IL-23 Gene and Protein Expression in Childhood Asthma

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ABSTRACT

Background: Asthma is the chronic inflammation of airways characterized by eosinophilic infiltration, mucus overproduction, airway hyper-responsiveness and airway remodeling. These changes are induced mostly by cytokines which are produced by T helper (Th) 2 cells. Recently, the role of interleukin-23 (IL-23) in the pathogenesis of adultallergic asthma has been studied. **Objective**: To explore IL-23 serum levels and its expression in persistent asthma compared with healthy children younger than five years old. **Method:** Blood samples of 40 children with mild and severe persistent asthma were compared to 34 healthy children regarding IL-23 serum levels and gene expression using enzyme-linked immunosorbentassay (ELISA) and real time quantitative polymerase chain reaction (PCR). **Results**: The IL-23 gene expression level was significantly different in the 25 children with mild persistent asthma and the 15 children with severe persistent asthma compared to the control group (p=0.001).There was no significant difference in IL-23 gene expression level between the two groups of patients with mild and severe persistent asthma. A significant difference was seen in IL-23 serum levels between the 25 children with persistent asthma and control group (p=0.002).**Conclusion:** For pre-school children with history and physical exam in favor of asthma which cannot be tested by spirometry, IL-23 serum levels may be an auxiliary biomarker for the diagnosis of asthma.

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INTRODUCTION

Asthma is a chronic inflammatory disease which usually leads to reversible obstruction of the airways. It's diagnosis is based on clinical findings and does not have a specific diagnostic test. Prevalence of the disease is increasing and currently more than 300 million individuals are estimated to have the disease worldwide. Approximately, 20 percent of the world's population has some form of allergic asthma. As western lifestyle and urbanization becomes more prevalent the incidences of asthma increases (1).

Chronic inflammatory disease of the airways in most patients with asthma is characterized by eosinophilic hyper-infiltration, overproduction of mucus in airways, hyper-reactivity and eventually remodeling of airways. Pathological changes in asthma are heterogeneous and these non-specific changes are mostly induced by T-helper 2 (Th2) cells and their related-Cytokines including Interleukins (IL) 4, 5 and 13(2).In addition to eosinophils, in other asthma phenotypes like severe asthma, neutrophils can also accumulate in the airways (3).

Depending on the number of various cells in the sputum induced by hypertonic saline, asthma can be classified into four phenotype: eosinophilic asthma (more than 1% sputum eosinophils), neutrophilic (sputum neutrophils more than 60%), mixed granulocytic asthma (both neutrophils and eosinophils increase in number) and asthma with normal sputum eosinophil and neutrophil which is also referred as paucigranulocyte (4,5).

Different subtypes of effector T cells produce different cytokines upon stimulation (6). IL-23 is a hetero-dimeric cytokine which is consisted of a P19 (IL-23α chain) a subunit specific for IL-23 and the p40 subunit of IL-12. P19 is specific, however P40 chain also exists in the structure of IL-12 and is a shared subunit between the two interleukins (7).

IL-23 receptor (IL-23R) is expressed on T cells (especially Th17), natural killer cells, monocytes and dendritic cells (8). This hetero-dimeric receptor is made up from two parts including IL-12RB1 (shared between IL-23 and IL-12) and another receptor known as IL-23Rα which is specific for IL-23(9, 10).

Activated macrophages and dendritic cells of peripheral blood produce a significant amount of IL-23 (7). The most commonly presumed function of IL-23 is to expand differentiated Th17 cells or maintain IL-17 production (11). IL-23 may function on dendritic cells to modulate their activity on Th2 cell differentiation; Therefore, IL-23 not only differentiates Th17 cells but also helps in Th2 cell differentiation (12).

IL-23 has a substantial role in causing eosinophilic inflammation in the airways; however, still few studies have been conducted on IL-23 serum levels and its association with in pediatric asthma (13). This study was conducted to investigate the association of IL-23 in serum and expression level in lymphocytes in children with persistent asthma compared to healthy children.

MATERIALS AND METHODS

1. Patient Selection. Forty children from one to five years of age with persistent asthma (diagnosed by allergist) referred to tertiary centers: Imam Reza allergy clinic and the pediatric ward of Namazi hospital in Shiraz, Iran from October 2014 to October 2015.

Diagnosis of persistent asthma was confirmed based on medical history and physical examination. Informed and written consent were obtained from guardians of patients for examination and for inclusion in the study.

