Expression of Serum MicroRNAs 221, 222, 15a and Level of VEGF-A in Children with Bronchial Asthma

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Bronchial asthma (BA) remains the most common chronic respiratory disease in children and is characterized by reversible airway obstruction and airway hyperresponsiveness. MicroRNAs (miRNAs) are a class of small, highly conserved non-coding RNA molecules that regulate gene expression at the posttranscriptional level. MiRNAs can either augment or minimize the allergic inflammation in asthma. It has been noted that a group of miRNAs may affect the development of asthma and related inflammatory response. The vascular endothelial growth factor (VEGF) is produced by different types of cells and has a major role in both, physiological and pathological angiogenesis. Our aim was to study serum expression of three microRNAs; 221, 222,15a and Vascular Endothelial Growth Factor A (VEGF-A) levels in children with bronchial asthma. The study included 30 children with BA and a control group of 20 apparently healthy, age and sex matched, children. Quantitative reverse transcription (q RT) polymerase chain reaction (PCR) was performed to examine the expression levels of miR-221, 222 and 15a in patients' sera. Levels of serum VEGF-A were quantified utilizing an ELISA technique. Serum levels of both miRNA-221 and miRNA-222 were not significantly different between pediatric asthmatic patients versus controls (*P*=0.76 and 0.52, respectively), but showed increase with the disease severity (persistent versus intermittent) (*P*= 0.09, 0.07, respectively). Serum miRNA-15a expression was significantly down-regulated in asthmatic patients versus the control group (*P*=0.03). miRNA-15a expression did not differ among various grades of BA (*P*=0.33) and was not correlated with serum levels of VEGF-A (*P*=0.56). The level of VEGF-A was significantly increased in serum of pediatrics with bronchial asthma in comparison to the control group (*P*=0.026). In conclusion, miRNA-15a and VEGF-A may have a role in BA pathogenesis, while miRNA-221 and 222 may reflect BA disease severity.

Bronchial asthma (BA) is a clinically prevalent heterogeneous condition that represents the pathologic consequence of exaggerated type 2 immune responses [1]. Circulating microRNAs (miRNAs) are highly conserved small dynamic endogenous RNAs that regulate gene expression [2]. They regulate allergic immune responses with the ability to classify disease severity and inflammation through the differential expression of miRNAs in different biological samples; sputum supernatants and plasma [3,4].

A diversity of miRNAs was evaluated in bronchial asthma with altered expression in normal and affected tissues in response to various allergen exposures [5-10]. The aberrant expression of these miRNAs could reflect the allergic responses which is represented by influx of inflammatory cells, reactive epithelial and stromal changes. Thus, miRNAs might have a potential role as applicable diagnostic/therapeutic non-invasive biomarkers for asthma management [11].

miR-221 and microRNA 222 share a similar seed region and are encoded by a common gene cluster [12]. Moreover, both miR-221 and miR-222 under the effect of Tumor growth factor b (TGF-b) are potential regulators of airway smooth muscles (ASM) growth, in addition to the proliferation and hyperplasia of vascular smooth muscle cells [13,14]. As such, miR-221 and 222 could play a role in the pathogenesis of asthma [15,16].

Vascular endothelial growth factor (VEGF) is a well-known angiogenic factor that plays a key role in the pathogenesis of asthma through the proliferation of airway smooth muscle (ASM) cells, neovascularization, vascular remodeling, tissue hypoxia and inflammation [17-20]. Multiple miRNAs, including miR-15a, miR-15b, miR-16, miR-20a and miR-20b can affect the expression of VEGF [21]. mir-15a, a member of the miR-15/miR-16 family, down-regulate VEGFA mRNA expression [22,23].

Our aim was to study the serum expression of three microRNAs; 221, 222, 15a and to investigate the correlation between serum level of both microRNA15a and VEGF-A in a cohort of children with bronchial asthma in a case control study.

Patients and Methods

The present study, conducted at the Allergy and Pulmonology Clinic, Beni-Suef University Hospital, included a group of 30 children with bronchial asthma and a control group of 20 apparently healthy, age and sex matched, children (Table 1). The study protocol was reviewed and approved by Faculty of Medicine, Beni-Suef University, Research Ethical Committee (00015574, February 16, 2016). In addition, we obtained an informed consent from one of the parents after clarifying the purpose and the nature of the study to them.

