

Stratum corneum TARC level is a new indicator of lesional skin inflammation in atopic dermatitis

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To cite this article: Morita E, Takahashi H, Niihara H, Dekio I, Sumikawa Y, Murakami Y, Matsunaka H. Stratum corneum TARC level is a new indicator of lesional skin inflammation in atopic dermatitis. *Allergy* 2010; DOI: 10.1111/j.1398-9995.2010.02361.x.

Keywords

atopic dermatitis; stratum corneum; tape-stripping; thymus and activation-regulated chemokine.

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Accepted for publication 4 February 2010

DOI:10.1111/j.1398-9995.2010.02361.x

Edited by: Thomas Bieber

Abstract

Background: Management of atopic dermatitis (AD) requires judging the symptoms of local skin lesions and prescribing a suitable treatment. However, no method has been established in which objective measures can be used to evaluate the severity of local symptoms. We established a method for measuring thymus and activation-regulated chemokine (TARC) levels in the stratum corneum (scTARC), and examined whether the scTARC can be used as an indicator of the severity of local skin lesions in patients with AD.

Methods: Stratum corneum was obtained from patients with AD by tape-stripping, and scTARC was evaluated using a TARC-specific antibody followed by image analysis. The scTARC was examined to determine correlation with the severity of local skin lesions (the severity of erythema, edema/papule, oozing/crusts, excoriations, lichenification, and xerosis) as well as with the severity scoring of atopic dermatitis (SCORAD) index, serum TARC level, serum IgE level, serum lactate dehydrogenase (LDH) level, interleukin (IL)-4-producing T cell ratio (Th2 cell ratio), and blood eosinophil count.

Results: The scTARC was correlated with the severity of local skin lesions, especially with the erythema, edema/papule, and oozing/crusts score. The scTARC in the most severe lesions was also correlated with the SCORAD index, serum TARC level, serum IgE level, and blood eosinophil count. The scTARC was not, however, correlated with the serum LDH level and Th2 cell ratio.

Conclusion: An immunofluorescent technique combined with tape-stripping was used to measure scTARC. The scTARC can be used as an indicator of the severity of local acute inflammation in patients with AD.

Thymus and activation-regulated chemokine/CCL17 (TARC) is a chemokine involved in lymphocyte migration. It has chemotactic activity specific to type 2 helper T (Th2) cells in which the TARC receptor CCR4 is expressed. TARC is overproduced in the skin of patients with atopic dermatitis (AD) and is believed to attract Th2 cells into the skin from circulating blood. This may trigger and/or exacerbate the inflammatory process of AD (1). The serum TARC levels are significantly and selectively higher in patients with AD than in those with other inflammatory skin diseases and these levels increase in proportion to the severity of AD (2). Determination of the serum TARC level is thus used in clinical settings as an objective indicator of AD because it is more sensitive and accurate than conventional laboratory

measures such as the serum IgE level or blood eosinophil count (3, 4).

Atopic dermatitis management requires judging the symptoms of local skin lesions to evaluate the condition and prescribe suitable treatment. To evaluate the severity of the AD symptoms, scoring methods, such as those based on the eczema area and severity index (EASI) have been used (5). However, no method has been established in which objective measures can be used to evaluate the severity of local symptoms.

We measured the amounts of TARC in extract of scales collected from patients with AD and found high amounts of TARC in the scales of the affected skin but not in the stratum corneum obtained from intact sole skin (6). In addition, a

significant correlation was observed between the amount of TARC in the scales and the serum IgE level. These observations suggest that the TARC levels in the stratum corneum (scTARC) could serve as an objective marker that reflects the severity of lesional symptoms of AD. Therefore, we established a method in which a fluorescent-based detection technique was used after immunostaining samples with a TARC-specific antibody. We also examined whether the scTARC correlates with the severity of local skin lesions in patients with AD.

Methods

Subjects

Sixty-one patients (17–54 years; mean age: 29.7 years) who fulfilled the Diagnostic Criteria for AD established by the Japanese Dermatological Association were enrolled in the study (7). Of the 61 patients, 20 were men (21–44 years; mean age: 28.6 years) and 41 were women (17–54 years; mean age: 30.2 years). A total of 19 healthy subjects (22–40 years; mean age: 28.4 years) consisting of 7 men (22–40 years; mean age: 29.6 years) and 12 women (23–34 years; mean age: 27.8 years) formed the control group. The inclusion criteria for healthy subjects were as follows: (i) those without any inflammatory skin diseases, (ii) those whose physical findings were normal, (iii) those with no past history or family history related to allergies, and (iv) those whose laboratory values were within the normal range.

