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Exosomal MicroRNAs as Biomarkers in Allergic Asthma

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ABSTRACT

Exosomes are extracellular vesicles that are involved in intracellular communication and different biological processes. Recently, the importance of microRNAs (miRNAs) in exosomes has been considered as biomarkers in asthma diagnosis. This study aimed to determine the expression of selective miRNAs from plasma-derived exosomes in moderate and severe asthmatic patients compared with healthy controls.

Forty-six subjects including 22 patients with severe and mild to moderate allergic asthma and 24 healthy controls have entered this study. MiRNAs were extracted from the plasma exosomes and selective miRNAs (miR-21, miR-16, miR-Let7, miR-148a, miR-155, miR-125, miR-150, miR-146a, miR-223, miR-126) expressions levels were determined; using quantitative polymerase chain reaction (qPCR).

In this study, we found a significant up-regulation of miR-223 and miR-21 in moderate asthmatic patients compared to the healthy controls (p=0.002, p=0.006). MiR-223 and miR-21 had the probability of 83% and 76% diagnosis estimation in moderate asthmatic patients respectively. Therefore, they could be used as biomarkers in these patients. No expression of miR-125, miR-126, and miR-155 was found in plasma exosomes by qPCR in this study. The other miRNAs had no significant expression between different groups.

Based on our findings, miR-223 and miR-21 may be considered biomarkers or used for targeted immunotherapies in asthma.

Keywords: Asthma; Exosomes; MicroRNAs; Plasma

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INTRODUCTION

Asthma is an inflammatory disease in the respiratory system that comprises different phenotypes with different mechanisms and triggers. As available diagnostic tools to assess versatile phenotypes are not warranted, there is a clear need for the identification of biomarkers to diagnose asthma endotypes.[1] Analysis of exosome contents is a new approach as a diagnostic biomarker in different diseases. Exosomes are small 30–100 nm membrane-enclosed vesicles that contain different molecules such as DNA, mRNA, microRNAs (MiRNAs), lipids, proteins. Exosomes are considered as mediators that deliver messages through their contents, from cell to cell and distinct target cells, this delivery system regulates the function of target cells.[2] MiRNAs are single-stranded RNA molecules of 19-25 nucleotides that get involved in post-transcriptional gene silencing of target genes. These molecules suppress gene expression by pairing to the 3'untranslated region (UTR) of the target mRNA and this leads to mRNA degradation or translational repression depending on the level of complementarily to the intended regions. MiRNAs are secreted into the blood and other body fluids in a cell-free state which is associated with lipoproteins (HDL) or forming complexes with argonaute RISC catalytic component 2 (AGO2) protein to protect from blood RNases. Also, miRNAs can be packaged within extracellular vesicles including exosomes and microvesicles.[3]

Previous studies have shown miRNAs content of exomes may vary in pathological conditions.[4]Altered expression levels of miRNAs might be important in regulating inflammatory reactions in asthmatic patients compared with healthy subjects. Increasing expression levels of miRNAs including miR-125b, miR-126, miR-21, miR-16, miR-223, miR- let7, miR-148a, and miR-146a have been implicated in most studies. On the contrary, downregulation of miR-150 and miR-155 have been reported in asthmatic subjects.[1]

In this study, the expression of 10 selected exosomal miRNAs based on previous studies (miR-21, miR-16, miR-Let7, miR-148a, miR-155, miR-125, miR-150, miR-146a, miR-223, miR-126) were studied to find out whether they expressed differentially in asthmatic patients compared with normal subjects. This may provide further information that can be used to develop a potential tool for the diagnosis of asthma.

