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ORIGINAL ARTICLE

CCR4 Ligands (thymus and activation-regulated chemokine 'TARC/CCL17 and macrophage-derived chemokine 'MDC' /CCL22 ') as Disease Severity Markers in Atopic Dermatitis Patients

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ABSTRACT

Background: *Atopic dermatitis (AD) is a pruritic and chronically relapsing inflammatory skin disease with predominant infiltration of T helper 2 (Th2) cells as a hallmark of the disease. Th2 lymphocytes express CCR4 receptors. CCR4 ligands (thymus and activation-regulated chemokine 'TARC- CCL17 and macrophage-derived chemokine 'MDC' CCL22 ') direct trafficking and recruitment of Th2 cells into lesional skin in AD. These chemokines appear to be useful inflammatory markers for assessing severity of AD.* ***The objectives*** *were to establish the relationships between TARC , MDC, and clinical picture of AD as disease severity markers.* ***Methodology****: This study included 25 patients with AD in addition to 25 age and sex matched healthy subjects as a control group. Patients were classified into mild (n =3), moderate (n =13) and severe (n =9) according to the SCORing AD (SCORAD) index. Serum concentrations of CCR4 ligands were determined from all patients and controls by Enzyme- linked immunosorbent assay (ELISA). CCR4 expression was quantitated by real time RT-PCR* ***Results:*** *A highly significant increase was found in the serum TARC and MDC levels in AD patients compared to controls. There was significant positive correlation of TARC (r= 0.81, p<0.001). and MDC levels (r=0.53, p<0.006) with severity of the disease as determined by SCORing Atopic Dermatitis (SCORAD) score . There was more expression of CCR4 in AD patients than control with statistically significant difference (p<0.001).* ***Conclusion****: serum CCR4 ligands may be useful markers for assessing AD severity. Among the adjuvant therapeutic strategies of AD, further attention to these biomarkers will help with the development of novel targeted therapeutics as well as assessment of therapy response.*

INTRODUCTION

Atopic dermatitis (AD) is one of the most common chronic relapsing inflammatory skin diseases in children and adults and is characterized by pruritic skin lesions in distinctive body areas. AD causes substantial morbidity and greatly influences eminence of life of affected individuals and their families [1]

The exact pathogenesis of AD remains indefinable. Though, it is, evident that the pathogenesis of AD is complex and involves various exogenous and endogenous factors, comprising allergens and microbial antigens as triggers of acute flare ups of the disease as well as risk factors for severe persistent progressions. In addition, a multitude of genetic modifications impact on disease manifestation, resulting in alterations in keratinocyte differentiation [2]

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Lesional AD skin as well as clinically non-lesional skin of AD patients exhibit multiple histologic along with immunologic differences as compared to the skin of healthy individuals[3]. One of those differences is macrophage and T-cell infiltration, which predominantly T-helper (Th) 2-type cells expressing such cytokines as interleukin,IL-4, IL-5 and IL-13[4]. Many different chemoattractants are known to attract the lymphocytes and eosinophils to the skin, among these; are the chemokines[5]

Chemokines have been identified as chemotactic cytokines that mediate cellular trafficking, leukocyte maturation, homing of lymphocytes and the development of lymphoid tissues. More than 50 different chemokines and 20 chemokine receptors have been introduced in humans [6]. Depending on the position of the first two N-terminal cysteine residues, they are divided into four subfamilies, CX, CC, CX3C and C chemokines. The CC and CX chemokines are known to be inflammatory chemokines while the C and CX3C chemokines are known to be immune chemokines. These chemokines are produced locally in the tissues

and act on leukocytes through specific receptors. All the known chemokine receptors are seven transmembrane-type receptors, hence a systematic nomenclature has been created[7]

A large number of chemokines are found to be upregulated in lesional skin and serum of AD patients and several chemokine-chemokine receptor pairs have been related to the pathogenesis of AD. In AD, chemokines regulate the emigration of dendritic cells to the draining lymph node as well as the attraction of activated T cells, eosinophils, basophils and mast cells to the site of inflammation [8]. Among the various T-cell subsets, CC chemokine receptor 4 (CCR4) is predominantly expressed by Th2 cells as well as cutaneous lymphocyte antigen-positive skin-homing T cells and Treg cells .Thus, CCR4 attracts much attention for its possible clinical applications in diseases involving these T-cell subsets. CCR4 is the receptor for two CC chemokine ligands (CCLs)-CCL17 (also called thymus- and activation-regulated chemokine; TARC) and CCL22 (macrophage-derived chemokine; MDC). They induce staffing of Th2-type lymphocytes to the site of allergic inflammation in the course of AD[9].

