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Decreased Filaggrin Level May Lead to Sweat Duct Obstruction in Filaggrin Mutant Mice

Journal of Investigative Dermatology (2017) 137, 248–251; doi:10.1016/j.jid.2016.07.036

TO THE EDITOR

Although sweat is considered to be one of the major aggravating factors of atopic dermatitis (AD), recent studies have indicated the beneficial effects of sweat on host defense and anti-inflammatory mechanisms (Murota et al., 2015; Watabe et al., 2013). Several studies have demonstrated that sweating is attenuated in patients with AD (Matsui et al., 2014a, 2014b), but the precise underlying mechanisms remain unclear.

Filaggrin (FLG) is a key protein involved in skin barrier function that is known to be decreased in the epidermis of patients with AD (Otsuka et al., 2014; Palmer et al., 2006). FLG contributes to stratum corneum (SC) hydration and skin pH. FLG also plays an important role in keratinocyte integrity, because a decreased FLG level gives rise to SC disintegration and abnormal keratinocyte differentiation (Brown and McLean, 2012; Pendaries et al., 2014). We consider that a decreased FLG expression in keratinocytes may affect the structure and function of sweat ducts that go through keratinization, which may eventually lead to a decrease of sweat secretion.

To examine this hypothesis, we performed an iodine starch test, which involves the subcutaneous injection of acetylcholine into the footpads of wild-type (C57BL/6N (B6)) and *Flg* mutant mice (for further details of our materials and methods, see [Supplementary Materials and Methods](#) online, and the study protocol was approved by the Animal Experimentation Committee of Kyoto University). Of note, the *Flg* mutant mice harbor only an *Flg* mutation that was segregated from flaky tail mice (double-homozygous *Flg* and *matted* [*ma*] mutations), and were backcrossed more than 10 times with wild-type B6 mice. The number of sweat spots (represented by black dots) was significantly decreased in *Flg* mutant mice compared with wild-type mice (Figure 1a and b). In addition, SC conductance, which is an indicator of SC hydration, after subcutaneous acetylcholine injection, gradually increased in nonanesthetized mice. The increment of SC conductance of the *Flg* mutant mice was significantly lower compared with that of the wild-type B6 mice (Figure 1c). We confirmed that the skin conductance level from the abdomen, where free of sweat glands,

exhibited a significantly lower skin conductance compared with that of the footpads ([Supplementary Figure S1](#) online). Next, to confirm that sweating is decreased in *Flg* mutant mice, we analyzed the water content and main chemical compositions of the skin at various depths using in vivo confocal Raman spectroscopy as reported previously (Amano et al., 2015). After subcutaneous acetylcholine injection, the percentage change from the baseline for water content was significantly higher in wild-type mice compared with *Flg* mutant mice (Figure 1d). Interestingly, lactate, which is abundant in sweat (Watabe et al., 2013), was also significantly decreased near the skin surface in *Flg* mutant mice compared with wild-type mice (Figure 1e). These findings suggest that *Flg* mutation causes the decrease in sweating in the murine model.

Next, we histologically evaluated the acrosyringia, the intraepidermal part of the sweat duct, using a standard hematoxylin and eosin staining. The number of obstructed acrosyringia was pronouncedly higher in *Flg* mutant mice compared with wild-type mice (Figure 2a and b). To evaluate the nature of the obstructed material seen in the sections, we performed immunofluorescence staining using an antibody against mouse keratin 6 that is expressed only in the sweat ducts and not in the eccrine glands (Taylor et al., 2012). Interestingly,

Abbreviations: AD, atopic dermatitis; FLG, filaggrin; SC, stratum corneum

Accepted manuscript published online 31 August 2016; corrected proof published online 7 October 2016

