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

I. Nemoto-Hasebe,* M. Akiyama,* T. Nomura,*† A. Sandilands,† W.H.I. McLean† and H. Shimizu*

*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
†Epithelial Genetics Group, Division of Molecular Medicine, University of Dundee, Colleges of Life Sciences and Medicine, Dentistry & Nursing, Dundee, U.K.

Correspondence
Masashi Akiyama.
E-mail: akiyama@med.hokudai.ac.jp

Accepted for publication
4 June 2009

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.

DOI 10.1111/j.1365-2133.2009.09406.x

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 (χ^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

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.

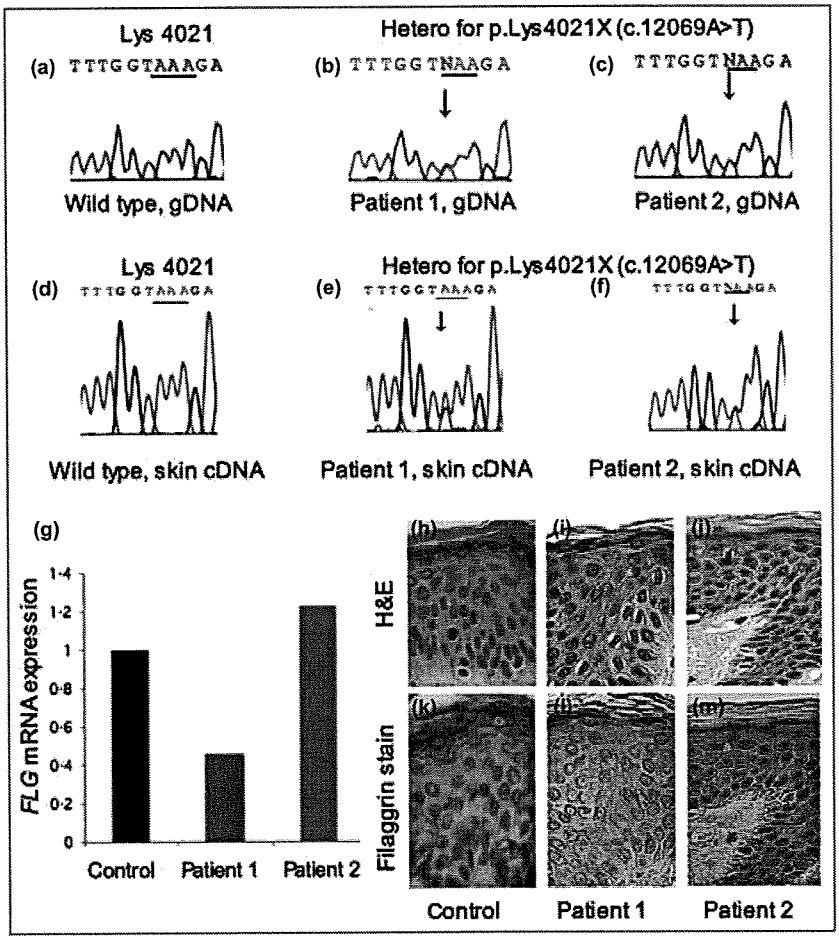

Fig 1. *FLG* mutation analysis and expression of p.Lys4021X mutant alleles. (a–c) Identification of the *FLG* mutation. Direct sequence analysis of *FLG* was performed on genomic DNA from peripheral leucocytes. (a) Normal control sequence from filaggrin repeat 11 in exon 3. (b, c) A heterozygous transition 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).

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 χ^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

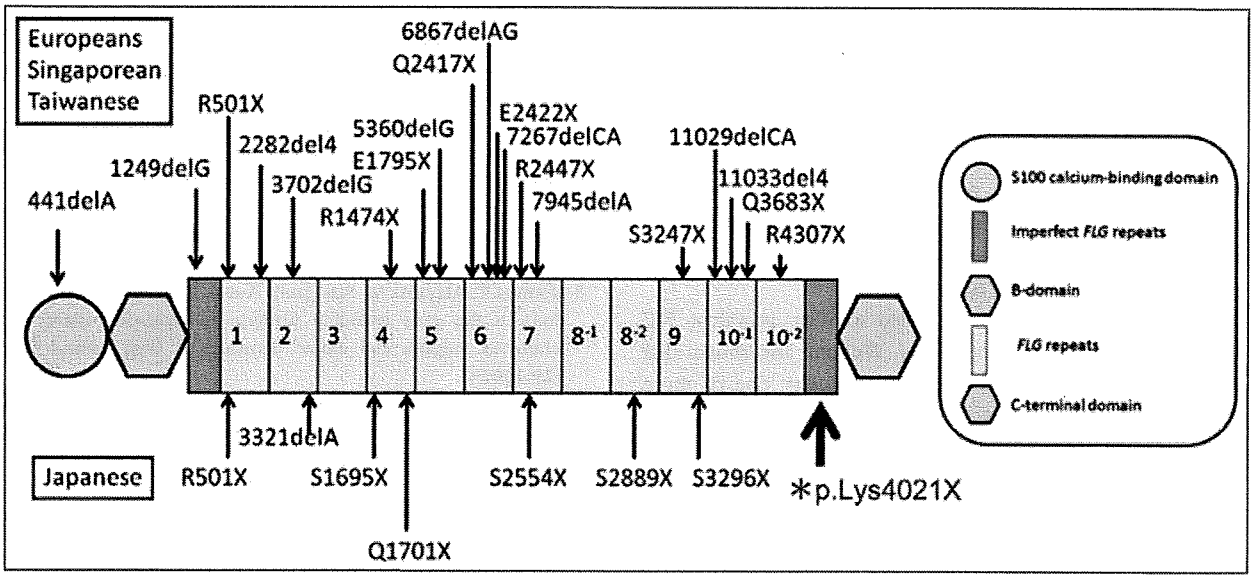

Fig 2. The present and previously reported *FLG* mutations are shown in a scheme of profilaggrin peptide. Some individuals have a duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8^{-1}, 8^{-2}, 10^{-1} and 10^{-2}. *Indicates the present mutation 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, $\chi^2 = 29{\cdot}218$, $P = 6{\cdot}50 \times 10^{-8}$; Fisher's exact test odds ratio = 9·94 (95% confidence interval 3·77–26·2, $P = 2{\cdot}35 \times 10^{-8}$).

(χ^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.

Acknowledgments

We thank the patients and their families for their participation. We also thank Ai Hayakawa for fine technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M.A. (Kiban B 20390304). Filaggrin research in the McLean laboratory is supported by grants from the British Skin Foundation, the National Eczema Society, the Medical Research Council (reference number G0700314), A*STAR, Singapore and anonymous donations from families affected by eczema in the Tayside Region of Scotland.

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ORIGINAL ARTICLE

Clinical Severity Correlates with Impaired Barrier in Filaggrin-Related Eczema

Ikue Nemoto-Hasebe[1], Masashi Akiyama[1], Toshifumi Nomura[1], Aileen Sandilands[2], WH Irwin McLean[2] and Hiroshi Shimizu[1]

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.

Journal of Investigative Dermatology (2009) **129,** 682–689; doi:10.1038/jid.2008.280; published online 25 September 2008

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

[1]*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan and* [2]*Epithelial Genetics Group, Division of Molecular Medicine, Colleges of Life Sciences and Medicine, Dentistry and Nursing, University of Dundee, Dundee, UK*

Correspondence: Dr Masashi Akiyama or Dr Hiroshi Shimizu, Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan.
E-mails: akiyama@med.hokudai.ac.jp or shimizu@med.hokudai.ac.jp

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*

Received 26 February 2008; revised 30 June 2008; accepted 30 July 2008; published online 25 September 2008

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).

