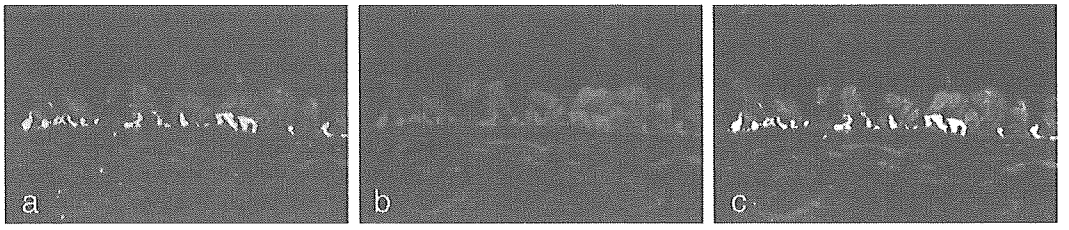


**Fig. 4** Co-localization of the 29A antigen and LR in normal placental amnion. (a) The 29A antibody intermittently stained the cytoplasm of normal human amniotic cells by an indirect immunofluorescence staining. (Green): FITC-conjugated anti-mouse IgM antibody. (b) Anti-LR antibody also intermittently 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.

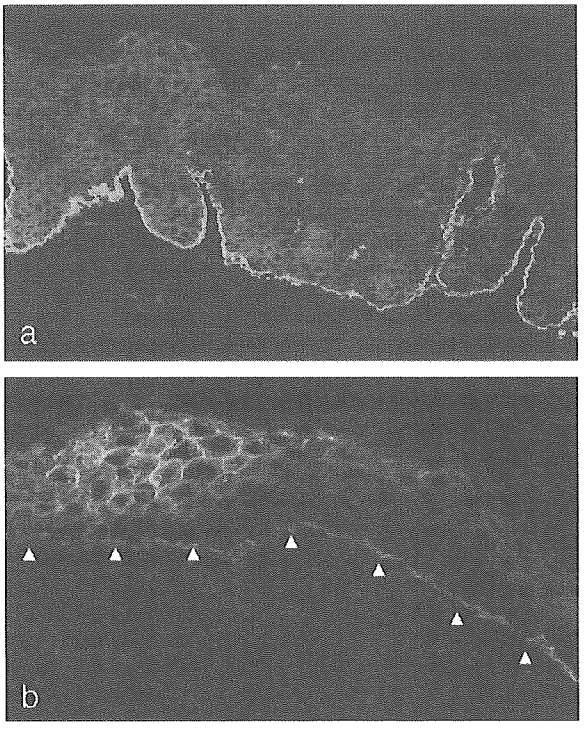


**Fig. 5** The 29A antibody stained the basement membrane zone in normal human skin. (a) Indirect immunofluorescence staining of normal skin showed that the 29A antigen linearly stained the dermal–epidermal junction. (b) A monoclonal antibody for LR, MLuC5, partially stained keratinocyte cell membranes 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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Letter to the Editor

# Possible role of endoplasmic reticulum stress in the pathogenesis of Darier's disease

KEYWORDS
Sarco/endoplasmic reticulum $Ca^{2+}$ ATPase;
*ATP2A2*;
Chaperone;
Keratinocytes

Darier's disease (DD, keratosis follicularis; OMIM 124200) is an autosomal dominant genodermatosis characterized by persistent, greasy, scaly papules which show abnormalities in keratinocyte adhesion and differentiation including acantholysis, suprabasal clefting, and unusual dyskeratosis. Mutations within the *ATP2A2* gene encoding the sarco/endoplasmic reticulum $Ca^{2+}$ ATPase type 2 (SERCA2) are found in DD patients, indicating that SERCA2 plays an important role in keratinocyte adhesion and differentiation [1]. However, the precise mechanisms underlying the histological hallmarks in DD have not yet been fully elucidated.

The endoplasmic reticulum (ER) may serve specialized functions including the post-translational modification, folding, and assembly of newly synthesized secretory proteins. Various conditions can interfere or disrupt ER function, and these are collectively grouped into ER stress-associated diseases. ER stress provokes an ER stress response, which includes upregulation of ER chaperones, inhibition of gene translation, degradation of the misfolding proteins, and induction of a transcription factor C/EBP homology protein (CHOP) that leads to cell apoptosis [2].

