



MicroRNA-21 as a novel biomarker in diagnosis and response to therapy in asthmatic children



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ABSTRACT

Background: The underlying molecular mechanisms leading to asthma remain largely unclear. MicroRNAs (miRNAs) are short noncoding RNAs exert powerful effects on immunological function by tuning networks of target genes that orchestrate cell activity. However, the role of miRNAs, specifically microRNA-21 (miRNA-21), in the regulation of allergic airway inflammation is not well defined. Our aim was to investigate the serum miRNA-21 expression levels as potential biomarker in childhood asthma [with, without inhaled corticosteroid (ICS) therapy, and steroid resistant (SR)]; and their possible contributions in disease status, its molecular target interleukin-12 (IL-12) p35, and response to therapy.

Materials and methods: This study included 175 children; 95 were asthmatic patients subdivided into 3 groups [40 asthmatic children without ICS, 40 steroid sensitive (SS) asthmatic children and 15 steroid resistant (SR) asthmatic children] and 80 were healthy children as healthy controls. The miRNA-21 expressions levels were determined by quantitative real-time polymerase chain reaction (qRT-PCR) in all children. Serum IL-12p35 and total IgE levels were measured using enzyme-linked immunosorbent assay (ELISA). **Results:** The expression levels of miRNA-21 were significantly higher in the asthmatic children than in control group ($P < 0.001$); with significantly higher levels in asthmatic patients without ICS or in SR patients compared to SS children ($P < 0.001$). On contrast, serum IL-12p35 levels were significantly decreased in asthmatic patients without ICS therapy or in SR asthmatic patients as compared to SS patients ($P < 0.001$). Our data revealed that serum miRNA-21 expression levels was significant negatively correlated with serum IL-12p35 levels and FEV1, while it was positively correlated with both sputum and blood eosinophils. Importantly, serum miRNA-21 had a predictive value in differentiating SS from SR patients, with an AUC value of 0.99, specificity of 86.7%, sensitivity of 97.5% and $P < 0.001$.

Conclusion: This study suggested that serum miRNA-21 is stable and detectable in serum of asthmatic children, which could promise potential biomarker in diagnosis as well as in response to therapy of asthma.

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1. Introduction

Asthma is a chronic inflammatory disease of the lungs, characterized by airway inflammation, hyper-reactivity, mucus hyper-secretion, and airflow obstruction, resulting in a broad spectrum of problems, ranging from mild respiratory symptoms to severe respiratory distress (Tarlo and Malo, 2006). The incidence of asthma continues to increase in all age groups, and it is the most common chronic disease in children (Moorman et al., 2011).

Despite great progress in treatment, there is no way to prevent the initial onset of asthma and there is no cure for this disease (MacDonald et al., 2007). The difficulty in developing a more effective therapeutic strategy may reflect the fact that the fundamental causes of asthma are not completely understood (Seguin and Ferrari, 2009).

The discovery of microRNAs (miRNAs) may bring fundamental changes in the understanding and therapeutic strategies in complex human diseases such as asthma (Wang et al., 2011). miRNAs are a class of small, noncoding, single-stranded RNAs that regulate gene expression by binding to their target mRNA and triggering either repression of protein translation or RNA degradation. miRNAs regulate development, differentiation, stem-cell differen-

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tiation, growth control, apoptosis and immune functions (Wang et al., 2011; Ambros, 2004). miRNAs may play an important role in asthma as it is characterized by marked changes in gene and protein expression in the lung (Konigshoff et al., 2008; Kuperman et al., 2005). Notably, miRNA-21 has been found to be up-regulated in many pathological conditions including cancer and cardiovascular diseases (Jazbutyte and Thum, 2010), and are involved in the pathogenesis of asthma (Rodriguez et al., 2007; Chiba and Misawa, 2010). Interestingly, miRNAs can represent a new class of drug target and their therapeutic modulation is possible by the use of over-expression or antisense inhibition approaches (Garbacki et al., 2011a).

Circulating nucleic acids are extracellular and are found in serum and other body fluids of healthy subjects and patients (Fleischhacker and Schmidt, 2007). The ability to detect and quantify specific DNA and RNA sequences has opened up the possibility of diagnosis and monitoring of diseases (Mitchell et al., 2008). The miRNAs in serum and other body fluids are stable under harsh conditions including boiling, low/high pH, extended storage and multiple freeze-thaw cycles (Chen et al., 2008; Perry et al., 2015).

Although relatively few studies have been carried out to date, there is evidence that miRNAs are implicated in the asthmatic disease process (Perry et al., 2015). For example, in a study by Lu et al. (2009), investigated expressions profiles of 579 miRNAs in transgenic mice; they revealed that miRNAs were differentially expressed upon induction of experimental asthma following treatment with doxycycline. Additionally they suggested that miRNA-21 was the most up-regulated miRNA (Lu et al., 2009). Several studies have attempted to identify the role of miRNA in human asthmatic subjects. An initial study by Williams et al. (2009), found no significant difference in the expressions of 227 miRNAs in the airway biopsy specimens obtained from mild asthmatic patients with compared with healthy subjects. A subsequent study by Liu et al. (2012), revealed up-regulation of miRNA-221 and miRNA-485-3p in the peripheral blood of 6 asthmatic children compared with control subjects. In a separate study, they subsequently confirmed the up-regulation of miRNA-221 in the peripheral blood of an asthmatic patients compared with 4 additional healthy controls (Qin et al., 2012). Recently, Perry et al. (2015), suggested that circulating miRNAs may be useful potential biomarkers of asthma status or response to therapy. However; the potential role of miRNAs as biomarkers in asthma therapy remains to be determined.