Measuring the disease activity clinically is a challenge owing to the clinical manifestations of AD, such as erythema and lichenification. Moreover, AD has a high placebo response rate which is a great drawback when enterprise clinical trials of therapeutics. So, a set of biomarkers is required, both for disease activity, and for successful treatment response, to allow accurate assess reversal of the core disease pathology [10].

This study aimed to establish the relationship between CCR4 ligands (TARC/CCL17 and MDC /CCL22) and clinical picture of AD as disease severity markers

METHODOLOGY

The study group comprised 25 people with atopic dermatitis. Atopic dermatitis was diagnosed with Hanifin's and Rajka's criteria [11]**.** People with other allergic diseases (i.e. rhinoconjunctivitis, bronchial asthma), ever suffering from immunodeficiencies, malignancies or psychiatric diseases, having any skin infection during the 3 months preceding the study were excluded. Treatment with antihistamines or systemic or topical corticosteroids was not allowed before inclusion into the study. Severity of AD was assessed according to the SCORAD "SCORing Atopic Dermatitis" (ranging from 0 to 103 points) with assessment of extensity and localization of the skin lesions and the intensity of objective and subjective symptoms [12]. The control group consisted of 25 age and sex matched healthy people with a negative history of allergic diseases, confirmed by negative results of skin prick tests and the normal level of total serum IgE. All participants gave their consent before blood sample extraction. This study was approved by the local institutional review board (Zagazig University IRB).

Venous blood samples (10 ml) were obtained from 50 subjects (25 AD patients and 25 controls), 2.5 ml of each sample was centrifuged at 1300 g at 4^{o}C for 10 min, next, the separated serum samples were kept frozen at -20^{o}C until measurements.

The serum concentrations of TARC and MDC were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (pg/ml; R&D Systems Inc., Minneapolis, MN, USA; detection thresholds – 7 and 62.5 pg/ml, respectively) and the serum concentration of total IgE (IU/ml, Allergopharma; detection threshold – 1.0 IU/ml).

The drawn blood (7.5 ml) was placed in the presence of anticoagulant and PBMCs were separated using lymphocyte separation medium (Ficoll-paque™-plus) as described before [13].

RNA Isolation was done according to the manufacturer's instructions by using Ambion *mir*Vana Isolation Kit (USA,Cat: AM1561). Conversion of mRNA to cDNA consists of denaturation at 70°C for 10 min followed by amplification at 42°C for 60 min followed by 72°C for 15 min. .For RT-PCR, the primers and PCR conditions for amplification of CCR4 were used as described previously [14]. Specific primers and probe of CCR4 were used: Forward primer
5-CACACATACTGCAAAACCCAGTA-3,
Reverse primer 5-TCCAGGGAGCTGAGGACTT-3, (369 bp, X85740) and
Probe 5-TCGGTCAACTCGACCACGT-3. The probe used for detection in real-time PCR was labeled with 6-carboxyfluorescein (FAM) at their 5-terminal. TaqMan assay for CCR4 was obtained from Applied Biosystems. TaqMan PCR assays were performed as one-step RT-PCR using the EZ-RT-PCR Reagent Kit (Applied Biosystem) and 40 ng of total RNA, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer,
5'-GGTGGTCTC-CTCTGACTTCAACAG-3'; reverse primer, 5'-GTTGTTGTAGCCA-AATTCGTTGT-3')
was used as an internal standard. PCR reactions were performed at 94°C, 1 minute, 60°C, 1 minute, 72°C, 1 minute for 30 cycles (Mx3005 P Stratagene). The relative CCR4 gene expression was normalized on the basis of GAPDH. Results were expressed as an x-fold difference relative to the calibrator.

Statistical analysis

The collected data were encoded and analyzed using SPSS version 17 Quantitative variables were given as mean ± SD, median and range. Anova test, Kruskal Wallis, Mann-Whitney U test, independant t test and Spearsman correlation were used for data analysis. For qualitative data, number and percentage, chi-square test were used. p value <0.05 means significant and p value <0.001 means highly significant.

RESULTS

The study group consisted of 14 females and 11 males with mean age of 25.28 years (SD 5.12), while the control group comprised 25 age and sex matched control persons with mean age of 26.64 years (SD 5.73). Difference in mean age between the two groups was not statistically significant (t=0.88, P= 0.38).The values of SCORAD index were ranging from 18-93 (median 45+/-25.12), according to scores, patient group was divided into 3 subgroups: mild (n=3), moderate (n=13) and sever (n=9).

