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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 in epidermal 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  $\mu$ m 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'-aagtggctcagtgggttg-3'; antisense primer 5'-aaccccttccctcactct-3'. For PCR amplification, approximately 200 ng of genomic DNA, 40 pmol of each primer, 0.5 mM MgCl<sub>2</sub>, 20  $\mu$ mol of each dNTP and 1.25 U of Taq polymerase were used in a total volume of 50  $\mu$ l. The amplification conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 55-60°C for 45 s and 72°C for 45 s, and extension at 72°C 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 amphotopack-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  $\pm$  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 successfully 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 interfere with the  $\alpha$ -chain polypeptide structure and allow the formation of abnormal triple helix structures affecting the other, normal  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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.

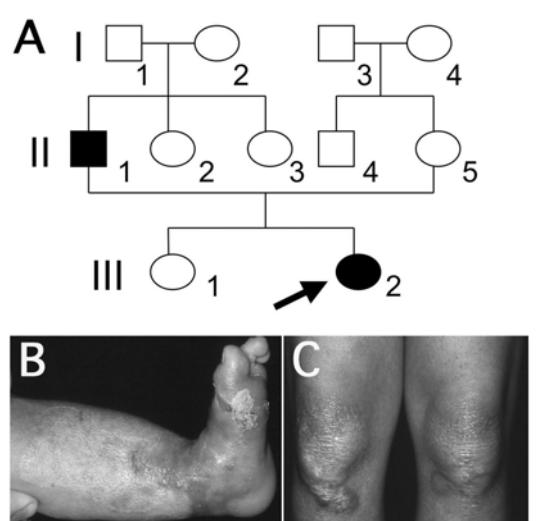


Fig 1 Sawamura et al

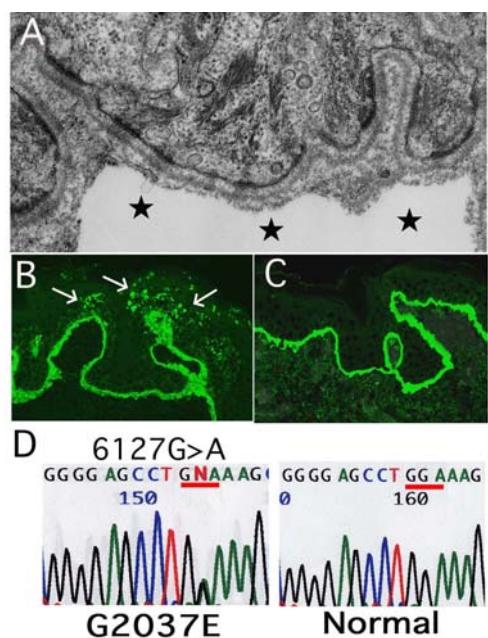


Fig 2 Sawamura et al

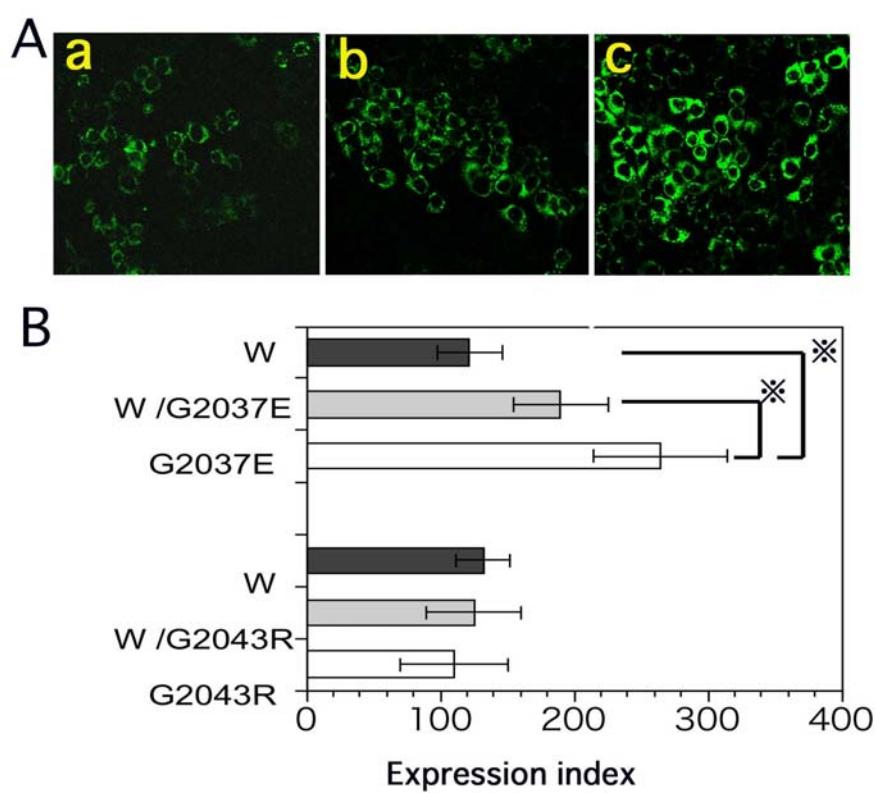


Fig 3 Sawamura et al