The severity of persistent asthma in patients was determined based on the Export Panel Report 3 (EPR3) classification (14).

Inclusion and Exclusion Criteria. Children younger than five years of age with persistent asthma of different severity were randomly included in the study with their parents' consent. Patients with inflammatory and/or auto-immune diseases, cystic fibrosis, bacterial pneumonia, immunodeficiency, congenital heart disease and those consuming oral steroids in the past three months before their examination, were excluded from the study.

Blood samples of 34 healthy children less than 5 years old were obtained as control group. Samples of asthmatic patients were obtained from 40 children with asthma who were not previously or simultaneously treated with steroids.

2. Sampling. Blood (plus EDTA and clot) samples, were taken from both patient and control groups. Blood samples were first kept for one to two hours in room temperature to be clotted, they were then centrifuged for 10 minutes and the serum was extracted. Serum samples were frozen at minus eighty degrees centigrade and after collecting all samples, IL-23 serum levels were measured in both patients and control group using the enzyme-linked immunosorbent assay (ELISA) (Human IL-23 ELISA Ready –Set –Go, eBioscience, USA) method according to the manufacturer's recommendations and instructions.

The following steps were used to study the level of expression of IL-23 gene by real time quantitative polymerase chain reaction (PCR).

In the EDTA blood samples following steps were conducted: Preparing oviding Buffy coat, Leukocytes count, Ribonucleic acid (RNA) extraction, Complementary deoxyribonucleic acid (cDNA) synthesis and at the end performing real time quantitative PCR.

In order to extract the RNA, RNA RNX (Cina Gen co., Iran) was used and to produce cDNA, M-MULV Reverse Trans criptase (Cina Gen Co. in Iran) was used.

Level of expression of IL-23 gene was measured using real time quantitative PCR (SYBR Green Master Mix, Takara Co., Japan).

In the interpretation of results of real time quantitative PCR we had two threshold cycles (CTs) for each sample. One of the CTs was related to the target gene (IL-23) and the other was related to the internal control gene Glyceraldehyde phosphate dehydrogenase (GAPDH) and data of primers of IL-23 and GAPDH showed in Table 1.

The value of ΔCT for each sample was then measured by determining the difference between the target gene CT and the CT of the internal control gene GAPDH. This calculations was done for both patients and the healthy control group, after which using the Pffafl equation ($2^{-\Delta\Delta CT}$) the fold change of the IL-23 gene expression was evaluated(15).

Pffafl equation:

ΔCT (case) = CT target - CT reference gene

ΔCT (calibrator) = CT target - CT reference gene

ΔΔCT (case) = ΔCT (case) - Mean ΔCT of control

Fold change (of the related gene) = $2^{-\Delta\Delta CT}$

Table 1. Sequences of IL-23 and GAPDH* primers.

Gene	Gene Id	Primer Sequence	Product Length
IL-23	NM_016584.2	F:5'AGTGGAAGTGGGCAGAGATTC R:5'CAGCAGCAACAGCAGCATTAC	115 bp
GAPDH	NM_00128974501	F:5'TGAGAAGTATGACAACAGCC R:5'TCCTTCCACGATACCAAAG	109 bp

*-BLASTn (www.ncbi.nlm.gov./BLASTn USA).

Statistical Analysis. Analysis of data was done using the statistical package for the social sciences (SPSS) Software (Version 22). For comparison of IL-23 serum levels the Mann-Whitney non-parametric test was used. For comparison of IL-23 expression levels the Binomial non-parametric test was used. Also, binomial non-parametric test was utilized to compare the level of expression of IL-23 gene in patients with severe asthma and mild asthma compare with the control group. A p-value<0.05 was considered as significant. Graph Pad Prism 6 was used to draw graphs.

RESULTS

In this study, we compared expression levels of IL-23 in 40 children with persistent asthma and 34 healthy children less than 5 years of age. The average age was 27 months in patients and 25 months in healthy control groups. Among the 40 children with persistent asthma, 30 children (75%) were boys and 10 children (25%) were girls and among the control group 17 (50%) were boys and 17 children (50%) were girls. Twenty five (62%) of the 40 children with persistent asthma had mild persistent asthma and 15 children (38%) had severe persistent asthma. In the present study, 29 individuals (72%) had allergic rhinitis and 16 individuals (40%) presented with atopic dermatitis along with asthma. In our study among 40 children with asthma, 16 patients (40%) experienced one to three asthma attacks in the past year and were hospitalized. In this study, 11 of the children' parents had asthma and 11 of them were cigarette smokers.