Prior to initiating this study, the details were fully explained to each subject or his/her guardian, and written informed consent was obtained. This study was approved by the ethical committee of the Shimane University Faculty of Medicine (Approval No. 459).

Assessment of the clinical conditions

Clinical severity of AD

The clinical severity of AD was evaluated as very mild, mild, moderate, or severe according to the Diagnostic Criteria for AD set by the Japanese Dermatological Association (7). The severity of the disease in the 61 patients with AD who participated in this study was scored as follows: 45 patients had very mild or mild AD, 14 patients had moderate AD, and two patients had severe AD.

Scoring of the skin symptoms

The severity of the eruptions on the lesion was evaluated on a 4-point scale (0: none, 1: mild, 2: moderate, or 3: severe) by scoring the symptoms, erythema, edema/papule, oozing/crust, excoriation, lichenification, and xerosis. The severity scoring of atopic dermatitis (SCORAD) index was calculated by the equation $A/5 + 3.5B + C$. In this equation, A is the percentage of body area with a skin rash; B is the total score of erythema, edema/papule, oozing/crust, excoriation, lichenification, and xerosis, each of which was evaluated on a 4-point scale (0: none, 1: mild, 2: moderate, or 3: severe); and C is the visual analog scale (VAS) (a scale from 0 to 10) for subjective assessment of both itching and sleep loss (8).

Laboratory tests

A total of 33 patients with AD (17–54 years; mean age: 29.2 years) consented to the blood tests. Of these, 19 were men (21–44 years; mean age: 28.9) and 14 were women (17–54 years; mean age: 29.6 years). The serum TARC level, serum total IgE level, serum lactate dehydrogenase (LDH) level, and peripheral blood eosinophil count were measured by laboratory tests. Percentage of IL-4-positive and IFN- γ -negative cells in peripheral blood CD4⁺ T cells (Th2 cell ratio) was determined as previously described (9). Briefly, CD4⁺ T cells were purified using the sheep red blood cell resetting technique and negative selection via anti-CD8 and anti-CD16 monoclonal antibodies (BD Pharmingen, Tokyo, Japan). The CD4⁺ T cells were stimulated with phorbol 12-myristate 13-acetate (10 mg/ml) and ionomycin (1 μ g/ml) for 3 h in the presence of Brefeldin A (10 μ g/ml, Sigma, Tokyo, Japan). The cells were then fixed with 1% paraformaldehyde, permeabilized with 0.5% saponin, stained with phycoerythrin-conjugated anti-IL-4 and fluorescein isothiocyanate-conjugated anti-IFN- γ monoclonal antibodies and analyzed using a FACSCalibur flow cytometer (BD Immunocytometry Systems). The results of the 33 patients were as follows: the serum TARC level was 1497.9 ± 2810.3 pg/ml; serum IgE level 3336.2 ± 6402.7 IU/ml; serum LDH level 243.9 ± 94.8 IU/l; blood eosinophil count 405.9 ± 261.5 cells per μ l; and Th2 cell ratio $0.47 \pm 0.32\%$.

Measurement of the scTARC

A tape-stripping method was used to collect the stratum corneum from the cubital fossa, and unaffected and affected sites on the trunk (10–12). The affected site was the area with the most severe eruptions. The study sites were cleansed prior to the tape-stripping. Cellophane tape (24 mm $\times$ 50 mm, Serotape[®], Nichiban, Tokyo, Japan) was applied to the skin, pressed for approximately 10 s and then gently removed. The stratum corneum obtained by the tape-stripping was kept at a temperature of -20°C until further analysis. The tape was attached to a slide glass (Matsunami Glass, Osaka, Japan), and the slide glass was immersed in n-hexane (Nacalai, Tokyo, Japan). It was allowed to stand overnight so that the adhesive tape would be removed spontaneously. The slide glass was immersed in cold acetone (Nacalai) for 10 min, and the cells were fixed on the slide glass and blocked with 1.0% bovine serum albumin. After washing with PBS (–), the cells were allowed to incubate overnight with an anti-TARC antibody (R&D Systems, Minneapolis, MN, USA) at a temperature of 4°C . The cells were then incubated with FITC-anti-mouse IgG (Plysciences, Warrington, PA, USA) for 2 h at room temperature while being protected from light. After mounting, the cells were observed under a fluorescence microscope (BZ-8000, Keyence, Tokyo, Japan). The bright-field and fluorescent images were photographed, and the fluorescent intensity and cell area were determined using PHOTOSHOP[®] software. The scTARC was defined as the fluorescent intensity per cell area. The fluorescent intensity was separately measured in

