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Role of microRNA-21 and microRNA-155 as biomarkers for bronchial asthma

Sabrin Mohamed Mohamed Abd Elhamed ElKashef[1], Sohair El-Attar Ahmad[1], Youssef Mohamed Amin Soliman[2] and Marwa Salah Mostafa[1]

Abstract

MicroRNA (miRNA)-21 and miRNA-155 are important regulators of gene expression of different immunological molecules. This study aimed to investigate the role of miRNA-21 and miRNA-155 as biomarkers in asthma by comparing their serum expression levels in asthmatic patients to those in healthy controls and correlating their levels with serum IL-4. The expression levels of miRNA-21 and miRNA-155 were evaluated by quantitative RT-PCR. Serum levels of IL-4 were determined using ELISA. Asthmatic patients showed significantly higher serum miRNA-21 and miRNA-155 expression levels compared to controls. A statistically significant positive correlation between the expression levels of miRNA-21 and IL-4 serum levels in asthmatic patients was detected. Nonetheless, no correlation was detected between miRNA-155 expression and each of IL-4 and miRNA-21. A receiver operating characteristic curve analysis showed that at a cut-off value of 1.37, the sensitivity of miRNA-21 as an asthma biomarker was 100% and the specificity was 95%. At a cut-off value of 1.96, the sensitivity of miRNA-155 as an asthma biomarker was 100% and the specificity was 100%. It can be concluded that miRNA-21 and miRNA-155 are potential non-invasive biomarkers in the diagnosis of eosinophilic asthma and its response to therapy.

Keywords

Asthma, IL-4, miRNA-21, miRNA-155, pathogenesis

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Introduction

Asthma is a heterogeneous disease, usually described as chronic inflammatory airway illness associated with hyper-secretion of mucus and airway obstruction, which results in highly diverse clinical presentations varying from mild symptoms to tremendous respiratory distress.[1,2]

The search for genes linked to the development of asthma has focused on four major areas: production of allergen-specific IgE (atopy), generation of inflammatory mediators, expression of airway hyperresponsiveness and determination of the ratio between Th1 and Th2 immune responses.[3] Airway inflammation in asthma may represent a loss of normal balance between the two 'opposing' types of Th cells. Th1 cells secrete IL-2 and IFN-γ. On the other hand, Th2 cells produce IL-4, IL-5, IL-6 and IL-13 which are responsible for the allergic immune response.[4] A possible consequence of the overproduction of Th2 cytokines is eosinophilic inflammation and the subsequent manifestations of asthma.[5] Based on the predominant immunological pathway, two distinguishable phenotypes of asthma have been identified: Th2 asthma and non-Th2 asthma. In Th2 asthma, patients show an excellent response to corticosteroids, whereas non-Th2 asthma is more liable to exhibit resistance to conventional corticosteroid therapy.[6]

[1]Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Egypt
[2]Department of Chest Disorders, Faculty of Medicine, Cairo University, Egypt

Corresponding author:
Marwa Salah Mostafa, Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Kasr Al-Ainy St., Cairo 11562, Egypt.
Email: marwa75_jan@yahoo.com

The recent scientific literature involving microRNAs (miRNAs) and their functions may convey essential influences in the comprehension and management approaches in a vast majority of human diseases, including asthma. MiRNAs represent small, non-coding, single-stranded RNAs that have a crucial role in the regulation of gene expression through interacting with their target messenger RNA and inducing either suppression of protein synthesis or degradation of RNA.[7] MiRNAs are involved in a wide range of biological functions, including immune cell maturation, activation, differentiation and preservation of immune homeostasis.[8,9] More evidence suggests that asthma is under the control of a diverse range of miRNAs as demonstrated by noticeable changes in gene expression and protein synthesis in the airways. Exploring the biological functions of miRNAs in the immuno-pathogenesis of asthma can also facilitate the discovery of novel therapeutic targets.[9,10]

It has been established that miRNA-21 is up-regulated in inflammatory disorders such as eczema and autoimmune diseases.[11,12] It has been found to be involved in the pathogenesis of asthma and appears to be a biomarker for Th2-type allergic diseases.[13]

