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ORIGINAL ARTICLE

GENOTYPE AND PHENOTYPE CORRELATION OF DYSTROPHIC EPIDERMOLYSIS BULLOSA

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Abstract Dystrophic epidermolysis bullosa (DEB) is a heritable mechanobullous skin disease derived from mutations in the type VII collagen gene (*COL7A1*). DEB cases are divided into dominant dystrophic EB (DDEB) and recessive dystrophic EB (RDEB). Most of the DDEB cases are induced by glycine substitution (GS) mutation because of its dominant negative effect, although there are silent GS which are not pathogenic without combination of other mutation in *COL7A1*. To know the relations between clinical features and *COL7A1* mutations, we examined *COL7A1* gene mutation in two Japanese families with DEB, one is dominantly inherited and another is sporadic.
We identified three mutations; 8068del17ins2 in case 1, G2395D/152delG in case 2. Case 1 is DDEB, which does not result from GS but from insertion/deletion mutation. In case 2, GS does not result in DDEB but causes recessive DEB (RDEB) with the combination of premature termination codon (PTC) in non-collagenous amino-terminal region (NC-1). This study demonstrates novel *COL7A1* mutations for DEB and furthers our understanding of genotype-phenotype correlation displayed in DEB patients.

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Key words: dystrophic epidermolysis bullosa; type VII collagen; glycine substitution; gene mutations.

原 著

優性栄養型表皮水疱症における臨床型と遺伝子型の関連

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抄録 栄養障害型表皮水疱症，Dystrophic epidermolysis bullosa (DEB) は，機械的刺激により水疱を生じる遺伝性皮膚疾患で，VII型コラーゲン遺伝子 (*COL7A1*) の変異が原因となる．栄養障害型表皮水疱症は，優性栄養障害型 dominant dystrophic EB (DDEB) と劣性栄養障害型 recessive dystrophic EB (RDEB) とに分けられる．ほとんどのDDEBは，グリシン置換変異 (GS) の dominant negative effect によって生じる．しかし，GSの中には，silentといって，*COL7A1* 他の変異と組み合わせられないと，病的意義をもたないものもある．*COL7A1* における臨床症状と遺伝子型の関係を明らかにするため，DEBの優性変異型，単発型2家系について，*COL7A1* geneの変異検索を行った．症例1に8068del17ins2を，症例2にはG2395Dと152delGの新規変異を同定した．症例1はDDEBであるが，GSではなくinsertion/deletion変異によって発症し，症例2のGSは，DDEBではなく，非コラーゲンアミノ酸領域 (NC-1) の早期終止コドン変異との組み合わせでRDEBを生じていた．本研究はDEBの新規変異を明らかにするとともに，本症のおける臨床と遺伝子変異の理解をさらに深めた．

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Introduction

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) or recessive (RDEB) diseases, each form having a different specific clinical presentation and severity[1)]

Typically, DDEB is less severe than RDEB, and blister formation decreases with age. RDEB has wide range of clinical severities. In the Hallopeau-Siemens type (RDEB-HS), the most severe form of RDEB, extracutaneous involvement such as strictures of the esophagus, erosion of the anus, and digital fusions resulting from repeated blister formation accompany severe blistering. The other end of the clinical spectrum of RDEB shows only localized skin involvement.

On electron microscopic examination of DEB patients' skin, anchoring fibrils, attachment structures between the lamina densa and dermis, are absent or diminished[2)]. Because type VII collagen is the major component of anchoring fibrils, the corresponding gene, *COL7A1*, was proposed as the candidate for DEB. In 1994, *COL7A1* was cloned[3,4)], and elucidation of its genomic structure has led to identification of multiple, specific mutations in *COL7A1* in many DEB cases. To date, almost two hundred pathogenic mutations within the collagenous and noncollagenous domains of type VII collagen gene have been identified in different forms of DEB[5-8)], but the detailed genotype-phenotype correlations have not been elucidated yet.

To understand precise genotype-phenotype correlation in DEB patients, the present study examined *COL7A1* mutations in two Japanese families, one with DDEB and the other with RDEB.

