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Genetic studies of 20 Japanese families of dystrophic epidermolysis bullosa

Daisuke Sawamura, Maki Goto, Kana Yasukawa, Kazuko Sato-Matsumura, Hideki Nakamura, Kei Ito, Hiroyuki Nakamura, Yuki Tomita, Hiroshi Shimizu.

Departments of Dermatology, Hokkaido University Graduate School of Medicine, Japan;

Running title: *COL7A1* mutation

Corresponding author: Daisuke Sawamura

Department of Dermatology

Hokkaido University Graduate School of Medicine

North 15 West 7, Kita-ku, Sapporo 060-8638, Japan

Voice: 81-(0)11-706-7387

Fax 81-(0)11-706-7820

E-mail: smartdai@med.hokudai.ac.jp

Abbreviations:

BMZ: basement membrane zone, DDEB: dominant dystrophic epidermolysis,

DEB: dystrophic epidermolysis bullosa, HS: Hallopeau-Siemens type, n-HS:

non-Hallopeau-Siemens type, RDEB: recessive dystrophic epidermolysis,

Abstract

Dystrophic EB (DEB) is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy, and is caused by mutations in the *COL7A1* gene encoding type VII collagen. DEB is inherited in either an autosomal dominant (DDEB) or recessive (RDEB) fashion. DDEB basically results from a glycine substitution mutation within the collagenous domain on one *COL7A1* allele, while a combination of mutations such as premature stop codon, missense, splice-site mutations on both alleles causes RDEB. This study performed mutation analysis in 20 distinct Japanese DEB families (16 RDEB and 4 DDEB). The result demonstrated 30 pathogenic *COL7A1* mutations with 16 novel mutations, which included 4 missense, 5 nonsense, 1 deletion, 2 insertion, 1 indel, 3 splice-site mutations. We confirmed that Japanese *COL7A1* mutations were basically family specific although 3 mutations 5818delC, 6573+1G>C, E2857X were recurrent based on previous reports. Furthermore, Q2827X mutation found in two unrelated families would be regarded as a candidate recurrent Japanese *COL7A1* mutation. The study furthers our understanding of both the clinical and allelic heterogeneity displayed in Japanese DEB patients.

Key Words: type VII collagen, mutation, *COL7A1*, blister, glycine substitution

Introduction

Epidermolysis bullosa (EB) comprises a group of cutaneous hereditary mechano-bullous disorders that can be classified into three major categories, the simplex, the junctional, and the dystrophic forms, on the basis of the level of tissue separation within the basement membrane zone (BMZ) (Fine *et al*, 2000). Dystrophic EB (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 at the level of the anchoring fibrils. It occurs as either an autosomal dominant (DDEB) or recessive (RDEB) trait, each form having a different specific clinical presentation and severity (Fine *et al*, 2000).

Both DDEB and RDEB are caused by mutations in the *COL7A1* gene encoding type VII collagen, the major component of anchoring fibrils (Uitto *et al*, 1995; Fine *et al*, 2000). The most severe RDEB subtype, the Hallopeau-Siemens type (HS), 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 comparatively milder than those of RDEB. To date, several hundred pathogenic mutations within the collagenous and noncollagenous domains of type VII collagen gene have been identified in different forms of DEB (Uitto *et al*, 1995; Christiano *et al*, 1995; Shimizu *et al*, 1996; Pulkkinen and Uitto, 1999, Whittock *et al*, 1999). Although particular molecular and phenotypic characteristics of DEB have been elucidated, we cannot always expect DEB clinical manifestations precisely from genetic information of *COL7A1*. Furthermore, no systematic study has thus far revealed detailed delineation of *COL7A1* mutations in Japanese DEB patients apart from several recurrent *COL7A1* mutations (Tamai *et al*, 1999; Murata *et al*, 2004).

In this study, we performed mutational analysis of 20 Japanese DEB families and have demonstrated the characteristic features of *COL7A1* mutations in Japanese DEB patients.

Materials and Methods

Subjects

Twenty unrelated Japanese DEB families, who have been referred to the special clinic for inherited skin disorders of Hokkaido University Hospital during January 2000 to December 2004, were studied (Table 1). DEB was at first clinically diagnosed and later confirmed by immunofluorescence antigen mapping demonstrating tissue separation beneath the lamina densa as mentioned below. Also clinical features and inheritance modes can help to differentiate most, though not all, cases into recessive or dominant DEB subtypes. Immunofluorescence expression of type VII collagen was of significant diagnostic value in determining HS-RDEB and nHS-RDEB, respectively.

