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Figure legends

Figure 1
Generation of *COL17*$^{m-/-}$ mice. (a) The targeting vector was constructed by disrupting exon 2 in which there is the ATG initiating codon. (b) PCR reaction with primers YA1 and N1 amplified a 562 bp mutant band, whereas YA1 and WT1 revealed a 496 bp fragment corresponding to the wild type genomic COL17 DNA, respectively. (c) RT and subsequent PCR reactions using RT-F and RT-B primers amplified 488 bp fragment. The primers for mouse GAPDH (NM:001001303), 5'-TTAGCCCCCCTGGCCAAGG-3' (mGAPDH-F) and 5'-CTTACTCCTTGGAGGCCATG-3' (mGAPDH-B), which amplified 541 bp fragment,

were used as a control. Northern (d) and Western (e) blotting showed definite disruption of COL17 mRNA and its transcript in *COL17* $^{m-/-}$ mouse keratinocytes.

Characteristic findings in *COL17* $^{m-/-}$ mice. (f) Epidermal detachment after gentle friction (arrows), and blister development on the paw. (g) Eight-week old *COL17* $^{m-/-}$ mouse. Note the white hair around the face and head, small hemorrhagic blisters and scar formation on the paw and digits, nail loss, and erosions within the genital area (arrowhead). (h) White hair growth over the entire body of a 12-week old *COL17* $^{m-/-}$ mouse. (i) Light microscopic findings from *COL17* $^{m-/-}$ mouse skin reveals blister formation in the sub-epidermis (stars). (j) Immunofluorescence study of *COL17* $^{m-/-}$ and wild type mice skin. BP180 failed to show any immunoreactivity (mNC16A antibody) along the dermal-epidermal junction in *COL17* $^{m-/-}$ mouse. (k) Electron microscopic findings. In wild type mouse skin, hemidesmosomes were composed of prominent inner plaques (IP), outer plaques (OP), and sub-basal dense plates (small arrow). Anchoring filaments (white arrow head) span the lamina lucida (LL), and anchoring fibrils (black arrowheads) extend from lamina densa (LD) to the papillary dermis. (l) *COL17* $^{m-/-}$ mouse skin shows a reduced number of hypoplastic (small) hemidesmosomal inner and outer attachment plaques with poor keratin filament attachment and less prominent anchoring filaments, while anchoring fibrils and the lamina densa (LD) are both well preserved.

Figure 2
(a) *COL17* $^{m-/-}$ mouse and rescued COL17-humanized mouse (*COL17* $^{m-/-, h+}$). Compared with *COL17* $^{m-/-}$ mouse, COL17-humanized mouse (*COL17* $^{m-/-, h+}$) showed no abnormal clinical manifestations. (b) Immunofluorescence studies using antibody against human COL17 (D20) and murine COL17 (mNC16A) showed that in human skin, only human BP180 was positively stained (A), while murine BP180 failed to stain (E). By contrast, murine COL17 was positively stained only in mouse skin (B, F). In human COL17 transgenic (*COL17* $^{m+/+, h+}$) mouse skin, not only human COL17 but also murine COL17 was positively stained (C, G). Note, in the rescued COL17-humanized mouse skin, murine COL17 was absent while human COL17 was positive (D, H). (c) Electron microscopic findings from the rescued COL17-humanized mouse skin. Note the distinct normal appearing hemidesmosomes with clear keratin filament attachment (arrow heads) adjacent to the lamina lucida (LL), lamina densa (LD), and anchoring fibrils (arrows).

(d) COL17-humanized mouse injected with pathogenic IgG (total IgG dose: 2mg/g body weight) showed diffuse erythema and the epidermis was frequently detached from the dermis. (e) Histological findings in the IgG injected mice skin. There were no abnormal findings in control mouse skin (A), while mouse skin injected with the whole IgG fraction (2 mg/g body weight) showed subepidermal blisters as well as mononuclear inflammatory cells and neutrophils (arrows) (B). Furthermore, mouse

skin injected with COL17-affinity purified IgG (50 μg/g body weight) showed a greater degree of subepidermal blistering with inflammatory cells (arrows) (C). (f) Direct immunofluorescence microscopy showed linear IgG deposition along the dermal-epidermal junction. (g) Electron microscopy of rescued mouse skin injected with BP IgG reveals skin separation or putative blisters (stars) developing within the lamina lucida adjacent above the lamina densa (LD).

Figure 3

(a) Scheme of the antigenic epitope of COL17, NC16A domain, and therapeutic recombinant proteins. The 77 amino acid NC16A domain is located between the first extracellular 15th collagenous domain (COL15) and transmembrane residues. R1 to R6 refers to the recombinant proteins that are complementary to different portions and different lengths of the corresponding NC16A domain. (b) *In vitro* BP autoantibody ELISA percent index value after immunoadsorption with 20 μg of recombinant proteins, R1 and R2 (including GST-fusion forms of R1-GST, and R2-GST), and 10 μl of serum from BP patients. Note R1-GST suppressed BP autoantibody index values most effectively (n=6, p=0.0038). (c) R1-GST protein suppresses BP antibody index values in a dose dependent manner, whereas control GST protein shows no therapeutic effect (n=5). (d) Control COL17-humanized mouse injected with pathogenic BP autoantibody with GST peptide alone developed clinical and histological skin detachment (stars) associated with IgG deposits at the BMZ (arrows). In contrast, R1-GST treated mouse showed little clinical or histological findings as seen in the control mice, and IgG deposition along the dermal-epidermal junction was reduced (arrowheads). (e) Recombinant peptide, R1-GST, carrying COL17 antigenic sequences remarkably suppressed blister formation in experimental mice injected with BP autoantibody *in vivo*. (f) BP autoantibody ELISA index values were also significantly reduced in the R1-GST treated mice.

