2.2. Antibodies used in this study

The monoclonal antibody of interest, termed 29A, was purified from ascites fluids of the SCID mice by HiTrap IgM Purification HP (Amersham) according to the manufacturer's instructions, as the 29A antibody was of the IgM subclass. A monoclonal antibody MLuC5 against laminin receptor (LR) was from Quartett (Berlin, Germany). The polyclonal anti-LR antibody FD4818 derived from rabbit raised against the polypeptide corresponding to amino acids 3–15 of LR [10] was kindly provided by Dr. Seiji Takashima (Osaka University Graduate School of Medicine, Osaka, Japan). Another polyclonal antibody against the polypeptide corresponding to amino acids 279–295 of LR was produced by rabbit immunizations. The following commercial secondary antibodies were obtained: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM antibody from Biosource International, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG antibody from Dako, and peroxidase-conjugated second antibodies from Jackson ImmunoResearch Laboratories Inc.

2.3. Immunofluorescence staining

Immunofluorescence staining of 5 μm cryosections of normal human placental amnion and normal human skin were carried out using standard methods. Briefly, after being washed with a PBS/BSA mixture, the tissues were incubated with the first antibodies overnight at 4 °C, and then incubated with FITC- or TRITC-conjugated second antibodies for 1 h at room temperature. Following extensive washing, fluorescence was observed with confocal laser scanning microscope (Olympus Fluoview FV300, Tokyo, Japan).

2.4. Western blotting

Normal human amnion was extracted with Laemmli sample buffers with or without 2-mercaptoethanol. Protein samples were separated on SDS-PAGE with polyacrylamide gels, and then transferred to nitorocellulose membranes. After the membranes were incubated for 1 h in a blocking buffer of 2% fat-free milk in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl (tris-buffered saline; TBS). The first antibody was applied overnight at room temperature. After washes with 0.05% Tween-20 in TBS, the membranes were incubated for 2 h with peroxidase-conjugated second antibody diluted 1:1000 in blocking buffer. After the membranes were washed, the signals were detected with chemiluminescence (Amersham Inc., Amersham, UK).

2.5. Immunoscreening

Normal human keratinocyte cDNA library (Clontech) was screened with the 29A antibody. Briefly, single *E. coli* Y1090 colony was grown at 37 °C in 10 ml LB medium (10 g/l trypton, 5 g/l bacto-yeast extract, 10 g/l NaCl, and 1 mM NaOH) supplemented with 10 mM $MgSO_4$, 0.4% maltose, and 50 μg/ml ampicillin until the OD_{660} reached 0.3. The cells were harvested by centrifugation and resuspended in 10 mM $MgSO_4$. Phage solution containing approximately 10^4 pfu phages in SM buffer (0.1 M NaCl, 50 mM Tris–HCl, pH 7.5, 10 mM $MgSO_4$, 0.01% gelatin) was incubated with 200 μl of Y1090 cell suspension at 37 °C for 20 min, and then plated on the 100 mm LB–agar plates containing 50 μg/ml ampicillin with an LB soft top agarose. After the plates were incubated at 42 °C for 4 h, nitrocellulose filters presoaked in 10 mM isopropyl-B-thiogalactopyranoside were overlaid on the plates and incubated for 4 h at 37 °C. After being washed and blocked with milk proteins, the filters were incubated with the 29A antibody. The positive signal was detected using diaminobenzidine. Positive plaques were picked up on the original plate, and eluted in SM buffer containing chloroform. After repeated isolation and screening of these clones, candidate clones were obtained. As a control, normal mouse IgM (Sigma) was used instead of the 29A antibody.

2.6. Sequence analysis

The insert cDNA of positive phage clones were sequenced on a Perkin-Elmer 3100 sequencer using the forward primer; 5′-GAA GGC ACA TGG CTG AAT ATC GAC GGT TTC-3′ and reverse primers; 3′-CAG CGA TGG TAA TGG TCA ACC AGA CCA CAG-5′, and the sequence results were searched on the BLAST site (http://www.ncbi.nlm.nih.gov/BLAST/).

2.7. Recombinant LR protein generated in the baculovirus

Recombinant LR was expressed in baculovirus-infected insect cells using Bac-to-Bac baculovirus expression system (Invitrogen, Tokyo, Japan). A full-length cDNA encoding the human LR was cloned by RT-PCR using mRNA isolated from normal human skin into pFastBac plasmid (Invitrogen). For construction of the donor plasmid, a pair of the gene-specific primers was designed; 5′-TAG GAA TTC TCA CAA TGT CCG GAG CCC TT-3′ (spacer and *Eco*R I site are included) and 5′-CTA CTC GAG TTA AGA CCA GTC AGT GGT TG-3′ (spacer and *Xho* I site are included). The PCR product was digested with *Eco*R I and *Xho* I (Takara Bio, Otsu, Japan), separated by agarose-gel

electrophoresis and purified from the gel with QIAquick Gel Extraction Kit (Qiagen). The purified DNA was ligated into a pFastBac plasmid predigested with *Eco*R I and *Xho* I, and then sequenced. The expression cassette of the plasmid was incorporated into the baculovirus genome (bacmid DNA) in DH10Bac competent cells (Invitrogen) by using Tn7 site-specific transposition according to the manufacturer's instruction. The resulting recombinant bacmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen) and transfected into *Spodoptera frugiperda* Sf9 cell (Invitrogen) using CellFECTIN (Invitrogen). After incubation at 27 °C for 72 h in Sf-900 II SFM medium (Invitrogen), the resultant recombinant baculovirus was harvested. A control baculovirus was prepared in the same way by using pFastBac. Viral titers were determined with a conventional viral plaque assay.

10^7 Sf9 cells were seeded in a 150 mm dish and infected with above recombinant baculoviruses with a multiplicity of infection of 10. After incubation in serum-free Sf-900 II SFM medium at 27 °C for 96 h, the generated recombinant LR protein was collected from the Sf9 cells. The cell pellets were homogenized in ice-cold 50 mM Tris—HCl buffer (pH 7.2) containing protease inhibitors and centrifuged at 15,000 × *g* for 30 min. Then the samples were concentrated with Amicon Ultra YM-30 and stored at −80 °C until use.

2.8. Dot binding assays

The extracted recombinant LR protein was eluted in TBS buffer and dotted onto nitrocellulose membranes using a vacuum manifold [11]. After blocking with 2% milk protein in TBS, the filters were incubated with the first antibody followed by peroxidase-conjugated second antibody. Bound antibodies were revealed using chemiluminescence.

3. Results

3.1. Isolation of the 29A antibody

The monoclonal antibody, which one of the single-cloned hybridoma cells secreted, the 29A antibody, intermittently stained the cytoplasm of amniotic cells using immunofluorescence staining (Fig. 1). We were interested in this unique staining pattern; therefore, we analyzed the amniotic protein extracts by western blotting. However, no positive band was shown under both reduced and non-reducing conditions (data not shown). These results indicated that the 29A antibody probably recognized a conformational epitope on a low level-expressed cytoplasmic protein in amniotic cells.

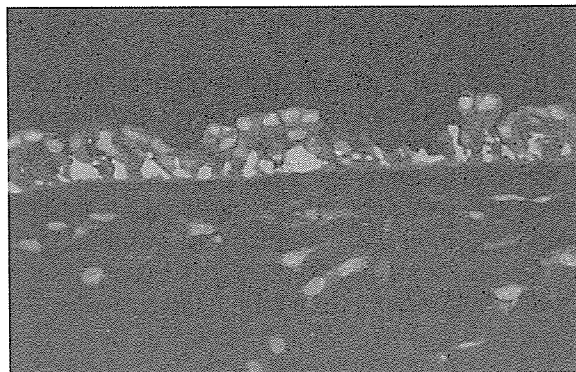

Fig. 1 Isolation of the 29A antibody. The monoclonal 29A antibody intermittently stained the cytoplasm of normal human amniotic cells using an indirect immunofluorescence staining technique. (Green): FITC-conjugated anti-mouse IgM antibody; (Red): propidium iodide, nuclear stain.

3.2. Immunoscreening using the 29A antibody

For the purpose of cloning of the 29A antigen, we used a keratinocyte cDNA expression library and immunoscreened it. Recombinant phages were plated with *E. coli* Y1090, and plaques were immunoscreened with the 29A antibody. Upon the first screening of 8×10^6 clones, we obtained three candidate clones after repeated screening and isolations. Each cDNA insertion was sequenced and found to contain one continuous open reading frame. BLAST research of these sequences revealed identical cDNA fragments of laminin receptor (Fig. 2).

3.3. The 29A antibody reacted with recombinant LR

To confirm that the 29A antibody recognizes the LR protein, full-length recombinant LR protein was expressed in insect cells. The size of the generated recombinant LR protein was 37 kDa under non-reducing conditions. The 29A antibody reacted with the

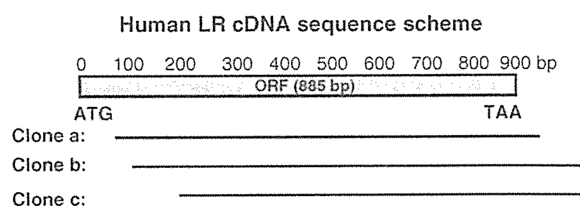

Fig. 2 The schema selecting positive clones for immunoscreening. Upon the first screening of 8×10^6 clones, finally three candidate clones (a—c) were obtained. Each cDNA insertion contained one continuous open reading frame, and their sequences revealed identical cDNA fragments encoding for the laminin receptor (LR).

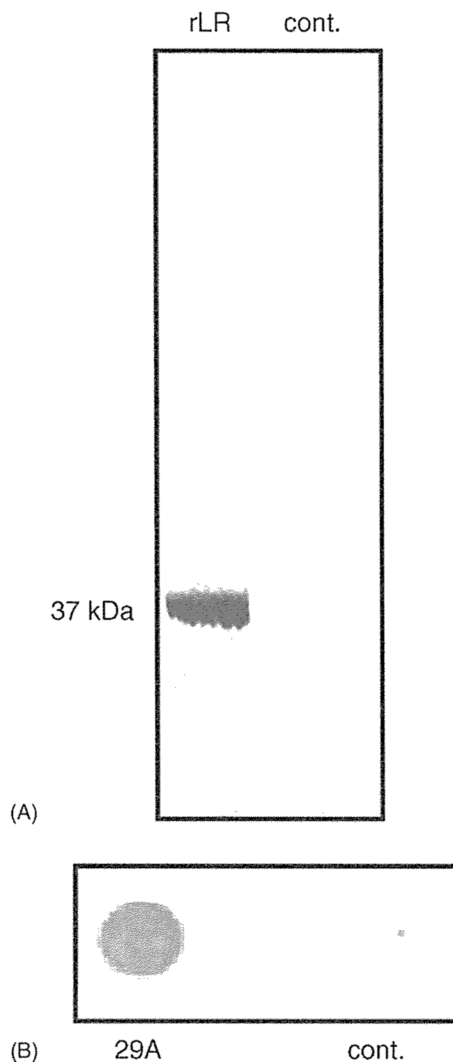

Fig. 3 Reactivity of the 29A antibody with human LR. Full-length recombinant human LR was expressed in insect cells. (A) Western blotting. Generated recombinant LR (rLR) was present in a 37 kDa form under non-reducing condition. This was confirmed using the anti-LR antibody. (B) Dot binding assay. The 29A antibody reacted with recombinant LR. Cont., control baculovirus-infected insect cell lysate.

recombinant LR by a native dot binding assay (Fig. 3).

