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Increased Epidermal Filaggrin in Chronic Idiopathic Urticaria is Associated with Severity of Urticaria

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INTRODUCTION

Chronic idiopathic urticaria (CIU) is defined as the repeated occurrence of transient (≤ 24 hours) wheals and/or angioedema lasting for more than 6 weeks without an eliciting cause (1). Similar to atopic dermatitis (AD), CIU is characterized by immune aberrations, severe pruritus and unpredictable aggravations which lead to a substantial impact on quality of life (2-5). As the prevalence of CIU has been reported to reach 1.0% in the general population, the socioeconomic burden of this disease is remarkable (5). Recent guidelines (6) recommend to identify and to avoid underlying causes of CIU as the main goal for treatment. However, it is very difficult to find and eliminate the causes of CIU. Further understanding of the pathophysiology in CIU patients with severe and refractory to conventional treatments is still required.

Although the cutaneous manifestation of CIU, i.e. fleeting wheals is distinct from AD, some features are shared by these two common skin diseases. Severe pruritus, perivascular inflammatory infiltration and epidermal involvement are observed in both CIU and AD (7, 8). The epidermal barrier defects associated with filaggrin deficiency play a crucial role in the AD pathogenesis (9). Not only does genetic impairment lead to skin barrier protein abnormalities and immune dysregulation, but also continuous physical stimulation to the skin by itch and scratch vicious cycle can cause chronic inflammation in patients with AD (10). However, epidermal barrier defects in CIU have not been studied. The goal of this

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Author contributions
YMY wrote the first draft of the manuscript, designed experiments and performed experiments. BEK designed experiments, performed experiments, interpreted data and revised manuscript. YSS collected skin samples and measured skin pH. HSP collected samples and designed experiments. DYML designed experiments, interpreted data and revised manuscript.

study was to compare the expression of filaggrin in skin from CIU, AD and normal controls and to investigate whether altered filaggrin expression is associated with CIU severity.

METHODS

Subjects

Participants with CIU, AD and non-atopic normal controls, aged 20 to 70 years, were enrolled at Ajou University Hospital in Suwon, Korea and National Jewish Health in Denver, Colorado. Subjects included 14 Korean normal controls (mean age, 37.5±8.7 years) with no history of allergic and skin diseases, 16 Korean patients with CIU (mean age, 41.7±10.2 years; mean urticaria activity score (UAS) 12.1±2.9), and 11 patients with AD (8 Korean and 3 European American; mean age, 34.9±11.1 years) whose onset were after the age of 20. Table 1 shows clinical characteristics of the three study groups. None of the subjects had received systemic corticosteroids or immunomodulators including cyclosporine, methotrexate and anti-IgE previously, and none had received antihistamines or topical corticosteroid prior to enrollment in our study. The study was approved by the institutional review board at the Ajou University Medical Center and National Jewish Health. All subjects gave written informed consent before participation in the present study.

To investigate whether increased filaggrin expression can be associated with physiologic function of epidermis, transepidermal water loss (TEWL, Tewameter® TM300, Courage +Khazaka electronic GmbH, Germany) and skin surface pH (PH900®, Courage+Khazaka electronic GmbH, Germany) on the lesional skin of CIU and AD patients and normal controls were obtained. Two-millimeter punch biopsies were collected from wheals of CIU patients and eczematous AD lesions and uninvolved skin of the same patients with CIU and AD, and normal control skin. The skin biopsies were submerged immediately in either Tri-Reagent (Molecular Research Center, Inc, Cincinnati, OH) or 10% buffered formalin for real-time RT-PCR and immunohistochemical studies, respectively.

Quantitative real-time RT-PCR

Total RNA was isolated from 2-mm skin biopsy samples by chloroform: phenol extraction and isopropanol precipitation according to the manufacturer's guidelines (Molecular Research Center, Inc). RNeasy Mini Kits (Qiagen, Inc) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to purify RNA from skin biopsies further. One microgram of RNA was reverse-transcribed in a 20-mL reaction containing Random Primers (500 mg/mL; Invitrogen, Carlsbad, CA), dNTP (10 mmol/L; Invitrogen), 5X First Strand Buffer (Invitrogen), DTT (0.1 mol/L; Invitrogen), Superscript III enzyme (200 U/mL; Invitrogen) and RNase inhibitor (10 U/mL; Invitrogen). Real-time PCR was performed and analyzed by the dual-labeled fluorogenic probe method by using an ABI Prism 7300 sequence detector (Applied Biosystems). Primers and probes for human 18sRNA and filaggrin were purchased from Applied Biosystems. Amplification reactions were performed in MicroAmp optical tubes (Applied Biosystems) in a 25-mL volume as previously described (11). Relative expression levels were calculated by the relative standard curve method as outlined in the manufacturer's technical bulletin. A standard curve was generated using the fluorescent data from the 10-fold serial dilutions of total RNA of

the highest expression sample. This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in test samples were normalized to the corresponding 18sRNA levels in cultured keratinocytes and skin biopsies.

Filaggrin immunohistochemical staining

Paraffin-embedded tissues were cut at 5 mm and placed on frosted microscope slides. Slides were deparaffinized by using a series of xylene and ethanol washes and then stained using the Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN). And tissue sections were then blocked with serum blocking reagent G (R&D Systems) for 1 hour. Slides were stained overnight with mouse monoclonal antibodies against human filaggrin (Vector laboratories (Burlingame, CA) and Abcam (Cambridge, MA) at a 1:500 dilution. We also performed immunohistochemical staining for loricrin, which is another epidermal barrier protein, with mouse monoclonal antibodies (Abcam, Cambridge, MA) at a 1:500 dilution. The secondary antibody was added according to the manufacturer's protocol (R&D Systems). Antibody specificity was confirmed by using purified nonimmune mouse IgG (Southern Biotechnology, Birmingham, AL). All slides were coded before analysis and read blindly to ensure patient anonymity. The intensity of the immunostaining was estimated with the use of microscopy with SlideBook 5.0 (Intelligent Imaging Innovations, Santa Monica, CA).