Table 1
Result of passive transfer of IgG from 5 BP patients into COL17-humanized mice. The COL17-humanized mice that were injected intra-peritoneally with whole IgG fractions and COL17-affinity purified (underlined) IgG fraction developed distinct skin fragility (positive Nikolsky sign) at 48 h. In contrast, control IgG from healthy volunteers resulted in no detectable abnormal skin lesions (n=5, all mice were injected with total IgG because COL17-affinity IgG was not obtainable). Neg; negative. N.D.; not done.

Table 1 **Characteristics of serum and IgG from BP patients with the extent of disease in neonatal mice**

Patient	Serum from BP patients: IIF titer Human skin	IIF titer Mouse skin	BP180 ELISA titer	BP230 ELISA titer	Passive transfer of IgG into COL17-humanized mice: IgG / COL17-affinity IgG dose (mg/body weight (g))	Skin detachment
1	x320	Neg	171.4	Neg	2	5/6
					1	7/7
					0.5	3/6
					0.25	0/7
					0.05	5/5
2	x320	Neg	162.5	27.06	2	7/7
					1	7/7
					0.5	3/5
					0.25	2/6
					0.05	5/5
3	x320	Neg	142.4	Neg	2	5/5
					1	5/5
					0.5	3/6
					0.25	2/6
					0.05	5/5
4	x320	Neg	184.4	Neg	2	5/6
					1	6/8
					0.5	5/7
					0.25	4/5
					0.05	N.D.
5	x40	Neg	125.5	Neg	2	5/5
					1	5/5
					0.5	5/6
					0.25	2/5
					0.05	N.D.
healthy control (n=5)	Neg	Neg	Neg	Neg	2	0/5
					1	0/5
					0.5	0/5
					0.25	0/5
					0.05	0/5

Supplementary Methods

Screening of recombinant clones and genotyping of *COL17* $^{m-/-}$ mice by PCR
Primer, YA1 (5'-CATACCAGGGCCAACTTTGA-3') was designed downstream outside the region used to generate the targeting construct. The primer N1 (5'-TTGTGTAGCGCCAAGTGCCA-3') and WT1 (5'-CCTTATATCCCTTGACTGCC-3') was designed at the 5' end of the Neo cassette and within the region used to generate the targeting construct, respectively.

RT-PCR
For RT-PCR, total RNA from tissues was extracted using ISOGEN, according to manufacturer's recommendations (Nippon Gene, Tokyo, Japan). Three micrograms of total RNA was used for cDNA synthesis in SuperScript III reverse transcriptase according to manufacturer's instructions (Invitrogen, Carisbad, CA). The following primers specific for mouse BP180 chain sequences (NM: 007732) were used for RT-PCR: 5'-AGAAGAAAAGCATCCGAGGG-3' (RT-F); and 5'-TGGTTGAAGAAGAGGCGAGT-3' (RT-B).

Northern blotting
For northern analysis, denatured RNA gels using 13 μg of total RNA per lane were transfer to Zetaprobe membrane (Bio-Rad, Hercules, CA) and Northern blotting was carried out as described elsewhere [1, 2]. Membranes were hybridized with 5' labeled probes (residues 351 690) amplification products of the mouse COL17 cDNA by use of a digoxigenin DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

Western blotting
Western blotting was performed as previously described [3]. Tissue extracts were subjected to SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked with 1% non-fat dry milk in PBS, probed with rat monoclonal antibodies against mouse COL17 NC16A domain (mNC16A mAb, 1:250,000; a gift from T Tanaka, Shiga Medical University, Japan), and then allowed to react with goat anti-rat IgG antibody coupled with horseradish peroxidase.

Histology and electron microscopy
The tissue samples were fixed in 10% buffered formalin followed by paraffin embedding. Five-μm sections were stained with hematoxylin-eosin. Transmission electron microscopy was performed as previously described [4], using a transmission electron microscope (Hitachi, H-7100).

Immunofluorescence

Five -μm cryosections of OCT-embedded skin were cut and placed onto microscope slides and subjected to indirect immunofluorescence. The following primary antibodies and dilutions were used: mNC16A mAb (1:5000) and mouse monoclonal antibodies against human COL17 (D20, 1:160; a gift from K Owaribe, Nagoya University, Japan). The following secondary antibodies were used, FITC-conjugated donkey anti-rat IgG, FITC-conjugated goat anti-mouse IgG (Jackson, West Grove, PA).

Generation of recombinant COL17 NC16A peptide

77 amino acids covered with full-length of COL17 NC16A domain (R1, amino acids 490-566), and its 45 amino acids N-terminus half (R2, amino acids 490-534) were both synthesized as GST-fusion proteins using expression vector pGEX2-T (Amersham Biosciences, Japan) and bacteria BL21 (Amersham Biosciences, Japan) as reported previously [5]. In this study, we used both native GST-fusion forms, R1-GST and R2-GST, and GST-cleaved ones (R1, and R2). Other variably sized and distributed amino acids R3 (amino acids 490-525), R4 (amino acids 526-559), R5 (amino acids 490-509), and R6 (amino acids 506-525) peptides were chemically synthesized (R3 and R4; Greiner Bio-one, Japan, R5 and R6; Nippi, Inc, Japan).

***In vitro* immunoadsorption study**

To assess the therapeutic potential of recombinant peptides carrying COL17 antigenic sequences, preliminary *in vitro* immunoadsorption studies were performed. Briefly, 20 μg of each of the recombinant peptides was incubated with 10 μl of serum from BP patients in 37°C for 30 minutes, followed by testing using a BP180 ELISA kit according to manufacturer's instructions (MBL, Japan). Preliminarily immunoadsorption study of the suppression rate (%) compared with control (GST) showed; R1: 29.02±17.18 (p=0.009), R1-GST: 60.18±22.35 (p=0.004), R2: 7.6±11.54 (p=0.16), R2-GST: 33.89±13.1 (p=0.001), R3: 10.38±5.47 (p=0.01), R4: -0.44±3.87 (p=0.791), R5: 12.03±5.05 (p=0.002), R6: 0.2±3.91 (p=0.907). From these data, we selected to use the R1-GST peptide for the *in vivo* decoy therapy studies.