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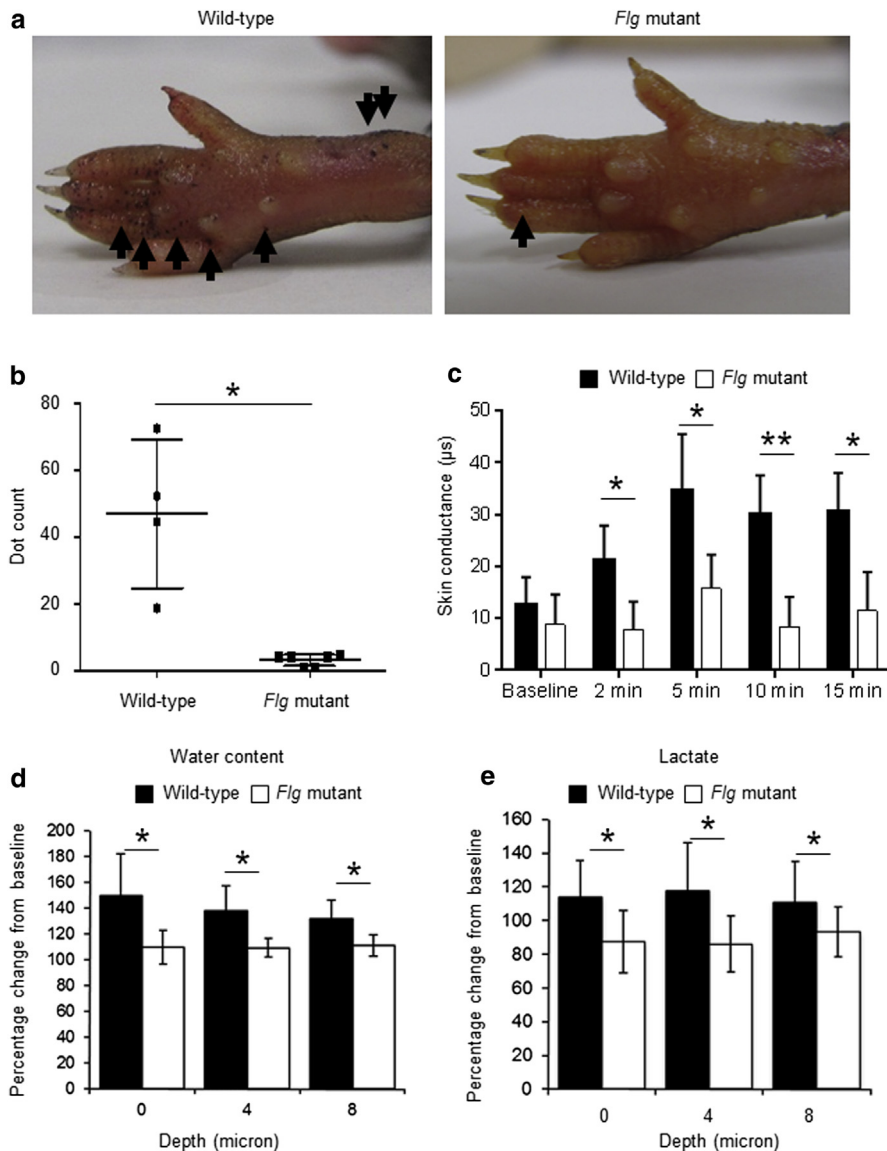


Figure 1. *Flg* mutant mice showed sweating impairment on stimulation with acetylcholine (ACh) injection. (a) Iodine starch test: after injections with ACh into the hind paws, black dots, representing sweat spots (arrows), can be observed. (b) The number of sweat spots was significantly decreased in *Flg* mutant mice. (c) Skin conductance representing stratum corneum hydration: ACh was injected into the footpads and skin conductance levels at baseline, 2, 5, 10, and 15 minutes were obtained. (b, c) Statistics were analyzed by the unpaired *t*-test, Mann-Whitney test, and multiple *t*-tests, respectively. The data shown are the representative of three independent experiments. In vivo confocal Raman spectrometry measurements of the mice food pads after an injection with ACh. (d) Water content; the percentage change from the baseline of water content was higher in wild-type mice than in *Flg* mutant mice. (e) Lactate; percentage change from the baseline was significantly higher in *Flg* mutant mice than in wild-type mice. Statistical analysis was conducted by the unpaired *t*-test. Number of mice = 13 in each group; **P* < 0.05, ***P* < 0.01.

the amorphous substances were stained positively with the anti-mouse keratin 6 (Figure 2c). These findings suggest that keratinous material in the sweat ducts might give rise to the obstruction.

