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Original article

Serum levels of squamous cell carcinoma antigens 1 and 2 reflect disease severity and clinical type of atopic dermatitis in adult patients

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LDH, lactate dehydrogenase;

IgE, Immunoglobulin E; IL-4, interleukin-4;

IL-13, interleukin-13; ELISA, enzyme-linked immunosorbent assay

ABSTRACT

Background: Recent studies have indicated that serum levels of squamous cell carcinoma antigen (SCCA) 1 and 2 induced by type 2 cytokines such as IL-4 and IL-13, are increased in patients with atopic dermatitis (AD). However, no clinical studies have analyzed serum levels of SCCA2 in larger series of AD patients or their association with various clinical characteristics. This study was performed to clarify whether serum levels of SCCA2 are associated with disease severity and clinical phenotypes of adult AD patients.

Methods: An enzyme-linked immunosorbent assay was performed to examine serum SCCA2 levels in 240 adult patients with AD and 25 healthy controls in this study. Serum SCCA2 levels were analyzed with clinical characteristics and laboratory parameters including thymus and activation-regulated chemokine (TARC), lactate dehydrogenase (LDH), blood eosinophils, total IgE, and specific IgE (*Japanese cedar pollen*, *Dermatophagoides farina*, *Candida*, *malassezia*, *Staphylococcal enterotoxin B*). Expression of SCCA2 in AD eruption was examined by immunohistochemistry. The effect of treatment on serum SCCA2 was also assessed.

Results: Serum SCCA2 level showed a positive correlation with disease severity, levels of TARC, LDH, eosinophil counts, and IgE levels. Robust expression of SCCA2 was detected in the supra basal keratinocytes in the epidermis of AD patients. Serial measurements of serum SCCA2 revealed decreased levels of SCCA2 after treatment for AD.

Conclusions: Serum SCCA2 levels reflected disease severity and clinical type of AD. Serum SCCA2 may thus be a relevant biomarker for AD.

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Introduction

Squamous cell carcinoma antigen (SCCA) is known as a tumor biomarker for various squamous cell tumors. Total SCCA comprises 2 nearly identical proteins, SCCA1 and SCCA2. Both SCCA1 and 2 belong to the ovalbumin-serpin proteinase inhibitor family.¹

Although SCCA1 and 2 are closely homologous at the amino acid level, they are active against different proteases.

SCCA1 inhibits parasite-derived cysteine proteases,² and staphylococcal cysteine proteases,³ and cysteine proteases such as papain, cathepsin K, L, and S¹. On the other hand, SCCA2 inhibits serine protease such as cathepsin G, human mast cell chymase, and cysteine protease *Der p 1*.^{1,4} These findings indicate that SCCA proteins play some roles in the defense mechanisms against extrinsic proteases.

Recent studies have identified some associations between SCCA and allergic diseases. It is reported that SCCA1 and SCCA2 are induced by type 2 cytokines, such as IL-4 and IL-13, in human bronchial epithelial cells.⁵ Increased serum levels of SCCA proteins

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have been detected in patients with bronchial asthma⁵ and chronic allergic rhinitis.⁶

A previous report showed a high correlation between SCCA1 and transepidermal water loss (TEWL) in human study.⁷ Furthermore SCCA1 content showed a very high correlation with the number of parakeratotic cells in the cornified layer of the skin.⁷ Recently it was reported that SCCA2 is also related to barrier dysfunction and the early inflammatory response following cutaneous allergen exposure in mice.⁸ In addition, severe AD patients have higher serum levels of SCCA proteins.^{9,10} Not only in the circulation but also in the lesional skin of AD patients, the expression of SCCA seems to be up-regulated.¹⁰ Furthermore, our previous study showed that SCCA2 levels in the horny layer correlated significantly with serum IgE level in AD.¹¹ It was also shown that both SCCA1 and SCCA2 are induced by IL-4 and IL-13 in cultured keratinocytes.¹⁰ These findings imply that SCCA may play some roles in type 2 immunity, and that it might be useful as a biomarker in certain clinical contexts.

