

Expression of mRNA for IL-13 and DNMT-1 in children with Atopic Dermatitis

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Back ground: Atopic dermatitis (AD) is a chronic inflammatory skin disease frequently associated with an increased serum level of IgE. IL- 13 is one of the cytokines that are produced by Th2 lymphocytes and it can induce the production of IgE. DNA methylation is known to play an important role in gene transcription and alteration of methylation (eg. hypomethylation) is set to be responsible for the development of certain disorders such as an autoimmune diseases and atopy.

Objectives: To evaluate the role of IL- 13 in the pathogenesis of AD and to investigate the DNA methylation in AD patients.

Methods: Messenger RNA (mRNA) level of IL- 13 and DNA methyltransferase- 1 (DNMT- 1) [the enzyme used for DNA methylation] in peripheral blood mononuclear cells (PBMC) were examined using a real- time quantitative polymerase chain reaction method (RT- PCR). IgE level was assayed by Chemiluminescence method..

Results: IL- 13 mRNA was significantly expressed in patients with AD as compared to normal controls ($P < 0.01$). IL- 13 mRNA showed higher level of expression in severe AD group in comparison with mild - moderate group ($P < 0.01$). Serum levels of IgE showed highly significant increase in patients with AD as compared with control group ($P < 0.01$) and it correlated with disease severity. There was a highly significant positive correlation between serum level of IgE and IL-13 mRNA expression in both AD groups ($r=0.616$).

The level of DNMT-1 mRNA was significantly lower in atopic patients compared to controls ($P < 0.01$). There was a significant negative correlation between DNMT-1 mRNA expression and IL-13 mRNA expression in both AD groups ($r=-0.712$). Also there was a significant negative correlation between DNMT-1 mRNA expression and the level of IgE in both AD groups ($r=-0.868$).

Conclusion:- The high level of IL-13 mRNA expression in AD and its correlation with serum level of IgE and with severity of disease indicates that IL-13 is involved in the pathogenesis of AD and it is an important in vivo IgE inducer. As DNA hypomethylation leads to IL-13 over expression so decrease of DNMT-1 might be related to the pathogenesis of AD.

Key words:- Atopic dermatitis, IgE, IL-13 mRNA and DNMT-1 mRNA.

I. Introduction

Atopic dermatitis (AD) is a chronic inflammatory fluctuating pruritic skin disease in which hereditary, environmental and immunological factors play a major role. Its clinical presentation varies from acute eczematous eruption in early life to a characteristic lichenified dermatitis in older patients¹⁻³. The chronicity of AD intensely affects the quality of life of patients and their families. Its diagnosis is usually based on clinical criteria and lacks the laboratory evidence that can confirm or rule out its existence⁴. The pathogenesis of AD is multifactorial including the differentiation of helper T cells, pattern of local cytokine expression, multiple role for IgE, infectious agents, and super antigens⁵. The aetiology of AD as an IgE- mediated disorder is complex and still not completely understood. IgE contributes to the inflammatory cell infiltration in AD by several mechanisms, including a biphasic immediate/ late phase reaction, allergic presentation of IgE- bearing langerhans cells, allergen- induced activation of IgE- bearing macrophages, and IgE auto reactivity to human proteins⁵. IL-13 is an immunoregulatory cytokine generated predominately by activated Th2 cells and it shares many functional properties with IL-4⁶. Similar to IL-4, IL-13 induces Ig class switching and IgE secretion, upregulates the expression of MHC class II molecules and the low affinity receptor for IgE (CD23) on B cells and induces the expression of VCAM-1 on endothelium⁷. IgE productions by B cells depends mainly on secreted IL- 13, and to a lesser extend, on IL-4 of cutaneous lymphocyte- associated antigen (CLA)+ memory T cells. Accordingly, IL-13 produced by the skin selective homing T cells in high amount may have an important role in the pathogenesis of AD⁸. DNA methylation is known to play an important role in gene transcription and alterations of methylation can contribute to the development of certain disorders such as autoimmune diseases⁹. Hypomethylation has been considered to be related to hyperresponsiveness against autoantigens and exogenous antigen in certain autoimmune diseases as well as in allergic disorders¹⁰. For DNA methylation in mammalian

cells, DNA methyltransferase -1 (DNMT-1); one of the essential member of this family is supposed to be responsible for the promotion of gene methylation¹¹. The aim of this work is to study IL-13 mRNA and DNMT-1 mRNA expression in peripheral blood in patients with different degrees of AD in order to evaluate their role in the pathogenesis of the disease and to correlate their profile with the serum level of IgE and with the clinical severity of the disease.

