Original Article

mRNA expression analysis of interleukins 17A and 17F in bronchial asthmatic patients from Northern Indian population

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Abstract

Introduction: Asthma being a chronic inflammatory disease concerning to the airways involves genetic and environmental factors. It is known to develop a clinical condition of airway hyper-responsiveness, which induces frequent symptoms in patients such as breathlessness, chest congestion, coughing, and wheezing, particularly during night hours or during early morning hours. The cytokine, Interleukin 17F (IL17F), is important in mediating allergic reactions in the body and regulating the pathophysiology and pathogenesis of asthmatic attacks, as well as airway inflammation, respectively. The Interleukin 17A (IL17A) is involved in increasing the biosynthesis of interleukins IL-6 and IL11. In contrast, IL17F enhances the expression of interleukin IL11 and tumor growth factor, TGF-β. **Methodology:** Standard procedures were followed for collection and processing of blood samples from the subjects (controls and patients, 104 each), isolation of mRNA and to determine the quantities of IgE, and the interlukins (IL17A and IL17F) in the serum. The Real-time PCR and ELISA techniques were employed for synthesis of cDNA and determination of interleukins, respectively, using standard protocols. Early diagnosis of asthma is still a challenge to meet. **Results:** The statistical analysis of the data reflected a positive correlation between each of the interleukins (IL-17A and IL17F) and IgE ($p = 0.001$ and $r = 0.41$), ($p = 0.004$ and $r = 0.077$). The results indicated the upregulation of expression of IL17A and IL17F genes in the patients suffering from asthma. **Conclusions:** This study has indicated that the blood levels of IL-17A and IL17F could be utilized as viable clinical markers for early diagnosis, timely treatment, and proper management of asthma.

Keywords: Asthma, ELISA, IL-17, IL-17F, mRNA, real-time PCR

Introduction

Asthma recognized as a chronic airway inflamatory disorder, is characterized by excess mucus production, as well as hyper-responsiveness of airways (AHR) involving remodeling of airways. This disease with high frequency has been found to affect the people of all age groups such as children, young, adults including old aged subjects with increased morbidity and death.[1] Mainly T-cells have been found to be involved to drive the asthmatic disorder. However, the pathophysiology of asthma is significantly regulated by two subpopulations of T helper cells, i.e., Th1 and Th2. In additions, another subset of T helper cells, i.e., Th17 cells, is also involved in this process but it exhibits functions different from those of Th1 and Th2 cells. Th17 cells produce another interleukin, i.e., IL17. The IL17 has been shown to be implicated in regulation of the asthma's pathophysiology[2] and also the progression of several other inflammatory and autoimmune diseases.[3] The available evidences suggest that an increase in the levels of IL17 is firmly associated to the varied inflammatory clinical conditions, such as inflammatory bowel diseases, rheumatoid arthritis and psoriasis.[4] In the patients suffering from asthma,

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Received: 07-01-2020 **Revised:** 12-03-2020
Accepted: 08-04-2020 **Published:** 31-05-2020

Access this article online

Quick Response Code:

Website: www.jfmpc.com

DOI: 10.4103/jfmpc.jfmpc_35_20

How to cite this article: Pandey R, Prakash V. mRNA expression analysis of interleukins 17A and 17F in bronchial asthmatic patients from Northern Indian population. J Family Med Prim Care 2020;9:2258-63.

there is overexpression of IL17 causing increase in mucus and sputum in lungs.[5]

Rouvier *et al.*[6] who get the credit of identification of IL17 for the first time, demonstrated that IL17 was chiefly expressed by activated CD4 +ve T-cells. Later on, it was observed by other workers that in addition to these T cells, other blood cells such as eosinophils and neutrophils, were also producer of IL17A.[7] The fibroblasts of asthmatics were found to show that IL17A might enhance the generation of the IL-6 and IL11. On the other hand, IL17F was observed to induce the expression levels of IL11 and TGF-β. The results of the experiments conducted by Molet *et al.* (2001) suggested that IL17 was upregulated in the moderate to severe asthma patients as compared to those with mild asthma or control subjects.[8,9]

Keeping in view the lack of a suitable molecular biomarker for early diagnosis of asthma, we have carried out this study to detect the mRNA expression levels of the two interleukins, i.e., IL17A and IL17F in the whole blood of asthmatics and the healthy controls. The change in the levels of these interleukins was assessed by the real-time polymerase chain reaction (RTPCR), for evaluating whether these cytokines could be utilized as viable parameters toward the early diagnosis and timely start of treatment of asthma.