The disease severity was categorized according to the Global Initiative for Asthma (GINA) guidelines [24]; 15 patients were classified with an intermittent form of the disease, while 15 patients suffered from persistent bronchial asthma who were further subdivided into mild (n=4), moderate (n=8) and severe (n=3). Note that 21 of the pediatric bronchial asthma patients were under corticosteroids therapy. Moreover, pediatrics with bronchial asthma were categorized into 21 atopic and 15 non atopic patients.

Methodology

Venous blood samples were collected from all study children. Serum isolated from each child was stored at -20°c in 4 labelled aliquots for processing in PCR and ELISA.

- Assessment of microRNAs 221, 222 and 15a by quantitative reverse transcription (q RT) real time PCR

Extraction and purification of microRNA was performed using the miRNeasy Serum/Plasma Kit Qiagen, Germany (Catalog no. 217184), according to the manufacturer's instructions. In brief, 500 µl QIAzol lysis reagent were added to 100 µl of each serum sample, mixed by vortex and incubated at room temperature for 5 min. Then, 100 µl Chloroform were added and centrifuged for 15 min at 12,000 x *g* at 4°C. The upper aqueous phase was transferred to a new tube and 1.5 volumes of 100% ethanol were added and mixed thoroughly. An aliquot of 300 µl sample, including any precipitate, were pipetted into a RNeasy MinElute spin column in a 2 ml collection tube, and centrifuged at 8000 x *g* for 15 s at room temperature, then the flow-through was discarded. A volume of 700 µl Buffer RWT was added to the RNeasy MinElute spin column, centrifuged for 15 s at 8000 x *g*, and the flow-through was discarded. An aliquot of 450 µl Buffer RPE was added onto the RNeasy MinElute spin column, centrifuged for 15 s at 8000 x *g* and the flow-through discarded. A volume of 450 µl of 80% ethanol was added to the RNeasy MinElute spin column, centrifuged for 2 min at 8000 x *g*, and the flow-through and the collection tube were discarded. The RNeasy MinElute spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 5 min to dry the membrane. The flow-through and the collection tube were discarded. The RNeasy MinElute spin column was placed in a new 1.5 ml collection tube and 14 µl RNase-free water were added directly to the center of the spin column membrane and centrifuged for 1 min at full speed to elute the RNA.

Quantitative reverse transcription (q RT) real-time PCR analysis of microRNA 221, 222, 15a, with normalization with the endogenous control (SNORD 68), was performed using miScript PCR System, Qiagen, Germany. The miScript PCR System enables quantification of miRNA expression using SYBR® Green-based real-time PCR technique in two main steps. In the first step microRNA was transcribed into cDNA, using miScript II RT Kit, Qiagen, catalogue number (218161), according to the manufacturer's instructions. Briefly, in a 20 µl tube the following were added 1.5 µl of extracted template RNA, and the reverse-transcription master mix prepared as follows: 4 µl of 5x miScript HiSpec Buffer, 2 µl 10x miScript Nucleics, 10.5 µl RNase-free water, 2 µl miScript Reverse Transcriptase. The mixture was shortly

centrifuged and kept on ice. The tube was then incubated for 60 minutes at 37°C followed by incubation for 5 minutes at 95°C to inactivate miScript Reverse Transcriptase mix. RNase-free water (200 µl) was added to the 20 µl reverse-transcription reaction for cDNA dilution before PCR.

The Second step included Real-time PCR detection of mature micro RNAs 221, 222, 15a with SNORD 68 as the endogenous control for normalization. Both miRNA-specific miScript Primer Assay (product number 218300) which is a specific forward primer for each microRNA and the control in addition to the miScript SYBR Green PCR Kit (product number 218073), which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. A total reaction volume of 20ul was prepared for each microRNA quantified as follows: 2 µl template cDNA acquired from the reverse transcription reaction was added to 10 µl 2x QuantiTect SYBR Green PCR Master Mix in combination with 2 µl 10x miScript Universal Primer in addition to 2 µl10x miScript Primer Assay and 4 µl RNase-free water. The thermal cycling conditions for real-time PCR assay; initial activation step for 15 minutes at 95°C which was followed by 40 cycles of denaturation for 15 seconds at 94°C, annealing for 30 second at 55°C and extension at 70°C for 30 seconds. Relative quantification (RQ) of microRNAs was calculated by the comparative method (ΔΔCT method) using 2-ΔΔct equation where SNORD 68 was used for normalization of the target microRNAs.

