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REVIEW

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The roles of ABCA12 in keratinocyte differentiation and lipid barrier formation in the epidermis

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Key words: ABCA12, congenital ichthyosiform erythroderma, harlequin ichthyosis, lamellar granules, lamellar ichthyosis

Abbreviations: ABC, ATP-binding cassette; ABCA12, ATP-binding cassette transporter sub-family A member 12; CIE, congenital ichthyosiform erythroderma; HDL, high-density lipoprotein; HI, harlequin ichthyosis; LG, lamellar granule; LI, lamellar ichthyosis; PPAR, peroxisome proliferator-activated receptor

ABCA12 is a member of the large superfamily of ATP-binding cassette (ABC) transporters, which bind and hydrolyze ATP to transport various molecules across limiting membranes or into vesicles. The ABCA subfamily members are thought to be lipid transporters. ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids in lamellar granules to the apical surface of granular layer keratinocytes. Extracellular lipids, including ceramide, are thought to be essential for skin barrier function. *ABCA12* mutations are known to underlie the three main types of autosomal recessive congenital ichthyoses: harlequin ichthyosis, lamellar ichthyosis and congenital ichthyosiform erythroderma. *ABCA12* mutations lead to defective lipid transport via lamellar granules in the keratinocytes, resulting in malformation of the epidermal lipid barrier and ichthyosis phenotypes. Studies of ABCA12-deficient model mice indicate that lipid transport by ABCA12 is also indispensable for intact differentiation of keratinocytes.

Introduction

ABCA12 is a member of the large superfamily of ATP-binding cassette (ABC) transporters,[1] which bind and hydrolyze ATP to transport various molecules across limiting membranes or into vesicles.[2] The ABCA subfamily members are thought to be lipid transporters.[3] The ABC transporter A12 (ABCA12) is known to be a key molecule in keratinocyte lipid transport (**Fig. 1**).[4-6] ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with the transport of lipids in lamellar granules to the apical surface of granular layer keratinocytes.[4] This article reviews the importance of *ABCA12* as a keratinocyte lipid transporter in the context of keratinocyte differentiation and skin lipid barrier formation.

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ABCA12 and Other ABCA Transporters

Several genetic diseases have been shown to be caused by mutations in ABCA subfamily genes. The ABCA subfamily, of which the *ABCA12* gene is a member, comprises 12 full transporters and one pseudogene (ABCA11) that are essential for lipid transport and secretion.[7] Three ABCA genes of the same subfamily as ABCA12 have been also implicated in the development of genetic diseases affecting cellular lipid transport. In the phylogenetic tree of ABCA subfamily proteins, ABCA3 is very close ABC12.[1] ABCA3 is known to aid lipid secretion from alveolar type II cells via lamellar granules,[8] and an ABCA3 deficiency recently was reported to underlie a fatal lung surfactant deficiency in newborns,[9] a condition that often leads to death shortly after birth.

Another important member of the ABCA subfamily is ABCA1. Mutations in the human ABCA1 gene underlie familial alpha-lipoprotein deficiency syndrome (Tangier disease), which suggests that ABCA1 is a major regulator of high-density lipoprotein metabolism.[10-12]

ABCA2, ABCA3 and ABCA7 mRNA levels were reported to be upregulated after sustained cholesterol influx,[13,14] suggesting that ABCA transporters are involved in the transmembrane transport of endogenous lipids.[15] From these facts, transporters in the ABCA subfamily are thought to be involved in the transmembrane transport of cholesterol.[16-18] Interestingly, ABCA3, a member of the same protein superfamily as ABCA12, functions in pulmonary surfactant lipid secretion through the production of similar lamellar-type granules within lung alveolar type II cells.[8,9]

The Role of ABCA12 in the Transport of Lipids into Lamellar Granules

Extracellular lipids, including ceramide, are thought to be essential for skin barrier function.[19] Mutations in the ABCA12 gene (*ABCA12*) were reported to underlie the devastating phenotype seen in harlequin ichthyosis (HI) patients,[4,20] the most severe keratinization disorder. ABCA12 mutations underlying HI are thought to have major disruptive defects on ABCA12 lipid

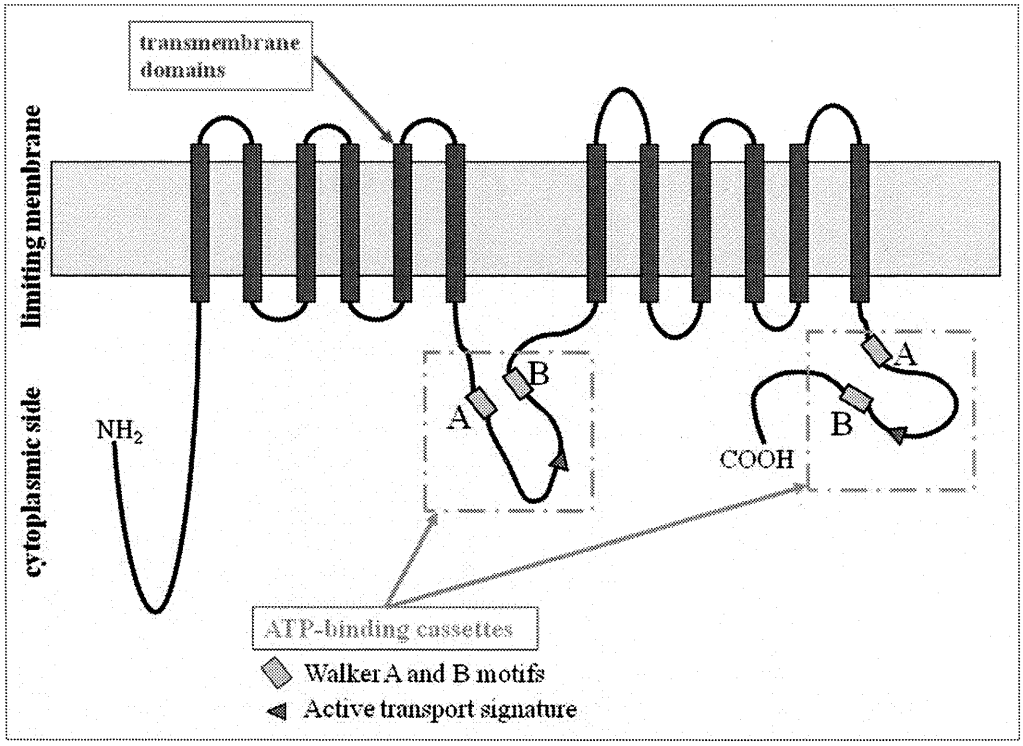

Figure 1. ABCA12 protein structure and domains. Analysis of the predicted structure of the ABCA12 protein reveals features typical of ABCA transporters.[1]

transporter function, resulting in the HI phenotype.[4] We reported that ABCA12 is localized in lamellar granules (LGs) in the granular layer keratinocytes and might work in lipid transport through LGs to form the intercellular lipid layers in the stratum corneum.[4] We have analyzed the epidermal localization of ABCA12 in comparison with the localization of Golgi apparatus markers and LG-associated proteins together with transglutaminase 1, because LGs are thought to be a part of the continuous tubular network that originates from the Golgi apparatus and extends to the cell membrane.[5] We employed antibodies to well-established marker molecules of each part of the Golgi apparatus-LG-cell membrane network, i.e., the GM130, anti-TGN-46 and anti-transglutaminase 1 antibodies (B.C1), as markers for cis-Golgi, trans-Golgi and cell membrane, respectively. Our results show that ABCA12 localizes throughout the entire Golgi apparatus to LGs at the cell periphery, mainly in the granular layer keratinocytes. These results suggest that ABCA12 works in the transport of lipids from the Golgi apparatus to LGs in the granular layer cells.[5] Double-labeling immunofluorescence staining of cultured keratinocytes clearly indicates that ABCA12 is localized from the Golgi apparatus (colocalized with cis-Golgi marker GM130 and trans-Golgi marker TGN-46) to the cell periphery (close to the plasma membrane stained with transglutaminase 1). ABCA12 fails to colocalize with TGase1, a cell membrane-bounding protein, both in vivo and in cultured keratinocytes and ABCA12 is thought to distribute only very sparsely on the cell membrane.[5]