MATERIALS AND METHODS

Subjects

Twenty-two asthmatic and twenty-four healthy controls were enrolled in this study. The severity of asthma was diagnosed based on the recommendations of the Global Initiative for Asthma (GINA, www.ginasthma.org), their clinical symptoms, spirometry, and physician's confirmation. Age, sex, body mass index (BMI), allergens (based on skin prick test), outcome measures of lung function included FEV_1 and forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) are summarized in Table 1. Twenty-four sex and age-matched healthy control subjects were investigated in this study with a negative history of chronic illness, asthma, other allergic and respiratory tract diseases, and exposure to smoking. Four ml blood was collected of asthmatic and healthy subjects in sterile EDTA-containing tubes and centrifuged at 960× g for 3 min to isolate plasma. Plasma samples were kept frozen at −70°C until extraction of exosomes.

Adult patients with allergic asthma aged 25-50 years old were included in the study. They had asthma symptoms for at least 6 months at the time of sampling. Patients with a history of congenital heart diseases, other immunologic diseases, acute upper or lower respiratory tract infections, smoking, pregnancy, and negative skin prick test were excluded from the study.

Characterization of Plasma-derived Exosomes

Plasma exosomes were isolated according to the Exo-spin™ kit (Cell Guidance systemExospin Cambridge, UK) protocol. Isolated particles were assessed with Scanning electron microscopy (SEM) and dynamic light scattering (DLS) to confirm efficient isolation of exosomes with acceptable quality regarding their size range and morphology.

MiRNAs extracted from exosomes by miRCURY™ RNA Isolation Kit (Exiqon, Denmark) according to the manufacturer's instruction. MiRNAs concentration and purity were measured based on A260/280 absorbance by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Extracted miRNAs were reverse transcribed by Bon miR microRNA cDNA Synthesis Kit (Stem Cell Technology, Iran).

Real-time PCR was performed using the SYBR Green Master Mix kit (Ampliqon, Denmark) in StepOnePlus™ Real-Time PCR System (Applied

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Biosystem, US). RNU6B was used as an endogenous control to normalize the expression levels of target miRNAs. Relative quantification (Rq) of miRNA expression was calculated using the $2^{-\Delta\Delta CT}$ threshold cycle method.[5]

Statistical Analysis

R 3.5.2 software (R Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project.org/) was used for statistical analysis. Kruskal Wallis test was performed to compare data obtained from three independent groups, including patients with moderate asthma, severe asthmatic patients, and healthy controls, followed by Dunn's multiple comparison tests. Results with a p-value less than 0.05 were considered significant.[6] The ROC (Receiver Operating Characteristics) curve was plotted to evaluate the diagnostic potency of the significantly expressed miRNA in each groups.[7]

Table1. The demographic and clinical characteristics of asthmatic patients

P	Age (y)	Gender	Sensitized allergens	BMI	FEV1		FEV1/FVC		Type of Asthma
					Pre	Post	Pre	Post	
1	50	male	Grasses	25.1	1.7	1.6	83.2	81.4	MA
2	33	male	Food Grasses	22.5	3.34	3.36	83.7	87.5	SA
3	33	female	Grasses	26.9	3.57	2.71	97.8	98.5	MA
4	50	female	Food	33.4	1.8	1.79	84.1	82.8	MA
5	41	female	Grasses	29.4	2.97	3.22	85.6	82.1	MA
6	45	female	GrassesTree	28.3	3.36	3.34	82.5	86.6	SA
7	21	male	food	24.9	2.9	3.18	84.6	81.1	MA
8	50	male	Food	24.6	4.3	4.12	88.3	87.1	SA
9	31	female	grasses	35.7	3.2	3.1	82.7	87.2	MA
10	38	male	Grasses	24.6	1.56	1.62	61.4	59.5	SA
11	36	female	grasses	26.8	2.29	2.59	97.1	96.3	MA
12	34	female	Tree Grasses	28.6	3.32	3.33	83.8	87.3	MA
13	29	male	Food	30.5	3.38	3.05	78.1	70.6	SA
14	43	female	Tree Grasses	36.3	2.31	2.62	97.9	97.1	MA
15	38	male	food	33.2	2.4	2.6	96.4	96.1	MA
16	33	male	grasses	29.7	3.87	4.38	87.2	83.3	MA
17	40	male	Tree Grasses Food	25.4	3.48	4.05	83.1	88.2	SA
18	51	female	Tree Grasses Molds	27.1	2.46	2.43	90.1	85.8	SA
19	50	female	Grasses	28.2	2.35	2.35	94.6	97.9	MA
20	36	male	Grasses	25.4	3.2	3.69	80.1	77.2	MA
21	31	male	Grasses	24.3	3.3	3.71	80.9	77.5	MA
22	32	female	Tree Grasses	30.7	1.01	2.91	79.2	83.2	MA