MiRNA-155 is expressed in large quantities in various organs, including the spleen, thymus, liver, lungs and kidneys.[14] Therefore, it is believed to have a major role in the regulation of the immunological responses.[15] MiRNA-155 is necessary for the maturation of dendritic cells (DCs) and for provoking these cells to stimulate Ag-specific T-cell activation.[16] It is also an important regulator for scavenging of pathogens as well as apoptotic cells. The effector role of miRNA-155 is mediated via three pathways: (a) miRNA-155 is needed for Ab production and activation of the germinal centre of B cells, crucial for B-cell maturation, differentiation and immunoglobulin class switching;[17] (b) miRNA-155 has a protective role against infections and tumours in cytotoxic T cells and NK cells;[18] and (c) miRNA-155 is abundantly expressed by macrophages, the main scavenger cells, after LPS stimulation.[19]

The aim of this work was to investigate the role of miRNA-21 and miRNA-155 as biomarkers in asthma by comparing their serum expression levels in asthmatic patients to those in normal individuals. The correlations between miRNA-21 and miRNA-155 serum expression levels with serum IL-4 levels were also assessed.

Methods

Subjects

The present study was conducted on 30 asthmatic patients attending the Outpatient Asthma Clinic and Chest Disorders Department at Faculty of Medicine, Cairo University, as well as 30 healthy non-asthmatic individuals who served as the control group. All individuals in the patient and control groups were selected with an average body mass index of 20–25 kg/m^2 at the time of sample collection. Serum samples were collected from all patients and control subjects during the period from October 2017 to April 2018. The study was approved by the Research Ethics Committee of the Institutional Review Board, Faculty of Medicine, Cairo University, and informed consent was obtained from each participant.

Patients who had upper and lower respiratory tract diseases or other allergic conditions within the previous 2 mo, chronic heart or lung disease and asthmatic patients on systemic corticosteroids, theophylline, leukotriene receptor antagonists or antihistamines within at least 4 wk before sample collection were excluded from the study.[10] Asthmatic patients were diagnosed by history taking, clinical examination and the existence of reversible airway obstruction, which was identified as a forced expiratory volume in the first second elevated by 12% or more, provided its elevation was $\geq$ 200 ml, 15 min after 400 μg salbutamol inhalation via a spacer.[2] All patients were in acute exacerbation at the time of sample collection. Asthma exacerbation was diagnosed clinically by acute or subacute progressively increasing shortness of breath, cough, wheezes, chest tightness and a progressive decline in lung function that needs medical treatment.[20] All patients were atopic with eosinophilic inflammation. There were no exacerbations secondary to a bacterial infection, but viral infections could not be excluded.

Serum samples from patients and controls were used for the measurement of serum levels of IL-4 as well as expression levels of miRNA-21 and miRNA-155. The laboratory tests were performed at the Molecular Biology Unit of the Medical Biochemistry Department, Faculty of Medicine, Cairo University.

Sera

Peripheral venous blood (5 ml) was withdrawn from each participant by venepuncture under complete aseptic conditions and collected in sterile tubes. Each tube was labelled with the patient's name and date of collection and then allowed to clot spontaneously at room temperature before being centrifuged at 1000 *g* for 10 min. Serum samples were divided into two portions: 2 ml serum for miRNA extraction and subsequent measurement of miRNA-21 and miRNA-155 expression levels by quantitative RT-PCR, and the remainder for measuring IL-4 levels by ELISA. Sera were stored at –80°C until processing.

Detection of IL-4 serum levels by ELISA

Semi-quantitative detection of serum levels of IL-4 in both patients and controls was performed using a Human IL-4 ELISA Kit (Sunlong Biotech, Hangzhou, PR China), which uses a semi-quantitative sandwich enzyme immunoassay technique. The test was performed according to the manufacturer's instructions.

Amplification and quantification of miRNA using quantitative RT-PCR

Isolation of miRNA from each sample was performed using a Favorgen miRNA Isolation Kit (Taiwan Biotech, Taoyuan, Taiwan) according to the manufacturer's instructions. The purified miRNA was stored at −80°C. Assessment of the quality and concentration of the isolated miRNA was performed with a ScanDrop Nano-volume spectrophotometer (Analytik Jena, Jena, Germany) which was used to measure the absorbance of the isolated miRNA at 260 and 280 nm. The purity of the extract was assessed by calculating the ratio of A260/A280. A ratio of 1.8–2.1 was considered acceptable.

