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Differential expression of miR-155 and Let-7a in the plasma of childhood asthma: Potential biomarkers for diagnosis and severity

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ABSTRACT

Background: MicroRNAs (miRNAs) are emerging gene expression regulators and their expression has been linked to various biological processes. However, the role of miRNAs in the regulation of allergic inflammatory disease still not clearly understood.

Aim: Our study was designed to investigate circulating miR-155 and Let-7a expression levels in the plasma of asthmatic children and healthy controls. Also, to correlate their expression levels to degree of severity of asthma as well as to IL-13 level and lung function parameters.

Method: Our study included 100 asthmatic children and 100 healthy children as control group. Plasma miR-155 and Let-7a expression levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: MicroRNA-155 expression was significantly increased in the plasma of asthmatic children than in control children. While, Let-7a expression was significantly lower in asthmatics than in control children. The relative levels of miR-155 and Let-7a were associated with degree of asthma severity. Receiver operating characteristic (ROC) curve analysis demonstrated that the levels of miR-155 and Let-7a were helpful for diagnosis of childhood asthma (AUC were 0.91 and 0.92, respectively), and in prediction of the severity of disease (AUC were 0.83 and 0.80, respectively). Plasma miRNA-155 was correlated positively with Il-13 levels and correlated negatively with FEV1and FVC. While, Let-7a was correlated negatively with Il-13 and correlated positively with FEV1 and FVC.

Conclusion: MicroRNA-155 and let-7a could be used as serological non-invasive biomarkers for diagnosis of asthma and degree of severity. Our results could be used for exploring the pathogenesis of asthma and help in selecting promising therapeutic modalities.

1. Introduction

Asthma is a common chronic lung disorder with inflammation in the airways, that is characterized by reversible obstruction of the airflow, and hyper-responsiveness of the bronchi, with various clinical presentation including recurrent attacks of wheezing, breathing difficulty, cough, and tightness in the chest [1].

The altered immune response in asthmatic individuals could be attributed to both genetic and epigenetic alterations. MicroRNAs (miRNAs) are small, non-coding, RNA molecules that are highly conserved and control the gene expression process by targeting mRNAs causing mRNA degradation, cleavage or inhibition of protein translation [2]. MicroRNAs are responsible for controlling different cell processes, such as differentiation, proliferation, and apoptosis and their dysregulation has been related to various pathological disorders [3]. In allergic airway diseases, the role of miRNAs was evident by influencing general inflammatory and tissue responses [4].

MicroRNA-155, is a multifunctional non-protein coding miRNA, that participate in multiple biological processes including inflammation, immunity and hematopoiesis [5,6]. Moreover, miR-155 has a significant role in the pathogenesis of different allergic diseases, such as allergic rhinitis [7], and atopic dermatitis [8] in addition to autoimmune disease as rheumatoid arthritis [9].

Overexpression of miR-155 has been demonstrated to be involved in asthma development and in activation of allergy promoting cells. MicroRNA-155 regulates Th2 immune responses and gene signaling [10]. A study of miR-155 knock out mice, demonstrated that deficiency of miR-155 resulted in lowering of activated CD4+ T cells, resulting in reduced production of Th2 cytokines (IL-4, IL-5, and IL-13) in the airway [11].

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Let-7 microRNAs belongs to a family of highly conserved microRNA consisting of 12 genes which encodes 9 different micro-RNAs named let-7a to let-7i. The let-7 family is the most abundant miRNAs in the lungs [12]. Let-7a inhibits the expression of IL-13 by direct targeting the 3′ UTR of the IL-13 transcript thus represent a major regulatory process for modulating IL-13 secretion and thereby airway allergic inflammation [13,14].

IL-13, is a cytokine secreted mainly by Th2 cells. It is an important mediator of allergic inflammation in different diseases including asthma [15]. IL-13 in inflammatory conditions acts as central regulator of hyperplasia of goblet cell, IgE synthesis, mucin hyper-secretion, bronchial hyper-responsiveness, chitinase up-regulation and fibrosis [16].

Actually, the use of animal models to study miRNAs role in the pathogenesis of asthma has gained insight into particular facets of the disease and its relevance to humans. So, we conducted this study to examine the use of plasma miR-155 and Let-7a expression as diagnostic non-invasive biomarkers of asthma in Egyptian children and their relation to IL-13 level and lung function parameters and asthma severity.

2. Material and methods

2.1. Study participants

Our study is a case-control study that comprised 100 allergic asthma children (47 males and 53 females, aged 6–10 years) admitted to Zagazig University Children's Hospital between March 2017 and March 2018. From all participants, grouping were considered according to degree of severity of asthma; mild (45 cases), moderate (31 cases), and severe (24 cases). In the participated children, diagnostic criteria for asthma and degree of severity were based on Global Initiative for Asthma guidelines (GINA) [17].

