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*Molecular Pathogenesis of Genetic and Inherited Diseases*

# Flaky Tail Mouse Denotes Human Atopic Dermatitis in the Steady State and by Topical Application with *Dermatophagoides pteronyssinus* Extract

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**The barrier abnormality, a loss-of-function mutation in the gene encoding filaggrin (*FLG*), which is linked to the incidence of atopic dermatitis (AD), is a recently discovered but important factor in the pathogenesis of AD. Flaky tail (*Flg$^{ft}$*) mice, essentially deficient in filaggrin, have been used to investigate the role of filaggrin on AD. However, the relevancy of *Flg$^{ft}$* mice to human AD needs to be determined further. In this study, we observed the clinical manifestations of *Flg$^{ft}$* mice in the steady state and their cutaneous immune responses against external stimuli, favoring human AD. Under specific pathogen-free conditions, the majority of *Flg$^{ft}$* mice developed clinical and histological eczematous skin lesions similar to human AD with outside-to-inside skin barrier dysfunction evaluated by newly devised methods. In addition, cutaneous hapten-induced contact hypersensitivity as a model of acquired immune response and a mite extract-induced dermatitis model physiologically relevant to a human AD were enhanced in *Flg$^{ft}$* mice. These results suggest that the *Flg$^{ft}$* mouse genotype has potential as an animal model of AD corresponding with filaggrin mutation in human AD.** *(Am J Pathol 2010, 176:2385–2393; DOI: 10.2353/ajpath.2010.090957)*

Atopic dermatitis (AD), which affects at least 15% of children in developed countries, is characterized by eczematous skin lesions, dry skin, and pruritus.[1–3] Although the precise pathogenic mechanism of AD is as yet unknown, several accumulated lines of evidence suggest that a defective skin barrier to environmental stimuli may contribute to its pathogenesis. It has long been thought that the barrier abnormality in AD is not merely an epiphenomenon but rather is the "driver" of disease activity.[4] The evidence for a primary structural abnormality of the stratum corneum in AD is derived from a recently discovered link between the incidence of AD and loss-of-function mutations in the gene encoding filaggrin (*FLG*). Individuals carrying the *FLG* null allele variants tend to develop AD.[5–7]

Filaggrin protein is localized in the granular layers of the epidermis. Profilaggrin, a 400-kDa polyprotein, is the main component of keratohyalin granules.[8–10] In the differentiation of keratinocytes, profilaggrin is dephosphorylated and cleaved into 10 to 12 essentially identical 27-kDa filaggrin molecules, which aggregate in the keratin cytoskeleton system to form a dense protein-lipid matrix.[10] This structure is thought to prevent epidermal water loss and impede the entry of external stimuli, such as allergens, toxic chemicals, and infectious organisms. Therefore, filaggrin is a key protein in the terminal differentiation of the epidermis and in skin barrier function.[11]

Because AD is a common disease for which satisfactory therapies have not yet been established, understanding the mechanism of AD through animal models is an essential issue.[1,12] Flaky tail (*Flg$^{ft}$*) mice, first introduced in 1958, are spontaneously mutated mice with

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abnormally small ears, tail constriction, and a flaky appearance of the tail skin, which is most evident between 5 and 14 days of age.[13] Mice of the *Flg*$^{ft}$ genotype express an abnormal profilaggrin polypeptide that does not form normal keratohyalin F granules and is not proteolytically processed to filaggrin. Therefore, filaggrin is absent from the cornified layers in the epidermis of the *Flg*$^{ft}$ mouse.[14–16]

Recently, it has been revealed that the gene responsible for the characteristic phenotype of *Flg*$^{ft}$ mice is a nonsense mutation of 1-bp deletion analogous to a common human *FLG* mutation.[15] These mice developed eczematous skin lesions after age 28 weeks under specific pathogen-free (SPF) conditions[17] and enhanced penetration of tracer perfusion determined by ultrastructural visualization,[16] and were predisposed to develop an allergen-specific immune response after epicutaneous sensitization with the foreign allergen ovalbumin (OVA).[15,17] On the other hand, general immunity through intraperitoneal sensitization with OVA was comparable between *Flg*$^{ft}$ mice and control mice.[15,17]

Despite these recent advances, there still remain several issues with *Flg*$^{ft}$ mice to be addressed. For example, serial close observation of clinical manifestations in reference to human AD will be informative. It is of value to evaluate the responses to external stimuli relevant to human AD, such as mite extracts, instead of OVA that has been used previously. A comparative study on the skin-mediated contact hypersensitivity (CHS) response and non-skin-mediated delayed-type hypersensitivity response is important to evaluate the impact of barrier dysfunction on immune responses *in vivo*. In addition, although it has now been determined that the barrier dysfunction is a key element in the establishment of AD, there is no established method to evaluate the outside-to-inside barrier function quantitatively.

In this study, we found that *Flg*$^{ft}$ mice showed spontaneous dermatitis with skin lesions mimicking human AD in a steady state under SPF conditions: serial occurrence of manifestations as scaling, erythema, pruritus, and erosion followed by edema in this order. We also successfully evaluated outside-to-inside barrier dysfunction in *Flg*$^{ft}$ mice quantitatively using a newly developed method. In addition, we determined that the Th1/Tc1-mediated immune response was enhanced by immunization through skin but not through non-skin immunization. Last, we induced severe AD-like skin lesions in *Flg*$^{ft}$ mice by application of mites as a physiologically relevant antigen for human AD, which will be an applicable animal model of AD.

## Materials and Methods

### Mice

C57BL/6NCrSlc (B6) mice were purchased from SLC (Shizuoka, Japan). Flaky tail (STOCK *a/a ma ft/ma ft*/J; *Flg*$^{ft}$ mice) mice have double-homozygous filaggrin (*Flg*) and matted (*ma*) mutations.[13,14] We used B6 mice as a control of *Flg*$^{ft}$ mice because *Flg*$^{ft}$ mice were described to be outcrossed onto B6 mice at The Jackson Laboratory (Bar Harbor, ME)[13,14] (of note, although the strain was crossed with B6, it is not a B6 congenic strain but rather a hybrid stock that is probably semi-inbred). Female mice were used in all experiments unless otherwise stated; they were maintained on a 12-hour light/dark cycle at a temperature of 24°C and at a humidity of 50 + 10% under SPF conditions at Kyoto University Graduate School of Medicine. Routine colony surveillance and diagnostic workup verified that mice were free of Ectromelia virus, lymphocytic choriomeningitis virus, mouse hepatitis virus, Sendai virus, *Mycoplasma pulmonis*, cilia-associated respiratory bacillus, *Citrobacter rodentium* [*Escherichia coli* O115a,c:K(B)], *Clostridium piliforme* (Tyzzer's organism), *Corynebacterium kutscheri*, *Helicobacter hepaticus*, *Pasteurella pneumotropica*, *Salmonella* spp., parasites, intestinal protozoans, *Enterobius*, and ectoparasites. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

### Clinical Observation and Histology

The clinical severity of skin lesions was scored according to the macroscopic diagnostic criteria that were used for the NC/Nga mouse.[18] In brief, the total clinical score for skin lesions was designated as the sum of individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe), for the symptoms of pruritus, erythema, edema, erosion, and scaling. Pruritus was observed clinically for more than 2 minutes.

For the histological portion of the study, the dorsal skin of mice was stained with H&E. Toluidine blue staining was used to detect mast cells, and the number of mast cells was calculated as the average from five different fields of each sample ×40 magnification).

### Flow Cytometric Analysis and Quantitative RT-PCR

Cells from the skin-draining axillary and inguinal lymph nodes (LNs) and from the spleen were analyzed with flow cytometry. Fluorescent-labeled anti-CD4 and anti-CD8 antibodies were obtained from eBioscience (San Diego, CA) and used to stain cells. The total number of cells per organ and the number of cells in each subset were calculated through flow cytometry using the FACSCanto II system (Becton Dickinson, San Diego, CA). Quantitative RT-PCR was performed as described previously, using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a control.[19]

### Total and Mite-Specific Serum IgE

Total serum IgE levels were measured with a mouse IgE ELISA Kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocols. For the measurement of mite-specific IgE levels, the same type of mouse IgE ELISA Kit was used with slightly modifications. Specifi-

cally, plates were coated and incubated with 10 μg/ml *Dermatophagoides pteronyssinus* (Dp) (Biostir, Kobe, Japan) diluted with coating buffer for 60 minutes. After a blocking period of 30 minutes, 100 μl of 5× diluted serum was added into each well and incubated for 2 hours. Anti-mouse IgE-horseradish peroxidase conjugate (1:15,000; 100 μL) was used to conjugate the antigen-antibody complex for 60 minutes at room temperature; from this point on the ELISA Kit was used according to the manufacturer's protocol. Absorbance was measured at 450 nm. The difference between the sample absorbance and the mean of negative control absorbance was taken as the result.

### Skin Barrier Function

The dorsal regions of the skin were shaved in all mice before measurement. To evaluate inside-to-outside barrier function, transepidermal water loss (TEWL) was measured with a Tewameter Vapo Scan (Asahi Biomed, Tokyo, Japan) at 24°C and 46% relative humidity.

Outside-to-inside barrier function was assessed by means of fluorescein isothiocyanate isomer I (FITC) (Sigma-Aldrich, St. Louis, MO). The shaved dorsal skin of mice was treated with 100 μl of 1% FITC diluted in acetone and dibutyl phthalate (1:4); 3 hours later, this area was tape-stripped (Scotch tape, 3M, St. Paul, MN) nine times to remove the stratum corneum containing the remnant of FITC. The painted area (1.2 cm × 1.2 cm) was removed, and FITC concentration was measured. Each skin sample was soaked in PBS at 60°C for 10 seconds, after which the dermis and epidermis were separated. The epidermis was soaked in 500 μl of PBS, homogenized, and spun down at 2200 × *g*. The supernatant was collected, and fluorescence was measured at an excitation wavelength of 535 nm and an emission wavelength of 460 nm using an Arvo SX 1420 counter (Wallac, PerkinElmer, Waltham, MA). The fluorescence value was compared with a standard curve using FITC serial dilutions.

For the evaluation of fluorescence intensities of FITC penetrated into the epidermis, a 1 × 1 cm skin sample was taken after tape stripping, and a 10-μm Tissue-Tek (Sakura Finetek, Tokyo, Japan)-embedded section was analyzed using a BZ-9000 Biorevo digital microscope (Keyence, Osaka, Japan) at the same time exposure.

An *in situ* dye permeability assay with toluidine blue was performed using embryos at 18 days (littermates). Unfixed, untreated embryos were dehydrated by a 1-minute incubation in an ascending series of methanol (25, 50, 74, and 100%) and rehydrated with the descending same methanol series, washed in PBS, and stained with 0.01% toluidine blue.