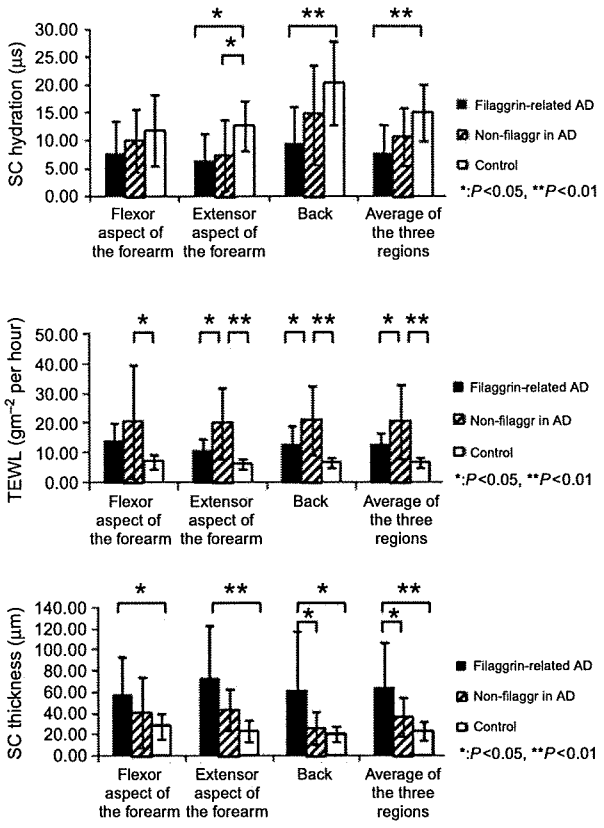

Figure 1. SC hydration, TEWL, and SC thickness on the flexor and extensor aspects of the forearm, on the back and average of the three regions. (Top) Comparison of SC hydration (μs) between filaggrin-related AD, non-filaggrin AD and the control group. (Middle) Comparison of TEWL ($g\,m^{-2}$ per hour) 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

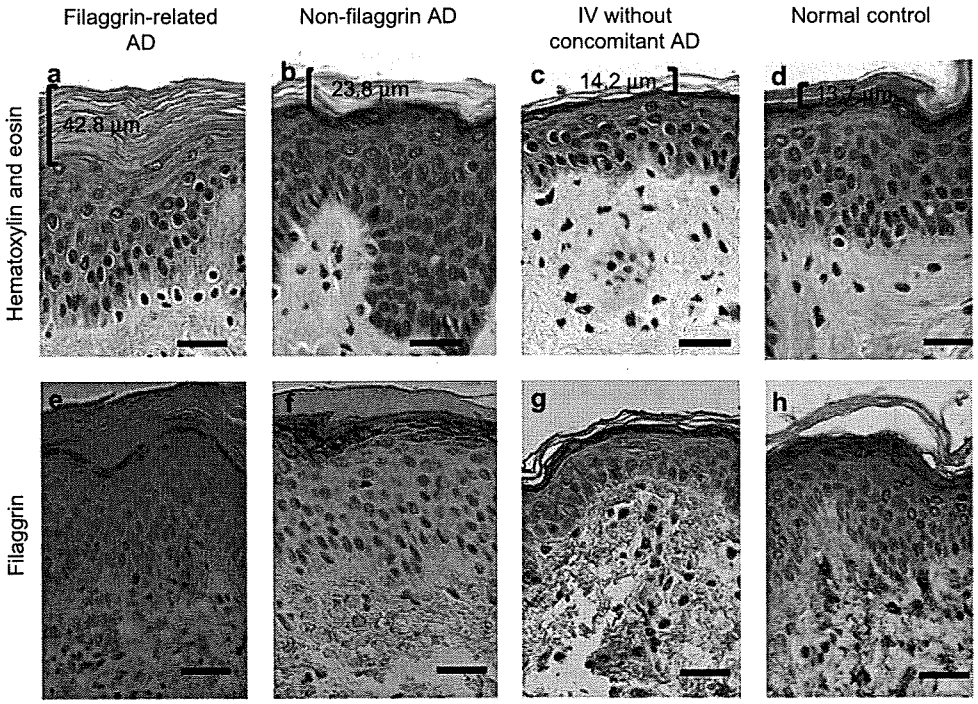

Figure 2. SC thickness measured by conventional histological methods and filaggrin immunostaining. Note remarkably increased SC thickness in filaggrin-related AD (**a**) due to an increased number of cornified cell layers. Immunohistochemical staining with anti-filaggrin monoclonal antibody against an epitope conserved in all filaggrin repeat peptides revealed a marked reduction of filaggrin staining in the epidermis of filaggrin-related AD (**e**) and IV without concomitant AD (**g**). (**a–d**) Hematoxylin and eosin staining; (**e–h**) filaggrin immunostaining. (**a**, **e**) Patient number 3 with compound heterozygous *FLG* mutations, c.3321delA, and p.Ser2554X (filaggrin-related AD); (**b**, **f**) patient number 21 without any *FLG* mutation (non-filaggrin AD); (**c**, **g**) patient number 27, IV without concomitant AD, harboring heterozygous *FLG* mutation p.Ser2554X; (**d**, **h**) normal control. Bars, 25 μm.

scores for any antigen between filaggrin-related AD and non-filaggrin AD.

Neither EOS, total serum IgE nor serum LDH showed any apparent association with the OSCORAD in either filaggrin-related AD or non-filaggrin AD by Wilcoxon rank sum test. Although no significant correlation was obtained between EOS, total serum IgE, LDH, and the OSCORAD in either filaggrin-related AD or in non-filaggrin AD by simple regression test, the OSCORAD and specific IgE for several allergens in filaggrin-related AD revealed a significant correlation coefficient, such as between OSCORAD and IgE for house dust (correlation coefficient $r=0.66$, $P<0.05$) in filaggrin-related AD, between the OSCORAD and IgE for mite allergen (correlation coefficient $r=0.53$, $P<0.05$) in filaggrin-related AD, and between the OSCORAD and IgE for cat dander (correlation coefficient $r=0.64$, $P<0.05$) in filaggrin-related AD. IgE for the other allergens in filaggrin-related AD did not show any correlation with the OSCORAD. IgE MAST scores for none of the allergens exhibited any correlations with the OSCORAD in non-filaggrin AD.

DISCUSSION

Atopic dermatitis is thought to comprise a group of patients with heterogeneous pathogenic factors. For a long time, abnormalities in the immune system have been highlighted as causative factors underlying AD. Recently, *FLG* mutations were found to be an important predisposing factor for AD and epidermal barrier defects have been attracting attention as an important pathomechanisms leading to AD.

The epidermal barrier function, in ichthyosis patients measured by SC hydration, thickness, and TEWL are known to be associated with the severity of the disease (Tomita *et al.*, 2005). Additionally, in AD patients, TEWL was reported to be increased, although controversy still remains as to whether the defective barrier function in AD patients is a primary cause of AD or a secondary consequence following dermatitis (Leung, 2000). Several studies have proven that there is a close correlation between clinical severity assessed using the SCORAD and skin barrier dysfunction in AD patients (Chamlin *et al.*, 2002; Sugarman *et al.*, 2003).

Stratum corneum hydration was marginally lower in filaggrin-related AD compared to non-filaggrin AD, although no statistical significance was obtained between our two AD groups. In contrast, TEWL was higher in non-filaggrin AD compared to in filaggrin-related AD. Low SC hydration in filaggrin-related AD might be indirectly related to barrier defects, but could primarily be related to a deficiency of water-binding filaggrin breakdown products (natural moisturizing factor) within the SC of filaggrin-related AD patients.