3.4. The 29A antigen and LR co-localized in the amnion

The presence of LR in the amnion was examined using the polyclonal anti-LR antibody. It stained the partial cytoplasm of amniotic cells. Moreover, double staining with the 29A antibody and the anti-LR antibody revealed co-localization of the 29A antigen and LR in the amnion (Fig. 4).

3.5. The 29A antibody reacted to the basement membrane zone in the normal human skin

We examined human skin tissue with the 29A antibody. Unexpectedly the 29A antibody reacted in a linear fashion along the BM of normal human skin by IF staining (Fig. 5a). However, the cytoplasm of keratinocytes was not stained. Conversely, we investigated the localization of LR in the skin using MLuC5 monoclonal antibody. In normal human skin, MLuC5 partially stained keratinocyte cell membranes but only very weakly along the BM (Fig. 5b).

4. Discussion

In this study, we have produced and characterized a novel monoclonal 29A antibody against human placental amnion. The 29A antibody stained the cytoplasm of selected amniotic cells at intervals in a unique manner. However, the 29A antigen was not detected by western blot analysis, therefore it is considered that the 29A probably recognizes a conformationally sensitive epitope. We cloned this antigen using an immunoscreening method, and identified the candidate gene of interest as a laminin receptor. We confirmed that the 29A antibody actually reacted with the recombinant LR protein by dot binding assay. In addition, we proved that the 29A antigen showed the same staining pattern as LR in the placental amnion by double staining IF microscopy analysis. We therefore concluded that the 29A antibody recognized the LR-related protein. To further confirm these findings, we have repeatedly tried to isolate the 29A antigen by affinity chromatography and immunoprecipitation using amniotic extracts. However, it was impossible to isolate this specific protein by the affinity chromatography or immunoprecipitation methods using this 29A antibody (data not shown).

LR was originally identified as a 67 kDa protein that binds to laminin 1 and is expressed in a colon cancer cell line [12]. It seems that the evidence for LR being a receptor was based on the finding that it was retained in a laminin column [13], and in addition to laminin, the known ligands for LR are type IV collagen and fibronectin [14,15]. LR cDNA encodes for only 295 aa (37 kDa), and it is still unknown if cells make the 67 kDa LR through the addition of extra glycoprotein moieties or via other post-translational modifications. Castronovo et al. reported that transfection of the full-length LR cDNA into COS-7 cells induced an increase in the synthesis of the 37 kDa form but not of the 67 kDa form [16]. In

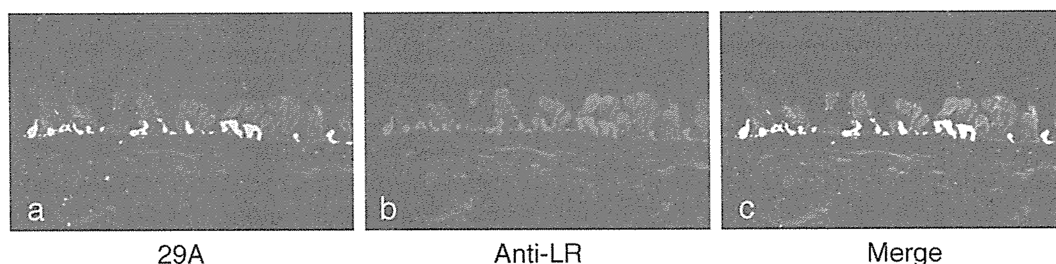

Fig. 4 Co-localization of the 29A antigen and LR in normal placental amnion. (a) The 29A antibody intermittently stained the cytoplasm of normal human amniotic cells by an indirect immunofluorescence staining. (Green): FITC-conjugated anti-mouse IgM antibody. (b) Anti-LR antibody also intermittently stained the cytoplasm of normal human amniotic cells by an indirect immunofluorescence staining. (Red): TRITC-conjugated anti-rabbit IgG antibody. (c) Double immunofluorescence staining with the 29A antibody and anti-LR antibody revealed co-localization of the 29A antigen and LR within the placental amniotic cells.

addition, we could only produce a 37 kDa recombinant LR protein generated in insect cells. Various studies have revealed that LR is expressed on cell surfaces in various tissues [17], but little is currently known about the specific function of LR.

Interestingly, the 29A antibody failed to react with the cytoplasm of keratinocytes by IF staining, but reacted in a linear fashion along the dermal—epidermal junction of normal human skin. Previously characterized cutaneous dermal—epidermal junction component proteins are also present in placental BM, therefore the 29A antigen has a unique distribution. Whether the reaction of the 29A monoclonal antibody with both amnion and skin was due to the presence of the same protein or just a similar or related epitopes needs to be determined. First the cDNA of human LR was cloned by RT-PCR using mRNA isolated from normal human skin. The sequence of the cloned cDNA was identical to the published human LR cDNA (data not shown). The anti-LR monoclonal MLuC5 antibody also stained the epidermal BM, albeit weakly, we used two different polyclonal anti-LR antibodies, which were produced against N-terminal (FD4818) or C-terminal peptides of LR to confirm the presence of LR in the dermal—epidermal junction. However, these polyclonal antibodies failed to stain not only dermal—epidermal junction but also keratinocyte cell membranes (data not shown). The shed form of LR has also been detected [18,19]. In the normal glomerulus, it was revealed that mesangial cells produced and secreted LR into extracellular matrix, and it was demonstrated that LR was present as an adhesion molecule in the glomerular BM [19]. The epidermal BM comprises a highly organized network of supramolecular components, including the hemidesmosome-anchoring filament complex and anchoring fibrils, and these structures firmly anchor the epidermal keratinocytes to the dermis and dermal components. Many other molecular components form a network of strong interactions that contribute to the dermal—epidermal adhesion [20]. In such a complicated structure, each molecule is closely associated with its adjacent component and antigenic epitopes are often masked.

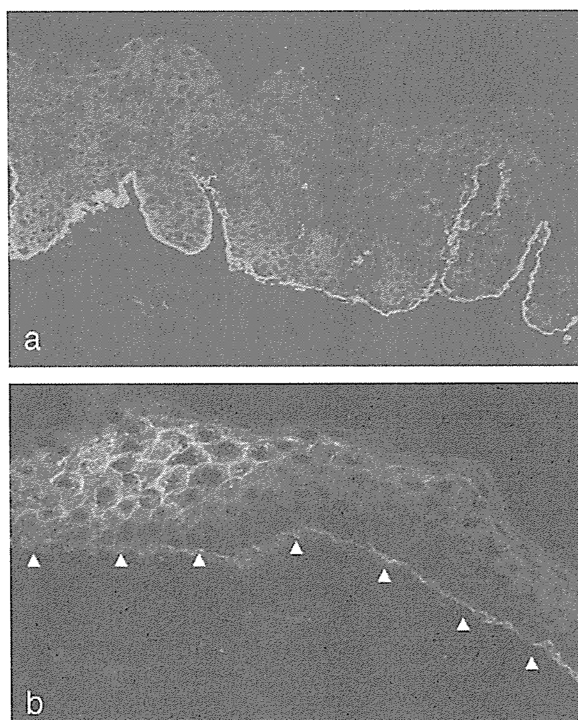

Fig. 5 The 29A antibody stained the basement membrane zone in normal human skin. (a) Indirect immunofluorescence staining of normal skin showed that the 29A antigen linearly stained the dermal—epidermal junction. (b) A monoclonal antibody for LR, MLuC5, partially stained keratinocyte cell membranes but only very weakly dermal—epidermal junction. The arrows point to the basement membrane zone.

The production of monoclonal antibodies against epithelial component molecules has been indispensable for investigations into normal and abnormal epithelial conditions. Although our 29A antibody has

been difficult to characterize biochemically, this monoclonal antibody might be a useful tool for investigations into epithelia biology and further in dermatological research.

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References

[1] Laurie GW, Leblond CP, Martin GR. Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. J Cell Biol 1982;95:340–4.
[2] Paulsson M. Basement membrane proteins: structure, assembly, and cellular interactions. Crit Rev Biochem Mol Biol 1992;27:93–127.
[3] Oyama N, Bhogal BS, Carrington P, Gratian MJ, Black MM. Human placental amnion is a novel substrate for detecting autoantibodies in autoimmune bullous diseases by immunoblotting. Br J Dermatol 2003;148:939–44.
[4] Katz SI. The epidermal basement membrane zone—structure, ontogeny, and role in disease. J Am Acad Dermatol 1984;11:1025–37.
[5] Fleishmajer R, Perlish JS, Macdonald II ED, Schechter A, Murdoch AD, Iozzo RV, et al. There is binding of collagen IV to beta 1 integrin during early skin basement membrane assembly. Ann N Y Acad Sci 1998;857:212–27.
[6] Liotta LA. Tumor invasion and metastases—role of the extracellular matrix. Cancer Res 1986;46:1–7.
[7] Timpl R. Macromolecular organization of basement membranes. Curr Opin Cell Biol 1996;8:618–24.
[8] Costell M, Gustafsson E, Aszódi A, Mörgelin M, Bloch W, Hunziker E, et al. Perlecan maintains the integrity of cartilage and some basement membranes. J Cell Biol 1999; 147:1109–22.
[9] Hattori N, Fujiwara H, Maeda M, Fujii S, Ueda M. Epoxide hydrolase affects estrogen production in the human ovary. Endocrinology 2000;141:3353–65.
[10] Asano Y, Takashima S, Asakura M, Shintani Y, Liao Y, Minamino T, et al. Lamr1 functional retroposon causes right ventricular dysplasia in mice. Nat Genet 2004;36:123–30.
[11] Heckmann M, Aumailley M, Hatamochi A, Chu ML, Timpl R, Krieg T. Down-regulation of alpha 3(VI) chain expression by gamma-interferon decreases synthesis and deposition of collagen type VI. Eur J Biochem 1989;182:719–26.
[12] Yow H, Wong JM, Chen HS, Lee C, Steele Jr GD, Chen LB. Increased mRNA expression of a laminin-binding protein in human colon carcinoma: complete sequence of a full-length cDNA encoding the protein. Proc Natl Acad Sci USA 1988;85:6394–8.
[13] Wewer UM, Liotta LA, Jaye M, Ricca GA, Drohan WN, Claysmith AP, et al. Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin. Proc Natl Acad Sci USA 1986;83:7137–41.
[14] Iwabuchi K, Nagaoka I, Someya A, Yamashita T. Type IV collagen-binding proteins of neutrophils: possible involvement of L-selectin in the neutrophil binding to type IV collagen. Blood 1996;87:365–72.
[15] Narasimhan S, Armstrong MY, Rhee K, Edman JC, Richards FF, Spicer E. Gene for an extracellular matrix receptor protein from *Pneumocystis carinii*. Proc Natl Acad Sci USA 1994;91:7440–4.
[16] Castronovo V, Claysmith AP, Barker KT, Cioce V, Krutzsch HC, Sobel ME. Biosynthesis of the 67 kDa high affinity laminin receptor. Biochem Biophys Res Commun 1991; 177:177–83.
[17] Ardini E, Sporchia B, Pollegioni L, Modugno M, Ghirelli C, Castiglioni F, et al. Identification of a novel function for 67-kDa laminin receptor: increase in laminin degradation rate and release of motility fragments. Cancer Res 2002; 62:1321–5.
[18] Karpatova M, Tagliabue E, Castronovo V, Magnifico A, Ardini E, Morelli D, et al. Shedding of the 67-kD laminin receptor by human cancer cells. J Cell Biochem 1996;60:226–34.
[19] Hu C, Oliver JA, Goldberg MR, Al-Awqati Q. LRP: a new adhesion molecule for endothelial and smooth muscle cells. Am J Physiol Renal Physiol 2001;281:F739–50.
[20] Aumailley M, Battaglia C, Mayer U, Reinhardt D, Nischt R, Timpl R, et al. Nidogen mediates the formation of ternary complexes of basement membrane components. Kidney Int 1993;43:7–12.

