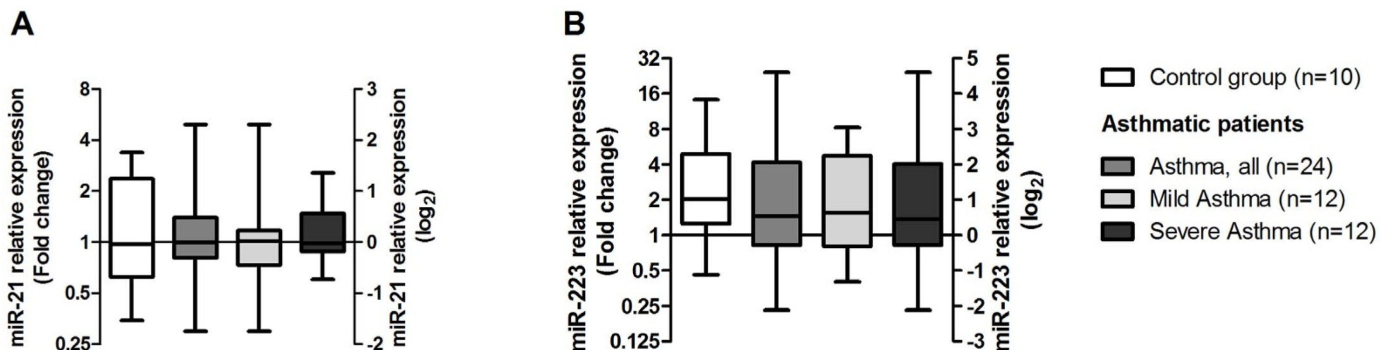

Figure 2 | Analysis of (A) miR-21 and (B) miR-223 expressions in bronchial biopsies from control individuals and patients with varying asthma severity. Data are presented as medians with ranges and interquartile ranges.

severity and inflammation[3,4,13,14,19]. Therefore, miRNAs were not only shown to affect several mechanisms in asthma, but also show potential utility as novel targets for therapy[3,4,10,21–23].

Depending on asthma severity, different doses of therapy are required and this raises a question about therapy influence on miRNA expression in our study. Reduced let-7a and non-altered miR-21 and miR-223 could be due to therapy; however, Williams et al. demonstrated that different treatment regimens and different doses of inhaled corticosteroids do not affect miRNA expression[19]. In addition to the relatively small sample size, one concern regarding our study design might be related to the appropriate choice of controls. Our study investigated miRNA expression in bronchial biopsies, and as controls we included 10 patients in whom bronchoscopy was indicated as part of the diagnostic procedure. In all controls, chronic lung disease was excluded by various routine tests. Six were diagnosed as GERD and four of them as hemoptysis with normal radiologic, endoscopic, and lung function findings. Even though the controls used in our study were not healthy, and both conditions might be potentially associated with lung inflammation, we believe these controls represent a valid control group for our experiments. MiRNA expression did not vary significantly between different controls, specifically between patients with GERD and hemoptysis. Furthermore, Smith et al. analyzed miRNA expression in GERD patients and found that none of the miRNA analyzed in our study were differentially expressed[43]. Even though the results of our preliminary study are promising, additional prospective studies and larger sample sizes are needed to improve our knowledge of asthma pathogenesis and to elucidate the mechanisms that lead to the development of this inflammatory disease. This will eventually lead to new insights for the development of targeted therapies for more specific and effective treatment.

In summary, our results show that let-7a is down-regulated in patients with severe asthma. These results could contribute to understanding the severe asthma mechanism and let-7a could potentially be used as a biomarker to discriminate between different asthma phenotypes. Most importantly, let-7a might be a promising target for novel therapeutic approaches for a group of patients that can-not be adequately controlled despite the use of all currently available therapeutic approaches[1–6], especially because it was shown that inhibiting let-7a could tone down asthma symptoms[10]. Many studies already performed in this field, as well as a number of preclinical and clinical studies, predict exponential growth of RNA-interference–based methods as possible new target therapies to regulate gene expression. Nevertheless, RNA-interference–based gene silencing approaches have proven to be successful in treating several disorders, including retinal degeneration, dominantly inherited brain and skin diseases, cancer, and metabolic disorders[44].