Quantification of miRNA-21 and miRNA-155 was done by quantitative RT-PCR using the TaqMan MicroRNA Assay. In the reverse transcription step, the cDNA was reverse transcribed from total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using a microRNA-specific RT primer (Megaplex RT Primer Human Pool B v3.0; Applied Biosystems). Amplification of the target cDNA of miRNA-21 and miRNA-155 was performed using TaqMan microRNA assays for hsa-miRNA-21-3p (assay ID 002438; Applied Biosystems) and hsa-miRNA-155-3p (assay ID 002287; Applied Biosystems), respectively. Each PCR contained 10 µl TaqMan Universal PCR Master Mix, 1 µl 20× TaqMan microRNA assay, 1.33 µl template cDNA and 7.76 µl nuclease-free water to complete the reaction mixture to 20 µl. The prepared reaction mixtures were processed in the Applied Biosystems Step One thermal cycler with software v3.1 (Applied Biosystems) for amplification and analysis. The cycling conditions were as follows: one hold cycle at 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, then 60°C for 1 min. Data were normalised to *snRNA U6* which was used as an endogenous control gene. The $2^{-\Delta\Delta ct}$ method was used to calculate relative miRNA expression (RQ).

Statistical analysis

Data were analysed using IBM SPSS Statistics for Windows v25 (IBM Corp., Armonk, NY). In case of quantitative variables, data were expressed using the mean and *SD* for parametric data, whereas the median and range were used for non-parametric data. Frequency and relative frequency were used for categorical variables. Comparisons between non-parametric quantitative variables were done using the Mann–Whitney test. The chi-square test was used to compare categorical data. Correlations between serum levels of IL-4, expression levels of miRNA-21 and miRNA-155 in cases and controls were done using Spearman's correlation coefficient (r_s). Multivariate stepwise linear regression analysis was conducted to predict the risk factors that affect each of IL-4 serum levels and the expression levels of miRNA-21 and miRNA-155. P Values of ≤ 0.05 were considered statistically significant.

Results

Demographic data of asthmatic patients and control subjects

The present study was conducted on 30 asthmatic patients and 30 healthy subjects. The patient group included 10 (33.3%) males and 20 (66.7%) females, while the control group included 14 (46.7%) males and 16 (53.3%) females. The patient group included six (20%) smokers, while the control group included seven (23.3%) smokers. Both groups were matched regarding age, sex and smoking status (Table 1).

MiRNA-21, miRNA-155 and IL-4 levels in asthmatic patients and controls

The expression levels of both miRNA-21 and miRNA-155 were significantly higher in asthmatic patients (median = 2.99, range 0.82–6.02; median = 2.57, range 1.17–4.95, respectively) compared to controls (median = 1.01, range 0.84–1.76; median = 1.01, range 0.89–1.58, respectively; $P < 0.001$). The levels of IL-4 were also significantly higher in asthmatic patients (median = 114.65, range 24.2–184.2) compared to controls (median = 31.25, range 18.9–58.2; $P < 0.001$; Table 2).

A receiver operating characteristic (ROC) curve analysis was performed to assess the values of IL-4, miRNA-21 and miRNA-155 as biomarkers for asthma (Figure 1). At a cut-off value of 60.3, the sensitivity of IL-4 as an asthma biomarker was 96.7% and the specificity was 100%, with an area under the curve (AUC) of 0.97 (95% confidence interval (CI) 0.912–1.028, $P < 0.001$). At a cut-off value of 1.37, the sensitivity of miRNA-21 as an asthma biomarker was 100% and its specificity was 95%, with an AUC of 0.993 (95% CI 0.978–1.009, $P < 0.001$). At a cut-off value

of 1.96, both the sensitivity and specificity of miRNA-155 as an asthma biomarker were 100%, with an AUC of 1 (95% CI 1–1, $P < 0.001$).

Correlations between miRNA-21, miRNA-155 and IL-4 levels in asthmatic patients

There was a statistically significant positive correlation between the expression levels of miRNA-21 and IL-4 serum levels in asthmatic patients ($r_s = 0.609$, $P < 0.001$; Figure 2). On the other hand, no statistically significant correlation was detected between the expression levels of miRNA-155 and IL-4 levels in asthmatic patients ($r_s = 0.278$, $P = 0.136$; Figure 3). There was also no correlation between the expression levels of miRNA-21 and miRNA-155 in asthmatic patients ($r_s = 0.188$, $P = 0.321$).