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Novel ALDH3A2 Heterozygous Mutations in a Japanese Family with Sjögren–Larsson Syndrome

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TO THE EDITOR

Sjögren–Larsson syndrome (SLS: OMIM No. 270200) is an autosomal-recessive hereditary disorder characterized by congenital ichthyosis, mental retardation, and spastic diplegia or tetraplegia (Rizzo, 1993). Rizzo *et al.* (1988) revealed that long-chain fatty alcohol was abnormally accumulated in cultured fibroblasts, white blood cells and serum in SLS patients. In 1996, De Laurenzi *et al.* (1996) reported that mutations in the fatty aldehyde dehydrogenase (FALDH) gene (*ALDH3A2*) underlie SLS. In the present study, we report novel compound heterozygous mutations in *ALDH3A2* in a Japanese family with SLS.

A 2-year-old Japanese girl was suffering from congenital ichthyosis, mental retardation, spastic tetraplegia, and recurrent epileptic seizures. The patient was the first child of non-consanguineous, healthy Japanese parents. Ichthyosis was not seen in any other family members. She was born as a pre-term baby at 34 weeks 3 days of gestation (body weight, 2392 g), but not a collodion baby. Physical examination revealed fine scales and slight erythema over her whole body (Figure 1). Hyperkeratosis was also seen on the palms and soles. Her hair, nails, and teeth were normal. The skin manifestations were consistent with non-bullous congenital ichthyosiform erythroderma. Her extremities were hypertonic. T2-weighted magnetic resonance imaging demonstrated a high-intensity area in her brain periventricular white matter. Ophthalmologic examination revealed retinal crystals, generally referred to as "glistening white dots".

To elucidate the precise genetic abnormality in the family, mutational analysis was performed in the affected girl and her parents. Briefly, genomic DNA isolated from peripheral blood cells was subjected to PCR amplification, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (ABI Advanced Biotechnologies, Columbia, MD). Oligonucleotide primers and PCR conditions used for amplification of all exons and exon–intron borders of *ALDH3A2* were originally derived from the report by Rizzo *et al.* (1999) and were partially modified for our study (Shibaki *et al.*, 2004). The entire coding region including the intron/exon boundaries for both forward and reverse DNA strands from the patient, her parents and 100 healthy Japanese controls were sequenced. In the patient, a combination of two novel heterozygous mutations, 332G>A in exon 2 and 636T>G in exon 4, were identified (Figure 2a). The mutation 332G>A was present in her father, and the mutation 636T>G was demonstrated in her mother. The presence of both mutations was excluded in 200 alleles of 100 normal unrelated Japanese individuals. The medical ethical committee of the Hokkaido University approved all described studies. These studies were conducted according to the Declaration of Helsinki Principles. The patient's parents gave their written informed consent.

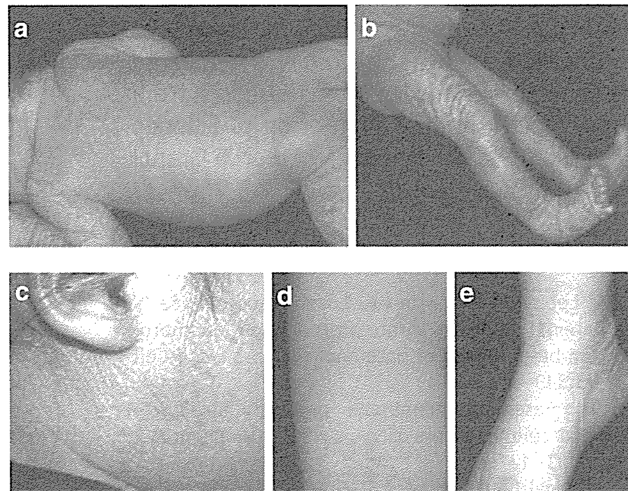

Figure 1. Clinical features of the SLS patient. (**a, b**) In the perinatal period, xerotic skin, and fine scales were apparent over the (**a**) proband's trunk and (**b**) hyperkeratosis was severe on her legs. (**c, d, e**) At 2 years of age, fine, whitish scales were seen on her (**c**) cheek, (**d**) thigh, and the (**e**) dorsal foot.

The paternal mutation 332G>A in exon 2 leads to an alteration of the tryptophan residue at codon 111 into a stop codon (nonsense mutation W111X) and this premature translation termination eliminates approximately 80% of the length of FALDH polypeptide (loss of 77.3% length of the FALDH major splice variant). Thus, this nonsense mutation is expected to seriously abolish FALDH function.

The maternal mutation 636T>G in exon 4 resulted in an alteration of a serine at codon 212 to arginine (S212R). FALDH amino-acid sequence alignment shows that this serine residue at codon 212 is conserved among several diverse species (reference sequences: CR457422, XP 511337, NP 113919, CAI25890, NP 001016537, AAK49120) (Figure 2b).

In addition, according to a comparison of 145 full-length aldehyde dehydrogenase-related sequences by Perozich *et al.* (1999), this serine is

Abbreviations: FALDH, fatty aldehyde dehydrogenase; SLS, Sjögren–Larsson syndrome

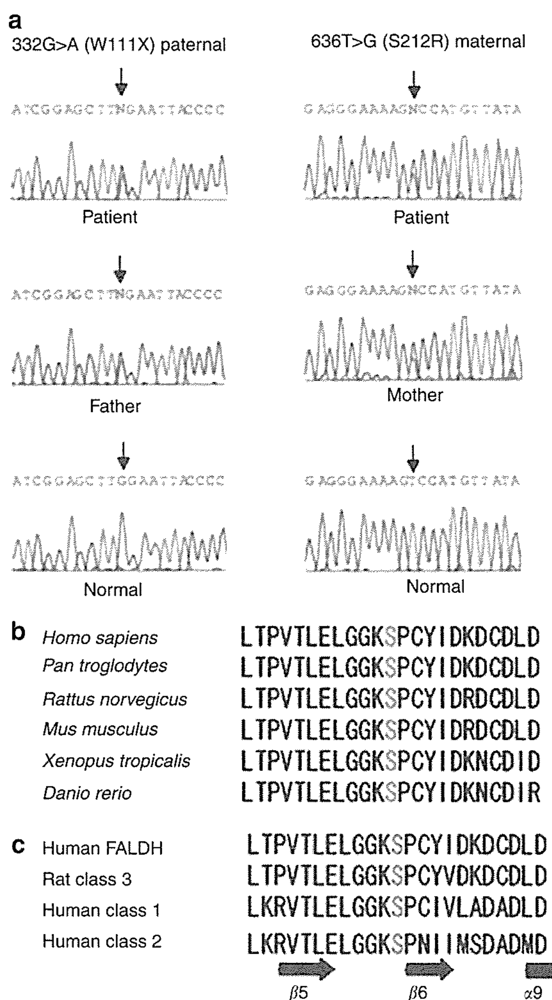

Figure 2. *ALDH3A2* mutations in the present SLS patient and sequence alignments around the missense mutation. (**a**) Sequence analysis of the *ALDH3A2*. A combination of heterozygous mutations derived from their mother (332G>A (W111X) in exon 2) and father (636T>G (S212R) were detected. (**b**) FALDH amino-acid sequence alignment shows the level of conservation in diverse species of the amino-acid S212 (red characters), which was altered by the missense mutation in the present family. (**c**) A sequence alignment between the FALDH, rat class 3 and human class 1 and class 2 ALDHs showing the relative locations of key residues in these enzymes. Serine residue at codon 212 of FALDH is strictly conserved. Secondary structure components found in the class 3 rat ALDH structure are presented with a bar and arrows. The bar represents an α-helix and arrows represent β-strands. (Modified from the paper by Liu *et al.*, (1997).)

highly conserved among many of the ALDH family members, and participates in one of the 10 most conserved sequence motifs in ALDHs (Figure 2c). In addition, analysis of the crystallized three-dimensional structure of the related class 3 rat cytosolic ALDH revealed that this serine is located adjacent to the first β-strand, β6, of the six parallels of β-strands, comprising a significant portion of the catalytic domain of the molecule (Liu *et al.*, 1997) (Figure 2c). These findings strongly suggest that the serine at codon 212 is important for connecting the α/β structure of a dinucleotide-binding Rossmann fold (Freshney *et al.*, 1994) to the catalytic domain and/or for structural folding of the catalytic domain, and is therefore essential for the normal function of the FALDH protein. The fact that the present patient harboring the missense mutation S212R showed typical SLS phenotypic features strongly suggests that this serine residue is essential for FALDH enzymatic function.

FALDH is a microsomal NAD-dependent enzyme, which is necessary for the oxidation of long-chain aliphatic aldehydes into fatty acids (Kelson *et al.*, 1997). The FALDH gene (*ALDH3A2*) located on chromosome 17p11.2 (De Laurenzi *et al.*, 1996) comprises 11 exons, and is widely expressed in a variety of tissues (Chang and Yoshida, 1997; Rogers *et al.*, 1997). Until now, a number of mutations in *ALDH3A2* have been shown to be responsible for SLS over the world (Rizzo *et al.*, 1999). Founder effects were observed in certain areas and races (Rizzo *et al.*, 1999; Kraus *et al.*, 2000; Rizzo and Carney, 2005). In Japanese patients with SLS, one homozygous mutation 1157A>G (N386S) in *ALDH3A2* was reported in a patient of one family (Aoki *et al.*, 2000) and two other mutations, 481delA and 1087_1089delGTA, were reported in another family (Shibaki *et al.*, 2004). We have detected two additional mutations, 332G>A (W111X) and 636T>G (S212R), in the present family. All the mutations detected in Japanese families were distinct one another and we therefore speculate there is no founder effect in Japanese cases with *ALDH3A2* mutations causing SLS.

The pathogenesis of the ichthyosis in SLS includes abnormal lamellar or membranous inclusions in cornified cells, which were reported in a patient with SLS, although causative genetic abnormalities were not known in that particular case (Ito *et al.*, 1991). The inclusions were speculated to be lamellar granule-in-origin. Later, a deficiency in acyl-ceramides in the lipid layer in the stratum corneum was also reported in SLS patients (Paige *et al.*, 1994). In a previous report (Shibaki *et al.*, 2004), we observed ultrastructurally abnormal lamellar granules lacking normal lamellar contents in the upper spinous and granular layers. In addition, we showed some malformed lamellar granule components were secreted into the intercellular space in the stratum corneum (Shibaki *et al.*, 2004). These observations suggest defective lamellar granule formation in SLS. SLS is thought to be a form of ichthyoses caused by defective

lipid barrier in the stratum corneum (Akiyama, 2006).