From the results of the present study, SC thickness was significantly thicker in filaggrin-related AD than in non-filaggrin AD. In contrast, the TEWL increase observed in

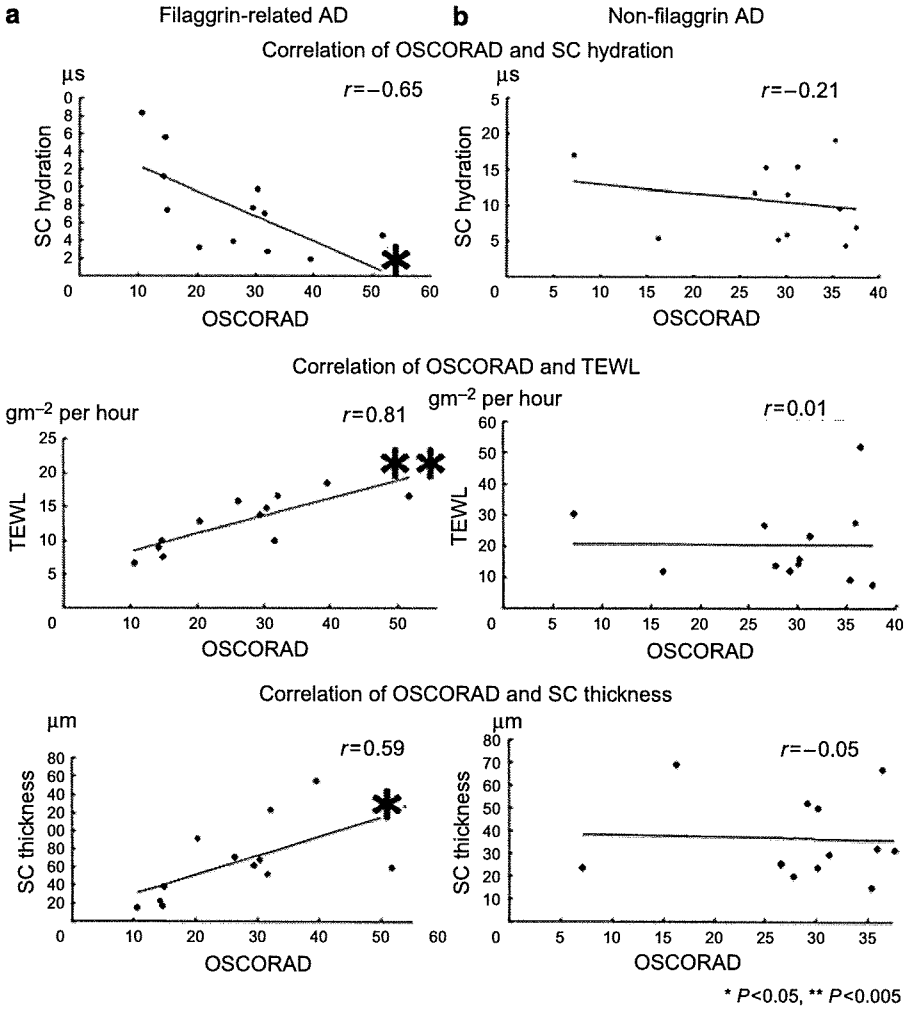

Figure 3. Correlation of clinical severity (OSCORAD) and SC hydration (average values of the three regions, μs), OSCORAD and TEWL (average values of the three regions, $g m^{-2}$ per hour), and OSCORAD and SC thickness (average values of the three regions, μm). (**a**) Filaggrin-related AD. A significant correlation was shown between OSCORAD and each parameter. Data with *P*-values $*P<0.05$ were evaluated as significant and $**P<0.005$ were evaluated as highly significant. (**b**) Non-filaggrin AD. No statistically significant correlation was shown between OSCORAD and any parameter.

non-filaggrin AD was significantly greater than that in filaggrin-related AD. It appears interesting that there seems to be a clear difference in barrier formation (SC thickness) and function (TEWL) between the two groups. In total, 4 patients out of 12 in filaggrin-related AD exhibited concomitant IV, although none of non-filaggrin AD patients had concomitant IV. Thus, we cannot exclude the possibility that remarkable SC thickness in filaggrin-related AD is related to the IV characteristics of patients, though SC was generally thinner in IV patients without AD in the present study. In addition, in our study, a negative correlation between the clinical AD severity (OSCORAD) and SC hydration and a positive correlation between the OSCORAD and TEWL were confirmed only in filaggrin-related AD. There was no significant correlation between the OSCORAD and SC hydration and between the OSCORAD and TEWL in non-filaggrin AD. However, according to a previous report studying French AD patients (Hubiche *et al.*, 2007), no significant difference was observed in the level of barrier function defects between filaggrin-related AD and non-filaggrin AD. It should be noted that in the previous study (Hubiche *et al.*, 2007), that the AD cohort was screened only for the two common filaggrin mutations, p.R501X, and c.2282del4 and was not screened for the many newly identified European filaggrin mutations, some of which are also prevalent in European populations (Sandilands *et al.*, 2007).

The OSCORAD scoring in the present study showed slight skewing between the two AD groups. Filaggrin-related AD seemed in general to have a relatively low OSCORAD score. This fact could have affected the interpretation of the other data. The effect of omitting treatments for 24 hours might also make non-filaggrin AD patient's disease worse than that of filaggrin-related AD patients. Thus, we checked this skewing using the Wilcoxon rank sum test and box-whisker plots, whereby the skewing was in fact revealed to be nonsignificant (Figure S1). No clinically relevant differences were seen between filaggrin-related AD and non-filaggrin AD in the present study.

From our results, increased SC thickness, TEWL, and reduced SC hydration are thought to be good indicators to evaluate the severity of filaggrin-related AD. Furthermore, in filaggrin-related AD, increased SC thickness and TEWL and reduced SC hydration might be useful to predict clinical course of the patients. In addition, given the strong correlations between the AD severity score (OSCORAD) and all the three parameters of skin barrier (TEWL, SC hydration, and thickness) obtained only in filaggrin-related AD, we are able to speculate that epidermal barrier defects may be one of the primary abnormalities in filaggrin-related AD.

Only in filaggrin-related AD, significant positive correlations were confirmed between the OSCORAD and IgE for house dust, between the OSCORAD and IgE for mite allergen, and between the OSCORAD and IgE for cat dander. There was no significant correlation between the OSCORAD and allergen-specific IgE in non-filaggrin AD. These results indicated the possibility of percutaneous sensitization for the allergens due to skin barrier defects in filaggrin-related AD. Mechanisms by which barrier defects due to *FLG* mutations contribute to the overall clinical end points of AD have yet to be completely clarified. Profilaggrin/filaggrin is crucial for maintaining the epidermal barrier function (Hudson, 2006). *FLG* mutations result in complete or incomplete loss of profilaggrin/filaggrin peptides and seem to be important in facilitation of allergic sensitization in AD patients. Defective epidermal barrier function provides easy access of allergens, antigens, and irritants through the epidermis, leading to stimulation of active T cells that contribute to more inflammation of the skin (Leung *et al.*, 1995).

We examined the histopathological features of three patients, one filaggrin-related AD, one non-filaggrin AD, and one IV without concomitant AD. Our results demonstrate that SC thickness measured in conventional histological slides correlated well to SC thickness as measured by a corneometer. Concordance of SC thickness measured by a corneometer and that measured by conventional histological methods was previously reported in ichthyosis patients (Tomita *et al.*, 2005). Thus, we feel that SC thickness measured by a corneometer without any further invasive procedures may be a useful and reliable parameter of hyperkeratosis. From histopathological observations, the thickening of SC in filaggrin-related AD (patient 3) appeared to be due to an increased number of cornified cell layers in SC. This observation suggests that hyperkeratosis in filaggrin-related AD might be caused by reduced desquamation of cornified cells, but not by diminished compaction of the corneocytes in the SC. These observations further emphasize the close pathophysiological relationship between ichthyosis vulgaris and filaggrin-related AD.

In our study, AD patients showing aggravation in summer were restricted to the AD group of the patients without *FLG* mutations, although the number of patients included in the present study was limited. Overexposure to sweat antigens was suggested to be an accelerating factor for AD eczema (Tanaka *et al.*, 2006). AD patients with summer aggravation might have other predisposing factors including sweat antigen exposure, but not epidermal barrier defects. Further study with a larger number of patients is needed to verify this hypothesis.