Risk factors that affect each of the serum IL-4 levels and miRNA-21 and miRNA-155 expression levels were studied using multivariate stepwise linear regression analysis. A significant regression equation was detected between IL-4 serum levels with each of the following risk factors: the presence of asthma ($P < 0.001$), age ($P = 0.023$), smoking ($P = 0.002$), miRNA-21 ($P < 0.001$) and miRNA-155 ($P = 0.015$). Therefore, the presence of asthma, age, smoking, miRNA-21 and miRNA-155 expression levels were found to be significant predictors of IL-4 serum levels. Serum levels of IL-4 were found to be a significant predictor of miRNA-21 expression levels ($P < 0.001$). On the other hand, the presence of asthma alone was found to be a significant predictor of miRNA-155 expression levels ($P < 0.001$).

Table 1. Demographic data of patients and control subjects.

Variable	Patients ($n = 30$)	Controls ($n = 30$)	*P* Value
Age (yr)			
Range	27–60	26–62	0.069
Mean ± SD	44.73 ± 7.63	40.6 ± 11.4	
Sex: *n* (%)			
Male	10 (33.3%)	14 (46.7%)	0.292
Female	20 (66.7%)	16 (53.3%)	
Smoking: *n* (%)			
Yes	6 (20%)	7 (23.3%)	0.754
No	24 (80%)	23 (76.7%)	

Discussion

We found that serum expression levels of miRNA-21 were significantly elevated in asthmatic patients compared to controls ($P < 0.001$). This finding is in agreement with that published by Sawant et al. who showed that miRNA-21 is higher in serum samples of asthmatic patients.[21] Furthermore, Lu et al. documented higher expression of miRNA-21 in the airways of asthmatic patients, presenting noteworthy evidence of the role of miRNA-21 in asthma.[22] In addition, a recent study by Hammad et al. showed that miRNA-21 is up-regulated in the plasma of asthmatic children and plays a role in the eosinophilic phenotype of asthma.[23] Elbehidy et al. reported significantly higher expression levels of miRNA-21 in serum samples of children with asthma compared to controls. Moreover, miRNA-21 was

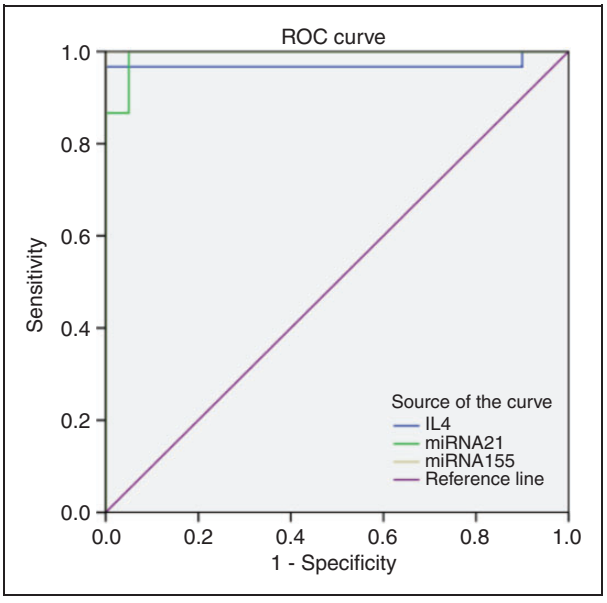

Figure 1. Receiver operating characteristic curve analysis showing the sensitivity and specificity of IL-4, miRNA-21 and miRNA-155 as biomarkers for asthma.

Table 2. Comparison between asthmatic patients and controls regarding miRNA-21, miRNA-155 and IL-4 levels.

	Cases			Controls			
	Median	Minimum	Maximum	Median	Minimum	Maximum	*P* Value
MIRNA-21 (RQ)	2.99	0.82	6.02	1.01	0.84	1.76	< 0.001
MIRNA-155(RQ)	2.57	1.17	4.95	1.01	0.89	1.58	< 0.001
IL-4 (pg/ml)	114.65	24.2	184.2	31.25	18.9	58.2	< 0.001

RQ: relative expression measured by quantitative RT-PCR.