As illustrated here, mutation analysis of the *ALDH3A2* gene is a highly sensitive method to confirm a diagnosis of SLS, which does not require a skin biopsy and can complement or replace FALDH enzymatic assays or analysis in SLS.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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REFERENCES

Akiyama M (2006) Harlequin ichthyosis and other autosomal recessive congenital ichthyoses: the underlying genetic defects and pathomechanisms. *J Dermatol Sci* 42:83–9

Aoki N, Suzuki H, Ito K, Ito M (2000) A novel point mutation of the FALDH gene in a Japanese family with Sjögren–Larsson syndrome. *J Invest Dermatol* 114:1065–6

Chang C, Yoshida A (1997) Human fatty aldehyde dehydrogenase gene (ALDH10): organization and tissue-dependent expression. *Genomics* 40:80–5

De Laurenzi V, Rogers GR, Hamrock DJ, Marekov LN, Steinert PM, Compton JG *et al.* (1996) Sjögren–Larsson syndrome is caused by mutations in the fatty aldehyde dehydrogenase gene. *Nat Genet* 12:52–7

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J *et al.* (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* 78:1039–49

Ito M, Oguro K, Sato Y (1991) Ultrastructural study of the skin in Sjögren–Larsson syndrome. *Arch Dermatol Res* 283:141–8

Kelson TL, Secor McVoy JR, Rizzo WB (1997) Human liver fatty aldehyde dehydrogenase: microsomal localization, purification, and biochemical characterization. *Biochim Biophys Acta* 1335:99–110

Kraus C, Braun-Quentin C, Ballhausen WG, Pfeiffer RA (2000) RNA-based mutation screening in German families with Sjogren–Larsson syndrome. *Eur J Hum Genet* 8: 299–306

Liu Z-J, Sun Y-J, Rose J, Chung YJ, Hsiao CD, Chang WR *et al.* (1997) The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD and the Rossmann fold. *Nat Struct Biol* 4:317–26

Paige DG, Morse-Fisher N, Harper JI (1994) Quantification of stratum corneum ceramides and lipid envelope ceramides in the hereditary ichthyoses. *Br J Dermatol* 131: 23–7

Perozich J, Nicholas H, Wang B-C, Lindahl R, Hempel J (1999) Relationships within the aldehyde dehydrogenase extended family. *Protein Sci* 8:137–46

Rizzo WB (1993) Sjögren–Larsson syndrome. *Semin Dermatol* 2:210–8

Rizzo WB, Carney G (2005) Sjögren–Larsson syndrome: diversity of mutations and polymorphisms in the fatty aldehyde dehydrogenase gene (*ALDH3A2*). *Hum Mutat* 26: 1–10

Rizzo WB, Carney G, Lin Z (1999) The molecular basis of Sjögren–Larsson syndrome: mutation analysis of the fatty aldehyde dehydrogenase gene. *Am J Hum Genet* 65: 1547–60

Rizzo WB, Dammann AL, Craft DA (1988) Sjögren–Larsson syndrome. Impaired fatty alcohol oxidation in cultured fibroblasts due to deficient fatty alcohol: nicotinamide adenine dinucleotide oxidoreductase activity. *J Clin Invest* 81:737–44

Rogers GR, Markova NG, De Laurenzi V, Rizzo WB, Compton JG (1997) Genomic organization and expression of the human fatty aldehyde dehydrogenase gene (FALDH). *Genomics* 39:127–35

Shibaki A, Akiyama M, Shimizu H (2004) Novel ALDH3A2 heterozygous mutations are associated with defective lamellar granule formation in a Japanese family of Sjögren–Larsson syndrome. *J Invest Dermatol* 123:1197–9

Novel Hairless *RET*-Transgenic Mouse Line with Melanocytic Nevi and Anagen Hair Follicles

Journal of Investigative Dermatology (2006) **126,** 2547–2550. doi:10.1038/sj.jid.5700444; published online 15 June 2006

TO THE EDITOR

The c-*RET* proto-oncogene encodes a receptor-tyrosine kinase and glial cell line-derived neurotrophic factor ligands, including glial cell line-derived neurotrophic factor, neurturin, artemin, and persephin, have been reported to be ligands of RET (Takahashi, 2001). *RFP/RET* is a hybrid oncogene between c-*RET* and *RFP* that was isolated by NIH3T3 transfection assays (Takahashi *et al.*, 1985). Previously, we established a metallothionein-I/*RFP-RET* (*RET*)-transgenic mouse line (242) that spontaneously develops systemic skin melanosis without macroscopic tumors (Iwamoto *et al.*, 1991; Kato *et al.*, 1999). Generally, most hair follicles in adult wild-type mice are in telogen (Kato *et al.*, 2001). It is basically impossible to induce continuous anagen hair follicles in adult wild-type mice, although temporal anagen hair follicles are inducible by shaving hairs (Kato *et al.*, 2001). Interestingly, adult *RET*-transgenic mice have continuous anagen hair follicles with hyper melanin production (Kato *et al.*, 2001, 2004). Moreover, hair growth of adult transgenic mice was promoted compared with that of control C57BL/6 mice (Kato *et al.*, 2001). These results suggest that a continuous anagen phase of hair follicles plays an important role in hair growth.

We also established another *RET*-transgenic mouse line (304/B6) (Kato

Gene Corner

Glycine substitution mutations by different amino acids at the same codon in *COL7A1* cause different modes of dystrophic epidermolysis bullosa inheritance

DOI: 10.1111/j.1365-2133.2006.07388.x

Dystrophic epidermolysis bullosa (DEB) is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy, in which patients exhibit tissue separation beneath the lamina densa close to dermal anchoring fibrils. Tissue separation occurs in both autosomal dominant (DDEB) and recessive (RDEB) forms of the disease, each form having a different specific clinical presentation and severity.[1] The most severe RDEB subtype, the Hallopeau–Siemens type (HS), typically shows a complete lack of expression of type VII collagen while some collagen expression is found in the non-Hallopeau–Siemens type (nHS). Clinical features of DDEB are generally milder than those of RDEB.

Both DDEB and RDEB are caused by mutations in the *COL7A1* gene encoding type VII collagen. To date, almost 300 pathogenic mutations within the collagenous and noncollagenous domains of the type VII collagen gene have been identified in different forms of DEB[2–5] and the basic genotype–phenotype correlations have been elucidated. In this study, we examined a Japanese boy with generalized blistering from birth and found interesting *COL7A1* mutational findings.

Case and methods

A 23-month-old boy with skin fragility from birth was referred to our hospital. Examination revealed generalized blisters and erosions over his whole body (Fig. 1). Healing occurred with minimal scarring and formation of occasional milia. His hands and feet showed complete nail loss and fusion of fingers and toes. The mucous membrane of his mouth was also severely eroded. The patient was an only child and there was no family history of skin fragility or nail dystrophy.

Histologically, the patient showed a subepidermal blister. Direct immunofluorescence using the monoclonal antibody LH 7.2 recognizing collagen VII revealed reduced linear immunoreactivity at the dermal–epidermal junction (Fig. 2). Electron microscopic examination showed sublamina densa epidermal detachment and the presence of some fibrillar anchoring fibril-like structures below the lamina densa (Fig. 2).

To identify the underlying type VII collagen mutations, we used polymerase chain reaction (PCR) and direct automated DNA sequencing.[6] Genomic DNA was isolated from peripheral blood lymphocytes of the proband and his unaffected parents. Sequencing of the PCR products corresponding to exons 70 and 105 from the patient's DNA led to the identification of compound heterozygous mutations in the *COL7A1* gene.

Results and discussion

The paternal mutation was a single base-pair deletion of a cytosine nucleotide in exon 70, designated 5818delC, resulting in a downstream premature termination codon (PTC) (Fig. 3). The maternal mutation was identified as a G to A transition located at 7867, and led to the substitution of a glycine with a serine at position 2623, designated G2623S (Fig. 3). Sequence analysis of 100 alleles from Japanese normal individuals could not detect any nucleotide change at this codon in *COL7A1*.

An increasing number of DEB mutations has enabled elucidation of the general genotype–phenotype correlations.[5,7] Patients with DDEB typically harbour glycine substitution mutations within the collagenous domain on one *COL7A1* allele. On the other hand, patients with HS-RDEB have PTC mutations on both alleles. In addition, nHS-RDEB can be caused by a combination of any mutation such as PTC, missense (including glycine substitution) or splice-site mutations on both alleles. Thus, *COL7A1* glycine substitution mutations can cause either DDEB or RDEB, but the detailed mechanism of the difference has not as yet been shown.

This affected boy showed mucocutaneous blistering followed by scarring and nail dystrophy, and tissue separation beneath the lamina densa close to anchoring fibrils consistent with DEB. Furthermore, the expression of type VII collagen indicated a diagnosis of DDEB or nHS-RDEB. *COL7A1* mutation analysis detected paternal 5818delC and maternal G2623S, resulting in the final diagnosis of nHS-RDEB. *COL7A1* mutation 5818delC is a recurrent mutation in Japanese patients with RDEB, while G2623S is a novel glycine substitution mutation.[6,8] Interestingly, at the same amino acid position, the substitution of glycine with cysteine was reported in a Chinese family with the pretibial form of DDEB.[9]

A similar report showed the mutations G2028R and G2028A in families with EB pruriginosa and the classical type of DDEB, respectively, and demonstrated that different glycine substitution mutations at the same codon could lead to a variety of

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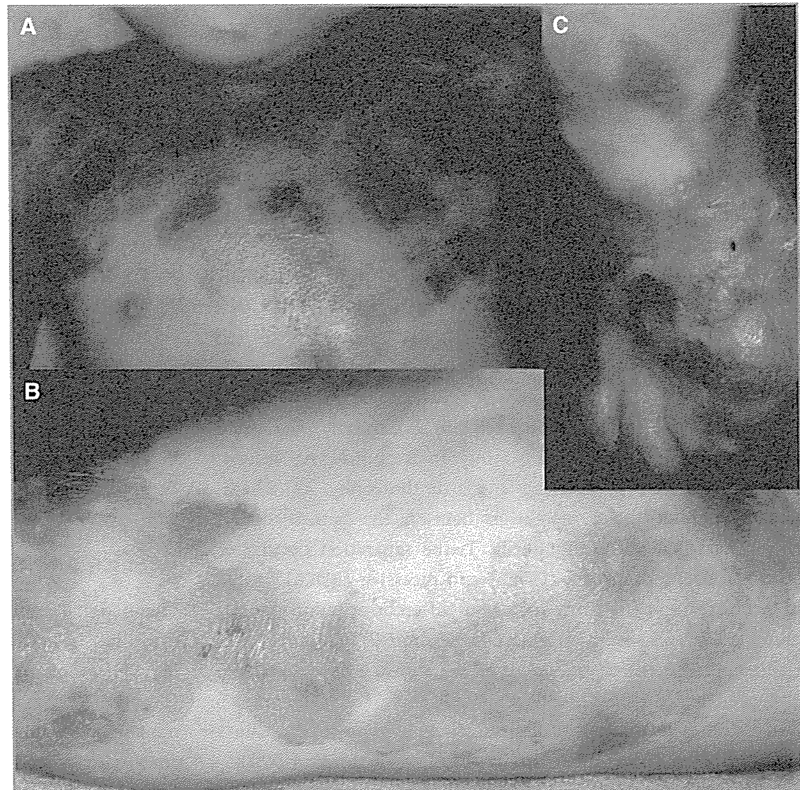

Fig 1. Clinical features. (A,B) Generalized blisters and erosions were found over the patient's entire body. Healing occurred with minimal scarring and formation of occasional milia. (C) His feet showed complete nail loss and fusion of the toes.