Immunohistochemical analysis

Skin biopsies were taken from DEB patients and subjected to a routine immunofluorescence antigen mapping study (Shimizu et al, 1996). The specimens were embedded in OCT compound, and 10 μ m thick sections were cut. The following monoclonal antibodies (mAbs) against BMZ components were used: mAbs HD1-121 for plectin; GoH3 and 3E1 (Chemicon International, CA) for the α 6 and β 4 integrins, respectively; GB3 (Sera-lab, Cambridge, UK) for laminin 5; LH7.2 (Sigma, St. Louis, MO) for type VII collagen; S1193 and HDD20 for BPAG1 and BPA2, respectively. The antibodies GoH3, S1193 and HDD 20 were kind gifts from Dr. Sonnenberg A, the Netherlands Cancer Institute. The antibodies HD1-121 was also a kind gift from Dr. Owaribe K, Nogyo University. The bound antibodies were detected with FITC-conjugated goat anti-mouse IgG antibody. In some cases, nuclei were counterstained with propidium iodide.

All DEB patients in this study were evaluated by several experienced dermatologists. This study was approved by the Ethical Committee at Hokkaido University Graduate School of Medicine. Informed consent was obtained from individual patients or their parents.

Mutation analysis

Genomic DNA was isolated from peripheral 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 (1997) (GenBank numbers L02870,

L23982). The PCR products were examined on 2% agarose gel and subjected to direct automated nucleotide sequencing using the BigDye Terminator System (Applied Biosystems, Foster City, CA).

Results and Discussion

An increasing number of DEB mutations have elucidated some general genotype-phenotype correlations (Jarvikallio et al, 1997; Pulkkinen et al, 1999). DDEB patients basically harbor glycine substitution mutations within the collagenous domain on one *COL7A1* allele, leading to disruptions in anchoring fibril assembly and relatively mild clinical features. On the other hand, patients with RDEB in its most severe form, the Hallopeau Siemens variant (HS-RDEB), frequently have premature termination codon (PTC) mutations on both alleles. These mutations characteristically lead to nonsense-mediated mRNA decay that manifests as a complete absence of type VII collagen protein and total loss of anchoring fibrils. On the other hand, patients with the non-Hallopeau Siemens variant (nHS-RDEB) show milder phenotype and type VII collagen can be generally detected immunohistologically. This DEB subtype is caused by a combination of mutations such as PTC, missense, splice-site mutations on both alleles.

The routine immunofluorescence antigen mapping study in a blister site showed that all BMZ antigens were located in the roof of the blister, indicating tissue separation beneath the lamina densa. Also, linear type VII collagen expression was found along the dermal epidermal junction in nHS-RDEB and DDEB patients whereas HS-RDEB cases showed no expression (Table 1). We found retention of type VII collagen within epidermal keratinocytes in a DDEB (Family 17, data not shown).

Examination of 40 alleles of 20 families (10 nHS-RDEB, 6 HS-RDEB, 4DDEB) identified 30 pathogenic *COL7A1* mutations including 16 novel mutations (Table1). *COL7A1* mutations of nHS-RDEB included 5 missense mutations G1595R(4783G>A), G1815R(5443G>A), R1957Q(5870G>A), G2366C(7096G>T), C2875F(8627G>T), 5 nonsense mutations R236X(706C>T), R1340X(4018C>T), R1978X(5932C>T), Q2827X(8479C>T), E2857X(8569G>T), 1 insertion-deletion mutations 5818delC, and 4 splice site mutations 5604+2G>C,

6573+1G>C, 8109+2T>A, 8358+1G>T. HS-RDEB patients showed 4 nonsense mutations, R137X(409C>T), Q641X(1921C>T), R1683X(5047C>T), R2261X(6781C>T), and 4 insertion-deletion mutations 434insGCAT, 1474del8 and 5818delC, 6081insC. As predicted by previous DEB mutation reports, all combinations of PTC mutations caused HS-RDEB, while nHS-RDEB resulted from compound heterozygous *COL7A1* mutations except homozygous nonsense PTC/PTC mutations. Although we could not find positional effect of PTC mutations as suggested by the previous report (Tamai et al, 1999), final conclusion needs further accumulation of the RDEB patients with PTC mutations.

In DDEB patients, we identified three dominant glycine substitution mutations G2034R (6100G>A), G2037E (6110G>A), G2064E (6191G>A). These glycine substitution mutations were previously reported, and interestingly the nucleotide changes also were identical to those in previous reports (Kon et al, 1997; Rouan et al, 1998; Jonkman et al, 1999; Whittock et al, 1999; Lee et al, 2000). Glycine residues within the collagenous domain are critical for proper triple helix formation. Some *COL7A1* glycine substitution mutations, which cause RDEB in association with a second mutation on the other allele, are silent in patients with a normal *COL7A1* allele. In addition, heterozygous glycine substitution mutations can cause DDEB through dominant negative interference of the collagen triple helix. Although this study also identified both dominant and recessive glycine substitution mutations (Table), we could not clarify positional effect of glycine substitution on the inheritance mode. A single indel mutation 8069del17insGA was novel. The 17 nucleotide deletion from 8069 to 8084 with GA insertion resulted in a 15 nucleotide deletion within the collagenous domain, which failed to change an open reading frame of *COL7A1* but interfered with the collagen triple helix (Gly-X-Y). This mutation causes a DDEB phenotype probably in a dominant negative fashion.