SERCA2 actively transports $Ca^{2+}$ from the cytosol back into the ER lumen to maintain the correct $Ca^{2+}$ concentration in the ER. ER stress can be induced by a decrease in $Ca^{2+}$ concentration within the ER. These suggest that SERCA2 abnormalities in DD are caused by the low $Ca^{2+}$ in the ER lumen, resulting in ER stress. This study was designed to address a hypothesis that ER stress is involved in formation of the characteristic histological features of DD.

The lesional skin specimens and cultured keratinocytes obtained from a 17-year-old female with DD were used for study. Diagnosis of DD was determined by dermatologists based upon clinical and histopathological features (the mutation C318R in *ATP2A2* was previous reported [3]). The normal human keratinocytes and skin specimens were obtained from a normal adult female. In order to observe the ER stress response in keratinocytes, we examined the expression of calreticulin [4], BiP/GRP78 [5] and CHOP/gadd153 [6]. The primary keratinocyte cultures were grown in serum-free keratinocyte growth medium (KGM, Clonetics) and then treated with the stressors, 1.0 μM thapsigargin [7] or 1.0 mM *S*-nitro-*N*-acetyl-DL-penicillamine (SNAP) [8] for 48 h.

The specimens were embedded in OCT compound, and 10 μm thick sections were cut. The treated keratinocytes and cryosections were stained with rabbit polyclonal antibodies against calreticulin (Stressgen), rabbit polyclonal antibodies against or CHOP/GADD153 (Santa Cruz) or goat polyclonal antibodies against BiP/GRP78 (Stressgen), followed by treatment with FITC-conjugated secondary antibodies. To semiquantify the keratinocytes expression of calreticulin, CHOP/GADD153 and BiP/GRP78, respectively), we measured fluorescence intensity of each cell using the digital photograph (Gel Plotting Macros; NIH Image; provided in the public domain by the National Institutes of Health, Bethesda, MD, and available at http://rsb.info.nih.gov/nih-image/). We graded the fluorescence intensity of stained cells as follows: Level 1: poor staining; Level 2: moderate fluorescence levels; Level 3: bright cytoplasmic fluorescence. Typical staining pattern of each Lever was shown in Fig. 1A. Intensity value was estimated from; (the number of level 1 cells × 1) + (the number of level 2 cells × 2) + (the number of level 3 cells × 3)/the total number of the cells, and repeated the same experiment four times. Finally, the expression ratio