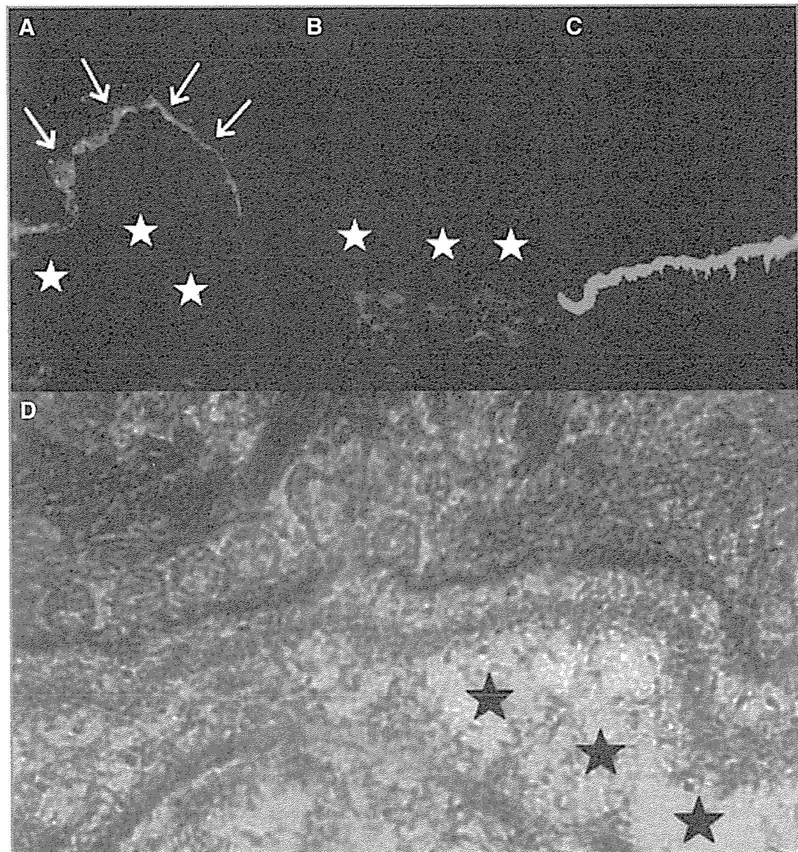

Fig 2. Immunofluorescence and ultrastructural analyses. (A–C) Direct immunofluorescence using monoclonal antibody LH 7.2 recognizing collagen VII. (A) Proband: reduced, but positive linear staining at the dermal–epidermal junction (arrows); (B) negative control sample without the primary antibody; (C) positive control sample from normal individual. (D) Electron microscopic examination showed detachment within the dermis in the sublamina densa region and some fibrillar anchoring fibril-like structures below the lamina densa. Stars indicate position of blister.

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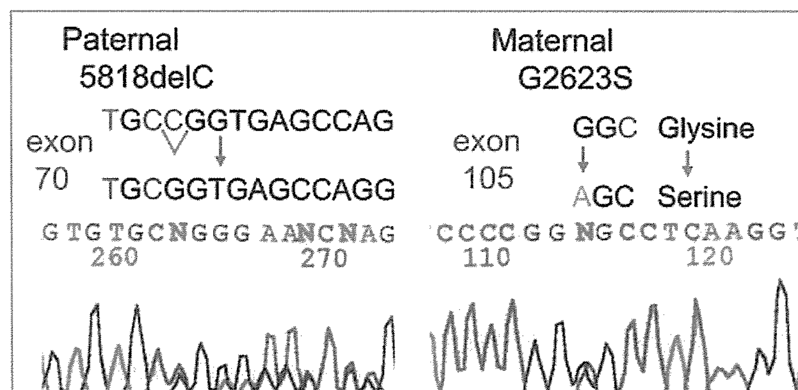

Fig 3. Mutational analysis of *COL7A1*. Sequencing of the polymerase chain reaction products corresponding to exons 70 and 105 led to the identification of compound heterozygous mutations in the type VII collagen gene. The paternal mutation was a single base-pair deletion of a cytosine nucleotide in exon 70, designated 5818delC, resulting in a downstream premature termination codon. The maternal mutation was identified as a G to A transition located at 7867, and led to the substitution of a glycine with a serine at position 2623, designated G2623S.

heterogeneous clinical phenotypes.[10] Furthermore, we recently reported that the mutation G2028R could cause simple toenail dystrophy in addition to the classical DDEB subtype as well as EB pruriginosa as mentioned above.[11]

The process of collagen VII dimer assembly is central to the formation of anchoring fibrils as the dimers overlap in an antiparallel fashion at their C-termini. The dimers are stabilized by a disulphide bond formed between C-terminal cysteine residues, and the cysteine at 2625 is known to be critical for dimer formation.[12,13] In fact, the analysis of *COL7A1* mutations in DEB supports the significance of the region adjoining this cysteine.[12] In this study, the substitution of glycine with serine is a recessive mutation as the mother who is heterozygous for G2623S has shown neither skin fragility nor nail dystrophy during her life. Conversely, the substitution with cysteine at the same amino acid position was previously reported to cause DDEB.[9] Thus, substitution with cysteine disturbs collagen VII assembly more than that with serine in this codon. In general, cysteine has an almost similar destabilizing effect to serine as a substituted residue within the collagen triple helix.[14] We hypothesize that the substituted cysteine at 2623 might form a disulphide bond at the C-termini and generate abnormal dimer formation, resulting in dominant inheritance.

We are the first to have demonstrated that glycine substitution mutations by different amino acids at the same codon are able to cause different modes of inheritance of DEB. This result furthers our understanding of both the clinical and allelic heterogeneity displayed in patients with DEB as well as genetic disorders caused by mutations in other collagen genes.

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References

1 Fine JD, Eady RA, Bauer EA *et al.* Revised classification system for inherited epidermolysis bullosa: report of the Second International Consensus Meeting on Diagnosis and Classification of Epidermolysis Bullosa. *J Am Acad Dermatol* 2000; **42**:1051–66.

2 Uitto J, Hovnanian A, Christiano AM. Premature termination codon mutations in the type VII collagen gene (*COL7A1*) underlie severe recessive dystrophic epidermolysis bullosa. *Proc Assoc Am Physicians* 1995; **107**:245–52.

3 Christiano AM, Morricone A, Paradisi M *et al.* A glycine-to-arginine substitution in the triple-helical domain of type VII collagen in a family with dominant dystrophic epidermolysis bullosa. *J Invest Dermatol* 1995; **104**:438–40.

4 Shimizu H, McGrath JA, Christiano AM *et al.* Molecular basis of recessive dystrophic epidermolysis bullosa: genotype/phenotype correlation in a case of moderate clinical severity. *J Invest Dermatol* 1996; **106**:119–24.

5 Pulkkinen L, Uitto J. Mutation analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol* 1999; **18**:29–42.

6 Sawamura D, Goto M, Yasukawa K *et al.* Genetic studies of 20 Japanese families of dystrophic epidermolysis bullosa. *J Hum Genet* 2005; **50**:543–6.

7 Jarvikallio A, Pulkkinen L, Uitto J. Molecular basis of dystrophic epidermolysis bullosa: mutations in the type VII collagen gene (*COL7A1*). *Hum Mutat* 1997; **10**:338–47.

8 Tamai K, Murai T, Mayama M *et al.* Recurrent *COL7A1* mutations in Japanese patients with dystrophic epidermolysis bullosa: positional

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effects of premature termination codon mutations on clinical severity. *J Invest Dermatol* 1999; **112**:991–3.
9 Christiano AM, Lee JY, Chen WJ *et al.* Pretibial epidermolysis bullosa: genetic linkage to *COL7A1* and identification of a glycine-to-cysteine substitution in the triple-helical domain of type VII collagen. *Hum Mol Genet* 1995; **4**:1579–83.
10 Murata T, Masunaga T, Shimizu H *et al.* Glycine substitution mutations by different amino acids in the same codon of *COL7A1* lead to heterogeneous clinical phenotypes of dominant dystrophic epidermolysis bullosa. *Arch Dermatol Res* 2000; **292**:477–81.
11 Nakamura H, Sawamura D, Goto M *et al.* The G2028R glycine substitution mutation in *COL7A1* leads to marked inter-familiar clinical heterogeneity in dominant dystrophic epidermolysis bullosa. *J Dermatol Sci* 2004; **34**:195–200.
12 Brittingham R, Colombo M, Ito H *et al.* Single amino acid substitutions in procollagen VII affect early stages of assembly of anchoring fibrils. *J Biol Chem* 2005; **280**:191–8.
13 Colombo M, Brittingham RJ, Klement JF *et al.* Procollagen VII self-assembly depends on site-specific interactions and is promoted by cleavage of the NC2 domain with procollagen C-proteinase. *Biochemistry* 2003; **42**:11434–42.
14 Persikov AV, Pillitteri RJ, Amin P *et al.* Stability related bias in residues replacing glycines within the collagen triple helix (Gly-Xaa-Yaa) in inherited connective tissue disorders. *Hum Mutat* 2004; **24**:330–7.

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Gene Corner

A novel indel *COL7A1* mutation 8068del17insGA causes dominant dystrophic epidermolysis bullosa

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Dystrophic epidermolysis bullosa (DEB) is characterized clinically by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy, and shows blister formation beneath the epidermal lamina densa at the level of the anchoring fibrils. It occurs in autosomal dominant (DDEB) and recessive (RDEB) forms, each form comprising different subtypes with variable clinical presentation and severity.[1] Based on the genetic background, both DDEB and RDEB are caused by mutations in the *COL7A1* gene encoding type VII collagen, the major component of anchoring fibrils.[1] Examination of the *COL7A1* mutations in DEB has so far disclosed certain genotype–phenotype correlations.[2] Patients with DDEB usually harbour glycine substitution mutations within the collagenous domain on one *COL7A1* allele, leading to disruptions in anchoring fibril assembly and relatively mild clinical features. This study describes the first indel (insertion/deletion) mutation[3] in the *COL7A1* gene that causes a clinical DDEB phenotype.

Case and methods

The proband was a 21-year-old Japanese man with blistering mainly on his knees and elbows after minor trauma since infancy. The healing lesions left scars and milia. Although the frequency of blister formation gradually diminished with age, severe pruritus started at around the age of 13 years. Afterwards, excoriations and skin lesions appeared over the whole body. There were nine other affected family members with skin fragility, but they failed to show any severe pruritus (Fig. 1a). Examination revealed multiple erosions, scarring papules and lichenoid plaques on the proband's trunk and extremities, and some tense blisters on his legs (Fig. 1b,c). Laboratory tests showed IgE levels elevated to 149 667 U mL^{-1}. Skin biopsies from the proband revealed subepidermal blister formation. Further immunostaining with the monoclonal antibody LH7:2 recognizing type VII collagen demonstrated bright linear staining along the basement membrane zone and on the roof of the blister (Fig. 2a), and electron microscopy showed tissue separation beneath the lamina densa within the dermal sublamina densa region (Fig. 2b). The combination of topical betamethasone and oral antihistamines was moderately effective for the pruritus and prurigo.

Total genomic DNA from the proband was examined to detect the precise *COL7A1* mutational defects. The *COL7A1* DNA segments including all 118 exons, all intron–exon borders and the promoter region were amplified by polymerase chain reaction (PCR) using pairs of oligonucleotide primers synthesized on the basis of intronic sequences according to Christiano *et al.*[4] The PCR products were subjected to automated nucleotide sequencing using an ABI 3100 Genetic Analyzer (Perkin Elmer, Warrington, U.K.).

This study was approved by the Ethical Committees at Hokkaido University Graduate School of Medicine and Nara Medical University School of Medicine. Informed consent was obtained from the proband.

Results and discussion

Direct DNA sequencing detected a heterozygous indel *COL7A1* mutation 8068del17insGA in exon 109. The 17-nucleotide deletion from 8068 to 8084 with a GA two-base insertion resulted in a 15-nucleotide deletion within the collagenous domain, which failed to change the downstream *COL7A1* open reading frame (Fig. 2c). The other affected and unaffected family members refused DNA analysis.

