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Serum Exosomal Expression of miR-155 and miR-221 in Moderate-to-severe Asthmatic Patients

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ABSTRACT

The cardinal features of asthma include airway inflammation, airway hyper responsiveness (AHR) and airway remodeling. Exosomes help orchestrate the immune response and contain microRNAs (miRNAs) such as miRNA-155 and miRNA-221 which play significant roles in the pathogenesis and exacerbations of severe asthma. In this study, we aimed to investigate the exosomal expression of miRNAs (155, 221) in the serum of severe asthma patients.

Eighteen moderate-to-severe asthma patients and eighteen healthy subjects were recruited for this study. Serum exosomes were isolated and characterized according to their shape, size, and exosomal markers by transmission electron microscopy, dynamic light scattering (DLS) and flow cytometry, respectively. Exosomal miRNA extraction and quantitative real-time PCR (qRT-PCR) were used to measure miR-155 and miR-221. Besides the forced expiratory volume in 1 second and forced vital capacity (FVC) were evaluated in the patient groups.

Round exosomes with a mean size of 25.8 nm were isolated from serum of asthmatic patients. Flow cytometry shows high expression of CD63 and CD81 on isolated exosomes. Serum exosomes from severe asthma patients and healthy donors contained miR-155 and miR-221 but miR-155 and miR-221 expression levels were significantly increased in severe asthma patients. There was a positive correlation between miR-221 expression and FVC).

Receiver operating characteristic (ROC) analysis indicated that miR-155 and miR-221 had an excellent diagnostic efficiency in predicting asthma (AUC=0.91 and AUC=0.76, respectively). Serum exosomal miR-155 and miR-221 may be a potential biomarker for severe asthma.

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However, the results need to be validated in another cohort, and further studies with larger samples size should be conducted on the effects of these miRNAs on effector cells.

Keywords: Exosome; miRNA-155; miRNA-221; Serum; Severe asthma

INTRODUCTION

Asthma, characterized by chronic airflow obstruction and airway inflammation, represents a significant global health burden.[1] Despite advancements in treatment, severe asthma presents considerable therapeutic challenges, marked by persistent symptoms despite high-dose corticosteroid therapy.[2]

Notably, severe asthma is distinguished by increased airway smooth muscle (ASM) mass and subepithelial fibrosis compared to non-severe asthma [3] . MicroRNAs (miRNAs), crucial regulators of gene expression, have garnered attention for their roles in asthma pathophysiology.[4] Specifically, miR-155 and miR-221 have been implicated in processes such as epithelial-mesenchymal transition (EMT) and airway remodeling.[5,6] While miR-155 contributes to Th2 cell-mediated inflammation and lung airway remodeling, miR-221 regulates ASM cell proliferation and cytokine release, particularly in severe asthma.[6,7] Recently, the role of extracellular vesicles as key players in the pathogenesis of the disease has been significantly appreciated.[8] In the majority of cases, exosomes contain molecular cargo, including miRNAs, which may play an important role in intercellular communication and therapeutic delivery systems in asthma.[9] A number of miRNAs have been implicated in asthma pathophysiology, where they affect gene expression and protein synthesis in the airways.[10] For example, miR-155 and miR-221 play an important role in EMT and remodeling.[11,12] Mice lacking miRNA-155 display increased lung airway remodeling,[13] and miR-155 is essential in T helper 2 (Th2) cell-mediated eosinophilic inflammation in ovalbumin (OVA) mouse models of asthma.[14] In contrast, miR-221 regulates airway smooth muscle (ASM) cell proliferation and IL-6 release in patients with severe asthma.[6] Thus, in the current study, we hypothesize that dysregulation of miR-155 and miR-221 occurs in severe asthma and can be detected in circulating exosomes, shedding light on their diagnostic and therapeutic implications in severe asthma management.