In normal human epidermis, ABCA12 is expressed throughout, but mainly in the upper spinous and granular layers.[5] Immunofluorecent double labeling reveals that the majority of ABCA12 colocalizes with glucosylceramide in the cytoplasm within the upper spinous and granular cells (**Fig. 2**).[5] Immunofluorescence labeling on ultrathin cryosections clearly reveals localization of ABCA12 and glucosylceramide. In immunofluorescence labeling under light microscopy, ABCA12 and glucosylceramide staining almost completely overlap within the granular layer keratinocytes.[5] Post-embedding immunoelectron microscopy reveals both ABCA12 and glucosylceramide in the LGs of the uppermost granular layer keratinocytes.[5] Under immunoelectron microscopy using ultrathin cryosections, glucosylceramide labeling is seen with the lamellar structures in the LGs. ABCA12 immunogold labeling is observed on or close to the membrane surrounding LGs in the uppermost granular layer cells.[5]

We can hypothesize that ABCA12 is likely to be a membrane lipid transporter that functions in the transport of lipids from the trans-Golgi network to LGs at the keratinocyte periphery (**Fig. 3**).[4,5] Recently, it was confirmed biochemically that ABCA12 deficiency impairs glucosylceramide accumulation in lamellar granules and that ABCA12 transports glucosylceramide to the inner side of lamellar granules.[6] In addition, ceramide was reported to upregulate ABCA12 expression via PPAR delta-mediated signaling pathway, providing a substrate-driven, feed-forward mechanism for regulation of this key lipid transporter.[21] More recently, studies using *Abca12*$^{-/-}$ mice suggested that ABCA12 plays an important role in the normal differentiation of epidermal keratinocytes.[22]

ABCA12 Mutations and Ichthyoses

ABCA12 mutations are known to underlie the three main types of autosomal recessive congenital ichthyoses: harlequin ichthyosis

(HI), lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE). Harlequin ichthyosis is the most severe ichthyosis subtype. Affected patients show plate-like scales over the whole body, severe eclabium and ectropion.

In 2010, a review of the literature was performed to identify all known *ABCA12* mutations in patients with ARCI and 56 *ABCA12* mutations were described (online database: www.derm-hokudai.jp/ABCA12/) in 66 unrelated families, including 48 HI, 10 LI and 8 CIE families.[23] Mutations have been reported among autosomal recessive congenital ichthyosis patients with African, European, Pakistani/Indian and Japanese backgrounds in most parts of the world. Of the 56 mutations, 36% (20) are nonsense, 25% (14) are missense, 20% (11) comprise small deletions, 11% (6) are splice site, 5% (3) are large deletions and 4% (2) are insertion mutations. At least 62.5% (35) of all the reported mutations are predicted to result in truncated proteins. There is no apparent mutation hot spot in *ABCA12*, although mutations underlying the LI phenotype are clustered in the region of the first ATP-binding cassette.[24]

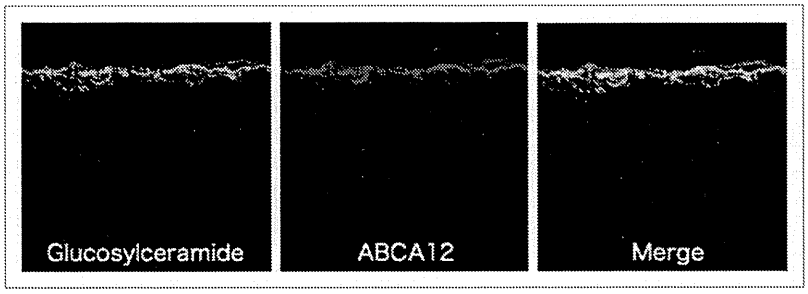

Figure 2. Immunofluorescence labeling using ultrathin cryosections as substrates reveal that glucosylceramide (green) and ABCA12 (red) overlap in the granular layers (derived from ref. 5).

In HI-affected epidermis, several morphologic abnormalities have been reported, including abnormal lamellar granules in the keratinocyte granular layer and a lack of extracellular lipid lamellae within the stratum corneum.[25-28] Lack of ABCA12 function subsequently leads to disruption of lamellar granule lipid transport in the upper keratinizing epidermal cells, resulting in malformation of the intercellular lipid layers of the stratum corneum in HI.[4] Cultured epidermal keratinocytes from an HI patient carrying *ABCA12* mutations demonstrate defective glucosylceramide transport, and this phenotype is recoverable by in vitro *ABCA12* corrective gene transfer.[4] Intracytoplasmic glucosylceramide transport has been studied using cultured keratinocytes from a total of three patients harboring *ABCA12* mutations. One patient was homozygous for the splice site mutation c.3295-2A>G[4] and another was compound heterozygous for p.Ser387Asn and p.Thr1387del.[29] Only one heterozygous mutation, p.Ile1494Thr, was identified in the other patient.[30] Cultured keratinocytes from all three patients showed apparently disturbed glucosylceramide transport, although this assay is not quantitative.

In addition, defective lamellar granule formation was observed in the skin of two CIE patients with *ABCA12* mutations.[30] Electron microscopy revealed that, in the cytoplasm of granular layer keratinocytes, abnormal, defective lamellar granules are assembled with some normal-appearing lamellar granules.[30]

Formation of the intercellular lipid layers is essential for epidermal barrier function. In ichthyotic skin with ABCA12 deficiency, defective formation of the lipid layers is thought to result in a serious loss of barrier function and a likely extensive compensatory hyperkeratosis.[31]

One hypothetical pathomechanism for ABCA12 deficiency in autosomal recessive congenital ichthyosis is explained by the "differentiation defect theory," which is derived from the clinical features of HI patients. Fetuses affected with HI start developing the ichthyotic phenotype while they are in the amniotic fluid, where stratum corneum barrier function is not required. According to this theory, barrier defects cannot be involved directly in the pathogenesis of the HI phenotype, at least during the in utero fetal period. In light of this, disturbed keratinocyte differentiation is speculated to play an important role in the pathogenesis of the HI phenotype. In fact, three-dimensional culture studies reveal that HI keratinocytes differentiate poorly according to morphologic criteria and show reduced expression of keratin 1 and defective conversion from profilaggrin to filaggrin.[32]

In an *ABCA12*-ablated organotypic co-culture system, which is an in vitro model of HI skin, the expression of keratinocyte late differentiation-specific molecules is dysregulated.[33] The expression of specific proteases associated with desquamation (kallikrein 5 and cathepsin D) is dramatically reduced in the ABCA12-ablated organotypic co-culture system.[33] In this model system, *ABCA12* ablation results in a premature terminal differentiation phenotype.[33] Furthermore, in mutant mice carrying a homozygous spontaneous missense mutation, loss of Abca12 function leads to the premature differentiation of basal keratinocytes.[34] In contrast, in our *Abca12*$^{-/-}$ HI model mice, immunofluorescence and immunoblotting of *Abca12*$^{-/-}$ neonatal epidermis revealed defective profilaggrin/filaggrin conversion and reduced expression of the differentiation-specific molecules (loricrin, kallikrein 5 and transglutaminase 1), although their mRNA expression is upregulated.[22] These data suggest that ABCA12 deficiency may lead to disturbances in keratinocyte differentiation during fetal development, resulting in an ichthyotic phenotype at birth. These observations suggest that ABCA12 deficiency might have global effects on keratinocyte differentiation, resulting in both impaired terminal differentiation and premature differentiation of the epidermis.