This study examined a 21-year-old Japanese man with skin fragility. The blister formation beneath the lamina densa and the presence of multiple affected members in his family suggested DDEB, the diagnosis of which was confirmed by the identification by mutational analysis of a *COL7A1* mutation in one allele. The characteristic clinical feature of this patient was his severe itching, resulting in scratching and prurigo formation, similar to the EB pruriginosa phenotype.[5,6]

EB pruriginosa is a form of DEB characterized by prurigo-like or lichenoid lesions associated with scarring. Blistering is usually confined to the shins and forearms. The mode of inheritance is variable. McGrath *et al.*[5] described eight unrelated families with EB pruriginosa, of which five were sporadic, two were autosomal dominant and one was autosomal recessive. In the present family, nine affected family members, unlike the proband, showed no severe pruritus or prurigo formation. Therefore, considering the elevated IgE levels, we made the diagnosis of DDEB with atopic dermatitis, not EB pruriginosa. As atopic dermatitis may show familial aggregation, we highlight here the difficulty in differential diagnosis of EB pruriginosa and EB associated with atopic dermatitis. In

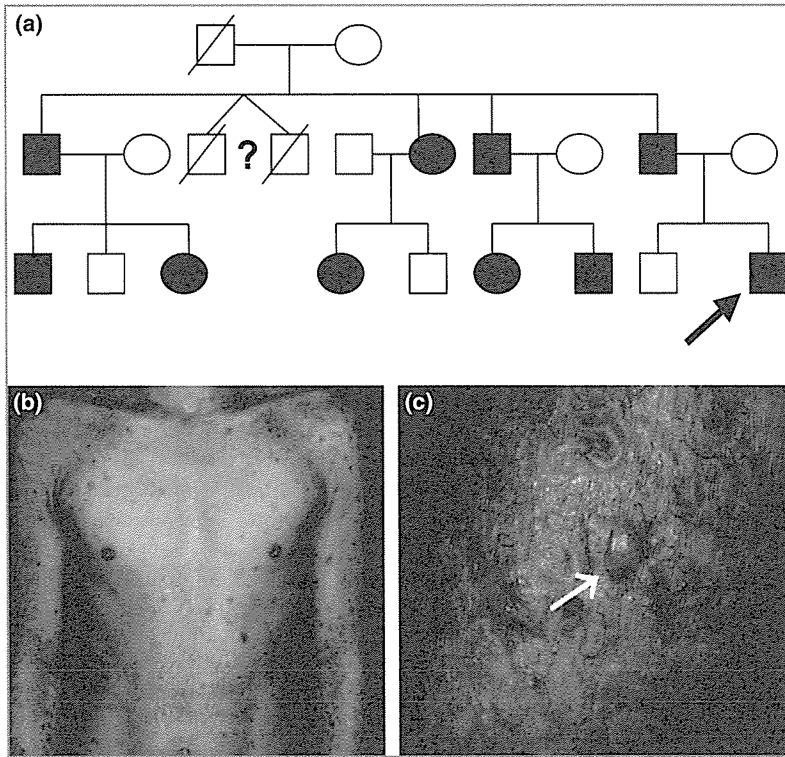

Fig 1. (a) Family pedigree. The proband is indicated with an arrow. The other nine family members affected with skin fragility failed to show severe pruritus. (b) Multiple erosions, scarring pruriginous papules and lichenoid plaques on the trunk and arms. (c) Tense blisters on the leg (white arrow).

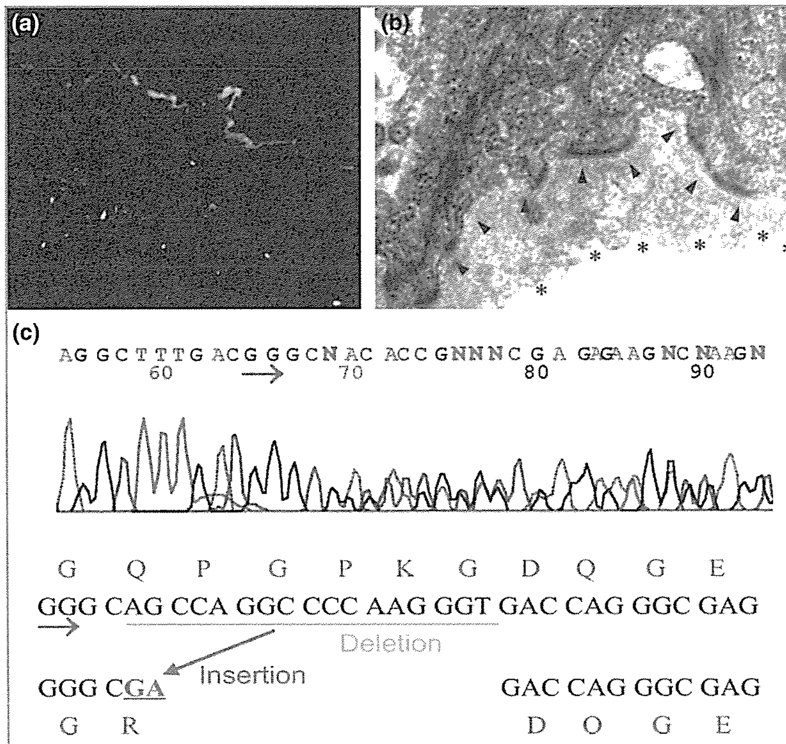

Fig 2. Immunohistochemical, ultrastructural and mutation analyses of the proband. (a) Immunostaining with monoclonal antibody LH7:2 to type VII collagen demonstrated positive, linear staining along the basement membrane zone and on the roof of the blister. (b) Electron microscopy showed tissue separation in the dermal side of the basement membrane: arrowheads, lamina densa; asterisks, blister site. (c) Direct DNA sequencing detected a heterozygous indel *COL7A1* mutation 8068del17insGA in exon 109. The 17-nucleotide deletion (green underlined area) from 8068 to 8084 with a GA insertion resulted in a 15-nucleotide deletion within the collagenous domain, which did not change the open reading frame of *COL7A1*, but interfered with the collagen triple helix (Gly–X–Y repeat). Red arrows start from G, nucleotide 8064.

this case, instability in the epidermal basement membrane zone caused by anchoring fibril dysfunction might have accelerated the tendency for prurigo formation.

The mutation 8068del17insGA is a novel DEB mutation. An indel *COL7A1* mutation is extremely rare, as only two indel mutations have been reported in RDEB patients among

approximately 300 previously reported *COL7A1* mutations according to the Human Gene Mutation Database (http://hgmd.cf.ac.uk). The 17-nucleotide deletion with a GA two-base insertion resulted in a 15-nucleotide deletion within the collagenous domain, which failed to change the downstream *COL7A1* open reading frame. Consequently, the deletion of 15 nucleotides (five amino acids) interfered with the collagen triple helix (Gly–X–Y repeat) and caused this DDEB phenotype probably in a dominant negative fashion. If the deleted nucleotide number had not been a multiple of 3, then a premature stop codon would have appeared downstream, resulting in a RDEB pattern mutation; therefore, this family would not have been affected.

Patients with DDEB usually harbour glycine substitution mutations within the collagenous region of collagen VII. As far as we know, only six dominant mutations other than glycine substitution mutations have been reported in the literature. Of these, three mutations, 4084–1G → C,[7] 6899A → G[8] and 8045Ae → G,[9] were one-nucleotide substitutions and the others were gross deletion mutations, 6847del27,[10] 6863del16[6] and 6081del28.[10] Thus, we have demonstrated for the first time that indel mutations may result in the DDEB phenotype and have therefore further extended the body of evidence implicating a full range of *COL7A1* gene mutations in DEB.

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References

1 Fine JD, Eady RA, Bauer EA *et al.* Revised classification system for inherited epidermolysis bullosa: report of the Second International Consensus Meeting on diagnosis and classification of epidermolysis bullosa. *J Am Acad Dermatol* 2000; **42**:1051–66.

2 Pulkkinen L, Uitto J. Mutation analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol* 1999; **18**:29–42.

3 den Dunnen JT, Antonarakis S. Nomenclature for the description of human sequence variations. *Hum Genet* 2001; **109**:121–4.

4 Christiano AM, Hoffman GG, Zhang X *et al.* Strategy for identification of sequence variants in *COL7A1*, and a novel 2 bp deletion mutation in recessive dystrophic epidermolysis bullosa. *Hum Mutat* 1997; **10**:408–14.

5 McGrath JA, Schofield OM, Eady RA. Epidermolysis bullosa pruriginosa: dystrophic epidermolysis bullosa with distinctive clinicopathological features. *Br J Dermatol* 1994; **130**:617–25.

6 Mellerio JE, Ashton GH, Mohammedi R *et al.* Allelic heterogeneity of dominant and recessive *COL7A1* mutations underlying epidermolysis bullosa pruriginosa. *J Invest Dermatol* 1999; **112**:984–7.

7 Christiano AM, Fine JD, Uitto J. Genetic basis of dominantly inherited transient bullous dermolysis of the newborn: a splice site mutation in the type VII collagen gene. *J Invest Dermatol* 1997; **109**:811–14.

8 Jiang W, Bu D, Yang Y, Zhu X. A novel splice site mutation in collagen type VII gene in a Chinese family with dominant dystrophic epidermolysis bullosa pruriginosa. *Acta Derm Venereol (Stockh)* 2002; **82**:187–91.

9 Kon A, Pulkkinen L, Ishida-Yamamoto A *et al.* Novel *COL7A1* mutations in dystrophic forms of epidermolysis bullosa. *J Invest Dermatol* 1998; **111**:534–7.

10 Sakuntabhai A, Hammami-Hauasli N, Bodemer C *et al.* Deletions within *COL7A1* exons distant from consensus splice sites alter splicing and produce shortened polypeptides in dominant dystrophic epidermolysis bullosa. *Am J Hum Genet* 1998; **63**:737–48.

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Key words: *COL7A1*, *epidermolysis bullosa pruriginosa*, *pruritus*, *type VII collagen*

Conflicts of interest: None declared.

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ORIGINAL ARTICLE

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COL7A1 mutation G2037E causes epidermal retention of type VII collagen

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Abstract *COL7A1* glycine substitution (GS) mutations result in dominant and recessive dystrophic epidermolysis bullosa (DDEB and RDEB). Here, we report a DDEB family in which retention of type VII collagen by epidermal keratinocytes was observed for a female proband. Mutational analysis detected a GS mutation, G2037E, in the proband and her affected father. To demonstrate direct association of G2037E and type VII collagen retention we introduced this mutated *COL7A1* gene into cultured keratinocytes using retroviral methods. This mutation was dominant, so we transferred a 1:1 mixture of wild-type (unaffected) and G2037E-mutated *COL7A1*, together, in addition to the unaffected gene or the mutated gene alone. The increase in type VII collagen cytoplasmic staining in the G2037E/wild transfectant cell samples was compared with that for control/wild-type cells. Intracellular collagen VII staining in the G2037E (alone)-transfected cells was even stronger than for the G2037E/wild transfection sample. These results indicate that the G2037E *COL7A1* mutation leads to increased epidermal retention of type VII collagen in vivo, and also suggests that homozygotes carrying this dominant GS mutation may have more severe phenotypes than heterozygotes. This study furthers our understanding of GS *COL7A1* mutations in DEB.