Methods

Clinical features

Case 1: The proband (Fig. 1A-a) is a 46-year-old male. Since he was 10 years old, he has periodically developed blisters with itching on his lower legs, elbows, and knees. On recent examination, there were ulcers and scarring on bilateral lower legs and some of his nails are deformed (Fig. 1B-a, b, c). The mother, elder sister, and elder brother of this patient were affected but to a lesser extent, indicating a dominant pattern of inheritance (Fig. 1A-a). Electron microscopy of uninvolved skin from his forearm revealed splits just beneath the basal lamina, and anchoring fibrils were diminished in number (Fig. 1B-d). The patient was diagnosed as having DDEB.

Case 2: The proband, a 1-month-old boy, was the first child of healthy, non consanguineous parents (Fig. 1A-b). There was no family history of related skin disease or similar genetic disorders. At birth, a large skin defect over the right leg and left foot, small erosions on the face and fingers, and hypogrowth of right fourth toe were observed (Fig. 1B-e,f). Transmission electron microscopy showed blistering just beneath the basal lamina (Fig. 1B-g). From the clinical and microscopic features of this patient, a DEB variant that is relatively severe, but less severe than RDEB-HS, was suspected. These phenotypic manifestations are consistent with a diagnosis of RDEB, although diagnosis of mode of inheritance could not be decided at this point.

Mutation detection

Genomic DNA was used as a template for amplification of genomic sequences of *COL7A1*. We synthesized 67 pairs of oligonucleotide primers, which spanned all 118 exons of the gene [3,4)], on the basis of the intronic sequences. The PCR products were screened by heteroduplex

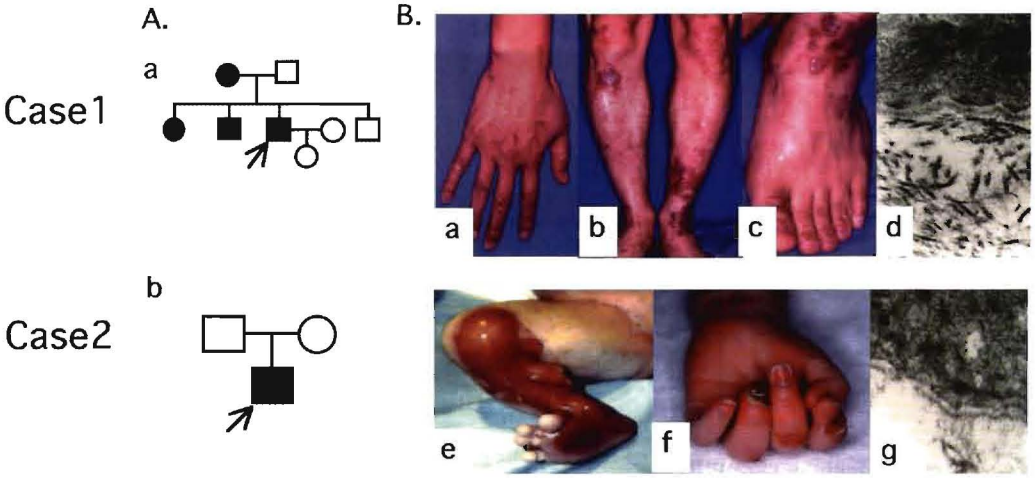

Fig. 1 The family pedigrees, clinical features, and electron microscopic features of patients. A: The pedigree of two families. The proband is indicated with an arrow. Affected individuals are indicated by solid symbols. The family of Case 1 (a) comprises several affected individuals. The parents of Case 2 (b) are clinically unaffected. B: Dorsal right hand (a), bilateral lower legs (b), and dorsal left foot (c) of Case 1. Right leg (e) and left hand (f) of Case 2. Electron-microscopic features of epidermal-dermal border in clinically unaffected skin of Case 1 (d), and Case 2 (g).

analysis utilizing conformation-sensitive gel electrophoresis (CSGE). PCR products with altered mobility (heteroduplexes) detected were then directly sequenced. When the mutation led to gain or loss of a restriction enzyme site, the PCR product was subjected to digestion with the appropriate restriction enzyme to verify the mutation (PCR-restriction enzyme fragment length polymorphism (PCR-RFLP).