Statistical analysis

Descriptive statistics are presented to characterize all subjects included in the analysis. Categorical data are presented as enumerations and percentages. Continuous data are presented as arithmetic mean ± standard deviation or as median (25^{th} percentile, 75^{th} percentile) if the distribution of the data is skewed.

Statistical analysis of gene expression and immunohistochemical staining was conducted by using Graph Pad Prism, version 4.03 (San Diego, CA). Statistical differences between groups were determined by using an unpaired t test with significant differences conferred when $P<0.05$. In cases in which multiple groups were compared with a control, data were analyzed by a 1-way ANOVA, and significant differences were determined by a Dunnett's T3 test.

RESULTS

Filaggrin expression is increased in CIU skin

We compared *FLG* gene expression among three groups using real-time RT-PCR. As illustrated in Figure 1a, relative *FLG* expression was significantly increased in lesional CIU as compared to non-lesional CIU skin ($P<0.01$) with a significant correlation between lesional and non-lesinal *FLG* expression (Spearman's rho = 0.829, $P<0.001$). In comparison with AD lesions, *FLG* gene expression was significantly increased in lesional skin from CIU patients ($P<0.01$). In contrast, *FLG* gene expression was significantly decreased in both lesional and non-lesional skin from AD patients as compared with normal controls ($P<0.01$,$P<0.01$, respectively). The *FLG* expression in lesional CIU skin could be classified as two groups (Figure 1a); one group with high *FLG* expression (greater than the mean plus

2 standard deviation of *FLG* gene expression in normal controls) and one group with normal *FLG* expression. The high *FLG* gene expression group had significantly higher UAS as compared to normal *FLG* gene expression group (13.9 ± 0.90 vs 11.4 ± 3.0, $P<0.05$). In addition, the patients with the higher *FLG* gene expression in non-lesional skin also had higher UAS as compared to normal *FLG* gene expression group (14.25 ± 1.0 vs 11.42 ± 2.8, $P = 0.042$).

Next, filaggrin protein expression was analyzed in skin biopsies from normal controls, CIU and AD patients using an immunostaining technique with an antibody directed to filaggrin protein. A significant increase of filaggrin protein expression was observed in lesional CIU skin, without increased expression in non-lesional skin (Figure 1b). The composite data for filaggrin immunostaining in all samples are shown in Figure 1c. The staining intensity of filaggrin was significantly increased in lesional skin from CIU patients as compared to skin from normal controls ($P<0.01$) and lesional AD ($P<0.001$). Filaggrin expression was decreased in both lesional and non-lesional skin of AD patients (Figure 1c). The staining intensities of filaggrin in lesional ($P<0.001$) and non-lesional ($P<0.001$) skin from AD patients were significantly decreased as compared with normal controls.

Filaggrin expression is significantly correlated with clinical urticaria activity

To examine whether filaggrin expression is associated with UAS, we measured UAS in patients with CIU and investigated correlations between filaggrin expression and UAS. There was a significant positive correlation between UAS and filaggrin staining intensity of lesional CIU skin (correlation coefficient= 0.538, $P<0.05$, Figure 2).

Epidermal physiologic function is enhanced in CIU compared to AD

It has been reported that the deficiency of filaggrin and its acid metabolites results in decreased epidermal hydration and increased TEWL as well as skin pH of stratum corneum (12). Therefore, to investigate whether increased filaggrin expression has an influence on the physiologic function of CIU epidermis, we measured TEWL and pH in the skin from CIU and AD patients. As shown in figure 3a, TEWL was significantly increased in lesional skin of AD patients compared to both skin from normal controls ($P<0.01$) and lesional skin from CIU patients ($P<0.01$). No significant difference was noted in TEWL between normal controls and CIU patients. To investigate whether an increase of filaggrin leads to decreased skin pH, we measured skin pH in normal controls, CIU and AD patients. The mean skin pH was significantly decreased in lesional skin from CIU patients compared to skin from normal controls and AD patients ($P<0.01$, $P<0.001$, Figure 3b).

DISCUSSION

The current study demonstrates, for the first time, that filaggrin expression is increased in lesional CIU skin at both the protein and RNA level. To confirm the validity of our data, we performed immunostaining for filaggrin with two different monoclonal antibodies from Vector Inc. and Abcam Inc. (data not shown), and found that the results were the same. The *FLG* expression in both lesional and non-lesional CIU skin was classified as two groups (high and normal), and UAS was higher in high *FLG* groups. Interestingly, we also found

that protein level of filaggrin was positively correlated with urticaria severity, suggesting that filaggrin may play a role in driving CIU severity. Additionally, we found that TEWL is increased in AD patients compared with normal controls, and there is no difference in TEWL between CIU patients and normal controls.