### Scratching Behavior

Scratching behavior was measured in detail using the Sclaba Real system (Noveltec, Kobe, Japan). Mice were put into the machine 20 minutes before measurement to allow them to adapt to the new environment. Ointment was then applied, and the number and duration of scratching sessions were counted according to the manufacturer's protocol for 15 minutes.[20]

### Dermatitis Models

For the assessment of irritant contact dermatitis, 20 μl of 0.2 mg/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) was applied to both sides of the ears. Ear thickness change was measured at 1, 3, 12, and 24 hours as well as 5 days after application.

To induce a CHS response, 25 μl of 0.5% 1-fluoro-2.4-dinitrobenzene (DNFB) (Nacalai Tesque, Kyoto, Japan) was painted on the shaved abdomens of mice for sensitization. Five days later, the ears were challenged with 20 μl of 0.2% DNFB, and ear thickness change was measured at 24 and 48 hours after application. Nonsensitized mice were used as a control. A delayed-type hypersensitivity response model was established using OVA (Sigma-Aldrich). Mice were sensitized with 200 μl of 0.5 mg/ml of OVA in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) intraperitoneally and challenged 5 days later with an injection of 20 μl of 1 mg/ml of OVA in incomplete Freund's adjuvant (Difco Laboratories) into the hind footpads. Footpad thickness was measured before and 24 hours after challenge. Nonsensitized mice were used as a control. Footpad swelling was calculated by (footpad thickness change of sensitized mice) − (footpad thickness change of nonsensitized mice). To induce murine AD-like skin lesions, 40 mg of 0.5% Dp in white petrolatum was topically applied to the ears and upper back twice a week for 8 weeks. Petrolatum without Dp was used as a control. One gram of Dp body product (Biostir) contained 1.78 mg of total protein with 2.47 μg of Dp protein (Der p1). Ear thickness and clinical scores were measured every week. Mite-specific IgE levels, TEWL, and histological appearance of eczematous skin were observed 12 hours after the final application.

### Statistical Analysis

Data were analyzed using an unpaired two-tailed *t*-test. $P < 0.05$ was considered to be significant.

## Results

### Spontaneous Dermatitis of $Flg^{ft}$ Mice in the Steady State under SPF Conditions

As described previously,[14,15] the expression of the filaggrin monomer was barely detectable by Western blotting in the dorsal skin of $Flg^{ft}$ mice compared with that of B6 mice (data not shown). Here, we investigated the clinical manifestations seen in the skin of $Flg^{ft}$ mice raised in a steady state under SPF conditions and found that $Flg^{ft}$ mice developed spontaneous dermatitis (Figure 1A). The clinical severities of skin lesions, including pruritic activity, erythema, edema, erosion, and scaling, were scored. The total clinical scores of $Flg^{ft}$ mice increased with age

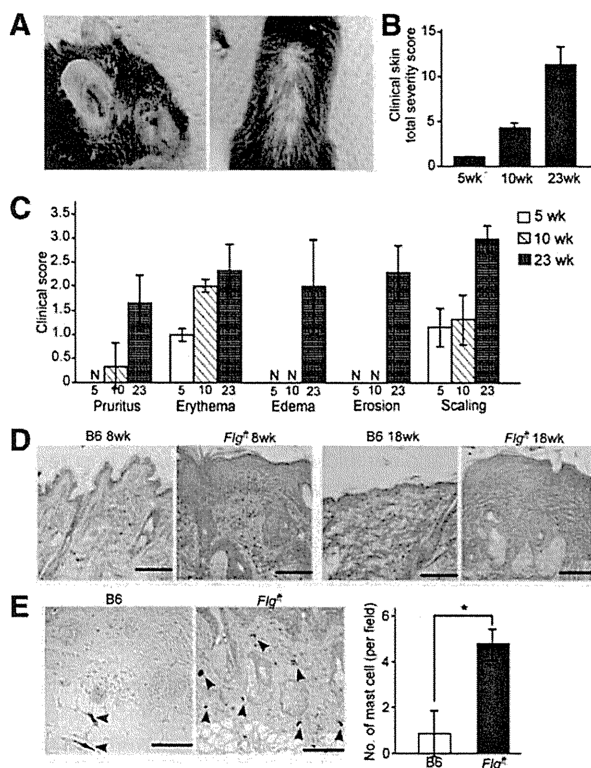

**Figure 1.** Spontaneous dermatitis in $Flg^{ft}$ mice in SPF. **A:** Clinical photographs of 20-week-old $Flg^{ft}$ mice. Total clinical severity scores (**B**) for each particular item (**C**) in 5-, 10- and 23-week-old $Flg^{ft}$ mice. N, none. **D:** H&E-stained sections in 8- and 18-week-old mice. Scale bar = 100 μm. **E:** Toluidine blue staining of the skin from 8-week-old B6 and $Flg^{ft}$ mice and the numbers of mast cells (**arrowheads**) per field are shown. $^*P < 0.05$.

(Figure 1B). The first manifestations to appear when mice were young were erythema and fine scaling; pruritic activity, erosion, and edema followed later (Figure 1C). In contrast, no cutaneous manifestation was observed in either B6 mice, studied as a control, or heterozygous mice intercrossed with *Flg*^ft^ and B6 mice kept under SPF conditions throughout the experimental period (data not shown). In addition, there was no apparent difference in terms of clinical manifestations between the genders of $Flg^{ft}$ mice throughout the period (data not shown).

Histological examination of skin from $Flg^{ft}$ mice revealed epidermal acanthosis, increased lymphocyte infiltration, and dense fibrous bundles in the dermis in both younger (8-week-old) and older (18-week-old) $Flg^{ft}$ mice; none of these were observed in B6 mice (Figure 1D). In addition, toluidine blue staining to detect mast cells showed an increased number of mast cells, especially degranulated mast cells in the upper dermis, in $Flg^{ft}$ mice (Figure 1E). No mouse or human mite bodies were detected in the sections. These data support the diagnosis of spontaneous clinical dermatitis in $Flg^{ft}$ mice in the steady state under SPF conditions.

## *Defect of Skin Barrier Function in* Flg^ft^ *Mice*

Because barrier dysfunction is a common characteristic of AD,[4–7,21] we measured TEWL, an established indicator

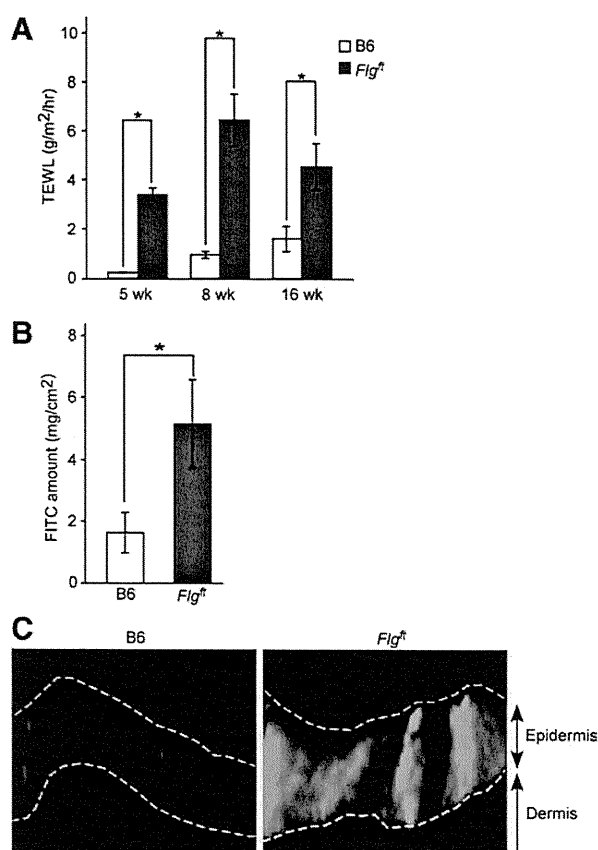

**Figure 2.** Skin barrier dysfunction in $Flg^{ft}$ mice. **A:** TEWL through dorsal skin of 5-, 8-, and 16-week-old B6 and $Flg^{ft}$ mice. **B:** Amount of FITC in the skin of B6 and $Flg^{ft}$ mice after topical application. **C:** Fluorescence intensities of FITC of the skin after topical application. **Dashed white lines** indicate the border between the epidermis and the dermis, and the top of the epidermis. $^*P < 0.05$.

of barrier function.[21] TEWL was significantly higher in $Flg^{ft}$ mice than in B6 mice from an early age (4 weeks) to an older age (16 weeks) (Figure 2A). Because TEWL is only a measure of water transportation through the skin from the inside to the outside of the body, another experimental method was necessary to evaluate outside-to-inside barrier function from the perspective of invasion of external stimuli. To address this issue, we measured FITC penetration through the skin from the outside. FITC solution was applied to the shaved dorsal skin of 8-week-old female mice; 3 hours later, the epidermis was separated and homogenized so that the FITC content could be measured with a fluorometer. The epidermis of $Flg^{ft}$ mice contained a higher amount of FITC than that of B6 mice (Figure 2B). Neither group had FITC in the dermis after this procedure, however (data not shown). In addition, observation of fluorescence intensities in the epidermis of both mice showed stronger fluorescence in $Flg^{ft}$ mice (Figure 2C). To further analyze the skin permeability, we examined the mouse embryos by toluidine blue solution and showed that the $Flg^{ft}$ embryo was entirely dye-permeable compared with the control littermate (Supplemental Figure S1, see *http://ajp.amjpathol.org*). These data strongly indicate a defect in the skin barrier of $Flg^{ft}$

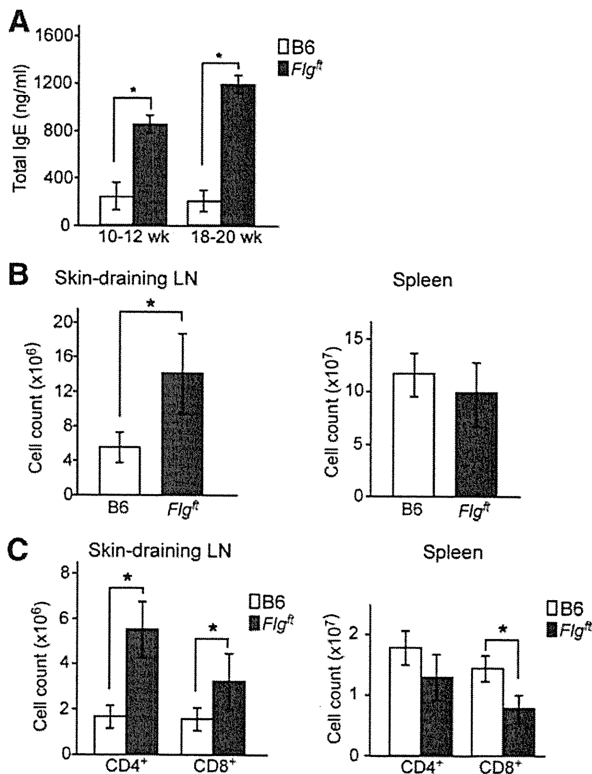


**Figure 3.** The immune status of *Flg*$^{ft}$ mice in a steady state. **A:** Total serum IgE levels of B6 and *Flg*$^{ft}$ mice as measured by enzyme-linked immunosorbent assay. **B** and **C:** Numbers of total cells (**B**), CD4$^+$ cells, and CD8$^+$ cells in the skin-draining LN and spleen (**C**). *$P < 0.05$.