Asthma were considered in children if they have repeated wheezing and sharp breath without evidence of common cold, and if treated with inhalation of a B2 agonist, their forced expiratory volume in 1 s (FEV1) increased by 12% in comparison to prior to the inhalation. All were positive for skin prick test. Exclusion criteria were children having heart, liver, kidney diseases or children having other lung diseases associated with chest tightness, sharp breath, and/or cough. Also, patients who were using concomitant anti-asthmatics or anti-allergic drugs for at least 3 weeks before diagnosis were excluded.

The degree of severity of asthma was evaluated by using FEV1 levels as mild (FEV1, > 80%), moderate (FEV1, 60% - 80%), or severe (FEV1, < 60%).

Additionally, 100 healthy children (55 males and 45 females, aged 6–10 years) with no history of respiratory diseases or chronic illness were selected as the control group.

Approval on the study was obtained from the Ethical Committee of the Faculty of Medicine, Zagazig University and informed consent was obtained from all participated children's parents.

2.2. Lung function assessment

Forced expiratory volume in 1 s (FEV1), and forced vital capacity (FVC) of children in the asthma group were assessed using portable pediatric spirometer (Masterlab Jaeger, Wurzberg, Germany). The patient was instructed to stop using short-acting bronchodilators for a minimum eight hours prior to the test. The results of lung function test were presented as a percentage of that predicted.

2.3. Blood sampling

Peripheral blood samples (4 ml) were collected from participated children under complete aseptic conditions and divided into two portions: Two ml were collected into EDTA-tubes, then the tubes were centrifuged for 10 min (1900 × *g* at 4 °C) and the plasma phases were carefully transferred into RNase-free tubes and then centrifuged again for 10 min (12,000 × g at 4 °C). The other portion (2 ml) was left to clot at room temperature for thirty to sixty minutes and then being centrifuged for 10 min (1000 × *g*) for preparation of the serum. Plasma and serum samples were stored at −80 °C until further analysis.

Table 1
Demographic characteristics, biochemical profiles in healthy controls and asthmatic patients.

Parameters	Asthmatic patients	Healthy control	p
Age (years)	8.2 ± 3.4	7.4 ± 2.9	$t = 1.7$ $p = 0.07$
Gender			
Male	47 (47%)	55(55%)	$P = 0.32$
Female	53 (53%)	45 (45%)	
Family history of asthma			
Positive	70 (70%)	18 (18%)	p = 0.0001
Negative	30 (30%)	82 (82%)	
Asthma severity			
Mild	45(45%)		
Moderate	31(31%)		
Severe	24(24%)		
FEV1(%)	88.8 ± 20.7	99.3 ± 14.1	$t = 4.1$ p = 0.0001
FVC (%)	90.2 ± 12.8	120.1 ± 10.7	$t = 17.9$ $p = 0.0001$
Il-13 level (pg/ml)	10.48 ± 3.54	4.38 ± 1.48	$t = 15.8$ p = 0.0001
miR-155 expression level	6. 4 ± 0.9	3.7 ± 0.5	$t = 26.2$ p = 0.0001
Let-7a expression level	1.9 ± 0.77	3.2 ± 0.69	$t = 12.5$ p = 0.0001

FEV(%) = forced expiratory volume in 1 s, FVC(%) = forced vital capacity.

2.4. Measurement of serum IL-13 levels

IL-13 levels in human serum were immunoassayed using the human IL-13 enzyme-linked immunosorbent assay commercial kit (ELISA kit) (Bender MedSystems, Heidelberg, Germany) following the manufacturer's instructions and the sensitivity of assessment was 1.5 pg/ml.

2.5. Measurement of serum immunoglobulin E (IgE) levels

Total IgE levels were measured using human ELISA AccuBind IgE Kits (Lake Forest, California, USA) following the manufacturer's instructions.

2.6. MicroRNA primer design for RT-qPCR analysis

Specific primers for RT-qPCR of the studied miRNA-155 and Let-7a were designed according to miRBase database (http://microrna.sanger.ac.uk) using miRprimer Software (https://sourceforge.net/projects/mirprimer/). The primers for miR-155 were: forward 5′-GATACTCAT AAGGCACGCGG- 3′ and reverse 5′-GTGCAGGGTCCGAGGT-3′. While the primers for Let-7a were: forward 5′-GCAGTGAGGTAGTAGGTTG-3′ and reverse 5′-GGTCCAGTTTTTTTTTTTTTTAACTATAC-3′.

Also, the housekeeping small RNA (SNORD-68) was used as internal control and its primers sequence were: forward 5-CTCGCTTCGGCAGC ACA-3 and reverse 5–AACGCTTCACGAATTTGCGT-3.