Filaggrin and its precursor profilaggrin are essential structural proteins in the epidermis (12). It has been well known that they play multiple roles in skin homeostasis and barrier formation against water loss as well as penetration of allergens and microbes (12). Profilaggrin has a unique structure containing 10-12 filaggrin repeats. It is dephosphorylated and proteolytically processed to release monomeric filaggrin during terminal differentiation of the epidermis. Then filaggrin, as a histidine-rich protein, is deamidated and finally degraded to its component amino acids, including histidine and glutamine. Subsequently histidine and glutamine are metabolized to *trans*-rocanic acid (*tran*-UCA) and pyrrolidone-5-carboxylic acid (PCA), respectively. These organic acids, UCA and PCA serve as “natural moisturizing factor (NMF)” which plays an important role maintain epidermal hydration and acidic environment (13). In addition, filaggrin deficiency causes high skin pH in AD patients (14). Therefore, we measured pH in the lesional skin from patients with AD and CIU and compared it with normal controls to examine whether increased filaggrin expression is related to skin pH in CIU patients. Importantly, we found that the skin pH was significantly higher in AD patients and lower in CIU patients compared to normal controls. This decreased skin pH in CIU lesions supports a physiologic role for increased filaggrin expression resulting from an increase of filaggrin breakdown products. Additionally, it may account for reduced staphylococcal colonization in CIU patients as compared to AD patients (15) despite frequent excoriation in both of these allergic skin diseases.

There have been previous reports evidence that *cis*-UCA is involved in cutaneous inflammation. (16-18). It has been reported that *cis*-UCA is a potent inducer of prostaglandin (PG) E2 and cytokines, such as tumor necrosis factor (TNF)-α, IL-6, and IL-8 (19). Furthermore, *cis*-UCA has a synergistic effect with histamine on PGE2 production from human keratinocytes (20). As PGE2 has been shown to induce an EP3 receptor-dependent mast cell degranulation and to increase microvascular permeability and infiltration of inflammatory cells into tissues, an increase of PGE2 may contribute to wheal reaction. It has also been reported that *cis*-UCA stimulates mast cell degranulation directly as well as indirectly by inducing neuropeptide release from peripheral sensory nerves in skin (21). On the other hand, histidine, the major component amino acid of filaggrin, is a substrate of mast cell histidine decarboxylase (HDC) producing histamine. HDC has been reported to be induced by various stimuli, not only in mast cells and basophils, but also in neutrophils, T lymphocytes, macrophages and keratinocytes (22, 23). As HDC has been observed to be increased in CIU skin (24), greater histidine release may result in the more histamine production in wheals from CIU patients. We also confirmed a significant increase of HDC expression at the levels of both protein ($P<0.05$ for normal and AD) and mRNA ($P<0.05$ for normal, $P<0.01$ for AD) in the lesional skin from CIU as compared to normal controls and AD skins (data not shown).

Taken together, we speculate that increased expression of filaggrin can result in more *cis*-UCA and histamine production, which increases the disease activity of CIU patients. Even though filaggrin is a structural protein, it turns over quickly and takes only 6 hours to be fully degraded in the skin (25). Therefore, a transient increase of filaggrin in CIU patients seems to be well correlated with temporary and migrating characteristics of wheals, which don't last for more than 24 hours at the same skin region of CIU patients (1).

However, the present study has not answered the question why filaggrin expression is increased in wheals from CIU patients yet. These results obtained from a small number of CIU samples will require further replication and studies to validate their mechanisms and clinical relevance through further studies. We just observed that there was an accelerated skin differentiation with upregulation of epidermal differentiation markers, such as filaggrin and loricrin, in the wheals from CIU patients. Evans *et al.*(26) reported that a positive correlation between extracellular calcium and endogenous PGE2 synthesis during the differentiation of keratinocytes. As calcium is a strong inducer of filaggrin production, an increase of extracellular calcium following PGE2 generation stimulated by *cis*-UCA can enhance filaggrin expression in CIU skin.

Histamine, a major inflammatory mediator in the pathogenesis of CIU, has been found to have an influence on keratinocyte differentiation (27-30). Treating keratinocytes with a lower concentration of histamine (0.1 μM – 0.1mM) enhances expression of human beta-defensin D2 and D3, which are late differentiation markers and inhibits proliferation through an increased cAMP and subsequent activation of H2 receptor which leads to calcium increase and keratinocyte differentiation (27, 29, 30). However, incubating keratinocytes with a higher concentration of histamine (10 μM – 1mM) reduces skin barrier formation via activation of H1 receptor (28). In addition, a previous study noted that even with high concentrations of histamine, the expression of differentiation markers is not influenced (28).

Taken together, our study suggests future directions to study the potential role of filaggrin in CIU as shown in Fig 4. Filaggrin expression may also be a novel biomarker for CIU severity. The altered filaggrin expression has physiologic effects on TEWL and pH in skin of CIU patients and indicates enhanced barrier function compared to AD patients. Considering that filaggrin breakdown products may contribute to increased urticaria severity, modulating filaggrin expression should be investigated as an alternative treatment modality for severe CIU patients.