There was a significant difference in IL-23 gene expression level between case and control groups (Figure 1, p=0.001).

Further analysis showed a significant difference between IL-23 gene expression in patients with mild and severe persistent asthma compared to control group (p=0.001 and p=0.01 respectively, Figure 2). The median of IL-23 gene expression in patients with severe persistent asthma was greater than patients with mild persistent asthma. But, there was no significant difference in IL-23 gene expression level between the two groups of patients with mild and severe persistent asthma.

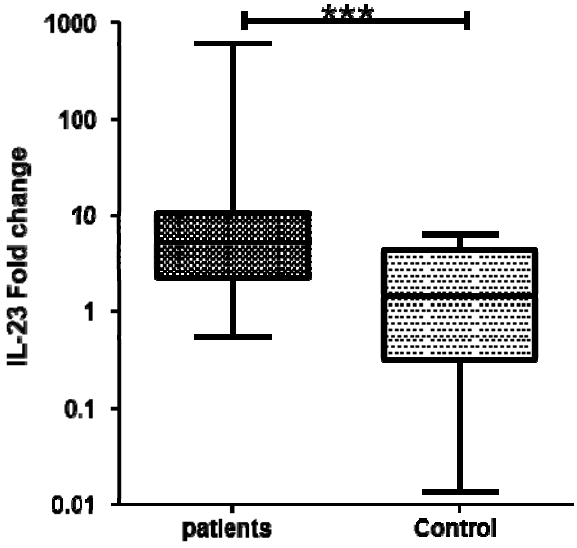

Figure 1. Real time quantitative PCR of IL-23 gene expression level between control group and all asthma patients showed a significant difference.

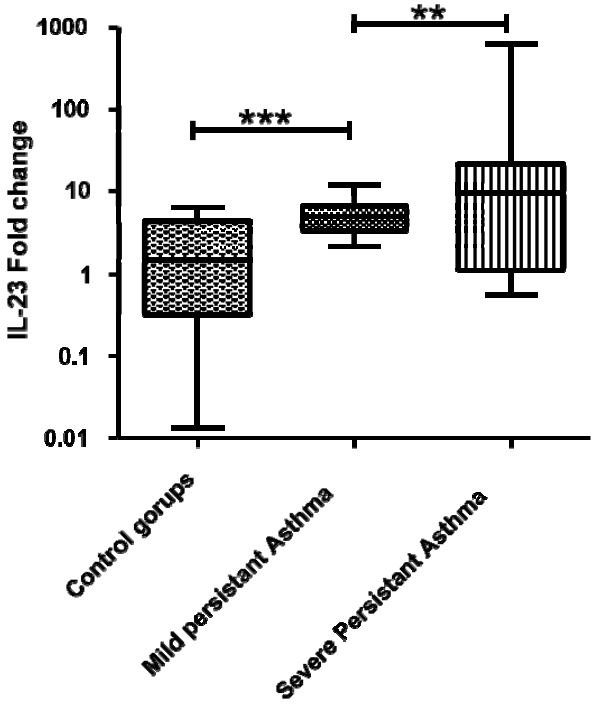

Figure 2. Real time quantitative PCR showed significant differences between IL-23 gene expression in patients with mild and severe persistent asthma in comparison to control groups.

For IL-23 level assay, sera of 25 patients and 25 healthy children less than five years old were compared. The number of available patients'sera declined from 40 to 25 due to sampling problems. The average age was 27 months for patients and 25 months for the control group. Regarding gender in the patients group, 16 individuals (65%) were boys

and 9 individuals (35%) were girls. In the control group there were 15 boys (60%) and 10 girls. IL-23 serum levels of patients with persistent asthma compared to the IL-23 serum levels of the control group suggests a significant difference (p=0.002, Figure 3). But, as in IL-23 gene expression there was no significant difference in IL-23 serum level between the patients with persistent asthma. The average IL-23 serum level was 40pg/ml and 14pg/ml in the case and control groups, respectively.