Results

Examination of DNA from the proband and the mother of Case 1 revealed a heteroduplex band when the PCR product spanning exons 109 and 110 of *COL7A1* was examined (Fig. 2A). Sequencing of the PCR product demonstrating the heteroduplex revealed a 17-bp deletion (AGCCAGGCCCCAAGGGT) and 2-bp insertion (GA) (Fig. 2B). This mutation, 8068del17ins2, deletes a restriction enzyme site for Fnu4HI, and digestion with this enzyme demonstrated that both the proband and his mother were heterozygous for this mutation (Fig. 2C).

For Case 2, heteroduplex analysis of exon 94 revealed heteroduplex bands in the patient and his father (Fig. 3A). Sequencing of the PCR product disclosed a G-to-A transition at nucleotide position 7184, which was designated G2395D (Fig. 3B). This mutation eliminates a restriction enzyme site for BglI, and digestion with this enzyme demonstrated that both the proband and his father were heterozygous for the G2395D mutation (Fig. 3C). Further heteroduplex analysis of Case 2 revealed heteroduplex bands in exon 2 from the patient and his mother (Fig. 3D). Sequencing of the PCR product disclosed a deletion of G at nucleotide position 152, introducing premature termination codon (PTC) at N-terminal region of the molecule (Fig. 3E). This mutation, 152delG, deletes a restriction enzyme site for EaeI, and digestion with this enzyme demonstrated that both the proband and his mother were heterozygous for this mutation (Fig. 3F). In light of the clinical features and these findings, we diagnosed RDEB in this patient, although we need further

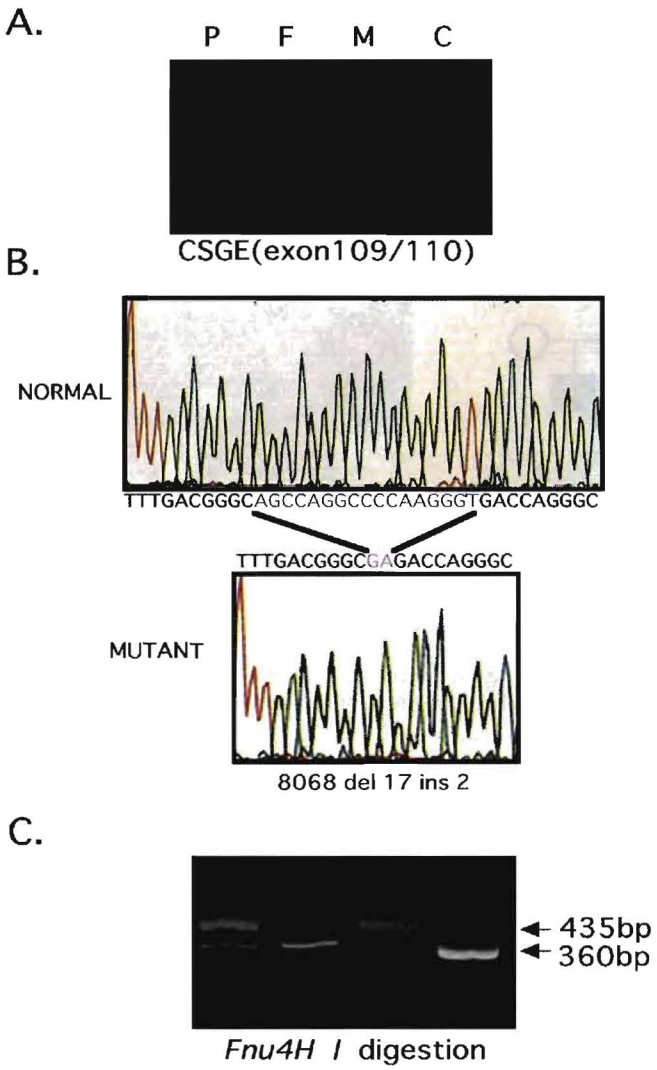

Fig. 2 Identification and verification of insertion/deletion mutation 8068del17ins2 in the *COL7A1* gene of Case 1.