Our results showed remarkable barrier dysfunction (increased TEWL) in AD patients who did not have any *FLG* mutations. Jakasa *et al.* (2007) reported altered penetration of polyethylene glycols into uninvolved AD patient skin. There are likely a variety of mechanisms that modulate barrier integrity, other than the profilaggrin/filaggrin system, although it should be noted that there may be other filaggrin mutations still remain undetected in the Japanese population. Other structural molecules may also contribute to skin barrier formation by as yet unknown mechanisms.

MATERIALS AND METHODS

Patients

We selected 24 patients with AD according to criteria proposed by Hannifin and Rajka (1980), 14 males and 10 females, with a mean age of 21.36 years (range, 6–33 years). Twelve patients harbored *FLG* mutations (filaggrin-related AD) and the other twelve patients had no apparent *FLG* mutation (non-filaggrin AD). They were all treated by topical steroid ointment ranged from moderate to very strong, topical tacrolimus, moisturizer (heparinoid), or oral anti-histamines. We interviewed patients about disease duration of AD, presence or absence of family history of AD, and other atopic disorders including asthma or allergic rhinitis, and seasonal difference in AD severity. Patient numbers 3, 4, 10, and 11 had features of concomitant IV including hyperlinearity in the palms and scales on the lower legs. Patient numbers 1, 2, 5, 6, 7, 8, 9, 12, and 13–24 had dry skin, but no apparent concomitant IV features. In addition, three typical IV patients without concomitant AD harboring *FLG* mutations were included in the present study.

Age-matched healthy volunteers were included in the present study as controls. The control group consisted of 12 healthy individuals aged 6–30 years (eight male and four female) without any past or present skin disease.

The present study was approved by the Institutional Ethical Committee of Hokkaido University Graduate School of Medicine. This study was conducted according to all the Declaration of Helsinki Principles. Participants or their legal guardians gave their written informed consent.

Filaggrin genotyping

FLG mutation analysis was performed in patients and their family members. Briefly, genomic DNA isolated from peripheral blood was subjected to PCR amplification, followed by direct automated sequencing using ABI PRISM 3100 or 3730 genetic analyzers (Applied Biosystems, Foster City, CA). Mutations p.Ser2554X, p.Ser2889X, and p.Ser3296X were screened using restriction enzyme digestion of PCR products (Nomura *et al.*, 2007, 2008). Mutation c.3321del was screened by fluorescent PCR (Nomura *et al.*, 2007). Primers and PCR conditions were as described previously (Nomura *et al.*, 2007, 2008).

Disease severity

The SCORAD (severity scoring of AD, score range 0–103; European Task Force on Atopic Dermatitis, 1993) utilizes the rule of nines with

six clinical features of AD disease intensity: erythema/darkening, edema/papulation, oozing/crust, excoriations, lichenification/prurigo, and dryness. Dryness was evaluated on noninflamed skin. The other features were assessed on a representative area for a given intensity item, also on a scale of 0–3. To measure AD clinical severity, we employed the objective SCORAD (OSCORAD; score range 0–83; Holm *et al.*, 2006). In the OSCORAD, which is often used in clinical trials, two subjective symptoms (itch and sleep loss) were excluded from the conventional SCORAD.

Measurement of stratum corneum hydration, TEWL, and stratum corneum thickness

Measurements were performed under standardized conditions, that is, at a room temperature of 22–25 °C and a humidity level of 40–55%. Before the measurements, patients were given time to adapt to room conditions without covering the measurement sites with clothes. All the measurements were performed by one investigator (INH). Almost all patients were taking anti-histamines, and treated by topical steroids, topical immunosuppressants, emollients (heparinoid), which were all kept maintained. However, from 2100 hours on the day before the investigation, nothing was applied to the skin to be examined. To exclude the bias of different dermatitis severities in the examination sites, three body sites, clinically normal areas in the extensor and flexor aspects of the forearm and on the back, were selected for examination. All measured values were expressed as the median of three recordings to avoid measuring inaccuracies. SC hydration was measured as (low-frequency susceptance) × (square root of electrode distance)/(square root of low frequency conductance) by using noninvasive methods (Yamamoto, 1994) with a Corneometer ASA-M2 (ASAHI BIOMED, Yokohama, Japan). ASA-M2 evaluated conductance of two different electric currents with low frequency and high frequency. The low-frequency current was limited to the superficial SC and the high-frequency current penetrated the highly moist region immediately below the SC. Thus, SC thickness was calculated from low-frequency susceptance and high-frequency admittance by the corneometer as (square root of low-frequency susceptance)/(high-frequency admittance; Yamamoto, 1994). TEWL was measured using Evaporimeter AS-TW1 (ASAHI BIOMED, Yokohama, Japan). AS-TW1 utilizes the ventilated chamber method of measuring TEWL. Its hygrometer measures the humidity of incoming air and of outgoing air that has passed over the test area of skin, and TEWL is calculated from the difference. TEWL measurements were done on the extensor and flexor sides of the forearm that were observed clinically normal. All the measurements were performed three times for each skin spot.

Immunohistochemical staining

Immunoperoxidase staining of paraffin-embedded sections was performed using the ChemMate Peroxidase/DAB system (Dako Cytomation, Hamburg, Germany). Mouse monoclonal antibody 15C10 (Novocastra, Newcastle, UK) was used to detect the human filaggrin repeat unit. Antigen retrieval was performed by heating sections under pressure for 10 to 15 minutes in 10 nmol l^{-1} citrate buffer, pH 6.0.

Laboratory tests

Peripheral blood EOS count (number × 100 per μl; normal 40–440), serum LDH (IU l^{-1}; normal 119–229), total serum IgE (IU ml^{-1}; normal 0.0–400.0), and allergen-specific IgE (SRL Inc., Tokyo, Japan) were measured. Allergen-specific IgE were estimated by fluoroenzyme immunoassays for house dust, mite allergen, grass pollen (*Tancy*), cedar pollen, fungal allergen (*Candida*), animal dander, and foods. Concerning to the sensitivity for detection of specific IgE, 100 lumicount and values greater than or equal to 100 lumicount were considered positive (+).

Statistical analysis

Statistical analysis was performed using Excel 2000 (Microsoft, Redmond, WA) with the add-in software Statcel 2 (OMS, Saitama, Japan) and JMP 6.0.3 (SAS Institute, Tokyo, Japan). Wilcoxon rank sum and Turkey–Kramer's honestly significant difference tests were used to compare the continuous variables between the group of total AD patients and normal controls, and between filaggrin-related AD and non-filaggrin AD. Data with *P*-values less than 0.05 were evaluated as significant. We interpreted *P*-values less than 0.01 as highly significant.

Simple regression analyses were also used to identify significant associations of SC hydration, thickness, or TEWL to OSCORAD. Data with *P*-values less than 0.05 were evaluated as significant. We interpreted *P*-values less than 0.005 as highly significant. Wilcoxon rank sum test and simple regression analyses were performed to assess the association or correlation between different biological markers including IgE, LDH, EOS, and the OSCORAD.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We thank Dr James R. McMillan for his critical reading of this paper. We thank Ms Akari Nagasaki, for her fine technical assistance on this project. Filaggrin/ichthyosis/eczema research in the McLean laboratory is supported by grants from the British Skin Foundation, National Eczema Society (UK), and the UK Medical Research Council (reference number G0700314) and donations from anonymous families affected by eczema in the Tayside Region of Scotland. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M. Akiyama (Kiban B 18390310 and Kiban B 20390304).

SUPPLEMENTARY MATERIAL

Table S1. Clinical information on the patients and FLG mutations.

Table S2. Patients' clinical severity (OSCORAD), SC hydration, TEWL, and SC thickness.