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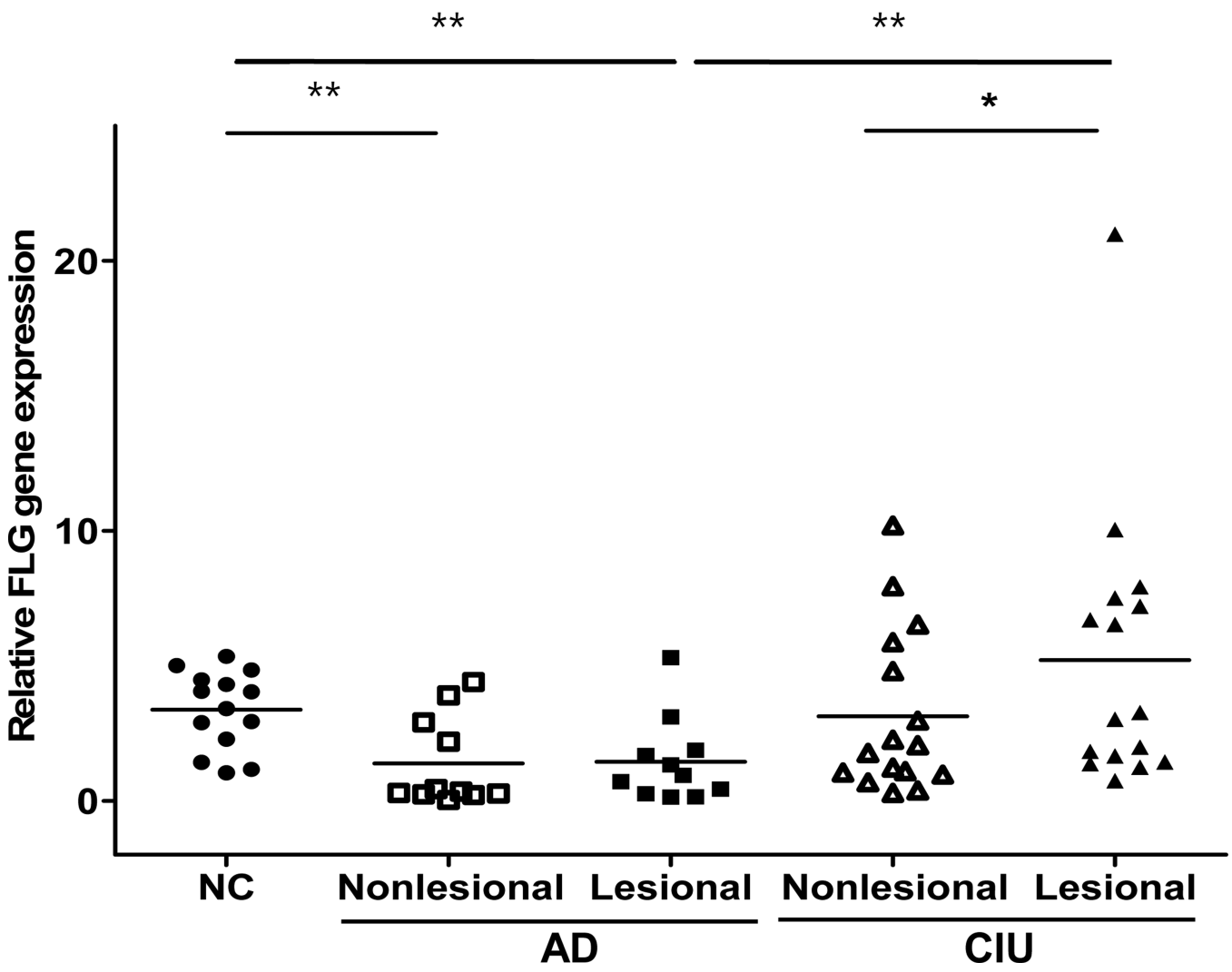

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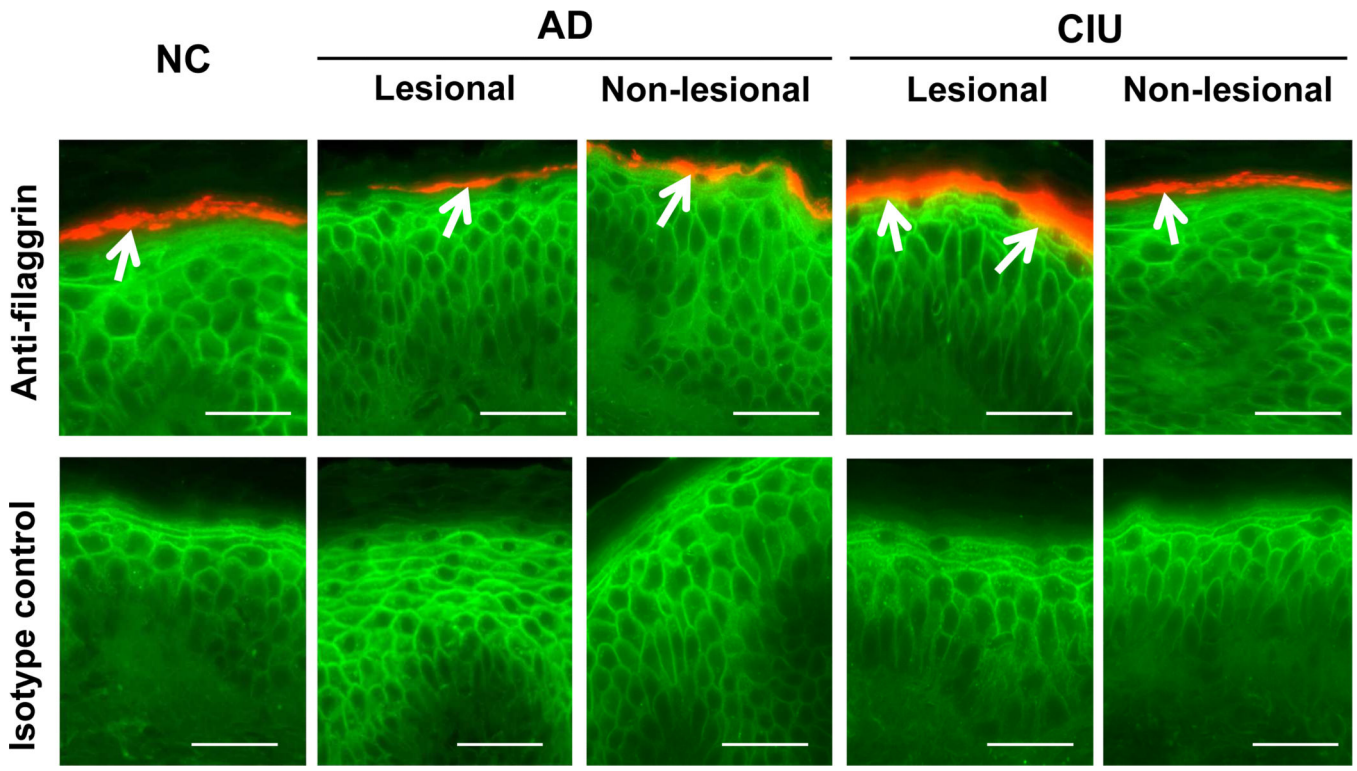