In this study, the serum levels of TARC/CCL17 and MDC/CCL22 were increased in AD patients compared to the control subjects with highly statistical significant difference (MW=6.05 &5.64 respectively, P value < 0.001) (Table 1).

Serum TARC/CCL17 and MDC /CCL22 levels correlated to SCORAD (table 2). It was found a significant positive correlation between serum TARC levels and SCORAD (r= 0.81, p<0.001). Also, there was a significant positive correlation between serum MDC levels and SCORAD (r=0.53, p<0.006) in AD patients.

To investigate whether an increased serum TARC and MDC correlates with an enhanced expression of its receptor, we analyzed the expression of CCR4 in the PBMC of AD patients by real-time PCR: Mean (SD) was 1.95±0.45 and range was 0.98 – 2.87in AD patients comparing Mean (SD) of 0.91±0.15 and range 0.74–1.35 in controls. There was a highly significant difference between AD patients and controls (t=10.95, p <0.001), the increased expression of TARC and MDC was associated with an elevated mRNA expression of CCR4 indicating an influx of CCR4 Th2 cells into pathogenesis of AD (Table 2). It was found a highly significant difference on comparing expression of CCR4 to different AD severity groups (table 3).

Table 3 shows serum TARC/CCL17,MDC /CCL22 and IgE levels and CCR4 expression in AD patients with different grades of severity. The difference in serum TARC, IgE, CCR4 expression between the three groups was highly statistically significant (p<0.001) and in serum MDC levels was significant (p<0.05)

Table 1: Serum TARC/CCL17, MDC /CCL22 and IgE level in AD patients and control group.

	Atopic dermatitis ***Mean (SD)*** ***Range*** ***Median (IQR)***	***Control group*** ***Mean (SD)*** ***Range*** ***Median (IQR)***	***MW***	***P***
TARC (pg/ml)	3081.6 ± 1709.85 600 – 9200 2700 (2075-3525)	334.8 ± 144.6 200 – 890 300 (250 – 350)	**6.05**	**<0.001****
MDC (pg/ml)	802.8 ± 341.79 340 – 2000 780 (560-945)	320 ± 106.8 200 – 650 300 (225 – 370)	**5.64**	**<0.001****
Total IgE (IU/ml)	519.76 ± 160.17 246– 800 500 (375-631)	75.44 ± 44.3 25 – 145 85 (30.5 – 114.5)	**6.06**	**<0.001****

Table 2: Spearsman correlation coefficiences between the measured indices

	TARC		***MDC***		***IgE***		***SCORAD***		***CCR4***	
	r	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**
TARC	---	---	0.41	0.04*	0.88	<0.001**	0.81	<0.001**	0.87	<0.001**
MDC	0.41	0.04*	---	---	0.42	0.04*	0.53	0.006	0.31	0.13
IgE	0.88	<0.001**	0.42	0.04*	---	---	0.75	<0.001**	0.68	<0.001**
SCORAD	0.81	<0.001**	0.53	0.006**	0.75	<0.001**	---	---	0.85	<0.001**
CCR4	0.87	<0.001**	0.31	0.13	0.68	<0.001**	0.85	<0.001**	---	----

Table 3: Relation between serum TARC, MDC, CCR4 and IgE and severity of the disease:

	Mild ***Mean ± SD*** ***Median*** ***Range***	***Moderate*** ***Mean ± SD*** ***Median*** ***Range***	***Sever*** ***Mean ± SD*** ***Median*** ***Range***	***Test***	***P value***
TARC	1083.3 ± 436.84 1200 600 – 1450	2530 ± 485.92 2500 1900 – 3600	4544.4 ± 1992.4 3600 3000 – 9200	K 17.38	<0.001**
MDC	483.33 ± 124.33 550 340 – 560	739.23 ± 215.97 780 430 – 1200	1001.1 ± 433.08 990 560 – 2000	K 6.89	0.03*
Ig E	280.76 ± 52.44 255 246 – 341	461.46 ± 86.98 455 332 – 590	683.67 ± 88.05 652 585 – 800	F 31.79	<0.001**
CCR4	1.16 ± 0.19 1.14 0.98 – 1.36	1.86 ± 0.26 1.93 1.2 – 2.25	2.34 ± 0.30 2.24 1.9 – 2.87	F 22.93	<0.001**