HI patients often die in the first week or two of life. However, those that survive beyond the neonatal period phenotypically improve within several weeks after birth. To clarify the mechanisms of phenotypic recovery, we studied grafted skin and keratinocytes from *Abca12*-disrupted (*Abca12*$^{-/-}$) mice.[22] *Abca12*$^{-/-}$ skin grafts kept in a dry environment exhibited dramatic improvements in all the abnormalities seen in the model mice. Increased transepidermal water loss, a parameter of barrier defect, is remarkably decreased in grafted *Abca12*$^{-/-}$ skin. Ten-passage sub-cultured *Abca12*$^{-/-}$ keratinocytes show restoration of intact ceramide distribution, differentiation-specific protein

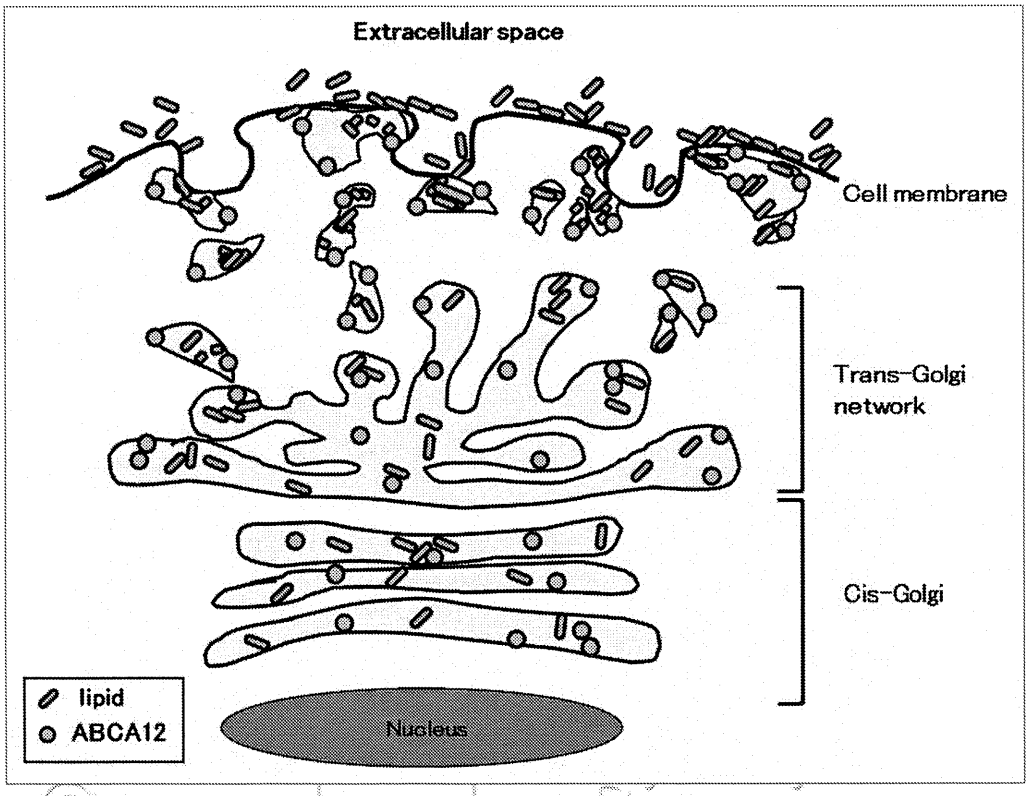

Figure 3. Scheme of ABCA12 distribution from the cis-Golgi, trans-Golgi network to lamellar granules in the upper spinous and granular layer keratinocytes (derived from ref. 5).

expression and profilaggrin/filaggrin conversion, which are defective in the primary-culture.[22] These observations suggest that, during maturation, *Abca12*$^{-/-}$ epidermal keratinocytes regain normal differentiation processes, although the exact mechanisms of this restoration remain unknown.[22]

ABCA12-deficient Animal Models

Recently, bioengineered disease models were established to investigate the ichthyotic pathomechanisms that result from defective ABCA12 function and to aid the development of innovative treatments for ichthyosis with ABCA12 deficiency.

We transplanted cultured keratinocytes from patients with HI and succeeded in reproducing HI skin lesions in immunodeficient mice.[35] These reconstituted HI lesions show similar changes to those observed in HI patients' skin. In addition, we generated *Abca12*-disrupted (*Abca12*$^{-/-}$) mice that closely reproduced the human HI phenotype, showing marked hyperkeratosis with eclabium and skin fissures.[36] Lamellar granule abnormalities and defective ceramide distribution were remarkable in the epidermis. Skin permeability assays of *Abca12*$^{-/-}$ mouse fetuses revealed severe skin barrier dysfunction after the initiation of keratinization. Surprisingly, the *Abca12*$^{-/-}$ mice also demonstrated lung alveolar collapse immediately after birth. Lamellar bodies in alveolar type II cells from *Abca12*$^{-/-}$ mice lack normal lamellar structures.[36] The level of surfactant protein B, an essential component of alveolar surfactant, is reduced in the *Abca12*$^{-/-}$ mice.[36] Another group independently developed *Abca12*$^{-/-}$ mice and these also had the clinical features of HI.[37]

A study in one *Abca12*-disrupted HI model mouse indicates that a lack of desquamation of skin cells, rather than enhanced proliferation of basal-layer keratinocytes accounts for the five-fold thickening of the *Abca12*$^{-/-}$ stratum corneum determined by in vivo skin proliferation measurements.[37] It was suggested that this lack of desquamation is associated with a profound reduction in skin linoleic esters of long-chain omega-hydroxyceramides and a corresponding increase in their glucosylceramide precursors. Omega-hydroxyceramides are required for correct skin barrier function, and these results from HI model mice establish that ABCA12 activity is required for the generation of the long-chain ceramide esters that are essential for the development of normal skin structure and function.[37]

In addition, a mouse strain carrying a homozygous spontaneous missense mutation was reported to show skin manifestations similar to ichthyosis.[34] Lipid analysis of *Abca12* mutant epidermis revealed defects in lipid homeostasis, suggesting that *Abca12* plays a crucial role in maintaining lipid balance in the skin.[34] The cells from the *Abca12* mutant mouse have severely impaired lipid efflux and intracellular accumulation of neutral lipids.[34] Abca12 was also demonstrated as a mediator of Abca1-regulated cellular cholesterol efflux.[34] Injection of a morpholino designed to target a splice site at the exon 4/intron 4 junction to block *Abca12* pre-mRNA processing induced altered skin surface contours, disorganization of the melanophore distribution, pericardial edema