However, the above-mentioned studies did not include sufficiently large numbers of AD patients, and no clinical study has analyzed the serum levels of SCCA1 and 2 as related to various clinical characteristics. This prompted us to attempt to clarify the clinical significance of SCCA by examining serum SCCA levels as correlated with various phenotypes of AD.

Methods

Subjects

Two hundred and forty adult AD patients and 25 healthy controls (HCs) were included in this study. They were diagnosed with AD by dermatologists. The diagnosis of AD was made based on the criteria of Hanifin Rajka.¹² Patient characteristics are shown in Table 1. All patients were treated with topical corticosteroids and oral antihistamines according to the guidelines for management of atopic dermatitis.¹³ Some of the AD patients had a history of asthma, but none showed active asthma during this study. The study protocol was approved by the Ethics Committee of Yokohama City University School of Medicine (Approval No. B141106011). All patients provided written informed consent for the study.

The severity of skin eruptions was determined as mild, moderate, severe, and very severe according to the Japanese guidelines for atopic dermatitis proposed by the Research Group established by the Ministry of Health, Labor and Welfare of Japan.¹³ Briefly, mild: skin involvement limited to mild eruption, moderate: <10% surface area involvement by eruption with severe inflammation, severe: 10% but <30% skin involvement by severe eruption, very severe: ≥30% of body involvement by severe eruption. AD patients were further grouped as having the erythroderma type, widespread combinations of various types, prurigo type, limb type, or head/face/neck/chest/back type based on the individual clinical characteristics according to the modified criterion published by the JDA.¹³

Table 1
Clinical characteristics of AD patients.

	AD patients	Healthy controls	P-values
Number, n	240	25	—
Age (years), median (IQR)	32 (16.3)	30 (16)	0.19
Males/Females, n	137/103	14/11	—
TARC levels (pg/ml), median (IQR)	2050 (5756.3)	254 (81)	<0.001
Eosinophils (counts/ul), median (IQR)	632.7 (800.8)	188 (161)	<0.001
LDH levels (IU/L), median (IQR)	260.5 (135.3)	194 (56)	<0.001
IgE levels (ng/ml), median (IQR)	4549 (12000.5)	234 (157)	<0.001

Measurement of serum SCCA levels

Serum samples were obtained from 240 adult AD patients and age- and sex-matched healthy subjects and then immediately stored at −80 °C. Serum SCCA1 and SCCA2 levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA). ELISA was carried out as previously described.⁹ Capture antibody was coated on the ELISA plate. After blocking with a blocking buffer (0.5% casein in Tris-buffered saline, pH 8.0) overnight at 4 °C, the plate was incubated with 50- to 1000-fold diluted samples for 18 h at 25 °C. Biotin-labeled SS8G (50 ng/mL) for SCCA2 was added, followed by incubation for 2 h at 25 °C. After incubation with 15,000-fold diluted peroxidase-conjugated streptavidin (Stereo-specific Detection Technologies, Baesweiler, Germany) for 90 min at 25 °C, substrate solution (0.64 mmol/L 3,3', 5,5'-tetramethylbenzidine, 2.5 mmol/L H₂O₂) was added, followed by incubation for 10 min at 25 °C. The reaction was stopped with 0.7 N HCl. SCCA2 concentrations in the serum were calculated from the measurements of recombinant SCCA standards. We performed ELISA assays on duplicate samples.

Laboratory parameters

Serum levels of TARC and total IgE were measured using commercial ELISA kits according to the manufacturers' instructions. These included a human TARC ELISA kit (Shionogi, Osaka, Japan) with a lower detection limit of 125 pg/mL, and a human IgE sandwich electrochemiluminescence immunoassay assay kit (Roche Diagnostics GmbH, Mannheim, Germany) with a lower detection limit of 0.10 IU/mL. Serum LDH levels and blood eosinophil counts were also measured.