microRNAs (miRNAs) belong to a small class of endogenous, single-stranded noncoding RNAs of approximately 19 to 24 nucleotides that regulate gene expression at the post-transcriptional level.[15,16] miRNAs play critical roles in many biological processes, such as cell proliferation, apoptosis, and cell differentiation.[17,18] Dysregulated miRNA production has been reported in an increasing number of chronic inflammatory diseases.[19-20] Exosomes are small (10–150 nm) membrane-bound vesicles. They can be released from cells upon activation and play a crucial role in intercellular signaling and communication.[22,23] Exosomes contain proteins such as HSP70, CD9, CD81, CD63 and CD82 which represent exosomal markers.[22,24-26] They are secreted by all types of cells and shuttle DNA, RNA, miRNAs and proteins between cells and significantly affect the normal and pathological states of recipient cells.[25,27]

Exosomes derived from various cells and modified exosomes have the ability to deliver many kinds of cargo to the target cell.[28,29] Thus, exosomes are professional transporters and a possible tool for the therapeutic delivery of small interference RNAs (siRNAs), miRNAs and, potentially acting as anti-inflammatory agents.[22,30] Furthermore, miRNA signatures from both unfractionated whole serum, urine, and saliva[30] and from exosomes[31] could be diagnostic for diseases or therapeutic interventions. Exosomal packaging of miRNAs protects them from digestion by RNases and enhances their stability.[32]

During EMT, a decline in epithelial cell surface markers and an upregulation of mesenchymal proteins results in an accumulation of myofibroblasts and fibroblasts in airways.[11,33,34] We hypothesize that the airway expression of these two miRNAs is dysregulated in severe asthma and that this dysregulation is detectable in circulating exosomes. Thus, we evaluated miR-155 and miR-221 expression levels in serum exosomes from severe asthma patients.

MATERIALS AND METHODS

Patients

Eighteen patients diagnosed with moderate to severe asthma, aged (43.11±9.13 years old, mean±SD) were recruited at the Masih Daneshvari hospital (Tehran, Iran) between April 2018 and April 2019. Asthmatic subjects had a history of asthma for at least 6 months, as defined by the clinical guidelines of the Global Initiative for Asthma (GINA, 2019, https://erj.ersjournals.com/content/53/6/1901046). Patient demographics and treatment are shown in Table 1. Subjects suffering from infections, including pneumonia, smokers, and those with any type of systemic disease or neoplasms were excluded from the study [33]. No patient with asthma had suffered an exacerbation in the 2 months prior to recruitment. Eighteen healthy subjects (34.23±12.24 years old, mean±SD) with a negative history of shortness of breath or wheezing were also recruited as a healthy comparator group (Table 1). Inclusion and exclusion criteria were based on recent GINA guidelines (2019).[33] Forced expiratory value in one score (FEV1) and forced vital capacity (FVC) were determined in all patients. None of the subjects in either group were smokers.

Serum Exosome Isolation

Whole blood samples (5 mls) were collected in anti-coagulant-free tubes from all study participants. Serum was prepared by centrifugation at 1600 x g for 10 minutes at room temperature) and exosomes were isolated using a total exosome isolation kit (Invitrogen Life Technologies, CA, USA) following the Manufacturer's protocol. In brief, serum samples were centrifuged at 2000 x g for 30 minutes to remove cells and debris. The serum supernatant (600 μL) was transferred to a new tube without disturbing the pellet and 100μl of total exosome isolation reagent was added and vortexed. After incubation at 2-8°C for 30 minutes, the samples were centrifuged at 10,000 x g for 10 minutes at room temperature. The supernatant was aspirated and the remaining pellet contained the exosomes. The pellet was resuspended in a 200 μL of 1X PBS and stored at -70°C until analyzed.

Exosome Characterization

Transmission Electron Microscopy

Electron microscopy studies were used to identify the isolated multivesicular bodies (MVBs) from serum and to verify their size and characteristic cup-shaped morphology as described previously.[35,36] Briefly, purified exosome pellets were resuspended in 2% paraformaldehyde and loaded onto formvar carbon-coated grids and dried for 20 minutes at 40°C. The exosome-loaded grids were washed in PBS, fixed in 1% glutaraldehyde and subsequently rinsed in distilled water several times. The grids were transferred to 4% uranyl-oxalate solution for 5 minutes and finally embedded in a mixture of uranyl acetate (4%) and methyl cellulose (2%) on ice. Mounted exosomes were then examined by electron microscopy.