Interleukin-12 (IL-12) is a heterodimeric cytokine produced primarily by activated inflammatory cells (monocytes, macrophages, neutrophils, microglia and dendritic cells), and formed of a 35-kDa light chain (known as p35) and a 40-kDa heavy chain (known as p40) to form the biologically active IL-12 (also called p70) (Gately et al., 1998). Several evidences suggest that bioactive IL-12p35 is critical for the synthesis of bioactive IL-12p70 (Trinchieri, 2003; Chen et al., 2014). IL-12 represents an essential link between innate and adaptive immunity. It is a pro-inflammatory cytokine that induces the production of Interferon gamma (IFN- γ) and favors the differentiation of T helper type 1 (Th1) cells. IL-12 has been shown to markedly suppress IL-4-induced IgE production by human mononuclear cells (PBMCs) in vitro (Qi et al., 2011). Notably, Lu et al. (2009), confirmed that IL-12p35 is a putative molecular target of miRNA-21. Interestingly IL-12, through its capability of inducing Th1 cells, might be capable of down-regulating pathological Th2 responses (Chen et al., 2014). This suggests that miR-21 may potentially regulate a number of processes involved in allergic airway inflammation (Gately et al., 1998).

To the best of our knowledge, there are no previous studies investigating the role of serum miRNA-21 expression level as potential biomarkers of childhood asthma and response to therapy. In light of this deficiency, the aim of the present study was to evaluate the potential role of miRNA-21 expression profiles as novel

molecular biomarkers of childhood asthma status, and response to therapy. We further explored the relationships of miRNA-21 quantitative expressions with serum IL-12p35 levels, FEV1, blood and sputum eosinophils (EOS) counts and percent; to elucidate their possible contributions in disease status and response to therapy. Knowledge of patients' expression profiles is expected to provide a clear understanding of how aberrant expression of miRNA-21 can aid in the development of more effective and safer therapeutic strategies for childhood asthma.

2. Subjects and methods

2.1. Subjects

This study consisted of 175 children who were unrelated and lived in Sharkia governorate. Ninety five children were diagnosed as having allergic asthma (39 boys and 56 girls) admitted to pulmonology and allergy unit, Zagazig University Children's Hospital, Egypt. In addition, 80 healthy children (36 boys and 44 girls), who were randomly selected from the same geographical regions, were matched to cases by gender, age, and exposure to tobacco smoke. They control subjects had negative skin prick tests, normal total IgE values, and normal lung function tests. None of control subjects had history of chronic illness, asthma or other allergic diseases, or respiratory tract diseases.

Asthma was diagnosed on the basis of the recommendations of the Global Initiative for Asthma (GINA) (GINA, 2014). Childhood asthma patients are defined as having asthma if they have visited the hospital within the past 12 months due to wheezing without evidence of common cold, and if their forced expiratory volume in 1 s (FEV1) after inhalation of a β 2 agonist increased by 12% compared to prior to the inhalation. They all underwent skin prick testing and the results were positive. The asthmatic children were included: 40 inhaled corticosteroid (ICS) sensitive (SS) asthmatic patients presented with less evident asthmatic symptoms after ICS therapy. Fifteen steroid resistant (SR) asthmatics defined as less than 15% improvement in baseline FEV1 after 14 days course of high-dose oral steroids. The remaining 40 cases were not received ICS therapy.

None of the participants had history suggesting upper and lower respiratory tract diseases within 2 months, chronic heart or lung, diabetes mellitus, tumors, and immunodeficiency diseases. Moreover, none of the participants received antihistamine, systemic corticosteroids, theophylline, long-acting β 2-agonists, or leukotriene receptor antagonists' antihistamines within 4 weeks prior to clinical evaluation.

The current study was approved by the Medical Ethics Committee of Faculty of Medicine, Zagazig University. Written informed consent was obtained from all children parents.

2.2. Pulmonary function tests assay

FEV1 and forced vital capacity (FVC) were measured using a portable spirometer (Masterlab Jaeger, Würzburg, Germany). Short-acting bronchodilators were stopped at least 8 h before the test. All pulmonary function data were collected at a single visit. A minimum of 3 results within 10% of each other were recorded, and the result with the highest FEV1 was analyzed, according to the standards of the European Respiratory Society (European Respiratory Society, 1993). The participants were not suffering from asthma exacerbations or other acute illnesses at the time of the measurement of pulmonary function. The lung function test results were expressed as a percentage of that predicted.

2.3. Sputum induction and processing

After baseline FEV₁ measurements, subjects were pre-treated with inhaled salbutamol (200 µg) to minimize bronchoconstriction during the induction procedure. 10 min later nebulization was done with hypertonic (3%) sterile saline solution for three periods of 5 min at most by nebulizer (DeVilbiss 65 ultrasonic nebulizer; DeVilbiss, Somerset, PA, USA). The subjects were instructed to cough sputum into containers. If any symptom occurred or fall in FEV₁ of >20% compared to baseline values; the nebulization was discontinued. The volume of the selected sputum was measured and 0.1% dithiothreitol (Sigma Chemicals, Poole, United Kingdom) added to the sputum in a 4:1 ratio to break up the disulphide bonds and disperse the cells. The cell suspension was aspirated until homogenized and filtered to remove any remaining debris. Phosphate-buffered saline (PBS) was then added to the cell suspension (Brightling, 2006; Pignatti et al., 2002). Staining of cell was done by trypan blue; the cell count and viability were determined. The cell suspension is centrifuged at 400 × g for 10 min and cytopspins made and stained by May-Gruinwald Giemsa stain (Bio Optica Milano s.p.a). Four hundred non-squamous cells are counted: an adequate sample is defined as less than 50% squamous cells. The eosinophils count is then expressed as a percentage of the total cell count as it is more accurate than absolute count (Fantin et al., 2001).