Immunohistochemical staining

Skin biopsy samples obtained from adult AD patients and healthy volunteers were fixed in 10% neutral-buffered formalin, and embedded in paraffin. For immunohistochemistry, the 5 μm-thick-paraffin sections were deparaffinized with xylene and graded ethanol. Antigen retrieval was performed by microwave in EDTA buffer (pH 9) for 15 min. Endogenous peroxidases were blocked using 0.3% hydrogen peroxidase in PBS for 30 min at room temperature. After washing with PBS, sections were incubated at 37 °C for 10 min with protein block serum free (Dako, CA, USA). Sections were incubated at room temperature overnight with monoclonal rat antibody against SCCA2 (clone SS8G, 30 μg/mL). An appropriate isotype control was used as a control. After washing with PBS, the sections were incubated with horseradish peroxidase-conjugated goat anti-rat IgG (GE Healthcare, Little Chalfont, UK) as the secondary antibody. The peroxidase reaction was visualized by incubating the sections with substrate chromogen (Dako). Sections were then counterstained with hematoxylin, washed, and mounted.

Statistical analysis

Comparisons between two groups were tested for statistical significance using the Mann–Whitney *U* test as appropriate. Sensitivity and specificity calculations and ROC analysis were used to estimate the best cut-off point.

The Kruskal–Wallis test was used to test for overall group differences, and Dunn's multiple comparison test was used to determine between-group differences.

Correlations between two groups were evaluated by Spearman's rank correlation coefficient. *p* < 0.05 was considered statistically significant.

Results

Serum SCCA levels in patients with AD and disease severity

SCCA1 and SCCA2 levels in AD patients and those in healthy controls are shown in Figure 1a, b. Both SCCA1 and SCCA2 levels were significantly higher in AD patients (SCCA1; median (interquartile range; IQR, Q1–Q3), 3.24 (1.48–8.10) ng/mL, SCCA2; 7.42 (2.83–17.95) ng/mL) than in healthy controls (SCCA1; 0.74 (0.58–0.84) ng/mL, SCCA2; 0.71 (0.42–0.84) ng/mL, $p < 0.001$). According to the ROC analysis, the optimal cut-off value of SCCA1 for AD was measured as more than 1.085 ng/mL, with 86.3% sensitivity and 96.0% specificity. When using the SCCA2 cut-off value at 0.995 ng/mL, the sensitivity and specificity for AD were 92.9% and 92.0% respectively.

A similar tendency was seen between the serum levels of SCCA1 and those of SCCA2. Then, we examined the correlation between SCCA1 and SCCA2. Figure 1c shows the extremely strong correlation between the serum levels of SCCA1 and SCCA2 ($r = 0.98$, $p < 0.001$). As SCCA2 has a wider range of titers compared to SCCA1, we focused on SCCA2 to examine the association between SCCA and various phenotypes of AD.

We next examined whether the serum levels of SCCA2 correlated with the clinical severity of AD. SCCA2 levels in mild AD were significantly higher than those of healthy controls. Though there was no significant difference in SCCA2 levels between AD patients with mild and moderate, severe and very severe disease, higher SCCA2 levels were observed in parallel with progression of disease severity (Fig. 2; median (IQR); mild 4.25 (1.92–5.96) ng/mL, moderate 5.35 (2.70–12.26) ng/mL, severe 11.16 (4.53–25.29) ng/mL, very severe 17.45 (5.07–36.09) ng/mL), indicating that the SCCA2 level reflects disease severity in AD patients.

Clinical types of AD and serum SCCA2 levels

To determine whether the clinical types of AD influence serum SCCA2 levels, AD patients were further classified according to

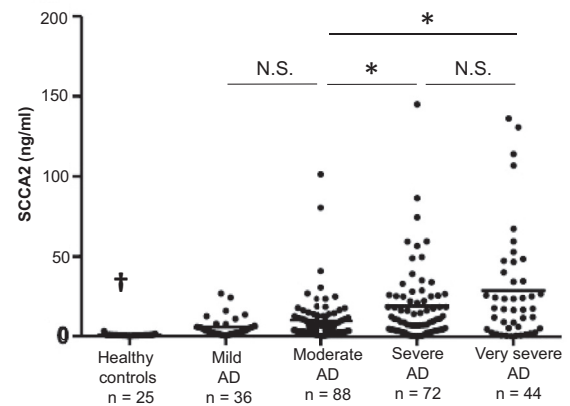


Fig. 2. Serum levels of SCCA in association with disease severity. SCCA levels were determined in AD patients with mild ($n = 36$), moderate ($n = 88$), severe ($n = 72$), and very severe ($n = 44$) disease activities. Horizontal bars indicate the median values. * $p < 0.01$, † $p < 0.001$ significant with regard to all other groups. NS, not significant.