- Quantitative determination of serum VEGFA by enzyme linked immunosorbent assay (ELISA)

Serum Vascular endothelial growth factor (VEGF or VEGF-A, VEGF165), initially identified as vascular permeability factor (VPF) was measured by a 4.5-hour solid phase quantitative sandwich-based ELISA technique using Quantikine® Human VEGF ELISA kit which is manufactured and distributed by R&D Systems, Inc, USA.

Briefly, the procedure included the addition of 100 µl of Assay Diluent RD1W per well. Then 100 µl of one of the following; standard, control, or serum sample were pipetted to each well. The plat was covered with adhesive strip provided and incubated for 2 hours at room temperature. Each well was aspirated and washed with 400 µl Washing Buffer. The washing step was repeated twice for a total of three washes. Then, the plate was incubated and covered for 2 hours at room temperature after adding 200 µl of Human VEGF Conjugate to each well. The aspiration/wash step was repeated as previously described. The substrate solution (200 µl) was added per well in the plate and incubated away from light for 25 minutes at room temperature. Finally, the Stop Solution (50 µl) was added to each well. The optical density of each well was determined using a microplate reader set to 450 nm within 30 minutes. For more accuracy, the plate was also read at 540 nm and readings at 540 nm were subtracted from the readings at 450 nm.

Statistical Analysis

Data was statistically analyzed using an IBM Statistical Package for the Social Sciences (SPSS) program version 25 (IBM Corp. in Armonk, NY). Quantitative data was described as mean, SD, percentage and range. The unpaired t-test was used to compare the means of 2 groups of quantitative variables when distributed in a parametric way, while the nonparametric data were analyzed using the Mann–Whitney U test. Association between parameters were analyzed using correlation tests. One-way Annova test was utilized to compare quantitative data when allocated in more than 2 groups. A Statistical significance was considered for *P* value less than 0.05.

Table 1. Demographic and clinical data of bronchial asthma patients and the control group.

	Control N=20	Bronchial asthma N=30	*P* value
Gender, Number (%)			
Male	13 (65.0%)	22 (73.3%)	NS
Female	7 (35%)	8 (26.7%)	
Age (year)			
Range	3.0-15.0	2.50-16.0	NS
Mean (SD)	9.10 (4.17)	7.15 (4.24)	
Allergic rhinitis no (%)	1 (5%)	21 (70%)	0.001
Positive family history no. (%)	0 (0%)	20 (66.7%)	0.00

P>0.05 is not significant (NS).

Results

Expression of MicroRNAs 221, 222 and 15a in serum of both pediatric patients with bronchial asthma and the control group: Serum level of microRNA 15a was significantly lower in asthmatic pediatrics when compared with the control group (*P*=0.03) (Figure 1). There was no significant difference between level of microRNAs 221 and 222 in the serum of the patient group versus the control group (*P*=0.76 and 0.52, respectively) (Table 2) (Figure 2).

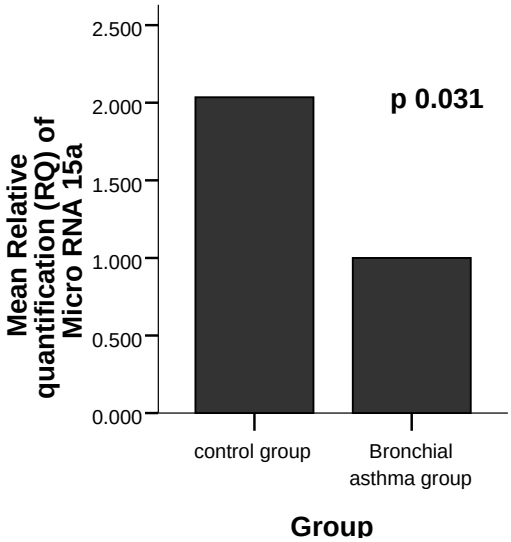

Figure 1. Mean relative quantification (RQ) value of microRNA 15a among control and bronchial asthma groups.