Table 1. Demographic information of study participants

	Non-asthmatic healthy controls	Patients with Severe Asthma
NO	18	18
Age (year), mean±SD	34.23±12.24	43.11±9.13
Sex, (Male/Female)	12/6	10/8
FEV1 (%), mean±SD	Not Determined	49.39±22.09
FVC (%), mean±SD	Not Determined	63.39±20.49
Atopic (%)	None	66.6
Therapies	None	ICS as part of combination therapy (Fluticasone/salmeterol, (Seretide^{R}) at a dose 1000/100 μg

FEV1: Forced expiratory value in one second; FVC: Forced vital capacity; ICS, Inhaled Corticosteroids; CTLA-4: Cytotoxic T-lymphocyte associated protein 4

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Dynamic Light Scattering (DLS) Analysis of Exosomes

DLS was used to determine the size distribution of the isolated exosomes using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) as described earlier.[35]

Exosome Bead Coupling and Flow Cytometry

The isolated exosomes were fixed on latex microbeads (Invitrogen, Carlsbad, CA) of a size that falls within the range of flow cytometry detection (3.9 μM) overnight as described earlier (35). After washing with PBS and staining with anti-CD81/FITC and anti-CD63/PE antibodies (BioLegend (San Diego, CA, USA). Isotype controls (mouse IgG/FITC and mouse IgG/PE) were used to eliminate fluorescent signals caused by any nonspecific binding (BD Biosciences, San Jose, CA, USA).

Exosomal RNA Isolation

Total RNA was isolated from exosomes using the *mir*Vana™ miRNA Isolation Kit (Life Technologies, CA) according to the manufacturer's instructions. The eluate (which contains the RNA) was collected and stored at -80°C (37).

miRNA Detection by qRT-PCR

For the evaluation of exosomal miR-155 and miR-221, total RNA was measured in a nanodrop 2000 spectrophotometer (Thermo Scientific, Rochester, New York**,** USA) and 200 ng was reverse-transcribed to cDNA using the Universal cDNA Synthesis Kit following the manufacturer's protocol (miRCURY LNA RT Kit-QIAGEN, Maryland, USA).

qRT-PCR was performed under the following conditions: 95°C for 10 minutes, 50 cycles of 95°C for 10 seconds, 60°C for 60 seconds, and 72°C for 5 seconds. The U6 gene was used as an endogenous control. The relative expression of each miRNA was calculated using the 2-ΔΔCt method as described before.[37] miRNA primers for miR-221 were purchased from BonYakhte (Tehran, Iran) with Stem Loop: 5′GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACGTTTCC-3′, Forward: 5′-CGCGCAAGCTACATTGTCTGCT-3′ and Reverse: 5′-CCAGTGCAG GGTCCGAGGTA-3′ and for miR155 (Has-miR-155) from Qiagen (catalog number YP00204308). Quantitative PCR was performed using a Roche Real-time PCR System (Roche, Manheim, Germany).

Statistical Analysis

The qRT-PCR data were analyzed using the 2^-ΔΔCt method. The statistical analyses were conducted using the GraphPad Prism 6 software. The D'Agostino-Pearson normality test was applied to determine whether the data follows a normal distribution in which case a student's t-test was applied. For data with a non-parametric distribution, the Mann-Whitney *U* test was used. For all assays $p<0.05$ was considered statistically significant.

RESULTS

Characterization of Serum Exosomes

Transmission electron microscopy (TEM) confirmed the round shape of the isolated exosomal vesicles. The exosome diameter ranged from 30-100 nm in both healthy control and severe asthma patients which was consistent with the characteristic size range of exosomes (Figure 1A and B). Size characterization of exosomes using DLS confirmed that the size of the isolated serum exosomes from the healthy control and severe asthmatic patients was 25-85nm (Figure 1C and D). The exosomal markers CD63 (Figure 2A) and CD81 (Figure 2B) were present on isolated serum exosomes from severe asthmatic patients.