clinical type, such as erythroderma, widespread type, prurigo type, limbs type and head/face/neck/chest/back type. AD patients with erythroderma type showed significantly higher levels of SCCA2 compared to the others ($p < 0.001$), followed by the widespread type. On the other hand, AD patients whose lesions were not distributed systemically, such as those with limb type, had lower levels of SCCA2 (Fig. 3a, Supplementary Fig. 1).

Lichenification is one of the barometers of chronic inflammation in the skin lesions of AD. We compared SCCA2 levels in patients with AD in the presence and absence of lichenification in $>5\%$ of total body surface area. As shown in Figure 3b, AD with widespread lichenification demonstrated significantly higher levels of SCCA2 than those with less extensive lichenification [median (IQR); 10.80 (3.62–22.16) ng/mL, 4.25 (1.67–8.90) ng/mL, respectively, $p < 0.001$].

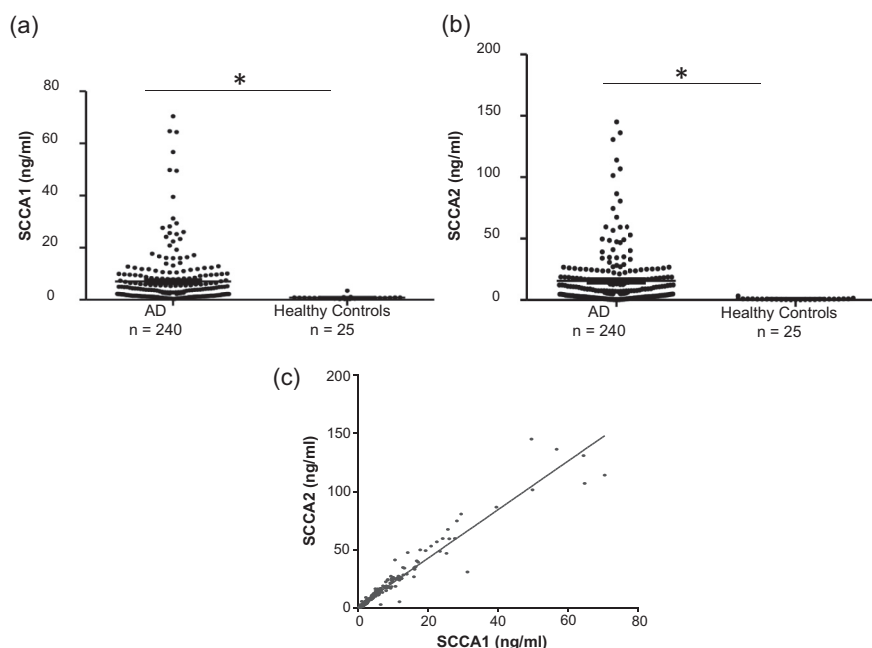


Fig. 1. Serum SCCA levels in AD patients. SCCA levels were determined using a specific enzyme-linked immunosorbent assay in AD patients ($n = 240$) and HCs ($n = 25$). (a) SCCA1, (b) SCCA2, Horizontal bars indicate the median serum SCCA values. * $p < 0.001$ versus HCs. (c) Correlation between SCCA1 and SCCA2 ($n = 240$, $r = 0.98$, $p < 0.001$).

