Gene Expression and Serum *IL-23* in Asthmatic Iraqi Children

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Abstract

Asthma is a non-communicable inflammatory airway disorder in which patients present with recurring bouts of breathlessness and wheezing. *IL-23* is a proinflammatory cytokine its biological functions have been well known for the capability to enhance Th17 cell functions. Current study aimed to estimate the gene expression and serum level of *IL-23* and to investigate whether *IL-23* plays pivotal roles in the development of asthma in asthmatic Iraqi childhood . This study was conducted on two groups: seventy five asthma patients (27 female and 48 male) and twenty five apparently healthy as a control group (10female and 15 male).The age of the samples ranged from (1-10) years old. Recruited from admitting the Central Teaching Hospital Pediatrics and Alzahra'a Center for Asthma Allergy in Baghdad during the period extended from October /2019 to last February/2020. Subject information's were collected using a specific questionnaire form as a descriptive study; on the other hand, the present study was approved by the council of institute of genetic engineering and biotechnology for post graduate studies / University of Baghdad. The RNA was extracted from the blood sample of asthma patients and apparently healthy subjects by using TransZol Up Plus RNA Kit (blood) .The acceptable purity of RNA in asthmatic patient is range between 1.84-1.99 and for apparently healthy group is range between 1.84-1.96.mRANA expression were determined by real time PCR assay and detect the concentration of *IL-23* using ELISA technique. For *IL-23* gene expression that showed the Ct of asthma patient group (22.82) and control group (23.33) and the 2-ΔΔCt of asthma patient group (5.35) and control group (3.70) and the fold of gene expression was statistically significantly (P≤0.05).

in the asthmatic group than healthy non asthmatic group that show in ratio (1.44: 1.00) . Human *IL-23*concentration was estimated by ELISA, the results were statistically significant (P≤0.002), the level of *IL-23* in asthma patient group (451.80 ± 91.80pg/ml) while the level of *IL-23* in Control group(182.36 ± 58.21 pg/ml) as well as increase concentration of *IL-23* in severe asthma patients than mild form asthma 726.91 ± 142.98 and 133.72 ± 79.56 respectively.

Key Word: *Asthma Childhood,* **IL-23***, qRT-PCR.*

Introduction

Asthma of childhood is, a complex chronic disease, characterized by sever and chronic inflammation of airway, airflow limitation, , wheezing, recurrent coughing , shortness of breath and chest tightness. Symptoms may occur several times in a day or week in affected individuals, and for some people become worse during physical activity or at night. During an asthma attack, the lining of the bronchial tubes swell, causing the airways to narrow and reducing the flow of air into and out of the lungs. Recurrent asthma symptoms frequently cause sleeplessness, daytime fatigue, reduced activity levels and school absenteeism. Asthma has a relatively low fatality rate compared to other chronic diseases[1]. Asthma onset in children usually occurs before their fifth birthday. The majority of children with asthma are sensitive to household allergens and irritants, and they can benefit from a smoke-free, dust-free and pet-free environment [2,3]. According to World Health Organization estimates that more than 339 million people had Asthma globally in 2016 [4]. (*IL-23*) a member of the *IL-12* family of cytokines, is a heterodimeric cytokine. It is composed of subunits p40 (shared with *IL-12*) and p19 (an *IL-12* p35-related subunit) and is secreted by several types of immune cells, such as natural killer

cells and dendritic cells[5]. And is a protein consists of 189 amino acids with a molecular mass of 20730Da, and its gene lies on human chromosome 12q13.3, *IL-23* may contribute to the differentiation of macrophages, it has been shown that the enforced expression of *IL-23* in the lung enhances not only antigen-induced *IL-17A* production and neutrophil recruitment, but also antigen-induced Th2 cytokine production and eosinophil recruitment in the airways[6]. *IL-23* Induces Memory T Cell Secretion of *IL-17,* the Memory (CD4 CD45RO) T cells secrete *IL-17* in normal peripheral blood after activation in vitro. Addition of *IL-23* alone to cultured memory (CD4 CD45RO) T cells induces a slight increase in *IL-17* secretion. However, when other factors, such as activating anti-CD3/anti-CD28, are added together with *IL-23*, the level of *IL-17* secreted from memory T cells significantly increases consistently as in, intracellular *IL-17* mRNA levels significantly increase in these conditions[7].