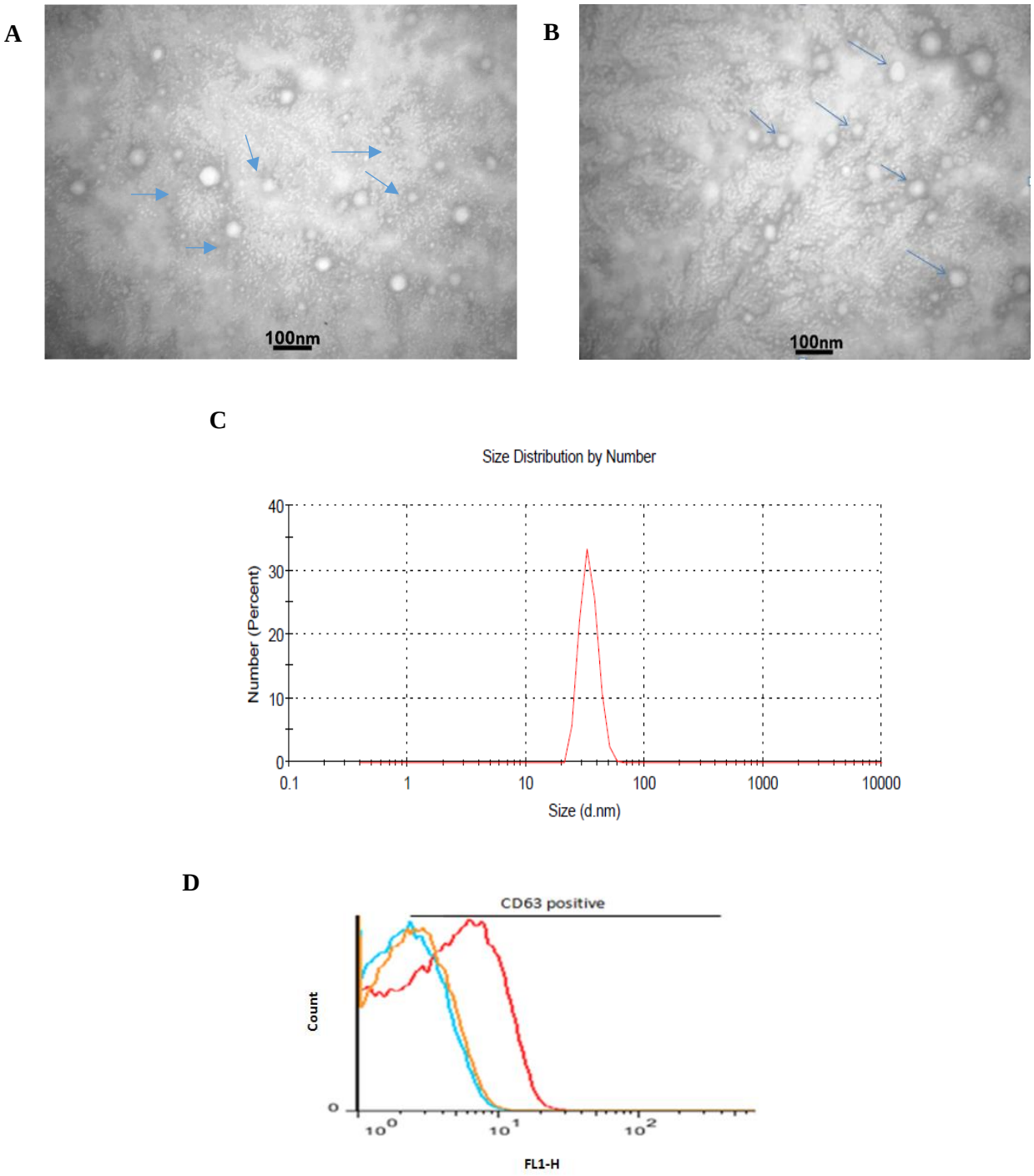

Figure 1. Evaluation of exosomes isolated from asthmatic patients. Representative electron microscopy image of serum-derived exosomes from Healthy control and severe asthma patient (B). The size range of approximately 30-100nm in results are representative of three independent experiments. The bar shows size of 100 nm in two healthy control (C) and severe asthma patients (D)Results from a representative dynamic light scattering (DLS) analysis of exosomes isolated from the serum of a severe asthmatic patient.