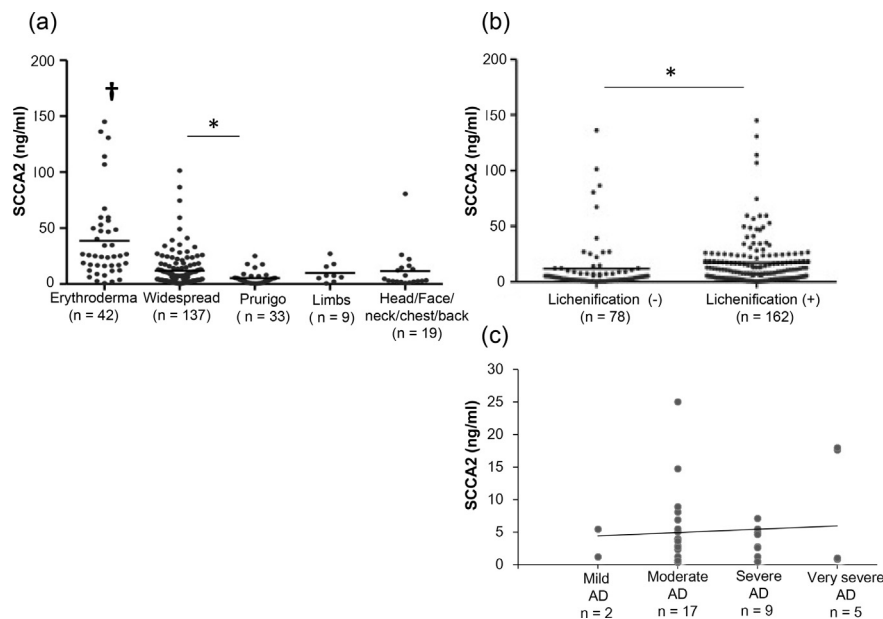


Fig. 3. Serum SCCA2 levels in association with clinical type of AD patients. (a) SCCA levels are shown in AD patients with erythroderma type ($n = 42$), widespread type ($n = 137$), prurigo type ($n = 33$), limb type ($n = 9$), and head/face/neck/chest/back type ($n = 19$). Horizontal bars indicate the median serum SCCA values. * $p < 0.001$. † $p < 0.001$ significant with regard to all other groups. (b) Levels of serum SCCA2 in AD patients in the absence or presence of lichenification ($n = 78$, 162, respectively). Horizontal bars indicate the median values. * $p < 0.001$. (c) Correlation between SCCA2 and severity in AD patients with prurigo type ($n = 33$, $r = 0.07$, $p = 0.66$).

As compared with other types, the serum levels of SCCA2 were relatively low in prurigo type even in the presence of widespread distribution [median (IQR); 3.64 (1.21–5.50) ng/mL]. It showed no correlation with the severity in prurigo type (Fig. 3c). In the patients with severe and very severe conditions, the ratio of the prurigo type in AD patients with lower levels of serum SCCA2 (≤ 2.8 ng/mL; 25% percentile) was significantly higher than the ratio in patients with higher levels of serum SCCA2 (> 2.8 ng/mL), 25% (8 of 32 patients) and 7% (6 of 84 patients) respectively ($p < 0.001$).

Immunohistochemical expression of SCCA in skin specimens obtained from AD patients

To examine SCCA2 expression in AD skin, we performed immunohistochemical analysis using skin specimens obtained from AD patients. Representative images are shown in Figure 4. Slight expression of SCCA2 was detected in the epidermis of normal human skin (Fig. 4a). In contrast, SCCA2 was strongly expressed in the epidermis, especially in the stratum spinosum to granular layers of lesional skin, in atopic dermatitis (Fig. 4b). Expression of SCCA2 was detected in the cytoplasm.

Correlation between SCCA2 levels and laboratory parameters in AD patients

Next, the relationship between serum SCCA levels and laboratory parameters was examined. SCCA2 showed strong correlations with the serum levels of TARC ($n = 228$, $r = 0.673$, $p < 0.01$), and LDH ($n = 234$, $r = 0.614$, $p < 0.01$), and eosinophil counts ($n = 232$, $r = 0.557$, $p < 0.01$). A weak correlation was also detected between SCCA2 and IgE levels ($n = 235$, $r = 0.311$, $p < 0.01$) (Fig. 5). We also examined the correlations between SCCA2 and specific IgE levels of *Dermatophagoides farina* (*D. farina*), *Candida*, *malassezia*, and Staphylococcal enterotoxin B (SEB) in AD patients (data not shown). Weak correlations were also detected between SCCA2 and specific IgE levels of *D. farina* ($n = 226$, $r = 0.20$, $p < 0.01$), *Candida* ($n = 218$, $r = 0.27$, $p < 0.001$), *malassezia* ($n = 224$, $r = 0.20$, $p < 0.01$) and SEB ($n = 166$, $r = 0.24$, $p < 0.01$). Otherwise, no correlation was noted

between serum SCCA2 levels and Japanese cedar pollen-specific IgE ($n = 223$, $r = 0.045$, $p = 0.50$).