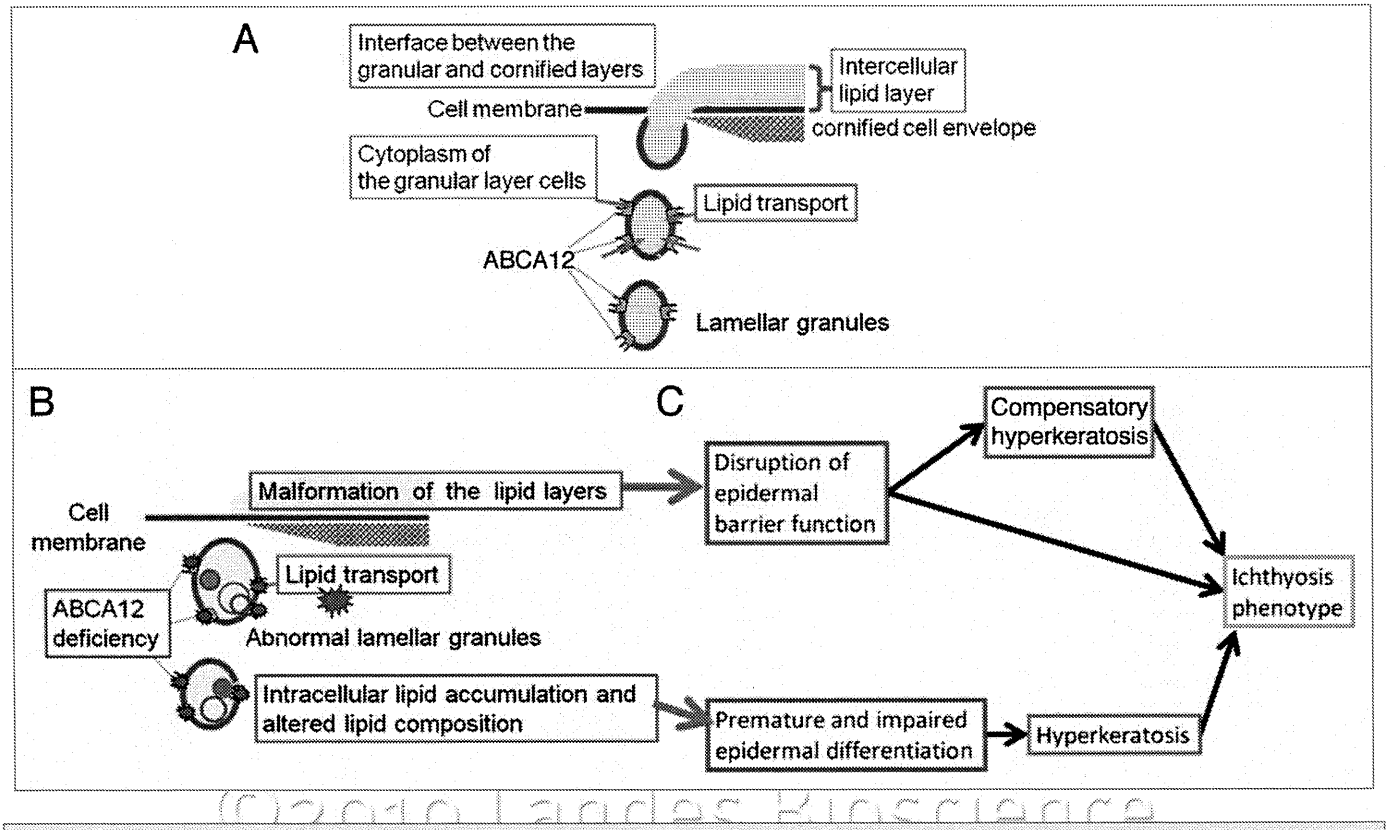

Figure 4. Physiological role(s) of ABCA12 in lipid trafficking of epidermal keratinocytes and the model of pathogenetic mechanisms in ichthyosis phenotypes caused by ABCA12 deficiency. (A) Model of how ABCA12 transports lipids in epidermal keratinocytes. (B) Model of how loss of ABCA12 function leads to lipid abnormality and lipid barrier malformation in the upper epidermis. (C) It is hypothesized that the combination of lipid barrier defects and disturbed keratinocyte differentiation cause hyperkeratosis and the ichthyosis phenotype (derived from ref. 20).

and enlargement of the yolk sac at 3 days post-fertilization in the larvae of zebrafish. It was also associated with premature death at around 6 days post-fertilization. These results suggest that *Abca12* is an essential gene for normal zebrafish skin development and provide novel insight into the function of ABCA12 (reported at the Annual Meeting of the Society for Investigative Dermatology 2010; Abstract, Frank et al. J Invest Dermatol 2010; 130:86).

Using our Abca12$^{-/-}$ HI model mice, we tried fetal therapy with systemic administration to the pregnant mother mice of retinoid or dexamethasone, which are effective treatments for neonatal HI and neonatal respiratory distress, respectively. However, neither of these improved the skin phenotype nor extended the survival period.[36] Retinoids were also ineffective in in vivo studies using cultured keratinocytes from the model mice.[22]

Conclusion

ABCA12 is apparently localized in the membrane of the trans-Golgi network and lamellar granules in the upper epidermis, mainly in the uppermost spinous and granular layer cells. Our own studies and a review of the literature suggest that ABCA12 works in the transport of lipids into the trans-Golgi network and lamellar granules, to accumulate lipids that are essential to skin barrier formation. Consequently, the lipids packed in lamellar granules are secreted to the extracellular space to form intercellular lipid layers in the stratum cornuem, which is important for skin barrier function (**Fig. 4**). In addition, model mouse studies indicate that lipid transport by ABCA12 is indispensable for intact differentiation of keratinocytes. To elucidate the mechanisms of ABCA12 in keratinocyte differentiation/proliferation, further accumulation of data is needed.

Acknowledgements

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REVIEW ARTICLE

Filaggrin Gene Defects and the Risk of Developing Allergic Disorders

Rinko Osawa[1], Masashi Akiyama[2] and Hiroshi Shimizu[1]

ABSTRACT

Filaggrin is a key protein that facilitates terminal differentiation of the epidermis and formation of the skin barrier. Mutations in the gene encoding filaggrin (*FLG*) have been identified as the cause of ichthyosis vulgaris (IV) and have been shown to be major predisposing factors for atopic dermatitis (AD). Approximately 40 loss-of-function *FLG* mutations have been identified in patients with ichthyosis vulgaris (IV) and/or atopic dermatitis (AD) in Europe and Asia. Major differences exist in the spectra of *FLG* mutations observed between different ancestral groups. Notably, prevalent *FLG* mutations are distinct between European and Asian populations. Many cohort studies on *FLG* mutations in AD have revealed that approximately 25-50% of AD patients harbour filaggrin mutations as a predisposing factor. In addition, *FLG* mutations are significantly associated with AD-associated asthma. The risk for developing allergic rhinitis is also significantly higher with a *FLG* mutation, both with and without accompanying AD. Recent studies have hypothesized that skin barrier defects caused by *FLG* mutations allows allergens to penetrate the epidermis and to interact with antigen-presenting cells, leading to the development of atopic disorders including asthma. The restoration of skin barrier function seems a feasible and promising strategy for prophylactic treatment of AD patients with *FLG* mutations.

KEY WORDS

allergic rhinitis, asthma, atopic dermatitis, atopic eczema, filaggrin, *FLG*, ichthyosis vulgaris

INTRODUCTION

Filaggrin, which is processed from profilaggrin, is a key protein that facilitates terminal differentiation of the epidermis and formation of the protective skin barrier. In the outer granular layer of the epidermis, filaggrin is associated with keratin intermediate filaments and it aids their packing into bundles. In terminally differentiated keratinocytes, filaggrin is cross-linked to the cornified cell envelope, which constitutes an insoluble barrier in the stratum corneum, protecting the organism against environmental agents and preventing epidermal water loss.[1] Mutations in the filaggrin gene (*FLG*, GenBank accession number NM_002016) have been identified as the underlying cause of the relatively common genetic keratinization disorder ichthyosis vulgaris (IV; OMIM 146700), which is clinically characterized by scaling, especially on the extensor limbs, and by palmoplantar hyperlinearity.[2-4] Although *FLG* is very difficult to analyse because of its large size (>12 kb) and highly repetitive nature, a polymerase chain reaction (PCR) strategy that permits routine and comprehensive sequencing of the entire coding region has recently been developed.[5] Until now, around 40 *FLG* mutations have been reported, and the prevalent *FLG* mutations are distinct in each population.[6] Based on the information of population-specific *FLG* mutations, many cohort studies on *FLG* mutations in atopic dermatitis (AD) have been performed and approximately 25-50% of patients with AD were revealed to harbour *FLG* mutations as a predisposing factor.[7] In several studies, these mutations also demonstrated strong association with other allergic phenotypes, including asthma and allergic rhinitis.[8] This article gives an overview of *FLG* population genetics with respect to AD, asthma and allergic rhinitis.