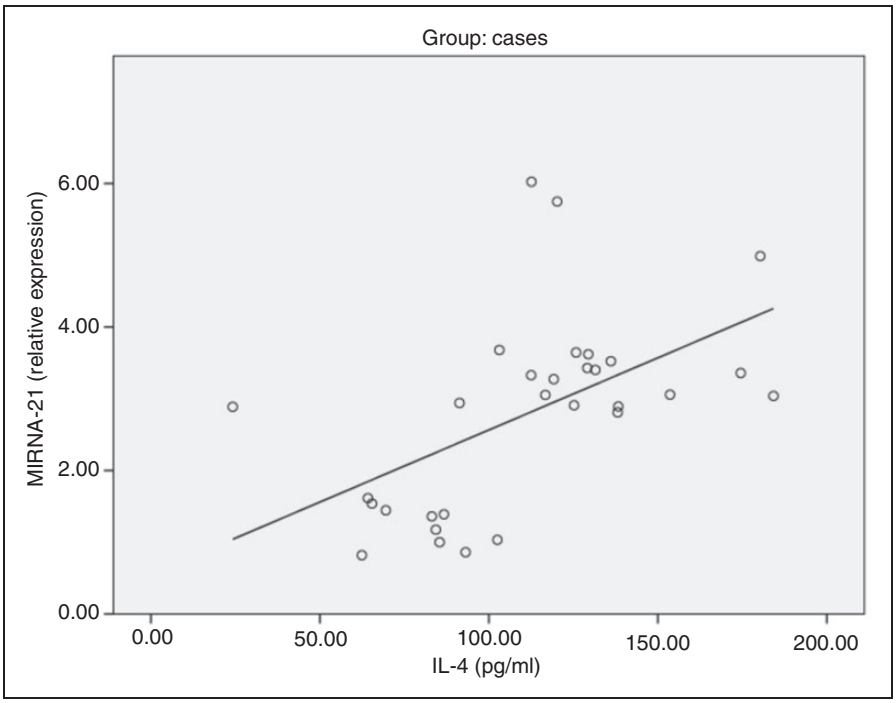

Figure 2. Correlation between miRNA-21 and IL-4 in asthmatic patients. Scatter plot curve showing a positive correlation between miRNA-21 and IL-4 levels ($r_s = 0.609$, $P < 0.001$).

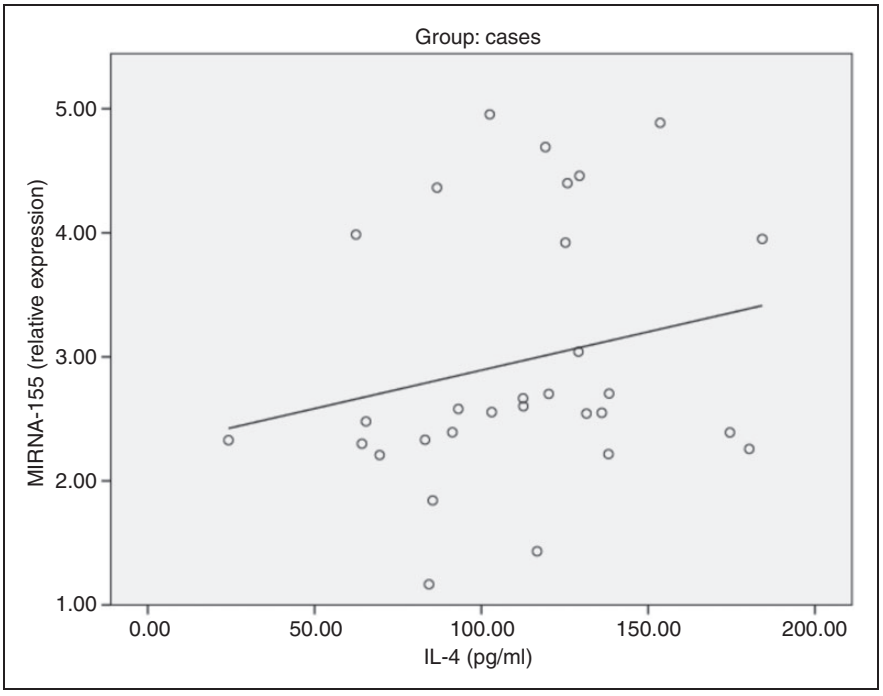

Figure 3. Correlation between miRNA-155 and IL-4 levels in asthmatic patients. Scatter plot curve showing no correlation between miRNA-155 and IL-4 levels ($r_s = 0.278$, $P = 0.136$).