P: Patient, Y: Year, MA: Mild to moderate Asthma, SA: Severe Asthma, BMI: Body Mass Index, FEV1: Forced Expiratory Volume in 1 second, FVC: Forced Vital Capacity.

Bioinformatic Analysis

To find functional targets of miR-223, two databases were taken into consideration; TargetScan comprising of potential targets[8] and miRTarBase containing experimentally-validated targets.[9] To find biological pathways associated with these genes, the Enrichr web tool was used (The Database for Annotation, Visualization and Integrated Discovery, DAVID). All data preprocessing and analysis were done using Bash scripting and R 3.5.2 software. Significantly enriched pathways visualized with an adjusted p value$\leq$0.05.

Ethics

This study was accepted by the Research Committee of Immunology, Asthma and Allergy (IAARI) and National Institute for Medical Research Development (NIMAD) and approved by the Ethics Committee of IAARI (IR.TUMS.IAARI. REC.1396.4899). All patients have answered the questionnaire and signed the written informed consent.

RESULTS

Subjects

Twenty-two asthmatic patients (mean age 38, 11 male/11 female) and 24 healthy controls (mean age 35, 13 male/11 female) were entered this study. Seven patients had severe asthma and 15 patients had mild to moderate asthma based on clinical and paraclinical tests. All patients had allergic reactions to various allergens (aero and food allergens such as Mugwort, rough pigweed, Russian thistle, ash, mustard, walnut, and strawberry, etc). Plasma exosomes from the samples were morphologically confirmed by SEM and DLS. Exosomes are spherical particles with an average size of 70.52 nm, as shown by SEM analysis, (Figure 1A). The results of SEM demonstrated that exosomes were uniform in size to those measured by DLS (Figure 1B).

Increased Expression Level of MiR-223 and MiR-21 in Patients with Moderate Asthma

Statistically significant up-regulation of miR-223 (p=0.002) and miR-21 (p=0.006) were found in moderate asthmatic patients compared to controls. The expression of miR-21 was significantly decreased in severe asthmatic patients compared to controls (p=0.02) (Figure 2).

According to the ROC curve and AUC (Area under the curve) estimation, miR-223 and miR-21had 83% and 76% probability of asthma diagnosis in patients with moderate asthma compared to controls; respectively (Figure 3A and 3B). Moreover, these miRNAs also were positively correlated in patients with moderate asthma (p=0.01, r=0.4) (Figure 3C).