Methodology

Selection of asthmatics and control subjects

In this study, 104 cases and 104 controls were enrolled, cases were patients with asthma patients attending OPD of Pulmonary and Critical Care medicine in King George Medical University and healthy controls were individuals'visiting KGMU for blood donation camps. This study got approval from the Institutional Ethical Committee (IEC) of KGMU-Lucknow. The informed consents were taken from both the asthma patients and/or their respective guardians as well as from the healthy controls.

The diagnosis of asthmatics was made by the physicians based on their clinical assessment. The data concerning their demographic and clinical status were recorded in structured questionnaire.

Blood samples

Blood samples were drawn by vein puncture and 500 µl of the blood was added into the Tri BD reagent Blood RNA tubes. The samples thus collected were used or kept stored at −20°C till further analysis.

RNA isolation and cDNA synthesis

The isolation of RNA from the whole blood was done by using the Blood RNA kits (Ref no 52304 Qiagen) and following the procedure as described in the manufacturer's protocol. The assessment of RNA integrity was made by performing formaldehyde agarose gel electrophoresis[10] and also spectrophotometrically by monitoring absorption ratio at 260/280 nm. The reverse transcription reaction was carried out in a total reaction volume of 20 µl employing the high capacity RNA to cDNA Reverse Transcription Kits, as per the instructions provided by the manufacturer. The quality of the constructed cDNA product was analyzed by the electrophoresis on a 1% agarose gel containing ethidium bromide. The quantity of transcripts of IL17 was determined by using a standard curve of GAPDH as a reference.

Assay of real-time-polymerase chain reaction (RT-PCR)

For carrying out real-time RT-PCR assays, the synthesized cDNAs (2µl) was amplified using the gene-specific primers. Each of the assays were carried out in duplicates. For each sample, the calculation of the threshold cycle (ct) was automatically made by the 7500 fast Real-Time PCR software. The analysis of the data was done using a comparative CT method for the gene expression in relation to GAPDH.[11]

Determination of the level of IL17 A and IL17F

The specific enzyme-linked immunosorbent assay (ELISA) kits supplied by the Ray Biotech Inc., Norcross, Georgia, USA were used for quantitative determination of the IL17A and IL17F.

Statistical analysis

The results were analyzed using the Graphpad (Prism). The asymmetric and non-normal distribution of the IL17A and IL17F mRNA prompted us to use the one way ANOVA analysis of variance test for assessing the differences in the expressions of IL17A and IL17F in asthmatics and the control subjects. In order to evaluate the correlation between levels of mRNAs of IL17A and IL17F, the Pearson's correlation test was performed.

Results

Recruitment of subjects in the study

For this study, a total of 104 asthma patients and 104 controls were recruited. Both groups (cases and control) had similar number of age.

The demographic profile of patients suffering from asthma and the controls have been summarized in Table 1. Our

Table 1: Demographic profile of asthmatics and control

Age group (Years)	Group analysis	Groups		Total
		Cases	Controls	
20-35 years	Count	78	78	156
	% within Groups	75%	75%	75%
36-50 years	Count	23	24	47
	% within Groups	22.1%	23%	22.5%
51-60 years	Count	3	2	6
	% within Groups	2.8%	1.9%	2.8%
Total	Count	104	104	208
	% within Groups	100.0%	100.0%	100.0%

Descriptive Statistics has been followed in analysis of the data.

study included 104 cases consisting 78 cases between 20 and 35 years, 23 cases between 36 and 50 years, and 3 cases between 51 and 60 years of age. The control group included 78 controls between 20 and 35 years, 24 controls were between 36 and 50 years and 2 controls between 51 and 60 years.

In the present study, we observed that tobacco smoking habits were similar in the cases and control. The status of smokers is displayed in Table 2. The types of smokers have been demonstrated in Figure 1.

The group for asthmatic patients contained higher number of smokers using biddi and cigarette and higher number of alcohol users of alcohol [Figure 1].

Clinical symptoms in study population

The occurrence of breathlessness, cough, headache, disturbed sleep, chest tightness, and wheezing were recorded to be 86.5%, 72.1%, 66.3%, 76.9%, 91.0%, and 88.4%, respectively, in the patients [Table 3]. The values of the clinical indices for above parameters in the cases are also shown.