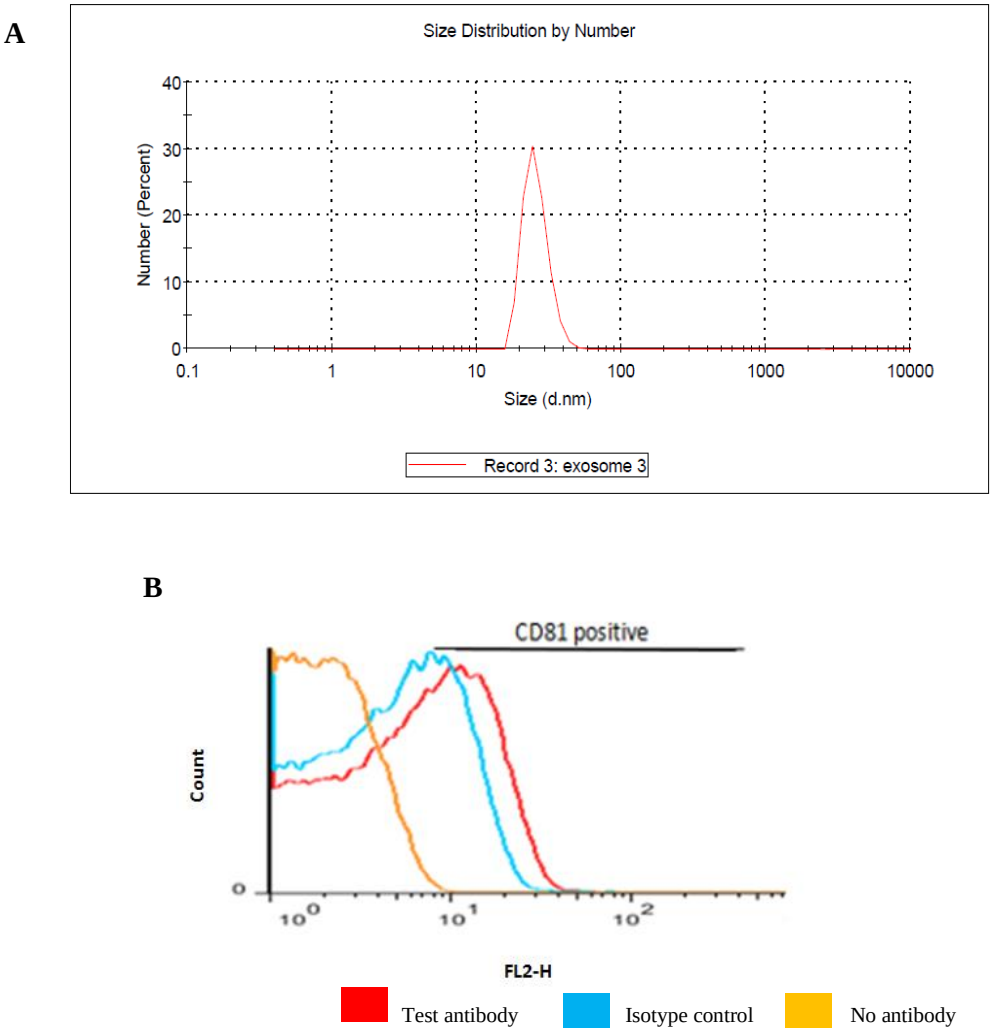

Figure 2. Flow cytometric analysis of CD63 and CD81 expression in isolated exosomes from severe asthmatic patients
(A) Representative flow cytometric plot of CD63 (red curve) staining intensity of serum-derived exosomes. The intensity of the isotype control (blue curve) and no antibody control (orange) is also shown. (B) Flow cytometry analysis of CD81 expression by serum-derived exosomes. Serum-derived exosomes were stained with CD81 (red curve) and compared with an isotype (blue curve) or no antibody (orange) control.