To examine this, we employed the clear, unobstructed brain imaging cocktails and computational analysis (CUBIC) method, a tissue-transparentizing protocol (Susaki et al.,

2014), to better visualize and measure the acrosyringial wall thickness. The acrosyringial wall thickness was significantly thicker in *Flg* mutant mice (Figure 2d and e). This observation indicated that a reduced FLG level caused structural changes in the sweat duct and contributed to the attenuation of sweating. The proposed mechanisms of decreased sweating include obstruction

in the acrosyringium caused by abnormalities in the sweat duct structures and/or common sudomotor nerves that controlled sweating. Thus, we evaluated sweat duct peripheral nerve innervation/distribution in the experimental mice by immunohistochemistry with S100 staining, and positive staining nerves were observed in both wild-type and *Flg* mutant mice. The number of nerves identified did not show significant differences (Figure 2f and g). These results suggested that the decrease in sweat response might not be due to a reduction in the number of peripheral nerve bundles supplying the sweat glands.

In this study, we demonstrate that the reduced FLG level but not skin eczema may contribute to acrosyringium obstruction that finally leads to reduced sweating. These findings suggest that FLG controls the functions or contributes to the integrity of the acrosyringial wall, where, consistently, a high expression level of FLG is detected in healthy human skin (Ishida-Yamamoto et al., 1994). Several studies have confirmed that sweating is reduced in individuals with AD (Matsui et al., 2014a, 2014b), and that a reduction in epidermal FLG occurs irrespective of *Flg* mutation status in patients with AD because T helper type 2 cytokines are known to decrease FLG expression (Howell et al., 2009). Therefore, an acquired deficiency of FLG may as well contribute to a decreased sweating in AD.

Importantly, the size of sweat glands varies considerably and largely correlates with the difference in the rate of sweating in humans and mice (Sato and Sato, 1983). The sweat glands and/or ducts in *Flg* mutant mice appeared to be morphologically different from those in wild-type mice: they were larger in size and more crooked. Thus, sweat secretion in *Flg* mutant mice could be impaired by the increased luminal pressure due to such a morphological change. In addition, myoepithelial cells surrounding secretory coils that serve as mechanical support are particularly important in sweating response. Further studies to investigate the functional defects of myoepithelial cells in *Flg* mutant mice are of great interest. Although the precise relationship between the acrosyringial wall and the obstructed material should