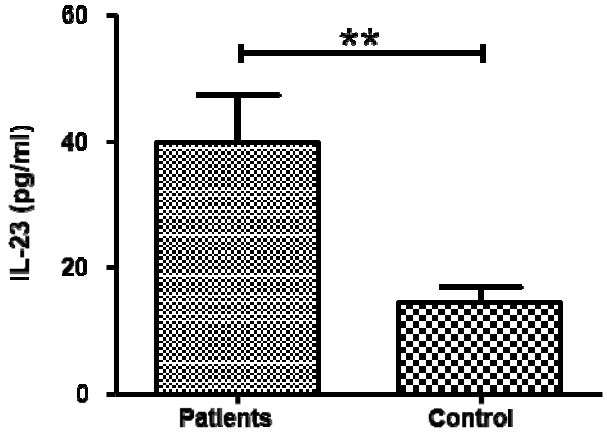

Figure 3. ELISA serum levels of IL-23 in persistent asthma patients compared to control group show significant difference.

DISCUSSION

Based on the few recent studies (mostly conducted in animal models) persistent asthma is associated with IL-23 serum level (13,16). The purpose of our study was to explore the relationship between the gene expression and the serum levels of IL-23 in patients with persistent asthma compare to healthy children and compared to gene expression and serum level of IL23 between several types of persistent asthma themselves (mild to severe). The best method to do this was to conduct a spot test for example a Broncho-alveolar lavage; however, as it was an invasive procedure, we investigated the serum titer and expression levels of the IL-23 mRNA.

In our study the average age of patients was 27 months and the average serum level of IL-23 was 40 pg/mg; however, in the study of Ciprandi *et al.* (13) the average age of patients was 108 months (9 years) and the serum levels of IL-23 was 111 pg/ml. This could be due to the lower age of our patients which resulted in lower IL-23 serum levels.

In our study, differences between the patients' group and the control group were investigated regarding IL-23 serum levels and its gene expression. In fact, the two methods were complementary to one another; however, Ciprandi *et al.* studied and compared the relationship between IL-23 serum levels and forced expiratory volume in one second (FEV1)in spirometry and found a strong and inverse relationship(13). In the study by Cuppari *et al,* IL-23 serum levels were compared to forced expiratory volume in one second /forced vital capacity (FEV1/FVC)ratio andforced expiratory flow at

25,75% (FEF25-75)that revealed strong and inverse relationship between IL-23 serum levels and both FEV1/FVC and FEF_{25-75} ratio that were complementary to each other(16).

In the study by Ciprandi *et al,* IL-10, IL-23 and IL-4 serum levels were assessed in eighty children with asthma before and tree months after treatment by inhaled steroid (Budesonide 200 mcg two times a day). IL-23 and IL-4 serum levels at first point were higher and IL-10 serum level of patients was lower than that of healthy individuals. Three months after starting treatment, IL-23 and IL-4 serum levels significantly decreased and IL-10 serum levels significantly increased (17).

In our study in addition to an increase of IL-23 in children with persistent asthma, seventy two percent of patients presented with both asthma and allergic rhinitis and 42% of them with a concomitant atopic dermatitis. There was a significant difference ($p=0.001$) in both number of patients and IL-23 serum levels between these patients (those with concomitant allergic rhinitis or atopic dermatitis) and those without allergic rhinitis and atopic dermatitis. The same finding in the study by Leonard *et al.* that was a significant relationship between an increase in IL-23 serum levels and allergic rhinitis (18). Therefore, we suggest that measuring IL-23 could be considered as a marker of atopic march.

Due to association of IL-23 levels and neutrophilic asthma especially in adult and higher gene expression in more severe asthma, IL-23 may be helpful in conditions like Aspirin exacerbated respiratory disease (AERD) that have more severe asthma without exact etiology and even as a marker in aspirin desensitization (19). Genetic factors are important in asthma phenotyping and the controversy of our results with previous studies may be due to race and different asthma phenotype (20).

Limitations of our study were small sample sizes and inability to assign a before and after treatment data point to investigate IL-23 variability by treatment. Finally there was a significant rise in gene expression and higher titer of serum IL-23 in patient with persistent asthma compared to healthy children (Figure 1-3), but there was no significant difference by asthma severity between asthmatic patients.

Our findings show significant relationship between IL-23 serum levels and its gene expression and children's persistent asthma. Our findings suggest measurement of IL-23 serum levels as a complementary marker along with clinical criteria for diagnosis of asthma in children less than five years of old.

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