Figure S1. Box-whisker plots of OSCORAD in the two AD groups. Wilcoxon rank sum test and box-whisker plots revealed no significant difference in OSCORAD scores between filaggrin-related AD and non-filaggrin AD.

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Novel mutation p.Gly59Arg in *GJB6* encoding connexin 30 underlies palmoplantar keratoderma with pseudoainhum, knuckle pads and hearing loss

I. Nemoto-Hasebe, M. Akiyama, S. Kudo,* A. Ishiko,* A. Tanaka,† K. Arita and H. Shimizu

Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan
**Department of Dermatology, Keio University School of Medicine, Tokyo, Japan*
†Department of Dermatology, Kameda General Hospital, Chiba, Japan

Correspondence
Ikue Nemoto-Hasebe.
E-mail: i-nemoto@med.hokudai.ac.jp

Accepted for publication
31 January 2009

Key words
Bart–Pumphrey syndrome, Clouston syndrome, gap junction, pseudoainhum, Vohwinkel syndrome

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2009.09137.x

Summary

Background Connexins, components of the gap junction, are expressed in several organs including the skin and the cochlea. Mutations in connexin genes including *GJB2* (Cx26), *GJB3* (Cx31), *GJB4* (Cx30.3), *GJB6* (Cx30) and *GJA1* (Cx43) are responsible for various dermatological syndromes and/or inherited hearing loss, frequently showing overlapping phenotypes.

Objectives To clarify the spectrum of clinical phenotypes caused by connexin mutations.

Methods We report a 32-year-old Japanese woman with mild palmoplantar keratoderma (PPK) with severe sensorineural hearing loss, knuckle pads and pseudoainhum of her toes.

Results Direct sequencing revealed no mutation in *GJB2*, but a novel heterozygous missense mutation p.Gly59Arg in *GJB6*. Electron microscopy revealed no apparent morphological abnormality of gap junctions in the patient's lesional epidermis.

Conclusions The patient harboured the novel *GJB6* missense mutation p.Gly59Arg in the first extracellular loop of Cx30. Mutations in glycine 59 of Cx26 are associated with PPK–deafness syndrome, and the similar phenotype here supports the observed heteromeric channel formation; the dominant nature of the mutation suggests an effect on gap junctions similar to that of the comparable mutation in Cx26.

Gap junctions are cell-to-cell connecting structures containing clusters of intercellular channels that allow intercellular passage of ions and molecules of up to 1 kDa. These channels are oligomeric assemblies of family members of related proteins called connexins. Six connexin polypeptides assemble into a connexon, a hemichannel that interacts with its counterpart in an adjacent cell membrane to form a complete intercellular channel.[1] All the connexins in an individual connexon may be of the same type (homomeric), or heteromeric connexons may be formed by oligomerization between different connexins. Connexin 26 (Cx26) and connexin 30 (Cx30) are known to form heteromeric connexon hemichannels.[2]

Connexins are expressed in several organs including the skin and the cochlea. Mutations in connexin genes including *GJB2* (Cx26), *GJB3* (Cx31), *GJB4* (Cx30.3), *GJB6* (Cx30) and *GJA1* (Cx43) are responsible for several hereditary skin disorders associated with hearing loss.[3] Cx30 mutations are typically associated with Clouston syndrome[4] in which palmoplantar keratoderma (PPK) is only occasional and not usual, although cases resembling keratitis–ichthyosis–deafness (KID) syndrome have also been reported.[5] Various mutations affecting Cx26 cause PPK–deafness syndrome.[6] PPK–deafness syndromes are typically *GJB2* associated. However, as Cx26 and Cx30 interact, one might expect more phenotypic overlap as exemplified by the report of Jan *et al.*[5]

Here, we report a Japanese woman with clinical features resembling those of Vohwinkel syndrome and Bart–Pumphrey syndrome. We found a novel heterozygous missense mutation p.Gly59Arg in *GJB6*. As far as we know, this is the first reported patient with PPK with pseudoainhum, knuckle pads and hearing loss, harbouring a *GJB6* gene mutation.

Patients and methods

Patient

The patient was a 32-year-old Japanese woman. She was diagnosed with congenital sensorineural hearing loss when she

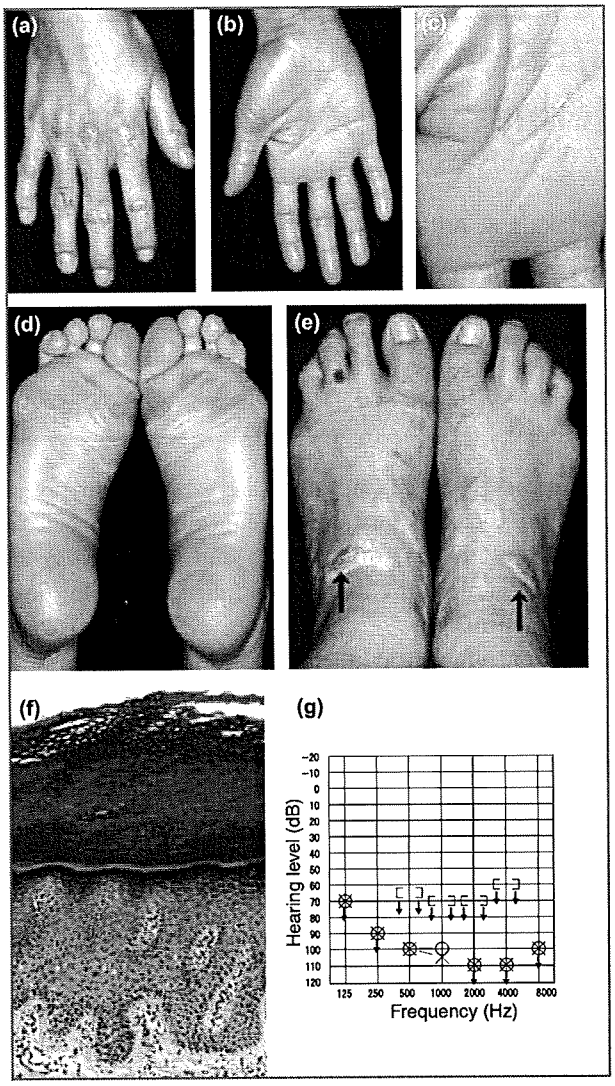

Fig 1. (a–e) Clinical features of the patient's skin. (a) Knuckle pads on the dorsa of the fingers of the patient; (b–d) diffuse palmar and plantar hyperkeratosis without honeycomb features; (d, e) amputation due to constriction bands on the fifth toe of each foot; extensive hyperkeratosis was seen on the ankle joints. (f) Skin biopsy from the sole revealed marked orthohyperkeratosis with hypergranulosis (haematoxylin and eosin; original magnification × 100). (g) Patient's pure tone audiogram showed pronounced sensory hearing loss: air conduction indicated by cross/round marks (cross, left ear; round, right ear); bone conduction indicated by bracket marks ([, right ear;], left ear); arrows pointing downwards indicate the loudest tone that was not heard.

was 3 years old. She also had diffuse PPK without a honeycomb hyperkeratosis appearance and hyperkeratotic plaques over the knuckles (Fig. 1a–c). An audiogram obtained at 24 years of age showed pronounced sensorineural hearing loss (Fig. 1g). At the age of 26 years, the fifth toe on each foot was surgically amputated due to pseudoainhum (Fig. 1d,e). Her fingers did not show mutilation. Extensive hyperkeratosis was seen in areas exposed to mechanical stress, such as the extensor (ventral) aspect of her ankle joints (Fig. 1e) probably because of folding the legs in the Japanese sitting or kneeling style. She had no features of ichthyosis on her trunk or extremities. Her hair, teeth and nails were normal and she had no ocular involvement. There was no family history of skin disorders or auditory dysfunction, or consanguinity in her family. All members of the family, including her parents and her elder sister, were generally healthy and were without PPK or hearing loss.