The increase in *IL-17* concentration is accompanied by the enhanced concentration of *IL-23* which is a critical regulator of *IL-17* [8].

As to our knowledge, this is the first study about the genetic aspect of child asthma disease in genetic engineering and biotechnology institute so that. The present study aims to increase knowledge about the occurrence of Asthma in child in Iraq to determining the gene expression of *IL-23* in patient by Real time PCR as well as determine serum level of *IL-23* using ELISA technique.

Subjects, Materials and Methods

Study consist of two groups, Seventy Five patients (27 female and 48 male) and twenty five as apparently healthy subjects (control) and personal information such as: age, season, gender, family history, sensitivity ,incidence intensity, onset of disease, other diseases, the samples were admitting the Central Teaching Hospital Pediatrics and Alzahra'a Center for Asthma and Allergy in Baghdad during the period between October /2019 to February /2020. The study design was approved by the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/ University of Baghdad. Writing informed consents were obtained from all patients and apparently healthy control group; all patients were diagnosed according to clinical examination by a chest physician and selected according to the criteria of the global initiate of asthma.

Genomic RNA extraction and determine level *IL-23* in serum

Three ml of peripheral venous blood samples were collected from the asthma patients and apparently healthy control using disposable latex gloves and syringes. Then,1 ml of blood was kept in EDTA anticoagulant tubes and then converted to 0.25 ml of EDTA blood in Eppendorf tubes that contain1ml Trizol and freezer at -20°C to be a source for RNA extraction. The RNA was extracted from the samples of blood of asthma patients and apparently healthy subjects by using TransZol Up Plus RNA Kit (blood) company of kit (Transgen) Then, RNA concentration and purity were measured by nanodrop. The acceptable purity asthmatic patient of samples of RNA is range between 1.84-1.99 and for healthy group samples of RNA purity is range between 1.84-1.96. and level of *IL-23* determining in serum of asthma patients and apparently healthy by using *IL-23* ELISA kit, ELISA kit. MY BIOSOURSE COMPANY was used in this study.

cDNA synthesis for mRNA

Total RNA was reversely transcribed to complementary DNA (cDNA) using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix. The procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions. The total RNA volume to be reversely transcribed was (20µl).

Real time PCR Primers for *IL-23* Gene:

The primer sequence of this study[9].

Interleukin 23: Forward 5 ' AGTGGAAGTGGGCAGAGATTC-3

Reverse5 ' CAGCAGCAACAGCAGCATTAC-3

GAPDH: Forward 5 ' TGAGAAGTATGACAACAGCC-3

Reverse 5 ' TCCTTCCACGATACCAAAG-

Components of quantitative real-time PCR used in *IL-23* (Transgen / China).

Template DNA	3 µl
Reagent Master Mix	25 µl 1 rxn
PCR grade water	7.5
2xqPCR Master Mix	12.5 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl

Real Time PCR Program

The expression levels of *(IL-23),* and housekeeping genes (*GAPDH)* were estimated by RT-PCR To confirm the expression of target gene, quantitative real time RT-PCR SYBR Green assay was used. Primers sequences for *(IL-23),* as housekeeping gene (GAPDH*)* were prepared according to[9]. Optimal annealing temperature of qPCR reaction was found out after several try to 64˚C with a total volume of 50 µl. The reaction components are described in table (1).