Supplementary Fig. 1 Growth and Kaplan-Meier survival curves of wild type, *COL17* $^{m-/-}$, and rescued COL17-humanized mice.

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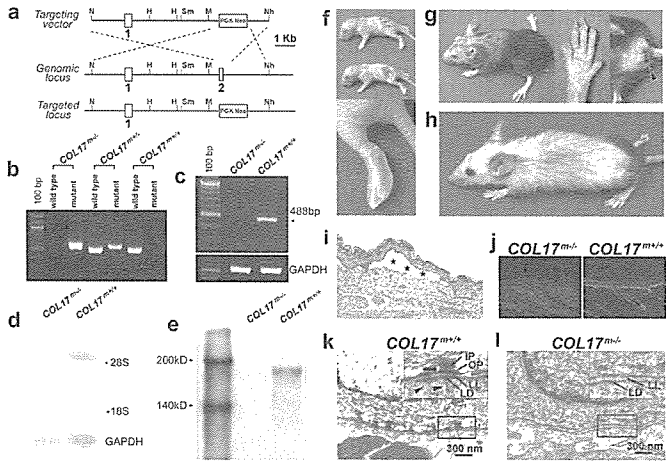


Fig 1 Nishie et al

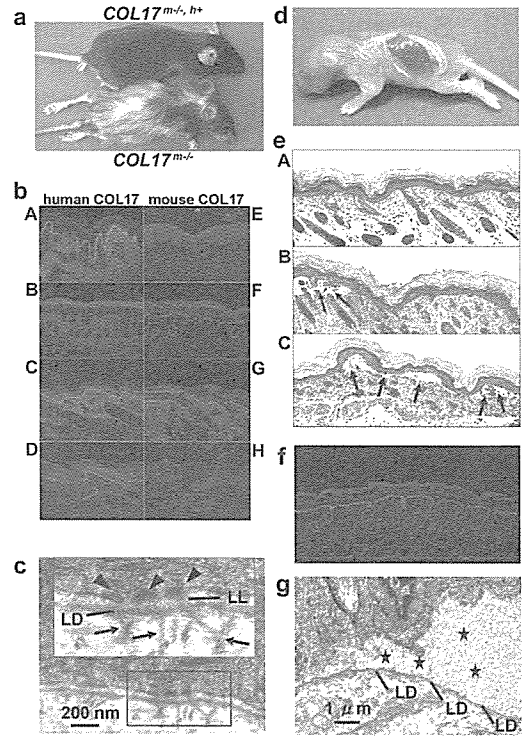


Fig 2 Nishie et al

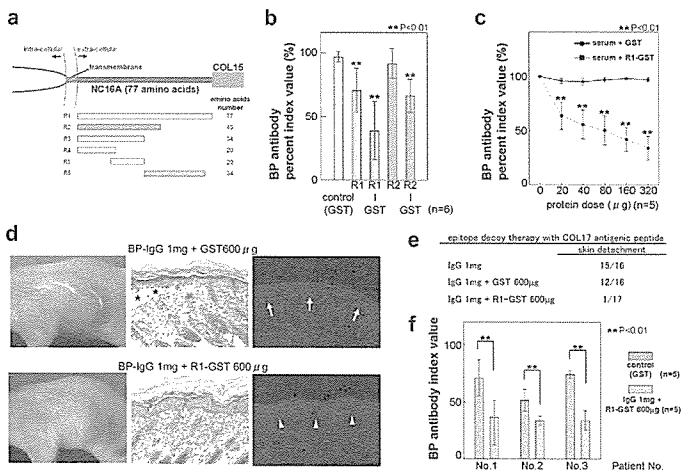
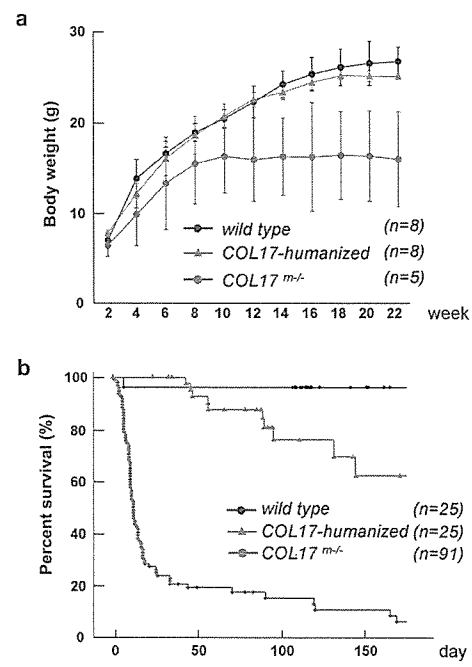


Fig 3 Nishie et al



Supplementary Fig 1 Nishie et al

[Q3] *Rapid publication*

Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis

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Background: Filaggrin is a key protein involved in skin barrier [Q5] function. Recently, mutations in the filaggrin gene, FLG, were identified in European families with ichthyosis vulgaris (IV) and shown to be an important predisposing factor for atopic dermatitis (AD).
Objective: To study the role of FLG mutations in IV/AD in Japan.
Methods: The known filaggrin mutations were studied by genotyping and new mutations identified by DNA sequencing.
Results: The European-specific mutations R501X and 2282del4 were absent from 253 Japanese individuals. We therefore sequenced the FLG gene in 4 Japanese families with IV and identified 2 novel mutations, 3321delA and S2554X. Immunohistologic and ultrastructural observations indicated that both truncation mutations lead to a striking reduction of keratohyalin granules in the epidermis. We screened 143 Japanese patients with AD for these null FLG mutations and identified them in 8 patients with AD (5.6%), including S2554X in 6 patients (4.2%) and 3321delA in 2 patients (1.4%). Both null variants were absent from 156 unrelated Japanese nonatopic and nonichthyotic controls, giving a significant statistical association between the FLG mutations and AD (χ^2 *P* value, .0015). This is the first report of FLG mutations in a non-European population.
Conclusion: Our data indicate that FLG mutations in Japan are unique from those found in European-origin populations.
Clinical implications: Filaggrin null variants are also significant predisposing factors for AD in Japan and, on the basis of the recent European studies, may predict a more severe and persistent form of atopy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Filaggrin, eczema, ichthyosis, keratinization, skin barrier, atopy, skin, genodermatosis, genetics, mutation*