Mutation detection

After fully informed, written consent, peripheral blood samples were obtained from the patient and genomic DNA was extracted (Qiagen, Hilden, Germany). The entire coding region and exon/intron boundaries of GJB2 and *GJB6* were amplified by polymerase chain reaction (PCR) using the specific primers described previously.[7,8] PCR products were sequenced and mutation was confirmed by enzyme digestion with BtgZI restriction enzymes (New England Biolabs, Ipswich, MA, U.S.A.). Reference cDNA for *GJB6* was cDNA sequence GenBank accession number NT_009799.

Morphological observations

A skin biopsy was taken from the right sole of the patient with fully informed consent. The biopsy specimen was processed for routine histological analysis and for ultrastructural observations as previously described.[9]

Results

Mutation analysis

Analysis in GJB2 revealed no mutation, although a common polymorphism p.Val27Ile was found. We identified a heterozygous 175G>C transversion in *GJB6* (Fig. 2a). This novel nucleotide alteration leads to the replacement of glycine 59 (neutral, hydrophilic residue) with a positively charged hydrophilic arginine residue (p.Gly59Arg) in the first extracellular loop. The mutation introduces a single BtgZI restriction site in the gene. We confirmed the presence of the mutation in the patient's genomic DNA by restriction enzyme BtgZI digestion (Fig. 2b). This nucleotide change was not detected in 100 unrelated, healthy Japanese individuals (200 alleles).

Histological evaluation of the patient's skin

A biopsy specimen from the patient's plantar skin revealed compact orthohyperkeratosis with hypergranulosis and acanthosis in the epidermis (Fig. 1f).

Electron microscopic findings

Ultrastructurally, keratinocytes in the epidermis of the patient's plantar skin assembled gap junctions showing normal morphology with a typical pentalaminar structure, 20 nm in width (data not shown).

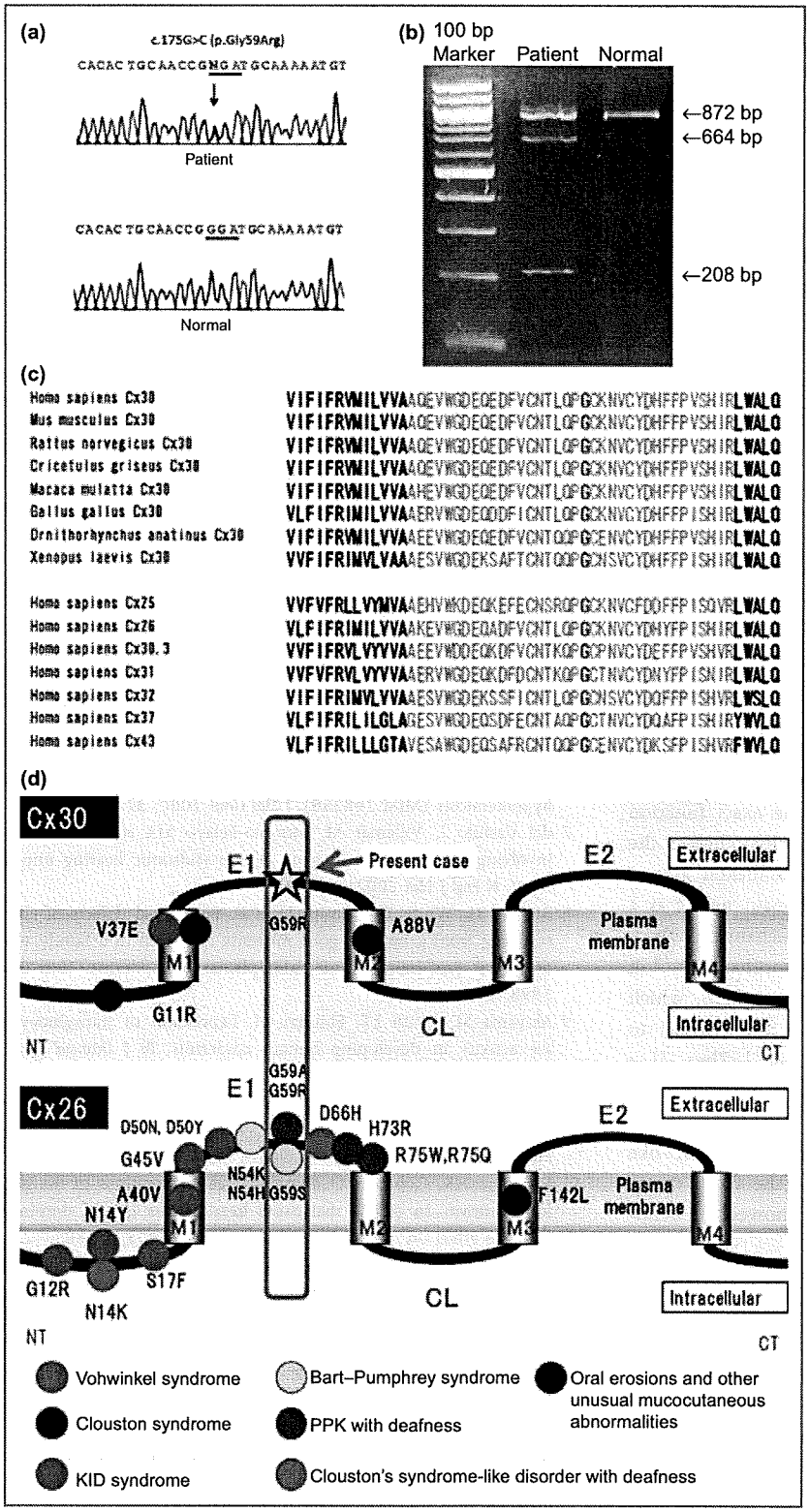

Fig 2. (a) Sequence chromatograms of GJB6 from the patient (upper) showed the heterozygous transition c.175G>C at codon 59 (p.Gly59Arg). (b) Confirmation of the presence of the mutation p.Gly59Arg in the patient by BtgZI restriction digestion. An 872 bp polymerase chain reaction fragment from the mutant allele was digested into 664 bp and 208 bp fragments, whereas that from the wild-type allele was not cut. Thus, 872 bp, 664 bp and 208 bp bands were seen in the patient harbouring the heterozygous p.Gly59Arg mutation, although only a 872 bp band was detected in the normal control. (c) Comparison of amino acid sequences of Cx30 from diverse species and other members of the human connexin family. Glycine residue at codon position 59 (red) is located in the centre of the first extracellular domain (blue) and is highly conserved in diverse species and other members of the human connexin family. (d) Cx30/Cx26-associated syndromes and reported causative mutations in Cx30/Cx26. M1–M4, transmembrane domains 1–4, respectively; E1 and E2, extracellular domains 1 and 2, respectively; CL, cytoplasmic loop; PPK, palmoplantar keratoderma; KID, keratitis–ichthyosis–deafness.

Discussion

We herewith report, as far as we know, the first case of PPK–deafness caused by a mutation affecting the E1 domain of Cx30. Glycine 59 in Cx30 is located in the first extracellular loop (Fig. 2c,d), which exhibits high sequence conservation in homologous proteins from different species (Fig. 2c). The first extracellular loop is thought to be essential for the

interaction between a connexon and its counterpart in an adjacent cell to form a complete intercellular channel. Three mutations in Cx26, p.Gly59Ala, p.Gly59Trp and p.Gly59Ser, occur at glycine 59 which is orthologous to glycine 59 in Cx30. These were reported in syndromes comprising sensorineural hearing loss and PPK[6,10] (Fig. 2d).

Cx30 and Cx26 form heteromeric junctions both in the skin and the inner ear and functional data suggest a dominant interaction between the two.[2,11] From these facts, we think it reasonable to speculate that missense mutations in the first extracellular loop domain in either Cx26 or Cx30 can lead to similar phenotypes. However, this hypothesis does not explain why the phenotypes look so similar or why, for example, p.Gly11Arg in Cx30 does not lead to KID syndrome whereas p.Gly12Arg in Cx26 does. There may be a genetic background effect which contributes to the phenotype in each patient.