Table (1)Thermal profile of *IL23* gene expression

Step	Temperature	Duration	Cycles
Enzyme activation	94ºC	30 sec	Hold
Denature	94ºC	5 sec	40
Anneal	64ºC	20 sec	
extend	72ºC	20 sec	
Dissociation	1min /95 ºC-30 sec /60ºC-30sec/95 ºC		

Real Time RT-PCR analysis of *IL-23*gene expression:

1. DCT

The expression ratio was calculated without a calibrator sample 2-DCt according to the following equation:

DCT (test) = CT gene of interest (target, test) – CT internal control

Finally, the expression ratio was calculated according to the formula

2^{-DCt} = Normalized expression ratio

2. DD CT

To compare the transcript levels between different samples the 2^{-DDCt} method was used[10].

The CT of gene of interest was normalized to that of internal control gene. And the CT was calculated as the following formula:

DCT (test) = CT gene of interest (target, test) – CT internal control

DCT (calibrator) = CT gene of interest (target, calibrator) – CT internal control. The calibrator was chosen from the control samples.

CT values ≥ 38 were considered unreliable and neglected

The DCT of the test samples was normalized to the DCT of the calibrator:

DD CT was calculated according to the following equation:

DD CT=DCT (test)- DCT(calibrator)

Finally, the expression ratio was calculated according to the formula

2^{-DDCt} = Normalized expression ratio.

Determination of *IL-23* titer using Enzyme-Linked Immunosorbent Assay kit (My biosoure/USA)

Statistical analysis

According to[11]. (SAS) Program was used to determine the fold of gene expression. Least significant difference –LSD test (Analysis of Variation-ANOVA) or T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Result and Discussion

RNA Extraction

Total RNA was successfully extracted from all samples. The concentration of total RNA ranged from 83-188ng/ µl and from 81-182 ng/µl in healthy group (non asthmatic). And the purity of total RNA samples ranged from 1.84-1.99ng/µl in the patients asthmatic group, and from 1.84-1.96 ng/µl in the healthy non asthmatic group. With p-value 0.74.

There was no significant difference between the concentration of the total RNA of the two study groups, p=0.366. There was no significant difference between the RNA purity of the two study groups as well.

Real time PCR quantification of *IL-23* Expression:

The mean Ct value of *IL-23* cDNA amplification were (22.82) in the patients asthmatic group, and the mean Ct healthy non asthmatic group were **(23.33)**. The results are shown in table (2). There was a significant difference in the mean Ct values between the different study groups (**(P≤0.05).**

This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *IL-23*cDNA amplification of each single case and that of the *GAPDH*.

Table (2) shows the mean of ΔCt (normalization Ct values) of each study group. ΔCt means in asthmatic patient group(**0.57).** And healthy non asthmatic group was **(1.1)**. A significant difference was noticed between the study groups **(P≤0.05)**

Results of $2^{-\Delta Ct}$ revealed significantly higher results for the asthmatic patient group from the healthy non asthmatic group **(P≤0.05**), mean of $2^{-\Delta Ct}$ for asthmatic patient group **(0.673**) while In the healthy non asthmatic group a mean of $2^{-\Delta Ct}$ was **(0.466)**.

To calculate the gene expression folds in relation to the housekeeping genes the result of $2^{-\Delta Ct}$ of each group was measured in relation to that of Healthy non asthmatic group .The results are shown in table (2). The fold of gene expression in asthmatic patient group was higher than Healthy non asthmatic group in **1.44** times. While the fold number in healthy non asthmatic group was**1.00** times. as in table (2). These results indicate a significantly increase expression of *B-actin* gene in these groups.

Table (2): Fold of *IL-23*expression Depending on 2-ΔCtMethod

Groups	Means Ct of IL23	Means Ct of GAPDH	ΔCt (Means Ct ofIL23 - Means Ct of GAPDH)	2-ΔCt	experimental group/ Control group	Fold of gene expression
Asthma patient group	22.82	22.25	0.57	0.673	0.673/0.466	1.44 ± 0.07
Healthy group	23.33	22.23	1.1	0.466	0.466/0.466	1.00 ± 0.00
T-test	--	--	--	--	--	0.288 *
P-value	--	--	--	--	--	0.0372

* (P≤0.05).