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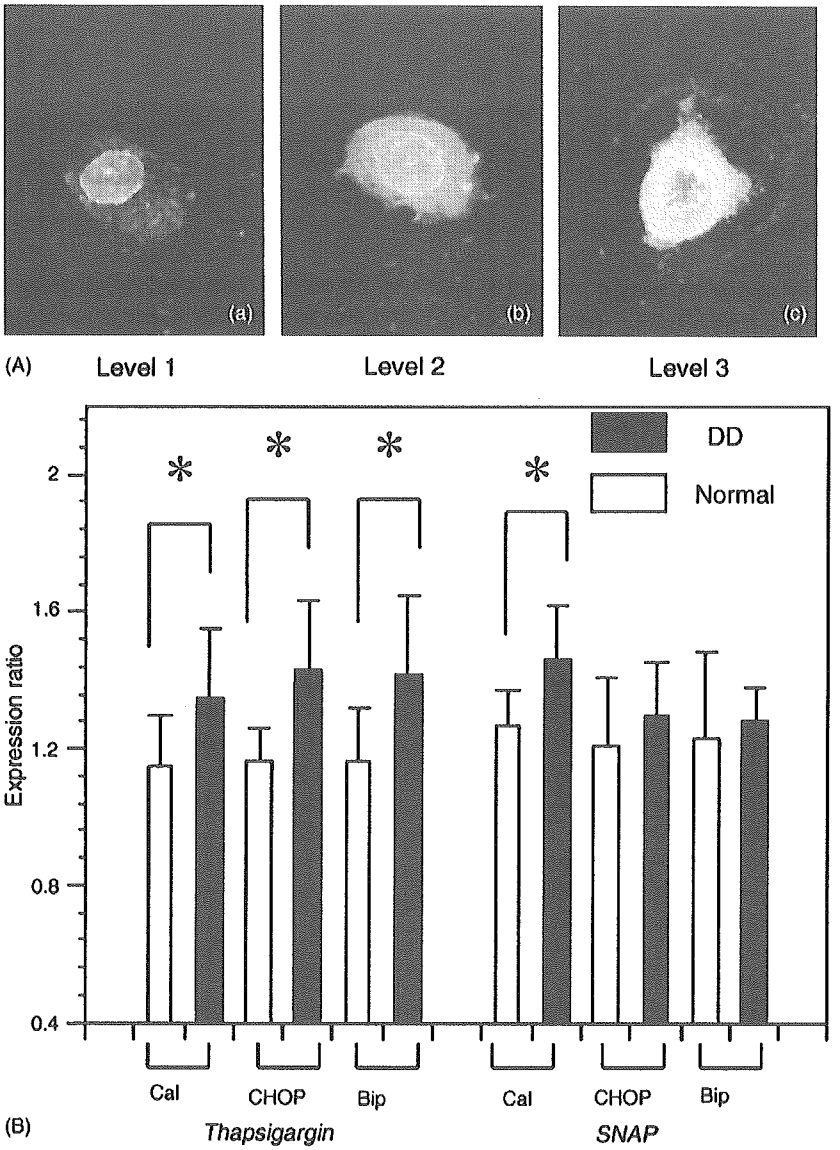


Fig. 1 Expression of calreticulin, CHOP/GADD153 and BiP/GRP78. DD (■) and normal (□) keratinocytes were treated with thapsigargin and SNAP for 48 h, and expression of calreticulin (Cal) CHOP/GADD153 (CHOP) and BiP/GRP78 (Bip) was scored and quantified as intensity value. (A) We counted to a hundred of the cells in a chamber, and classified as follows; Class 1: dark and poor staining in the cytoplasm (a); Class 2: moderate green deposit in the cytoplasm (b); Class 3: very bright cytoplasm with yellow colored (c). (B) DD keratinocytes induced expression of calreticulin, CHOP/GADD153 and BiP/GRP78 more than normal keratinocytes. Expression ratio was represented as the ratio of intensity values of stimulated to unstimulated cell samples. The results were plotted as a mean ± S.D. *Significant differences between DD and normal samples ($p < 0.02$).

was represented as the ratio of intensity values of stimulated to unstimulated samples.

The results of the culture cell study are shown in Fig. 1B. The expression of three molecules in DD keratinocytes was all higher than that in normal keratinocytes. Significant differences ($p < 0.02$) were found in thapsigargin-induced calreticulin, CHOP/GADD153 and BiP/GRP78 samples and SNAP-induced calreticulin sample.

Next, we examined the expression of calreticulin, CHOP/GADD153 and BiP/GRP78 in DD lesional skin. Hematoxylin and eosin sections clearly showed the dyskeratotic cells and corps ronds with the suprabasal cell layers. These cells stained with anti-calreticulin antibody (Fig. 2). We however failed to find immunostaining in any of the anti-Bip/GRP78 and CHOP/GADD153 in sections (data not shown). Skin section from normal control