Serial SCCA2 levels in AD

The serial samples from 25 AD patients were analyzed retrospectively. All patients were treated and considered as clinically improved after treatment. The SCCA2 levels at 2 different phases are shown in Figure 6. Serum SCCA2 was significantly decreased after treatment compared to baseline levels ($p < 0.001$). This result indicates that SCCA2 can serve as an index of disease severity and the effectiveness of treatment in patients with AD.

Discussion

Our purpose of this study was to clarify the clinical significance and usefulness of SCCA2 in AD patients. We showed that the serum levels of SCCA proteins including SCCA1 and SCCA2 in AD patients were higher than those in healthy controls, and the levels correlated with disease severity. The levels also correlated with previously reported biomarkers for type 2 inflammation of AD including blood eosinophil count, serum LDH, TARC levels, and IgE level. These results were consistent with those of previous reports that assessed fewer AD patients.^{9,10} As previously reported,⁹ serum SCCA1 and SCCA2 levels were strongly correlated and SCCA2 showed a wider range of titers as compared to SCCA1. Hence, we focused on SCCA2 in further studies.

On immunohistochemical staining, SCCA2 was highly expressed in the epidermis of AD patients. Type 2 cytokine production is well known to be increased in AD skin. IL-4 or IL-13 induces SCCA in cultured keratinocytes.¹⁰ In addition, allergen exposure induced SCCA2 expression in the skin, along with increased TEWL.⁸ Thus SCCA2 production in the atopic skin seems to be increased due to type 2 inflammation.

In the relevance of SCCA2 levels and clinical types of AD, the patients with erythroderma type showed significantly higher levels of SCCA2 compared to the others, followed by widespread type. On the other hand, AD patients whose lesions were not distributed

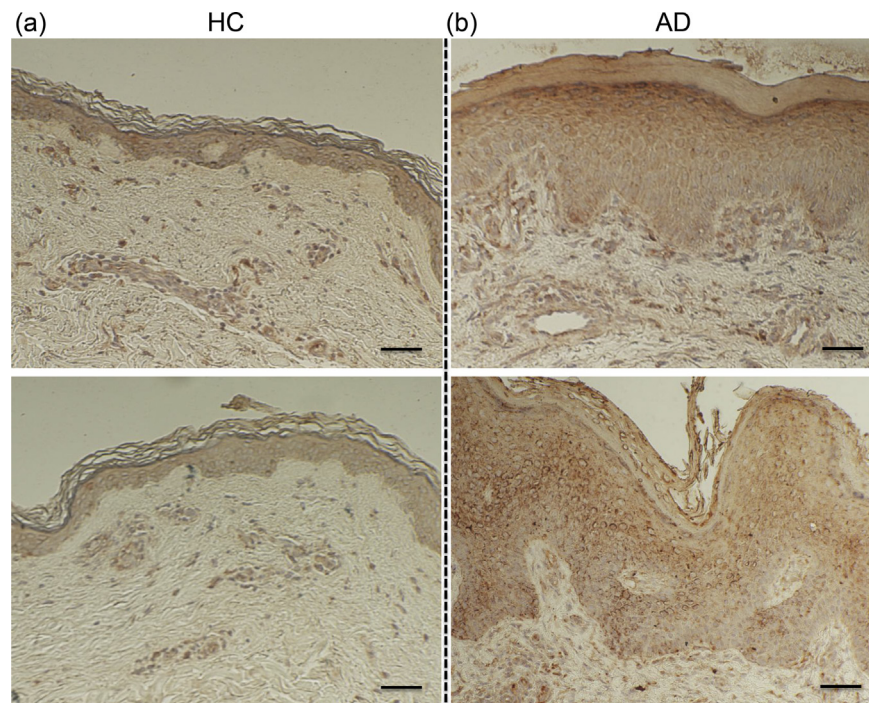


Fig. 4. Representative images of immunohistochemical stainings of SCCA2 in human skin obtained from two healthy controls (a) and AD patients (b) each. Magnification range: 100 $\times$. Scale bars = 100 μ m.