2.7. Extraction of miRNA from plasma samples

Total RNA was extracted from plasma samples using miRNeasy serum/plasma kit (Qiagen, Hilden, Germany). The extracted RNA purity and quality was confirmed by spectrophotometer through measuring the absorbance at 260 and 280 nm.

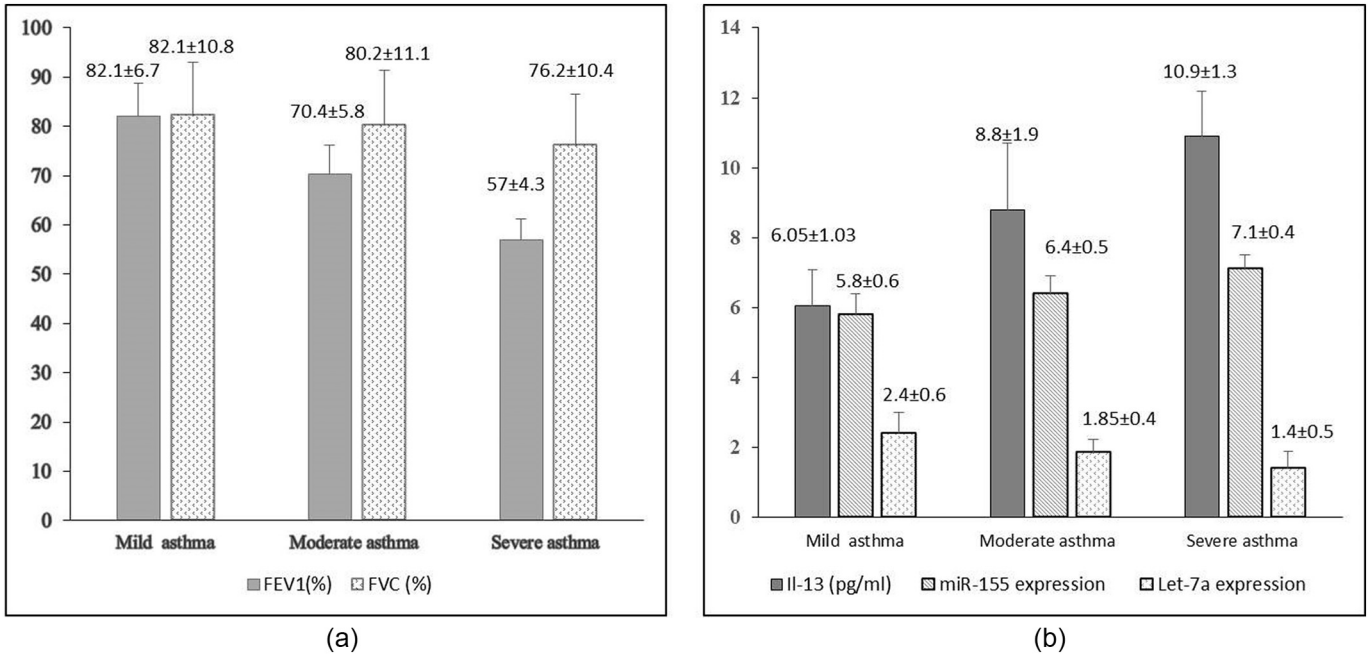

Fig. 1. Demographic characteristics, biochemical profiles in relation to varying degree of asthma (asthma severity) by one way analysis of variance test (ANOVA). Fig. (1a): Lung function parameters in relation to asthma severity ($p = 0.0001$for each). Fig. (1b): Il-13, mir-155 and Let7-a level in relation to asthma severity ($p = 0.0001$ for each).

2.8. Reverse transcription (RT) and quantitative PCR for miR-155 and Let-7a

cDNA was synthesized by reverse transcription reaction using miScript II RT Kit (Qiagen, Germany), according to the manufacturer's instructions and cDNA was stored −80 °C till use.

Quantitative real-time PCR (qPCR) was carried in Stratagene Mx3000p system (real-time PCR system, Agilent Technologies, Germany) using SYBR Green assay kit {mi-Script SYBR® Green PCR Kit with miScript Primer assays (Qiagen, Valencia, CA, USA)}. The reaction mixture was performed in a total volume of 20 μl containing 4 μl of cDNA (100 ng/ml), 300 nM of each primer, and 10 μl of SYBR Green Master Mix and completed to 20 μl with nuclease-free water. Thermal cycling condition was as follow: initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing (was adjusted according to melting temperature suitable for each primer set), and extension at 72 °C for 10 s.