Table 2. Comparison of microRNA 221, 222 and 15a expressed levels in control and bronchial asthma groups

	Control group Mean ±SD (range)	Bronchial asthma group Mean ±SD (range)	*P* value
RQ microRNA222	2.3 ± 2.5 (0.001-6.74)	1.8 ±2.3 (0.15-10.29)	NS
RQ microRNA221	1.2 ± 1.3 (0.005-3.93)	1.3 ±1.4 (0.19-5.31)	NS
RQ microRNA15a	2.0 ± 1.8 (0.001-5.60)	1.0 ±1.3 (0.00-4.5)	0.03

RQ is a relative quantification value of microRNAs using $2^{-\Delta\Delta ct}$ equation

P>0.05 is not significant (NS).

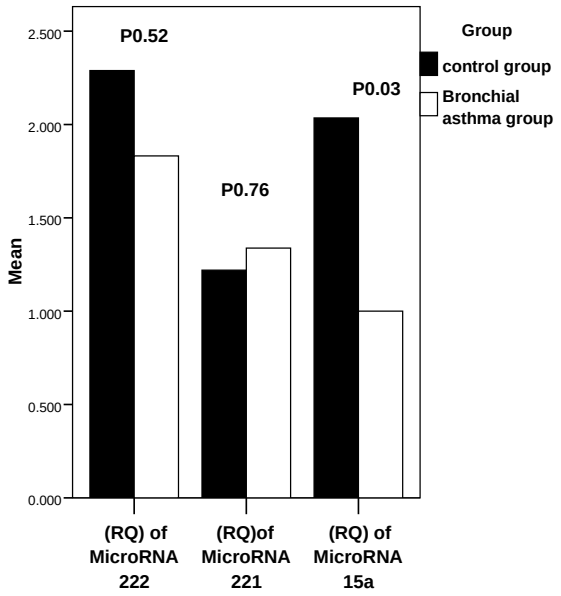

Figure 2. Mean relative quantification (RQ) value of Micro RNA 222, 221 and 15a among control and bronchial asthma groups.

The serum level of VEGFA was significantly increased in the pediatric group suffering from bronchial asthma in comparison to the control group (*P*=0.026) (Figure 3). There was no significant correlation between serum levels of VEGFA and microRNA15a (*P*=0.56).

The expression of all the studied microRNAs 221, 222 and 15a showed no significant difference when compared in sera of atopic versus sera of non-atopic bronchial asthma groups (*P*=0.44, 0.93 and 0.46, respectively). Patients on corticosteroid therapy demonstrated no difference in the serum levels of microRNAs 221, 222 and 15a when compared with patients not receiving corticosteroids (*P*=0.31, 0.72 and 0.40, respectively). The severity of bronchial asthma was not significantly affecting the serum level of microRNA 15a (*P*=0.30). Both micro RNA221 and 222 showed no significant difference (*P*=0.09 and 0.07, respectively) when serum level of each was measured in intermittent and persistent bronchial asthma (Table 3). Although microRNA 221 showed stepladder increase along the different grades of the disease severity [intermittent and persistent (mild, moderate and severe)], yet this difference did not reach statistical significance (*P*=0.34).

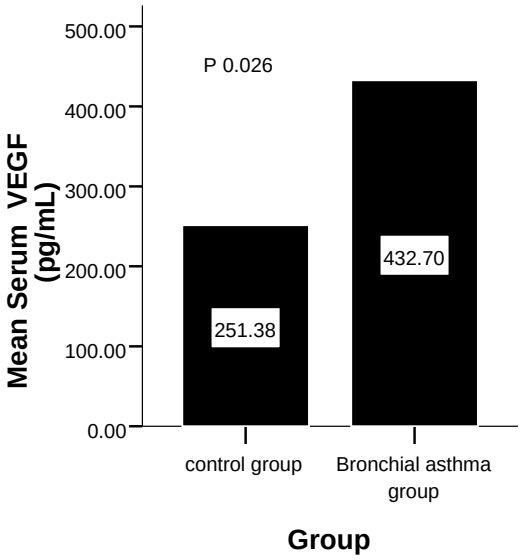

Figure 3. Mean serum VEGFA level among control and bronchial asthma groups.

Table 3. Mean relative quantification (RQ) of Micro RNA 222, 221, 15a and serum VEGFA among intermittent and persistent bronchial asthma sub-groups

	Intermittent Mean (SD)	Persistent Mean (SD)	*P* value
RQ microRNA222	1.04 (1.05)	2.62 (3.05)	NS
RQ microRNA221	0.91 (0.98)	1.75 (1.57)	NS
RQ microRNA15a	0.74 (1.14)	1.26 (1.35)	NS
VEGFA (pg/mL)	491.93 (339.3)	373.47 (286.84)	NS

P>0.05 is not significant (NS).