five fields to obtain the mean values, and the latter was taken as the scTARC of the site.

Data analysis

For statistical analysis, Student's *t*-test or Mann–Whitney's *U*-test was used for comparison between the two groups, Tukey's test was used for comparison among three or more groups, and Spearman's rank correlation test was used for correlation. The results were considered to be significant or correlated when $P < 0.05$.

Results

Measurement of scTARC

Immunofluorescent staining with the anti-TARC antibody revealed marked fluorescent emission because of TARC in the stratum corneum collected from the affected skin of patients with AD. Furthermore, only faint fluorescence was observed in the unaffected skin of patients with AD as well as in the skin of healthy subjects. Typical expression patterns are shown in Fig. 1. The mean fluorescent intensity was 28.5 ± 13.2 ($n = 61$) in the stratum corneum obtained from the affected skin of patients with AD, 20.7 ± 13.0 ($n = 33$)

in unaffected skin of patients with AD and 10.3 ± 2.7 ($n = 19$) in healthy subjects (Fig. 2). The scTARC was significantly higher in the affected skin of patients with AD than that in the unaffected skin of patients with AD as well as in the skin of healthy subjects. The scTARC was also significantly higher in the unaffected skin of AD than that in the skin of healthy subjects.

When the scTARC of the cubital fossa was compared among the groups that were divided according to the severity of AD, the mean scTARC value was 23.9 ± 8.2 ($n = 45$) in the very mild/mild AD groups, 31.7 ± 13.8 ($n = 14$) in the moderate AD group, and 42.9 ($n = 2$) in the severe AD group (Fig. 3). The scTARC of both the very mild/mild and moderate AD groups were significantly higher than that of the healthy control group.

Correlation of the scTARC and severity of skin symptoms

Correlation of the scTARC was assessed with the severity score of skin lesions (total score, erythema score, edema/papule score, oozing/crust score, excoriation score, lichenification score, and xerosis score) in the affected skin (trunk and cubital fossa) where the tape-stripping was done in patients with AD (Fig. 4). The scTARC correlated significantly with the total score of eruptions. When the correlation