2.9. Normalization of plasma miR-155 and Let-7a expression levels

The housekeeping miRNA SNORD68 (miScript PCR control, Qiagen, Germany) was selected as internal control according to previous studies [18,19]. The expression values of miR-155 and Let-7a were reported as the ΔCt value (calculated by subtracting the Ct values of miRNA SNORD-68 from the Ct values of the target miRNAs) and comparative cycle threshold method (ΔΔCt method). The comparative threshold method was used after a validation experiment demonstrated that efficiencies of target genes and reference (miRNA SNORD68) gene were approximately equal. Assessing the relative efficiencies of the target amplification and the reference amplification was achieved by running standard curves for each amplicon utilizing the same sample. In the present study, it is considered as a passing validation experiment because the absolute value of the slopv be of ΔCt vs. log input is < 0.1 (0.047 for miRNA-155 and 0.035 for Let-7a).

2.10. Statistical analysis

Data analysis was performed using the SPSS statistical software (version 23, IBM, NY, USA). Quantitative data was expressed as (mean ± SD). We used independent-samples *t*-test or one-way analysis of variance (ANOVA) with the least significant difference (LSD) test for comparison between two groups or multiple groups respectively. Spearman's linear correlation analysis was used for correlation between studied parameters. Diagnostic values of the studied miRNAs were analyzed by a receiver operating characteristic curve (ROC curve), with area under the curve (AUC) as the evaluation index. A statistically significant value was considered when $P \leq 0.05$.

3. Results

3.1. Demographic characteristics and biochemical analysis in asthmatic children and healthy controls

There were no significant differences in the age and gender distribution between asthmatic children and control group. However, statistically significant differences were found in family history of asthma, FEV1, and FVC ($P = 0.0001$ for each) between the two-studied groups. The miRNA-155 expression level was significantly elevated in asthmatic children in comparison with healthy children ($P = 0.0001$). Also, the serum level of IL-13 was significantly higher in asthmatic children when compared to its level in control group (P = 0.0001). However, the expression level of Let-7a was significantly decreased in asthmatic children than in healthy children ($P = 0.0001$) (Table 1).

3.2. Demographic characteristics and biochemical profiles in relation to varying degree of asthma (asthma severity)

The expression levels of miR-155 and IL-13 in peripheral blood significantly increased with severity of illness ($P = 0.0001$ for each), while Let-7a and lung function indices decreased significantly in relation to severity of asthma (P = 0.0001 for each) (Fig. 1).

3.3. Receiver operating characteristic curve (ROC curve) analysis (Fig. 2)

ROC curve analysis showed that miRNA-155 could differentiate asthmatic patients from healthy controls with an AUC of 0.91 (95% CI:

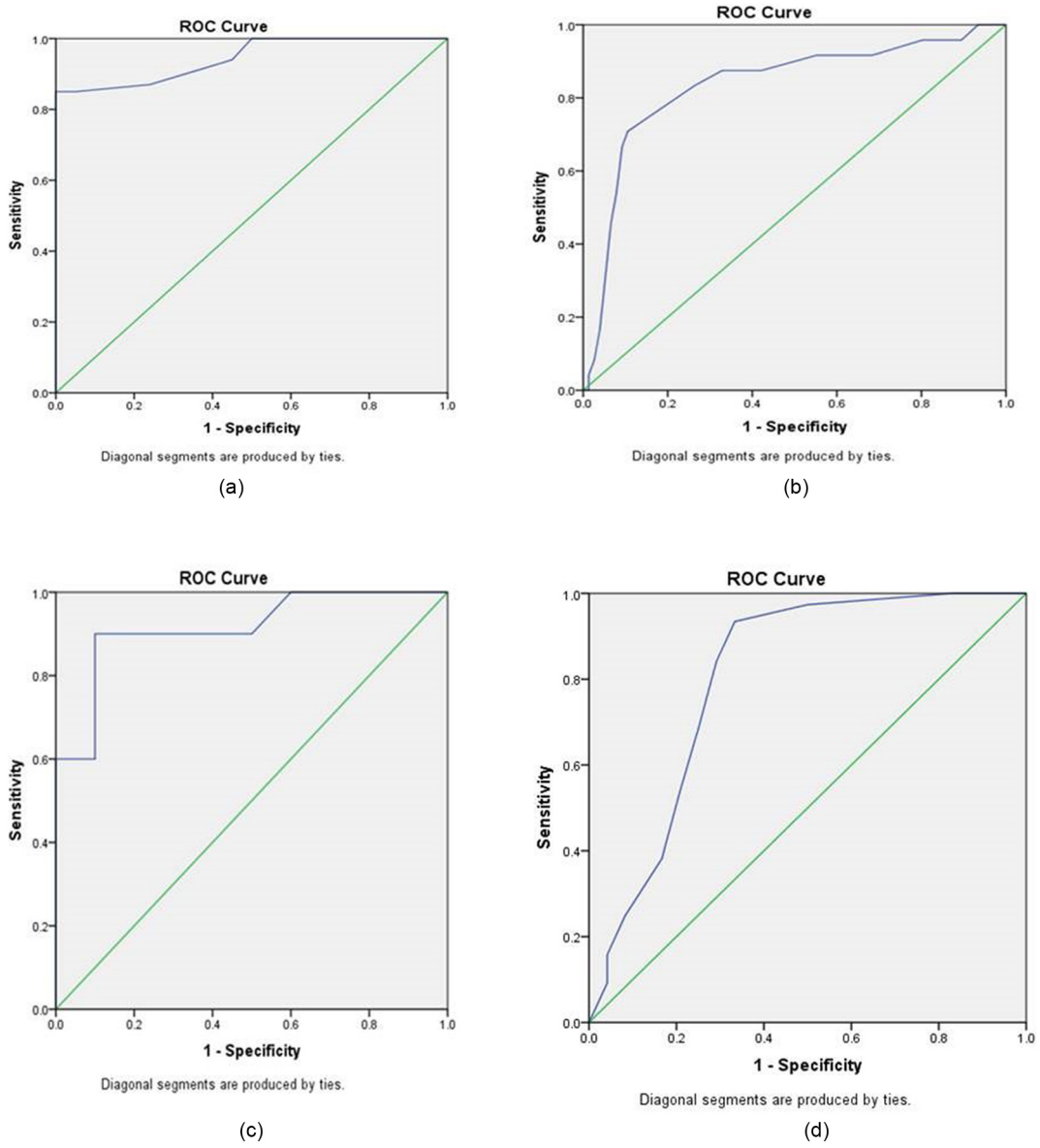

Fig. 2. Receiver operating characteristic (ROC) curve analysis. Fig. (2a): ROC curve for miRNA −155 diagnostic potential in differentiating asthmatic children and control, AUC of 0.91 (95% CI: 0.88–0.95, $P = .001$). Fig. (2b): ROC curve for miRNA-155 prediction of asthma severity, AUC of 0.83 (95% CI, 0.73–0.93, $P = .001$).Fig. (2c): ROC curve for Let-7a diagnostic potential in differentiating asthmatic children and control with an AUC of 0.92 for (95% CI, 0.87–0.95, P = .001). Fig. (2d): ROC curve for Let-7a prediction of asthma severity with an AUC of 0.80 (95% CI, 0.68–0.92, P = .001).

0.88–0.95, $P = 0.001$), with sensitivity and specificity of 80% (71.7–86.6) and 89% (81.3–93.7) respectively at a cutoff expression value > 3.2 and diagnostic accuracy of 84.5%.

We also performed ROC analysis of miRNA-155 for prediction of asthma severity. The AUC for miRNA-155 as severity marker was 0.83 (95% CI: 0.73–0.93, $P = 0.001$). At cutoff value 6.6, miRNA-155 provided sensitivity of 66.6% and specificity of 89.7% for differentiating patients with sever asthma from mild and moderate asthma with diagnostic accuracy 84.16%.

Regarding Let-7a expression our data revealed that let-7a could differentiate asthmatic children from healthy children with an AUC of 0.92 for (95% CI: 0.87–0.95, $P = 0.001$). The sensitivity and specificity to differentiate asthmatic children from controls were 85% (95% CI: 76.7–90.69) and 90% (95% CI: 82.5–94.7) at a cutoff expression value < 2.09 and diagnostic accuracy of 87.5%.

For asthma severity, ROC analysis showed that let-7a could be predictor of the severity of asthma with an AUC of 0.80 (95% CI: 0.68–0.92, P = 0.001). At cutoff value 1.6, let-7a showed sensitivity of 75% and specificity of 93.5% for identifying patients with sever asthma from mild and moderate asthma with diagnostic accuracy of 89.1%.

3.4. Correlation analysis of miR-155 and Let-7a with IL-13, FEV1 and FVC

Using Pearson correlation analysis, the miRNA-155 expression level was positively correlated with IL-13 levels ($r = 0.56$, $P = 0.001$). However, the miRNA-155 expression levels were significant negatively correlated with FEV1and FVC ($r = -0.63$, P = 0.001; $r = -0.59$, P = 0.001 respectively) (Fig. 3).

The expression level of Let-7a was negatively correlated with Il-13 levels ($r = -0.29$, P = 0.001). The expression levels of Let-7a showed significant positive correlations with FEV1and FVC ($r = 0.29$, $P = 0.006$; $r = 0.59$, P = 0.001 respectively) (Fig. 4).