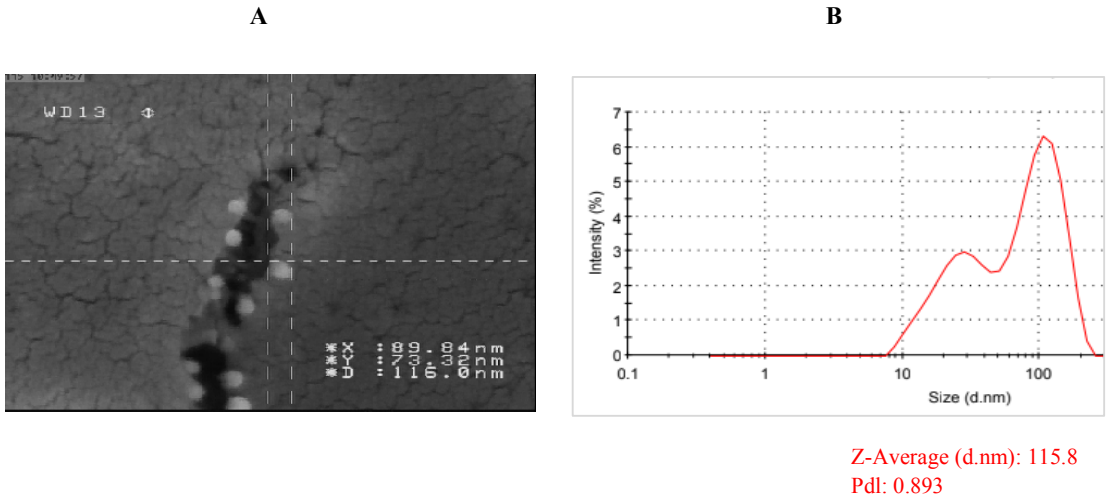

Figure 1. Characterization of the plasma exosomes: Scanning electron micrographs of exosomes isolated from plasma (A). The sample size distribution of exosomes by dynamic light scattering (DLS) in asthmatic patients and healthy control subjects (B). Z average (the hydrodynamic diameter), PDI (the polydispersity index).

Exosomal Absence of MiR-125, MiR-126, MiR-155 in Samples

Almost no expression of miR-125, miR-126, and miR-155 was found in plasma exosomes by Real-time PCR in this study. The quality of the qPCR data for 3 miRNAs was assessed by a single PCR and gel electrophoresis but the latter did not show any single product between 50 and 100 nucleotides in length.

The other 5 miRNAs (miR-16, miR-Let7, miR-148, miR150, miR-146) were not differentially expressed (p>0.05) in patients compared to healthy control samples (Figure 2).

Bioinformatics Data

Data processing and analysis showed that miR-223 is involved in biological pathways including MAPK, FOXO, cGMP-PKG, cAMP-Insulin, C- type lectin, AMPK, phosphatidylinositol signaling pathways which are related to allergic diseases. Involved miR-21biological pathways related to allergic diseases are MAPK, PI3K-AKT, RAS, Cytokine-cytokine receptor, FOXO, TNF, Hippo, HIF-1, Toll-like receptor, Jak-STAT, Focal adhesion, Chemokine, TGF-beta, cGMP-PKG, mTOR, Insulin, Th17, NF-kappa B, T cell receptor, IL17, ErbB, B cell receptor signaling pathways (Figure 4A and 4B).

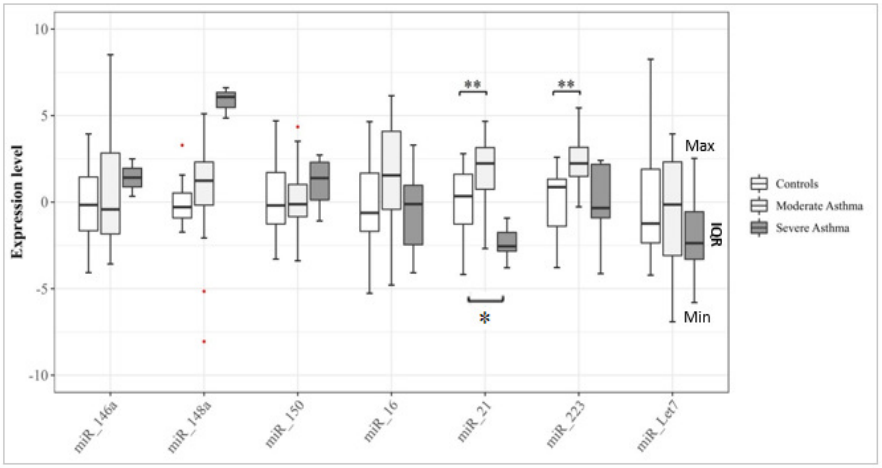

Figure 2. Boxplot with outliers (red points) comparing selective microRNAs (miRNAs) relative expression levels in 22 patients with moderate or severe asthma and 24 healthy control subjects.
***: p<0.05, **: p<0.01, Min: Minimum, Max: Maximum, IQR: Interquartile range**