II. Patients and Methods

This study included 15 patients with AD; 9 males and 6 females; selected from Outpatient Clinics of Dermatology and Venereology and Paediatrics of Al Zahraa University Hospital during the period from October 2009 to April 2010. Their ages ranged from 1-16 years.

Fifteen healthy volunteers were chosen as controls. They were 8 males and 7 females. Their ages ranged from 3-14 years. None of them had a history of skin or systemic disease.

The patients were not receiving systemic treatment for the present conditions for at least 4 weeks before enrollment in the study and no topical treatment for at least 2 weeks. Thorough history, especially the family history of atopic dermatitis was taken. History and clinical examination were done to detect other atopic manifestations. All patients fulfilled the diagnostic criteria of Hanifin and Rajka¹². The disease severity was assessed using the Nottingham Eczema Severity Score (NESS)¹³. The patients were classified according to NESS into three groups: Group A: Mild cases (3 AD patients with NESS total score 3-8), Group B: Moderate cases (4 AD patients with NESS total score 9-11), Group C: Severe cases (8 AD patients with NESS total score 12-15). For statistical issue as the numbers of the patients in mild and moderate groups are small they are gathered in a group termed mild- moderate. An informed written consent was obtained from the subjects or their legal guardians before their contribution in this study.

III. Methods

Five ml of venous blood were withdrawn from each subject and divided into two portions, the first portion 2 ml used for estimation of total IgE level, serum samples were obtained after clotting and centrifugation of blood and stored at -20 °C until the time of IgE assay

IgE was assayed by Immulite 1000 systems (chemiluminescence method) using kit supplied by Siemens Health-care Diagnostics products Ltd (united kingdom). IgE is a solid phase, two- site chemiluminescent immunometric assay. The solid phase is a polystyrene bead enclosed within a test unit containing the coated bead. An alkaline phosphatase conjugated to polyclonal goat anti IgE is also added to the test unit. After the wash and incubation steps, chemiluminescent substrate undergoes hydrolysis in the presence of alkaline phosphatase. The photon output as measured by the luminometer is related to the presence of IgE in the sample).

The second portion, 3 ml of venous blood was added to Heparin and mixed well and then put over tube containing 6 ml Ficoll-hypaque density gradient solution. (Biowaltker - Germany). The mononuclear cells (PBMC) were obtained by centrifugation at 1200 rpm for 20min. in a cooling centrifuge at 4 °C. Then the Buffy coat ring of mononuclear cells were aspirated at the interface and carefully transferred with Pasteur pipette and washed twice with HBSS (hank's salt balanced solution) using the cooling centrifuge for 20 min at 1200 rpm, then after the pellet of cells is suspended in a least amount of phosphate buffer saline (PBS) then subject it to the extraction procedure.

A – RNA extraction:

The extraction of mRNA from PBMC were performed according to QIAGEN (QIA amp) Spin column mRNA extraction kit (QIA gen Inc, Germany) catalog number 52904. 570 µl of lysis reagent + 200 µl of cell sustentation were dispensed in a sterile eppendorf tube (RNase, DNase free) and then incubated for one hour at 65 °C. 570 µl of absolute ethanol were added and then transferred to the column and spin at 8000 rpm for one minute. 500 µl of wash I was dispensed then the tube was spin for one minute at 8000 rpm. 500 µl of wash II was dispensed then the tube was spin for 3 min at 1400 rpm. Then dispense 80 µl of elution buffer and stay at room temp for 5 min. The tube was centrifuged for one minute at 8000 rpm, giving a final volume of 80 µl of purified RNA.

B – Amplification

Quantitative estimation of DNMT1 mRNA and IL13 mRNA in PBMC using Real time PCR:

the PCR master mix kit of QIAGEN was used (catalog number 1005) and consists of :

- 15.5 ul of universal MMX including 10x PCR buffer , 10 mM dNTPs Mix ,
- DNA polymerase enzyme, Random hexamers , 6 mM Mgcl2 and PCR grade water.
- 1 µl of superscript RT enzyme
- 0.5 µl of primer 1

- 0.5 µl of primer 2
- 0.5 µl of probe mix
- 7.0 µl of RNA template.

The tube containing the mixture (final volume was 25 µl) was put in real time thermal cycler for 30 minute at 48 °C .then the resultant cDNA was amplified at 95 °C for 15 second and finally at 60 °C for 30 sec. using Applied Biosystem 7300 sequence detection instrument [using applied Biosystem taqman PCR MMX Kit] the amplification was repeated for 45 cycle and the results were interpreted relating to threshold line.