Discussion

In bronchial asthma, miRNA could be an ideal biomarker that can identify disease phenotype, monitor disease activity and determine its prognosis [25,26] which highlights the importance of prioritizing individual miRNAs in the context of severe asthma.

In the current study, there was no significant difference in expression levels of miRNA-221 and miRNA-222 when comparing both the pediatric patients with the control group (*P*= 0.51 and 0.94 respectively) and patients with persistent to intermittent asthma (*P*=0.09 and 0.07 respectively). In agreement to our results, no significant difference in the expression of miRNA221was found by Solberg *et al.* [27] and Wiliam *et al.* [28] between the control and the bronchial asthma group. On the other hand, miRNA-221 was significantly over-expressed two-fold in pediatric asthmatics and three-fold in murine asthma models in the study of Liu *et al.* [29]. This discrepancy can be attributed to the difference in the analyzed samples; where the study by Liu *et al.* [29] separated lymphocytes in contrast to the isolated serum in ours, in addition to the difference in the percentage and degree of severity of the disease in the study population in the two studies.

Different miRNAs expression profiles were affected by the severity of asthma and inflammatory phenotype [3]. In the current study, although there was no significant difference in the expression of miRNA222 and 221 between the control and the patient groups, still the marginal over-expression of miRNA-221 in the persistent asthma could reflect the role of this marker in the pathogenesis and severity of bronchial asthma. However, further studies with larger number of patients to confirm such observation are needed.

Atopy and current steroid therapy did not affect the expression level of both miRNAs-221 and 222 in our study. Similarly, the expression of both miRNAs was not affected by corticosteroids in a study by Perry *et al.* [12] and by Williams *et al.* [28]. The results may highlight the role of mi-RNA 221 and 222 in the context of severe asthma, however, such role could not be expressed in atopic asthma or during current steroid therapy.

Many miRNAs have been involved in the process of angiogenesis [30-32]. miR-15a promotes anti-angiogenic effects through the downregulation of VEGFA mRNA expression [22,23]. To evaluate such relation, we estimated serum VEGF and miR-15a individually in children with bronchial asthma. miR-15a was significantly lower among pediatric patients with

bronchial asthma compared to the control group (*P*=0.02) while serum levels of VEGF-A were significantly (*P*=0.026) elevated in pediatric asthmatic patients with no significant correlation between serum levels of VEGF-A and expression of miR-15a (*P*=0.56). The aberrant expression of miR-15 may be considered as a regulator of occurrence of asthma [33].

It is interesting that the expression of mir-15a measured in CD4+ T cells isolated from the peripheral blood in asthmatic patients was related to the presence of atopy, but not to the usage of inhaled corticosteroids (ICSs) [33]. In our study, severity of asthma, atopy or the current corticosteroids therapy did not show significant effect on the serum expression of mir-15a

The significant increase in serum levels of VEGF-A in the current study among pediatric asthmatic patients may indicate its role in bronchial asthma through airways angiogenesis, hyperactivity and bronchial wall thickening [34]. Other studies reported, higher levels of serum, sputum, bronchoalveolar lavage or bronchial biopsies of VEGF were found between asthmatic patients and control groups [17, 34, 35]. Increased VEGF concentration was found to be proportional to the severity of asthma in children during exacerbations of asthma [36]. In contrast, in our results, the different grades of severity, atopy and current steroids therapy did not cause a significant difference in serum VEGF levels. In addition, no significant correlation was found between serum levels of VEGF-A and expression of microRNA15a among the study population. miR-15a may affect the pathogenesis of bronchial asthma by targeting VEGF [22,23].

However, this study carries some limitations related to the small number of asthmatic patients. Any contradictory results with previous studies could be related to the heterogeneity of patients, in terms of clinical severity, methods used to study miRNAs and samples tested. Studying serum expression levels of miRNA is simpler than levels in saliva, or biopsies of airway tissues which is more complex and less reproducible. However, still serum levels carry some contradictory results [37].

In conclusion, several miRNAs could be involved in the bronchial asthma among the pediatric population. miRNA-15a and VEGF-A may have a role in the pathogenesis, while further studies are needed to clarify the role of miRNA-221 and 222 in severity of bronchial asthma.

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