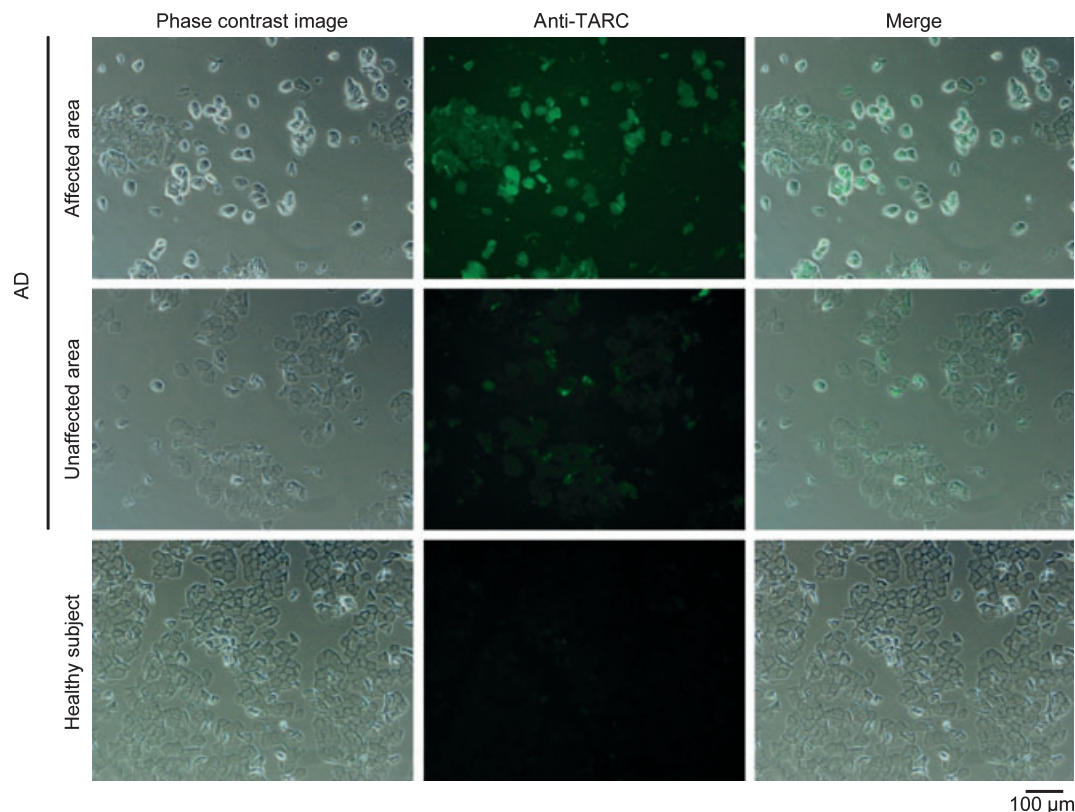


Figure 1 Immunoreactive thymus and activation-regulated chemokine (TARC) in the stratum corneum was visualized using a TARC-specific antibody in conjunction with an FITC-conjugated secondary

antibody. Typical results obtained from a patient with atopic dermatitis (AD) and a healthy subject are shown.

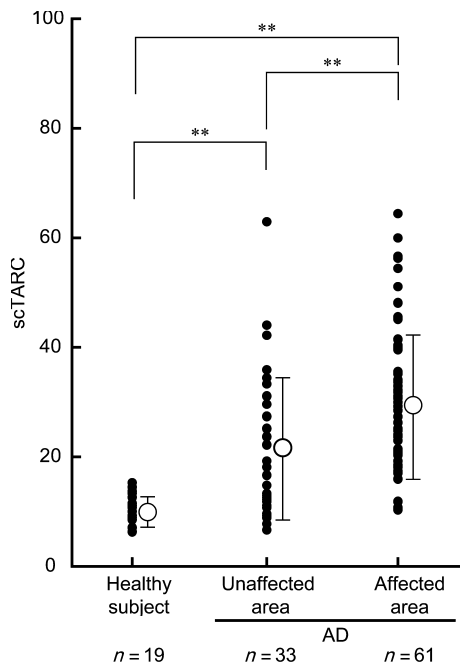


Figure 2 The scTARC of trunk in patients with atopic dermatitis (AD) and healthy subjects. In the patients with AD, the samples were taken from affected area and unaffected area of the trunk. (** $P < 0.01$).

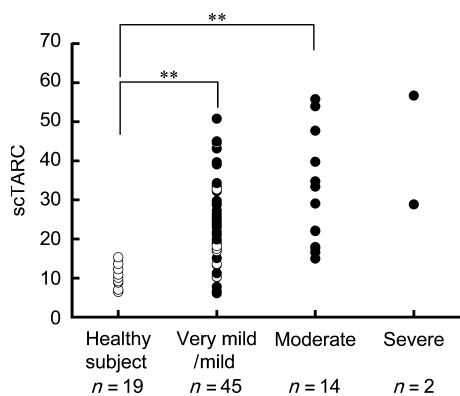


Figure 3 The scTARC of the cubital fossa were compared among the groups that had been divided according to the severity of atopic dermatitis (AD). Affected patients were presented with black points and unaffected patients were presented with white points. (** $P < 0.01$).

of the scTARC within each eruption parameter was studied, the scTARC was significantly correlated with the erythema score, edema/papule score, and oozing/crust score but was not significantly correlated with the excoriation score, lichenification score, and xerosis score.

Correlation of the scTARC and laboratory data

The scTARC of the affected skin (cubital fossa) of the patients with AD was examined to correlate with SCORAD

index, serum TARC level, serum total IgE level, serum LDH level, Th2 cell ratio, and peripheral blood eosinophil count (Fig. 5). The scTARC was positively correlated with the SCORAD index, the serum TARC level, the serum IgE level, and the peripheral blood eosinophil count. However, no significant correlation was observed between the scTARC and the serum LDH level and the Th2 cell ratio. The serum TARC level was significantly correlated with the SCORAD index, serum IgE level, blood eosinophil count, Th2 cell ratio, and serum LDH level (Table 1).