Keywords Gene transfer · Retrovirus · Glycine substitution · Dominant negative interference

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Abbreviations

DDEB	Dominant dystrophic epidermolysis bullosa
DEB	Dystrophic epidermolysis bullosa
GS	Glycine substitution
HS	Hallopeau–Siemens type
n-HS	non-Hallopeau–Siemens type
RDEB	Recessive dystrophic epidermolysis

Introduction

Type VII collagen, a non-fibrillar collagen, is a major component of anchoring fibril loop structures beneath the epidermal basement membrane (Uitto et al. 1992; Burgeson 1993). Mutations within the type VII collagen gene (*COL7A1*) are associated with dystrophic forms of epidermolysis bullosa (DEB) (Christiano et al. 1993). DEB is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy in which tissue dermal–epidermal separation is observed beneath the lamina densa at the level of the anchoring fibrils. It is inherited in either an autosomal dominant (DDEB) or recessive (RDEB) fashion, each form having a specific, slightly different clinical presentation and severity (Fine et al. 2000). Study of DEB mutations has revealed several general genotype–phenotype correlations (Pulkkinen and Uitto 1999).

Recessive dystrophic epidermolysis bullosa patients may harbor any type of *COL7A1* mutation including premature termination codons, missense, glycine substitution (GS), or splice site mutations on both alleles. GS mutations on one allele have been found in many DDEB patients and in-frame deletion mutations have been observed for a few patients. Thus, *COL7A1* GS mutations can cause both DDEB and RDEB subtypes (Christiano et al. 1995; Shimizu et al. 1996).

During the course of *COL7A1* DEB patient mutational analysis (Sawamura et al. 2005) we found a unique GS mutation which was associated with retention of type VII collagen in keratinocytes. Some, but not

all, GS *COL7A1* mutations result in intracellular accumulation of collagen VII (Hammami-Hauasli et al. 1998; Shimizu et al. 1999). To obtain direct evidence of whether G2037E leads to intracytoplasmic retention of type VII collagen we introduced the mutated *COL7A1* gene into cultured keratinocytes.

Materials and methods

Patient

A Japanese girl presented with erosions and blisters affecting her trunk and lower extremities that had persisted since birth. The blisters continued to appear, particularly at sites of trauma. Physical examination revealed bullae on her hands, feet, and abdomen (Fig. 1B). Healing occurred with minimal scarring and occasional milia formation. Her father had a similar history, and blister formation and the resulting scars were apparent, predominantly on the knees and elbows (Fig. 1C). A family tree is shown in Fig. 1A. Informed consent to the study and to publication of the clinical images were obtained from the family.

Ultrastructural and immunohistochemical studies

Skin biopsies were taken from the affected child, and processed for transmission electron microscopy and immunofluorescence microscopy, as described elsewhere (Shimizu et al. 1996). For ultrastructural examination, skin specimens were fixed in 5% glutaraldehyde, postfixed in 1% osmium tetroxide, and stained en-block in uranyl acetate. They were dehydrated in a graded series of ethanol solutions and then embedded in Araldite 6005. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) at 75 kV. For immunohistochemical examination the specimens were embedded in OCT compound and 5 μm thick sections were cut. The anti-human type VII collagen monoclonal antibody (LH7.2: kind gift from I. Leigh, UK) directed against the NC-1 amino terminal domain of the protein was used for experiments. The bound antibodies were detected with FITC-conjugated goat anti-mouse IgG antibody.

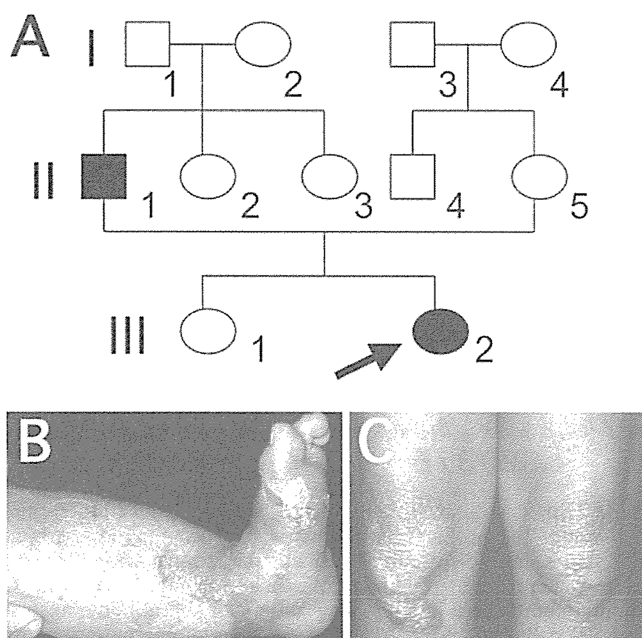

Fig. 1 Dystrophic epidermolysis bullosa pedigree. **A** The family tree. **B** The proband (III-2) is a Japanese girl with erosion, and blistering with scarring. **C** Her father (II-1) has a similar history; blister formation and scars are now predominantly apparent on the knees and elbows

Mutational analysis

Genomic DNA was isolated from peripheral blood lymphocytes of patients and their families, by using standard procedures. *COL7A1* segments including all 118 exons, all exon–intron borders, and the promoter region were amplified by PCR using pairs of oligonucleotide primers synthesized on the basis of intronic sequences in accordance with the report by Christiano et al. (1997) (GenBank numbers L02870, L23982). Specifically, to amplify exons 73 the primers used were: sense primer 5′-aagtggctcagtgggttgtg-3′; antisense primer 5′-aacccctcttccctcactct-3′. For PCR amplification, approximately 200 ng genomic DNA, 40 pmol of each primer, 0.5 $mmol^{-1}$ $MgCl_2$, 20 μmol of each dNTP, and 1.25 U Taq polymerase were used in a total volume of 50 μL. The amplification conditions were 94°C for 5 min, then 40 cycles of 94°C for 45 s, 55–60°C for 45 s, 72°C for 45 s, and extension at 72°C for 10 min in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR products were subjected to direct automated nucleotide sequencing using the BigDye Terminator System (Applied Biosystems).

Construction of retroviral *COL7A1* expression vectors and transfection

Normal human full-length *COL7A1* cDNA was constructed from several overlapping cDNA clones (Sawamura et al. 2002). *COL7A1* mutations 2037E, 6110G > A, and G2043R; 6127G > A were generated by an in-vitro mutagenesis technique using a Mutant-Super Express Km Kit (Takara, Otsu, Shiga, Japan). A retroviral vector pDON(Δ) was constructed by removing the SV-40 promoter and Neo gene from pDON-AI (Takara) and both the wild and mutated full-length *COL7A1* cDNAs were inserted into pDON(Δ) (Goto et al. 2006). The recombinant retroviruses were produced by transfecting the retroviral plasmids into the

amphotropic amphopack-293 packaging cells (Clontech, Mountain View, CA, USA) using a calcium–phosphate co-precipitation method. We also used the G protein of the vesicular stomatitis virus (VSV-G), a pseudotyped retrovirus vector (Clontech). The retroviral plasmids and plasmid pVSV-G were cotransfected into pantropic GP2-293 packaging cells (Clontech). We applied the mutated gene, wild-type (normal) *COL7A1* gene (control), and a 1:1 mixture of mutated and normal genes. The viral particles were recovered from the cell culture medium and ultracentrifugation was performed for concentration of viruses with both normal and mutated *COL7A1* constructs.

Expression of mutated type VII collagen

The HaCaT human keratinocyte cell line was maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. HaCaT cells were expanded to 60% of confluent density and then transduced with viral suspensions in 5 μg mL^{-1} polybrene. To increase attachment of virus to keratinocytes, we coated the surface of culture plates with 10 ng mL^{-1} retronectin (Takara; fibronectin fragment CH-296). After incubation for 24 h at 32°C we maintained the treated keratinocytes with fresh medium for another 72 h and immunostaining was performed using the monoclonal antibody LH7.2. Digital images were analyzed on an Apple G5 computer (Apple, Cupertino, CA, USA) using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://www.rsb.info.nih.gov/nih-image/). To semiquantify *COL7A1* expression the HaCaT cells were classified into low, medium, and high expression depending on pixel values. We evaluated 100 fluorescing cells and the expression index value was calculated by use of the formula: (low expression cell number)×1 + (medium expression cell number)×2 + (high expression cell number)×3. The expression index is shown with the mean ± SD of the expression values from five different areas.

Results

Diagnosis of dominant dystrophic epidermolysis bullosa

The proband and her father had suffered from skin fragility since birth. The severity of the father's skin lesions had improved with age and healing had occurred but with scarring. Routine ultrastructural examination showed skin separation occurred within the sublamina densa in the region of the anchoring fibrils (Fig. 2A), suggesting DEB. The number of anchoring fibrils was also reduced. Immunofluorescence study using LH7.2 detected a linear staining pattern in the basement membrane zone, which was not characteristic of HS-RDEB (Fig. 2B). We also observed retention of type VII collagen within epidermal keratinocytes in this patient (Fig. 2B). This pattern is a characteristic feature of DDEB and transient bullous dermolysis of the newborn; this is a rare form of dystrophic epidermolysis bullosa and also caused by *COL7A1* mutations (Fassihi et al. 2005). Patients with transient bullous dermolysis of the newborn present with neonatal skin blistering which usually improves markedly during early life or even remits completely. Because blister formation on this patient continued until approximately 2 years of age and her father's skin was still fragile, we opted for diagnosis of DDEB rather than transient bullous dermolysis of the newborn.

Mutational analysis of *COL7A1* revealed a heterozygous G to A transition at nucleotide position 6110 in the mutant allele converting a glycine to glutamic acid (G2037E) (Fig. 2D). This mutation was not found in the unaffected family members. This mutation was

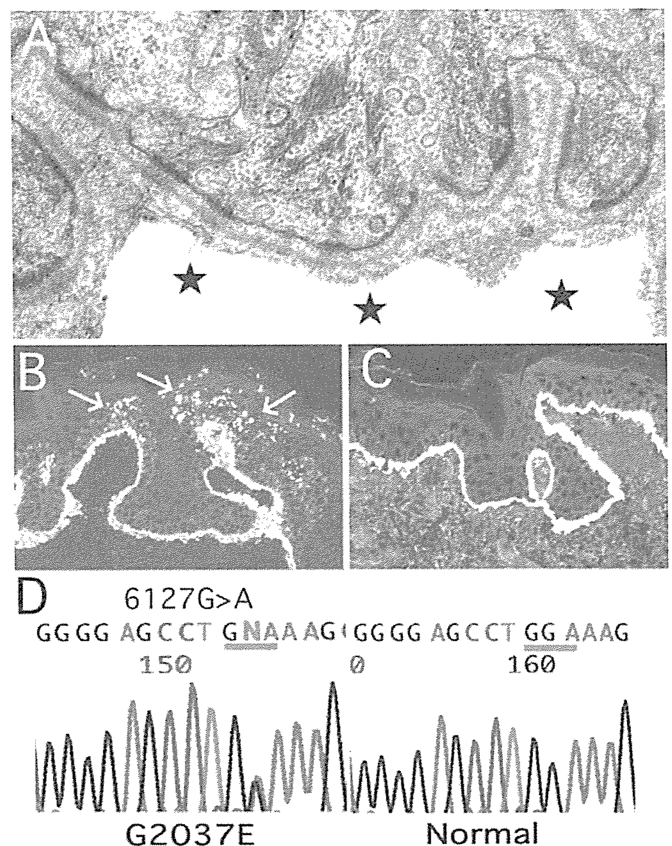

Fig. 2 Ultrastructural, immunohistochemical, and mutational analyses of the proband. **A** Ultrastructural examination showed that skin separation occurred beneath the lamina densa (★) and there were reduced numbers of anchoring fibrils. **B** and **C** Immunofluorescence study using monoclonal antibody against type VII collagen (LH7.2) detected a linear staining pattern along the basement membrane zone and retention of type VII collagen within epidermal keratinocytes (*arrows*) (**B**). Normal control individual collagen VII staining (**C**). **D** Mutational analysis of *COL7A1* revealed a heterozygous G to A transition at nucleotide position 6127 in the mutant allele converting a glycine to glutamic acid (G2037E)

confirmed by restriction enzyme digestion (data not shown). Thus, final diagnosis of DDEB was made on the basis of clinical and laboratory findings.