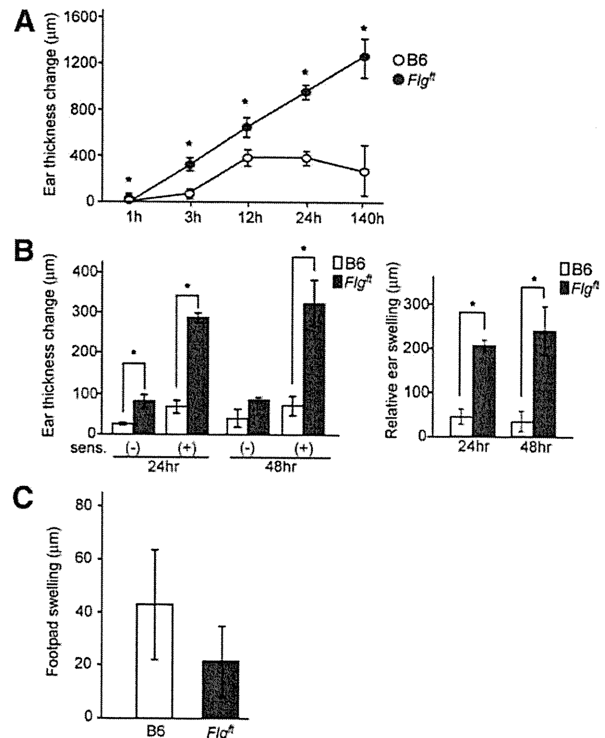


**Figure 4.** Enhanced cutaneous immune responses in *Flg*$^{ft}$ mice. **A** and **B:** Ear thickness change in B6 and *Flg*$^{ft}$ mice after topical application of PMA as a model of irritant contact dermatitis (**A**), after DNFB challenge on the ears with or without sensitization (**B, left panel**) and the relative ear swelling (**B, right panel**) as a model of CHS. **C:** Delayed-type hypersensitivity response. B6 and *Flg*$^{ft}$ mice were intraperitoneally sensitized with OVA, and challenged through subcutaneous injection to the footpad. Twenty-four hours later, footpad swelling change was measured. *$P < 0.05$.

mice, both from inside to outside and from outside to inside.

## *Immune Status in the Steady State*

To further elucidate the immune status of *Flg*$^{ft}$ mice in the steady state under SPF conditions, we measured the levels of total serum IgE, because increased severity of AD is known to be correlated with elevated serum IgE levels.[22] IgE levels were significantly higher in *Flg*$^{ft}$ mice than in age-matched B6 mice in the steady state under SPF conditions (Figure 3A). To investigate this matter in greater detail, single cell suspensions from the skin-draining inguinal and axillary LNs and from the spleen were analyzed. The total mononuclear cell number of the LNs was significantly higher in *Flg*$^{ft}$ mice than in B6 mice, but that of the spleen was comparable (Figure 3B). In addition, *Flg*$^{ft}$ mice exhibited significantly higher numbers of CD4$^+$ and CD8$^+$ cells in the skin-draining LNs, but not in the spleen (Figure 3C). Thus, an enhanced immune reaction seems to be induced in *Flg*$^{ft}$ mice by the condition of their skin.

To further analyze the immune condition of the skin, we measured the Th1 (interferon-γ [IFN-γ]), Th2 (interleukin [IL]-4 and IL-13), and Th17 (IL-17) cytokine mRNA levels of dorsal skin of 9-week-old mice in the steady state. The mRNA expression levels of IFN-γ, IL-4, and IL-13 were similar between *Flg*$^{ft}$ and B6 mice, but there was an enhancement in the IL-17 mRNA expression (data not shown) as reported previously.[17]

## *Enhanced Dermatitis in* Flg$^{ft}$ *Mice under External Stimuli*

To characterize the likelihood of various cutaneous immune responses, mice were exposed to various external stimuli. First, we studied the irritant contact dermatitis response to PMA as an irritant agent. When we applied PMA to the ears of B6 and *Flg*$^{ft}$ mice, *Flg*$^{ft}$ mice exhibited an enhanced ear swelling response compared with age-matched B6 mice throughout the experimental period (Figure 4A). Next, we measured the CHS response to DNFB. DNFB was applied to the abdominal skin for sensitization; 5 days later, the ears were challenged with the same hapten. The ear thickness change was more prominent in *Flg*$^{ft}$ mice than in B6 mice (Figure 4B, left panel). On the other hand, the ear thickness change of mice without sensitization was higher for *Flg*$^{ft}$ mice than B6 mice, suggesting that irritation contact dermatitis was enhanced in *Flg*$^{ft}$ mice as expected. To avoid the involvement of this irritation in CHS, we next analyzed the relative ear swelling by subtracting the ear thickness change without sensitization from the ear thickness change with sensitization. The relative ear swelling was more exten-

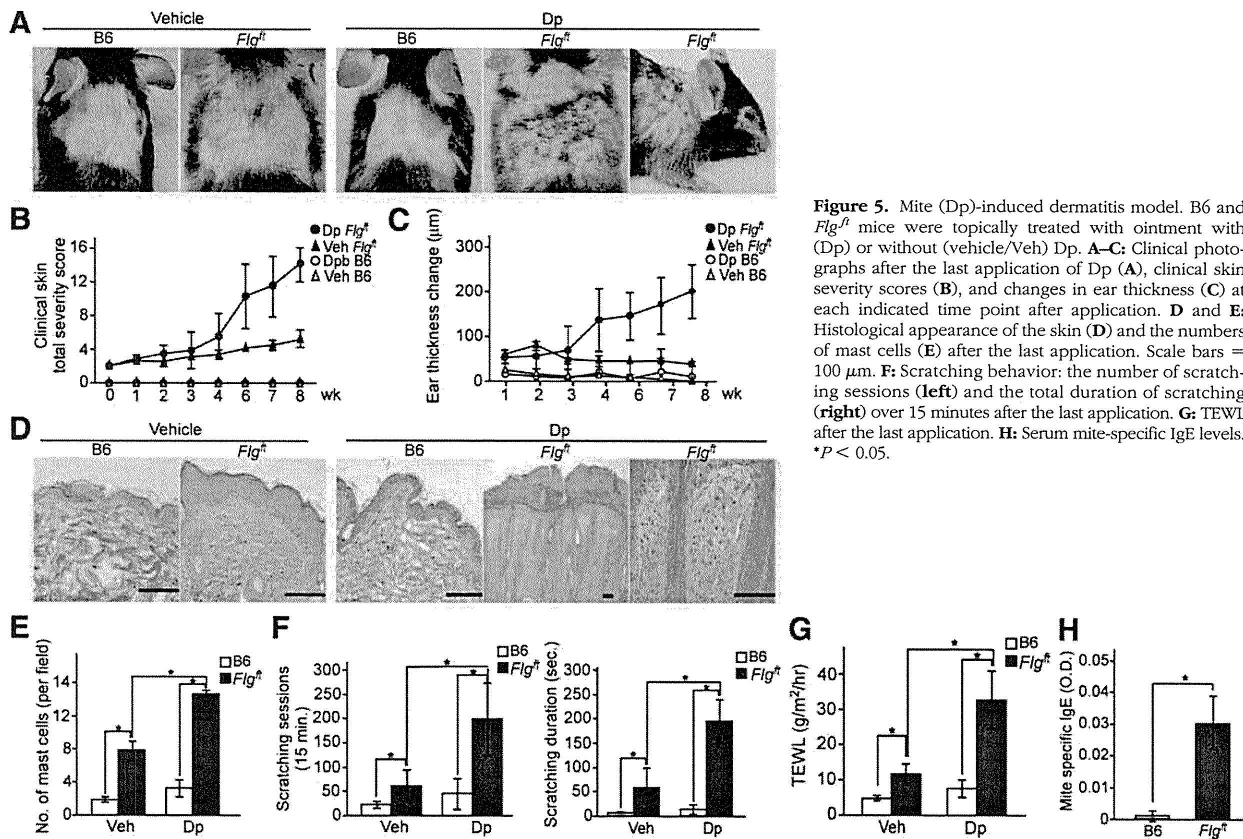


**Figure 5.** Mite (Dp)-induced dermatitis model. B6 and *Flg*$^{ft}$ mice were topically treated with ointment with (Dp) or without (vehicle/Veh) Dp. **A–C:** Clinical photographs after the last application of Dp (**A**), clinical skin severity scores (**B**), and changes in ear thickness (**C**) at each indicated time point after application. **D** and **E:** Histological appearance of the skin (**D**) and the numbers of mast cells (**E**) after the last application. Scale bars = 100 $\mu$m. **F:** Scratching behavior: the number of scratching sessions (**left**) and the total duration of scratching (**right**) over 15 minutes after the last application. **G:** TEWL after the last application. **H:** Serum mite-specific IgE levels. $^*P < 0.05$.

sive in *Flg*$^{ft}$ mice than in B6 mice (Figure 4B, right panel). We then measured the relative amount of mRNA for IFN-$\gamma$, as a representative Th1 cytokine, to GAPDH as an endogenous control. The relative amount of IFN-$\gamma$ was higher in the ears of *Flg*$^{ft}$ mice than in those of B6 mice 12 hours after the challenge (0.27 $\pm$ 0.13 versus 0.019 $\pm$ 0.013, $n = 3$). To further assess the immune responses of *Flg*$^{ft}$ mice, we elicited a delayed-type hypersensitivity response through nonepicutaneous sensitization and challenge. Mice were immunized intraperitoneally with OVA and challenged with a subcutaneous injection of OVA into the footpad. In contrast to the CHS response induced via the skin, the resulting footpad swelling in *Flg*$^{ft}$ mice was lower rather than higher than that in B6 mice (Figure 4C). We also examined the production of mRNA levels of the spleen 3 days after intraperitoneal OVA injection, and it showed a similar level of IFN-$\gamma$ between *Flg*$^{ft}$ mice and B6 mice (relative mRNA amount to GAPDH: 0.011 $\pm$ 0.005 versus 0.016 $\pm$ 0.006, $n = 5$). Thus, Th1/Tc1 immune responses were enhanced in *Flg*$^{ft}$ mice only when the stimuli operated via the skin, suggesting that the enhanced immune responses seen in *Flg*$^{ft}$ mice depend on skin barrier dysfunction.

It has been reported that *Flg*$^{ft}$ mice show an enhanced immune response to OVA.[15,17] Their reaction to clinically relevant allergens such as mites has not been evaluated, however. It has also been reported that BALB/c or NC/Nga mice develop an allergic cutaneous immune response to mite antigens when they are applied to the skin after vigorous barrier disruption by means of tape-stripping or SDS treatment.[23,24] Accordingly, we sought to determine whether skin lesions could be induced in *Flg*$^{ft}$ mice through the application of Dp ointment without any skin barrier disruption procedures to evaluate the physiological significance of filaggrin.