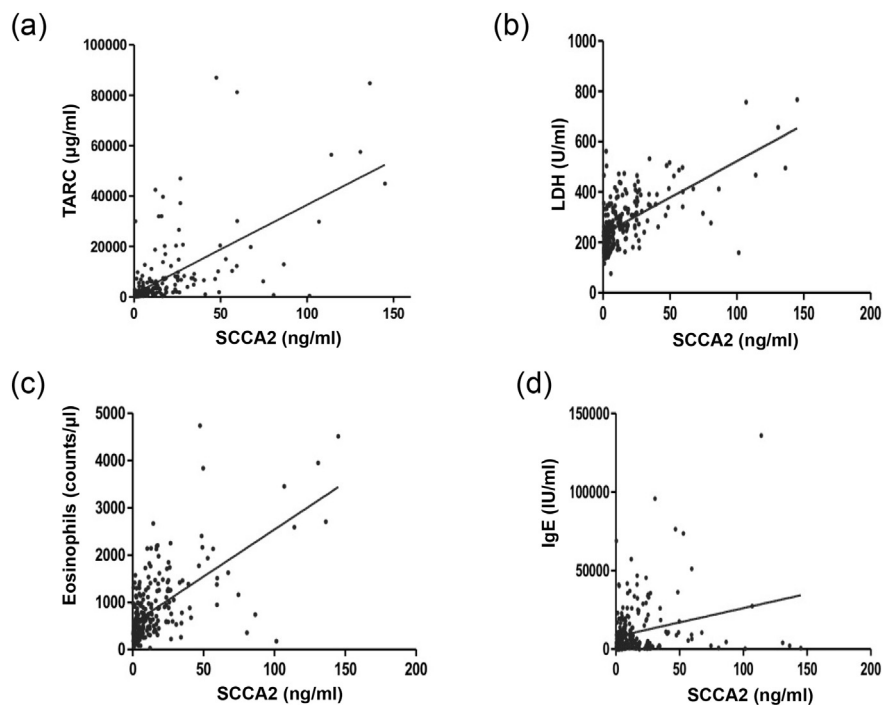


Fig. 5. Correlations between serum SCCA2 levels and laboratory parameters in AD patients. Correlations with serum TARC levels (a) ($n = 228$, $r = 0.673$, $p < 0.01$), blood LDH levels (b) ($n = 234$, $r = 0.614$, $p < 0.01$), eosinophil counts (c) ($n = 232$, $r = 0.557$, $p < 0.01$), and IgE levels (d) (SCCA2; $n = 235$, $r = 0.311$, $p < 0.01$) by Spearman's rank correlation test were shown.

widely, such as those with limb type, had lower levels of SCCA2. These findings indicate that serum SCCA2 levels in AD reflect the extent of lesional skin. In addition, AD with extensive lichenification demonstrated significantly elevated levels of SCCA2 as compared to those with less widespread lichenification.

Accordingly, the extent of chronic inflammation is likely to be better reflected by the level of SCCA2.

In prurigo type, the serum levels of SCCA2 were lower than in other types and had no correlation with the severity. Twenty of 116 patients with severe and very severe AD had low levels of serum

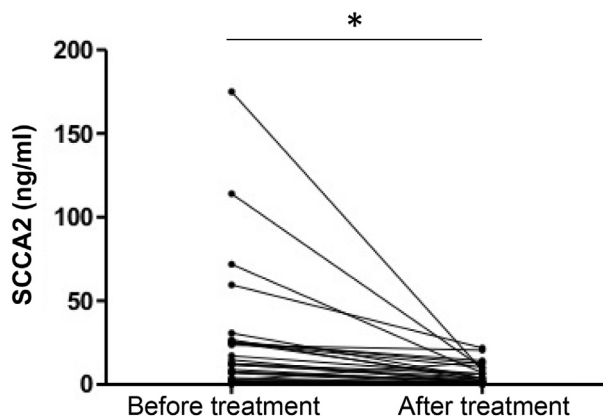


Fig. 6. Serial SCCA2 levels in AD patients. Serum SCCA levels were serially analyzed in 25 AD samples at baseline (before treatment) and at a follow-up visit after treatment. * $p < 0.01$.