significantly higher in untreated and steroid-resistant children compared to steroid-sensitive children. They concluded that miRNA-21 can be a promising biomarker for the diagnosis and follow-up of the response to inhaled corticosteroid therapy.[10] However, Wu et al. showed that the expression of miRNA-21 is significantly higher in the epidermis of the bronchial tree in adult patients suffering from asthma, regardless of treatment

with inhaled corticosteroids.[24] Another study showed that plasma miRNA-21 was up-regulated at least eight-fold in eosinophilic asthma patients.[25] A recent study conducted by Rodrigo-Muñoz et al. reported higher expression levels of a number of miRNAs in serum eosinophils, including miRNA-21 in asthmatics versus healthy controls. In addition, this study emphasised its ability to classify asthma according to disease severity.[26] On the contrary, Williams et al. did not show any disparity in miRNA-21 expression levels in airway tissue biopsies between healthy individuals and patients with mild asthma.[27] A report by Liu et al. reflected no significant difference between the expression profiles of miRNA-21 in alveolar tissues of healthy Chinese children and those with asthma.[28] These findings show that miRNA-21 is elevated in the systemic circulation of patients with eosinophilic asthma. However, this elevation may not be obvious in non-eosinophilic asthma.

Functionally, miRNA-21 can stimulate a Th2 response by two diverse mechanisms. The first mechanism is mediated by the ability of miRNA-21 to inhibit IL-12 gene expression, resulting in inhibition of Th1 functions, including decreased release of IFN-γ. Low IFN-γ levels lead to unrestricted Th2 activation and elevated Th2 cytokines. Th2 cytokines can additionally augment miRNA-21-mediated responses.[13] Second, miRNA-21 can directly induce differentiation of T cells towards Th2 lineage by escalating Gata3 and IL-4 expression immediately after T-cell activation.[21,29] Lu et al. showed that the key target of miRNA-21 is IL-12 p35, which is a component of the active form of IL-12, and therefore miRNA-21 inhibits IL-12 expression. IL-12p35 has a role in regulating Th2/Th1 balance, and reduced IL-12 expression to some extent causes the enhanced Th2 response detected in asthma.[22]

Deletion of miRNA-21 in mice has been found to provoke higher levels of the Th1 cytokine (IFN-γ), but it reduces lung eosinophilia after allergen exposure. Moreover, it has been proven that miRNA-21 deficiency induces DCs to release IL-12 after LPS stimulation and activates Th cells to generate more IFN-γ and less IL-4. These data emphasise that miRNA-21 is a chief regulator of Th1 and inducer of Th2 differentiation.[13] Therefore, it has also been suggested that miRNA-21 may be helpful as a prospective diagnostic biomarker for the diagnosis of asthma and as a predictor of responses to corticosteroid therapy.[10]

In the present study, we demonstrated that serum levels of miRNA-155 were significantly higher in asthmatic patients compared to controls ($P < 0.001$). Similarly, Daniel et al. discovered that miRNA-155 expression was higher in CD4+ T cells of asthmatic subjects compared to patients with allergic rhinitis and non-asthmatics.[30] Malmhall et al. also clearly demonstrated that miRNA-155 has a role in allergen-induced Th2-mediated eosinophilic airways inflammation. MiRNA-155 expression has been found to be significantly elevated in the airways of wild type mice after allergen challenge with the development of allergen-induced airway eosinophilia and mucus hyper-secretion. On the other hand, miRNA-155-deficient mice failed to develop allergen-induced airway eosinophilia and mucus hyper-secretion, both of which are dependent on Th2 cytokines.[31] Recently, Qiu et al. reported elevated plasma miRNA-155 levels in patients with severe asthma when compared to healthy participants and to mild/moderate asthmatics. They also reported that increased levels of plasma miRNA-155 have been observed in asthmatics with cockroach allergy compared to those without cockroach allergy.[32] MicroRNA-155 expression has been found to be up-regulated in patients with asthma, particularly the eosinophilic phenotype.[33]