Laboratory parameters in study population

The values of parameters of laboratory investigations of the cases and controls are depicted in Table 4. The levels of IgE, Eosinophil, AEC, TLC, and Hb (Mean ± SD) were observed to be 421 ± 102 IU/ml, 7.34 ± 4.02%, 543 ± 174 cell/cum, and 6585 ± 2457 cell/cum 11.19 ± 1.23 (gm/dl), respectively, in the cases. Except Hb, the values of other clinical indices were found to be significantly more than that of controls. The level of Hb, however, was lower (0.95 ± 0.1247 gm/dl) in the cases when compared with the control (13.05 ± 1.89 gm/dl).

mRNA expression analysis for IL-17A and IL-17F

In order to determine the level of mRNA expression for the interleukins IL-17A and IL-17F, the data obtained were statistically analyzed and presented in Table 5. The results indicated rise in mRNA expression in the cases as compared with the controls.

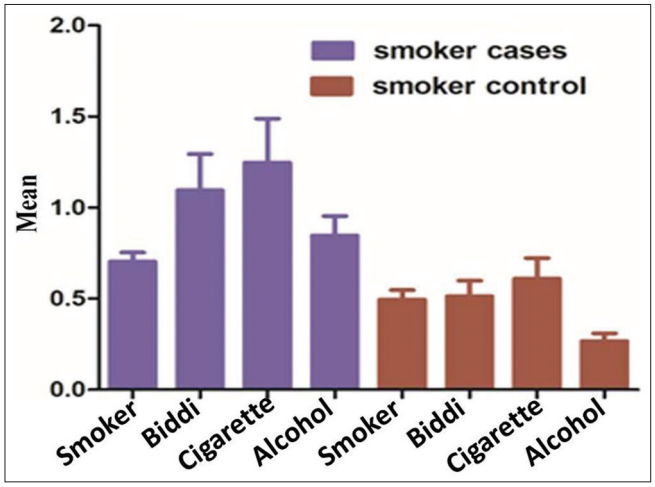

Figure 1: Types of smokers recruited in the study

Table 2: Status of smoking in controls and cases

Status of smoking	Group analysis indices	Groups		Total
		Cases	Controls	
Absent	Count	40	68	108
	% within Groups	38.4%	65.3%	51.9%
Present	Count	65	34	99
	% within Groups	62.5%	32.6%	47.5%
Total	Count	104	104	208
	% within Groups	100.0%	100.0%	100.0%

Descriptive statistics has been followed in analysis of the data

Table 3: Clinical symptoms recorded in the subjects under study

Symptoms	Cases (*n*=104) (%)
Breathlessness	86.5
Cough	72.1
Headache	66.3
Disturbed sleep	76.9
Chest tightness	91.0
Wheezing	88.4

Table 4: Clinical indices in cases and controls

Parameters	Groups	Number	Mean	Std. deviation	*P*
Hb (gm/dl)	Cases	104	11.19	1.23	<0.001*
	Controls	104	13.05	1.89	
Eosinophil (%)	Cases	104	7.34	4.02	<0.001*
	Controls	104	3.77	2.21	
TLC (cell/cum)	Cases	104	6585	2457	<0.001*
	Controls	104	9226.3	13510.7	
IGE (IU/mL)	Cases	104	421.00	102.00	<0.001*
	Controls	104	233.05	176.84	
AEC (cell/cum)	Cases	104	543.00	174.70	<0.001*
	Controls	104	421.60	204	
FEV1 (%)	Cases	104	18.97	5.23	<0.001*

Table 5: Statistical analysis of estimates of these interlukins (IL-17A, IL-17F) in the blood serum of thecases and the controls

Parameters	Values	*P*
IL-17F		
Mean±SEM of Cases	8.731±0.1614 *n*=104	
Mean±SEM of Control	5.031±0.1885 *n*=104	<0.001
Difference between means	3.700±0.2481	
95% confidence interval	3.214-4.186	
R^2	0.5167	
IL-17 A		
Mean±SEM of Cases	5.309±0.1902 *n*=104	<0.001
Mean±SEM of Control	2.362±0.0826 *n*=104	
Difference between means	2.947±0.2074	
95% confidence interval	2.541-3.354	
R^2	0.4926	

The levels of mRNA for the cytokines (IL17A and IL17F) in blood of asthmatics and controls have been determined in this study using RT-PCR. As demonstrated in Figure 2, the basal level of mRNA expression for IL-17F was higher as compared with IL-17A in controls. However, the levels of these interleukins were found to be higher in asthmatics than the controls showing up-regulation of these interleukins in asthma patients in comparison to the control subjects (P=<0.001and $P < 0.001$), respectively [Table 5]. The data obtained reflected that the detection of mRNA levels of IL17A and IL17F could prove to be useful indicators for the early diagnosis and hence treatment of asthma. However, the higher values of standard deviations (SD) of measurements indicated that in this context other factors would have also contributed to the computed values of IL17. The mRNA profile of the aforesaid interleukins and their up-regulations are summarized in Figure 2.

