Interleukin-17 Gene Expression and Serum Levels in Children with Severe Asthma

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ABSTRACT

Background: IL-17 is a major cytokine player in T cell mediated leukocyte associated inflammation. IL-17 is also recognized to participate in the pathophysiology of asthma. **Objective:** To determine the role of IL-17 in predicting severe asthma. **Methods:** We obtained serum samples from asthmatic children under the age of 5-year in three different groups of mild (n=33), moderate (n=28) and severe (n=32) persistent asthma. IL-17 serum concentrations and mRNA expression were determined by ELISA and real time PCR assays, respectively. **Results:** Serum IL-17 concentrations were significantly higher in patients with severe asthma than the other two groups of children with mild and moderate disease (p=0.00). Mean serum IL-17 values were 142.04 pg/ml in mild group, 180.4 pg/ml in moderate group and 251.25 pg/ml in severe group. IL-17 mRNA levels were also significantly elevated in severe asthmatic patients compared to mild and moderate asthmatic children (p=0.00). **Conclusion:** Our data reveal an increase in the serum IL-17 concentrations and IL-17 mRNA expressions in children with severe asthma compared to those with mild and moderate forms of the disease.

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INTRODUCTION

Asthma is the most common chronic respiratory disease (1) which is associated with significant morbidity and shows an increasing prevalence over time (2,3). In preschool-aged children, the diagnosis is most often based on symptom patterns, presence of risk factors and a favorable response to treatment. The preschool-aged children tend to be exacerbation-prone with relatively limited impairment, in contrast to older children who have more impairment-dominant disease (2).

Asthma is an airway disorder characterized by chronic airway inflammation, mucus production and airway hyper-responsiveness with airway remodeling (4). Multiple cytokines, chemokines and growth factors released from both inflammatory and structural cells in the airway tissues drive the resultant structural changes (5). The perception of asthma has changed from a pure inflammatory disease to a disease in which both inflammatory and structural changes are equally involved (5).

Severe asthma in children is a heterogenous and complicated disorder with distinct types of inflammatory processes (6). However affected children share many similar clinical features. Increased eosinophils and TH2-derived cytokines in the airways of these children on one hand and increased neutrophils and neutrophil activation biomarkers on the other hand are reported (7).

Airway inflammation in asthma usually involves polarization of the T lymphocyte response to the TH2 cells. But recent studies indicate that pathological mechanisms of asthma involve more than just a dichotomous TH1/TH2 inflammation (4,8,9). In this regard finding of another subset of effector helper T cells with distinct functions from TH1 and TH2 cells (named TH17 cells) has raised great interest. There have been reports of a significant increase in the frequency of TH17 cells in the peripheral blood of children with asthma compared with healthy controls (10). TH17 cells preferentially produce IL-17 family of cytokines. In adult asthmatic patients IL-17 mRNA expression has been shown to increase in sputum, lung cells, airways, bronchoalveolar lavage fluid and peripheral blood. In asthmatic airways, IL-17 correlates with the incidence of airway hyper-responsiveness and the severity of disease. Specifically, IL-17 expression is enhanced in severe and chronic asthma (11). It has been proposed that IL-17 may be involved in immunopathogenesis of asthma in children (12). Although, the pathogonomic features of asthma are considered to be mediated by eosinophils, mast cells and TH2 cells, the neutrophil counts are increased markedly in the airways of severe asthma. IL-17 signaling induces neutrophil recruitment into the airways and therefore can be an important cytokine in determination of the disease severity (4). Most of the studies , which detected IL-17 in asthma, have been performed on induced sputum or bronchoalveolar lavage fluid (13). The majority of these studies have been conducted on adult patients and there are only a few data in this regard in children. However, concepts and models of asthma derived from adult studies may not be applicable to common phenotypes of asthma in young children.

In this study, we compared the serum concentrations and mRNA expression of IL-17 in three distinct groups of mild, moderate and severe asthma in children to investigate any relationship between IL-17 with the severity of asthma in children.

MATERIALS AND METHODS

Patients and Controls. 93 children aged 1-5 years who referred to Imam Reza Clinic (Shiraz University of Medical Sciences, Shiraz, Iran) with asthma from June 2011 to June 2012, were included. All the patients were visited by two allergologists separately. They all fulfilled the criteria of persistent asthma. Then they were categorized in three distinct levels of mild, moderate and severe asthma.15 healthy, age-matched, non-asthmatic children were considered as the control group and included in the study. Asthma severity was evaluated on the basis of the last version of Expert Panel Report 3 guidelines (14). The inclusion criteria for severe asthma were:

1) Daytime symptoms throughout the day (most days for at least 3 months).
2) Nighttime awakening symptoms more than once a week.
3) Short-acting β-agonists used for symptoms≥ 3 $times\ a\ w$eek or several times a day.
4) Extreme limitation in normal activity.
5) Two or more exacerbations in 6 months requiring systemic corticosteroids or at least one admission to an ICU, or two hospital admissions requiring intravenous medications.