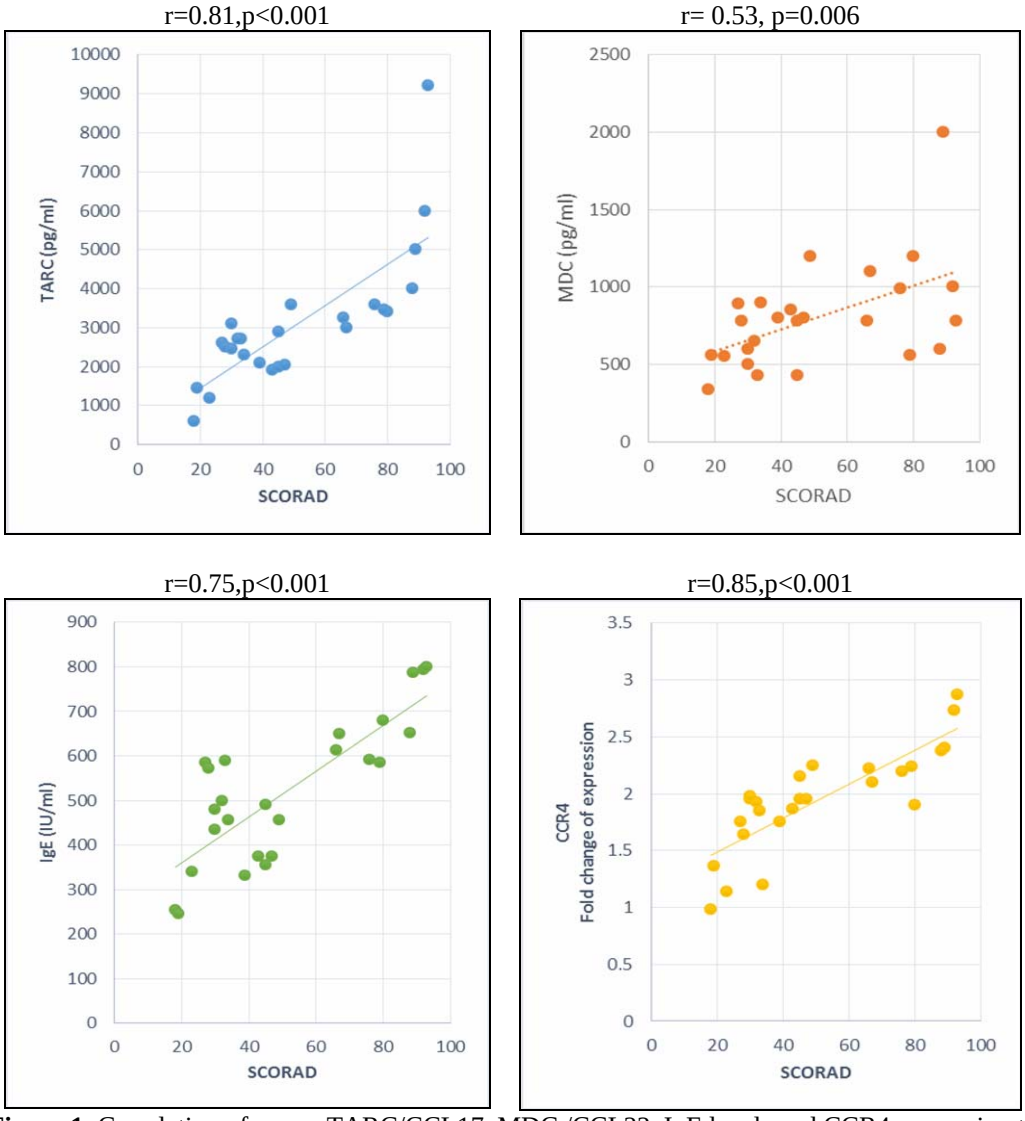

Figure 1: Correlation of serum TARC/CCL17, MDC /CCL22, IgE levels and CCR4 expression to disease activity in AD as assessed by SCORAD

DISCUSSION

Atopic dermatitis (AD) is a common, chronically relapsing, severely pruritic, eczematous skin disease [15]. The waxing and waning clinical course of AD associated with spontaneous or seasonal flare-up results in deterioration in patients' quality of life. The most important clinical symptom is intolerable itch. By scratching, patients easily fall into a vicious circle called the "itch-scratch cycle", resulting in chronic sleep disturbance [16]. Many research findings have provided a perception into the complex pathogenic mechanisms involved in this disease with special attention to biomarkers. These biomarkers will help with the development of novel targeted therapeutics and assessment of therapy response, with the promise of a more tailored therapy approach [10].