On the contrary, MiRNA-155 has been regarded as a Th2 suppressor in initial studies. MiRNA-155 over-expression in CD4+ T cells isolated from splenocytes has amplified Th1 responses following stimulation with IFN-γ, whereas its suppression has promoted Th2 differentiation in response to IL-4.[34,35] Similarly, Rodriguez et al. found that *in vitro* stimulation of CD4+ T cells lacking miRNA-155 with IL-4 induced Th2 differentiation and Th2 cytokine production. They concluded that Th2 immune responses as well as inflammatory and allergic episodes are hindered by miRNA-155.[15] Suojalehto et al. also reported down-regulation of five miRNAs, including miRNA-155, in nasal biopsies of asthma patients compared to controls.[36] Panganiban et al. showed that plasma miRNA-155 was significantly down-regulated in asthmatic patients compared to healthy subjects.[25] The potential role of miRNA-155 as a Th2 suppressor in *in vitro* CD4+ T cells as well as in asthmatic patients may indicate that other components of the immune system may interplay with miRNA-155 to potentiate a Th2 response. It may also reflect that its up-regulation during asthma exacerbations in our study group is a regulatory mechanism by which the immune system maintains the Th1/Th2 balance.

Nevertheless, the current studies have shown new proof that miRNA-155 plays an important role in the promotion, rather than suppression, of Th2 pathways. Anti-miRNA-155 can regulate Th2 inflammation through three main mechanisms: (a) decreased Ag presentation by DCs, (b) suppression of Th2 cell differentiation and (c) down-regulation of the production of Th2 cytokines. Therefore, anti-miRNA-155 can be seen as an emerging therapeutic approach for Th2-induced diseases, including asthma.[37]

Reduced levels of miRNA-21 may be associated with obesity.[38] It has been found to regulate adipogenic

differentiation,[39] and it has been linked to mass loss in severe obesity.[40] MiRNA-155 expression was higher in the adipose tissue of obese individuals compared to that of normal-mass subjects. Its over-expression also resulted in an increased inflammatory state in adipocytes.[41] As all patients and controls involved in this study had an average body mass index (BMI) of 20–25 kg/m^2 at the time of sample collection, it is unlikely that elevated miRNA-21 and miRNA-155 expression levels in our patients were influenced by their BMI.

MiRNAs are secreted into exosomes of most cell types, enter the bloodstream and travel to remote tissues to be utilised by distant cells.[42,43] Defects in miRNA transportation from the circulation to the airways together with the inability of lung tissues to express certain miRNAs may contribute to asthma pathogenesis.[25] The discrepancies of miRNA-21 and miRNA-155 levels among asthmatics compared to non-asthmatics can be explained by various factors, including: (a) different types of asthma (eosinophilic/non-eosinophilic), (b) degree of asthma severity, (c) different types of allergens and (d) different types of studied samples, that is, they are decreased in local tissues while increased in the circulation of asthmatic patients, as they may be depleted in asthmatic airways, or their expression can be repressed by negative feedback mechanisms. Failure of expression in local respiratory tissues may constitute a risk factor of asthma development.

In this study, the ROC curve analysis demonstrated high sensitivity and specificity of miRNA-21 (100% and 95%, respectively) at a cut-off value of $\geq$1.37 (AUC = 0.993), and of miRNA-155 (100%) at a cut-off value of $\geq$1.96 (AUC = 1). Elbehidy et al. reported that miRN-21 levels could differentiate asthmatic patients from controls at a lower cut-off value ($\geq$ 0.58) with a sensitivity of 92.6% and specificity of 77.2% (AUC = 0.8).[10] Approximate results regarding miRNA-155 were obtained by Karam et al. who reported that miRNA-155 can assist in the diagnosis of asthma and the prediction of disease severity.[33] These findings suggest that these miRNA molecules can serve as potential biomarkers for asthma diagnosis.

In this study, we demonstrated that serum levels of IL-4 were significantly higher in asthmatic patients compared to controls ($P < 0.001$). This is in line with another study by Cui et al. who revealed increased IL-4 and IL-6 levels and decreased IL-12 levels in the peripheral blood of asthmatics.[44] Similarly, Lee et al. suggested that acute asthmatics have significantly increased serum levels of circulating IL-4 compared to asymptomatic asthmatics and controls.[45] On the contrary, Hasegawa et al. showed that serum levels of IL-4 are significantly lower in asthmatic patients compared to healthy controls. However, they showed that the levels of Th2 cytokines, including IL-4, are significantly higher in atopic than in non-atopic asthmatics, specifically those with uncontrolled refractory asthma, and they concluded that atopic asthma is a subtype with increased serum Th2 cytokines and sputum eosinophils.[46]