The application of Dp ointment to shaved backs and ears induced no cutaneous manifestation in B6 mice throughout the experimental period (Figure 5, A and B), but the same treatment induced dermatitis in *Flg*$^{ft}$ mice, especially on the ears, face, and dorsal skin. Petrolatum alone, used instead of Dp ointment as a control, induced no skin manifestation (Figure 5, A–C). The clinical severity of Dp-induced dermatitis was scored; after 16 applications of Dp ointment over 8 weeks, *Flg*$^{ft}$ mice had developed a very severe skin condition in contrast with the control groups. Consistently, ear swelling in response to Dp ointment was most prominent in *Flg*$^{ft}$ mice (Figure 5C). Histological examination of H&E-stained sections of involved *Flg*$^{ft}$ skin after 16 applications showed acanthosis, elongation of rete ridges, and dense lymphocyte and neutrophil infiltration in the dermis (Figure 5D), accompanied by an increased number of mast cells in the dermis (Figure 5E). We also measured the scratching behavior of *Flg*$^{ft}$ mice treated with Dp using the Sclaba Real system. The number of scratching sessions and the total duration of scratching were significantly higher in *Flg*$^{ft}$ mice than in B6 mice, even among those mice that had not been treated with Dp ointment (Figure 5F); treatment of *Flg*$^{ft}$ mice with Dp ointment raised the number of scratching sessions and the total duration of scratching even higher.

We further evaluated barrier function by measuring TEWL in Dp-treated and untreated mice of each genotype; TEWL was higher in untreated *Flg*$^{ft}$ mice than in B6 mice, and Dp treatment of *Flg*$^{ft}$ mice raised TEWL even higher (Figure 5G). Finally, we examined mite-specific serum IgE levels after the last application and found that *Flg*$^{ft}$ mice had higher levels of Dp-specific IgE than B6 mice had (Figure 5H). Thus, the treatment of *Flg*$^{ft}$ mice with Dp ointment, even without prior barrier disruption, remarkably enhanced both the clinical manifestations and the laboratory findings that correspond to indicators of human AD.

## Discussion

Here, we demonstrated that *Flg*$^{ft}$ mice exhibit spontaneous dermatitis with lymphadenopathy, elevated IgE levels, and skin barrier disruption in a steady state under SPF conditions. These outcomes are compatible with the features of human AD, which include chronic eczema, pruritus, and dry skin with elevated TEWL and serum IgE levels.[1–4,25,26] In addition, *Flg*$^{ft}$ mice exhibit enhanced susceptibility to irritant contact dermatitis, CHS, and mite-induced dermatitis compared with B6 mice; these characteristics are also reminiscent of human AD. These results suggest that the barrier defect in this strain of mice leads to spontaneous dermatitis and enhances cutaneous immune responses and inflammation.

Since the first introduction of *Flg*$^{ft}$ mice in 1972,[13] there have been only a few reports of these mice. The first report demonstrated that *Flg*$^{ft}$ mice without the *ma* mutation showed flaky skin as early as postnatal day 2 but became normal in appearance by 3 to 4 weeks of age without spontaneous dermatitis except for their slightly smaller ears.[13] Later, the lack of filaggrin in the epidermis was proposed in the commercially available strain of *Flg*$^{ft}$ mice used in this study, which has both *Flg* and *ma* mutations, as a model of ichthyosis vulgaris, and therefore the cutaneous inflammatory conditions from the perspective of AD was not discussed.[14] There have been three recent studies using *Flg*$^{ft}$ mice as a model of filaggrin deficiency: Fallon et al[15] used *Flg*$^{ft}$ mice from which the *ma* mutation had been eliminated with four additional backcrosses to B6 mice, and others used the commercially available *Flg*$^{ft}$ mice.[16,17] The first report showed only a histological abnormality without clinical manifestations,[15] the second report demonstrated spontaneous eczematous skin lesions after 28 weeks of age,[17] and the third report did not indicate any spontaneous dermatitis in *Flg*$^{ft}$ mice.[16] In our experiment, we observed a spontaneous dermatitis as early as 5 weeks of age with mild erythema and fine scales. These symptoms gradually exacerbated, accompanied by scratching, erosion, and edema, respectively, and became prominent at the age of 23 weeks. The discrepancies among these results seem to be related to the presence or absence of the *ma* mutation and/or variation in the genetic backgrounds of the different strains used and to environmental factors. It has been reported that Japan has higher morbidity for AD than other countries,[27,28] possibly attributable to environmental factors such as pollen.

It has been reported that TEWL, an indicator of inside-to-outside barrier function, is high in both AD patients with the *FLG* mutation[29] and *Flg*$^{ft}$ mice.[15] In consideration of the immunological defense by the skin, however, it is more important to assess outside-to-inside barrier function rather than inside-to-outside barrier function. In fact, outside-to-inside barrier dysfunction has recently been proposed as the most important aspect in the pathogenesis of AD.[9,26] Scharschmidt et al[16] reported increased bidirectional paracellular permeability of water-soluble xenobiotes by ultrastructural visualization in *Flg*$^{ft}$ mice, suggesting a defect of the outside-to-inside barrier. However, the quantitative measurement of this parameter has not been addressed. Here, we propose a new method for evaluating outside-to-inside barrier function quantitatively by measuring the penetrance of FITC through the skin. This method has a parallel correlation with the qualitative measurement of FITC penetrated in epidermis and an established method for skin permeability assay, the *in situ* dye staining method. Therefore, by using this new method, we were able to detect outside-to-inside barrier dysfunction in *Flg*$^{ft}$ mice quantitatively.

The skin abnormality associated with AD is well known to be a predisposing factor to sensitive skin[30,31] and allergic contact dermatitis,[32,33] but patients with AD produce a tuberculin response similar to that of healthy control subjects.[34,35] In humans, sensitive skin is defined as reduced tolerance to cutaneous stimulation, with symptoms ranging from visible signs of irritation to subjective neurosensory discomfort.[30,31] The question of whether human AD patients are more prone to allergic contact dermatitis than nonatopic individuals is still controversial.[33] To address this question, we evaluated skin responsiveness to PMA as an irritant and found that irritant contact dermatitis was enhanced in *Flg*$^{ft}$ mice. In addition, *Flg*$^{ft}$ mice showed an increased skin-sensitized CHS reaction, a form of classic Th1- and Tc1-mediated delayed-type hypersensitivity to haptens, emphasized by increased IFN-$\gamma$ production. In contrast, when mice were sensitized intraperitoneally, no difference was observed between *Flg*$^{ft}$ and B6 mice *in vivo* or *in vitro*. This finding is consistent with the observation that humans with and without AD respond comparably to tuberculin tests[34,35] and suggests that skin barrier function regulates cutaneous immune conditions, which hints at a possible mechanism involved in human AD.

Clinical studies have provided evidence that a house dust mite allergen plays a causative or exacerbating role in human AD[36] and that a strong correlation exists between patients with *FLG* null alleles and house dust mite-specific IgE.[37] AD-like skin lesions can be induced by repeated topical application of a mite allergen in NC/Nga mice but not in BALB/c mice.[23] In the present study, we induced skin lesions that were clinically and histologically similar to AD, along with increased TEWL, increased scratch behavior, and increased levels of mite-specific IgE, in *Flg*$^{ft}$ mice through the application of Dp. Dp is a common aeroallergen that is frequently involved in induction of human AD. It has protease activities, spe-

cifically from Der p1, Der p3, and Der p9, which may activate protease-activated receptor-2 in human keratinocytes.[38,39] A recent report has shown that activation of protease-activated receptor-2 through Dp application significantly delays barrier recovery rate in barrier function-perturbed skin or compromised skin.[39] Therefore, Dp may play a dual role in the onset of AD, both as an allergen and proteolytic signal and as a perturbation factor of the barrier function, leading to the persistence of eczematous skin lesions in AD.[39,40]

To address the issue of variable genetic background, we observed immune responses in mice of other genotypes, such as BALB/c and C3H, as controls, but both of these lines exhibited much less severe CHS responses compared with those in *Flg*$^{ft}$ mice (data not shown), suggesting that the enhanced immune responses seen in *Flg*$^{ft}$ mice were not solely due to their genetic background. The effect of the *ma* mutation in relation to the *ft* mutation in commercially available *Flg*$^{ft}$ mice in the development of AD-like skin lesions needs to be clarified in future studies. Furthermore, our study showed that heterozygous mice intercrossed with *Flg*$^{ft}$ mice and B6 mice did not develop spontaneous dermatitis. In this way they are unlike human AD patients, most of whom are heterozygous for the *FLG* mutation. Not only human studies but also additional mouse studies are required to clarify these relationships.

In this study, we have shown that *Flg*$^{ft}$ mice exhibit spontaneous dermatitis resembling human AD, enhanced irritation dermatitis and a contact hypersensitivity response, and mite-induced AD-like skin lesions, which provide hints for possible mechanisms in the human disease. These results suggest that *Flg*$^{ft}$ mice have the potential to serve as an animal model of human AD and further accentuate the important role of filaggrin in skin barrier function in the pathogenesis of AD.

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CXCL8 in the NOD1 shRNA-expressing cells is mediated by such receptors.

In summary, to our knowledge this is the first report demonstrating that the intracellular receptor NOD1 is functional expressed in human keratinocytes, suggesting that NOD1 may be involved in cutaneous innate immunity. Further studies are needed to understand the contribution of intracellular innate immune receptors to cutaneous host defense.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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# Prevalent and Rare Mutations in the Gene Encoding Filaggrin in Japanese Patients with Ichthyosis Vulgaris and Atopic Dermatitis

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**TO THE EDITOR**

Mutations in the gene encoding filaggrin (*FLG*) were identified as the underlying cause of ichthyosis vulgaris (IV; OMIM #146700) and also shown to predispose to atopic dermatitis (AD; Palmer *et al.*, 2006; Smith *et al.*, 2006). Although *FLG* is considerably difficult to analyze because of its large size (>12 kb) and highly repetitive nature, PCR strategy that permits routine and comprehensive sequencing of the entire *FLG* has been developed recently (Sandilands *et al.*, 2007).