The up-regulation of mRNA expression for the interleukins (IL-17A and IL-17F) prompted us to determine the levels of them in the blood serum of the controls, as well as asthmatics and the results have been illustrated in Table 6. The data indicated the higher level of IL-17A and IL-17F proteins in cases compared with their corresponding controls [Figure 3].

Attempts were made to establish a correlation between IL-17A and IgE, IL-17F and IgE, as well as between these two interleukins (IL-17A, IL-17F). The statistical correlates are summarized in Table 7. The results indicated a positive correlation between the expression levels of mRNAof IL17A and IL17F in the cases included in this study [Figure 4a]. The statistical correlates for asthmatic patients were as $r = 0.073$, and $P < 0.005$.The statistical correlates between IL-17A and IgE were as following: $r = 0.077$, and $P < 0.004$ for patients [Figure 4b]. The correlation between IL-17F and IgE were highly significant ($r = 0.47$, $P < 0.001$ in cases [Figure 4c].

Table 6: Quantitative estimates of interlukins (IL-17A, IL-17F) proteins in the blood serum of controls and asthmatics

Proteins	Mean	Std. deviation	Std. error	*P*
IL-17 A (ng/l) cases	0.517	0.321	0.0309	*P*<0.0001
IL-17 A (ng/l) control	0.312	0.0776	0.0076	
IL-17 F (ng/l) cases	0.689	0.310	0.026	*P*<0.0001
IL-17 F (ng/l) control	0.318	0.143	0.012	

Table 7: Statistical correlates

The correlation between IL-17A, IL17F and IgE	In Cases
Correlation between IL-17A and IL-17F	
P	0.0053
Significant	Yes
R^2	0.07319
Correlation between IL-17A and IgE	
P	0.0041
Significant	Yes
R^2	0.07738
Correlation between IL-17F and IgE	
P	*P*<0.0001
Significant	Yes
R^2	0.473

Discussion

Asthma has been classified as allergic and non-allergic types. The characteristics of allergic asthma involve T2-type immune response. It induces allergic inflammation development through enhanced release of IgE, secretion of mucus and chemotaxis of eosinophils to the lungs.[12,13] In contrast, the non-allergic asthma exhibit only basal level secretion of the T2-type cytokines.[12,14] It has been documented that asthmatics show higher level of IgE in their blood serum and it has association with the release of IL-17; the family members of cytokines, i.e., IL-17A and IL-17F.[15] In

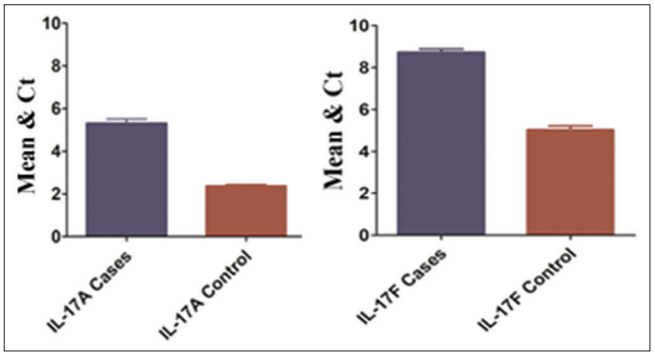

Figure 2: The mRNA expression levels of interlukins (IL-17A, IL-17F) in the blood asthmatics and controls

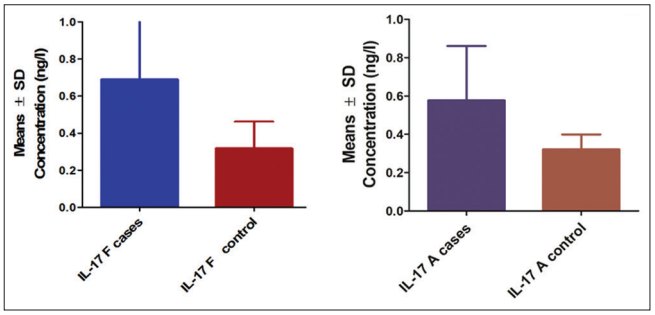

Figure 3: Levels of IL17A and IL17F significantly upregulated in the asthmatic patients