The *GJB2* keratoderma/deafness phenotypes are almost all caused by mutations clustering in the first extracellular loop domain.[12] The Cx26 mutation p.Gly59Arg results in a diffuse (although more severe) keratoderma, as does this mutation in Cx30 which is shown in the present patient. In contrast, mutations in the N-terminal cytoplasmic region in Cx26 are associated with KID syndrome and similar phenotypes. Likewise, p.Gly11Arg and p.Val37Glu in Cx30 in the N-terminal cytoplasmic region underlie KID-like syndrome or Clouston syndrome. We do not know the exact function of the N-terminal domain. There are two hypotheses: the first is that the N-terminus is involved in connexon assembly;[13] the second is that the N-terminal domain works as a plug in a vestibule of the connexon hemichannel, which physically blocks the channel (plug gating mechanism).[14] It was suggested that the mutation p.Asn14Tyr in Cx26, which is associated with a KID phenotype, causes the channel to be locked in a closed position by the plug.[15] Thus, if a similar phenomenon happens with the Cx30 mutation in a heteromeric channel composed of Cx30 and Cx26, Cx30 mutations in the N-terminus may lead to similar KID-like phenotypes. The present patient harbouring the Cx30 mutation in the first extracellular loop domain showed a phenotype distinct from KID syndrome.

Light microscopic observation of the lesional skin showed orthohyperkeratosis with hypergranulosis without any specific findings for our present patient. The mutation we found did not affect connexin morphology. Likewise, some Cx26 mutations in the E1 domain were examined for their effects on connexin morphology and apparently did not affect it.[11,16]

In conclusion, we present a novel *GJB6* missense mutation p.Gly59Arg in a patient who had PPK with pseudoainhum, knuckle pads and hearing loss. The present case expands the clinical spectrum of *GJB6* mutations and shows that, in PPK–deafness cases where we do not find *GJB2* mutations, we should check *GJB6*. Furthermore, our results suggest an interaction between Cx30 and Cx26 E1 domains.

Acknowledgments

We thank Ms Kaori Sakai, MS, Ms Megumi Sato and Ms Akari Nagasaki for their technical assistance on this project. We also thank Prof. James R. McMillan and Dr Heather Ann Long for proofreading this manuscript. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M.A. (Kiban B 20390304).

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Animal Models of Epidermolysis Bullosa

Ken Natsuga, MD*, Satoru Shinkuma, MD,
Wataru Nishie, MD, PhD, Hiroshi Shimizu, MD, PhD

KEYWORDS

• Epidermolysis bullosa • Transgenic mice
• Knock-out mice • Knock-in mice

For more than 2 decades, animal models have been used to clarify the pathogenic mechanisms of human diseases and develop new therapeutics for these diseases. Several therapies for human diseases have become available through trials using animal models.

Epidermolysis bullosa (EB) is one of the most severe inherited skin disorders, whose effective treatments have not been fully available. EB is characterized by abnormalities of the proteins that consist of the dermoepidermal junction (DEJ). EB has been classified into three major subtypes according to the level of skin cleavage: EB simplex (EBS), junctional EB (JEB), and dystrophic EB (DEB). To date, 13 genes have been shown to cause EB phenotype.[1,2] After the discovery of the causative genes responsible for each EB subtype, many researchers have tried to develop EB animal models by genetically manipulating the corresponding genes.[3]

CHARACTERIZATION OF ANIMAL MODELS OF EPIDERMOLYSIS BULLOSA, AND THERAPEUTIC EXPERIMENTS USING THESE MODELS

Table 1 summarizes previously described EB animal models whose genetic abnormalities are clarified. The characteristics of each EB animal model and therapeutic experiments using the models are described in this section.

Junctional Epidermolysis Bullosa

Six genes, *COL17A1*, *LAMA3*, *LAMB3*, *LAMC2*, *ITGA6*, and *ITGB4*, have been identified as responsible for the JEB phenotype. Twelve JEB animal models have been developed, although most of the models die perinatally. Recently, the authors' group developed a *Col17a1* knock-out (KO) mice that can survive for approximately 12 months.[15]

Defective type XVII collagen (COL17) encoded by mutated *COL17A1* causes non-Herlitz JEB (nH-JEB).[27,28] The authors recently developed *Col17a1* KO mice whose blistering phenotype is similar to that of human nH-JEB.[15] Notably, 20% of the *Col17a1* KO mice exhibited long survival, allowing for the therapeutic experiments, including bone marrow transplantation, cell therapy, protein therapy, and gene therapy.[15] The authors described transgenic rescue experiments for *Col17a1* KO mice using human *COL17A1* cDNA transgenic mice driven under the keratin-14 promoter, and the rescued mice were born without any skin defects and were able to reacquire reproductive ability.[15] In addition, the tooth abnormalities seen in *Col17a1* KO mice were also correctable after incorporation of human *COL17A1* cDNA.[29]

Herlitz JEB (H-JEB) is a severe form of EB with short-term survival expectancy. H-JEB is caused by mutations in the genes encoding laminin 332, which consists of the laminin α3, β3, and γ2 chains (*LAMA3/LAMB3/LAMC2*).[28,30–35] *Lama3* and *Lamc2* KO mouse models have been generated.[17,19] Furthermore, spontaneous mutant dog,[16] horse,[20,21] and mouse[18] models whose laminin genes are inactivated have also been described. Those animal models exhibited severe skin detachment with perinatal lethality. Among

Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan
* Corresponding author.
E-mail address: natsuga@med.hokudai.ac.jp (K. Natsuga).

Dermatol Clin 28 (2010) 137–142
doi:10.1016/j.det.2009.10.016
0733-8635/09/$ – see front matter

Table 1
Animal models of epidermolysis bullosa

Disease	Causative Gene	Species	Type	Survival	References
EBS	*KRT5*	Mouse	KO	Neonatal death	4
EBS	*KRT14*	Mouse	Tg	Neonatal death	5
EBS	*KRT14*	Mouse	KO	Neonatal death	6
EBS	*KRT14*	Mouse	KI	Neonatal death	7
EBS	*KRT14*	Mouse	KI (an inducible model)	Not mentioned	7
EBS-MD/EBS-PA	*PLEC1*	Mouse	KO	Neonatal death	8
EBS-MD/EBS-PA	*PLEC1*	Mouse	Conditional KO	Neonatal death	9
JEB-PA	*ITGA6*	Mouse	KO	Neonatal death	10
JEB-PA	*ITGB4*	Mouse	KO	Neonatal death	11
JEB-PA	*ITGB4*	Mouse	KO	Neonatal death	12
JEB-PA	*ITGB4*	Mouse	Partial ablation (expressing ectodomain of β4 integrin)	Neonatal death	13
JEB-PA	*ITGB4*	Mouse	Conditional KO	Not mentioned	14
nH-JEB	*COL17A1*	Mouse	KO	Prolonged survival in 20% of mice	15
nH-JEB	*LAMA3*	Dog	Naturally occurring	Not mentioned	16
H-JEB	*LAMA3*	Mouse	KO	Neonatal death	17
H-JEB	*LAMB3*	Mouse	Naturally occurring	Neonatal death	18
H-JEB	*LAMC2*	Mouse	KO	Neonatal death	19
H-JEB	*LAMC2*	Horse	Naturally occurring	Not mentioned	20
H-JEB	*LAMC2*	Horse	Naturally occurring	Not mentioned	21
RDEB-sev gen	*COL7A1*	Mouse	KO	Neonatal death	22
RDEB-O	*COL7A1*	Dog	Naturally occurring	Not mentioned	23
RDEB-O	*COL7A1*	Mouse	Hypomorphic	Prolonged survival	24
RDEB-O	*COL7A1*	Mouse	KO with transgenic rescue using mutated human cDNA	Prolonged survival	25
Kindler syndrome	*FERMT1*	Mouse	KO	Neonatal death	26