4. Discussion

miRNAs are large group of small regulatory molecules which have

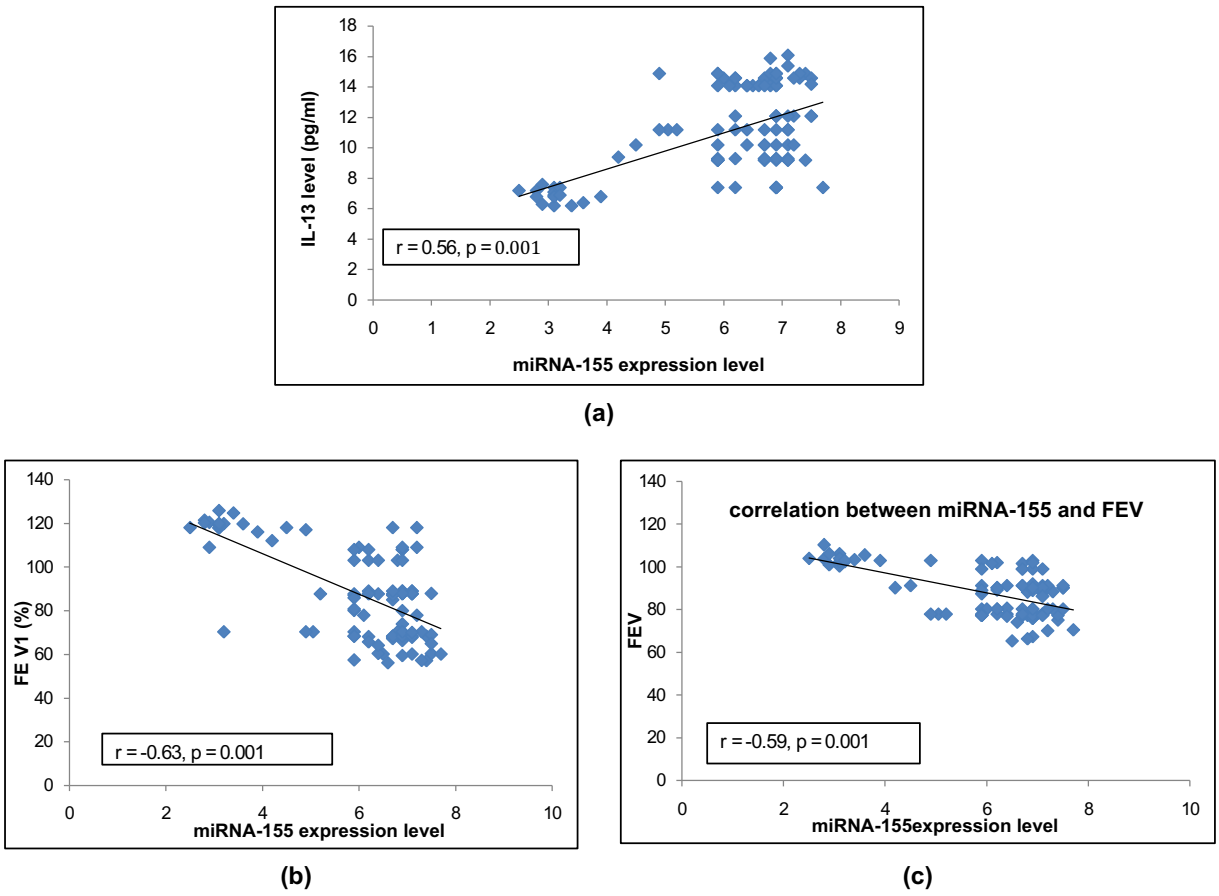

Fig. 3. Linear correlation analysis of miRNA-155 with serum IL-113 levels, FEV1 and FVC. Fig. (3a): Correlation between miRNA-155 & IL-13 level. Fig. (3b): correlation between miRNA-155 and FEV1 (%). Fig. (3c): correlation between miRNA-155 and FVC.

important roles in various cell processes and determination of cellular fate [20]. During asthma pathogenesis, miRNAs control the intensity of allergic immune response by regulating the Th2 survival, activation, proliferation, differentiation, and development [21,22].

Thus, our work aimed to investigate the role of plasma miR-155 and Let-7a expression as diagnostic non-invasive biomarkers of childhood asthma and severity and their relation to IL-13 level and lung function parameters.

Our study demonstrated that the miR-155 expression levels were significantly elevated in children with asthma than in healthy control children. At a cutoff value of > 3.2, miRNA-155 differentiate asthmatic children from control children as demonstrated by ROC curve analysis (AUC = 0.91, p = 0.001) with sensitivity and specificity of 80% and 89% respectively.

The miR-155 expression levels were significantly higher in severe asthmatic children than in mild and moderate asthmatic children (f = 48.3, P = 0.0001). ROC curve analysis showed that miRNA-155 could be used as predictor of the severity of asthma (AUC = 0.83, p = 0.001) and at cutoff value > 6.6, miRNA-155 expressed sensitivity of 66.6% and specificity of 89.7% for differentiating severe asthma from mild and moderate asthma. These results suggested that miR-155 participate in the pathogenesis and immune imbalance in asthmatic patients and could be used as a marker for diagnosis and prediction of severity of asthma.