Exosomal miR-155 and miR-221 Expression

The expression of both miR-221 (13.22±22.4 vs 3.06±7.5, $p \leq 0.005$, Figure 3A, Table 2) and miR-155 (12.5±20.66 vs 0.28±0.61, $p < 0.0001$, Figure 3B, Table 2) was upregulated in serum exosomes from severe asthmatic patients. Atopic status didn't affect expression of miR-155 ($p > 0.99$) or miR-221($p = 0.43$) by serum exosomes (Table 2). Receiver operating characteristic (ROC) analysis indicated that miR-155 (AUC=0.91, $p \leq 0.0001$, Figure 4A) has a greater diagnostic efficiency for predicting severe asthma than miR-221 (AUC=0.76, $p \leq 0.006$, Figure 4B).

Pearson Correlation Between miR-155, miR-221 Expression and FEV1, FVC in Patients

There was a positive correlation between miR-221 expression in serum exosomes and FVC (r=0.47, $p \leq 0.04$) but no correlation between FEV1, FEV1/FVC ratio or between miR-155 expression in serum exosomes and either lung function parameter was observed (Table 3).

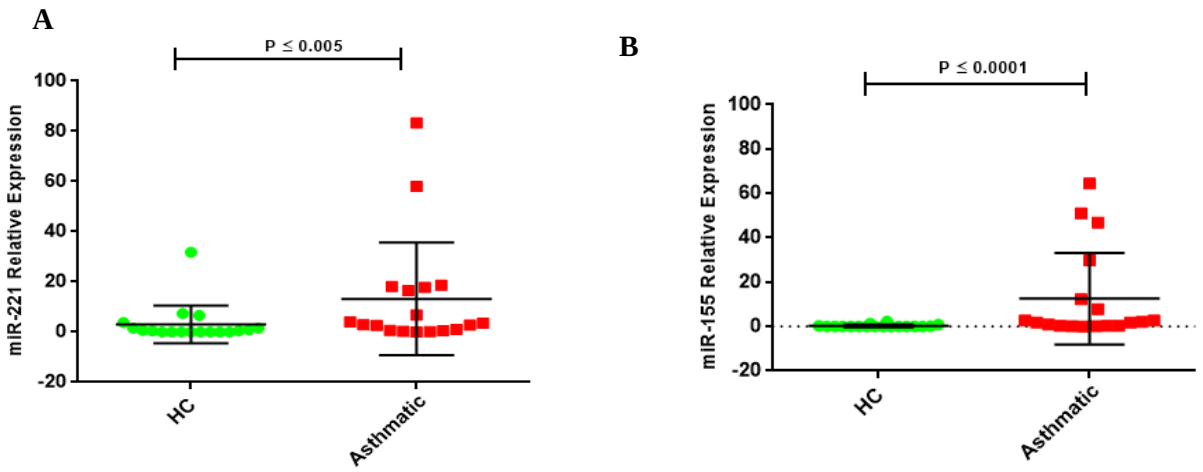

Figure 3. Expression of miR-221 and miR-155 by exosomes isolated from patients and healthy control subjects (HC). (A) The expression of miR-221 was upregulated in serum exosomes of severe asthmatic patients compared to HC. (B) Up-regulation of miR-155 expression in serum exosomes of severe asthmatic patients compared to HC. Significance values are as indicated on the graphs.