The selected primer (Invitrogen) for DNMT1 mRNA was the following sequence

Forward: 5'-CCTAGTTCGGTGGCTACGAGGAGAA-3'

Reverse: 5'-TCTCTCTCCTCTGCAGCCGACTCA-3'

And the Primer sequence for IL-13 mRNA was (Invitrogen),

Forward: 5'-TGAGGAGCTGGTCAACATCA-3'

Reverse: 5'-CAGGTTGATGCTCCATACCAT-3'

The Applied Biosystems taqman PCR MMX kit was used with 10 template in a final volume of 25 µl Gylceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as input control. The primer sequence (Invitrogen) of GAPDH was as follow:

Forward: 5'-TTCCAGGAGCGAGATCCCT-3'

Reverse: 5'-CACCCATGACGAACATGGG-3'

16S rRNA standard with known conc. is used to establish a standard curve as a reference for quantifying the results.**Statistical Analysis:-**

The statistical analysis of data was done by using statistical package for social science (SPSS) version 16 on windows XP.

The description of data was done as frequency and proportion for qualitative data mean ± SD for quantitative data. The analysis of data was done to test statistical significant difference for quantitative data using student's t test. For qualitative data [frequency & proportion] chi-square test was used. Measuring the mutual correspondence between two values was done using the pearson's correlation coefficient. P value was considered significant if ≤ 0.05 at confidence interval of 95%¹⁴.

IV. Results

The present study included 15 patients with AD and 15 healthy age and sex matched controls. The patients were 9 males and 6 females. Their aged ranged from 1-16 years with a mean of 7.40 ± 4.73 . They gave no past or present history of any other cutaneous or systemic disease. Eight patients had family history of atopy, 6 patients had a history of recurrent bronchial asthma. None of these patients had been receiving systemic steroids for at least 2 months before the study. Serum level of IgE was statistically significantly higher in atopic patients than in control group ($P < 0.01$). Also IL-13 mRNA showed statistically significant positive expression in patients with atopic dermatitis as compared to control group ($P < 0.01$) while DNMT-1 mRNA showed statistically significant negative expression in patients with atopic dermatitis ($P < 0.01$) as compared to control group (Table1). When comparing serum level of IgE in mild - moderate atopic patients to control group , there was significant increase in serum level of IgE in mild - moderate atopic patients than in control group ($P < 0.01$). Also the expression of IL-13 mRNA was significantly higher in mild - moderate atopic patients than in controls ($P < 0.01$) while the expression of DNMT-1 mRNA was significantly lower in mild - moderate atopic patients compared to controls ($P < 0.01$) (Table 2).

When comparing the serum level of IgE, the expression of IL-13 mRNA and DNMT-1 mRNA in severe cases of atopic dermatitis and controls, there was significant increase in both serum level of IgE and expression of IL-13 mRNA and significant decrease in DNMT-1 expression in severe form atopic patients compared to control group ($P < 0.01$) (Table 3).

On comparing IgE level, IL-13 mRNA and DNMT-1 mRNA expression in severe and mild -moderate groups of AD, there was no significant difference in serum level of IgE in both groups ($p > 0.05$) while there was a statistically significant increase IL-13 mRNA expression and statistically significant decrease in DNMT-1 mRNA expression in severe form of AD group when compared to mild - moderate AD group ($P < 0.01$) (Table 4).

A statistically significant positive correlation was found between IL-13 mRNA expression and serum level of IgE ($P \leq 0.05, r = 0.616$) and statistically significant negative correlation was found between DNMT-1 mRNA expression and serum level of IgE ($P \leq 0.05, r = -0.712$). Also we found statistically significant negative correlation between IL-13 mRNA expression and DNMT-1 mRNA expression ($P \leq 0.01, r = -0.868$) in all atopic dermatitis patients A statistically significant positive correlation was found between IL-13 mRNA expression and NESS score ($P < 0.01, r = 0.805$) and statistically significant negative correlation between DNMT-1 mRNA expression and NESS score ($P < 0.01, r = -0.961$) (Table5).