As shown in table (3), the mean of $2^{-\Delta\Delta Ct}$ values of asthmatic patient group and Healthy non asthmatic group it was (**5.35**) and (**3.70**) respectively. There was a significant difference between these groups regarding the mean $2^{-\Delta\Delta Ct}$, (p**≤0.05**).

Table (3): Fold of *IL-23* expression Depending on 2-ΔΔCt Method

Groups	Means Ct of IL23	Means Ct of GAPDH	ΔCt (Means Ct of IL23 - Means Ct of GAPDH	Mean ΔCt Calibrator (ctIL23-ct GAPDH	ΔΔCt	2-ΔΔCt	experimental group/ Control group	Fold of gene expression
Asthma patient group	22.82	22.25	0.57	2.99	-2.42	5.35	5.35/3.70	1.44 ± 0.07
Healthy group	23.33	22.23	1.1	2.99	-1.89	3.70	3.70/3.70	1.00 ± 0.00
T-test	--	--	--	--	--	--	--	0.288 *
P-value	--	--	--	--	--	--	--	0.0372

*** (P≤0.05).**

When calculating gene expression it was significantly higher in asthmatic patient group than Healthy non asthmatic group 1.44 times. as shown in table (3). The above results demonstrate the significant gene expression in Healthy non asthmatic group.

The mean Ct values in healthy non asthmatic group were higher than those of asthmatic patient group .This is important in reflecting the original mRNAs present in the samples. It is evident from these results that the patients group is associated with the highest copy number of mRNAs reflecting its higher expression.

The results show convergence of Ct values between asthmatic patient group and healthy non asthmatic group it is important evidence that *IL-23*gene expression so it is possible to use *IL-23* gene as a biomarker for the early detection of asthma disease.

These results agreed with[11]. Their result indicate *IL-23* levels were higher in severe asthmatics than in control group Thus, it is presumably that *IL-23* could be a suitable marker of allergic inflammation in asthma.

Figures (1),(2) show the amplification plots and dissociation curves for *IL-23*.