Discussion

In this study, we determined scTARC in patients with AD by immunostaining technique combined with the tape-stripping method and found that the scTARC was significantly correlated with the severity of the local skin lesions. These results indicate that the scTARC can be used as an indicator of the condition of local skin lesions, especially during the acute phase of inflammation, in patients with AD. This is supported by the fact that the scTARC correlated well with acute phase parameters of eruption such as erythema, edema/papule, and oozing/crusts but not with itching and excoriation or chronic parameters such as lichenification and xerosis. The results are in contrast to the previous studies by Yamaguchi et al. (12) that examined levels of nerve growth factor (NGF) in the stratum corneum by using tape-stripping method in patients with AD. They reported that the NGF levels in the stratum corneum correlated well with the pruritus scores, total skin scores, and xerosis scores, but not with the skin scores of papule, erosion/crust and lichenification, indicating a close association between the skin NGF levels and itching in patients with AD. These results suggest that differential expression of cytokines regulates the inflammatory process and the symptoms of the lesions in AD.

The scTARC reflects not only the lesional severity of the eruption but also, to a certain extent, the systemic disease severity of AD because the scTARC correlated with the SCORAD index, and laboratory data such as the serum IgE level and the blood eosinophil count both of which reflect the systemic severity of AD (Fig. 5). This is consistent with our previous findings that the amount of TARC in the scales obtained from the patients with AD is correlated with the serum IgE level (6). Yamaguchi et al. (12) also found a significant correlation between the NGF level in the stratum corneum and the blood eosinophil count as well as the serum LDH level.

The scTARC was also correlated well with the serum TARC level in patients with AD, suggesting that serum TARC originates from the TARC that is overproduced in the inflammatory skin lesions. The TARC detected in the stratum corneum is considered to be of keratinocyte origin, because an immunostaining technique has clearly shown that immunoreactive TARC is expressed in the keratinocytes of lesional skin in both patients with AD and NC/Nga mice exhibiting AD-like lesions (3, 13, 14). In addition, these findings are consistent with those of *in vitro* studies in which cultured human keratinocytes and a