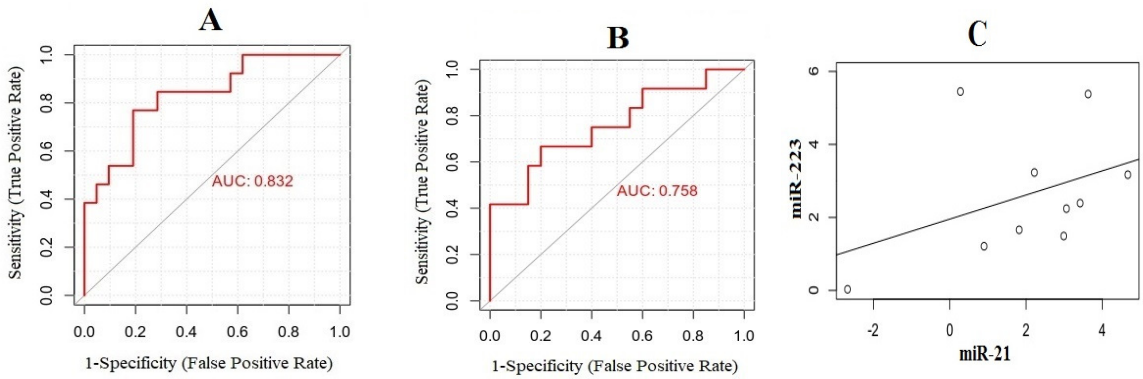

Figure 3. ROC curves for miR-223 (A) and miR-21 (B), and a scatter-graph presenting the correlation between miR-223 and miR-21 in 10 patients with moderate asthma (r=0.4) (C).