All the parents gave informed consent prior to sampling. The study was approved by the local ethical committee of the faculty of medicine, Shiraz, Iran.

Sampling and Buffy Coat Preparation. Oxalated blood samples were obtained from all of the children. The buffy coat was separated by centrifugation on Ficoll gradient.
Primer Design. The primer was designed by primer blast for IL-17 (NM_002190.2) and β-actin (NM_001101.3) as the internal control. The thermodynamic parameter and secondary structure were determined by mfold software. The primer position in relation to exon-exon domains were evaluated by Spidey Software (www.ncbi.nim.gov/Spidey USA) and their specificity was analyzed by BLASTn (www.ncbi.nlm.gov/BLASTn USA).

The primer sequences for IL-17A and β-actin were as follows:
IL-17 F: 5-TCTGGGAGGCAAAGTGCCGC-3
IL-17R: 5-GGGCAGTGTGGAGGCTCCCT-3

β-actin F: 5-GGGCGGCACCACCATGTACC-3
β-actin R: 5-GACGATGGAGGGGCCCGACT-3

RNA Isolation and cDNA Synthesis. According to the protocol for isolation of RNA from cells, total RNA was isolated by RNX plus (Cinnagen, Iran). The purity and integrity of RNA were determined by measuring the optical density ratio in 260 and 280 nm and agarose gel (1%) electrophoresis. Traces of the residual genomic DNA were removed by DNase digestion using RNase from DNA set (Roche, Germany). Subsequently one microgram of each RNA sample was reverse transcribed to cDNA by Reverse Transcriptase (Vivantis, Malaysia) and random hexamer.
Real Time PCR. Real time PCR was performed for the quantitative analysis of mRNA expression with a thermocycler (ABI, applied biosystems Step I plus, USA), and SYBR green Premix by Ex taq (Takara, Japan). The amplifications containing SYBR Green 1 Dye, forward and reverse primers, and template cDNA were carried out. The specificity

of amplification reaction was confirmed by a melting-curve analysis. The results for the target genes were measured as fluorescent signal intensity and normalized to the internal standard gene β-actin. The PCR conditions are shown in Table 1.

Table 1. PCR Conditions used for amplification of target DNA .

Stages and Steps	Temperature (°C)	Period of time (sec)	Cycle(s)
Denaturation	95	120	1
Denaturation	95	30	
Annealing	65	20	37
Melt curve	95 65 95	15 60 15	1

ELISA. The case group consisted of 93 patients (with characteristics mentioned before). Serum samples were isolated immediately and stored at -80℃ until required for an analysis by a commercial ELISA assay (R&D, USA, Cat No. DY317) according to the manufacturer's instructions.

Statistical Analysis. Data analysis was performed by using SPSS software (version 16). One way Anova was used to determine whether there were any significant differences between the mean of the results in the three studied groups. Fishers' LSD (Least Significant Difference) test was used as a method for comparing two groups of severity at each time (as a pairwise comparison of the means). A difference was considered to be significant when $p<0.05$.

RESULTS

To identify whether IL-17 rises with the severity of disease in asthmatic children, 93 children with an established diagnosis of asthma who were receiving asthma therapy but were not in acute exacerbation of disease, were selected. All of the patients were under 5 year old with a mean age of 37.5 months at the time of the study. The mean age of the patients in the three distinct groups was not different. The higher percentage of boys in the study was consistent with the epidemiology of asthma in this age group.

We established real time PCR protocol to evaluate IL-17 gene expression in our patients. GADPH and β-actin were used as the internal control. The obtained data were normalized by using β-actin. The $2^{-\Delta\Delta CT}$ was used to determine the relative changes in the expression of IL-17 gene. Mean IL-17 mRNA expression values were 9.5 (min=1.27, max=15.39) in mild group, 12 (min=6.35, max=16.5) in moderate group, and 14.24 (min=9.96, max=18.08) in severe group (Table 2).

Table 2. The expression of IL-17 in asthmatic children.

PCR						
Groups of patients	**N**	**Mean**	**Minimum**	**Maximum**	**Std Deviation**	**Std Error**
1	31	9.50	1.27	15.39	5.05	0.90
2	22	12.00	6.35	16.50	2.96	0.63
3	25	14.24	9.96	18.08	2.35	0.47
Total	78	11.73	1.27	18.08	4.26	0.48

1: mild group of asthmatic children;
2: moderate group of asthmatic children;
3: severe group of asthmatic children.