2.4. Blood sample collection

A 5 mL blood sample was taken under aseptic conditions and divided into 2 portions: 2 mL of whole blood was collected in sterile EDTA-containing tubes for RNA extraction and eosinophil counts, and the remainder was left for 30 to 60 min for spontaneous clotting at room temperature before being centrifuged at 1000 × g for 10 min. Serum samples were kept frozen at −20 °C for determination of IL12p35 and total IgE levels.

2.5. Measurement of total immunoglobulin E (IgE) levels

Total IgE level was measured by enzyme-linked immunosorbent assay (ELISA) using AccuBind IgE Quantitative Kits, (Lake Forest, California, USA).

2.6. Measurement of IL-12p35 serum levels

Serum levels of IL-12p35 were determined using the human ELISA kits of MyBioSource, Inc. (Minneapolis, MN, USA) according to instructions given by the manufacturer. The minimum detectable dose of IL-12p35 with these assay kits was 1 pg/ml.

2.7. Extraction of serum miRNA-21:

Total RNA containing miRNAs was extracted from serum using a commercial miRNeasy extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The miRNeasy Mini Kit combines phenol-/guanidine-based lysis of samples with silica-membrane-based purification of total miRNAs. Briefly, 500 µl QIAzol lysis reagent was added to 100 µl serum samples and the mixture was incubated for 5 min at room temperature. Then, chloroform (100 µl) was added and shook tube vigorously by hands for 15 s and incubated at room temperature for 5 mins. After centrifugation at 12,000 × g for 15 min at 4 °C, the upper aqueous phase of supernatant was transferred to a fresh tube and 1.5 times its volume of ethanol (100%) was added. 700 µl of this mixture was transferred into an RNeasy mini column and centrifuged at 8000 × g for 1 min at room temperature. After the mixture had completely passed the column, this step was repeated until all of the mixture had completely passed the column to the mini column. Next, the column

was washed two times with 500 µl RNA wash buffer (RWT buffer) centrifuged for 15 s at 8,000 × g at room temperature. Then, 500 µl buffer RPE was added to the column and centrifuged at 8000 × g at room temperature for 15 s. Another 500 µl buffer RPE was added to the column and centrifuged at 8000 × g at room temperature for 2 min. The column was transferred to a new 1.5 mL collection tube and 50 µl RNase-free water was pipetted directly onto the column and centrifuged for 1 min at 8000 × g to elute RNA. Finally, the quantity and purity of serum RNA was confirmed by the value of optical density (OD) at 260 and 280 nm using a spectrometer, with acceptable RNA purity ranging from 1.8 to 2.1.

2.8. Reverse transcription and quantification of serum miRNA-21

The miScript II reverse transcription kit (Qiagen, Valencia, CA, USA) was used for polyadenylation and reverse transcription of miRNA to complementary DNA (cDNA). After mixing with template RNA (100 ng), 5X miScript buffer, miScript reverse transcriptase mix and RNase-free water in a final volume of 20 µl, the mixture was centrifuged briefly and incubated for 60 mins at 37 °C. To inactivate the reverse transcriptase mix, the samples were incubated for 5 mins at 95 °C and then placed on ice for further PCR analysis. The diluted cDNA templates were either stored at −20 °C or used to precede with further to quantitative real-time polymerase chain reaction (qRT-PCR).

2.9. Expression of serum miRNA-21

Subsequently, the resultant cDNA was subjected to qRT-PCR to evaluate expression of serum of miRNA-21 using mi-Script SYBR® Green PCR Kit with miScript Primer assays (Qiagen, Valencia, CA, USA). This kit includes QuantiTect SYBR Green PCR Master Mix and the miScript Universal Primer (reverse primer that use to detect miRNAs) in combination with a miScript Primer Assay (Cat. no. MS00009079) that specifically recognizes the targeted miRNA-21. The housekeeping miScript PCR control, miRNA SNORD68 was selected as internal control. SNORD68 was reported to be expressed stably in different tissues (Mu et al., 2013) and of comparable stability to other small non-coding RNAs in several diseases (Shaffer et al., 2012) making its possible use as normalization control in miRNA PCR analysis. Notably, there are no reported results that SNORD68 alters its expression in asthmatic patients or by corticosteroids treatment. The real-time qPCR was performed on a Step One Plus™ System (Applied Biosystems Inc, Foster, CA). The PCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s and 70 °C for 34 s. The cycle threshold (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold in real-time PCR. The expression of miRNAs was reported as the ΔCt value, which was calculated by subtracting the CT values of miRNA SNORD68 from the CT values of the target miRNAs. Because there is an inverse correlation between ΔCt and miRNA expression levels, lower ΔCt values are associated with increased miRNA expression. The $2^{-\Delta\Delta\text{Ct}}$ method was used in the analysis of PCR data for relative quantification of miRNA-21.