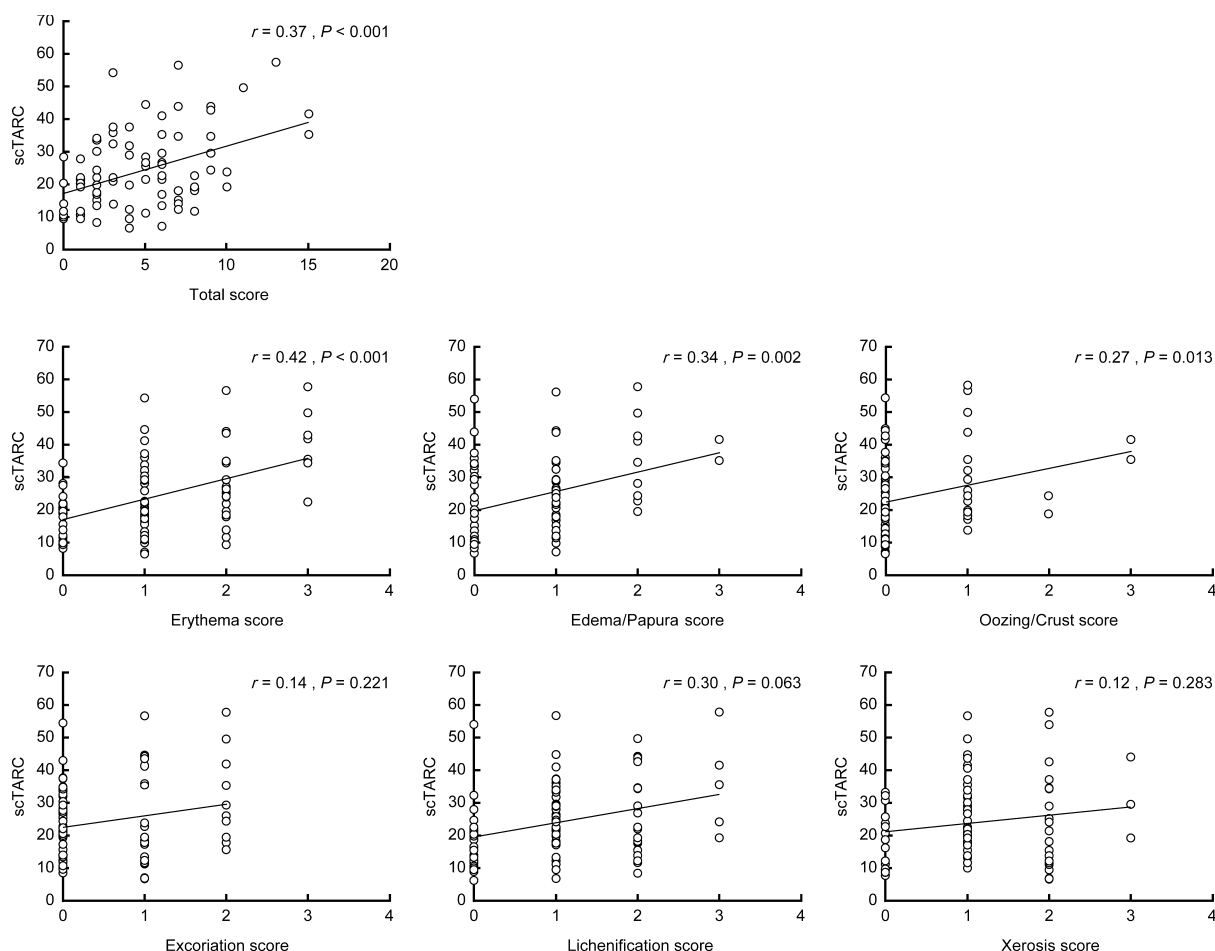


Figure 4 Correlation of the scTARC was assessed with severity score of skin symptoms in the affected skin where the tape-stripping was done in patients with atopic dermatitis (AD). The

results obtained from trunk and cubital fossa were presented simultaneously.

Table 1 Correlation between serum thymus and activation-regulated chemokine (TARC) level and other clinical data in patients with atopic dermatitis (AD)

Clinical data	Co-efficient	P
SCORAD	0.55	0.002
IgE	0.67	<0.001
Eosinophils	0.40	0.032
LDH	0.68	<0.001
Th2 cell ratio	0.47	0.008

SCORAD, severity scoring of atopic dermatitis; LDH, lactate dehydrogenase.

human keratinocyte model, HaCaT cells, produced TARC upon combined stimulation with IFN- γ and TNF- α (3, 13, 15). In contrast to these reports, Horikawa et al. (16) reported that primary cultured epidermal keratinocytes did not efficiently produce TARC, although they expressed TARC mRNA upon stimulation with IFN- γ . Human monocyte-derived dendritic

cells, which exist as a large population in the epidermis, have an ability to produce TARC; therefore it is conceivable that dendritic cells are the main TARC-producing cells in the epidermis (17).

The tape-stripping method is known to be an easy and safe method for collecting stratum corneum from both normal and inflamed skin in amounts that are sufficient for analysis of the cytokine content (10–12). The levels of pro-inflammatory cytokines, such as IL-1 α and IL-8, were measured in the stratum corneum collected from skin lesions directly by using specific ELISA and found to be associated with the severity of inflammation of these lesions. Prior to this study, TARC was extracted from the stratum corneum, and the amount was directly measured using a TARC-specific ELISA. However, it was impossible to determine the TARC levels because the sensitivity of the TARC-specific ELISA was limited. Therefore, in this study, stratum corneum cells collected by the tape-stripping method were directly mounted on a slide glass, and TARC present in the cells were then visualized by an immunostaining method