Using this methodology, we have identified four prevalent *FLG* mutations in Japanese patients with IV (Nomura *et al.*, 2008). We also demonstrated that *FLG* mutations were significantly associated with AD and the frequency of these *FLG* mutations observed in our Japanese AD cohort was about 20%. However, the frequency in our cohort

*Abbreviations: AD, atopic dermatitis; FLG, filaggrin; IV, ichthyosis vulgaris*

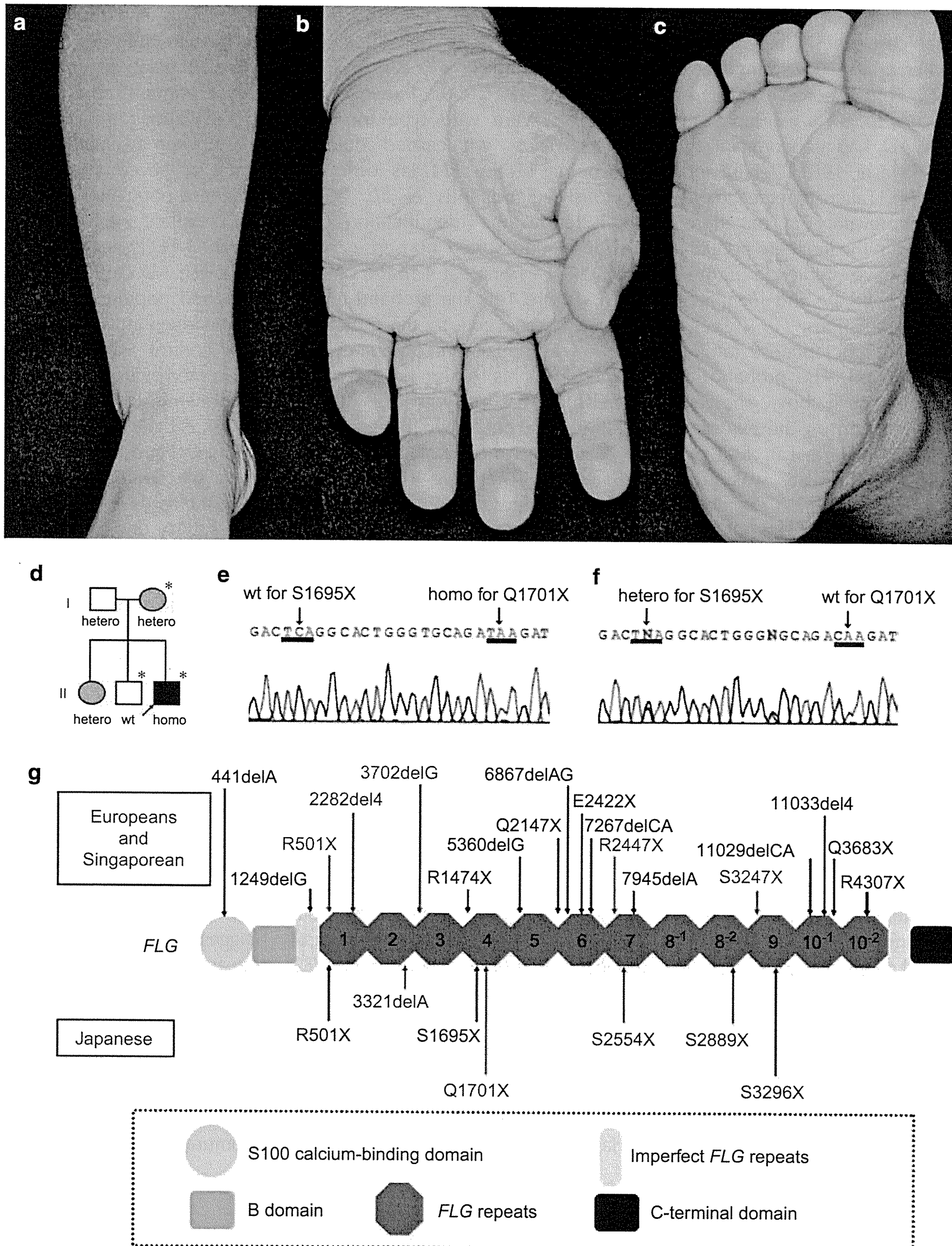


**Figure 1. Clinical features and results of mutation analysis.** (**a**) Fine scaling clearly visible on the proband's leg. (**b, c**) He also showed marked palmoplantar hyperlinearity. (**d**) A family tree of the ichthyosis vulgaris family shows the semidominant inheritance pattern. Solid symbols refer to the marked ichthyosis vulgaris presentation; cross-hatched symbols refer to the milder ichthyosis vulgaris presentation. In addition, the proband, his mother, and sister had concomitant dermatologist-diagnosed atopic dermatitis (*). wt, wild type for Q1701X; hetero, heterozygous; homo, homozygous. (**e, f**) A homozygous transition mutation c.5101C>T was identified in the proband, resulting in Q1701X. A heterozygous transition mutation c.5084C>G was identified in one Japanese individual in the control population, resulting in S1695X. Mutation S1695X is located only six amino acids upstream from Q1701X. (**g**) Loss-of-function *FLG* mutations are shown in a schematic of profilaggrin. Mutations shown in red are prevalent; those in black are rare. Some individuals have duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8-1, 8-2, 10-1, and 10-2.

was still lower than that seen in analogous European case series, where it is up to 48% (Barker *et al.*, 2007; Sandilands *et al.*, 2007). Furthermore, it was reported that up to 37% of Japanese patients with AD had concomitant IV (Uehara and Hayashi, 1981; Uehara and Miyauchi, 1984). Taken together, there might be further prevalent *FLG* mutations to be discovered in the Japanese population. Here we have studied a further Japanese family with IV and identified two further *FLG* mutations.

A newly recruited Japanese family with IV was studied. The proband, a one-year-old Japanese boy, showed marked scaly dry skin on the extensor limbs and trunk (Figure 1a). Marked palmoplantar hyperlinearity was also evident (Figure 1b and c). A diagnosis of IV was made from these clinical observations. His mother and sister also showed scaly dry skin and palmoplantar hyperlinearity, but the clinical severity was mild compared to the proband (Figure 1d). Therefore, the inheritance pattern seemed semidominant. The proband, his mother, and his brother had concomitant AD.

The medical ethical committee at Hokkaido University Graduate School of Medicine approved all the studies. The study was conducted according to the Declaration of Helsinki Principles. Participants or their legal guardians gave their written informed consent. Following informed consent, genomic DNA from all family members was extracted from peripheral blood according to standard procedures. Initially, all family members were screened for five *FLG* mutations identified in Japanese population so far, R501X, 3321delA, S2554X, S2889X and S3296X, by restriction enzyme digestion, fluorescent PCR, and direct DNA sequencing as described previously (Nomura *et al.*, 2007, 2008; Hamada *et al.*, 2008). However, all individuals were wild type for these variants. Thus, we carried out full sequencing of the *FLG* as described previously (Sandilands *et al.*, 2007), which led to the identification of a previously unreported nonsense mutation Q1701X in repeat 4 in the present family (Figure 1e). The proband turned out to be homozygous for this truncation mutation and his non-consanguineous parents and his sister heterozygous, whereas his brother wild type (Figure 1d). It was also confirmed that they carry no pathogenic mutations in the other *FLG* repeats. Then, we screened 118 unrelated Japanese patients with AD and 134 unrelated Japanese control individuals for Q1701X by direct DNA sequencing. The diagnosis of AD in our case series was made by experienced dermatologists, according to the AD diagnostic criteria by Hannifin and Rajka (1980). Notably, mutation Q1701X was also identified in two Japanese patients with AD (1.7%), which brings the total number of recurrent *FLG* mutations so far identified in Japanese population to five.

During the screening for Q1701X, we identified another previously unreported *FLG* mutation, S1695X, which is located only six amino acids upstream from Q1701X, in the general Japanese control population (Figure 1f). We screened 33 Japanese patients with IV and 118 with AD for S1695X, but all patients were wild type for this mutation. Only one heterozygote was identified in the control population. Therefore, S1695X seems to be an extremely rare *FLG* mutation in Japanese individuals. The control individuals had not been examined in relation to AD or IV status, that is, they were population controls rather than "hypernormal" controls, so no clinical details about the individual carrying S1695X are available. In total, there are at least seven *FLG* variants in the Japanese population, including five that are prevalent and two that are quite rare.

The *FLG* genotype data in the Japanese AD case series and ethnically matched population control series are summarized in Table 1. In this study, case–control association analyses were performed by using Pearson's $r^2$ statistics, as previously described (Palmer *et al.*, 2006). All alleles were observed to be in normal Hardy–Weinberg equilibrium. Here we demonstrate that about 25% of patients in our Japanese AD case series carry one or more of these seven *FLG* mutations (combined minor allele frequency = 0.127, $n = 236$) and these variants are also carried by 4% of general Japanese control individuals (combined minor allele frequency = 0.019, $n = 268$). There is significant statistical association between the seven *FLG* mutations and AD ($r^2$ $P = 1.75 \times 10^{-6}$). Moreover, AD was manifested in heterozygous carriers of these *FLG* mutations with a Fisher's exact test odds ratio for AD of 6.8 (95% CI 2.5–18.5, $P = 3.7 \times 10^{-5}$), implying a causal relationship between *FLG* mutations and AD. Taken together, these data strongly suggest that skin barrier impairment because of reduced filaggrin expression is important in the pathogenesis in AD.

To date, 24 *FLG* mutations, including the two identified in this study, have been reported in the European, Japanese, and Singaporean populations (Sandilands *et al.*, 2007; Chen *et al.*, 2008; Nomura *et al.*, 2008). Interestingly,

**Table 1. Atopic dermatitis case–control association analysis for *FLG* null variants in Japan**

| | R501X | | 3321delA | | S1695X | | Q1701X | | S2554X | | S2889X | | S3296X | | Combined | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypes | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases |
| AA | 134 | 118 | 133 | 113 | 133 | 118 | 134 | 116 | 133 | 112 | 132 | 105 | 134 | 114 | 129 | 91 |
| Aa | 0 | 0 | 1 | 5 | 1 | 0 | 0 | 2 | 1 | 6 | 2 | 13 | 0 | 4 | 5 | 24 |
| aa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Total | 134 | 118 | 134 | 118 | 134 | 118 | 134 | 118 | 134 | 118 | 134 | 118 | 134 | 118 | 134 | 118 |

For combined genotype, $r^2$ $P$=1.75 × $10^{-6}$; Fisher's exact test odds ratio=6.8 (95% CI 2.5–18.5).

mutations found in Japanese are different from those found in Europeans and Singaporean (Figure 1g), except in one case of the common European R501X mutation occurring as a very rare mutation on a different haplotype in the Japanese population (Hamada *et al.*, 2008). These observations imply that every population is highly likely to have a unique set of *FLG* mutations.

In conclusion, we have identified two further *FLG* mutations in the Japanese population. We also showed that at least about 25% of Japanese patients with AD carried one or more of *FLG* mutations. As we have sequenced more than 30 Japanese patients with IV, there is now little possibility that further highly prevalent mutations underlie the Japanese population. Taking the high frequency (up to 37%) of concomitant IV in patients with AD into account, however, it is still possible that there might be further multiple low-frequency *FLG* mutations to be discovered in the Japanese population. Further *FLG* mutation analysis will be necessary to understand the more precise genetic architecture of filaggrin-related AD in Japan.

### CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

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# "White" Nevi and "Red" Melanomas: Association with the RHC Phenotype of the *MC1R* Gene

*Journal of Investigative Dermatology* (2009) **129**, 1305–1307; doi:10.1038/jid.2008.378; published online 4 December 2008

### TO THE EDITOR

In 2002, we reported on three patients presenting with melanocytic nevi lacking pigmentation, which we named "white" dysplastic melanocytic nevi (DMN) due to their peculiar clinical appearance of white to pale red macules with accentuated skin markings and a silvery "shining" when observed with tangential light (Zalaudek *et al.*, 2002). Notably, all three patients had melanoma, and in one patient white DMN were associated with two primary amelanotic melanomas (AMMs).

We present herein a 25-year-old woman (skin type I, red hair, and blue eyes), who sought consultation for a mole check. Clinical examination revealed, besides approximately 30 slightly atypical light brown nevi on

*Abbreviations: AMM, amelanotic melanoma; DMN, dysplastic melanocytic nevi; RHC, red hair color*

# *FLG* mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema

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**Key words**
atopic dermatitis, atopy, ichthyosis, phenotype, profilaggrin

**Conflicts of interest**
W.H.I.M. has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

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## Summary

*Background* Mutations in the gene encoding filaggrin (FLG) have been shown to predispose to atopic eczema (AE).

*Objectives* Further to establish population genetics of FLG mutations in the Japanese population and to elucidate effects of FLG mutations to filaggrin biosynthesis in skin of patients with AE.

*Methods* We searched for FLG mutations in 19 newly recruited Japanese patients with AE. We then screened 137 Japanese patients with AE and 134 Japanese control individuals for a novel mutation identified in the present study. In addition, we evaluated FLG mRNA expression by real-time reverse transcription–polymerase chain reaction and profilaggrin/filaggrin protein expression by immunohistochemical staining in the epidermis of the patients carrying the novel mutation.

*Results* We identified a novel FLG nonsense mutation c.12069A>T (p.Lys4021X) in one patient with AE. Upon further screening, p.Lys4021X was identified in four patients with AE (2·9% of all the patients with AE). In total, there are at least eight FLG variants in the Japanese population. Here we show that about 27% of patients in our Japanese AE case series carry one or more of these eight FLG mutations and these variants are also carried by 3·7% of Japanese general control individuals. There is a significant statistical association between the eight FLG mutations and AE ($\chi^2$ $P = 6{\cdot}50 \times 10^{-8}$). Interestingly, the present nonsense mutation is in the C-terminal incomplete filaggrin repeat and is the mutation nearest the C-terminal among previously reported FLG mutations. Immunohistochemical staining for filaggrin revealed that this nonsense mutation leads to remarkable reduction of filaggrin protein expression in the patients' epidermis.

*Conclusions* We clearly demonstrated that FLG mutations are significantly associated with AE in the Japanese population. The present results further support the hypothesis that the C-terminal region is essential for proper processing of profilaggrin to filaggrin.

Filaggrin is a protein essential to skin barrier function. Mutations in *FLG*, the gene encoding profilaggrin/filaggrin, have been demonstrated as the underlying cause of ichthyosis vulgaris (IV; OMIM 146700) and have been shown to be an important predisposing factor for atopic eczema (AE).[1–4] The presence of population-specific *FLG* mutations in Europeans, Chinese-Singaporeans, Japanese and Taiwanese has been reported.[3,5–9] Recently, it was clarified that *FLG* mutations were found in approximately 25% of Japanese patients with AE.[6,8]

## Materials and methods

We searched for *FLG* mutations in 19 newly recruited Japanese patients with AE. All these patients had been diagnosed with AE based on widely recognized diagnostic criteria.[10] Initially, using genomic DNA, patients with AE were screened for seven *FLG* mutations previously identified in the Japanese population by restriction enzyme digestion, fluorescent polymerase chain reaction (PCR) and/or direct DNA sequencing as described previously.[8] Subsequently, for the patients with AE without

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any known *FLG* mutation, we sequenced the entire coding region of *FLG*. The medical ethics committee of Hokkaido University Graduate School of Medicine approved all the studies, which were conducted according to the Declaration of Helsinki Principles. The participants or their legal guardians gave written informed consent.

## Results

This sequencing revealed a novel nonsense mutation c.12069A>T (p.Lys4021X) in repeat 11 (imperfect filaggrin repeat) of one patient with AE (Fig. 1a–c). The nucleotide change was not detected in 50 unrelated, healthy Japanese individuals (100 alleles).

Subsequently, we screened for the newly identified *FLG* mutation p.Lys4021X in all 137 Japanese patients with AE we had collected to date and 134 unrelated Japanese control individuals. The 118 patients with AE and 134 control individuals were identical to those in a previous study,[8] and the data except for those on p.Lys4021X were reported by Nomura *et al.*[8] We identified p.Lys4021X in four patients with AE (2·9% of all the patients with AE) in our Japanese AE cohort.

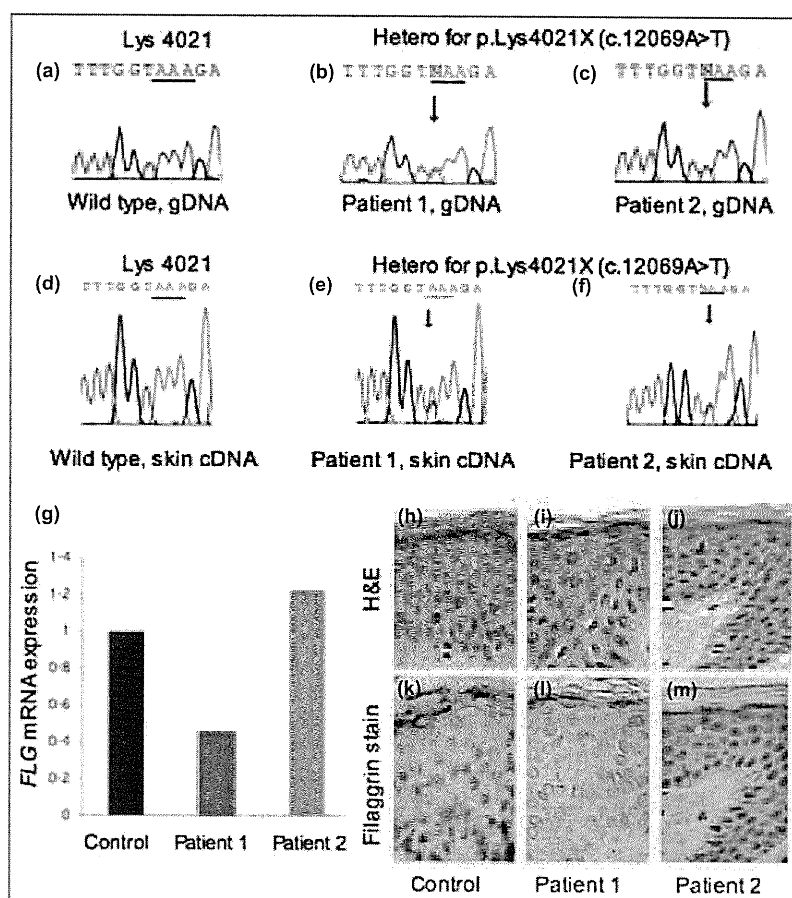


Fig 1. *FLG* mutation analysis and expression of p.Lys4021X mutant alleles. (a–c) Identification of the *FLG* mutation. Direct sequence analysis of *FLG* was performed on genomic DNA from peripheral leucocytes. (a) Normal control sequence from filaggrin repeat 11 in exon 3. (b, c) A heterozygous transition mutation c.12069A>T was identified in patient 1 (b) and patient 2 (c), resulting in p.Lys4021X. (d–f) Direct sequence analysis of *FLG* cDNA from mRNA expressed in skin samples from the back. (d) Normal control cDNA sequence derived from filaggrin repeat 11 in exon 3. (e, f) Expression of mRNA derived from both wild-type alleles and mutant alleles was confirmed in patient 1 (e) and patient 2 (f). The expression level of mRNA derived from the mutant allele was lower than that from the wild-type allele in patient 1 (e), although expression levels of mRNA from both mutant and wild-type alleles were roughly equal in patient 2 (f). (g) Real-time reverse transcription–polymerase chain reaction analysis of *FLG* mRNA expression in the skin. *FLG* expression was reduced in patient 1, but not in patient 2: patient *FLG* mRNA expression/control *FLG* mRNA expression = 0·46 for patient 1 and 1·23 for patient 2. mRNA expression of *FLG* in patients 1 and 2 was not significantly different from that in control skin. (h–m) Histological features of patients 1 and 2: (h–j) haematoxylin and eosin (H&E) staining; (k–m) immunohistochemical staining using antifilaggrin monoclonal antibody against an epitope conserved in all filaggrin repeat peptides. Patient 1 (i) and patient 2 (j) showed a lack of granular layers in the epidermis, where only a small amount of a basophilic substance, which resembled keratohyaline granules, was occasionally present. In contrast, normal control skin (h) had abundant keratohyaline granules in the granular layers. A marked reduction in staining for filaggrin was seen in the epidermis from both patient 1 (l) and patient 2 (m), relative to the strong staining in normal control skin (k).

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It was confirmed that these four patients with AE carry no other known *FLG* mutations. None of the control individuals had the p.Lys4021X mutation. The four patients with AE with the newly discovered mutation – three women and one man – were aged 12–31 years, and all four patients had severe AE symptoms. There was no specific clinical feature of AE characteristic to the four patients. None of the four patients had apparent clinical features or a family history of IV.

We investigated *FLG* mRNA expression by real-time reverse transcription (RT)-PCR and sequencing, and studied profilaggrin/filaggrin protein expression in the skin by immunohistochemistry in two of the patients with AE harbouring p.Lys4021X. Real-time RT-PCR analysis revealed that mRNA expression of *FLG* was not reduced significantly (Fig. 1g). The expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles (Fig. 1d–f). However, histopathological examinations of the patients' skin showed reductions in keratohyaline granules in the granular layers (Fig. 1h–j). Immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the patients' epidermis (Fig. 1k–m).