2.10. Statistical analysis

The data were tabulated and statistically analyzed using Statistical Package for Social Sciences (SPSS Inc. Chicago, IL, USA) version 17. Data are presented as the mean ± standard deviation (±SD), and percentages when appropriate. Comparison of continuous data was performed using student *t* test and Mann-Whitney test for two groups, while for more than two groups ANOVA test was used. The threshold value for optimal sensitivity and specificity of miRNA-21 was determined by receiver operating characteristics (ROC) curve. The correlation coefficients were calculated using Spearman

Table 1
Clinic-demographic characteristics, laboratory and miRNA-21 expression profiles in healthy controls and asthmatic patients.

Parameters	Healthy controls (n = 80)	Asthmatic patients (n = 95)	P value
Age, years	8.6 ± 2.9	9.0 ± 2.7	0.92
Gender, n (%)			
- Male	36(45)	39(41.1)	0.71
-Female	44(55)	56(58.9)	
Mean age at onset (years)	–	4.4 ± 1.2	–
Residence			
-Rural	34(42.5)	35(36.8)	0.54
-Urban	46(57.5)	60(63.2)	
Family history of asthma			
-Negative	71(88.8)	26(27.4)	<0.001*
-Positive	9 (11.2)	69(72.6)	
FEV1	90.3 ± 4	68.8 ± 5.7	<0.001*
Blood eosinophil count(cell/μl)	241.1 ± 93.5	384.7 ± 110.6	<0.001*
Sputum eosinophil (%)	–	10.5 ± 3.9	–
IL-12p35(pg/ml)	214 ± 27.4	47.4 ± 9.5	<0.001*
miRNA-21 expression levels	0.79 ± 0.25	2.8 ± 0.5	<0.001*

FEV1: forced expiratory volume in 1 s.

Quantitative data are represented as mean ± S.D., qualitative data are represented as number and frequency.

* Statistically significant from control group ($P < 0.05$).

correlation. Linear regression analysis was done to test possible association between miRNA-21 and other parameters. Multiple logistic regression analysis was performed to evaluate whether the biomarker miRNA-21 could predict the ICS response in asthmatic patients P -values less than 0.05 were considered statistically significant.

3. Results

3.1. Clinic-demographic characteristics, laboratory and miRNA-21 expression profiles in asthmatic patients and control group

Data represented in Table 1 showed that there were highly significant differences in terms of family history of asthma, FEV1, and blood eosinophil count ($P < 0.001$ for each), whereas no significant differences in the age, sex, and residence between the control group and asthmatic patients.

In current study, serum miRNA-21 expression was measured using qRT-PCR and normalized to SNORD68 as reference control. The expression level of miRNA-21 was significantly higher in asthmatic children in comparison with healthy controls ($P < 0.001$). In contrast, serum IL-12 p35 levels were significantly lower in asthmatic children compared to control group ($P < 0.001$).

3.2. Clinic-demographic characteristics, laboratory and miRNA-21 expression profiles in control group and asthmatic patients sub-groups

The findings presented in Table 2 demonstrated that there was a highly significant increase in the blood eosinophil count and a highly significant decrease of FEV1 in asthmatic patients without therapy, steroid sensitive (SS) asthma patients or steroid resistant (SR) asthma patients as compared to control group ($P < 0.001$ for each). Moreover, we found a significant decrease of blood eosinophil count and sputum eosinophil percent in SS children than SR children or asthmatic children without ICS or ($P = 0.009$, $P < 0.001$ respectively). A significant increase of FEV1 in SS asthma patients than SR patients or patient without ICS ($P = 0.001$, $P < 0.001$ respectively) was found. However, there were no significant differences

in family history of asthma age, sex, and residence were detected between SS, SR or asthmatic patients without ICS therapy.

Moreover, the quantitative expression of miRNA-21 in serum were significantly higher in SS, SR and patients without ICS therapy as compared to healthy controls (each, $P < 0.001$). In SR patients and in asthmatic patients without ICS therapy, serum expression levels of miRNA-21 were significantly higher than in SS children ($P < 0.001$ for each).

On the other hand, there were significant decreases of serum IL-12 p35 levels in SS, SR and patients without ICS therapy as compared to healthy children ($P < 0.001$ for each). Additionally, serum IL-12p35 levels were significantly lower in SR patients and in asthmatic patients without ICS therapy than SS patients ($P < 0.001$ for each).

3.3. ROC curve analysis

ROC analysis (Fig. 1A) revealed that miRNA-21 could differentiate asthmatic patients from healthy controls with an AUC of 0.80 for miRNA-21 (95% CI: 0.74–0.87, $P < 0.001$). The optimal sensitivity and specificity to differentiate asthmatic children from controls were (92.6% and 77.2% at a cutoff expression value > 0.58). This finding suggests that miRNA-21 could be a potential diagnostic biomarker in childhood asthma.

Moreover, ROC analysis (Fig. 1B) showed that miRNA-21 could predict the response to therapy with an AUC of 0.99 (95% CI: 0.98–1.09, $P < 0.001$). At cutoff value < 2.8 , miRNA-21 provided sensitivity of 97.5% and specificity of 86.7% for identifying patients responded to ICS treatment.