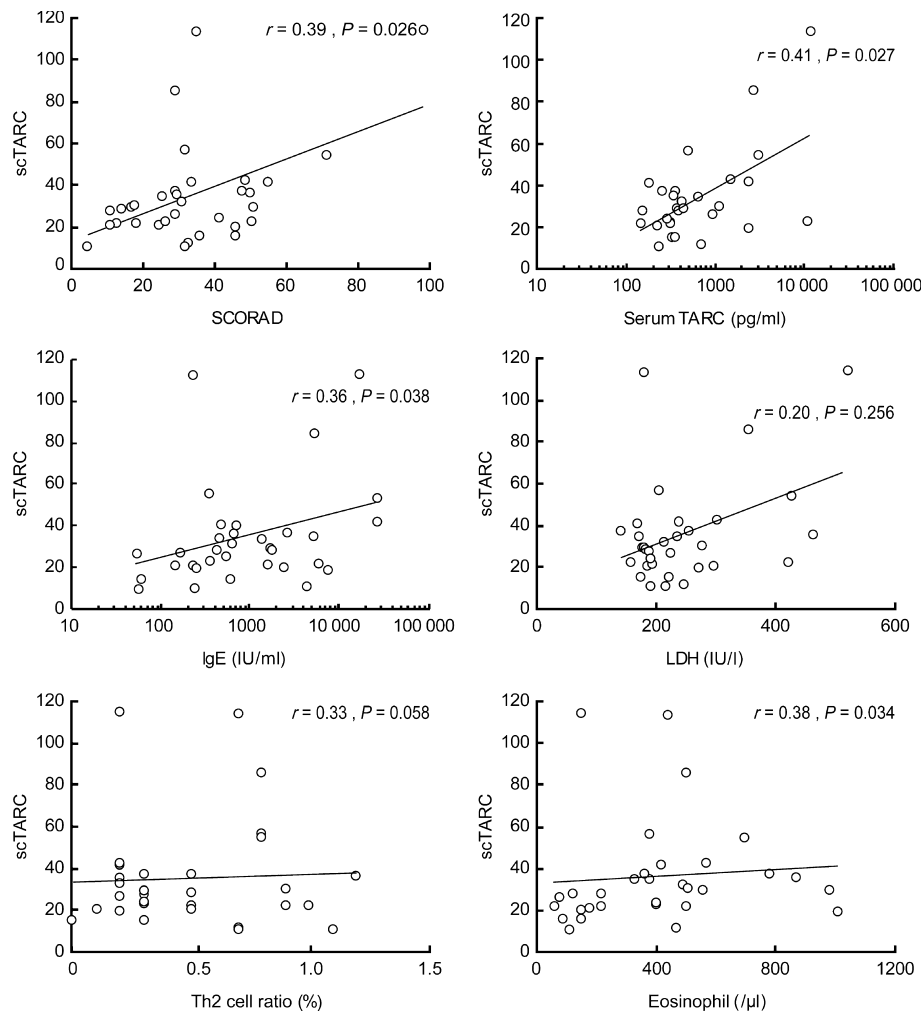


Figure 5 Correlation of the scTARC and severity scoring of atopic dermatitis (SCORAD) index, serum thymus and activation-regulated

chemokine (TARC) level, serum IgE level, serum lactate dehydrogenase (LDH) level, Th2 cell ratio, and blood eosinophil count.

with FITC-conjugated TARC-specific antibody (Fig. 1). When the fluorescent emission intensity per cell area was quantified by image analysis, the immunofluorescent technique was found to be more sensitive than the TARC-specific ELISA for measuring the scTARC.

Although TARC was hardly detected in the skin of non-atopic healthy subjects, a small but considerable amount of TARC was still detected in the clinically unaffected skin of patients with AD, suggesting that TARC overproduction in the epidermis is a basic abnormality and associated with hyperirritability commonly seen in AD. In the present study, we had no information on the effects of treatment on the scTARC. It is well recognized that serum TARC level is a sensitive and quick-response indicator of the severity of skin lesions in patients with AD (18). The normal serum TARC level is set as below 450 pg/ml. In addition, 700 pg/ml is set as the value that distinguishes AD of mild severity from AD of moderate or higher severity (18). The

serum TARC level is regarded as an objective indicator that reflects changes in the skin condition of patients with AD more accurately and sensitively than conventional laboratory measures. It will be necessary to determine the effect of topical treatment on the scTARC to determine whether the scTARC can be used as a reliable indicator of treatment. The fact that our immunofluorescent technique is a semi-quantitative evaluation for the scTARC represents a further limitation.

Many types of bioactive substances such as IL-1, IL-8, NGF, and β -defensin have been identified from the stratum corneum of patients with inflammatory skin diseases and have thus been considered to play an important role in the pathogenesis of skin diseases (10–12, 19). The immunofluorescent technique is a new method to detect small amounts of cytokines in the scales of lesions and may be useful in evaluating the effectiveness of therapies which objectively monitor the lesional severity.