We failed to detect one allelic mutation in Families 8, 9, 10 and 15 and both allelic mutations in RDEB Family 16 (Table 1). Thus, this study could demonstrate *COL7A1* mutations in 30 out of 36 alleles which were expected to have *COL7A1* mutations, and the resulting ratio of successful mutation detection was 83 %. Similar, large scale *COL7A1* mutation reports using Italian patient

data also failed to determine single allele mutations in 13 RDEB families out of a total of 49 families (Gardella et al, 2002). This suggests at least two possibilities that pathogenic mutations lie in the other parts of the *COL7A1* gene which were not examined in these studies, and that other genes except for *COL7A1* are responsible for DEB phenotype.

Although *COL7A1* mutations are generally family specific, some recurrent mutations have been reported in several populations: R2814X, R578R, 7786delG in British patients (Mellerio et al, 1997), 2470insG in Mexican patients (Salas-Alanis et al, 2000) and 8441-14del21, 4783-1G>A, 497insA, G1664A in Italian patients (Gardella et al, 2002). In Japanese patients, the mutations 5818delC (9 out of 50 cases: 18 %), 6573+1G>C (6/50:12%), and E2857X (9/50:18 %) are present only in individuals of Japanese ethnic origin (Tamai et al, 1999). The present study also detected 5818delC in 4, 6573+1G>C in 2 and E2857X in 1 families out of total of 20 unrelated families. Furthermore, the Q2827X mutation was found in 2 unrelated families, and this mutation should be regarded as a candidate recurrent Japanese *COL7A1* mutation. However, 16 mutations were novel out of a total of 30 pathogenic DEB mutations identified, indicating that Japanese *COL7A1* mutations are family specific. This result furthers our understanding of both the clinical and allelic heterogeneity displayed in Japanese DEB patients.

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The informed consents for both studies and for publication of the photographs were obtained from the both families in this study. We thank the

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Table 1 Clinical phenotype, type VII collagen expression and *COL7A1*

mutations in this study.

| Family | Age/Sex (proband) | Phenotype | VII expression | Mutation | Effect |
|--------|----------------------|-----------|-------------------|----------------------------------|---------|
| 1 | 1Y/F | nHS-RDEB | + | <u>R1340X/C2875F</u> | PTC/Mis |
| 2 | 44Y/F ¹⁾ | nHS-RDEB | + | <u>G1815R</u> /5818delC | GS/PTC |
| 3 | 40Y/M | nHS-RDEB | + | E2857X/ <u>5604+2G>C</u> | PTC/SS |
| 4 | 7Y/M ¹⁾ | nHS-RDEB | + | <u>G1595R/Q2827X</u> | GS/PTC |
| 5 | 1Y/F | nHS-RDEB | + | <u>8109+2T>A/6573+1G>C</u> | SS/SS |
| 6 | 9Y/M | nHS-RDEB | + | <u>8358+1G>T/G2366C</u> | SS/GS |
| 7 | 5Y/M | nHS-RDEB | + | <u>R1957Q/6573+1G>C</u> | Mis/SS |
| 8 | 37Y/M | nHS-RDEB | + | <u>Q2827X/ND</u> | PTC/ND |
| 9 | 7D/F | nHS-RDEB | + | R236X/ND | PTC/ND |
| 10 | 32Y/F | nHS-RDEB | + | R1978X/ND | PTC/ND |
| 11 | 2M/M ²⁾ | HS-RDEB | - | <u>434insGCAT/R2261X</u> | PTC/PTC |
| 12 | 1W/M | HS-RDEB | - | <u>R137X/Q641X</u> | PTC/PTC |
| 13 | 1Y/M | HS-RDEB | - | <u>1474del8/5818delC</u> | PTC/PTC |
| 14 | 3D/M | HS-RDEB | - | <u>R1683X/6081insC</u> | PTC/PTC |
| 15 | 3Y/F | HS-RDEB | - | 5818delC/ND | PTC/ND |
| 16 | 7Y/M | HS-RDEB | - | ND/ND | ND/ND |
| 17 | 1M/F | DDEB | +* | G2037E | GS |
| 18 | 4Y/F | DDEB | + | G2064E | GS |
| 19 | 23Y/M | DDEB | + | G2034R | GS |
| 20 | 26Y/M | DDEB | + | <u>8069del17insGA</u> | GS |

*: Retention of type VII collagen in keratinocytes. RDEB and DDEB: recessive and dominant dystrophic epidermolysis bullosa, Hallopeau-Siemens: HS, non-Hallopeau-Siemens: nHS, GS: glycine substitution mutation, PTC: premature stop codon mutation, mis: missense mutation, SS: splice site mutation. Novel mutations are underlined. ND: not detected. ¹⁾: Families 2 and 4 with unusual clinical phenotype was published in a case report (Sato-Matsumura et al 2002, Tomita et al 2003), ²⁾: Family 11 was also published (Sato-Matsumura et al. 2003).