SKIN BARRIER

The skin serves numerous functions, the most obvious being its primary protective or barrier function. The large surface area of the skin puts it in constant

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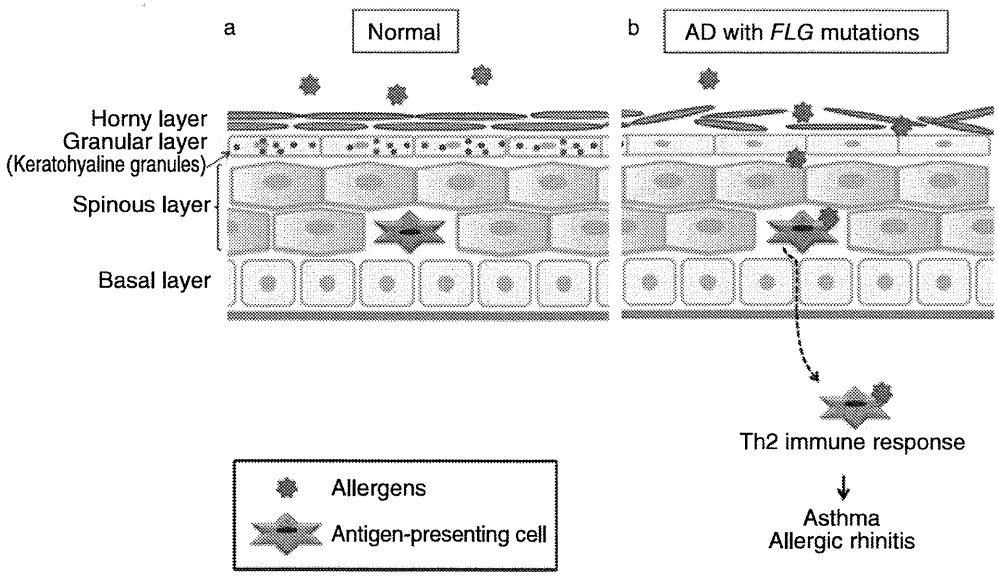

Fig. 1 Skin barrier function and allergic risk. (**a**) Normal skin: In the granular layer, keratohyaline granules composed of profilaggrin predominate. Upon terminal differentiation of keratinocytes, the products of degradation, filaggrins, aggregate keratin filaments and flatten the keratinocytes to form an effective barrier against external allergens. (**b**) In IV and AD with *FLG* mutation, there is a reduction or complete absence of filaggrin. The defective skin barrier allows external antigens to penetrate the epidermis, where they interact with antigen-presenting cells (Langerhans cells and dermal dendritic cells), which might further initiate the Th2 immune response and lead to the development of atopic disorders. (Modified from[3].)

contact with environmental pollutants, irritants, and allergens, and the horny layer of skin forms the major protective barrier between the body and the environment.

The terminal differentiation of keratinocytes (Fig. 1) results in the formation of an impenetrable barrier (the horny layer) that is the uppermost layer of the epidermis. The successive stages of keratinocytic differentiation in the epidermal layers are the basal cell, spinous cell, and granular cell layers. When spinous cells differentiate into granular cells, they begin to accumulate keratinocyte-specific proteins involved in terminal differentiation of the horny layer.

The skin barrier of the horny layer shows three key features: (i) intercellular lipid layers, (ii) the cornified cell envelope and (iii) the keratin filament network and keratohyaline granules.[9] Genetic defects in these components may result in various cutaneous disorders, such as ichthyosis, which is characterized by dry, thickened, scaly or flaky skin. The word "ichthyosis" is from the Ancient Greek, *ichthys*, meaning "fish".

The keratin filament network is an important basic structure for maintaining the integrity and dimensions of the cornified cell, and the degraded products of the keratohyalin granules, filaggrins, aggregate the keratin filaments in apoptosed keratinocytes into bundles and promote the flattening of dead-cell remnants.[10-13]

Abnormalities in the barrier function of the horny layer have been hypothesized as permitting epicutaneous allergen exposure in atopic and asthmatic patients. Furthermore, these alterations may, in part, help to explain the recent dramatic increase in atopic and asthmatic disorders in humans living in industrialized nations.

FILAGGRIN EXPRESSION AND FUNCTION

The term 'filaggrin' is derived from filament aggregation protein. A giant inactive precursor, profilaggrin is a large, complex, highly phosphorylated polypeptide that is the main constituent of the keratohyalin granules that are visible in the granular cell layer of the epidermis (Fig. 1). The profilaggrin/filaggrin gene (*FLG*) resides on chromosome 1q21 and consists of three exons (Fig. 2). Exon 3 is extremely large (>12 kb) and encodes most of the profilaggrin polypeptides with almost completely homologous 10, 11 or 12 repeats. Filaggrin is initially synthesized as profilaggrin, a >400-kDa, highly phosphorylated, histidine-rich polypeptide that comprises an S100 calcium-binding domain, a B-domain and two imperfect filaggrin-repeat domains flanking 10 to 12 essentially identical filaggrin repeats, as well as a C-terminal domain (Fig. 2).[14,15] On terminal differentiation of keratinocytes, profilaggrin is dephosphorylated and

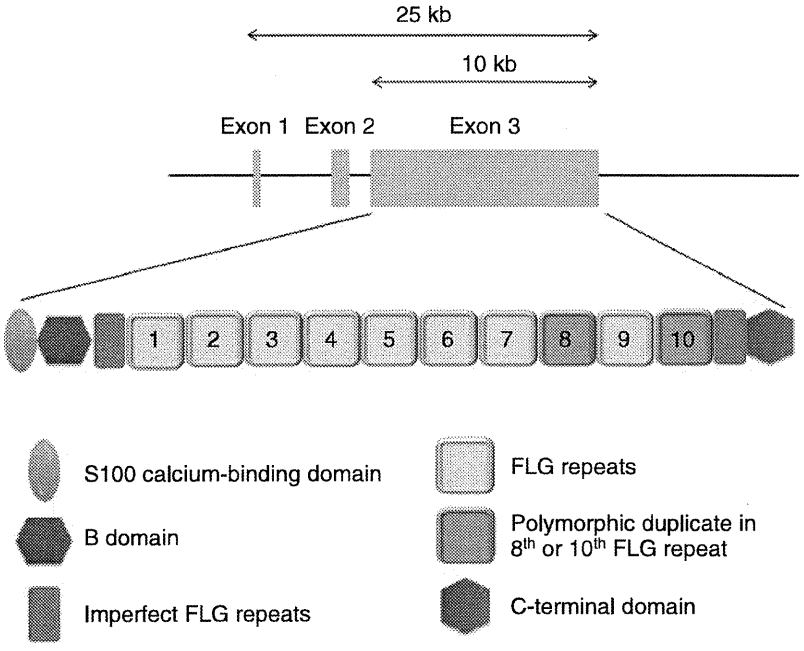

Fig. 2 The *FLG* gene, which is located within the epidermal differentiation complex on chromosome 1q21, comprises three exons and two introns. Exon 1 (15 bp) consists only of a 5' untranslated (UTR) sequence, exon 2 (159 bp) contains the translation initiation codon, and exon 3 contains a S100 calcium-binding domain, a B-domain and two imperfect filaggrin-repeat domains flanking 10 essentially identical filaggrin repeat domains, as well as a C-terminal domain. There exist polymorphic variations in the number of filaggrin repeats. Some individuals have duplication of the 8th and/or 10th filaggrin repeat(s).

cleaved into 10 to 12 essentially identical 37-kd filaggrin peptides. As mentioned above, the liberated filaggrin subsequently and highly efficiently aggregates the keratin filament, which causes the keratinocytes to collapse in the stratum corneum.[10,13] The collapsed crytoskeleton is crosslinked by transglutaminases to bind it to the cornified cell envelope. Filaggrin is subsequently degraded into amino acids that act to retain epidermal moisture.[13,16] Thus, filaggrin is a key protein during terminal differentiation and it is essential for the formation of a normal, intact, protective, and correctly moisturized skin barrier.[9,13]

FILAGGRIN DEFICIENCY CAUSED BY FLG MUTATIONS RESULTS IN ICHTHYOSIS VULGARIS (IV)

IV (OMIM 146700) is a common semidominant inherited skin disorder, estimated to affect 1 in 250 individuals. The onset is early childhood. It is characterized by generalized dry and scaly skin prominent on the extensor surfaces of limbs and on the lower abdomen, and it is associated with palmoplantar hyperlinearity (Fig. 3a, b).[2,17] The symptoms subside during the summer and aggravate during the winter, when the skin tends to dry. Histologically, a decrease in the size and number, or a complete absence, of keratohyalin granules in the epidermis is characteristic of IV. (Fig. 3c-f).[2,18] An association between IV and profilaggrin has long been suspected, but the gene that encodes profilaggrin, *FLG*, proved technically challenging to sequence. *FLG* resides on human chromosome 1q21 within the so-called epidermal-differentiation complex (EDC). The EDC is a dense cluster of genes involved in the terminal differentiation of the epidermis and the formation of the stratum corneum, the outermost dead cell compartment of the skin, where the main skin barrier function resides.