Eight *FLG* mutations including the present mutation p.Lys4021X have been identified in the Japanese population (Fig. 2). Case–control association analyses were performed for *FLG* mutations in Japanese patients with AE and normal controls using Pearson $\chi^2$ statistics, as previously described.[2] The *FLG* genotype data in the Japanese AE case series and ethnically matched population control series are summarized in Table 1. All alleles were observed to be in normal Hardy–Weinberg equilibrium. Here we demonstrate that about 27% of the patients in our Japanese AE case series carry one or more of the eight *FLG* mutations (combined minor allele frequency = 0·150, n = 274) and these variants are also carried by 3·7% of Japanese control individuals (combined minor allele frequency = 0·019, n = 268). There is a statistically significant association between the eight *FLG* mutations and AE

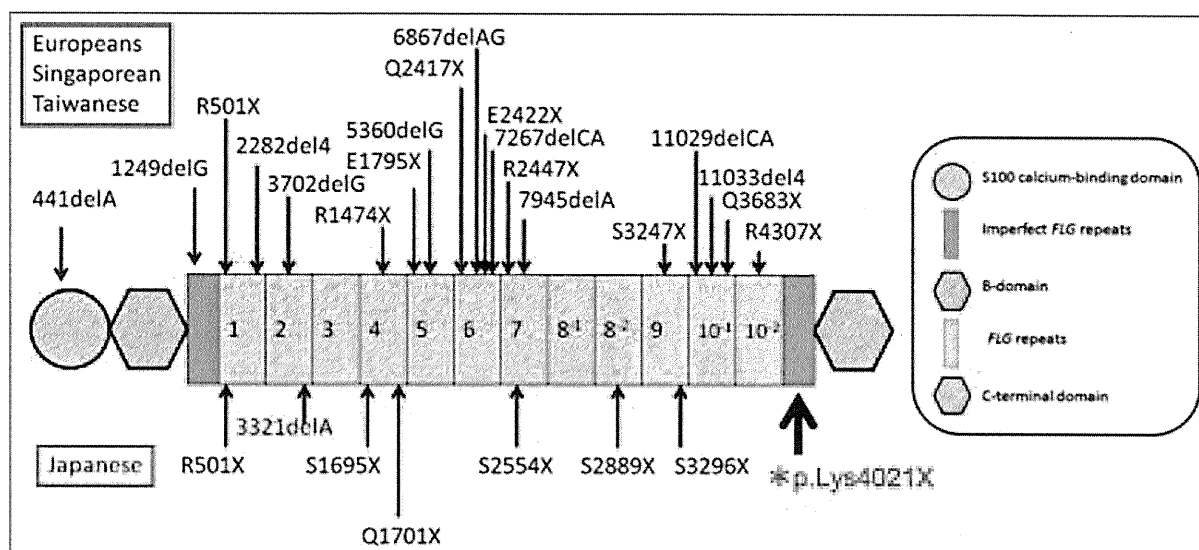


Fig 2. The present and previously reported *FLG* mutations are shown in a scheme of profilaggrin peptide. Some individuals have a duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as $8^{-1}$, $8^{-2}$, $10^{-1}$ and $10^{-2}$. *Indicates the present mutation p.Lys4021X. This mutation is the nearest to the C-terminus domain among all the reported mutations and is located in the C-terminal incomplete filaggrin repeat downstream of all the filaggrin repeats.

Table 1 Atopic eczema case–control association analysis for *FLG* null variants in Japan

| | R501X | | 3321delA | | S1695X | | Q1701X | | S2554X | | S2889X | | S3296X | | K4021X | | Combined | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypes | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls | Cases |
| AA | 134 | 137 | 133 | 131 | 133 | 137 | 134 | 134 | 133 | 129 | 132 | 122 | 134 | 132 | 134 | 133 | 129 | 96 |
| Aa | 0 | 0 | 1 | 6 | 1 | 0 | 0 | 3 | 1 | 8 | 2 | 15 | 0 | 5 | 0 | 4 | 5 | 33 |
| aa | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 |
| Total | 134 | 137 | 134 | 137 | 134 | 137 | 134 | 137 | 134 | 137 | 134 | 137 | 134 | 137 | 134 | 137 | 134 | 137 |

For combined genotype, $\chi^2$ = 29·218, P = 6·50 × $10^{-8}$; Fisher's exact test odds ratio = 9·94 (95% confidence interval 3·77–26·2, P = 2·35 × $10^{-8}$).

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($\chi^2$ P = $6{\cdot}50 \times 10^{-8}$). Moreover, AE was manifested in heterozygous carriers of these *FLG* mutations with a Fisher's exact test odds ratio for AE of 9·94 (95% confidence interval 3·77–26·2, P = $2{\cdot}35 \times 10^{-8}$), suggesting a causal relationship between FLG mutations and AE.

## Discussion

Filaggrin is synthesized initially as profilaggrin, an approximately 500-kDa polypeptide that contains two imperfect filaggrin-repeat domains flanking 10–12 essentially identical filaggrin repeats.[11]

Previous studies reported that *FLG* truncation mutations in both filaggrin repeats 1 and 7 lead to a severe filaggrin deficiency, despite the synthesis of a short N-terminal profilaggrin peptide.[1,3,12] The present mutation p.Lys4021X is in the C-terminal incomplete filaggrin repeat, and the truncation site is the nearest to the C-terminal among *FLG* mutations identified to date (Fig. 2). The longest truncated profilaggrin peptide containing all the complete filaggrin repeats may theoretically be produced from the mutant allele. However, our immunohistochemical staining revealed that profilaggrin/filaggrin peptides were remarkably reduced in the epidermis of the patients with p.Lys4021X, even though *FLG* mRNA expression was not reduced significantly and expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles. These results suggest that the truncated profilaggrin peptides are degenerated and are not processed to filaggrin peptides even when the mutation site is in the C-terminal incomplete filaggrin repeat.

In conclusion, we have identified another prevalent *FLG* mutation in the Japanese population. We have also shown that about 27% of Japanese patients with AD carry one or more *FLG* mutations. The present nonsense mutation p.Lys4021X in the C-terminal incomplete filaggrin repeat leads to filaggrin deficiency and our results further support the hypothesis that the C-terminal region is essential for proper processing of profilaggrin to filaggrin peptides.

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# Clinical Severity Correlates with Impaired Barrier in Filaggrin-Related Eczema

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Mutations in the gene-encoding filaggrin (*FLG*), a key molecule involved in skin barrier function, have been shown to be a major predisposing factor for atopic dermatitis (AD; eczema). To elucidate the pathomechanisms underlying filaggrin-related AD, we investigated stratum corneum (SC) hydration and transepidermal water loss (TEWL) as parameters of barrier function in AD patients harboring *FLG* mutations compared to AD patients without any *FLG* mutation. In filaggrin-related AD, SC hydration was both significantly reduced ($P<0.01$–$0.05$) and thicker ($P<0.01$–$0.05$) than that in healthy controls. TEWL was demonstrably increased in non-filaggrin AD compared to healthy controls ($P<0.01$–$0.05$). The objective score of atopic dermatitis (OSCORAD), a disease clinical severity index, significantly correlated with TEWL ($r=0.81$, $P<0.005$), SC hydration ($r=-0.65$, $P<0.05$), and SC thickness ($r=0.59$, $P<0.05$) in filaggrin-related AD. On the contrary, there was no correlation between these parameters and the OSCORAD in non-filaggrin AD. Furthermore, a significant correlation was obtained between the OSCORAD and specific IgE for house dust ($r=0.66$, $P<0.05$), mite allergen ($r=0.53$, $P<0.05$), and cat dander ($r=0.64$, $P<0.05$) in filaggrin-related AD, but not in non-filaggrin AD. All these data suggest that experimentally demonstrable skin barrier defects due to *FLG* mutations may play a crucial role in the pathogenesis of AD.

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## INTRODUCTION

Atopic dermatitis (AD; also known as atopic eczema) is a common skin disease that affects 15–20% of children in the developed world (Roll *et al.*, 2004). AD is thought to have a variety of heterogeneous etiologic factors including genetic predisposing factors and environmental factors. Recently, mutations within the gene-coding filaggrin (*FLG*) were reported to cause ichthyosis vulgaris (IV; Smith *et al.*, 2006) and to be a major genetic predisposing factor for AD (Palmer *et al.*, 2006; Sandilands *et al.*, 2007). Filaggrin is essential for the cell compaction process that precedes chemical cross-linking in the biogenesis of the stratum corneum (SC). Therefore, filaggrin is a key molecule in the initiation and maintenance of skin barrier function. Profilaggrin is the main protein component of the keratohyalin granules within the last living cell layers of the epidermis (Irvine and McLean, 2006). In addition, the terminal degradation products of filaggrin may act as a "natural moisturizing substance" (Rawlings and Harding, 2004). The fact that *FLG* mutations have been reported as an important predisposing factor for AD and secondary, less penetrant, atopic phenotypes such as atopic asthma, suggests that the skin barrier defect is a primary key event leading to allergic sensitization and development of AD and related allergic phenotypes (Weidinger *et al.*, 2006).

*FLG* null mutations are found from 15 to 55% of AD patients in European populations (Palmer *et al.*, 2006; Weidinger *et al.*, 2006; Sandilands *et al.*, 2007). Major differences exist in the spectra of *FLG* mutations observed between different ancestral groups. Specifically, *FLG* ancestral mutations p.R501X and c.2282del4 in the European population were not found in the Japanese population (Nomura *et al.*, 2007, 2008). However, very recently, we identified four unique *FLG* mutations p.Ser2554X, c.3321del, p.Ser2889X, and p.Ser3296X in Japanese IV families and clarified that these four mutations were found more than 24% of the Japanese AD patients (Nomura *et al.*, 2007, 2008).

Transepidermal water loss (TEWL) and SC hydration, which are measurements of skin barrier function, were reported to increase in AD patients due to their skin barrier insufficiency (Aalto-Korte, 1995; Chamlin *et al.*, 2002). Significant correlations were observed between penetration rates of a hydrophilic dye and elevated IgE levels in patients with severe AD (Hata *et al.*, 2002). In addition, percutaneous

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*Abbreviations: AD, atopic dermatitis; EOS, eosinophil;* FLG, *filaggrin gene; IV, ichthyosis vulgaris; LDH, lactate dehydrogenase; MAST, multiple antigen simultaneous test; SC, stratum corneum; SCORAD, score of atopic dermatitis; TEWL, transepidermal water loss*

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penetration of sodium lauryl sulphate was reported to be increased in uninvolved skin of patients with AD (Jakasa *et al.*, 2006). Taken together, these findings strongly support the hypothesis that patients with AD have a skin barrier defect.

In this context, we hypothesized that, in filaggrin-related AD, skin barrier defects caused by *FLG* deficiency is a primary abnormality leading to the AD symptoms. In the present study, to confirm this hypothesis, we evaluated skin barrier function in two AD patient groups divided by presence or absence of *FLG* mutations, by measurements of TEWL, SC hydration, and thickness that are useful markers of skin barrier function (Holm *et al.*, 2006).

## RESULTS

### Significant decrease of hydration and increase of TEWL in AD

We have summarized the details of clinical information from the patients and included *FLG* mutations (Table S1) and data on clinical severity as the objective score of atopic dermatitis (OSCORAD), SC hydration, TEWL, and SC thickness and in three representative regions (both the flexor and extensor aspects of the forearm, as well as the back; Table 1 and Figure 1). Scores for regions in each AD patient are shown in Table S2. SC hydration in AD patients was decreased in all the three regions of the body, as shown in Table 1 and Figure 1.

There were significant differences in SC hydration between filaggrin-related AD and normal controls on the back ($P<0.01$) and on the extensor aspect of the forearm ($P<0.05$), and to a lesser extent between non-filaggrin AD and normal control skin on the extensor aspect of the forearm ($P<0.05$). Average SC hydration values from the three regions were reduced in filaggrin-related AD patients compared with normal controls ($P<0.01$).