The analysis of results showed a significant difference between IL-17 mRNA expression in the group of severe asthmatic children compared to the other two groups with mild and moderate asthma (p=0.00 and 0.047, respectively; Table 3 and Figure 1).

Table 3. Comparison of IL-17 gene expression in different groups of severity of asthmatic children.

(I) Group	(J) Group	Sig.
1	2	0.021
	3	0.000
2	3	0.047

Group 1) patients with mild asthma
Group 2) Patients with moderate asthma
Group 3) patients with severe asthma

The relative changes in the expression of IL-17 gene in the severe group versus mild group based on $2^{-\Delta\Delta CT}$ was calculated as $2^{4.7}$ ^{4}. The relative change in the IL-17 gene expression in severe group versus moderate group was 2^{2} $^{.2}$ ^{4}. There was also a significant difference between mild and moderate groups in IL-17 gene expression, (p=0.02).

Evaluation of serum IL-17 concentrations in healthy control group and the three studied groups of asthmatic children revealed a significant increase in serum levels of children with severe asthma compared to the other three groups (p=0.00; Table 4). Mean serum IL-17 values were 11.87 pg/ml (min=9.1, max=16.4) in control group, 142.04 pg/ml (min=33.1, max=313.2) in the group of patients with mild disease, 180.4 pg/ml (min=37.95, max=277.6) in the group of patients with moderate disease, and 251.25 pg/ml (min=90.48, max=452.4) in the group of patients with severe disease.

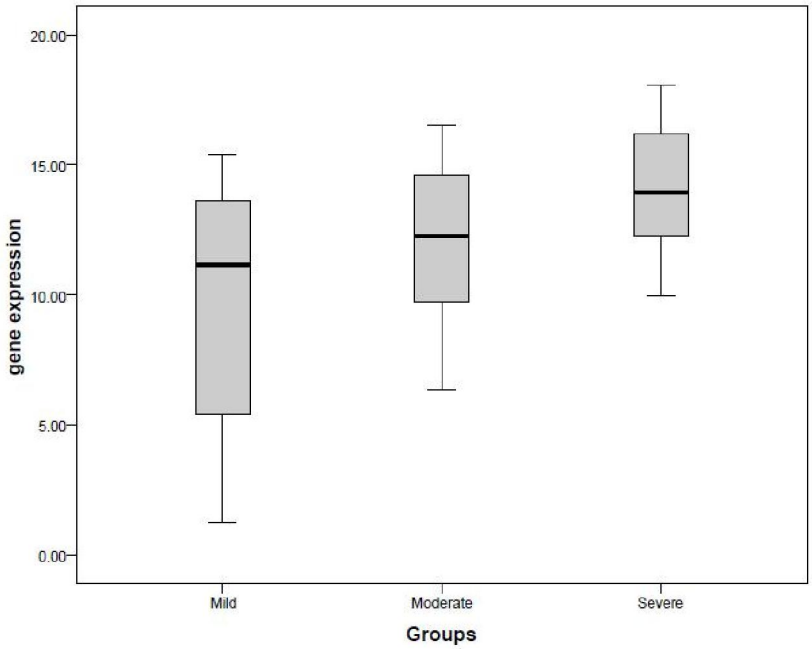

Figure 1. The comparison of IL-17 gene expression in the peripheral blood of different groups of asthmatic children with different severity of the disease.

There was a significant difference between the IL-17 serum concentrations in healthy control group and all the other three asthmatic groups of children (p=0.00).

Table 4. IL-17 levels in sera of patients with healthy controls.

	Number	Mean	Minimum	Maximum	Std.Deviation	Std.Error
1.00	27	142.04	33.10	313.2	92.48	17.79
2.00	26	180.40	37.95	277.6	83.85	16.44
3.00	25	251.25	90.48	452.4	92.21	18.44
4.00	15	11.87	9.1	16.4	2.24	0.57

1: Asthmatic children with mild disease;
2: Asthmatic children with moderate disease;
3: Asthmatic children with severe disease.
4: Healthy control group

There was no significant difference between mild and moderate groups (p=0.12) (Table 5 and Figure 2).

Table 5. Comparison of serum IL-17 levels in different groups of of asthmatic children based on the disease severity.

(I) Group	(J) Group	Sig.
1.00	2.00	0.093
	3.00	0.000
	4.00	0.000
2.00	3.00	0.003
	4.00	0.000
3.00	4.00	0.000

Group 1) Patients with mild asthma
Group 2) Patients with moderate asthma
Group 3) Patients with severe asthma
Group 4) Control group

Finally, we could not find any relationship in assessment of IL-17 mRNA expressions and serum concentrations in each group of patients with mild, moderate or severe asthma (p>0.05).