In congruence with our results, Malmahall et al., reported up-regulated expression of miR-155 in the lungs of ovalbumin (OVA)-challenged mice in comparison to non-challenged mice. Also, the lung of miR-155 Knock out (KO) mice showed significant decrease in eosinophils and mucous production after OVA treatment in comparison with wild type-mice [11]. Moreover, OVA-treated miR-155 KO mice showed marked alleviation of bronchial hyper-responsiveness [23]. These changes were accompanied by reduction in Th2 cells and cytokines suggesting that miR-155 acts as a key regulator in Th2 related allergic inflammation [11,23].

Another mechanism for involvement of miR-155 in allergic airway inflammation was demonstrated by Johansson, et al., proposing that miR-155 have an important regulatory role in type 2 innate lymphoid cell (ILC2s) and IL-33 mediated allergic inflammation [24].

Other than asthma, previous studies demonstrated miR-155 over-expression in atopic dermatitis individuals both in the plasma [25] and in skin lesions and in infiltrating immune cells [8]. Moreover, its expression level was shown to be significantly up -regulated in the nasal mucosa of allergic rhinitis and in positive skin prick test reactions patients [7].

Similar to our results, plasma miR-155 expression was significantly increased in asthmatics with cockroach allergy in comparison with patients without cockroach allergy. These increased plasma miR-155 levels were observed in severe asthma patients when compared with non-asthmatics or mild-to-moderate asthmatic patients [26].

In contrast, Malmahall, et al., observed reduced expression of miR-155 in lymphocytes of the airway of allergic asthma patients in pollen season compared to its post-season expression level [27]. This incongruity might be due to different asthma models used and/or different samples and different disease condition between our study and others.

Our study revealed that Let-7a expression level was significantly down regulated in asthmatic children than in control children (P = 0.0001). Also, ROC curve analysis demonstrated that Let-7a differentiated asthmatic from healthy controls (AUC = 0.9, P = 0.001) with sensitivity of 85% and specificity of 90% and cut-off value of Let-7a < 2.09. Accordingly, Let-7a plasma level could be used as probable