SCCA2. In these patients, the ratio of the prurigo type was much higher than the ratio in patients with higher levels of serum SCCA2. Numerous factors are thought to affect the development of prurigo nodules. Not only type 1 response but also type 2 response is thought to be relevant, since activated STAT6, which is induced by IL-4 and IL-13, is observed in the epidermis.¹⁴ Increased density of dermal substance P-positive nerve fibers might be important for the formation of the prurigo lesion.¹⁵ This might explain why no correlation was noted between the severity of prurigo type AD and serum levels of SCCA2 in this study.

Furthermore we analyzed the effect of treatment on SCCA2. Serum SCCA2 was significantly decreased after treatment in the recovered patients. This result shows that SCCA2 can serve as an indicator of the effectiveness of treatment in AD.

Recently several studies including our own reports have suggested that serum SCCA expressions are up-regulated in psoriasis as well.^{16,17} We reported that SCCA2 might be also generated as a downstream molecule of a type 17 response in keratinocytes in psoriasis.¹⁷ Compared to that study, the median serum level of SCCA2 in AD patients of the present study was higher than that in psoriasis vulgaris patients. This suggests that type 2 cytokines may be a stronger inducer of SCCA2 compared to type 17 cytokines in keratinocytes. Additional work is required to clarify the mechanisms and roles of SCCA in both atopic dermatitis and psoriasis.

In a previous study, serum SCCA levels correlated with the severity of rhinitis due to *D. farinae* allergy. Conversely, the SCCA levels and severity of rhinitis due to cedar pollen allergy did not correlate.⁶ Our results confirm the absence of any association between specific IgE levels of cedar pollen and the serum levels of SCCA2 in AD patients. This might be due to the fact that SCCA proteins play some roles in the defense mechanisms against extrinsic proteases such as serine protease in *Dermatophagoides* but do not react against pollen proteins. This characteristic seems to be useful to assess dermatitis not due to pollen allergy in pollen rhinitis patients.

Some other markers are also available for AD such as LDH, eosinophil counts, IgE, and TARC. Prior research has shown that TARC level is closely related with severity of AD in adults.^{18,19} In our study, serum SCCA2 level was related with the severity, as well. In addition, SCCA2 level does not correlate cedar pollen allergy as mentioned above, although TARC level increase in these patients. Therefore, measuring serum levels of SCCA2 seems to be useful especially in AD patients with pollen allergy.

TARC is good marker for AD, but is affected by patient age in children.²⁰ A recent study of ours suggested that the serum levels of

periostin reflect the severity of AD.²¹ However, the serum levels of periostin may be markedly affected by the bone metabolism in childhood, as periostin is produced by osteocytes and periosteal osteoblasts.²² SCCA production is not affected by patient age. A previous study suggested that serum SCCA levels increased during the acute phase of an asthma exacerbation in children regardless of age.²³ Therefore, SCCA may also become a good marker for AD in children.

In conclusion, the levels of serum SCCA2 reflected the disease severity and clinical type of AD. Our findings indicate that serum SCCA2 may become a novel and useful indicator for evaluating disease activity and treatment efficacy.

Appendix A. Supplementary data

Supplementary data related to this article can be found at doi: [10.1016/j.alit.2017.06.016](https://doi.org/10.1016/j.alit.2017.06.016)

Authors' contributions

To designed the study, analyzed the data, and wrote the manuscript. KK, YI, MK, SM, and TK contributed to sample collection. JO, YA, SO, and NK performed experiments. KI, MA, and YY wrote the manuscript, supported through critical reading, comments and corrections of the manuscript.

Conflict of interest

KI received a research funding from Shino-Test Corporation. JO and YA are employees of Shino-Test Corporation. The rest of the authors have no conflict of interest.

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