Ichthyosis vulgaris (IV; OMIM#146700) is a relatively [Q6] common genetic disorder of keratinization clinically characterized by scaling, especially on the flexor limbs, and palmoplantar hyperlinearity.[1] IV symptoms are most pronounced in winter or in cold, dry climates. Keratosis pilaris is also a feature and, importantly, atopic dermatitis (AD) is commonly seen in IV.[2] Histologically, a decrease in the size and number or a complete absence of keratohyalin granules in the epidermis is typically characteristic of IV.[1] AD is highly debilitating inflammatory skin disorder that is highly prevalent in the developed world, where it affects 15% to 20% of children.[3] Although there is clearly an environmental component to the pathogenesis of AD, the condition is highly heritable, and a number of susceptibility loci have been mapped, including the epidermal differentiation complex (EDC) on human chromosome 1q21, where a genetic predisposing factor for psoriasis has also been localized.[4] The EDC is a dense cluster of genes involved in the terminal differentiation of the epidermis and formation of the stratum corneum, the outermost dead cell compartment of the skin where the main skin barrier function resides.[5] Significantly, the FLG gene encoding filaggrin, one of the most abundant proteins in the outer epidermis, is located in the EDC.[6]

Keratohyalin granules in the granular layer of the epidermis are predominantly composed of a >400-kd polyprotein, profilaggrin.[7-9] On terminal differentiation of

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Abbreviations used
AD: Atopic dermatitis
DMSO: Dimethyl sulfoxide
EDC: Epidermal differentiation complex
IV: Ichthyosis vulgaris

keratinocytes, profilaggrin is dephosphorylated and cleaved into 10 to 12 essentially identical 37-kd filaggrin peptides. The liberated filaggrin subsequently aggregates the keratin filament cytoskeleton, causing the collapse of the granular cells into flattened squames. The collapsed cytoskeleton is crosslinked by transglutaminases to form the proteinaceous component of the cornified cell envelope. A secondary function has been suggested whereby degradation products of filaggrin contribute to moisture retention in the cornified layers of the epidermis.[10] Thus, filaggrin is a key protein in facilitating the terminal differentiation of keratinocytes and thereby in maintaining normal skin barrier function and hydration.

In 2006, two null mutations, R501X and 2282del4, in the FLG gene were identified in patients with IV from Scottish, Irish, and European-American populations.[11] Using the recently revised mutation nomenclature, these mutations are designated p.Arg501Term and c.2282_2285del4, respectively; however, the abbreviated names are used here for brevity and ease of reading. About half of patients with moderate to severe AD in the Irish population were also reported to harbor the same mutations, with strong associations in Scottish and Danish cohorts.[12] Therefore, FLG mutations have recently attracted great attention as a key predisposing factor for AD.[13] Subsequently, these 2 mutations were shown to predispose individuals to AD in a range of white European populations, with more than 20 forms of strongly positive replication study now in print.[14-20] The prevalent European mutations R501X and 2282del4, both of which are loss-of-function alleles leading to complete loss of filaggrin expression, have a combined allele frequency of about 0.045 in white populations.[12] However, FLG mutations have yet to be reported in non-European populations, and so the global contribution of this gene to AD remains to be evaluated.

Here, we have studied 4 Japanese families with IV and identified 2 novel FLG mutations. In addition, we have shown that this gene is significantly associated with genetic predisposition to AD in Japan.

METHODS

Clinical material

Blood samples were obtained from 7 patients with IV from 4 independent Japanese families and a further 143 Japanese AD patients from 140 independent families. The diagnosis of AD was made using the AD diagnostic criteria by Hannifin and Rajka.[21] For controls, DNA samples from 156 normal healthy, unrelated Japanese individuals were included in the current study. Patients gave written informed consent, which complies with all the Declarations of Helsinki Principles. This study was approved by the Medical Ethical Committees of the Hokkaido University, Sapporo, and Shiga Medical University, Shiga, Japan.

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 an ABI PRISM 3100 genetic analyzer (ABI Advanced Biotechnologies, Columbia, Md). Mutations R501X and 2282del4 were screened using restriction enzyme digest of PCR products. Mutation 3702delG was screened by fluorescent PCR. Primers and PCR conditions were as described previously.[11,16]

Mutation detection and screening for 3321delA

A PCR fragment of 3697 bp was amplified with forward primer FILF3 5′-GCT GAT AAT GTG ATT CTG TCT G-3′ and reverse primer RPT3P10R 5′-GAC CCC GAT TGT TCC TGT-3′ (Sandilands 2006). A total of 100 ng genomic DNA was amplified using High Fidelity PCR buffer (Roche, Penzberg, Germany) containing 1.5 mmol/L $MgCl_2$, 4% (vol/vol) dimethyl sulfoxide [Q7] (DMSO), and 1 unit Expand High Fidelity enzyme (Roche). PCR [Q8] amplification conditions were as follows: 1 cycle of 94°C for 5 min- [Q9] utes; 35 cycles of 94°C for 30 seconds, 61°C for 45 seconds, and 72°C for 3 minutes 20 seconds; and 1 cycle of 72°C for 5 minutes. The 3697-bp PCR fragment was sequenced with primer RPT2P20F 5′-CCA GAC AAT CAG GAA CTC C-3′.