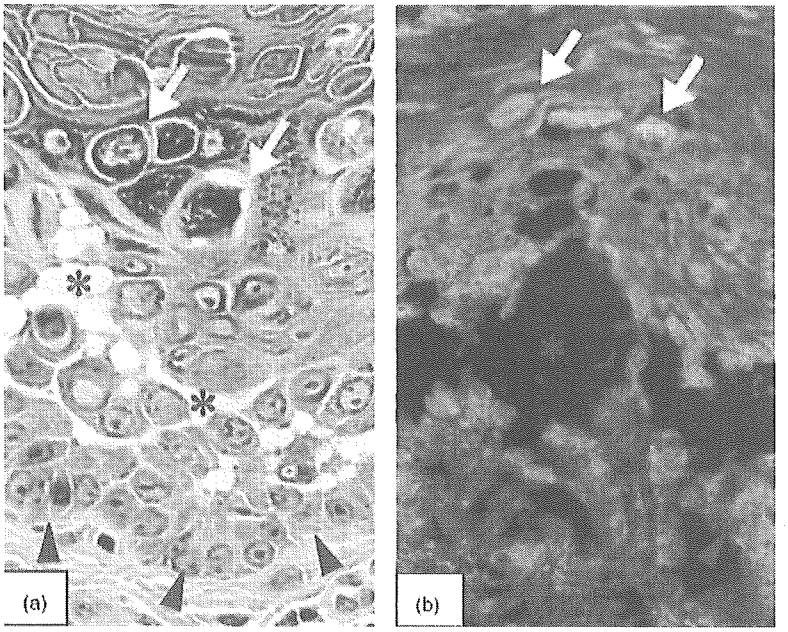


**Fig. 2** Immunohistochemical analysis of DD skin. (a) Dyskeratotic cells, corps ronds (arrows), suprabasal clefts (*) and acantholytic keratinocytes were observed (H&E staining). (b) Expression of calreticulin was detected in dyskeratotic cells (arrows). Blue arrowheads indicate the position of the basement membrane.

showed no immunoreactivities for any of those molecules.

The ER stress response is a mechanism by which cells protect themselves against ER stress. One response involves the up-regulation of genes encoding ER chaperone proteins to increase protein folding activity and to prevent protein aggregation. When the functions of the ER are severely impaired, apoptosis is induced via the transcriptional induction of CHOP/gadd153. The ER stress inducers, thapsigargin and SNAP were added to the keratinocyte cultures because both the DD and normal keratinocytes without any stressors showed a relatively low level of expression of above three ER chaperons. Those stressors induced ER stress by different mechanisms. This study showed that the levels of ER chaperons calreticulin, CHOP/gadd153 and Bip/GRP78 were increased in DD keratinocytes compared with normal control keratinocytes, suggesting that ER stress might be somehow involved with pathogenesis of Darier's disease.

Hakuno reported that the dissociation of intra- and extracellular domains of desmosomal cadherin and E-cadherin are characteristics of acantholytic cells in DD [9], this phenomenon might be explained by ER stress leading to important protein misfolding or misassembly. In addition, we found strong expression of calreticulin in dyskeratotic cells in DD lesional skins. Although we observed little or no detectable expression of CHOP/gadd153 in these cells, such dyskeratotic cells might result from apoptosis induced by ER stress.

This study suggests that the ER stress may be involved in the pathogenesis of DD. The treatment of DD has mainly included oral retinoids, or topical retinoids for localized DD. Recently some agents are shown to have an inhibitory effect on ER stress in the other organs [10], so we suggest that drugs which control ER stress in keratinocytes might hold significant potential for the treatment of DD.

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Original Article for Journal of Human Genetics

# *COL7A1* mutation G2037E causes epidermal retention of type VII collagen

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**Running title**: *COL7A1* mutation

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**Key Words:** gene transfer, retrovirus, glycine substitution, dominant negative interference

**Abbreviations-:**
DDEB: dominant dystrophic epidermolysis, DEB: dystrophic epidermolysis bullosa, GS: glycine substitution, HS: Hallopeau-Siemens type, n-HS: non-Hallopeau-Siemens type, RDEB: recessive dystrophic epidermolysis

## 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 a female proband showed retention of type VII collagen inepidermal keratinocytes. Mutational analysis detected a GS mutation; G2037E in the proband and her affected father. To demonstrate the direct association of G2037E and type VII collagen retention, we have 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. An increase in type VII collagen cytoplasmic staining in the G2037E/wild transfectant cell samples compared with the control/wild type cells. The G2037E (alone) transfected cells showed even stronger intracellular collagen VII staining than the G2037E/wild transfection sample. These results demonstrate 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 show more severe phenotypes than heterozygotes. This study furthers our understanding of GS *COL7A1* mutations in DEB.

## 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 the 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 patients exhibit tissue dermal-epidermal separation 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). An increasing number of DEB mutations thus far have elucidated several general genotype-phenotype correlations (Pulkkinen et al. 1999).