The initiation codon of the *FLG* gene is located in exon 2, although the bulk of the profilaggrin polyprotein in encoded by exon 3 (Fig. 2). Sequencing of exon 3 is problematic, not only because of its size (>12 kb) but also because it consists of between 10 and 12 tandemly arranged filaggrin repeat units. Some individuals have duplication of the 8th and/or 10th domain. The huge size, polymorphic variations in the number of filaggrin repeats, and highly repetitive nature prevent sequencing of the entire gene. Despite these difficulties, the improvement of PCR strategy by the use of long-range sequencing and multiple alignment techniques that permit comprehensive sequencing of the entire *FLG* gene have recently been

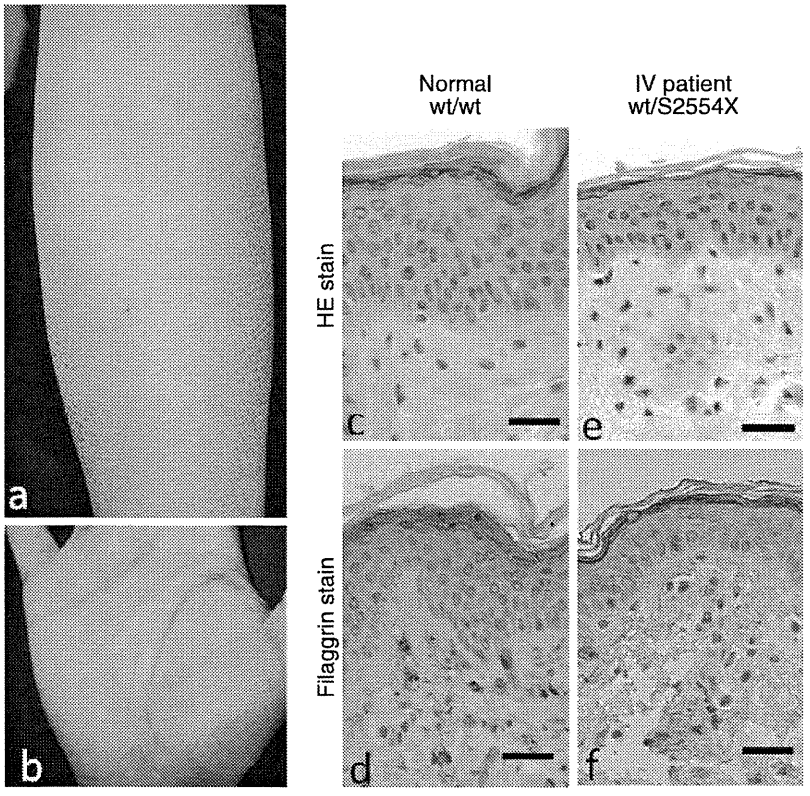

Fig. 3 Clinicopathological features of IV. (**a**) Marked, adherent scales are clearly visible on the pretibial region of this IV patient. (**b**) Marked plantar hyperlinearity is seen in this IV patient. (**c, e**) Hematoxylin and eosin staining. Normal control skin (**c**) shows abundant keratohyalin granules in the granular layers. In contrast, the IV patient who is heterozygous for S2554X (**e**) shows a lack of granular layers in the epidermis, where basophilic substances that resemble keratohyalin are present in only small amounts and only intermittently. (**d, f**) In immunohistochemical staining for filaggrin, normal control skin (**d**) stains strongly. The IV patient (**f**) shows a marked reduction in staining for filaggrin. Bar: 50 μm.

developed.[14,17] In 2006, two null mutations, R501X and 2282del4, in the *FLG* gene were first identified in patients with moderate or severe IV in 15 kindreds from Scottish, Irish, and European-American populations.[17] To date, approximately 40 loss-of-function *FLG* mutations have been identified in IV and/or AD in European populations and Asian populations (Fig. 4).[6,19] In addition, IV was found to exhibit semidominance, with incomplete penetrance (-90% in homozygotes). The homozygotes or compound heterozygotes had a severe form of IV, while the heterozygotes displayed mild or no phenotype.

The genotype/phenotype correlation in *FLG* mutations has not been clarified. *FLG* truncation mutations at any site within the profilaggrin peptide were reported uniformly to result in severe deficiency of profilaggrin/filaggrin processing.[14] Currently, it has been hypothesized that the profilaggrin C-terminal region is essential for proper processing of profilaggrin to filaggrin and, eventually, truncation at any site of profilaggrin results in abolishment of filaggrin/profilaggrin peptides. The hypothesis is supported by the finding of the nonsense mutation K4022X in the C-terminal incomplete filaggrin repeat. In the epidermis of patients carrying this mutation, profilaggrin/filaggrin peptides were remarkably reduced, even though *FLG* mRNA expression was not reduced significantly and the expressed mRNA included messages derived from both the wild-type alleles and the mutant alleles.[20] Histopathologically, however, the size of keratohyaline granules in the granular layers was decreased and immunohistochemically profilaggrin/filaggrin peptides were remarkably reduced in the patients' epidermis. These observations further support the hypothesis that the profilaggrin C-terminal region is essential for proper profilaggrin processing. It is now generally considered that all the truncation mutations lead to serious loss of filaggrin

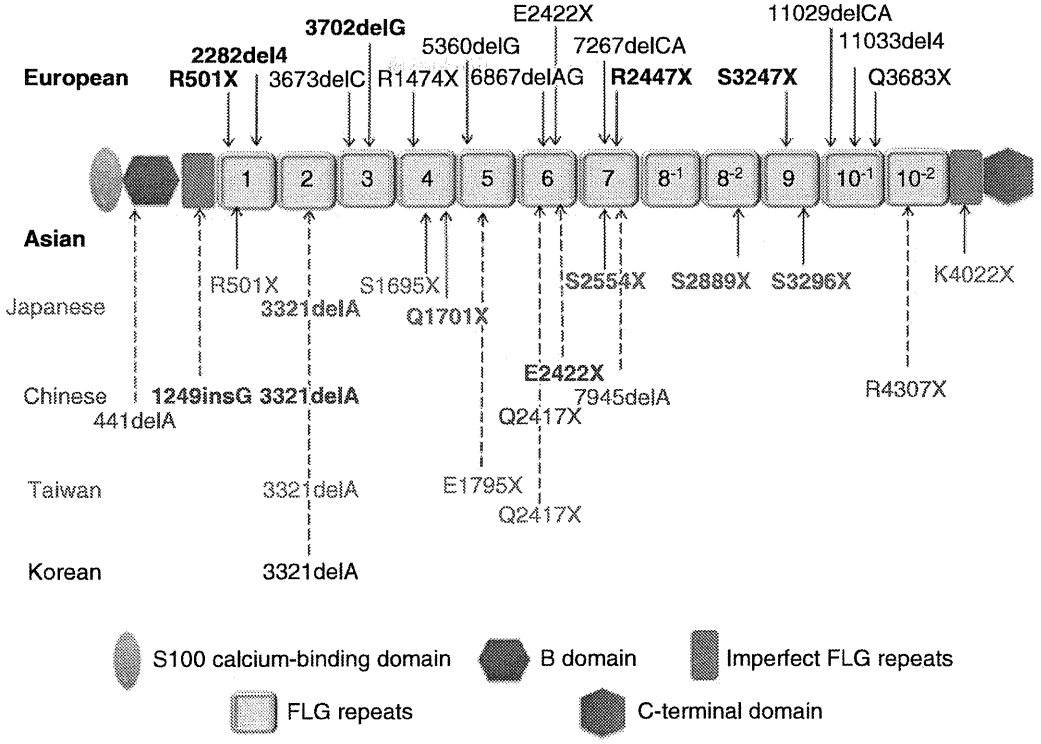

Fig. 4 Reported *FLG* mutations in a diagram of the profilaggrin peptide. Several of the mutations are rare, but a number of recurrent mutations have been identified (bold). Note that *FLG* mutations in the European and the Asian populations appear to be unique to each population. Only two mutations (R501X and E2422X) were reported in both European and Asian populations. The *FLG* mutations among Asian populations are shown (red = Japanese, blue = Chinese, brown = Taiwanese, black = Korean). Mutations are distributed widely in the profilaggrin sequence and the mutation K4022X is the most distal mutation in the C-terminal incomplete filaggrin repeat. The duplications of the 8th and 10th filaggrin repeats are represented as 8^{-1}, 8^{-2}, 10^{-1} and 10^{-2}.

peptides, resulting in the absence of genotype/phenotype correlations with regard to *FLG* mutations in IV and AD.