A: Heteroduplex analysis of the PCR product spanning exons 109 and 110 and flanking intronic sequences of the *COL7A1* gene revealed heteroduplex bands in the clinically affected proband (lane P) and mother (lane M), whereas the unaffected father (lane F) and normal control (lane C) each showed a single band. B: DNA sequence of exons 109 and 110 of the proband revealed deletion of AGCCAGGCCCCAAGGGT and insertion of GA at nucleotide position 8068 (8068del17ins2 mutation; wild-type sequence is shown on top). C: Verification of the mutation by digestion with Fnu4HI. All of the 450-bp PCR product from the father and the normal control is cleaved into digestion products of 90 bp (not shown) and 360 bp, but the patient- and maternally derived 435-bp product (the size reflects the deletion) remained uncut, indicating that the patient and his mother are heterozygous for 8068del17ins2.

continuous observation of clinical course.

Each of these results was confirmed by CSGE of samples from 50 normal controls.

Discussion

Like other collagen molecules, type VII collagen is a homotrimer, comprising three polypeptides. The central portion of the molecule consists of a collagen segment with Gly-X-Y repeat sequences, which folds into

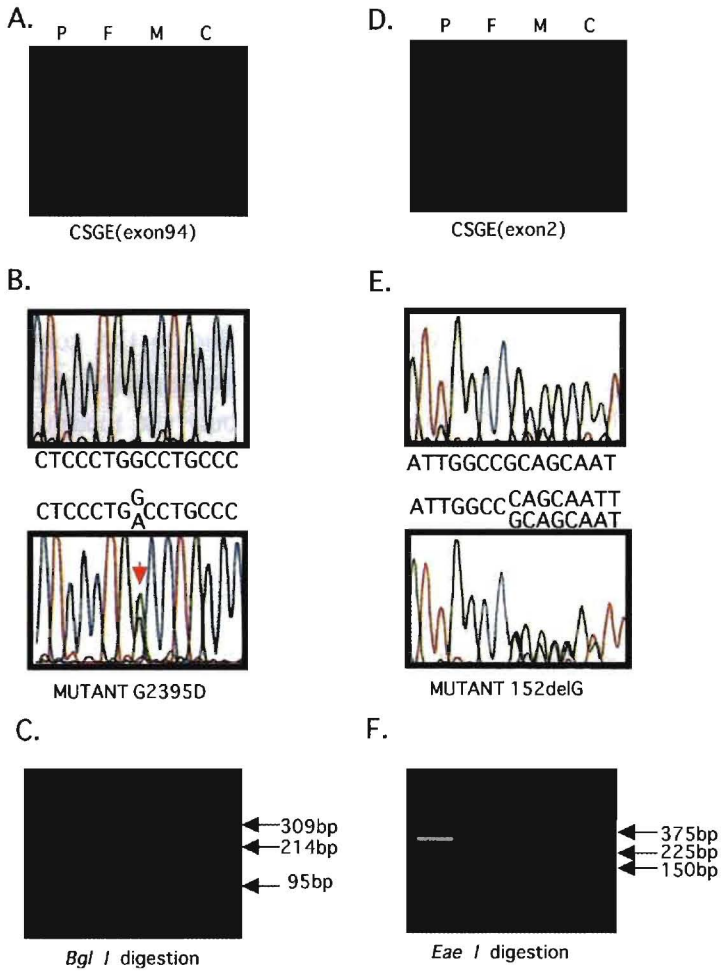

Fig. 3 Identification and verification of mutations in the *COL7A1* gene of Case 2.
A: Heteroduplex analysis reveals a shift in the patient- (lane P) and paternally derived (lane F) PCR products of exon 94 but not in corresponding samples from a normal control (lane C) or the patient's mother (lane M). B: Direct DNA sequencing of the exon 94 of the proband revealed a G-to-A transition at nucleotide position 7184, and the mutation was designated G2395D. C: Verification of the mutation by digestion with BglI indicated that all of the 309-bp PCR product from the normal control is cleaved into 214-bp and 95-bp fragments. D: In contrast, the patient- and paternally derived 309-bp PCR product remained uncleaved, indicating that the patient and his father are heterozygous for the G2395D mutation. Heteroduplex analysis of the PCR product spanning exon 2 revealed heteroduplex bands in the proband and his mother. E: The DNA sequence of exon 2 of the proband is unclear at nucleotide position 152 as compared with the wild-type sequence (shown on top), and there is deletion of G at nucleotide position 152. F: Verification of the mutation by digestion with EaeI. All the PCR product of 375 bp from the normal control is cleaved into 150-bp and 225-bp digestion products. In contrast, a portion of the 375-bp band from the patient and his mother remains, indicating that they are heterozygous for the 152delG mutation.