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

During the course of our *COL7A1* DEB patient mutational analysis (Sawamura et al. 2005), we found a unique GS mutation which was associated with a 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 demonstrate direct evidence whether G2037E leads to intracytoplasmic reteintion of type VII collagen, we have introduced the mutated *COL7A1* gene into cultured keratinocytes.

## Material and Methods

### *Patient*

A Japanese girl presented with erosions and blisters affecting her trunk and lower extremities that had persisted since birth (Fig 1A). The blisters continued to appear, however, 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 also had a similar history and now showed blister formation and the resulting scars, predominantly on the knees and elbows (Fig 1C). A family tree is shown in Fig 1C. The informed consents for studies and for publication of the clinical images were obtained from the family in this study.

***Ultrastructural and Immunohistochemical studies***

Skin biopsies were taken from the affected child, and processed for transmission electron microscopy and immunofluorescence microscopy, as previously described (Shimizu et al. 1996). For ultrastructural examination, skin specimens were fixed in 5% glutaraldehyde and postfixed in 1% osmium tetroxide, 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 75kv. 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, U.K.) 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.

***Mutational analysis***

Genomic DNA was isolated from peripheral blood lymphocytes of patients and their families 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 according to the report by Christiano, et al (Christiano et al. 1997) (GenBank numbers L02870, L23982). Specifically, to amplify exons 73, the following primers were used: sense primer 5'-aagtggctcagtgggttgtg-3'; antisense primer 5'-aacccctcttccctcactct-3'. For PCR amplification, approximately 200 ng of genomic DNA, 40 pmol of each primer, 0.5 mM $MgCl_2$, 20 μmol of each dNTP and 1.25 U of Taq polymerase were used in a total volume of 50 μl. The amplification conditions were 94℃ for 5 min, followed by 40 cycles of 94℃ for 45 s, 55-60℃ for 45 s and 72℃ for 45 s, and extension at 72℃ for 10 min in GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were subjected to direct automated nucleotide sequencing using the BigDye Terminator System (Applied Biosystems, Foster City, CA).

***Construction of retroviral COL7A1 expression vectors and transfection***

A 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, 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 in press). The recombinant retroviruses were produced by transfecting the retroviral plasmids into the amphotropic amphopack-293 packaging cells (Clontech) using a calcium-phosphate co-precipitation method. In addition, we utilized 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 Eagles medium (DMEM) with 10% fetal bovine serum (FCS). HaCaT cells were expanded up to 60 % of confluent density and then transduced with viral suspensions in 5 μg/ml polybrene. To increase attachment of virus to keratinocytes, we coated the surface of culture plates with 10 ng/ml 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) using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). To semiquantify the *COL7A1* expression, the HaCaT cells were classified into low, medium and high expression according to pixel values. We evaluated 100 fluorescing cells and the expression index value was calculated by the formula: (low expression cell number )X1 + (medium expression cell number)X2 + (high expression cell number)X3. The expression index is shown with the mean ± SD of the expression values from 5 different areas.

## Results

***Diagnosis of DDEB***

The proband and her father had suffered from skin fragility since birth, however, the severity of the father's skin lesions improved with age and healing occurred but with scarring. Routine ultrastructural examination showed skin separation occurred within the sublamina densa in the place of the anchoring fibrils (Fig 2A), suggesting dystrophic EB. The number of anchoring fibrils was also decreased. Immunofluorescence study

using LH7.2 detected a linear staining pattern at basement membrane zone, which was not characteristic of HS-RDEB (Fig 2B). Furthermore, we 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, which is 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 but which usually improves markedly during early life or even remits completely. Since this patient continued to show blister formation until around 2 years of age and her father still has skin fragility, we have opted for the 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 confirmed by restriction enzyme digestion (data not shown). Thus, the final diagnosis of DDEB was made by clinical and laboratory findings.