3.4. Correlation of miRNA-21 with serum IL-12p35 levels, FEV1, blood eosinophil count, and sputum eosinophil percent.

We further clarified the relations between the quantitative expression levels of miRNA-21 with serum IL-12 p35 levels, FEV1, blood eosinophil count, and sputum eosinophil percent among the asthmatic cases using Pearson correlation analysis. As shown in Fig. 2(A&B), the quantitative expression levels of miRNA-21 showed significant negative correlations with serum IL-12p35 levels and FEV1 ($r = -0.69$, $P < 0.001$; $r = -0.38$, $P < 0.001$ respectively). In contrast, miRNA-21 expression levels were positively correlated with both blood eosinophil count and sputum eosinophil percent ($r = 0.62$, $P < 0.001$; $r = 0.29$, $P = 0.005$ respectively (Fig. 2C&D).

3.5. Analysis of the power of miRNA-21 to predict ICS response in asthmatic cases

Logistic regression analysis was performed to evaluate whether the miRNA-21 could predict the ICS response in asthmatic patients. We found that miRNA-21 was an independent predictor for ICS response, with an odds ratio of 1.9 (1.3–2.7), $P = 0.002$, 95% CI: 1.3–2.7 and $P = 0.004$.

4. Discussion

The miRNAs are small ~22 nucleotide noncoding RNAs that inhibit gene expression by mRNA degradation, or translational repression. Importantly, cellular miRNAs can regulate cell functions, including proliferation, differentiation, immune reaction, and tumorigenesis. It is not surprising that circulating miRNAs display consistent profiles between healthy individuals and significantly altered expression in diseases (Allegra et al., 2012; Angulo et al., 2012). In addition, miRNAs also have an essential role in both the innate and adaptive immune system. Proper miRNA expression is required for correct differentiation of immune cells (Angulo et al., 2012; Lu and Liston, 2009). However, the functions of these miRNAs

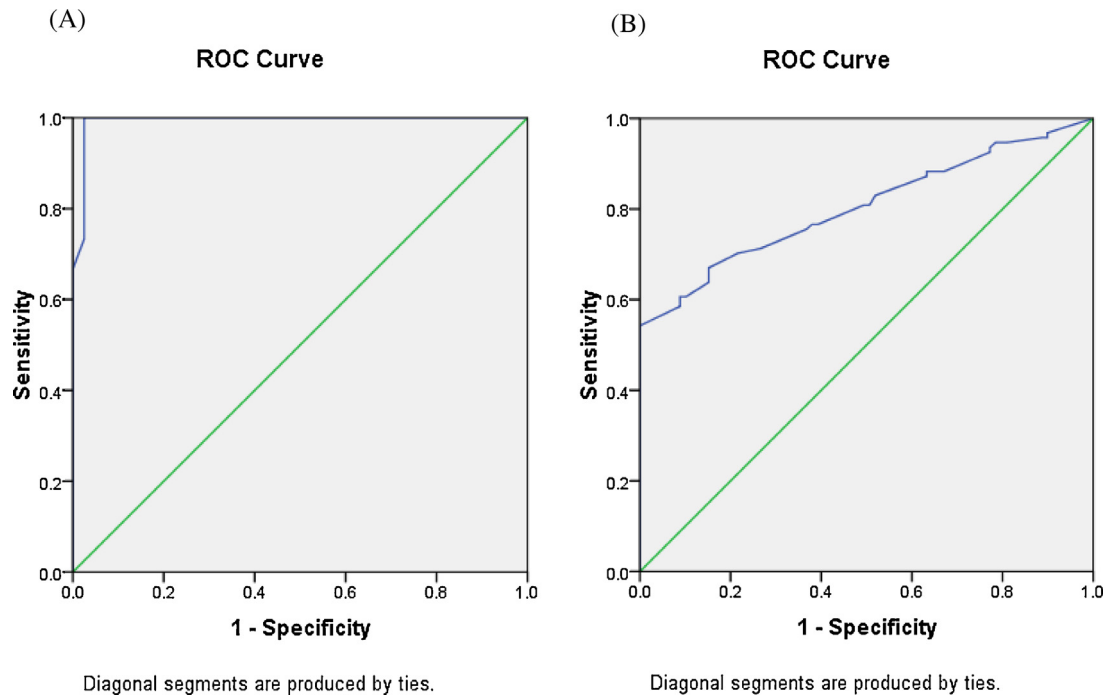


Fig. 1. (A) Receiver operating characteristic (ROC) curve for miRNA-21 expression in discriminating between asthmatic patients and controls. (B) Receiver operating characteristic (ROC) curve for miRNA-21 in differentiating between steroid sensitive and steroid resistant asthma patients.