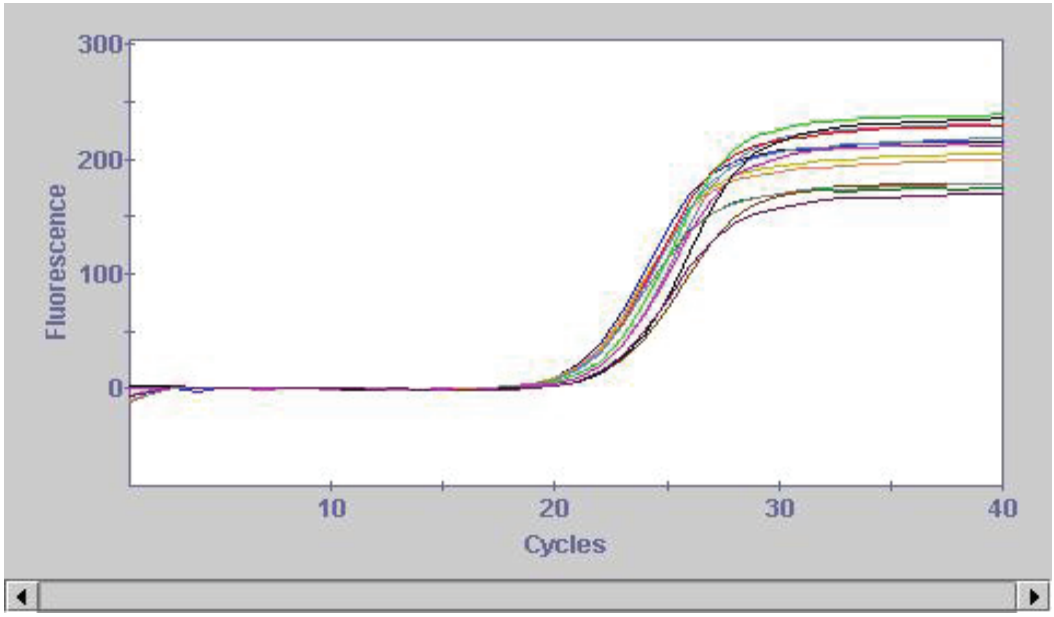

Figure (1): *IL-23* **amplification plots by qPCR Samples included all study groups.Ct values ranged from 21.97 to 23.75. The photograph was taken directly from Cephoidsmartcycler qPCR machine**

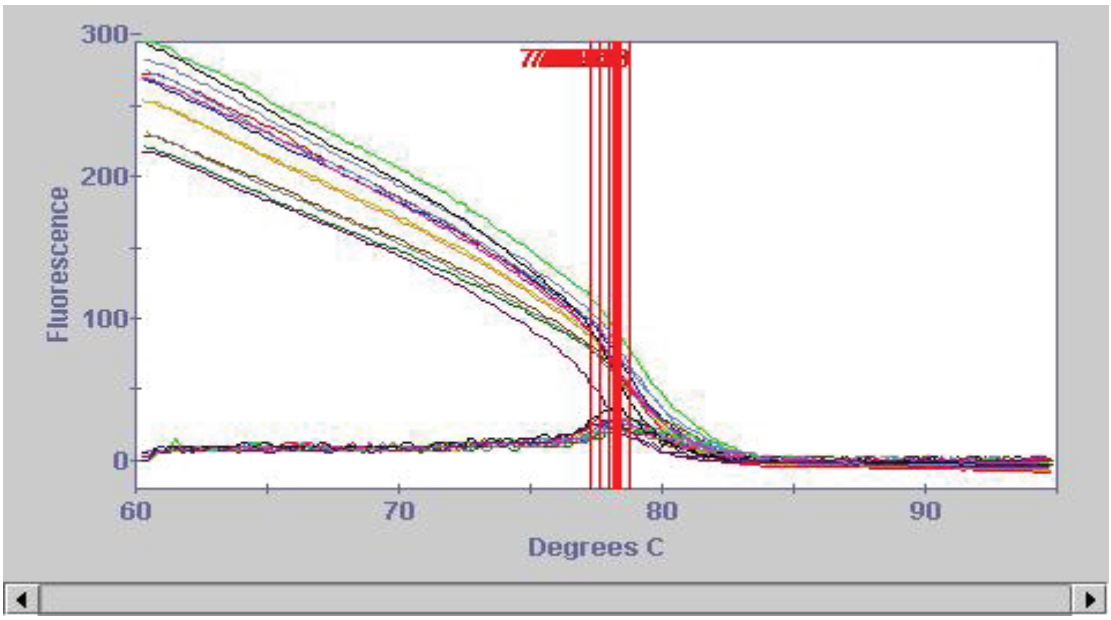

Figure (2): *IL-23* **dissociation curves by qPCR Samples included all study groups. Melting temperature ranged from 76.78°C to 79.96°C. The photograph was taken directly from Cephoid smart cycler qPCR machine**

Serological Study:

Concentrations of *IL-23* in blood serum of asthmatic patient and control group. that show significant between asthmatic patients and control group (P≤0.002). The Mean ± SE of asthma Patients(451.80 ± 91.80pg/ml) and Mean ± SE of Control group(182.36 ± 58.21 pg/ml).

This result is Similar to study [12]. That measure the level of *IL-23* in Seventy-eight asthmatic children and 40 healthy children by ELISA were evaluated *IL-23* levels were higher in asthmatic than in healthy children.