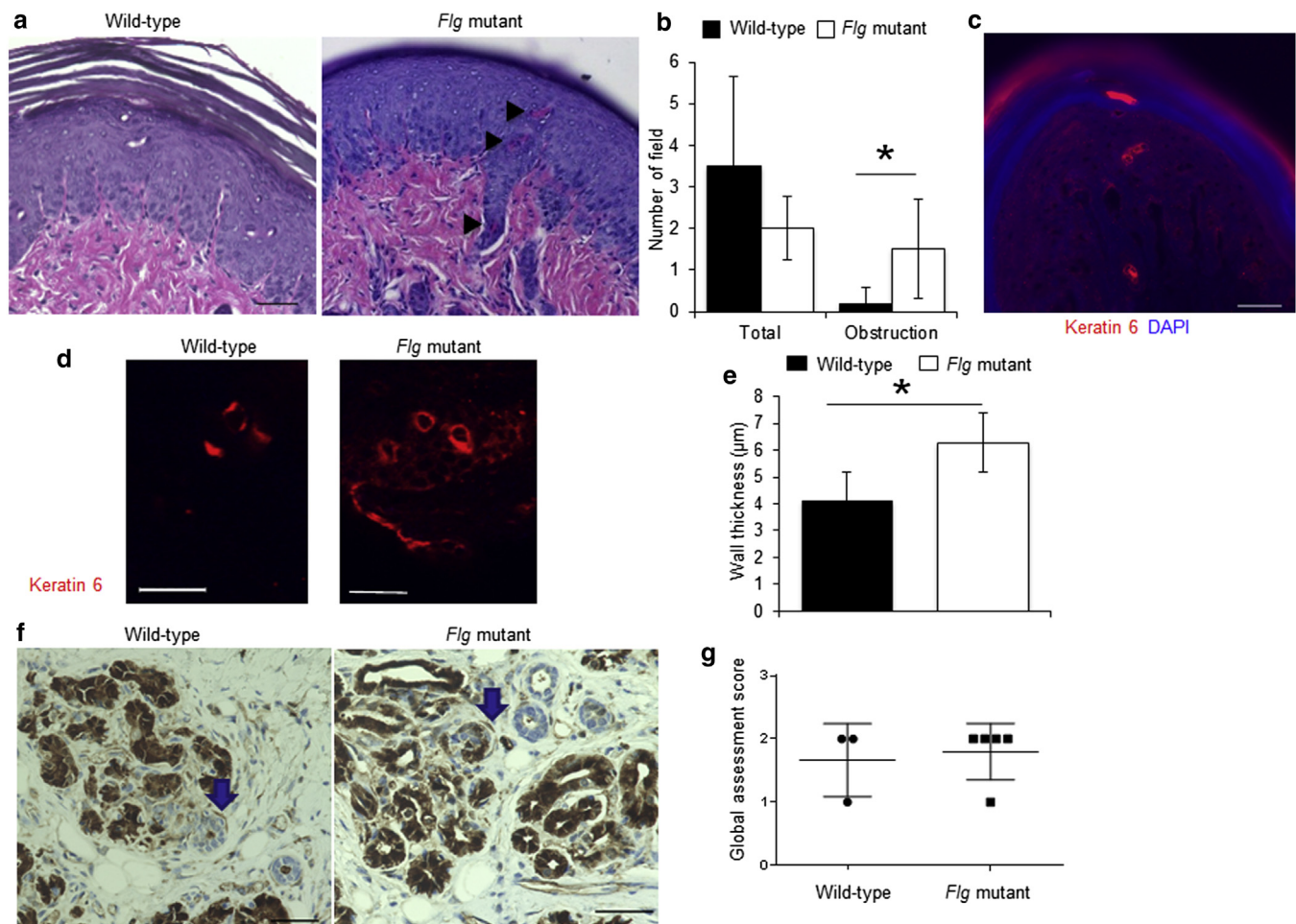


Figure 2. The blockage at the acrosyringium was detected in *Flg* mutant mice. (a) Hematoxylin and eosin sections show a blockage of acrosyringia in *Flg* mutant mice by an amorphous eosinophilic material (arrow head); original magnification: $\times 400$ and scale bar: $40\ \mu\text{m}$. (b) The ratio of total number to obstructed ducts was significantly higher in *Flg* mutant mice. The sections were examined by a certified dermatopathologist (JW). The total number of acrosyringia and the number of obstructed acrosyringia were counted under a magnification of $\times 100$ for three consecutive fields in each mouse. Statistical analysis was performed by the unpaired *t*-test and Mann-Whitney test. Wild-type, $n = 6$, and *Flg* mutant, $n = 8$. (c) Immunofluorescence staining using antibody against mouse cytokeratin 6 to identify sweat ducts. The amorphous substances were stained positively with the anti-mouse cytokeratin 6; cytokeratin 6 in red, DAPI for nuclear staining in blue. Original magnification: $\times 400$ and scale bar: $40\ \mu\text{m}$. (d) Photomicrographs show the cross sections of acrosyringia in wild-type and *Flg* mutant mice. The CUBIC method and two-photon microscopy were used to study the detailed structures of the sweat ducts. Images were analyzed by Imaris8.1 (Bitplane, Zurich, Switzerland). The courses of sweat ducts were traced using slice mode. The cross sections of acrosyringia were identified when the ducts became spirally aligned. Cytokeratin 6 in red; scale bars: $30\ \mu\text{m}$. (e) The acrosyringial wall thickness was significantly thicker in *Flg* mutant mice than in wild-type mice. The wall thickness (μm) was calculated by outer diameter (μm) – inner diameter (μm). Two to five ducts were measured in each mouse, $n = 5$ in each group. Statistical analysis was performed by the unpaired *t*-test and Mann-Whitney test. (f) Immunohistochemical staining using S100 was performed to identify the number of peripheral nerves in proximity to the sweat glands. Nerve bundles were detected in both types of mice; original magnification: $\times 400$ and scale bar: $40\ \mu\text{m}$. (g) The number of nerves in each mouse was assessed by a certified dermatopathologist (JW) employing a global assessment score (0 = no S100-positive nerve cells per sweat gland unit, 1 = 1–2 S100-positive nerve cells per sweat gland unit, 2 = 3–5 S100-positive nerve cells per sweat gland unit, 3 = more than 5 S100-positive nerve cells per sweat gland unit). There were no statistically significant differences between groups (wild-type, $n = 3$, and *Flg* mutant, $n = 5$). The statistics were analyzed by the Mann-Whitney test, $*P < 0.05$.