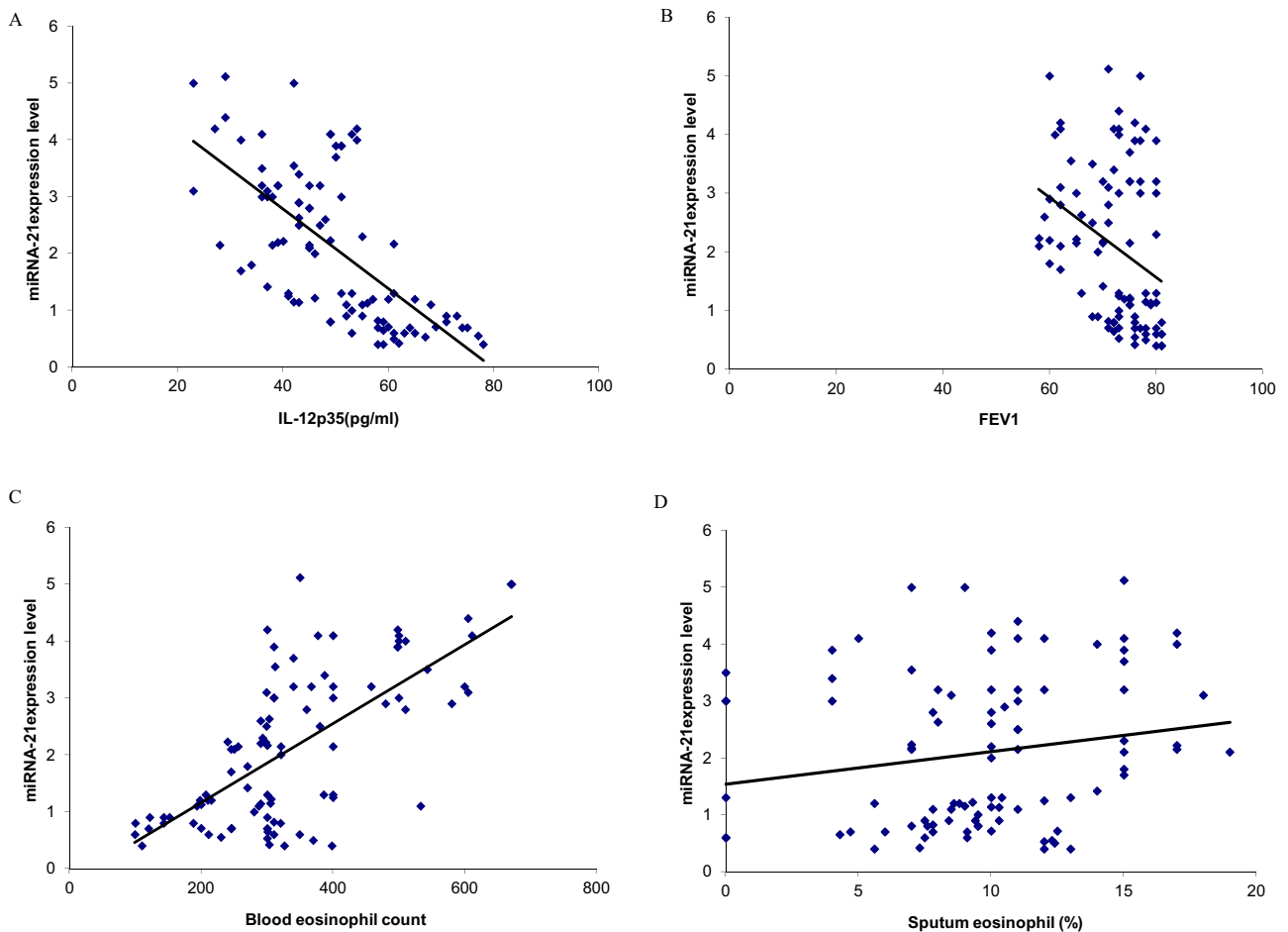


Fig. 2. (A) Correlation between quantitative expression levels of miRNA-21 with serum IL12 p35 levels. (B) Correlation between quantitative expression levels of miRNA-21 with FEV1 values. (C) Correlation between quantitative expression levels of miRNA-21 with blood eosinophil count. (D) Correlation between quantitative expression levels of miRNA-21 with sputum eosinophil (%).

Table 2

Clinic-demographic characteristics, laboratory and miRNA-21 expression profiles in control group and asthmatic patients sub-groups.

Parameters	Healthy controls (n = 80)	Asthmatic patients (n = 95)		
		Asthmatic patients without ICS (n = 40)	Steroid sensitive asthmatic patients (n = 40)	Steroid resistant asthmatic patients (n = 15)
Age, years	8.6 ± 2.9	9.0 ± 2.5	8.9 ± 2.7	9.1 ± 3.1
Gender, n (%)				
- Male	36(45)	17(42.5)	15(37.5)	7(46.7)
-Female	44(55)	23(57.5)	25(62.5)	8(53.3)
Mean age at onset (years)	–	4.6 ± 0.9	4.3 ± 1.2	4.2 ± 1.5
Residence				
-Rural	34(42.5)	16(40)	14(35)	5(33.3)
-Urban	46(57.5)	24(60)	26(65)	10(66.7)
Family history of asthma				
-Negative	71(88.8)	10(25)	12(30)	4(26.7)
-Positive	9 (11.2)	30(75)	28(70)	11(73.3)
FEV1	90.3 ± 4	65.6 ± 5.7 ^a	73.4 ± 6.3 ^{a,b}	67.3 ± 4.5 ^a
Blood eosinophil count(cell/μl)	241.1 ± 93.5	436.6 ± 118.7 ^a	324.6 ± 81.2 ^{a,b}	387.7 ± 63.3 ^{a,c}
Sputum eosinophil (%)	–	11.3 ± 4.2	9.5 ± 3.1 ^b	11.2 ± 3.2
IL-12p35(pg/ml)	214 ± 27.4	43.3 ± 12.3 ^a	60.3 ± 19.6 ^{a,b}	36.7 ± 10.5 ^{a,c}
miRNA-21 expression level	0.79 ± 0.25	3.62 ± 0.74 ^a	1.95 ± 0.54 ^{a,b}	3.57 ± 0.36 ^{a,c}

FEV1: Forced expiratory volume in 1 s. ICS: Inhaled corticosteroid therapy.

Quantitative data are represented as mean ± SD, qualitative data are represented as number and frequency.

^a $p < 0.05$ when compared each group to control group.^b $p < 0.05$ when compared steroid sensitive or steroid resistant asthmatic patients to asthmatic patients without ICS.^c $p < 0.05$ when compared steroid resistant to steroid sensitive asthmatic patient.

as potential biomarkers for diagnosis, prognosis and therapeutic applications remain to be clarified.