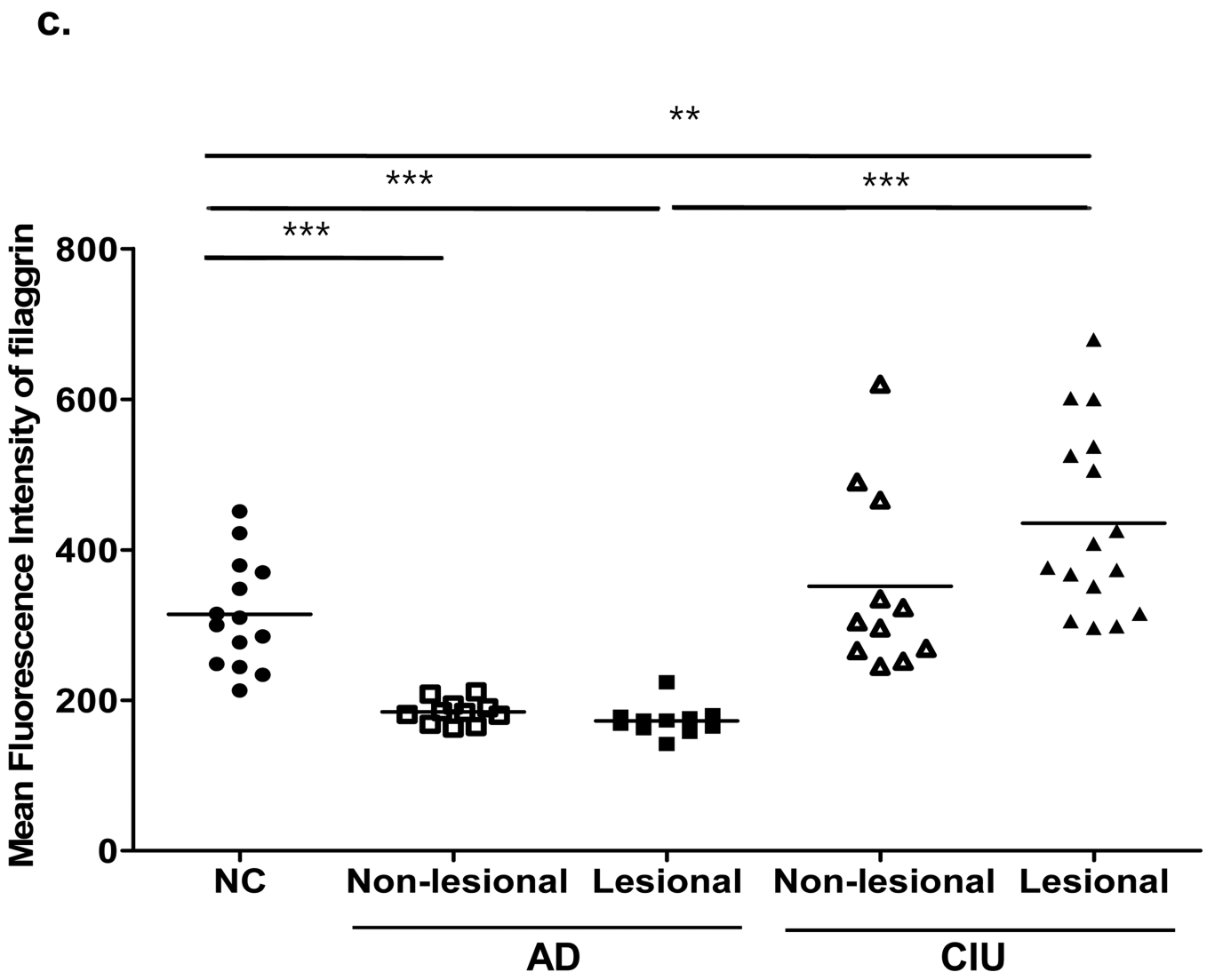


Figure 1. The expression of filaggrin in human skin

(a) The gene expression of filaggrin was evaluated by real-time RT-PCR. Relative filaggrin expression was calculated using 18s ribosomal RNA as a reference gene. (b) Representative paraffin embedded skin biopsies from normal controls and both lesions and non-lesions of patients with CIU and AD for filaggrin (red) are shown. Wheat germ agglutinin-conjugated fluorescein isothiocynate (green) stained the cytoskeleton. Images were collected at x 400 magnification. Arrows point to filaggrin expression. Bar = 50μm. (c) The mean fluorescent intensity of filaggrin is shown. Normal controls (NC, n=14) and both lesions (n=16) and nonlesions (n=11) of patients with CIU and AD (n=11). *P <0.05, **P <0.01.