Mutation 3321delA was screened by sizing of a fluorescently labeled PCR fragment on an Applied Biosystems 3100 or 3730 DNA [Q10] sequencer. Ten-microliter PCR reactions were performed by using primers delA.F1 5′-GTT TCT TAG TGA GGG ACA TTC AGA GGA G-3′ and FAM-delA.R 5′-ATG AGT GCT CAC CTG GTA GAT-3′ in AmpliTaq Gold buffer containing 1.5 mmol/L $MgCl_2$ (Applied Biosystems, Foster City, Calif), 10 nmol of each dNTP, [Q11] and 1 unit AmpliTaq Gold DNA polymerase (Applied Biosystems). Reactions were amplified as follows: 94°C (12 minutes), 1 cycle; [Q12] 94°C (30 seconds), 64°C (30 seconds), and 72°C (45 seconds), 31 cycles; and 72°C (5 minutes), 1 cycle. PCR products were typically diluted 1:50 and sized with ROX-500 size markers according to the manufacturer's recommended protocol (Applied Biosystems). Note that the sequence GTTTCTT was added to the unlabeled primer to promote plus-A addition during PCR and reduce stutter peaks; this therefore adds an additional 7 bp to the fragments amplified. Thus, the wild-type allele was identified as a 160-bp peak, and the 3321delA allele as a 159-bp peak.

Mutation detection and screening for S2554X

A 1233-bp PCR product containing repeat 7 of the FLG gene was amplified with forward primer RPT6P1F 5′-CCA CAC GTG GCC GGT CAG CA-3′ and reverse primer RPT8P1R 5′-CTA CCG AAT GCT CGT GGT GGT-3′. A total of 100 ng genomic DNA was amplified using High Fidelity PCR buffer (Roche) containing 1.5 mmol/L $MgCl_2$, 4% (vol/vol) DMSO and 1 unit of Expand High Fidelity enzyme (Roche). PCR amplification conditions were as follows: 1 cycle of 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, [Q13] 62°C for 30 seconds, and 72°C for 1 minute 10 seconds; then 1 cycle of 72°C for 5 minutes. The 1233-bp PCR fragment was sequenced with primer RPT6P1F. Mutation S2554X abolishes a Hpy188 I restriction enzyme site. A 512-bp PCR fragment was amplified from genomic DNA by using forward primer RPT6P1F and reverse primer RPT7P1R 5′-CTG GCT AAA ACT GGA TCC CCA-3′ using High Fidelity PCR buffer (Roche) containing 1.5 mmol/L $MgCl_2$, 4% (vol/vol) DMSO, and 1 unit Taq polymerase mix (Promega, Madison, Wis). PCR amplification conditions were as follows: 1 cycle of 94°C

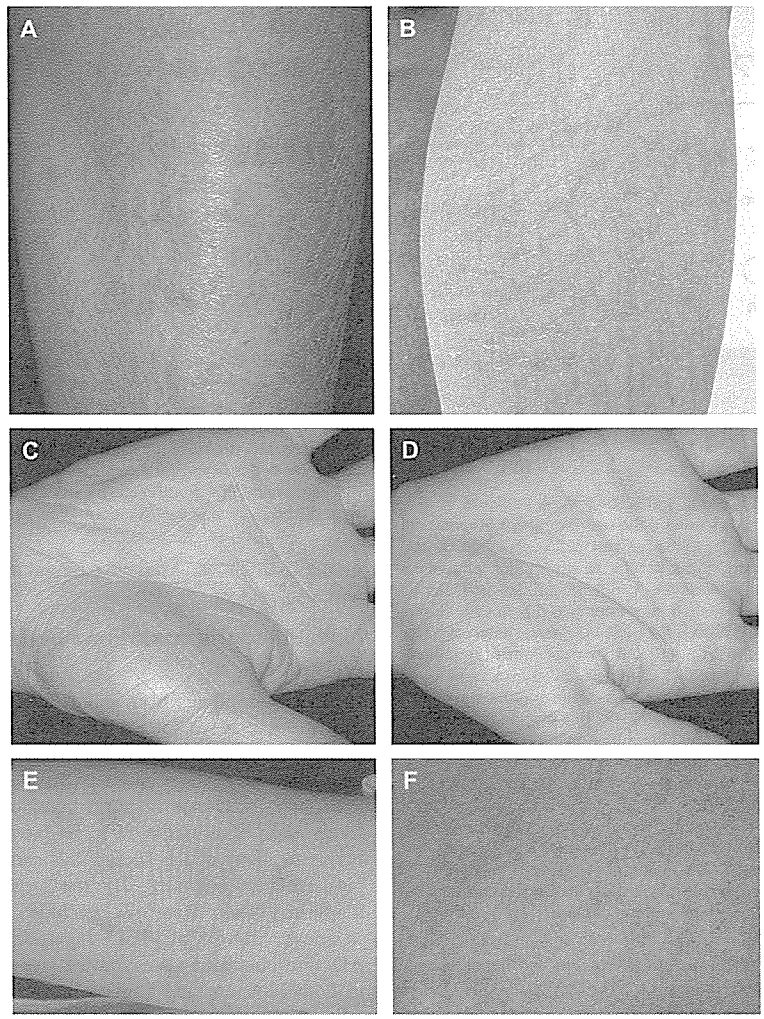

FIG 1. Clinical features of patients with IV. **A,** Marked, adherent scales were clearly visible on the pretibial region of a 3321delA heterozygote from family 3. **B,** Fine, pityriatic scales on the pretibial region of the proband, S2554X heterozygote, in family 1. **C,** Marked plantar hyperlinearity seen in the S2554X homozygote from family 1. **D,** In contrast, the S2554X heterozygote in family 1 presented with a less marked plantar hyperlinearity. This clearly shows that IV is inherited as a semidominant trait. **E,** Marked lichenification on the left antecubital fossa of a patient with AD who was heterozygous for 3321delA. **F,** Itchy excoriated papules on the trunk of a patient with AD who was heterozygous for S2554X.