The pathogenesis of asthma is influenced by both genetic factors and environmental triggers. The pathophysiologic changes leading to disease remain unclear and to date there are no fully effective therapies (Renz et al., 2011). Evidence is emerging that miRNAs are critical for lung development and for maintaining the lung in a disease-free state (Nana-Sinkam et al., 2009a; Tomankova et al., 2011). Notably, diagnosis of asthma is difficult due to a lack of accurate non-invasive biomarkers, and the regulatory mechanisms that govern cytokine expression are not well defined (Broide, 2008). Recent studies have shown that miRNA-21 target sites are located within IL-12 mRNA, therefore miRNA-21 can significantly inhibit the translation process after IL-12 transcription (Lu et al., 2009; Lu and Rothenberg, 2013).

Therefore, we hypothesized that the different expression of miRNA-21 between asthmatic patients and healthy individuals leads to alterations in its target, IL-12p35, in childhood asthma. This disturbance may lead to a disruption of homeostasis and the potential for enhanced airway inflammation mainly through IL-12p35. Additionally, miRNA-21 could be a novel predictor of ICS response which helps in decision-making and identify patients who are likely or unlikely to ICS therapy. Thus, it reduces the risk of side effects and spare patients from disappointment of treatment failure.

Our results revealed that the expression levels of miRNA-21 were significantly higher in asthmatic children as a whole and in SS, SR and asthmatic patients without ICS subgroups, more than in healthy controls. ROC analysis confirmed that miRNA-21 may be useful as potential diagnostic biomarkers for the diagnosis of childhood asthma (sensitivity: 92.6% and specificity 77.2% at a cutoff value >0.58). Moreover, the quantitative serum expression miRNA-21 levels are significantly high in SR asthmatic patients as compared to SS patients. ROC analysis showed that miRNA may be a predictive value for response to therapy (sensitivity: 97.5% and specificity 86.7% at a cutoff value >2.8). Thus, miRNA-21 can be useful for predicting patient drug responses before intake of ICS therapy, thereby reducing ineffective treatments. Similar to the present data, it has been recently reported that sera of patients with inflammatory disorders, such as eczema and psoriasis, in addition to autoimmune diseases, including systemic lupus erythematosus

and rheumatoid arthritis contain elevated levels of miRNA-21 in comparison to healthy controls (Schickel et al., 2008; Sonkoly et al., 2007; Nana-Sinkam et al., 2009b). Also our result are in accordance with an miRNA array analysis in murine models of acute and chronic asthma, which revealed that miRNA-21 expression in the lungs changed following exposure to allergens, and suggested that miRNA-21 may regulate the biological processes during the course of asthma development (Garbacki et al., 2011b). Interestingly, by using three different experimental animal models of asthma, OVA (ovalbumin), *Aspergillus fumigatus*, lung specific-IL-13 overexpression, Lu et al. have shown that miRNA-21 is the most up-regulated miRNA in asthma (Lu et al., 2009). Levänen et al. demonstrated also an altered expression of miRNA-21 of exosomes isolated from bronchoalveolar lavage fluid (BALF) between asthmatic patients and healthy subjects (Levänen et al., 2013). Recently, Sawant et al (Sawant et al., 2015). reported that miRNA-21 was elevated in the sera of both asthma and eosinophilic esophagitis patients, whereas they found no relation between serum miRNA-21 levels and atopy. In contrast, Williams and colleagues found no difference between the miRNA-21 expression profiles of healthy and mild asthmatic donors (Williams et al., 2009). Report by Liu et al., (2012). does not reflect any significant differences between miRNA-21 expression levels in lung tissues of Chinese asthmatic patients and healthy children. This discrepancy might be due to the different samples used between our study and others.

As the IL-12 p35 is a component of the functional active heterodimer form of IL-12 and the main target of miRNA-21 (Lu et al., 2009), we analyzed its level in asthmatic and healthy children. In our study, there were significant decreases of serum IL-12p35 levels in asthmatic children and asthma subtypes as compared to healthy children. Serum IL-12 p35 levels were significantly lower in asthmatic patients without ICS therapy and in SR asthmatic patients than SS asthmatic patients. In accordance with our data, Lu et al. reported that IL-12 p35 levels were significantly decreased during asthma in three experimental animal models (Lu et al., 2009). This was expected as IL-12 is a key cytokine derived from macrophages and dendritic cells involved in adaptive immune responses involving T helper cells (Th) 1 cell polarization (Trinchieri, 2003) and down-regulates Th2 responses (Jirapongsananuruk and Leung, 1997). Studies in asthma models concluded that the administra-

tion of IL-12 before and during the period of allergen challenge prevented allergen-induced airway eosinophilia, airway hyperresponsiveness, the production of Th2 cytokines, and the production of allergen-specific serum IgE (Sur et al., 1996), whereas it increased the production of interferon- γ and enhanced apoptosis of CD4⁺ T cells in allergic airway infiltrates (Kodama et al., 2003).