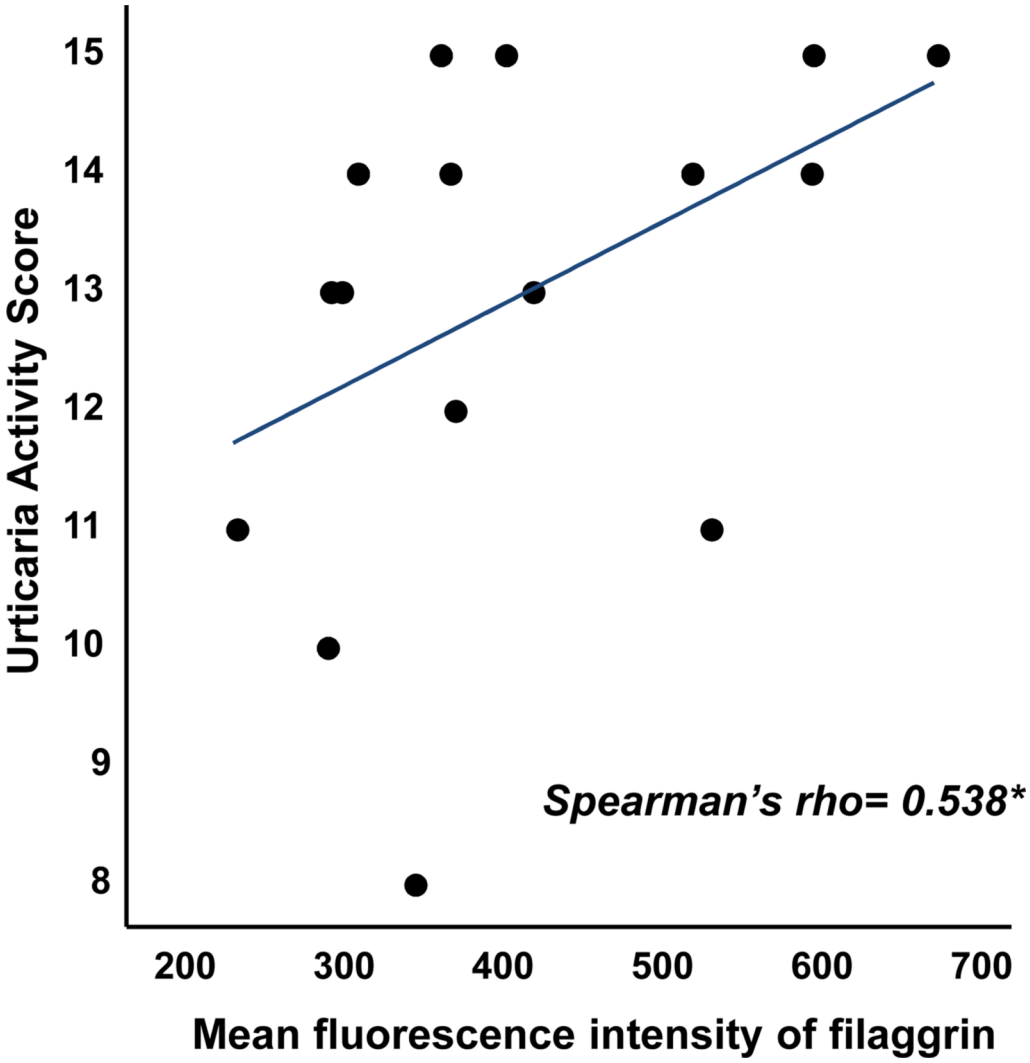
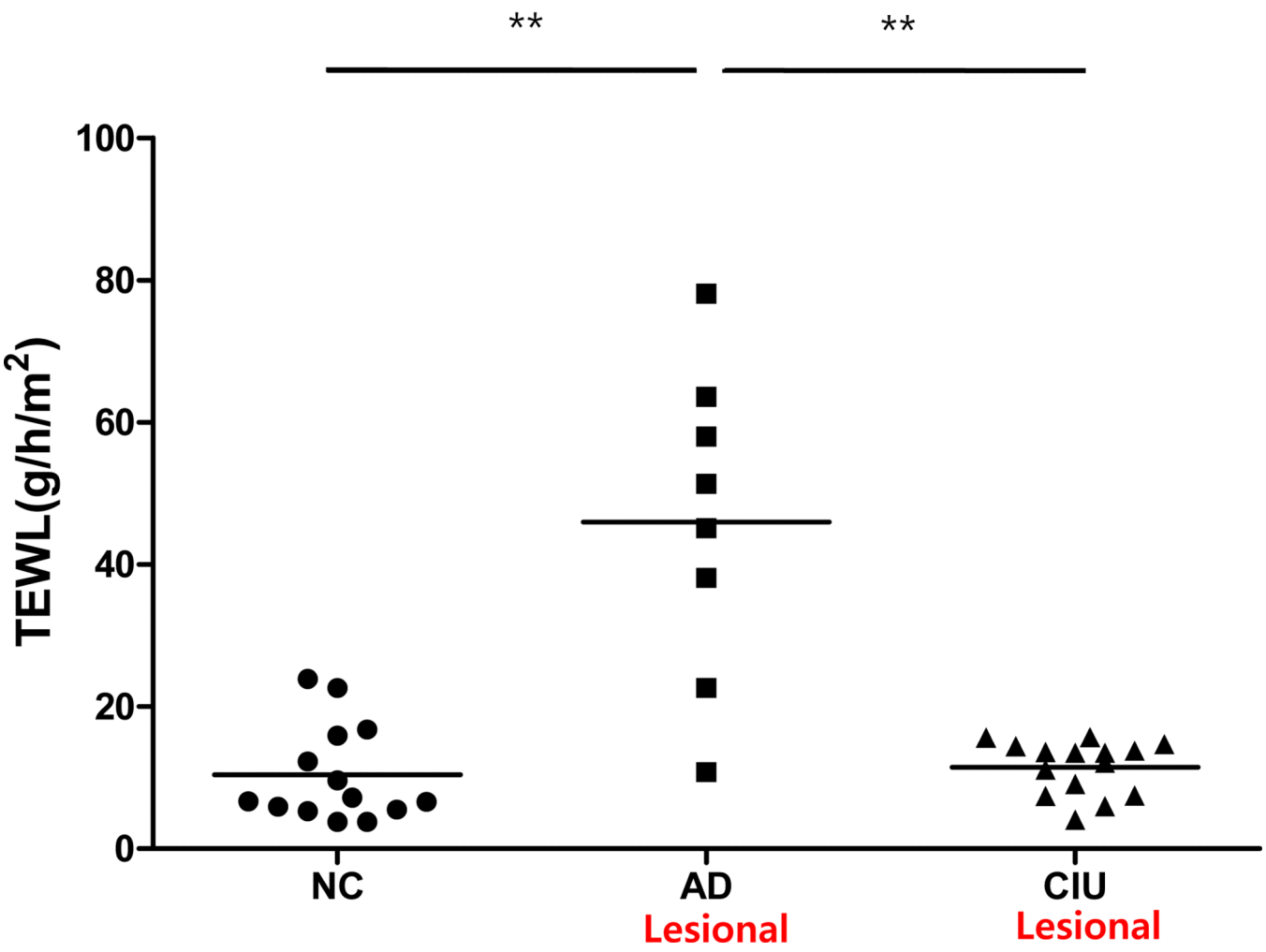