In our study, we demonstrated a statistically significant positive correlation between the expression levels of miRNA-21 and IL-4 serum levels in asthmatic patients ($r_s = 0.609$, $P < 0.001$). This result is in agreement with that published by Lu et al. who demonstrated that miRNA-21 deficiency affected the Th1/Th2 balance. Notably, its deficiency augmented IFN-γ and impaired IL-4 release.[13] Lu et al. previously reported over-expression of miRNA-21 in asthmatic airways of various mice models, including wild type mice exposed to IL-4, IL-13 or an allergen.[22]

On the other hand, the findings of this study demonstrated no correlation between the expression of miRNA-155 and IL-4 serum levels in asthmatic patients ($r_s = 0.278$, $P = 0.136$). Conversely, Suojalehto et al. found a weak correlation between miRNA-155 and Th2 cytokine levels in asthmatics.[36] A study by Malmhall et al. showed that Th2 cytokine production by the peri-bronchial cells of lymph nodes are significantly impaired in miRNA-155-deficient mice compared to wild-type mice.[31] In our study, no correlation was detected between the expression levels of miRNA-21 and miRNA-155 ($r_s = 0.188$, $P = 0.321$). Based on these results, the effect of miRNA-21 and miRNA-155 on Th2 response and cytokine production can be mediated by different mechanisms and pathways.

It is worth mentioning that miRNA-21 targets genes coding for TGF-β receptor type 1 (TGFBR1) and *s*ignal transducer and activator of transcription 3 (STAT3).[25] Interestingly, it was demonstrated that IgE alone can stimulate miRNA-21 expression which in turn promotes airway remodelling, signifying that its inhibition can be a therapeutic goal to decrease airway remodelling.[47,48] On the other hand, miRNA-155 targets TNF receptor associated factor 3 (*TRAF3)* and NF-κB p65 subunit (*RELA*) genes.[25] Administration of IL-33 has led to up-regulation of miRNA-155 in innate lymphoid murine cells that have a crucial role in Th2 activation and differentiation. It was found that miRNA-155 prevents apoptosis of innate lymphoid cells rather than affecting their expansion and cytokine release.[49]

One of the limitations of this study is the small sample size. However, power analysis using a *post hoc* test for continuous data was performed and revealed that the study power is 100%. Another limitation is that we were unable to study mRNA levels over time because most of the patients involved in the study do

not return to the outpatient clinic for routine follow-up on a regular basis. Therefore, we aimed to measure miRNA-21 and miRNA-155 in asthmatic patients during disease exacerbations, which can be considered a point of strength in this study, as the miRNA-21 and 155 expression levels during exacerbations may reflect the underlying mechanisms and the particular effect of the immuno-pathogenesis of asthma on miRNA-21 and miRNA-155. We aim in our future studies to investigate these molecules during and between exacerbations in order to determine if their baseline levels change during asthma exacerbations.

It can be concluded that both miRNA-21 and miRNA-155 expression levels are increased in serum samples of individuals with eosinophilic asthma compared to healthy non-asthmatics. Therefore, they have the potential to be used as non-invasive biomarkers in asthma diagnosis and response to therapy. Moreover, the positive correlation between serum miRNA-21 and IL-4 confirms the role of miRNA-21 in Th2 activation, allergic lung inflammation and asthma pathogenesis. On the other hand, the absence of a correlation between the expression levels of miRNA-155 and each of IL-4 and miRNA-21 indicates that the effect of miRNA-21 and miRNA-155 on asthma pathogenesis can be mediated by different mechanisms/pathways. It was found that several factors can affect IL-4 serum levels, including the presence of asthma, age, smoking and miRNA-21 and miRNA-155 expression levels. On the other hand, serum IL-4 was found to be the only significant predictor of miRNA-21 expression, and the presence of asthma itself was found to be the only significant predictor of miRNA-155 expression. The causal role of miRNA-21 and miRNA-155 in the pathogenesis of allergic asthma needs to be further illuminated. Therefore, future studies should be done on a larger scale, stratifying patients according to asthma phenotypes, severity, duration and type of allergen. The therapeutic potential of various strategies targeting miRNA-21 and miRNA-155 for the treatment of asthma needs to be investigated while taking into consideration the side effects associated with the disruption of the immune response.

Declaration of conflicting interests

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ORCID iD

Marwa Salah Mostafa https://orcid.org/0000-0002-8906-8938

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