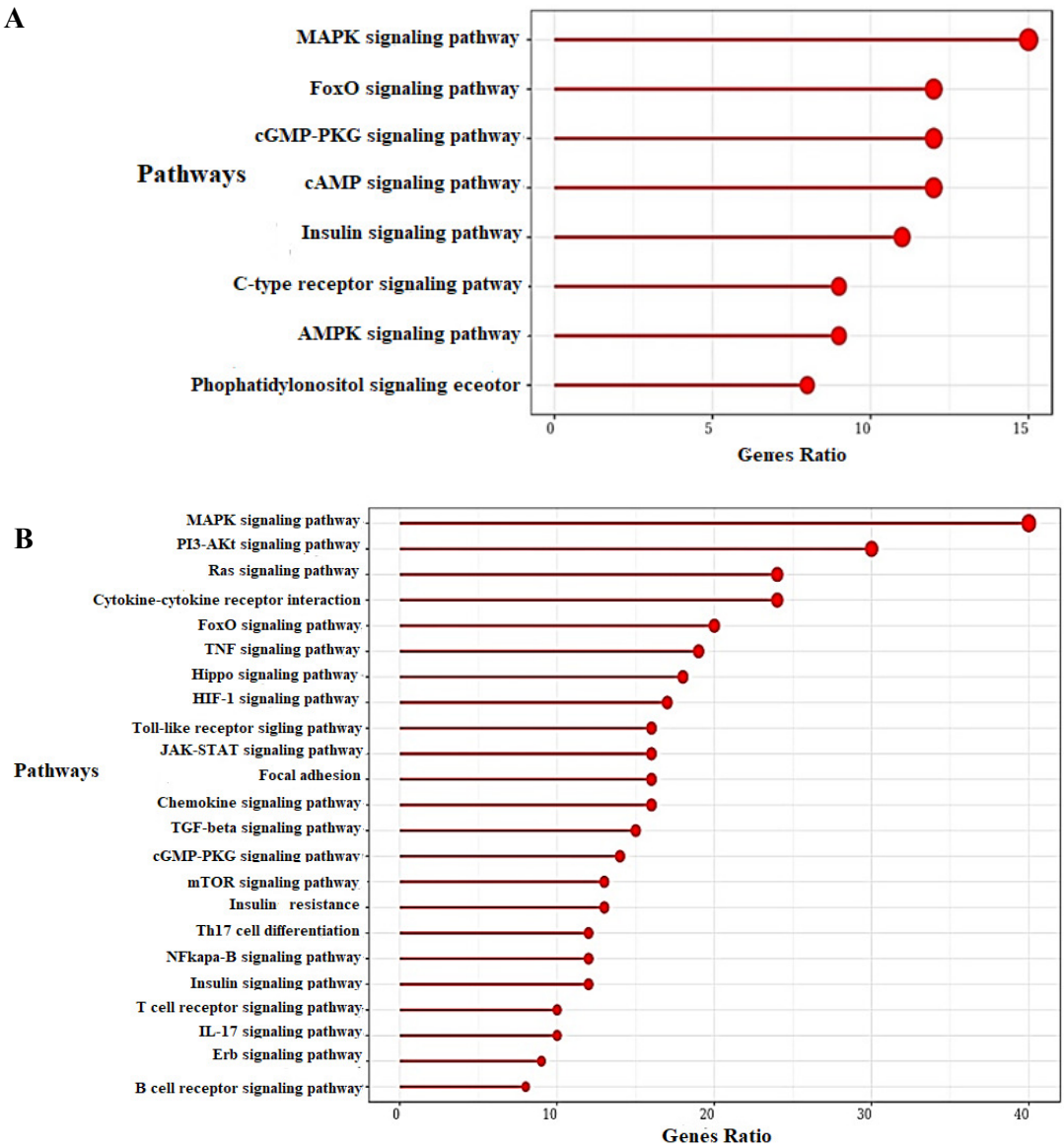

Figure 4. Allergic asthma-associated signaling pathways with miR-223 (A) and miR-21; using DAVID (https://david.ncifcrf.gov/) (B)

DISCUSSION

In this study, the expression of selected miRNAs from plasma-derived exosomes in patients with allergic asthma was compared with those of healthy control subjects.

Among selected miRNAs in the current study, significant upregulation of miR-223 and miR-21 were shown in moderate asthmatic patients. No significant differential expression of miR-223 was found between severe asthmatic patients and healthy controls. However, significant downregulation of miR-21 was shown in severe asthmatic patients compared to moderate asthma and control groups. The diagnostic potency of miR-223 and miR-21 and their positive correlation in patients with moderate asthma was demonstrated in our study.

Johnnidis et al, proved that miR-223 could physiologically regulate the generation and function of granulocytes. They showed increased numbers of neutrophils in the bone marrow and blood of mice lacking miR-223 with hyperplasia in BM and also neutrophil hyper-segmentation in blood.[10] The increase of eosinophil progenitor growth and delay in its differentiation was identified in mice lacking miR-223.[11] More studies in humans have been demonstrated high expression of miR-223 in hematopoietic cells and its contribution to neutrophil and macrophage differentiation and activation in inflammatory diseases.[12] Maes et al, reported upregulation of miR-223 in sputum samples from patients with severe neutrophilic asthma. They found a positive correlation (r=0.38) between miR-223 and neutrophil counts in severe asthmatic patients.[13] However, no significant differential expression of miR-223 was revealed in bronchial biopsies from severe asthmatic patients compared to patients with mild asthma and controls.[11]Complete overlap of miR-223 expression has been reported in mast cells from patients with allergic asthma and control subjects.[14] The study by Xiaoyiet al, has suggested that miR-223 could induce macrophage 2 (M2) (12). Pierre et al, have shown upregulation of CD206 and MHC II in M2 isolated from bronchoalveolar Lavage fluid (BALF) of asthmatic patients,[15] this feature might have a role in the development of Th2 response and asthma pathogenesis. Taking these findings together, it may be concluded that the induction of M2 by miR-223 results in Th2 response in allergic asthma. Overall, there is not enough validated data about miR-223 expression as diverse cells (epithelium, sputum, mast cells), plasma, and exosome (this study) have been investigated in different phenotypes of asthma.