***Transfection study***

Next, we 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 any significant difference in *COL7A1* staining between the G2043R, wild, and G2043R/wild treated sample groups. Semiquantitative analysis showed a similar result (Fig 3). In contrast, we detected an increase intracytomic type VII collagen staining in the G2037E/wild sample compared with the control wild type sample. The G2037E transfected sample also showed stronger intracellular collagen VII staining than the G2037E/wild transfection group (Fig3). This finding was confirmed by semiquantitative analysis, which demonstrated an expression index of G2037E and G2037E/wild samples were higher than that of wild samples by 2.2 and 1.6 fold, respectively compared to 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 was previously reported to induce type VII collagen retention in epidermal keratinocytes (Jonkman et al. 1999). However, no transfection study was employed to demonstrate the direct relevance of dominant GS mutations to increase intracellular type VII collagen retention although there were transfection studies

characterizing the recessive GS G2008R mutation (Chen et al. 2002). Therefore, we constructed *COL7A1* retroviral vectors with the 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 mutation G2037E mutation observed in our patient. The transfection efficacy of our retroviral system was almost 30% in HaCaT cells (Goto et al. in press). Since HaCaT cells show little or no intrinsic intracellular collagen VII expression, we predicted that any high level *COL7A1* expressing cells were likely to be sucessfully gene-transfected cells. Those mutations were dominant, so we also transferred a 1:1 mixture of wild and mutated *COL7A1* as well as 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 showed 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 through dominant negative interference of the collagen triple helix. The following theoretical explanation is proposed. These dominant mutations may mildly interferer with the α-chain polypeptide structure and allow 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 the formation of the α-chain so the mutated polypeptide cannot induce dominant negative effect in the normal chains. As far as we know, RDEB cases which are homozygous for certain DDEB GS mutations have thus far not been identified. In fact, heterozygous dominant GS mutations *in COL4A4* can cause Alport syndrome, whereas one healthy individual is homozygotes 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 form those of the homozygote (DePaepe et al. 1997). Thus, it is possible that DDEB GS homozygotes may not demonstrate any significant EB phenotype.

We applied the wild type (normal) *COL7A1* gene alone (control), 1:1 mixture of mutated (diseased) and wild type genes and the mutated gene only (positive control).

We failed to find a significant difference in collagen VII staining between the G2043R, wild, and G2043R/wild treatments. This mutation was not predicted to affect secretion but homotrimer formation. However, our results demonstrated that the G2037E mutation alone significantly affected collagen formation and this was more impaired than the combination G2037E/wild type gene transfected sample. This result indicates that homozygotes with the dominant GS mutation may show a more severe phenotype than the heterozygotes, suggesting that dominant GS mutations cause interference with the α-chain polypeptide structure itself as well as a dominant negative effect on the collagen triple helix.

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## Figure Legends

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

**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,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 keratinocyte (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).

**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* as well as 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), the G2037E/wild samples (b:W/G2037E) and G2037E samples (c: G2037E). B) These findings were confirmed by semiquantitative analysis, which demonstrated that 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. ※: $p < 0.01$ between W and W/G2037E, and between W/G2037E and G2037E.