[Q14]. for 5 minutes; 35 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute; then 1 cycle of 72°C for 5 minutes. PCR products were digested with 20 units Hpy188 I (New England Biolabs, Ipswich, Mass) at 37°C overnight. Digests were resolved on 3% (wt/vol) agarose gels. The mutant allele was resolved as 231-bp, 204-bp, and 77-bp fragments, whereas the wild-type allele gave fragments of 231 bp, 159 bp, 77 bp, and 45 bp.

Statistical analysis

In this study, case-control association analyses were performed by [Q15]. using Pearson χ^2 statistics, as previously described.[12] All alleles were observed to be in normal Hardy-Weinberg equilibrium.

Immunohistochemical and ultrastructural analysis

Immunoperoxidase staining of frozen and paraffin-embedded sections was performed by using the ChemMate Peroxidase/DAB system (DakoCytomation, Hamburg, Germany). Mouse mAb 15C10 (Novocastra, Newcastle, United Kingdom) was used to detect the human filaggrin repeat unit. Importantly, antigen retrieval of paraformaldehyde-fixed, paraffin-embedded [Q16]. was performed by heating sections under pressure for 10 to 15 minutes in 10 mmol/L citrate buffer, pH 6.0. For ultrastructural analysis, skin biopsy samples were fixed in 5% (vol/vol) glutaraldehyde solution, postfixed in OsO_4, dehydrated, and embedded in Epon 812. The samples were [Q17].

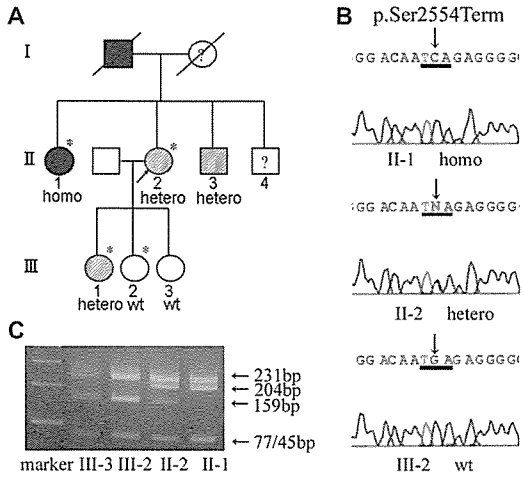

FIG 2. Detection of FLG mutation, S2554X. **A,** A pedigree of ichthyosis vulgaris family 1, showing the semidominant inheritance pattern. *Solid symbols* refer to the marked ichthyosis vulgaris presentation; *cross-hatched symbols* refer to the milder ichthyosis vulgaris presentation. In addition, 4 individuals also had atopic dermatitis. *Individuals with a dermatologist-diagnosed atopic dermatitis. *homo,* Homozygous for S2554X; *hetero,* heterozygous for S2554X; *wt,* wild type for S2554X. **B,** Normal control sequence from filaggrin repeat 7 in exon 3 identified in III-2 as shown in *A,* corresponding to codons 2552-2556. A heterozygous transition mutation c.7661C→G was identified in II-2 (the proband), resulting in S2554X. A homozygous transition mutation c.7661C→G in filaggrin repeat 7 in exon 3 was identified in II-1, resulting in nonsense mutation, S2554X. **C,** Screening for the mutation S2554X by Hpy188 I restriction digest. The mutant alleles were resolved into 231-bp, 204-bp, and 77-bp bands, whereas the wild-type alleles were resolved into 231-bp, 159-bp, 77-bp, and 45-bp bands. In this analysis, 45-bp and 77-bp alleles were resolved into a single band on a 3% agarose gel. The PCR product from the proband (heterozygote) in family 1 was resolved into 5 bands.

sectioned at 1-mm thickness for light microscopy and thin-sectioned for electron microscopy (70 nm thick). The ultrathin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope. For controls, normal skin samples were studied.

RESULTS

Case reports of IV families

In total, 4 Japanese kindreds with IV were studied. In families 1 and 2, the probands showed mild scaling on the extensor surfaces of the legs that became more pronounced during the winter season (Fig 1). Mild palmoplantar hyperlinearity was also seen in probands from both kindreds, and significantly, both probands also had a history of AD. In family 1, the proband's siblings also showed scaling, and especially her elder sister had marked palmoplantar hyperlinearity (Fig 1). The proband's deceased father was also reported to have shown a marked IV phenotype.

In families 3 and 4, both probands showed an even more marked IV phenotype. Distinct, adherent scales were clearly visible even in summer (Fig 1) and were extensively distributed, even on the trunk and flexor surfaces of the extremities. Marked palmoplantar hyperlinearity was also evident.

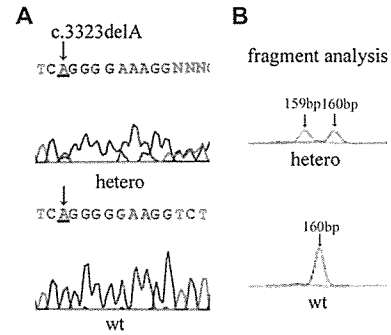

FIG 3. Detection of FLG mutation, 3321delA. **A,** A heterozygous deletion mutation in filaggrin repeat 2 in exon 3, 3321delA, was identified in the proband from family 3. Normal control sequence from filaggrin repeat 2 in exon 3, corresponding to codons 1105-1110. *hetero,* Heterozygous for 3321delA; *wt,* wild type for 3321delA. **B,** Screening for the mutation 3321delA by sizing a fluorescent labeled PCR fragment. The wild-type allele was identified as a 160-bp peak, and the 3321delA allele as a 159-bp peak.