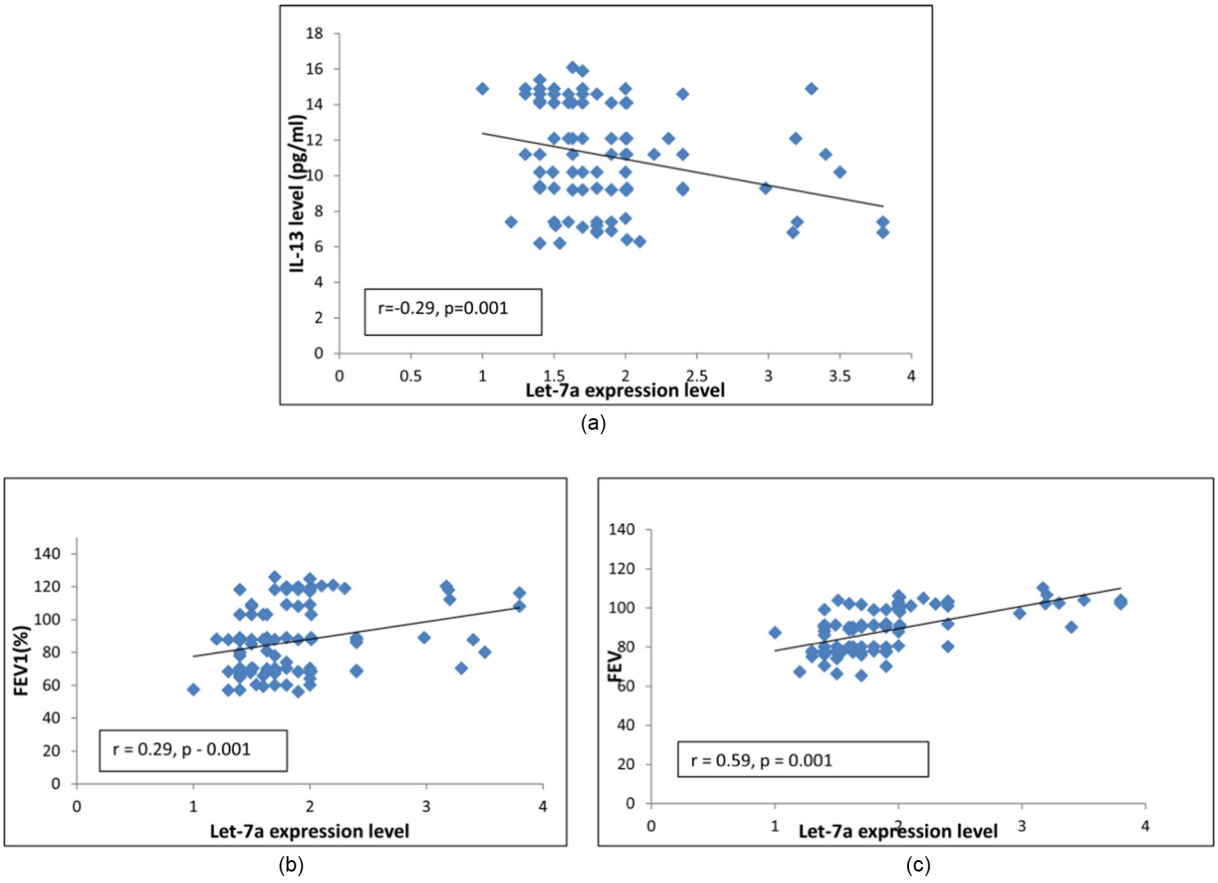

Fig. 4. Linear correlation analysis of Let-7a with serum IL-113 levels, FEV1 and FVC. Fig. (4a): Correlation between Let-7a & IL-13 level. Fig. (4b): correlation between Let-7a and FEV1 (%). Fig. (4c): correlation between Let-7a and FVC.

diagnostic biomarker in asthma patients.

In our study the Let-7a expression level was significantly reduced in patients with severe asthma as compared to mild and moderate asthma. ROC curve analysis showed that let-7a could predict the severity of asthma with an AUC of 0.80. At cut-off value < 1.6, Let-7a levels showed sensitivity of 75% and specificity of 93.5% for diagnosing severe asthma patients from mild and moderate asthma with diagnostic accuracy 89.1%. Thus Let-7a expression could be used as a potential biomarker for differentiating various phenotypes of asthma.

In accordance with our results, previous studies demonstrated reduced serum expression of Let-7a in patients with asthma [28] and also, in the airway epithelial cells [29]. Moreover, Pinkerton et al. found lower expression of let-7a miRNA in exhaled breath condensate of patients with asthma, when compared to patients having COPD and in control individuals [30]. Furthermore, examination of broncho-alveolar lavage fluid of asthmatic individuals by miRNA array analysis revealed altered expression of eight miRNAs, with down-regulated let-7a expression [31].

Interestingly, Rijavec et al., demonstrated that bronchial biopsies of severe asthmatic patients expressed reduced level of Let-7a in comparison to mild asthma patients and to control group [32].

In contrast to our results, study of Williams et al. demonstrated no differences in the expression of Let-7a miRNA in airway biopsies of patients with mild asthma compared to control subjects. They attributed this to mild inflammatory state of their asthma patients [33].

In our study, analysis of serum IL-13 was done by ELISA and its level was significantly increased in asthmatics than in control children ($P = 0.0001$) and was associated with the disease degree of severity.

Correlation analysis in our study showed that the expression level of miRNA-155 was correlated positively with Il-13 levels and negatively with FEV1and FVC.

In an experimental study by Johansson et al., upon allergen challenge, the lung of miR-155 −/− mice showed impaired IL-33 level and decreased ILC2s number compared with wild type mice [24]. ILC2 is a potent source of type 2 cytokines(IL-13 and IL-5) and the IL-33-challenged miR-155 −/− ILC2s showed impairment in their proliferation and IL-13 production [24].

Therapeutic strategy using small interfering RNA directed against miR-155 successfully decreased inflammation, eosinophilia, and level of Th2 cytokines (IL-13, IL-4, and IL-5) together with inhibition of airway hyper responsiveness [34]. These previous data highlighten the contribution of miR-155 to Th2 effector function.

In our study, the expression level of Let-7a was negatively correlated with Il-13 levels and positively correlated with FEV1and FVC.

Kumar and colleagues [13] reported that let-7 microRNAs target IL13 3′UTR, resulting in IL-13 inhibition with reduced airway inflammation, sub-epithelial fibrosis, and AHR. Using a combination of bioinformatics and molecular approaches in murine model of asthma, they demonstrated that the level of let-7 was inversely correlated to induced IL-13 levels in cultured T cells indicating the anti-inflammatory role of Let-7a in asthma [13]. Also, the anti-inflammatory role of let-7 has been suggested in cancer patients and in idiopathic pulmonary fibrosis patients [35,36].

In summary, miR-155 expression was significantly increased and let-7a expression was significantly decreased in the peripheral blood of asthmatic children and their levels were significantly correlated with degree of severity of illness, IL-13 level and lung function parameters.

We concluded that miR-155 and Let-7a could be used as non-invasive serological biomarkers for asthma diagnosis and severity. These results could contribute to understanding the pathogenesis of asthma

and help in selecting promising therapeutic modalities for this disease.

Conflict of interest

None.

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