Table 2. miR-155 and miR-221 expression in healthy subjects and patients

Healthy Control (HC)	Severe Asthma (SA)				*p* HC vs SA	*p* Atopic vs Non Atopic
		Atopic	Non atopic	Total		
NO	18	12	6	18		
miR-221 (mean±SD)	3.06±7.5	11.43±23.52	16.81±21.57	13.22±22.4	$p \leq 0.005$	0.43
miR-155 (mean±SD)	0.28±0.61	15.63±23.79	6.25±11.59	12.5±20.66	$p < 0.0001$	$p > 0.99$

Table 3. Pearson correlation between miR-155 and miR-221 expression and lung function in severe asthma patients

	FEV1		FVC		FEV1/FVC	
	r	*p*	r	*p*	r	*p*
miR-221	0.31	0.21	0.47	0.04	-0.11	0.64
miR-155	0.01	0.94	0.10	0.68	-0.19	0.43

FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second

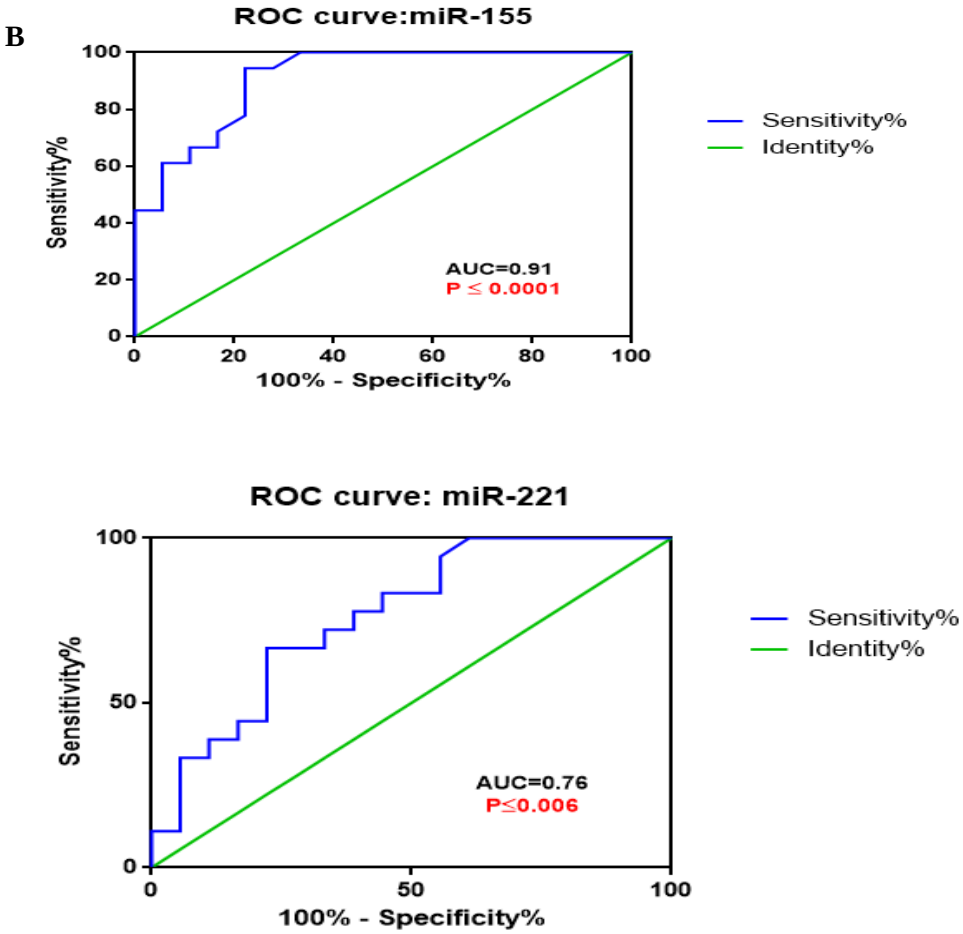

Figure 4. Receiver operating characteristic (ROC) curve for miR-155 (A) and (B) miR-221 expression in the severe asthma patients. ROC analysis indicated that miR-155 (AUC=0.91, P≤0.0001) has a greater diagnostic efficiency for predicting severe asthma than miR-221 (AUC=0.76, P≤0.006).