Novel filaggrin mutations in Japanese IV families

Seven individuals from 4 unrelated Japanese IV families were screened for the previously reported filaggrin mutations R501X and 2282del4 by restriction enzyme digestion, as previously described.[11] Furthermore, the third, apparently rare filaggrin mutation, 3702delG, was screened by fluorescent PCR, as previously reported.[16] However, none of the IV families carried these 3 European-specific mutations. By the same means, these 3 filaggrin variants were also excluded from 156 normal Japanese controls and 143 Japanese individuals with AD, thus confirming the reported lack of these mutations in both Japanese and Chinese populations.[12] We have recently developed specific PCR primers that allow routine diagnostic resequencing of the entire filaggrin coding sequence (Sandilands and McLean, paper in preparation). [Q18]. By these means, we fully sequenced the gene in Japanese IV families and identified 2 novel mutations, termed p.Ser2554Term and c.3321delA according to the current mutation nomenclature guidelines. For brevity, these mutations are refered to as S2554X and 3321delA. Both mutations lead to premature termination of profilaggrin translation in filaggrin repeat domains 7 and 2, respectively.

Specifically, the probands from families 1 and 2 were heterozygous carriers of the nonsense mutation S2554X (Fig 2). In family 1, DNA sequencing demonstrated that the proband's elder sister was homozygous for S2554X, whereas the younger brother and 1 of the proband's 3 daughters were heterozygous carriers (Fig 2). The mutation was also confirmed by restriction digestion (Fig 2). [F2-4/C]

The probands from families 3 and 4 were heterozygous carriers of the frameshift mutation 3321delA (Fig 3). This mutation leads to a premature termination codon 41 bases downstream that stops protein translation in filaggrin [F3-4/C]

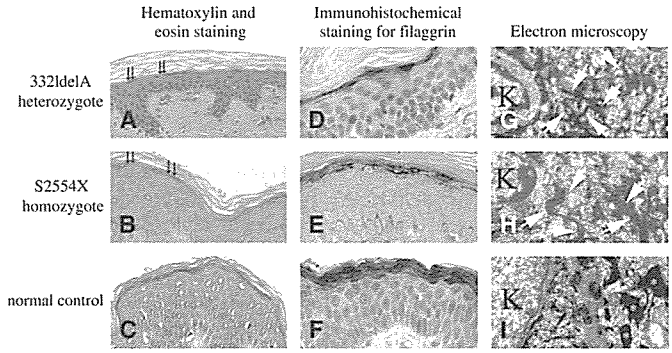

FIG 4. Histologic features of the patients with IV. **A-C,** Hematoxylin and eosin staining. Both the 3321delA heterozygote in family 3 *(A)* and the S2554X homozygote in family 1 *(B)* showed a lack of granular layers in the epidermis, where only a small amount of basophilic substance *(arrows)*, resembling keratohyalin, was occasionally present. In contrast, normal control skin *(C)* had abundant keratohyalin granules in the granular layers. **D-F,** Immunohistochemical staining using antifilaggrin mAbs against an epitope conserved in all filaggrin repeat peptides. Both the 3321delA heterozygote in family 3 *(D)* and the S2554X homozygote in family 1 *(E)* showed a marked reduction in staining for filaggrin, whereas normal control skin *(F)* stained strongly. **G-I,** Electron microscopic observation of the granular layers. Both the 3321delA heterozygote in family 3 *(G)* and the S2554X homozygote in family 1 *(H)* exhibited an apparent loss of keratohyalin granules, although small keratin filament aggregates *(arrows)* were observed in granular layer keratinocytes, perhaps corresponding to the residual keratohyalin seen histologically. In normal control skin *(I)*, a normal amount of keratohyalin granules *(*)* was confirmed.

repeat domain 2 (protein mutation nomenclature p.Ser 1107SerfsX1121). Again, this mutation was independently confirmed by restriction digestion (Fig 3).

[Q19]. These mutations were not found in 156 healthy, unrelated Japanese individuals (312 alleles) by sequence analysis. This, together with the fact that both variants are premature termination codon mutations, suggests that these are pathogenic mutations rather than harmless polymorphisms.

Absence of keratohyalin granules and lack of profilaggrin expression

Skin biopsy specimens from the S2554X homozygote in family 1 and the 3321delA heterozygote in family 3 revealed that a small amount of basophilic material was partially present in granular layer keratinocytes just below the stratum corneum by hematoxylin and eosin staining in

[F4-4/C] both patients (Fig 4). However, electron microscopic analysis showed an almost complete absence of keratohyalin granules, although tiny keratin filament aggregations could be observed in granular layer keratinocytes. Immunohistochemical staining using antifilaggrin mAbs showed that a conserved epitope in all filaggrin repeat peptides was present in the patient's epidermis but was greatly reduced, consistent with both the small amount of kerato-

[Q20]. hyalin visible by hematoxylin and eosin staining and the ultrastructural findings (Fig 4).

Novel FLG mutations are predisposing factors for AD in Japan

A total of 143 Japanese patients with AD were screened for mutation S2554X by restriction enzyme digestion and for 3321delA by sizing of fluorescently labeled PCR fragments (Figs 2 and 3). The presence of the mutations was independently confirmed by direct DNA sequencing of PCR products. The FLG genotype data in the Japanese AD and ethnically matched population controls are summarized in Table I. Interestingly, and as one would predict from the European studies, these 2 apparently Japanese-specific mutations found in IV families were also identified in the Japanese AD population. Mutations S2554X and 3321delA were carried by 6 (4.2%) and 2 (1.4%) of individuals in the Japanese AD cohort, respectively. The mutations were excluded from 156 unrelated Japanese control individuals who had no history of IV or AD, that is, this was a hypernormal cohort rather than a general population cohort. The association between the combined FLG null variant genotypes and AD was statistically significant. Comparing the AD cohort and hypernormal cohort for the 2 null alleles combined yielded a χ^2 P value of .0015 (Table I). [Q21].