We found that serum TARC and MDC levels in AD patients were higher than that of the control group with statistically significant difference. Regarding TARC levels, this result was in agreement with the reports of other studies; Hijnen et al.[17] who reported elevated serum levels of TARC as well as cutaneous T cell attracting chemokine in AD patients and they are disease- specific markers for AD. Horikawa et al.[18] found significant increase in plasma TARC level in AD patients. Similar finding was observed in the study of Hussein et al. [19] who found significant increase in plasma TARC level in Egyptian AD patients.

Kakinuma et al. [20] had declared the same results regarding TARC and in the following year, in another study, they found that serum MDC levels in AD patients were significantly higher than those in healthy controls and psoriasis patients[21]. Hashimoto et al. [22] found that the CCL22 level produced by dendritic cells obtained from AD patients reflects the disease activity and it may also play an important role regarding the production of CCL22 in the pathogenesis of AD. Also, the results obtained by Hirota et al. [23] strongly supported the important role of CCL22 in AD.

Shimada et al. [24] who concluded that Serum levels of TARC and MDC in AD patients were significantly higher than those found in normal controls. Expression of both, CCL17 as well as CCL22 is higher in skin or epidermal of patients with AD as compared to healthy skin or epidermal dendritic cells isolated from the epidermis of patients with other chronic inflammatory skin diseases such as psoriasis supporting the concept of CCL17 and CCL22 being crucial for the recruitment of Th2 cells, which predominate in particular in the acute phase of the disease

In the present study, a significant positive correlation was found between serum TARC and MDC levels in AD patients and severity of the disease based on SCORAD score. It was significantly elevated in severely affected group than in mild or moderate groups. This result agreed with that of Kakinuma et al.[20] who demonstrated that serum TARC level reflects the severity and therapeutic response in AD. Also, Shimada et al. [24] proved that serum level of TARC is closely related with disease activity. Hussein et al. [19] proved that TARC is one of the chemokines that are closely related to the disease activity and response to treatment in AD. Also, our results are in line with Shimada et al. [24] who found that Serum levels of TARC and MDC correlated positively with disease severity, total IgE levels, and peripheral eosinophilia in AD patients and with Oranje et al.[25] who revealed a significant correlation of CCL17 and CCL22 to the total value of SCORAD index.

Leung et al.[26] found that Serum MDC levels correlated with SCORAD as well as its extent and intensity components, while serum TARC concentration showed weaker correlation with extent and intensity of skin involvement but not SCORAD. Also, Kakinuma et al. [21] reported that increases in serum MDC levels in AD patients were greater in the severely affected group than in the moderate or mild groups.

Jahnz-Rozyk et al. [27] reported that TARC and MDC have a significant positive increase with severity of AD concluding that the peripheral immune responses of AD patients are skewed to a Th2 dominant bias. It is worth wide to be mentioned that Japanese medical insurance began to cover the monthly measurement of serum TARC/CCL17 levels in AD patients, monitoring TARC levels has been recognized as a highly valuable biomarker to objectively monitor disease activity of AD [28]. Yasukochi et al.[29] confirmed that wider range of TARC levels seems to be clinically more useful for monitoring AD severity.

Our results revealed that CCR4 expression was significantly higher in AD patients compared to controls and it correlates with the disease activity. These results are in line with the results obtained by Kakinuma et al.[20] who concluded that, CCR4 is a specific ligand for TARC/CCL17 and monocyte derived chemokine (MDC/CCL22), they examined CCR4 and CXCR3 expression on peripheral blood memory T cells and found that the percentage of CCR4 expression on memory T cells was significantly higher in AD patients compared with normal controls and psoriasis patients. Also, they found that CCR4 expression in the severe AD group was significantly higher than that seen in the mild group ($P < 0.05$). Another findings have been reported about the importance of CCR4 expression in AD. It has been shown that CCR4-expressing memory CD4+ T cells in blood are increased in AD patients [30]. The proportion of CLA+ CCR4+ lymphocytes is upregulated in peripheral blood in AD and CCR4-positive lymphocytes were infltrating lesions [31]. All these findings indicate that CCR4 expression is closely associated with the pathogenesis of AD.

CONCLUSION

Usually, physicians conduct an objective examination to decide whether their treatment has been effective, however, the objective examination results may not always be accurate as AD may follow non-regulated inflammatory fluctuating course caused by inadequate intermittent topical treatment. So, it is better to depend upon specific markers as TARC and MDC to reveal treatment outcome as well as disease severity.

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