Transepidermal water loss values in each group are summarized in Table 1 and Figure 1. TEWL in non-filaggrin AD patients was significantly increased compared with that in normal controls on the extensor aspect of the forearm, on the back and the average of the three regions ($P<0.01$) and slightly reduced on the flexor aspect of the forearm ($P<0.05$). There was a significant TEWL increase in non-filaggrin AD patients compared with filaggrin-related AD individuals on the extensor aspect of the forearms ($P<0.05$), on the back ($P<0.05$), and for the average TEWL in the three regions ($P<0.05$).

It was statistically confirmed that SC hydration was significantly lower and that the TEWL was significantly higher in the filaggrin-related AD compared to those of the non-filaggrin AD by using the Wilcoxon rank sum test and Turkey–Kramer's honestly significant difference test.

### SC thickness was significantly increased in filaggrin-related AD compared with that in non-filaggrin AD

Stratum corneum thickness in normal controls, filaggrin-related AD, and non-filaggrin AD is summarized in Table 1 and Figure 1.

Stratum corneum thickness in filaggrin-related AD was significantly increased compared to that of normal controls on the flexor aspect of the forearm ($P<0.05$), on the extensor aspect of the forearm ($P<0.01$), and on the back ($P<0.05$). Interestingly, there was a significant increase in SC thickness from filaggrin-related AD individuals compared with back skin from non-filaggrin AD patients ($P<0.05$). Average SC thickness was remarkably increased in filaggrin-related AD compared with normal controls ($P<0.01$) and compared to non-filaggrin AD patients ($P<0.05$).

Increased SC thickness in filaggrin-related AD was verified by conventional histology, as follows. SC thickness measured

**Table 1. Summary of the patients' SC hydration, TEWL, and SC thickness**

| Group | Flexor aspect of the forearm | Extensor aspect of the forearm | Back | Average of the three regions |
|---|---|---|---|---|
| *SC hydration (μs) (95% confidence interval)* | | | | |
| Filaggrin-related AD | 7.67 ± 5.98 (3.87–11.46) | 6.28 ± 5.00* (3.10–9.46) | 9.46 ± 6.63** (5.25–13.67) | 7.80 ± 5.17** (4.51–11.09) |
| Non-filaggrin AD | 9.93 ± 5.61 (6.37–13.49) | 7.39 ± 6.30* (3.38–11.39) | 14.65 ± 8.88 (9.01–20.29) | 10.66 ± 5.11 (7.41–13.91) |
| Control | 11.84 ± 6.40 (7.78–15.91) | 12.71 ± 4.48 (9.86–15.56) | 20.40 ± 7.43 (15.68–25.12) | 14.99 ± 5.06 (11.77–18.20) |
| *TEWL ($g m^{-2}$ per hour) (95% confidence interval)* | | | | |
| Filaggrin-related AD | 14.20 ± 5.58 (10.65–17.75) | 11.05 ± 3.70# (8.70–13.39) | 12.68 ± 6.49# (8.55–16.80) | 12.64 ± 3.90# (10.17–15.12) |
| Non-filaggrin AD | 20.44 ± 19.29* (8.18–32.69) | 19.94 ± 11.98** (12.32–27.55) | 20.87 ± 11.57** (13.52–28.22) | 20.42 ± 12.51** (12.47–28.37) |
| Control | 7.07 ± 2.45 (5.51–8.62) | 6.40 ± 1.77 (5.27–7.52) | 6.84 ± 1.47 (5.91–7.78) | 6.77 ± 1.74 (5.67–7.87) |
| *SC thickness (μm) (95% confidence interval)* | | | | |
| Filaggrin-related AD | 57.74 ± 34.90* (35.56–79.92) | 72.87 ± 50.27** (40.93–104.81) | 62.24 ± 55.61*,# (26.91–97.57) | 64.28 ± 42.28** (37.42–91.15) |
| Non-filaggrin AD | 40.92 ± 32.88 (20.03–61.81) | 42.92 ± 19.63 (30.45–55.39) | 26.14 ± 15.31 (16.41–35.87) | 36.66 ± 18.30 (25.03–48.29) |
| Control | 28.09 ± 12.55 (20.11–36.06) | 22.73 ± 10.10 (16.31–29.15) | 20.45 ± 7.59 (15.63–25.27) | 23.76 ± 8.88 (18.11–29.40) |

Abbreviations: AD, atopic dermatitis; SC, stratum corneum; TEWL, transepidermal water loss.
*$P<0.05$, **$P<0.01$ vs Control #$P<0.05$ vs non-filaggrin AD (Tukey–Kramer's honestly significant difference test).

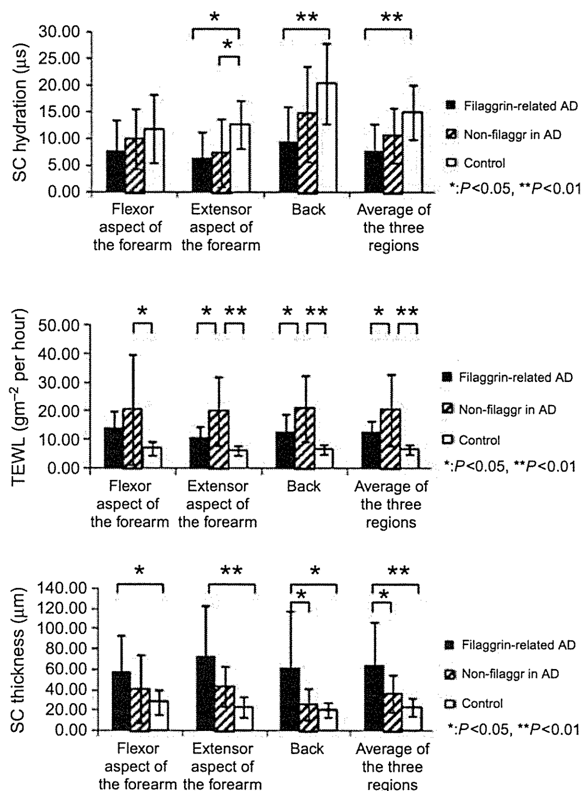


**Figure 1. SC hydration, TEWL, and SC thickness on the flexor and extensor aspects of the forearm, on the back and average of the three regions.** (Top) Comparison of SC hydration (μs) between filaggrin-related AD, non-filaggrin AD and the control group. (Middle) Comparison of TEWL ($g\,m^{-2}$ per hour) between filaggrin-related AD, non-filaggrin AD and the control group. (Bottom) Comparison of SC thickness (μm) between filaggrin-related AD, non-filaggrin AD and the control group. Data with *P*-values $^{*}P<0.05$ were evaluated as significant and $^{**}P<0.01$ were evaluated as highly significant.

in conventional histological slides was 42.8 μm in patient 3 (filaggrin-related AD), 23.8 μm in patient 21 (non-filaggrin AD), and 14.2 μm in patient 27 (IV without concomitant AD; Figure 2). SC thickness as measured by the corneometer was 47.6 μm in patient 3 (filaggrin-related AD), 25.3 μm in patient 21 (non-filaggrin AD), and 14.7 μm in patient 27 (IV without concomitant AD). These results confirmed that SC thickness data obtained using the corneometer are reliable and reflect the true SC thickness. Using hematoxylin and eosin-stained sections from patient 3 (filaggrin-related AD), additional layers of corneocytes in the SC were seen. Thus, the increase in SC thickness in filaggrin-related AD seems to be due to increased layers of corneocytes.

### AD clinical severity was correlated with SC barrier defects indicated by TEWL and SC hydration in filaggrin-related AD, but not in non-filaggrin AD

There was no significant difference in AD severity as indicated by OSCORAD between filaggrin-related AD and those of non-filaggrin AD using the Wilcoxon rank sum test and box-whisker plots; OSCORAD (interquartile range or interquartile interval: filaggrin-related AD, 14.71–31.93; non-filaggrin AD, 26.88–35.75; Figure S1).

In filaggrin-related AD, negative correlation was confirmed by simple regression analysis between the clinical AD severity indicated with OSCORAD and average SC hydration on all the three examined sites (correlation coefficient $r=-0.65$, $P<0.05$; Figure 3). Simple regression analysis revealed a significant, positive correlation between the OSCORAD and average TEWL on all the three examined sites (correlation coefficient $r=0.81$, $P<0.005$) and between the OSCORAD and average SC thickness (correlation coefficient $r=0.59$, $P<0.05$; Figure 3).

In non-filaggrin AD, simple regression analysis revealed that there was no significant correlation between the OSCORAD and average TEWL (correlation coefficient $r=0.01$, $P>0.5$), between the OSCORAD and average SC hydration (correlation coefficient $r=-0.21$, $P>0.5$), or between the OSCORAD and SC thickness (correlation coefficient $r=-0.05$, $P>0.5$; Figure 3).

### Significant correlation was obtained between the OSCORAD and specific IgE for house dust, mite allergen, and cat dander in filaggrin-related AD

We have shown the clinical history including the duration of AD, presence, or absence of AD family history, complication of asthma, rhinitis, and seasonal changes of disease activity, and laboratory data including peripheral blood eosinophil count (EOS), lactate dehydrogenase (LDH), total serum IgE, and allergen-specific IgE tests (IgE-multiple antigen simultaneous test (MAST), Table S1).

Atopic dermatitis family history was frequently observed in both AD patient groups (filaggrin-related AD, 10/12; non-filaggrin AD, 7/12). As complications, asthma (filaggrin-related AD, 5/12; non-filaggrin AD, 6/12), and rhinitis (filaggrin-related AD, 7/12; non-filaggrin AD, 9/12) were frequently seen. Patients whose skin lesions tended to get worse in winter were 3/12 in filaggrin-related AD and 2/12 in non-filaggrin AD. Patients whose skin lesions tend to get worse in summer were 0/12 in filaggrin-related AD and 4/12 in non-filaggrin AD. Due to the limited number of patients, it is difficult to draw firm conclusions about the clinical features including complications, family history, and seasonal changes in disease severity.

The IgE-MAST score of both AD groups showed high average, including IgE-MAST against house dust (filaggrin-related AD, 16.01; non-filaggrin AD, 24.00), mite (filaggrin-related AD, 63.41; non-filaggrin AD, 66.00), grass pollen (filaggrin-related AD, 23.46; non-filaggrin AD, 20.35), cedar pollen (filaggrin-related AD, 10.41; non-filaggrin AD, 11.66), fungal allergen (filaggrin-related AD, 6.33; non-filaggrin AD, 9.52), canine dander (filaggrin-related AD, 18.86; non-filaggrin AD, 37.07), feline dander (filaggrin-related AD, 29.64; non-filaggrin AD, 34.38), egg albumen (filaggrin-related AD, 4.21; non-filaggrin AD, 6.80), milk (filaggrin-related AD, 2.92; non-filaggrin AD, 2.32), wheat (filaggrin-related AD, 2.06; non-filaggrin AD, 2.30), and soy beans (filaggrin-related AD, 5.12; non-filaggrin AD, 2.90). No significant difference was seen in IgE-MAST