And when Comparison level of cytokine (*IL-23*) between asthma patient in different forms asthma (sever and mild) and control group the result appear significantly between these groups (P≤0.002), the concentration of *IL-23* in sever asthma patient (726.91 ± 142.98), mild asthma patient (133.72 ± 79.56) and control group(182.36 ± 58.22) Table (10), figure (3). The concentration of *IL-23* increase with severity form asthma more than mild form and these result identical with gene expression result that previously offered. This study is identical with several studies[13,14].

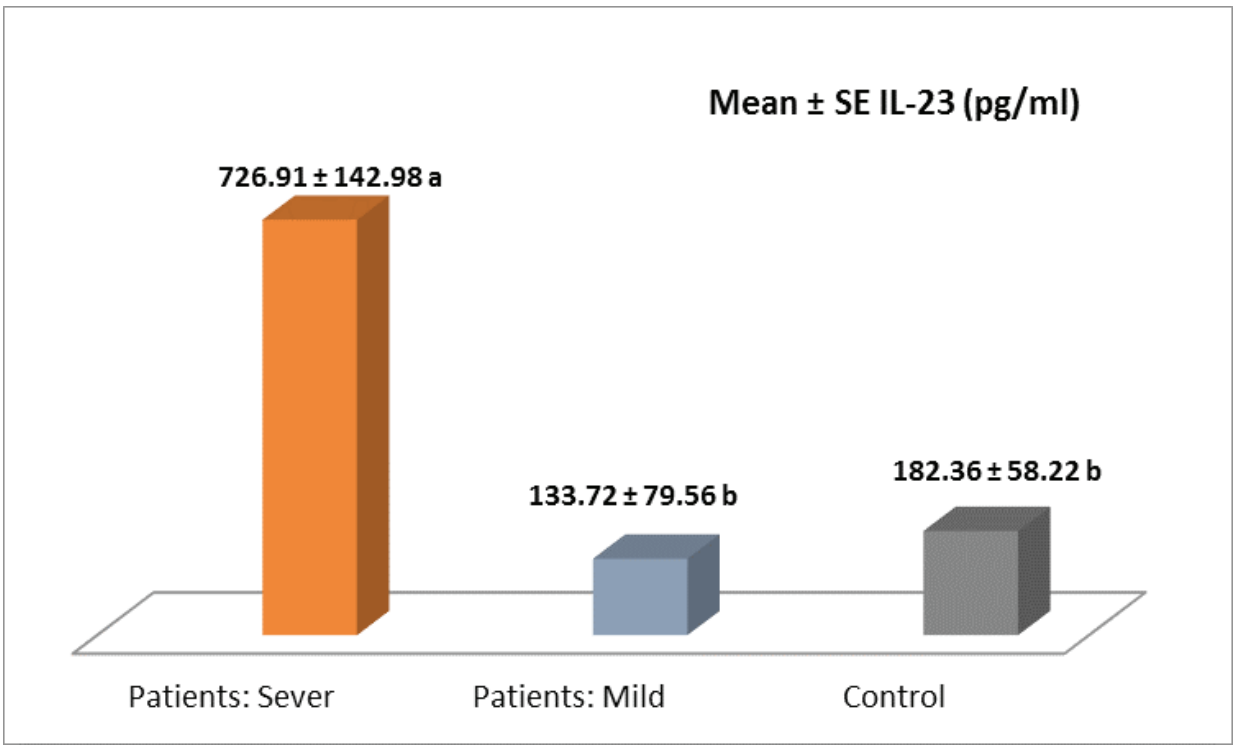

Figure (3): Comparison between patients (Sever, Mild) and control groups in concentration of *IL-23*.

Conclusion

Study revealed the an increase *IL-23* mRNA expressions and serum *IL-23* concentrations in children with severe asthma compared to that with mild of the disease and control group.

Recommendations: Study the gene expression and level of *IL-23* in asthma adult .

Acknowledgements: We are grateful to the persons for their involvement in this study.

Ethical Clearance: The principles and the experimental protocol in this study was approved by the medico – legal directorate, Ministry of Health, Baghdad, Iraq.

Source of Funding- Self

Conflict of Interest - nil

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