Figure 2. Correlation between filaggrin protein expression and urticarial activity

Filaggrin protein expression (Mean fluorescence intensity of filaggrin) in lesional skin from CIU patients was significantly correlated with urticaria activity score (0-15), which composed of the quantity, distribution range, mean diameter and duration of wheal and intensity of pruritus within the previous week of outpatient clinic visits.

a.



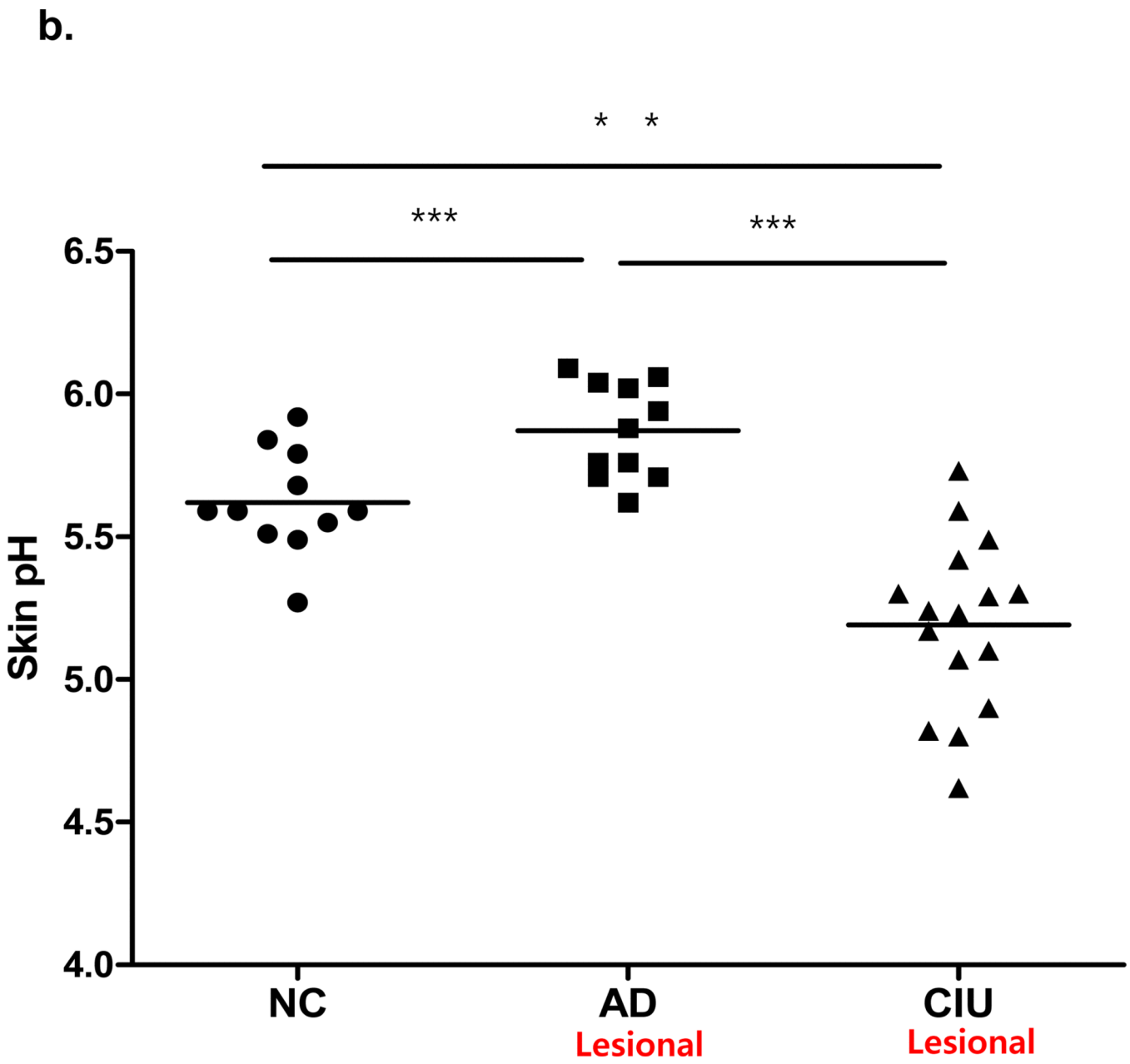


Figure 3. Epidermal physiologic function in CIU

Epidermal barrier function was evaluated by transepidermal water loss (TEWL) and skin surface pH in skin from normal controls (NC) and patients with CIU and AD. *P <0.05, **P <0.01, and ***P <0.001. (a) TEWL was significantly increased in lesional AD compared to both normal and lesional CIU skin (P= 0.008, P=0.006, Dunnett's T3 test). (b) The mean skin pH was significantly decreased in CIU lesions compared to normal and AD lesions (P= 0.007, P<0.001, Dunnett's T3 test, Figure 3b).