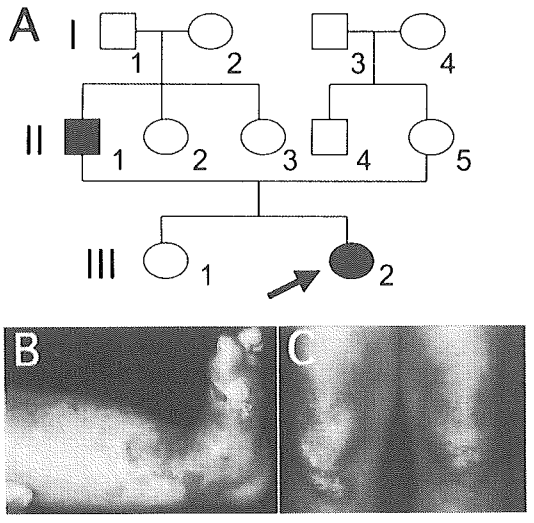


Fig 1 Sawamura et al

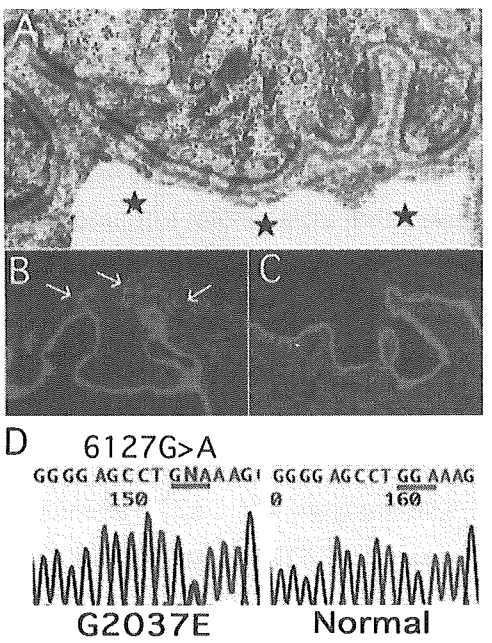


Fig 2 Sawamura et al

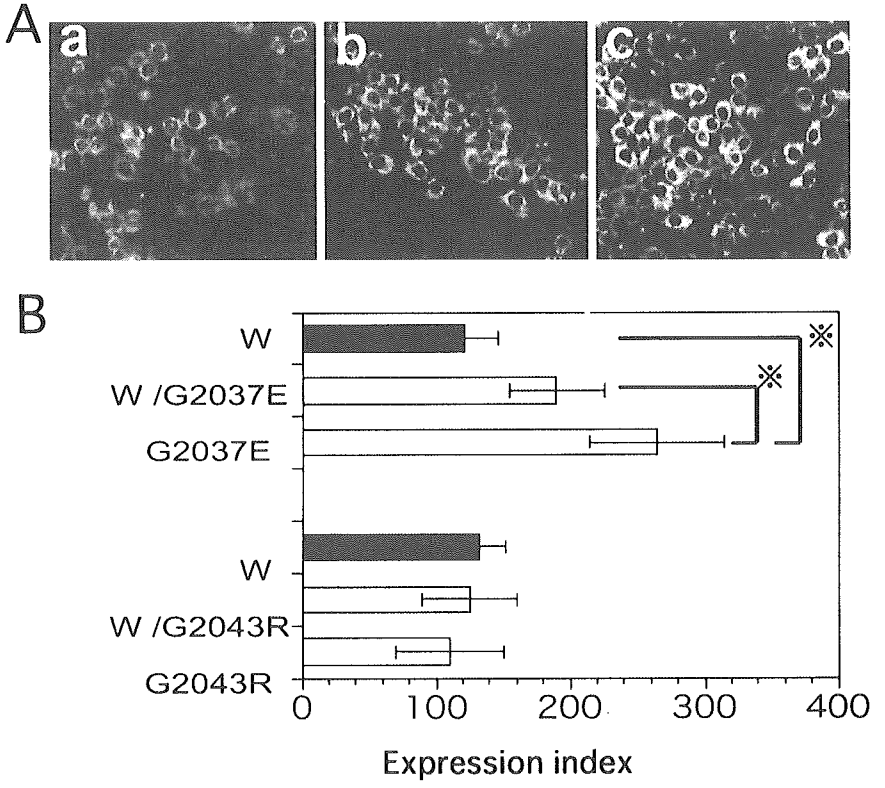


Fig 3 Sawamura et al

Key points

- Epidermolysis bullosa simplex (EBS) comprises a group of hereditary bullous diseases characterized by intra-epidermal blistering due to mechanical stress-induced degeneration of basal keratinocytes.
- Three major subtypes of EBS have been defined, based on the clinical severity: the Weber-Cockayne type (EBS-WC), the Koebner type (EBS-K), and the Dowling-Meara type (EBS-DM). All three EBS subtypes are caused by mutations in either keratin 5 or keratin 14, the major keratin intermediate filaments expressed in the basal cell of the epidermis. There has been significant correlation between the position of mutations within these proteins and the clinical severity of EBS.
- To identify additional EBS mutations for genotype/phenotype correlation studies in Oriental patients, we performed mutation analysis of the keratin genes *KRT5* and *KRT14* by direct sequencing in 17 Japanese and 2 Korean EBS cases and have also reviewed the previous reported mutations.
- We had identified 14 mutations, six of which were novel *KRT 5* missense mutations including the first mutation in the 2A domain. Most of these novel