PREVALENT FILAGGRIN MUTATIONS ARE DISTINCT IN EACH RACE

To date, approximately 40 loss-of-function *FLG* mutations have been identified in IV and/or AD in European populations and Asian populations (Fig. 4).[6,19] Mutations in *FLG* were initially identified in European families.[17,21,22] To establish baseline *FLG* mutation data for the Japanese population, we performed *FLG* mutation searches in more than 30 Japanese families with IV, after sequencing methods for the entire *FLG* coding region had been established. We carried out comprehensive sequencing of the entire *FLG* coding region using an overlapping PCR strategy and identified four Japanese-population-specific mutations in *FLG*: 3321delA, S2554X, S2889X, and S3296X.[23,24] In 2009, we reported two additional novel *FLG* mutations, S1695X and Q1701X, in the Japanese population.[25] Furthermore, we studied 19 newly recruited Japanese patients with AD and identified a novel *FLG* nonsense mutation, K4022X, in one patient with AD without any other known Japanese *FLG* mutation.[20] In addition, one of the common European mutations, R501X, was reported in a Japanese family.[26] The study was repeated in other Asian populations, including Chinese,[27] Taiwanese[28] and Korean populations.[29] Only two identical mutations (R501X and E2422X) were reported in both European and Asian populations.[26,27] Further haplotype analysis of the European-specific mutation R501X in the Japanese family showed that the mutation was not inherited from an European ancestor but occurred de novo in Japan.[26] Among Asian populations, 3321delA was found in all four East Asian populations[23,26-29] and Q 2417X was reported in Chinese and Taiwanese populations.[27,28] These results have revealed the differences in filaggrin population genetics between Europe and Asia (Fig. 4). As mentioned above, most *FLG* mutations are specific to a population, such as Europeans, Japanese, Singaporeans, Chinese, and Taiwanese. Major differences exist in the spectra of

FLG mutations observed between different ancestral groups. Prevalent *FLG* mutations are distinct in both the European and the Asian populations. In addition, there is a need to assess the ancestral admixture in geographical regions in order to know precisely the spectrum and preferential occurrence of *FLG* mutations in different populations. Every population is likely to have a unique set of *FLG* mutations. For mutation screening, we have to obtain information on prevalent *FLG* mutations in each population.

FILAGGRIN MUTATIONS CONFER STRONG GENETIC SUSCEPTIBILITY TO ATOPIC DERMATITIS

AD, one of the most common skin disorders, affects 15-20% of children in the developed world. AD often presents with IV. AD is a pruritic skin disease that typically starts early in life. The onset is during the first 6 months of life in 45% of affected individuals, the first year of life in 60% of affected individuals, and before 5 years of age in at least 85% of affected individuals.[30] The hallmark of the disease is a pruritic dermatitis that localizes in different areas depending on age. In infancy it tends to affect the face and extensors of the lower legs, and in childhood the flexural areas; in adulthood the eruption has a more diffuse distribution. Other important diagnostic indications include xerosis of the skin, early age of onset, and a chronic, relapsing course. The incidence and prevalence of AD decreases with increasing age. AD is thought to have various heterogeneous etiologic factors, including genetic predisposing factors and environmental factors. Despite considerable efforts to elucidate genes that confer susceptibility to AD and to clarify the genetic background of atopic disorders, until recently no strong and reproducible genetic factor has been identified.[31] Transepidermal water loss (TEWL) and SC hydration, which are measurements of skin barrier function, were reported to be increased in AD patients due to their skin barrier insufficiency.[32] Significant correlations were observed between penetration rates of a hydrophilic dye and elevated IgE levels in patients with severe AD.[33] In addition, percutaneous penetration of sodium lauryl sulphate was reported to be increased in uninvolved skin of patients with AD.[34] Taken together, these findings strongly support the hypothesis that patients with AD have a skin barrier defect. Three clues suggested that *FLG* mutations play an important role in AD pathogenesis. First, dermatologists have recognized that AD often occurs in patient with IV, although the pathophysiological mechanisms of this co-occurrence have not been fully clarified.[35-37] Second, the linkage of AD to the chromosome locus on 1q21, which contains the epidermal differentiation complex where *FLG* resides, has been reported.[38] Third, decreased filaggrin expression has been reported in the skin of patients with AD at both the mRNA and the protein levels.[39,40]

Palmer *et al.* first reported that decreased or absent *FLG* expression due to loss-of-function mutations leads to impaired barrier function which manifests as AD.[21] They found that AD was manifested in heterozygous carriers of two null *FLG* mutations, R501X and 2282del4, with a relative risk (odds ratio) for AD of 13.4, implying a causal relationship. Thereafter, about twenty case-control analyses and eight familial analyses investigated the association between filaggrin gene defects and AD. Most of the studies were on Western European populations, but three case-control studies and one family study were on a Japanese population and one case-control study was on a North American population.[14,41-47] In the Japanese population, there are at least eight *FLG* mutations. We showed that about 27% of the patients in our Japanese AD case series carried one or more of these eight *FLG* mutations (OR: 9.94; 95%; CI: 3.77-26.2) and that these variants were also carried by 3.7% of the Japanese general control individuals.[20] Meta-analysis *FLG* mutation studies on AD, focusing on the European-prevalent mutations (R501X or 2282 del4) found an overall OR of 4.78 (95%; CI: 3.31-6.92) from the case-control studies and a summary OR of 1.99 (95%; CI: 1.72-2.31) from the family studies.[8] The strong association between *FLG* mutations and AD marked a milestone in the genetic study of complex allergic disorders. It was confirmed that the strong effect of *FLG* mutations on AD risk exceeds that of any other candidate predisposing gene for AD identified so far. Based on the information of population-specific *FLG* mutations, many cohort studies of AD for *FLG* mutations were performed and approximately 25-50% of AD patients were revealed to harbour *FLG* mutations as a predisposing factor.[6]

As mentioned above, every population is likely to have a unique set of *FLG* mutations. Population differences highlighted by *FLG* mutations make it difficult to perform worldwide screening for *FLG* mutations in patients with AD. We cannot perform *FLG* mutation screening in one population using the *FLG* mutations reported in other populations. For example, we cannot use the prevalent European *FLG* mutations when we screen Asian patients with AE. For mutation screening, we have to obtain information on prevalent *FLG* mutations in each population. It is therefore important to establish global population genetic maps for *FLG* mutations.