References

- Nakatani T, Kaburagi Y, Shimada Y, Inaoki M, Takehara K, Mukaida N et al. CCR4 memory CD4⁺ T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. *J Allergy Clin Immunol* 2001;**107**:353–358.
- Tamaki K, Kakinuma T, Saeki H, Horikawa T, Kataoka Y, Fujisawa T et al. Serum levels of CCL17/TARC in various skin diseases. *J Dermatol* 2006;**33**:300–302.
- Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H et al. Thymus and activation-regulated chemokine in atopic dermatitis: serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001;**107**:535–541.
- Fujisawa T, Fujisawa R, Kato Y, Nakayama T, Morita A, Katsumata H et al. Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis. *J Allergy Clin Immunol* 2002;**110**:139–146.
- Hanifin JM, Thurston M, Omoto M, Cherill R, Tofte SJ, Graeber M. The eczema area and severity index (EASI): assessment of reliability in atopic dermatitis. EASI Evaluator Group. *Exp Dermatol* 2001;**10**:11–18.
- Morita E, Hiragun T, Mihara S, Kaneko S, Matsuo H, Zhang Y et al. Determination of thymus and activation-regulated chemokine (TARC)-contents in scales of atopic dermatitis. *J Dermatol Sci* 2004;**34**:237–240.
- Furie M, Saeki H, Furukawa F, Hide M, Ohtsuki M, Katayama I et al. Guidelines for management of atopic dermatitis. *J Dermatol* 2009;**36**:563–577.
- Stalder JF, Taïeb A. Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. *Dermatology* 1993;**186**:23–31.
- Berner B, Akça D, Jung T, Muller GA, Reuss-Borst MA. Analysis of Th1 and Th2 cytokines expressing CD4⁺ and CD8⁺ T cells in rheumatoid arthritis by flow cytometry. *J Rheumatol* 2000;**27**:1128–1135.
- Terui T, Hirao T, Sato Y, Uesugi T, Honda M, Iguchi M et al. An increased ratio of interleukin-1 receptor antagonist to interleukin-1α in inflammatory skin diseases. *Exp Dermatol* 1998;**7**:327–334.
- Perkins MA, Osterhues MA, Farage MA, Robinson MK. A noninvasive method to assess skin irritation and compromised skin conditions using simple tape adsorption of molecular markers of inflammation. *Skin Res Technol* 2001;**7**:227–237.
- Yamaguchi J, Aihara M, Kobayashi Y, Kambara T, Ikezawa Z. Quantitative analysis of nerve growth factor (NGF) in the atopic dermatitis and psoriasis horny layer and effect of treatment on NGF in atopic dermatitis. *J Dermatol Sci* 2009;**53**:48–54.
- Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA⁺ CCR4⁺ lymphocytes into lesional atopic dermatitis skin. *J Invest Dermatol* 2000;**115**:640–646.
- Vestergaard C, Yoneyama H, Murai M, Nakamura K, Tamaki K, Terashima Y et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J Clin Invest* 1999;**104**:1097–1105.
- Komine M, Kakinuma T, Kagami S, Hanakawa Y, Hashimoto K, Tamaki K. Mechanism of thymus- and activation-regulated chemokine (TARC)/CCL17 production and its modulation by roxithromycin. *J Invest Dermatol* 2005;**125**:491–498.
- Horikawa T, Nakayama T, Hikita I, Yamada H, Fujisawa R, Bito T et al. IFN-γ-inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epidermal keratinocytes and their roles in atopic dermatitis. *Int Immunol* 2002;**14**:767–773.
- Hashimoto S, Suzuki T, Dong HY, Nagai S, Yamazaki N, Matsushima K. Serial analysis of gene expression in human monocyte-derived dendritic cells. *Blood* 1999;**94**:845–852.
- Tamaki K, Saeki H, Kadono T, Sato S, Hatta N, Hasegawa M et al. Serum TARC/CCL17 levels as a disease marker of atopic dermatitis. *Jpn J Dermatol* 2006;**116**:27–39.
- Asano S, Ichikawa Y, Kumagai T, Kawashima M, Imokawa G. Microanalysis of an antimicrobial peptide, beta-defensin-2, in the stratum corneum from patients with atopic dermatitis. *Br J Dermatol* 2008;**159**:97–104.