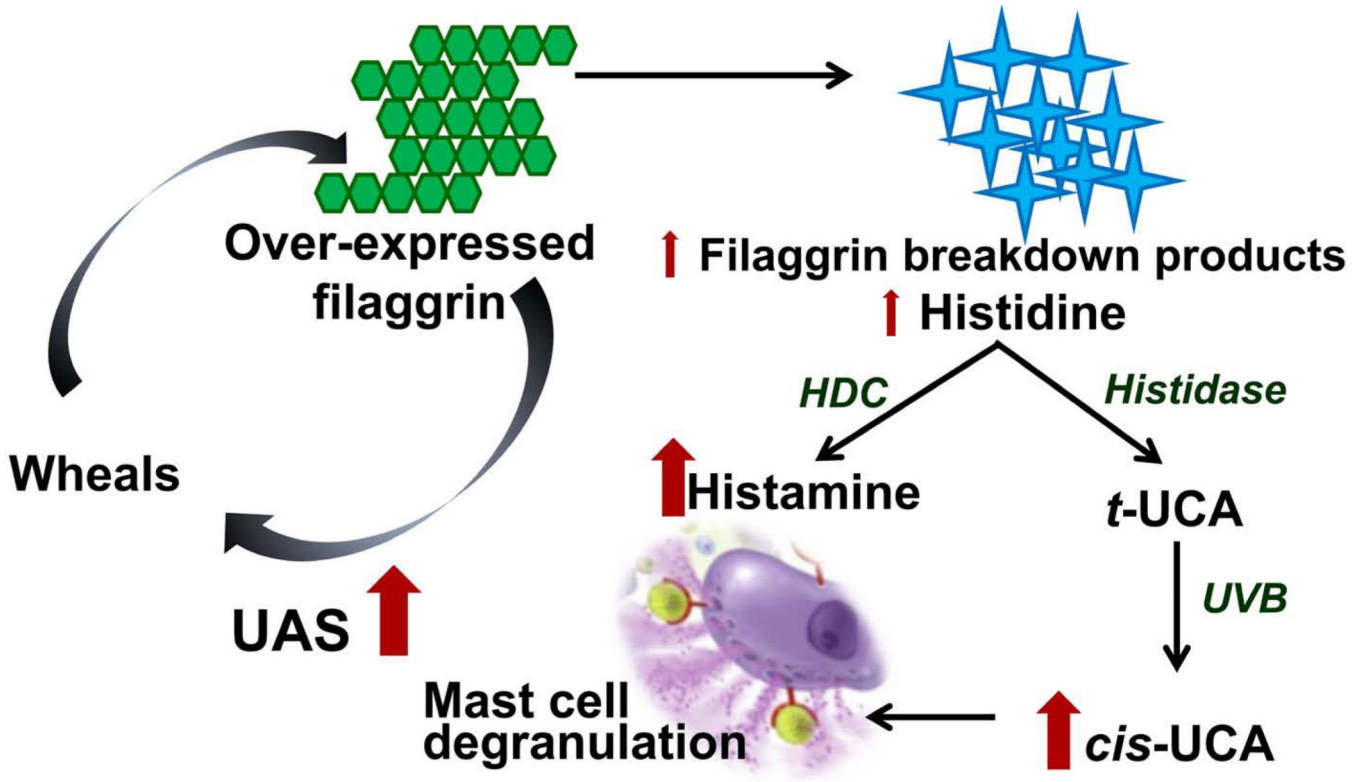


Figure 4. The potential role of filaggrin in CIU

Data in the present study demonstrate that filaggrin is overexpressed in the wheals from CIU patients. This may be the result of enhanced terminal differentiation of the epidermis induced by immune activation or mediator release. Histamine may increase filaggrin metabolism and filaggrin breakdown products to increase CIU severity. We are pursuing possibilities that an increase of histidine results in histamine overproduction mediated by histidine decarboxylase (HDC) and increased *cis*-urocanic acid (UCA) leads to mast cell degranulation. Filaggrin breakdown products therefore contribute to increase urticarial activity score.

Table 1

Clinical characteristics of study groups

	NC n=14	AD n=11	CIU n=16
Age (years)*	37.5±8.7	34.9±11.1	41.7±10.2
Women	12 (85.7%)	7 (63.6%)	9 (56.3%)
Skin Prick Test No. of positive allergens*Ť	0	5.6±4.7	1.2±1.8
Serum total IgE (KU/L)**	14.8 (6.6-99.3)	192.3 (19-3.829)	138.0 (26-1.188)
Urticaria Activity Score (0-15)	na	na	12.1±2.9

NC normal controls; AD atopic dermatitis; CIU chronic idiopathic urticaria.

*Values given are the mean±S.D.

**Values given are the median (min-max).

Ť*P*=0.014 for AD vs. CIU (Mann-Whitney U test).