be addressed in the future, our study may provide an insight into the mechanism of decreased sweating in patients with AD.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Hiromi Doi for her excellence in maintaining the mice and help with the laboratory techniques. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministries of Education, Culture, Sports, Science

and Technology, Japan Agency for Medical Research and development (AMED) and by Ministry of Health, Labor and Welfare Sciences (MHLW) research grants, and Precursory Research for Embryonic Science and Technology.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2016.07.036>.

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IRF4 Polymorphism Is Associated with Cutaneous Squamous Cell Carcinoma in Organ Transplant Recipients: A Pigment-Independent Phenomenon

Journal of Investigative Dermatology (2017) 137, 251–253; doi:10.1016/j.jid.2016.07.038

TO THE EDITOR

Cutaneous squamous cell carcinoma (cSCC) is the most common cancer in solid organ transplant recipients (OTRs), with a U.S. incidence of 1,355 per 100,000 (Garrett et al., unpublished data) and a 65- to 100-fold increased risk over the general population (Hartevelt et al., 1990; Jensen et al., 1999; Zwald and Brown, 2011). Primary risk factors for posttransplantation cSCC are white race, male sex, older age at transplantation, and extent and duration of immunosuppression (Gogia et al., 2013; Mansh et al., 2016; Singer et al., 2012; Zwald and Brown, 2011). An additional risk factor is Fitzpatrick skin type, a scale that describes patient ability to suntan versus sunburn (Fitzpatrick, 1988; Gogia et al., 2013).

Recently, a genome-wide association study (GWAS) was performed for cSCC (Asgari et al., 2016). This study reported increased cSCC risk associated with polymorphisms in six pigment-related loci and found that these influenced cSCC risk independent of pigment-related phenotypes. In this study, we investigated the association between pigment-related single-nucleotide polymorphisms (SNPs) and hazard for cSCC in organ transplant recipients (OTRs), independent of Fitzpatrick skin type.

Methods

Subjects in this study were white OTRs drawn from a previously described cohort and enrolled from patient-targeted outreach between 2004 and 2008 (Gogia et al., 2013). Subjects

consented for a cross-sectional survey and medical record review. Of the 694 subjects in the cohort, 639 (92%) were white; 453 gave blood for genotyping (71%). Subjects provided written informed consent according the procedures approved by the University of California, San Francisco Institutional Review Board.

Samples were genotyped using the Axiom Genome-Wide Human Array system, with the EUR array (Affymetrix, Santa Clara, California) used for the Kaiser Permanente Research Program on Genes, Environment and Health (Asgari et al., 2016; Hoffmann et al., 2011). Of 453 subjects, 44 (9.7%) failed quality control checks based on a call rate of less than 97%; 13 were removed on the basis of chromosome X heterozygosity requiring males to be less than 20% and females greater 10%; five were excluded for cSCC before transplantation (left-censoring), leaving 388 for analysis. We excluded 8,529 SNPs

Abbreviations: cSCC, cutaneous squamous cell carcinoma; GWAS, genome-wide association study; HR, hazard ratio; OTR, organ transplant recipient; SNP, single-nucleotide polymorphism

Accepted manuscript published online 24 August 2016; corrected proof published online 7 October 2016

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