Methods

Patients. Twenty-four patients treated at the University Clinic for Respiratory and Allergic Diseases Golnik from 2010 to 2012 with a diagnosis of asthma according to Global Initiative for Asthma (GINA) guidelines[1] were included. We divided them into two subgroups according to the asthma severity (GINA guidelines). Twelve patients were classified as having mild asthma with controlled asthma on low to moderate doses of inhaled corticosteroids (ICS). The other 12 patients had severe asthma, used high doses of ICS, and had frequent exacerbations that required systemic corticosteroids. They were in a stable phase of the disease, with no evidence of exacerbation in the past 4 weeks. As controls we used 10 patients with no known chronic disease; in six of them, bronchoscopy was indicated because of prolonged cough that was finally attributed as a consequence of gastro-esophageal reflux disease (GERD), and four of them had hemoptysis with normal radiologic, endoscopic, and lung function findings. The characteristics of the study groups are shown in Table 1.

Biopsy Procedures. Bronchial biopsies were taken during diagnostic procedures with a flexible bronchoscope and were immediately formalin-fixed and then paraffin-embedded using standard procedures. All patients were in a stable phase of the disease, with no evidence of exacerbation in the past 4 weeks. This study was conducted in accordance with the amended Declaration of Helsinki. All subjects gave written informed consent and the study was approved by the Slovenian National Medical Ethics Committee (approval number 95/06/13).

Selection of specific miRNAs involved in asthma pathogenesis. To select miRNAs involved in asthma pathogenesis, available publications[3,4,10–25] and several target prediction databases were searched, including miRanda (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl), TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), and miRbase (http://microrna.sanger.ac.uk/). The selection criteria were: miRNAs that were previously shown to be expressed in the lungs, and miRNAs associated with asthma and/or asthma-related genes/proteins that were reported in at least two published studies and/or target prediction databases. Based on these findings, three miRNAs involved in the pathway strongly associated with asthma, specifically let-7a, miR-21 and miR-223, were selected. Selected miRNAs were shown to have roles in asthma pathogenesis, either directly or indirectly repressing the translation of crucial factors such as STAT3, IL-6, IL-1β, IL-13, IFN-γ, TGF-β receptor, TLR4, and VEGF.

RNA extraction, reverse transcription, and real-time PCR. Total RNA was extracted from 10 FFPE tissue sections 5 μm thick using the miRNeasy FFPE Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Quantitative PCR was used to analyze the expression of selected miRNAs as previously described[45]. Isolated RNA was reverse transcribed using miRNA-specific RT primers (RNU6B, hsa-let-7a, hsa-miR-21, and hsa-miR-223) and a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). RT products were used in a real-time PCR reaction with the TaqMan miRNA assays (Applied Biosystems) and TaqMan Fast Advanced Master Mix (Applied Biosystems) according to the manufacturer's instructions. All samples were run in triplicate. The RNU6B was used as an endogenous control for normalization of the target miRNAs, and a sample from the control group was used as a calibrator. Relative expression was calculated using the ΔΔCt method. The fold change was determined by $2^{-\Delta\Delta Ct}$, where $\Delta Ct =$ (average of triplicate $Ct_{\text{Target miRNA}}$ − average triplicate Ct_{RNU6B}), and $\Delta\Delta Ct = (\Delta Ct_{\text{Sample}} - \Delta Ct_{\text{Sample from control group}})$. Real-time PCR was performed on an ABI PRISM 7500 Real-Time PCR System (Sequence Detection System instrument equipped with SDS version v2.0.5 software; Applied Biosystems).

Statistical Analyses. The distribution of data was determined using the D'Agostino and Pearson omnibus normality test. The strength of association between miRNA expression levels and other clinical variables was analyzed with the Mann–Whitney U-test, unpaired *t*-test, or Fisher's exact test, as appropriate. Statistical analyses were performed using GraphPad Prism 5 software (San Diego, California, USA), and probability values of $P < .05$ were accepted as significant.

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Acknowledgments

The authors are grateful to all the patients that participated in the study. This work was supported by the Slovenian Research Agency (grant no. P3-0360).

Author contributions

M.R., P.K., M.Ž. and M.M.M. contributed to designing the study, M.M.M. contributed to recruiting subjects, performing the bronchial biopsies, and collecting relevant data, M.R. and M.Ž. performed the expression experiments, M.R., M.M.M. and P.K. carried out the data analysis and data interpretation, I.K. contributed to performing the immunohistochemical staining and collecting relevant clinical data, and M.R., M.Ž. and M.M.M. wrote the main manuscript. All authors reviewed and approved the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Rijavec, M., Korošec, P., Žavbi, M., Kern, I. & Marc Malovrh, M. Let-7a is differentially expressed in bronchial biopsies of patients with severe asthma. *Sci. Rep.* **4**, 6103; DOI:10.1038/srep06103 (2014).

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