FLG MUTATIONS AND ASTHMA

The clinical cause of atopic disorders has been described as an atopic or allergic march. It involves sensitisation to food or aeroallergens, or both, in early life, progressing to eczema and wheezing within the first two years of life, and often leading to chronic asthma, rhinitis, and other clinical manifestations of atopic allergy in later life. Previous studies showed that 70% of patients with severe AD developed

asthma, compared with 30% of patients with mild AD, and approximately 8% of the general population.[48] Previous studies in European populations have reported that variants in the *FLG* gene are associated with eczema and concomitant asthma[41-45] or with eczema alone.[22] One recent meta-analysis study showed that *FLG* mutations are significantly associated with asthma (OR: 1.48; 95%; CI, 1.32-1.66). And strong effects for the compound phenotype of asthma plus eczema (OR: 3.29; 95%; CI, 2.84-3.82) were observed. In contrast, *FLG* mutations did not seem to be associated with asthma in the absence of eczema (OR: 1.11; 95%; CI: 0.88-1.41).[49]

To clarify whether *FLG* mutations are a predisposing factor for asthma in non-European populations, we studied 172 Japanese AE patients, 137 Japanese asthma patients and 134 unrelated Japanese control individuals. There is a statistically significant association between the eight *FLG* mutations and AE with asthma, and between the eight *FLG* mutations and AE without asthma. In the Japanese general asthma cohort, there was a statistically significant association between the eight *FLG* mutations and asthma with AE. There was no statistically significant association between the *FLG* mutations and overall asthma patients, nor between *FLG* mutations and asthma without AE. This Japanese cohort has a completely different *FLG* mutation spectrum from those in the European and the North American populations. However, our results clearly confirm the strong association of *FLG* mutations with our Japanese cohort of AE patients with asthma complications, and the association of *FLG* mutations and asthma patients with AE complications.[50]

The mechanism of the asthma risk associated with *FLG* null alleles is not yet fully understood. *FLG* is expressed in the skin and in the outer layers of the oral and nasal mucosae, but not in the respiratory epithelium of the nose or the lower airways.[51,52] Therefore it has been suggested that *FLG*-associated asthma is mediated by percutaneous priming[53] and/or secondary, possibly systemic, immunologic mechanisms stimulated through the impaired skin barrier. Recent studies hypothesized that skin barrier defects caused by *FLG* mutations allow allergens to penetrate the epidermis and to interact with antigen-presenting cells (Langerhans cells and dermal dendritic cells, which might further initiate Th2 immune response and lead to the development of atopic disorders including AD, asthma and allergic rhinitis.[53,54]

FILAGGRIN MUTATIONS AND ALLERGIC RHINITIS

Three case-control studies investigated the association between filaggrin gene defects and the risk of developing allergic rhinitis in people without AD.[42,55,56] Recent meta-analysis study showed that *FLG* mutations are significantly associated with allergic rhinitis without AD (OR: 1.78; 95%; CI: 1.16-2.73). In addition, the *FLG* mutations are significantly associated with allergic rhinitis with AD (OR: 2.84; 95%; CI: 2.08-3.88).[8] Filaggrin is expressed in the anterior vestibulum of the nose, but not in transitional and respiratory nasal epithelia.[56] Thus, it seems unlikely that *FLG* mutations exert organ-specific and localized effects in the upper airways. The mechanisms through which *FLG* mutations contribute to airway disease are not understood yet. Percutaneous priming and secondary immunologic effects from the induction of Th2 cytokines in epithelia are interesting hypotheses that need further investigation.

NOVEL TREATMENT FOR AD BASED ON RECENT FLG MUTATION STUDIES

The epidermal barrier dysfunction caused by *FLG* mutations has been recognized as a major contributor to the pathogenesis of AD over the past few years. The skin barrier defect is the primary event that initiates disease pathogenesis, allowing the entrance of numerous antigens into the epidermis in patients with AD. Thus, the restoration of skin barrier function seems a feasible and promising strategy for prophylactic treatment of AD patients with *FLG* mutation. There have been efficient clinical methods to restore skin barrier function, including the application of general moisturizers and specific lipid replacement therapy.[57] When used under nursing supervision, moisturizers have been shown to reduce topical steroid usage.[58] In addition, the topical application of ceramide-dominant lipid replacement therapy was proved effective in alleviating skin barrier defects and reducing AD severity significantly in childhood AD patients.[59]

Regarding the association between filaggrin deficiency and sensitization to specific antigens, allergen exposure during early life may increase the risk of AE, but the protective effect of reduction in allergen exposure remains uncertain. According to a population-based, longitudinal birth cohort study by Henderson *et al.*, eczema associated with *FLG* mutations presents in early life and is persistent.[60] In addition, a strong association was identified between *FLG* mutations and sensitisation to grass, house dust, mites, and cat dander. Our study revealed that AD disease severity and specific IgE for house dust, mite allergen, and cat dander were significantly correlated in *FLG* mutation-related patients with AD.[61] In light of this, if we select patients with *FLG* mutations and perform early intervention to reinforce/improve their skin barrier function and reduce sensitization to allergens, we may achieve a significant prophylactic effect against AD development. Further studies are required to clarify the preventive effect of early intervention against AD in high-risk, filaggrin-deficient children.

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Detection of Merkel cell polyomavirus in cutaneous squamous cell carcinoma before occurrence of Merkel cell carcinoma

To the Editor: In 2008, a previously unknown polyomavirus, Merkel cell polyomavirus (MCPyV), was identified in Merkel cell carcinoma (MCC) lesions and close association between MCPyV and MCC has been suggested.[1,2] However, to our knowledge, no previous reports have confirmed MCPyV infection in patients with MCC before the occurrence of MCC. We herein report a patient who developed squamous cell carcinoma (SCC) followed by MCC. MCPyV was detected in both tumors by polymerase chain reaction analysis.

A 78-year-old Japanese man who had been immunosuppressed as a result of diabetes mellitus noticed a nodule on his right cheek, and the tumor was simply resected (Fig 1, *A*). The tumor was diagnosed histopathologically as typical SCC. Coexisting MCC was not found anywhere in the resected specimen by either hematoxylin-eosin stain or immunostaining for cytokeratin 20. One year later, he presented with a subcutaneous nodule in his left axillary lymph node. Positron emission computed tomography showed a solid mass (Fig 1, *B*). Histopathological, immunohistochemical, and ultrastructural examination revealed that the tumor was a nodal MCC, although the primary lesion was not discovered. Fourteen months after the axillary dissection, he developed multiple metastatic MCC lesions and died 10 months later.

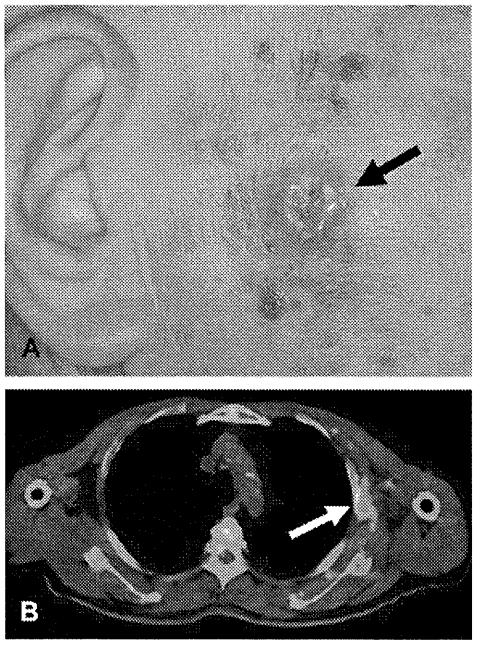

Fig 1. Squamous cell carcinoma (SCC) on right cheek (**A**) and Merkel cell carcinoma (MCC) in left axilla (**B**). **A**, Patient presented with crater-shaped nodule on right cheek (*arrow*). Resected tumor was typical, moderately differentiated SCC. **B**, Positron emission computed tomography showed solid mass 10 × 30 mm in size in left axilla (*white arrow*). Histopathologically, lesion was diagnosed as nodal MCC.

The VP1 region of MCPyV DNA was amplified from DNA samples both of the MCC and the SCC lesions (Fig 2, *A*). Copy numbers of MCPyV DNA large tumor (LT) domain were determined by quantitative real-time polymerase chain reaction using the β-globin gene as an internal control. The MCPyV-LT/β-actin in the MCC sample was $3.9 \times 10^4/4.8 \times 10^5$ ($=8.1 \times 10^{-2}$ copies per cell), and that in the SCC sample was $8.1 \times 10^2/2.0 \times 10^3$ ($=4.0 \times 10^{-3}$ copies per cell). Larger copy numbers of viral genome were obtained in the MCC lesion. Immunohistochemical staining demonstrated that the MCC cells were