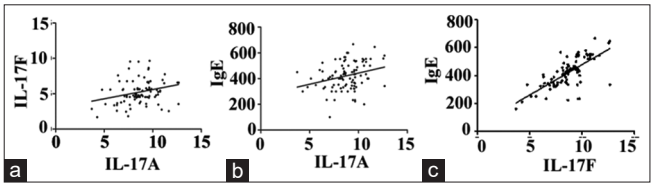

Figure 4: Statistical correlation between the (a) interleukins (IL-17A, IL-17F), (b) IL-17A and IgE and (c) IL-17F and IgE in the asthmatics. The panels a and b showed a positive correlation but panel c showed highly significant value (r = 0.47, $P < 0.001$ in cases)

the present study we have observed increased level of IgE in the blood serums of asthmatics. Interestingly, the statistical analysis of the data indicated positive correlations between IL-17A and IgE, as well as IL-17F and IgE with statistical values of correlations between IL-17A and IgE being (r = 0.0773, P < 0.004) and that between IL-17F and IgE being (r = 0.473, P < 0.0001) in asthamatic patients.

The IL17 family of cytokines produced by type 17 helper T cells has been correlated to several diseases.[4] The elevation in the levels of IL-17 has been shown to occur in the cases with chronic inflammatory diseases, and infections by bacterial.[16] The elevated IL-17A level has been observed in the synovial fluid taken out of the arthritis patients.[17] Similar results have been reported with the bronchoalveolar lavage fluid taken out from the asthmatics.[8] Some workers have shown that the level of IL17 at >20 pg/mL in an asthma patient could be considered as an index of risk factor for the occurrence of severe asthma.[18] Also, the level of IL-17A has been linked to neutrophilic inflammation in asthma.[19,20]

In the present study, the blood serum levels of mRNA of both IL17A and IL17F in asthmatics and controls were monitored and the results indicated that quantity of IL17A or IL17F transcripts were more in the asthmatics in comparison to that of controls. Although our results demonstrated considerable level of variations, but it remained smaller by several orders of magnitude than that of reference transcript (GAPDH), thereby suggesting it to be rare expressions. Several workers in their studies have examined the IL-17 gene expression in cell culture system. Most of these studies showed rise in expressions of IL-17A in asthma patients as compared with the healthy controls.[21-23] In this study, we have obtained similar results in the blood serum of the patients.

Earlier studies have demonstrated the absence of IL17 in some of the normal human tissues. Their expression was, however, restricted to neonatal tissues.[24] The increased level of expression of IL17F has been observed in the cells from the bronchoalveolar lavage fluid (BAL) of asthmatics upon stimulation by allergen. The cells challenged by the saline (control) did not show any change.[25] In this study, the workers observed very small levels of transcripts of either of IL17F or IL17A. These workers have shown very strong expression of IL17F only but not of IL17A in many tissues, such as fetal liver, lung, and ovary.[22] Our results, however, have shown that the levels of the mRNA transcripts of IL17A and IL17F got significantly up-regulated in the asthma patients.

The results from this study reflected a positive correlation between the extent of mRNA expression of IL17A and IL17F in the asthmatics. Some workers have shown that higher level of IL17F transcripts was correlated well with higher level of IL17A, and vice versa (r = 0.0731, P < 0.005).[22] Kawaguchi *et al.* (2006) have demonstrated that these results were expected because of location of both of the genes on the same chromosome (6p 12) in humans. Moreover, their promoters and the regions with conserved non-coding sequences undergo coordinated chromatin modifications.[22] Furthermore, both IL17A and IL17F act as homodimers or heterodimers, and both of them keep sharing similar biological functions and expression patterns in the asthma patients, thereby exhibiting a positive correlation between the expression of mRNA transcripts of IL17A and IL17F. These findings were found to be consistent with the other populations, where in the levels of IL17A and IL17F have been shown to be significantly up-regulated in the patients suffering from asthma.

Conclusion

Asthma being a chronic inflammatory disorder of airway involving genetic and environmental factors largely associates with roles of Interleukin 17F and 17A regulate pathophysiology of asthma. The blood samples from 104 asthmatic patients displayed positive statistical correlation between IL-17F and IL17A with IgE. The levels of gene expression of both of these interleukins got significantly up-regulated in the asthmatic patients. This study indicated that the monitoring of these cytokines may be exploited as viable indices for early diagnosis and treatment of asthmatics.[26]

Acknowledgements

RP is grateful to UPCST-Lucknow for providing financial support in the form of a fellowship for research.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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