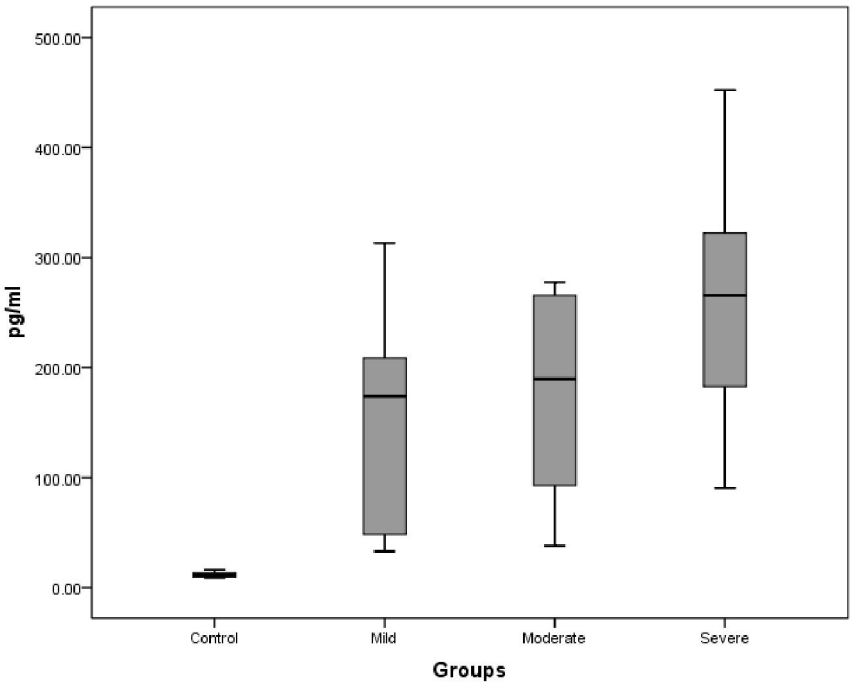

Figure 2. The comparison of serum IL-17 levels detected by ELISA in different groups.

DISCUSSION

In children affected by asthma, several pathophysiological effects have been related to presence or absence of immune mediators. A growing body of evidence suggests that severe asthma is associated with IL-17 production (15). IL-17 is a proinflammatory cytokine playing an important role in the induction and propagation of inflammation in asthma (16). IL-17 may be one of the major cytokines involved in exacerbation of bronchial asthma (17).

The objectives of our study were to investigate the serum concentrations and also mRNA expressions of IL-17 in children with asthma of varying severity. Because the assessment of IL-17 at the serum level would certainly simplify clinical evaluation as a less invasive and easier measurement compared to bronchoalveolar lavage fluid, lung cells or even sputum in children (4).

In the current study, we found a significant difference in IL-17 mRNA expression and IL-17 concentrations in the sera of children with severe asthma compared to healthy non-asthmatic children and also children with mild to moderate asthma. To our knowledge, most of the previous studies have evaluated IL-17 gene expression in asthmatic airways, bronchoalveolar lavage fluid, lung or sputum but not in serum. The majority of studies on serum specimens have just quantified the serum levels of IL-17 by ELISA assays.

It has been shown that IL-17 is clearly expressed in the airways of asthmatic individuals and its expression level correlates with disease severity (18). In some recent studies, the serum IL-17 levels were significantly higher in children with asthma, than that of healthy controls (19,20). A study conducted on 120 asthmatic children has concluded that CD4+IL-17A+ T cell counts and serum IL-17 levels in conjunction with augmented FeNO levels are systemic markers of childhood asthma (21). There are also reports of significant differences in the serum IL-17 concentrations during asthmatic exacerbations between mild and meso-severe attack groups with healthy controls (19). In another study conducted on adult asthmatics, serum IL-17 concentrations had been considered as an independent risk factor for severe asthma (20).

Recently, it has been shown that TH17 cells may induce steroid resistance. Steroid hyporesponsiveness in subjects with severe asthma may also relate to the presence of IL-17, which has been reported to affect structural (epithelial) cells and to stimulate the production of profibrotic cytokines and extracellular matrix proteins (9). Our observations in this study are in agreement with the previous studies. Our data are in accordance with the well-known biologic function of IL-17. Our results may suggest a role for IL-17 as a factor contributing in driving asthma severity.

In conclusion**,** higher serum levels and mRNA expression of IL-17 found in children with severe asthma are in accordance with the well-known biological function of IL-17. However, asthma is an extremely complex disease and the infiltration of different immune cells results in different pathological processes. Considering this, our study has provided a rationale for further evaluations of serum markers associated with asthma severity and control.

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