Transfection study

We next constructed retroviral expression vectors with mutations G2037E or G2043R as control, introduced them to keratinocytes and examined type VII collagen expression. In the G2043R transfection experiment we failed to find significantly different *COL7A1* staining among the G2043R-, wild-, and G2043R/wild-treated sample groups. Semiquantitative analysis gave a similar result (Fig. 3). In contrast, we detected more intracytomic type VII collagen staining in the G2037E/wild sample than in the control wild-type sample. Intracellular collagen VII staining was also stronger in the G2037E-transfected sample than in the G2037E/wild transfection group (Fig. 3). This finding was confirmed by semiquantitative analysis, which revealed the expression indices of G2037E and G2037E/wild samples were 2.2- and 1.6-fold, respectively, higher than that of wild samples when compared with wild-type transfected controls (Fig. 3).

Discussion

Some, but not all, dominant GS mutations in *COL7A1* result in intracellular accumulation of collagen VII (Hammami-Hauasli et al. 1998; Shimizu et al. 1999). The G2037E mutation has previously been reported to induce type VII collagen retention in epidermal keratinocytes (Jonkman et al. 1999). No transfection study was, however, used to demonstrate the direct relevance of dominant GS mutations to increased intracellular type VII collagen retention, although transfection studies had been performed characterizing the recessive GS G2008R mutation (Chen et al. 2002). We therefore constructed *COL7A1* retroviral vectors with G2037E or G2043R mutations, and transferred these genes into HaCaT cells. The reasons we selected the G2043R mutation as a control were that this defect is a known, recurrent DDEB mutation (Mellerio et al. 1998; Wessagowit et al. 2001), and that it was the closest to the dominant substitution G2037E mutation observed in our patient. The transfection efficacy of our retroviral system was almost 30% in HaCaT cells (Goto et al. 2006). Because little or no intrinsic intracellular collagen VII

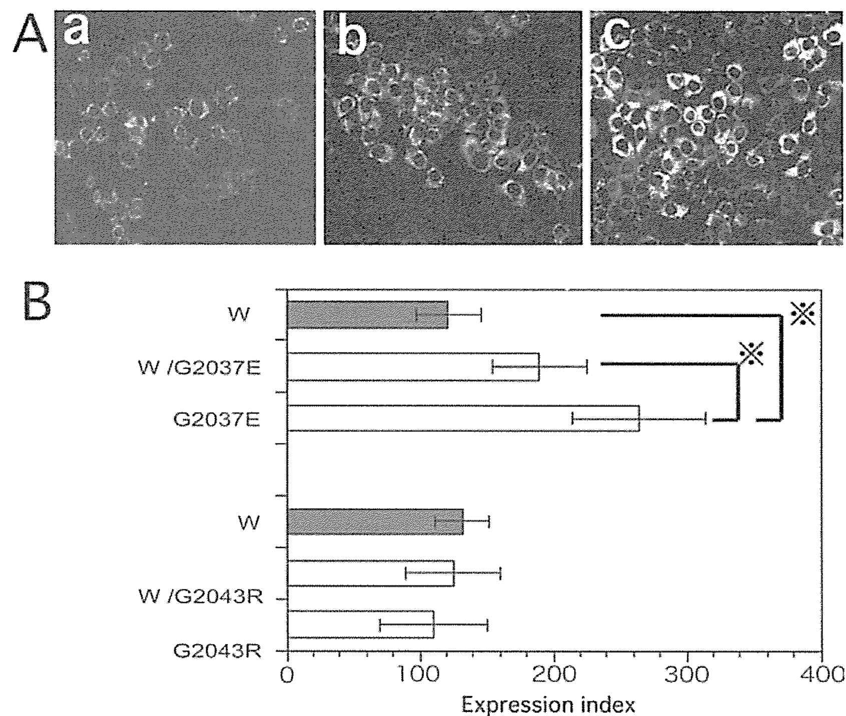

Fig. 3 The effect of the glycine substitution mutation on type VII collagen retention. We constructed *COL7A1* retroviral vectors with G2043R or G2037E mutations and introduced these genes into HaCaT cells. We transferred a 1:1 mixture of wild-type (normal) and mutated *COL7A1* and the wild-type gene alone or the mutated gene alone. **A** Type VII collagen staining showed that intracellular immunoreactivity was high, in the order wild-type control samples (*a*: W), G2037E/wild samples (*b*: W/G2037E), and G2037E samples (*c*: G2037E). **B** These findings were confirmed by semiquantitative analysis, which showed the G2037E and G2037E/wild samples expression indices were higher than that of the wild samples by 2.2- and 1.6-fold, respectively. The G2043R mutation, failed to show a significant difference between the G2043R, wild, and G2043R/wild samples in *COL7A1* staining. The *dotted cross symbol* indicates $P < 0.01$ between W and W/G2037E, and between W/G2037E and G2037E

expression is observed in HaCaT cells, we predicted that any high-level *COL7A1*-expressing cells were likely to be successfully gene-transfected cells. Those mutations were dominant, so we also transferred a 1:1 mixture of wild and mutated *COL7A1* and the wild-type *COL7A1* gene alone or the mutated gene alone. Transfection of G2037E mutation induced accumulation of type VII collagen in keratinocytes, whereas transfection of G2043R led to no abnormal findings. This proves that *COL7A1* mutation G2037E causes epidermal retention of type VII collagen.

Glycine residues within the collagenous domain are critical for proper triple-helix formation. Some *COL7A1* GS mutations, which cause RDEB in patients harboring a second mutation on the remaining allele, are silent in patients with one normal *COL7A1* allele. In addition, heterozygous GS mutations can cause DDEB by dominant negative interference with the collagen triple helix. The following theoretical explanation is proposed. These dominant mutations may mildly interfere with α-chain polypeptide structure and enable the formation of abnormal triple helix structures affecting the other, normal, α-chains. The change from glycine to the mutated residue is thus thought to result in disruption or destruction of the normal triple helical structure in a dominant, negative, manner. Conversely, the recessive GS mutations are thought to completely inhibit formation of the α-chain so the mutated polypeptide cannot induce this dominant negative effect in the normal chains. As far as we know, RDEB cases homozygous for certain DDEB GS mutations have not yet been identified. In fact, heterozygous dominant GS mutations in *COL4A4* can cause Alport syndrome, whereas one healthy individual is homozygous with these mutations (Boye et al. 1998). Also, in cases of *COL1A2* GS mutations, clinical and laboratory findings of the heterozygote was not significantly different from those of the homozygote (DePaepe et al. 1997). Thus, it is possible that DDEB GS homozygotes may not demonstrate any significant EB phenotype.

We used the wild-type (normal) *COL7A1* gene alone (control), a 1:1 mixture of mutated (diseased) and wild-type genes, and the mutated gene only (positive control). We failed to find significantly different collagen VII staining for the G2043R, wild, and G2043R/wild treatments. This mutation was not predicted to affect secretion but homotrimer formation. Our results demonstrated, however, that the G2037E mutation alone significantly affected collagen formation and this was more impaired than for the combination G2037E/wild-type gene transfected sample. This result indicates the phenotype may be more severe for homozygotes with the dominant GS mutation than for heterozygotes, suggesting the dominant GS mutation causes interference with the α-chain polypeptide structure itself and has a dominant negative effect on the collagen triple helix.

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References

Boye E, Mollet G, Forestier L, Cohen-Solal L, Heidet L, Cochat P, Grunfeld JP, Palcoux JB, Gubler MC, Antignac C (1998) Determination of the genomic structure of the COL4A4 gene and of novel mutations causing autosomal recessive Alport syndrome. Am J Hum Genet 63:329–340

Burgeson RE (1993) Type VII collagen, anchoring fibrils, and epidermolysis bullosa. J Invest Dermatol 101:252–255

Chen M, Costa FK, Lindvay CR, Han YP, Woodley DT (2002) The recombinant expression of full-length type VII collagen and characterization of molecular mechanisms underlying dystrophic epidermolysis bullosa. J Biol Chem 277:2118–2124

Christiano AM, Greenspan DS, Hoffman GG, Zhang X, Tamai Y, Lin AN, Dietz HC, Hovnanian A, Uitto J (1993) A missense mutation in type VII collagen in two affected siblings with recessive dystrophic epidermolysis bullosa. Nat Genet 4:62–66

Christiano AM, Morricone A, Paradisi M, Angelo C, Mazzanti C, Cavalieri R, Uitto J (1995) A glycine-to-arginine substitution in the triple-helical domain of type VII collagen in a family with dominant dystrophic epidermolysis bullosa. J Invest Dermatol 104:438–440

Christiano AM, Hoffman GG, Zhang X, Xu Y, Tamai Y, Greenspan DS, Uitto J (1997) Strategy for identification of sequence variants in *COL7A1*, and a novel 2 bp deletion mutation in recessive dystrophic epidermolysis bullosa. Hum Mutat 10:408–414

DePaepe A, Nuytinck L, Raes M, Fryns JP (1997) Homozygosity by descent for a COL1A2 mutation in two sibs with severe osteogenesis imperfecta and mild clinical expression in the heterozygotes. Hum Genet 99:478–483

Fassihi H, Diba VC, Wessagowit V, Dopping-Hepenstal PJ, Jones CA, Burrows NP, McGrath JA (2005) Transient bullous dermolysis of the newborn in three generations. Br J Dermatol 153:1058–1063

Fine JD, Eady RA, Bauer EA, Briggaman RA, Bruckner-Tuderman L, Christiano A, Heagerty A, Hintner H, Jonkman MF, McGrath J, McGuire J, Moshell A, Shimizu H, Tadini G, Uitto J (2000) Revised classification system for inherited epidermolysis bullosa: report of the second international consensus meeting on diagnosis and classification of epidermolysis bullosa. J Am Acad Dermatol 42:1051–1066

Goto M, Sawamura D, Ito K, Abe M, Nishie W, Sakai K, Shibaki A, Akiyama M, Shimizu H (2006) Fibroblasts show more potential as a target cells than keratinocytes for *COL7A1* gene therapy of dystrophic epidermolysis bullosa. J Invest Dermatol 126: (in press)

Hammami-Hauasli N, Schumann H, Raghunath M, Kilgus O, Luthi U, Luger T, Bruckner-Tuderman L (1998) Some, but not all, GS mutations in *COL7A1* result in intracellular accumulation of collagen VII, loss of anchoring fibrils, and skin blistering. J Biol Chem 273:19228–19234

Jonkman MF, Moreno G, Rouan F, Oranje AP, Pulkkinen L, Uitto J (1999) Dominant dystrophic epidermolysis bullosa (Pasini) caused by a novel GS mutation in the type VII collagen gene (*COL7A1*). J Invest Dermatol 112:815–817

Mellerio JE, Salas-Alanis JC, Talamantes ML, Horn H, Tidman MJ, Ashton GH, Eady RA, McGrath JA (1998) A recurrent GS mutation, G2043R, in the type VII collagen gene (*COL7A1*) in dominant dystrophic epidermolysis bullosa. Br J Dermatol 139:730–737

Pulkkinen L, Uitto J (1999) Mutation analysis and molecular genetics of epidermolysis bullosa. Matrix Biol 18:29–42