Table 1: Serum level of IgE and expression of both IL-13 mRNA and DNMT-1 mRNA in peripheral blood from AD patients and healthy controls

	AD Patients	Controls	P value
IgE Iu/ml	567.27 ± 437.83	34.60 ± 12.08	<0.01**
IL- 13 mRNA bp	3176926.00 ± 1846230.61	2783.00 ± 1278.34	<0.01**
DNMT- 1 mRNA bp	38830.40 ± 28381.54	529057.47 ± 179782.10	<0.01**

P ≤ 0.05 = Significant* P ≤ 0.01 = Highly**

Table 2: Serum level of IgE and expression of both IL-13 mRNA and DNMT-1 mRNA in peripheral blood from patients with mild to moderate AD compared to healthy controls

	Mild- Moderate AD	Controls	P value
IgE Iu/ml	514.14 ± 661.71	34.60 ± 12.08	< 0.01**
IL- 13 mRNA bp	1577122.00 ± 5436.15	2783.00 ± 1278.34	< 0.01**
DNMT- 1 mRNA bp	63409.43 ± 22773.92	529057.47 ± 179782.10	< 0.01**

P ≤ 0.05 = Significant* P ≤ 0.01 = Highly**

Table 3: Serum level of IgE and expression of both IL-13 mRNA and DNMT-1 mRNA in peripheral blood from patients with severe AD compared to healthy controls

	Severe AD patients	Controls	P value
IgE Iu/ml	613.75 ± 52.90	34.60 ± 12.08	<0.01**
IL- 13 mRNA bp	4576754.58 ± 1256814.73	2783.00 ± 1278.34	<0.01**
DNMT- 1 mRNA bp	17323.75 ± 5807.19	529057.47 ± 179782.10	<0.01**

P ≤ 0.05 = Significant* P ≤ 0.01 = Highly**

Table 4: Serum level of IgE and expression of both IL-13 mRNA and DNMT-1 mRNA in peripheral blood from patients with mild to moderate AD compared to severe AD patients

	Mild- Moderate AD patients	Severe AD patients	P value
IgE Iu/ml	514.14 ± 66.71	613.45 ± 52.90	>0.05
IL- 13 mRNA bp	1577122.00 ± 5436.15	4576754.50 ± 1256814.73	<0.01**
DNMT- 1 mRNA bp	63409.43 ± 22773.92	17323.75 ± 5807.19	<0.01**

P ≤ 0.05 = Significant* P ≤ 0.01 = Highly** P > 0.05 = Not significant

Table 5: Correlation between serum levels of IgE, IL-13 mRNA and DNMT-1 mRNA in Atopic patients.

	IgE (r value)	IL-13 (r value)	NESS Scoring
IL-13	0.616*		0.805**
DNMT-1	- 0.712*	- 0.868**	- 0.961**

V. Discussion

Atopic dermatitis is a multifactorial genetically based disease that affects 10- 15% of children in many parts of the world and constitutes a significant burden to patients and their families⁴. It is frequently seen in children with history of respiratory allergy and/ or family history of atopic disease¹⁵. Over all, atopy is familial hypersensitivity of skin and mucous membranes against environmental substances which is associated with increased IgE production⁵.

In the present study 6 patients had personal history and 8 patients had family history of atopic disease and this agreed with Hanifin and Chan¹⁶, who reported that most but not all persons with AD have a personal or family history of allergic rhinitis or asthma. Atopic dermatitis patients had significant elevation of serum IgE as compared to normal controls. This was in agreement with Leung and Soter⁵ who reported that only a small subgroup < 30% of atopic patients had a normal level of IgE. Atopy is associated with local infiltration of Th2 cells. Th2 cytokines play an important role in immune modulation of inflammatory diseases. It is hypothesized that several cytokines including IL-4, IL-5 and IL-13 are released from these cells¹⁷. IL-5 is a prominent Th2 cytokine that has important roles in immune modulation of inflammatory disease⁷. IL-4 and IL-13 induce immunoglobulin class switching and IgE secretion, upregulate the expression of MHC class II molecules and the low affinity receptor for IgE on B cells. These data suggest that IL-13 is a crucial mediator of immune

diseases, especially in atopic state¹⁸. Previous studies reported that this cytokine is significantly expressed in chronic lichenified lesion of AD patients than in the skin of healthy controls^{5,19}. IL-13 mRNA expression was also detected by RT-PCR in lesional and non lesional skin and PBMC from AD patients, but not in normal skin or PBMC from healthy control²⁰.

Present findings showed highly significant increase in expression of IL-13 mRNA in patients with different degrees of AD as compared to normal controls. This was in agreement with Katagiri et al²¹ and Metwally et al²² who found increased levels of IL-13 mRNA expression in PBMC in patients with AD. Obara et al²⁰ reported that IL-13 may play a local as well as a systemic role in the development of AD lesions. We also found that in more severe cases there was increase in the degree of expression of IL-13 mRNA and this was in agreement with Metwally et al²².