The upregulation of miR-21 in moderate asthmatic patients in this study was comparable to the results of previous studies. However, low expression of miR-21 was shown in severe asthmatic patients compared to healthy controls. It is speculated that activated NK cells could promote eosinophils apoptosis in patients with severe asthma.[16] Significant lower expression of miR-21 in severe asthmatic patients may be related to low levels of IL-13 and non-eosinophilic asthma as indicated in the other studies.[17]

Lu et al, have shown that miR-21 is the most upregulated miRNA in murine models of allergic asthma. Inhibition of IL-12 expression by miR-21 may increase Th2 response and IgE secretion and eosinophil activation to produce IL4, IL5, and IL13.[17] The significant high expression of circulating miR-21 was shown in children with recurrent wheeze or asthma.[18] Significant positive correlation was previously shown between miR-21 and IL-13 and eosinophil percentage.[16] Treatment with miR-21-specific antagomir or pan-PI3K inhibitor reduced PI3K activity and restored histone deacetylase expression and led to the suppression of airway hyper-responsiveness and steroid sensitivity to allergic airway disease, highlighting miR-21 as a novel therapeutic target for this asthma group.[19]

The positive correlation (r=0.4) of miR-223 and miR-21 was demonstrated in this study. In a murine model of asthma, upregulation of both miR-21 and miR-223 was shown in bone marrow-derived eosinophils.[4] Moreover, in deficient MiR-21 mice, an increased apoptosis rate was shown during eosinophils progenitors' maturation state while more eosinophil progenitor proliferation was demonstrated in miR223-deficient mice.[20]

Bioinformatics analysis showed that miR-223 is involved in eight different pathways that are associated with asthma. For example, miR-223 targets insulin-like growth factor 1 receptor (IGF-1R) and this suppresses RAS/mitogen-activated protein kinase (RAS/MAPK) and phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) pathways and promote mast cells apoptosis, and affect their degranulation.[21]

Involved miR-21 in twenty-three different biological pathways related to asthma have been shown in this study. MiR-21 inhibits PTEN expression and promotes AKT activity in MAPK and B cell receptor signaling pathways. On the other hand, AKT activates the mammalian target of the rapamycin (mTOR) signaling pathway which is effective in producing IgE.[22] Meanwhile; miR-21 activates the epidermal growth factor receptor/ AKT (EGFR/AKT) and ErbB signaling pathways and enhances hypoxia-inducible factor-1 alpha (HIF-1α) expression by targeting tumor suppressor genes such as PTEN and FOXO1. This, in turn, upregulate STAT3 and production of IL 17, IL-4, IL-5, IL-17, and CCL2/CCR2) in the lung, therefore, it may increase airway inflammation in asthmatic patients.[23,24]

The non-significant expression of selected miRNAs between patients and healthy controls may due to different endotypes of asthma and a limited number of

the patients in the current study. Moreover, the SYBR Green method may not be suitable for identifying all miRNAs. As extracted miRNAs from exosomes may not have enough concentrations in exosomes. The other important issue is the effect of the environmental factors which influence the epigenetics mechanisms and miRNAs expression.[25]

In this study overexpression of miR-223 and miR-21 and their correlation were shown in patients with allergic moderate asthma therefore, they could be used as potential biomarkers in the diagnosis of moderate asthma. Looking at miR-223 and miR-21 target genes will shed light on their molecular properties in asthma and may provide a novel therapeutic to control asthma.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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