[T1]

TABLE I. Japanese case-control study of filaggrin mutations in atopic dermatitis

Genotypes	3321delA		S2554X		Combined genotype	
AA	156	141	156	137	156	135
Aa	0	2	0	5	0	7
aa	0	0	0	1	0	1
Totals	156	143	156	143	156	143
χ^2	P = .139		P = .0017		P = .0015	

DISCUSSION

The human profilaggrin gene (FLG) is located on chromosome 1q21 and consists of 3 exons. Exons 1 and

2 are small, whereas exon 3 is unusually large (<12 kb) and encodes for most of the N-terminal S100 and B domains, as well as 10 to 12 filaggrin repeats. The homology between the repeats at the DNA level approaches 100%, making PCR and sequence analysis difficult. The profilaggrin protein is proteolytically cleaved into multiple filaggrin peptides that have been shown to aggregate the keratin cytoskeleton.[9] This is thought to bring about cell compaction and squame formation at the granular layer-stratum corneum transitional zone. Within the fully differentiated stratum corneum, an additional function has been suggested in which filaggrin is further degraded into amino acids and hygroscopic derivatives thereof that may contribute to moisture retention.[10] Therefore, loss or reduction of filaggrin expression correlates with excessively dry skin and impaired barrier function, which is variously manifest as ichthyosis and/or atopic disease.

The prevalent FLG mutations in the European population, R501X and 2282del4, were previously shown to lead to a complete loss of filaggrin peptide synthesis because these 2 mutations were located within the first filaggrin repeat.[11] A third mutation, frameshift 3702delG in repeat 3, has also been shown to lead to a severe IV phenotype,[16] showing that more distal mutations are likely to be of similar pathogenicity to the repeat 1 null variants. With the mutations identified here in the Japanese population, in filaggrin repeats 2 and 7, one might predict that truncated profilaggrin peptides could be produced, and this is what we indeed observed here (Fig 4). Specifically, electron microscopy and immunohistochemistry using skin biopsy from the probands in 2 families demonstrated that keratohyalin granules were greatly reduced in quantity.

The mouse model of IV, flaky tail, which lacks expression of mature, processed filaggrin, is thought to harbor a mutation within repeats 6 to 7 of the murine flg gene.[22] Affected homozygous ft/ft mice lack normal high-molecular-weight profilaggrin (500 kd) and instead express a smaller 220-kd form of profilaggrin.[22] This truncated mutant profilaggrin is not properly proteolytically processed into filaggrin.[22] Therefore, sequences close to the profilaggrin C-terminus appear to be necessary for proteolytic processing of profilaggrin.[16] Our observations here support this hypothesis and demonstrate that in human beings, as is the case in flaky tail mice, truncated profilaggrin species with as many as 6 intact repeats can be expressed at the protein level, but the marked IV phenotype of these patients implies that insufficient filaggrin is made from these mutant alleles and/or the mutant profilaggrin cannot be processed into functional filaggrin. Further biochemical analysis of patients carrying this class of more 3′ filaggrin mutation should clarify what effects these defects have on profilaggrin proteolysis.

Filaggrin is a key epidermal protein and essential for the formation of a normal, intact, and protective skin barrier. Null FLG mutations are a predisposing factor for AD in Japan, Europe, and quite possibly most other races worldwide to differing degrees. Impairment of the skin barrier has been regarded as a primary event in AD. However, studies on FLG mutations were previously performed only on people of European origin. Combining our study here with the published data, neither R501X nor 2282del4 was detected in 288 Japanese individuals (including 147 patients with AD) or 49 Chinese individuals. We also excluded the rare European mutation 3702delG from 156 Japanese individuals. Therefore, the European mutatons are either extremely rare or, more likely, completely absent from Oriental populations. We were, however, able to identify 2 specific FLG null mutations, 3321delA and S2554X, in 4 Japanese families with IV, and significantly, we have found these same mutations in 5.6% of a Japanese AD cohort. We also excluded the 2 Japanese variants from a cohort of 188 unrelated Irish people. Thus, these 2 novel mutations appear to be unique in people of Oriental origin and are carried by patients with AD as well as patients with IV. In contrast, we failed to find either of them in 156 unaffected control individuals of Japanese origin (312 normal control FLG alleles). In the Japanese population, there is significant statistical association between the newly identified FLG mutations and AD (χ^2 $P = .0015$). Although the frequency of FLG mutations observed in the Japanese AD cohort is lower than that seen in European populations, we cannot exclude the possibility that there are more common mutations in the Japanese population to be discovered. Analysis of further patients is ongoing in our laboratories. Because the filaggrin gene is large, repetitive, and difficult to sequence, the strategy we used in this study and in European populations of first sequencing patients with IV in whom there is a very high probability of a filaggrin mutation[11] and then screening for the mutations in AD and population cohorts[12] seems to be both efficient and successful in unravelling the global contribution of this problematic gene to atopy.

Because FLG mutations are a major predisposing factor for AD, the clinical importance of FLG mutations should continue to be emphasized. Impaired epidermal barrier function is also considered a key event in allergic sensitization. Disruption of the normal epidermal barrier allows allergens to penetrate into skin and leads to exposure to antigen-presenting cells, leading to allergic sensitization. In some patients with AD, the development of asthma and allergic rhinitis will follow, that is, the so-called atopic march.[23] Recently, FLG null mutations have been reported to predispose to allergic phenotypes involved in the atopic march.[12,18] Early intervention using topical emollients for the maintenance and repair of the epidermal barrier may be important to prevent the subsequent development of asthma and allergic rhinitis in infants with FLG mutations. Recently, a strong association with FLG mutations and early-onset AD persisting into adulthood has also been reported.[14] Therefore, identification of FLG null mutations is an indicator of a possible severe phenotype, and the screening for FLG mutations is helpful to predict a more accurate prognosis.[14]

In conclusion, we have uncovered the first FLG mutations unique to the Japanese population, and these data emphasize the importance of the screening for FLG mutations in Japanese patients with IV and/or AD.

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