Our data revealed that serum miRNA-21 expression levels was significant negatively correlated with serum IL-12 p35 levels and FEV1. In harmony with our findings, Lu et al. (2009). provided the first significant insight into the role of miRNA-21 in experimental asthma model by showing that miRNA-21 expression could be stimulated by IL-13 and that miRNA-21 targets IL-12p35 mRNA and lowers IL-12 protein expression. IL-12p35 plays a role in balancing the Th2/Th1 skewing of T-helper cells; less IL-12 expression would partially account for the exaggerated Th2 response seen in asthma (Lu et al., 2009). This research was expanded upon by studying the immune response of miRNA-21^{-/-} mice (Lu et al., 2011). Following OVA stimulation, these mice produced greater interferon γ and IL-12, whereas there was significantly less IL-4 compared with normal OVA-treated mice. Consequently, these mice had less influx of eosinophils into the lung but experienced greater Th1 hypersensitivity. Thus, Lu et al. have created an important explanation of how IL-12 controls the Th1/Th2 balance and how miRNA-21 in turn regulates IL-12 and promotes Th2 skewing (Lu et al., 2011). Importantly, Song-Quan et al. (2014) stated that the increased expression miRNA-21 levels were significantly correlated with decreased serum IL-12 levels in allergic asthmatic mice compared with those controls or asthmatic mice on dexamethasone groups. The miRNA-21's role in IL-12 regulation is not the only proposed activity for this miRNA. Sheedy et al. (2010) have proposed that miRNA-21 can have an anti-inflammatory/negative feedback regulatory role by targeting programmed cell death protein 4(PDCD4) (Wu et al., 2014). PDCD4 is a proinflammatory protein that down-regulates IL-10 production. This dual role for miRNA-21 demonstrates the complex nature of miRNA regulation, and both studies make the case for miRNA-21 being an immunomodulating molecule (Sheedy et al., 2010). These findings are not just of academic interest because anti-miRNA-21 administration could be promising treatment for asthma.

In the present study, we found significant decreased miRNA-21 expression levels and increased IL-12p35 concentrations in SS asthmatic patients compared to SR; but still significantly differ from normal controls. Our results are in accordance with Wu et al. (2014) who found that miRNA-21 expression was up-regulated in asthmatic adult bronchial epithelial cells regardless of treatment, but expression levels were decreased following ICS therapy (Sheedy et al., 2010). Song-Quan et al. (2014) reported also increased IL-12p35 concentrations and decreased miRNA-21 expression levels in dexamethasone treated asthmatic mice compared to non treated asthmatic group. This findings could be explained by re-establishment of the Th1/Th2 balance in asthmatic patients despite have not been sufficient to satisfactorily reach the normal values in healthy children (Song-Quan et al., 2014; Wu et al., 2014).

In contrast to our study, Williams et al. (2009). showed that changes in miRNA-21 expression are not associated with the mild asthma phenotype in adults or the anti-inflammatory action of the corticosteroid. This may be explained by the fact that the inflammatory changes were too mild, and the degree of cellular heterogeneity in airway biopsies (Williams et al., 2009).

Corticosteroids are potent anti-inflammatory agents that are known to interfere with a number of intracellular pathways including transcription factors such as nuclear factor kappa B (NF)- κ B and activator protein 1(AP-1) (Ito et al., 2006). NF- κ B-binding sites were located in miRNA-21 gene transcriptional elements and enhanced the binding of NF- κ B to the promoter of miRNA-21 and leads to upregulation of miRNA-21 (Shin et al., 2011). Additionally, the AP-

1 activates miRNA-21 transcription through binding to miRNA-21 promoter and enhanced its expression (Fujita et al., 2008). Taken together, the miRNA-21 was directly regulated by NF- κ B and AP-1. Also, this explained the decrease of miRNA-21 expression levels in asthmatic patients on ICS therapy because ICS therapy can down-regulate miRNA-21 expression.

It could be useful to further analyze correlations between serum miRNA-21 expression and FEV1, as well as blood and sputum eosinophils. In our study, there was significant positive correlation between miRNA-21 expression and both sputum and blood eosinophils. It is important to note that miRNA-21 up-regulation has commonly been associated with a variety of disorders with significant eosinophilia including asthma (Broide, 2008), ulcerative colitis (Wu et al., 2008), and eosinophilic esophagitis (Lu et al., 2012). Lu et al. (2012) found that miRNA-21 was among the most up-regulated miRNAs in patients with eosinophilic esophagitis and has the highest correlation with esophageal eosinophil levels. They demonstrated that miRNA-21 can regulate the development of eosinophils by influencing eosinophil progenitor cell growth (Lu et al., 2013). In a miRNA-21-null mouse model of asthma, allergen stimulation, decreases pulmonary eosinophil accumulation, increases IL-12 expression and increases Th1 response in comparison to a wild-type mouse model of asthma (Lu et al., 2011).

Interestingly, we observed a significant negative correlation between miRNA-21 expression levels and FEV1 in asthmatic children. Panganiban et al. found also the same relation between miRNA-21 expression and FEV1 (Panganiban et al., 2012). The significant correlations between serum miRNA-21 expression with FEV1, as well as blood and sputum eosinophils is an important observation, it indicates that cellular events occurring in the lungs are reflected systemically in the blood. It also raises the possibility that profiling of serum miRNA-21 levels may be utilized to assess a patient's disease status based on its severity.

5. Conclusion

The circulating extracellular miRNA-21 can be used as non invasive diagnostic biomarker in asthma. It is a promising severity biomarker, through its positive correlations with blood eosinophils count and sputum eosinophil percent, as well as its negative correlations with FEV1 and serum IL-12p35 levels. Finally, serum miRNA-21 has a high predictive therapeutic value in differentiating ICS sensitive from ICS resistant asthmatic patients.

Declaration of interest

The authors report no declarations of interest.

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