Abbreviations: EBS, epidermolysis bullosa simplex; EBS-MD, epidermolysis bullosa simplex with muscular dystrophy; EBS-PA, epidermolysis bullosa simplex with pyloric atresia; H-JEB, Herlitz junctional epidermolysis bullosa; JEB, junctional epidermolysis bullosa; JEB-PA, junctional epidermolysis bullosa with pyloric atresia; KI, knock-in; KO, knock-out; nH-JEB, non-Herlitz JEB; RDEB, recessive dystrophic epidermolysis bullosa; RDEB-O, generalized other recessive dystrophic epidermolysis bullosa; RDEB-sev gen, severe generalized recessive dystrophic epidermolysis bullosa; Tg, transgenic.

them, Swiatek and colleagues[18] reported spontaneous mutant mice in which the defective β3 chain of laminin-332 resulted from insertion of an intracisternal-A particle between the exon/intron junction of *Lamb3* (Lamb3^{IAP} mutant).[18]

Schneider and colleagues[36] described prenatal intra-amniotic human *LAMB3* cDNA delivery into LamB3^{IAP} mutant mice using adenovirus and adeno-associated virus vectors.[36] They showed that the defective β3 chain of laminin-332 was expressed in the skin of treated mice, although they found only a minor increase of the lifespan of these mice.[36]

JEB with pyloric atresia (JEB-PA) is caused by mutations in the genes encoding α6 integrin or β4 integrin (*ITGA6/ITGB4*).[28,37,38] α6 or β4 integrin null mice exhibited severe skin detachment with perinatal lethality.[10–12] Sonnenberg and colleagues[39] generated human *ITGB4* cDNA transgenic mice driven under keratin-5 promoter and then tried transgenic rescue for *Itgb4* KO mice using those transgenic mice, although the

rescued mice still exhibited skin fragility and high mortality.[39]

The same group generated *Itgb4* conditional knock-out mice, in which *Itgb4* was inactivated only in a small area of the skin. Those mice did not have obvious skin defects, although microscopy showed a small number of blisters in which β4 integrin had been deleted.[14] Giancotti and colleagues[13] generated mice carrying targeted deletion of the β4 integrin cytoplasmic domain.[13] Although those mice expressed truncated extracellular domain β4 integrin protein, their phenotypes were almost the same as mice with complete loss of β4 integrin.[13]

Dystrophic Epidermolysis Bullosa

Mutations in the gene encoding type VII collagen (*COL7A1*) are responsible for dystrophic EB (DEB).[40–42] *Col7a1* KO mice were developed as a severe generalized recessive DEB (RDEB) model by Uitto and colleagues.[22] *Col7a1* KO mice exhibited severe skin detachment with perinatal lethality.[22] Chen and colleagues[43] injected recombinant human type VII collagen (COL7) protein into *Col7a1* KO mice and found that the injected human COL7 was incorporated into the DEJ and that the treated mice exhibited longer survival rates than the controls.[43]

Tamai and colleagues[44] reported that embryonic transplantation with bone marrow cells derived from green fluorescent protein (GFP) transgenic mice through vitelline vein–alleviated skin phenotypes of *Col7a1* KO mice.[44] Tolar and colleagues[45] also showed that administration of hematopoietic stem-cell–enriched bone marrow cells derived from GFP transgenic mice ameliorated skin fragility and reduced lethality of newborn *Col7a1* KO mice.[45] However, the short survival time in which the treatment can show a significant effect in *Col7a1* KO mice is still an obstacle to new therapeutics for RDEB.

Bruckner-Tuderman and colleagues[24] developed a DEB hypomorphic mouse model in which the amount of mouse COL7 in skin was approximately 10% of that of wild-type mice and those mice had a prolonged survival.[24] Using this hypomorphic mouse model, they showed that injection of cultured fibroblasts derived from wild-type mice increased the expression of mouse COL7 at the DEJ above the treated area in the hypomorphic mouse skin.[24,46]

The authors' group showed transgenic rescue of the previously described *Col7a1* KO mouse using two transgenic mouse models comprising human *COL7A1* cDNA under keratin-14 promoter or type I collagen promoter.[22,25] They also used novel methods to develop a milder blistering phenotype in humanized RDEB model mice. They generated human *COL7A1* transgenic mice with a premature termination codon (PTC) expressing truncated human COL7 protein.[25] Then, transgenic rescue of *Col7a1* KO mice using PTC-causing human *COL7A1* transgenic mice resulted in a significantly milder clinical skin blistering manifestation resembling generalized other RDEB and prolonged survival.[25]

Epidermolysis Bullosa Simplex

Mutations in genes encoding keratin 5 and 14 (*KRT5/KRT14*) have been known to cause EBS, where an abnormal keratin network leads to blister formation.[47,48] Magin and colleagues[4] developed *Krt5* KO mice in which severe skin detachment and perinatal death were noted.[4] Fuchs and colleagues[5] generated transgenic mice expressing a truncated human keratin 14 protein.[5] Those mice exhibited severe skin detachment from the dominant negative effects of aberrantly expressed protein.[5] The same group generated *Krt14* KO mice that exhibited a milder phenotype than *Krt5* KO mice.[6] Although keratin-null mice showed skin fragility,[4,6] their mechanism of blister formation is different from that of human patients who have EBS, in which aberrant keratin protein from *KRT5/14* missense mutations interferes with the structural assembly of keratin filaments. Roop and colleagues[7] adopted a knock-in strategy to represent the dominant negative effects of abnormal protein encoded by *Krt14* harboring a missense mutation in the knock-in mice.[7] They showed that one amino acid substitution is enough to cause an EBS disease phenotype in the knock-in mice.[7] Furthermore, they generated an inducible animal model of EBS, in which topical application of a chemical inducer allows focal activation of a mutant keratin 14 protein in the epidermis of the treated area.[7]

Mutations in the gene encoding plectin (*PLEC1*) are responsible for EBS with muscular dystrophy[49–51] and EBS with pyloric atresia.[52,53] Wiche and colleagues[8] reported that mice with targeted inactivation of *Plec1* exhibited severe skin fragility and perinatal death.[8] They also succeeded in obtaining mice with conditional ablation of *Plec1* in the stratified epithelia,[9] although those mice also showed markedly short survival.[9]

Kindler Syndrome

The Third International Consensus Meeting on Diagnosis and Classification of EB proposed to include Kindler syndrome within the category of EB.[2] Kindler syndrome is caused by mutations in

the gene encoding Kindlin-1 or fermitin family homolog protein 1 (FFH1, *FERMT1, KIND1*).[54] Targeted ablation of *Fermt1* failed to show any skin fragility phenotype in mice, although skin specimens from those mice showed skin atrophy.[26]

FUTURE DIRECTIONS

Most EB subtypes already have been represented as animal models. Although perinatal death was a major obstacle in using genetically engineered EB animal models to develop new therapeutic strategies, several methods have been successful at overcoming the problem of short survival, and longer surviving animal models are suitable to observe the efficacy of therapeutic interventions. Several transgenic rescue trials have shown that successfully delivered human transgenes are able to function in vivo. In addition, cell therapy and protein replenishment used in many animal models have potential as future therapeutic options for patients who have EB.

RNA interference is a promising way to treat the dominant negative effects of mutated keratin genes in EBS and considerable work is underway in this field. In the future, safe, and efficient gene delivery systems will be developed for EB gene therapy to benefit patients. Researchers and clinicians should continue to explore new therapeutic options using animal models and test their efficacy for application to human diseases.

ACKNOWLEDGMENTS

We thank Dr James R. McMillan for reviewing and commenting on this manuscript and Mr Michael O'Connell for his proofreading.

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