a characteristic triple-helical conformation. Glycine, the smallest amino acid, plays a key role in this conformation. The central collagenous domain is flanked by a large, ~145-kD non-collagenous amino-terminal globular region (NC-1) and a smaller, ~20-kD carboxyl-terminal globular portion (NC-2)[3,4].

In DDEB, pathogenic mutations basically result from glycine substitutions (GS) within triple-helical domain, which is analogized to have

dominant negative effect on both type VII collagen assembly and anchoring fibril formation[9]. In this study, we first detected 8068del17ins2 mutation in the *COL7A1* gene of Case 1. The 17 nucleotide deletion with a GA two base insertion resulted in a 15 nucleotide deletion within collagenous domain which sustained a *COL7A1* open reading frame. Consequently, the 15 nucleotide deletion interfered with collagen triple helix (Gly-X-Y repeat) and caused this DDEB phenotype in a likely dominant negative fashion. As far as we know, only six dominant mutations except for glycine substitution mutations have been reported in the literature. Out of them, three mutations, 4084-1G>C[10], 6899A>G[11], 8045A>G [12], were one nucleotide substitutions and the others were gross deletion mutations 6847del27[9], 6863del16[13] and 6081del28[9]. Furthermore, only one insertion/deletion mutation 8068del17insGA was reported in Japanese DDEB patients [14], and was identical to the mutation of Case 1. However, the previous case showed severe itching and scratching, resulting in epidermolysis bullosa pruriginosa which was a specific EB form characterized by prurigo-like or lichenoid lesions associated with scarring[15]. On the other hand, clinical features of case 1 were those of typical DDEB with slight itching. It was reported that identical *COL7A1* glycine substitutions could cause remarkably heterogeneous clinical phenotypes including simple nail dystrophy, typical DDEB, and EB pruriginosa[16]. This report also reveals that *COL7A1* insertion/deletion mutation may cause clinically different phenotypes.

We identified novel *COL7A1* mutations in Case 2. The paternal mutation was GS of exon 94, 7184 G to A (G2395D). Case 2 was sporadic and firstly we detected this GS mutation in patient's DNA. When we encounter GS mutation in sporadic case of DEB, we cannot tell whether the case is de novo dominant or recessive. Soon after, we recognized the same GS in DNA of phenotypically unaffected patient's father. Thus, we concluded that this GS was silent and this case was recessively inherited. The maternal mutation (152delG) in Case 2 caused PTC in exon 3, and amino acid synthesis would stop in the middle of the NC-1 domain. Most of the reported cases of RDEB-HS are due to a PTC/PTC combination. However, the defect in Case 2 is due to the combination of GS and PTC. The consequence of the GS/PTC combination is that only the molecules containing GS assemble into anchoring fibril. Therefore, eventually only hypoplastic anchoring fibril will be produced.