Our results revealed statistically significant positive correlation between IL-13 mRNA expression and clinical severity of this disease and this is in agreement with that of Koning et al²⁴ and Metwally et al²² who reported that IL-13 correlated with the severity scoring of AD.

A statistically significant positive correlation was found between serum level of IgE and IL-13 mRNA expression and this finding is in agreement with Akdis et al⁸ who demonstrated that CLA⁺ T- cells are activated in AD and are capable of regulating IgE synthesis, mainly by IL-13 secretion and to a lesser degree on IL-4 of CLA⁺ T cells. Another reports suggested that IL-13 produced by Th2 cells is involved in IgE over production in AD patients^{24,25}.

Our results also are in agreement with Kaminishi et al²⁶ and Metwally et al²² who reported that patients having increase in IL-13 had higher serum IgE levels than those with normal IL-13 level, suggesting a direct association between serum levels of IL-13 and IgE.

Contrary to our results, Van Der Ploeg et al¹⁹ found no correlation between serum IgE levels and gene expression in the chronic lichenified lesions and they suggested that cells other than Th2 cells might be involved in IL-13 gene expression and maintenance of the disease. This controversy may be related to difference in IL-13 expression in blood from the skin.

DNA hypomethylation is supposed to promote hyperreactivity of Th2 cells to allergens and the resultant cytokine-mediated production of IgE. In fact, some reports have also suggested that IL-4 mediated production of IgE in patients with high serum levels is related to DNA hypomethylation in B cells^{27,28}. A previous study reported that IL-13 expression in Th2 cells is regulated by the methylation status of a region in the proximal promoter encompassing three Putative GATA -3 binding sites²⁹.

In this study we demonstrated that the expression of DNMT-1 mRNA was significantly lower in patients with different degrees of AD as compared to normal controls. And also statistically significant negative correlation was found between serum level of IgE and DNMT-1 mRNA expression. Nakamura et al⁹ in his study classify the AD patients into 2 groups; group A with high level of IgE who showed significant lower level of DNMT-1 mRNA expression compared to controls and supported the concept that the onset of AD is promoted by lower levels of DNMT-1 and group B with lower level of IgE also showed lower level of DNMT-1 mRNA expression compared to controls but without a statistically significant difference. Also he reported that there was no statistical significant difference of the DNMT-1 mRNA level between group A and B patients indicating that DNMT-1 may not be a factor solely influencing serum IgE. To clarify this issue, the studies such as evaluating the DNMT-1 levels in non-AD patients with high serum level of IgE would be necessary.

Several reports have suggested that DNA hypomethylation (especially in T cells) may contribute to the development of autoreactive cells in SLE³⁰ patients. Low DNMT-1 activity in SLE patients has also been suggested to relate to hyperresponsiveness to exogenous antigens (such as infectious agents) in addition to autoantigens. The fact that SLE patients are known to have a higher frequency of IgE-mediated allergic diseases (including AD) in their family history suggests certain similarities in both diseases, such as hyperresponsiveness to autoantigens and/ or exogenous antigens resulted from low DNMT-1 expression³¹.

The present study showed significant negative correlation between IL-13 mRNA and DNMT-1 mRNA expression in patient group. DNA methylation modifies gene expression through at least three mechanisms. Methylation of target sequences prevents the binding of some transcriptional activators, such as Sp1 and AP-2, thereby suppressing expression³². Methylated binding proteins can also prevent the binding of transcription factors and can suppress gene expression from a distance³³. Methylated binding proteins also attract chromatin remodeling complexes that modify adjacent histones, resulting in a condensed nucleosome structure, making the locus inaccessible to transcription factors³⁴. Moreover, it has been demonstrated that the expression of cytokine genes during T-cell differentiation is regulated by acetylation and methylation-dependent chromatin structural changes³⁵.

It can be concluded that

(1) IL-13 seems to be important in the pathogenesis of AD, the differences in IL-13 expression between groups indicates the association correlation of IL-13 with the severity of AD. The relation of IL-13 with IgE may indicate that IL-13 is responsible for regulating the in vivo synthesis of IgE in patients with AD.

(2) Disease activity- related increase of IL- 13 expression is associated with demethylation of DNA. These findings may provide a basis for the design of a new therapy aimed at reversing the epigenetic alterations of gene expression in AD patients.

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