DISCUSSION

Our study delves into the potential of serum exosomal microRNAs (miRNAs), specifically miR-155 and miR-221, as diagnostic biomarkers for severe asthma. Notably, we observed a significant upregulation of both miRNAs in exosomes isolated from the serum of severe asthma patients compared to healthy controls. Additionally, a positive correlation between miR-221 expression and forced vital capacity (FVC) in asthmatic patients was identified. These findings suggest that serum exosomal miR-155 and miR-221 may serve as promising diagnostic markers for severe asthma. Our results align with previous studies indicating dysregulated miRNA expression in asthmatic diseases. For instance, studies have reported increased miR-155 levels in various cellular compartments of asthmatic individuals, highlighting its role in asthma pathogenesis.[38] Moreover, miR-155 has been associated with modulating the anti-inflammatory effects of corticosteroids and regulating allergic airway inflammation, corroborating our findings of elevated miR-155 expression in severe asthma patients.[38,39]

Previous studies have demonstrated increased miR-221 expression in leukocytes and airway smooth muscle cells of individuals with asthma, suggesting its involvement in disease severity.[6,40] Consistent with our findings, these studies underscore the clinical relevance of miR-221 as a biomarker for assessing asthma severity and lung function impairment.

Moreover, receiver operating characteristic (ROC) analysis shows the diagnostic efficiency of serum

exosomal miR-155 and miR-221 in predicting severe asthma. These results are aligned with other studies that have explored the diagnostic potential of miRNAs in asthma. For instance, a study by Lu et al. demonstrated the diagnostic utility of miRNAs in distinguishing asthma phenotypes, emphasizing their role as non-invasive biomarkers.[41]

Despite the promising diagnostic value of serum exosomal miRNAs, several limitations warrant consideration. Our study underscores the need for replication in larger multi-center cohorts to validate our findings. Furthermore, elucidating the underlying mechanisms of exosomal miRNA dysregulation in severe asthma and exploring their therapeutic implications remain important areas for future research. The current study indicates that the levels of serum exosomal miRNA-155 and, to a lesser extent miRNA-221, may be a good predictor of severe asthma. This pilot study requires replication in a larger multi-center setting, alongwith further research to define the mechanism of expression of these exosomal miRNAs and whether they may have possible therapeutic applications. The amount and components of exosomal miRNAs vary between patients and controls. Therefore, exosomal miRNAs are useful as non-invasive biomarkers to indicate the degree of disease.

An important limitation of the current study was the absence of a mild and moderate asthma control group. Additionally, considering the potential for miR-221 to modulate airway remodeling, the lack of measures of airway reversibility or computed tomography. In addition, we did not formally test for atopic status and whether the presence of type 2 inflammation might affect the results. Thus, due to the above-mentioned critical points, the current study had a small size, and were evaluation of asthmatic patients only by FEV1 and FVC, therefor for the extending conclusion further studies should be done for the final outcome.

Given the limitations, such as the type of samples (blood), PCR methods (with own limitation) and sample size we could not able to claimed that miRNA-221 capable to in patients with severe asthma improve FVC by downregulation of ASM proliferation, thereby improving airflow obstruction and asthma control.

By corroborating previous findings and highlighting the clinical relevance of these miRNAs, our study paves the way for further research aimed at harnessing miRNAs as biomarkers for asthma diagnosis and disease severity assessment.

STATEMENT OF ETHICS

The Ethics Committee of the Dr. Masih Daneshvari Hospital approved the study and all subjects gave written informed consent (Ethics committee approval number: IR.SBMU.NRITLD.REC.139.083).

FUNDING

Not applicable

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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We acknowledge all study participants.

DATA AVAILABILITY

Describe how readers can access the data Upon reasonable request (author)

AI ASSISTANCE DISCLOSURE

If applicable, list any artificial intelligence (AI) tools used in preparing this manuscript.

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