Dominant negative GS mutations tend to cluster between amino acids 2003 and 2079, downstream of the largest non-collagenous interruption, especially in exon 73, according to the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). From these data, it is recommendable to start with this area in examining mutations in DDEB cases. In contrast, silent GS changes tend to be scattered over the entire triple-helical domain. In all GS detected in exon 73, dominant GS share 77.3%. Since identical positions of GS have not been shared between dominant and recessive inheritance so far reported, mode of inheritance for the sporadic DEB cases with GS in exon 73 tends to be dominant with almost 80% probability. Incidentally, talking of amino acid change from glycine, arginine (R) is the most frequent in both dominant negative GS and silent GS, and glutamic acid is slightly more in dominant negative GS, although no apparent difference is observed. This tendency suggests that positional effect of GS is more significant than amino acid change to regulate phenotype.

In conclusion, we have identified three *COL7A1* mutations in two cases of DEB with different phenotypes. The patient with DDEB has little difficulty in daily life, and we have to

keep observing case 2, because in many cases clinical feature changes as a patient grows up. Our findings will provide some insight into understanding the molecular mechanism of DEB.

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References

1) Fine JD, Eady RAJ, Bauer EA, Briggaman RA, Bruckner-Tuderman L, Christiano A, Heagerty A, 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) Hashimoto I, Schnyder UW, Anton-Lamprecht I, Gedde-Dahl T, Ward S. Ultrastructural studies in epidermolysis bullosa hereditaria. Arch Dermatol Res 1976;256:137-50.

3) Christiano AM, Greenspan DS, Lee S, Uitto J. Cloning of human type VII collagen: Complete primary sequence of the $\alpha 1$ (VII) chain and identification of intragenic polymorphism. J Biol Chem 1994;269:20256-62.

4) Christiano AM, Hoffman GG, Ghung-Honet LC, Lee S, Cheng W, Uitto J, Greenspan DS. Structural organization of the human type VII collagen gene (*COL7A1*), composed of more exons than any previously characterized gene. Genomics 1994;21:169-79.

5) 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 Phys 1995;107:245-252.

6) Christiano AM, Morricone A, Paradisi M, Angelo C, Mazzanti C, Cavalieri R, Uitto J. 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;104:438-440.

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

8) Sawamura D, Goto M, Yasukawa K, Sato-Matsumura K, Nakamura H, Ito K, Nakamura H, Tomita Y, Shimizu H. Genetic studies of 20 Japanese families of dystrophic epidermolysis bullosa. J Hum Genet 2005;50:543-546.

9) Sakuntabhai A, Hammami-Hauasli N, Bodemer C, Rochat A, Prost C, Barrandon Y, de Prost Y, et al. Deletions within *COL7A1* exons distant from consensus splice sites after splicing and produce shortened polypeptides in dominant dystrophic epidermolysis bullosa. Am J Hum Genet 1998;63:737-748.

10) 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;109:811-814.

11) 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 2002;82:187-191.

12) Kon A, Pulkkinen L, Ishida-Yamamoto A, Hashimoto I, Uitto J. Novel *COL7A1* mutations in dystrophic forms of epidermolysis bullosa. J Invest Dermatol 1998;111:534-537.

13) Mellerio JE, Ashton GH, Mohammedi R, Lyon CC, Kirby B, Harman KE, Salas-Alanis JC, et al. Allelic heterogeneity of dominant and recessive *COL7A1* mutations underlying epidermolysis bullosa pruriginosa. J Invest Dermatol 1999;112:984-7.

14) Sawamura D, Nizeki H, Miyagawa S, Shinkuma S, Shimizu H. A novel indel *COL7A1* mutation 8068del17insGA causes dominant dystrophic epidermolysis bullosa. Br J Dermatol 2006;154:995-997.

15) McGrath JA, Schofield OM, Eady RA. Epidermolysis bullosa pruriginosa: dystrophic epidermolysis bullosa with distinctive clinicopathological features.

Br J Dermatol 1994;130:617-25.

16) Nakamura H, Sawamura D, Goto M, Sato-Matsumura KC, LaDuca J, Lee JY, Masunaga T, Shimizu H. The G2028R glycine substitution mutation in *COL7A1* leads to marked inter-familiar clinical heterogeneity in dominant dystrophic epidermolysis bullosa. J Dermaol Sci 2004;34:195-200.