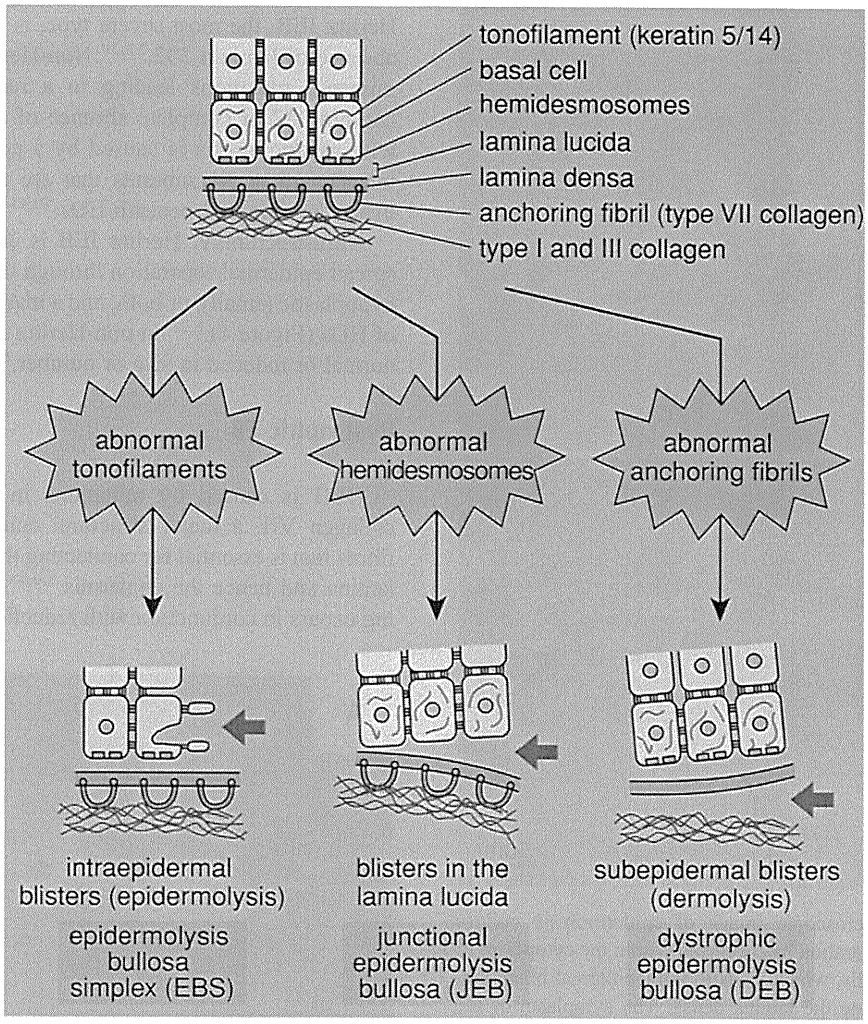

Fig. 2 The mechanism and the cleavage site of epidermolysis bullosa. (Adapted with permission from Shimizu H: Shimizu's Textbook of Dermatology: Blistering and Pustular Diseases. Sapporo, Japan: Hokkaido University Press/Nakayama Shoten Publishers. 2007:203p.).

antigen 1 (BPAG1e, BP230, also known as dystonin) hemidesmosomal antigens.[13-17] These two plakin protein family members, plectin and dystonin, form critical links in a continuous series of protein interactions bridging two distinct transmembrane molecular systems of the outer HD plaque, integrin $\alpha 6\beta 4$[18,19] and collagen XVII,[20,21] also known as 180-kDa bullous pemphigoid antigen 2 (BPAG2) or BP180.

Immediately beneath the keratinocyte plasma membrane lays an electron-lucent zone, the lamina lucida and an electron-dense layer comprising a closely packed fibrous network called the lamina densa.[7] Below the HD, there is a thin electron-dense line termed the subbasal dense plate, parallel to the plasma membrane that is visible in approximately one-third of HDs, depending on the precise orientation of the section.[10,22] Traversing the lamina lucida zone, subjacent to HDs, are thin anchoring filaments apparently inserting into the lamina densa. Laminin 332, one of the major epidermal laminins (formerly known as Kalinin, laminin 5), is found on the border between the upper lamina densa of HDs and lower lamina lucida at the base of anchoring filaments, which may comprise collagen XVII.[23,24] Beneath the lamina densa, most of the collagen VII molecules form semicircular loop structures called anchoring fibrils in which the amino (N-) terminals of the antiparallel collagen VII fibrils originate and terminate in the lamina densa.[3,25,26] In the dermis, anchoring fibrils may enable the lamina densa to link or encircle dermal collagen fibers or other components to provide basal lamina anchorage to the underlying structures.

Ultrastructural findings of EB

Epidermolysis bullosa simplex

The three major subtypes of EBS—Dowling-Meara (EBS-DM) (severe), other generalized (moderate), and the localized (mild) type—are caused by keratin 5 or 14 mutations that result in an abnormal keratin network leading

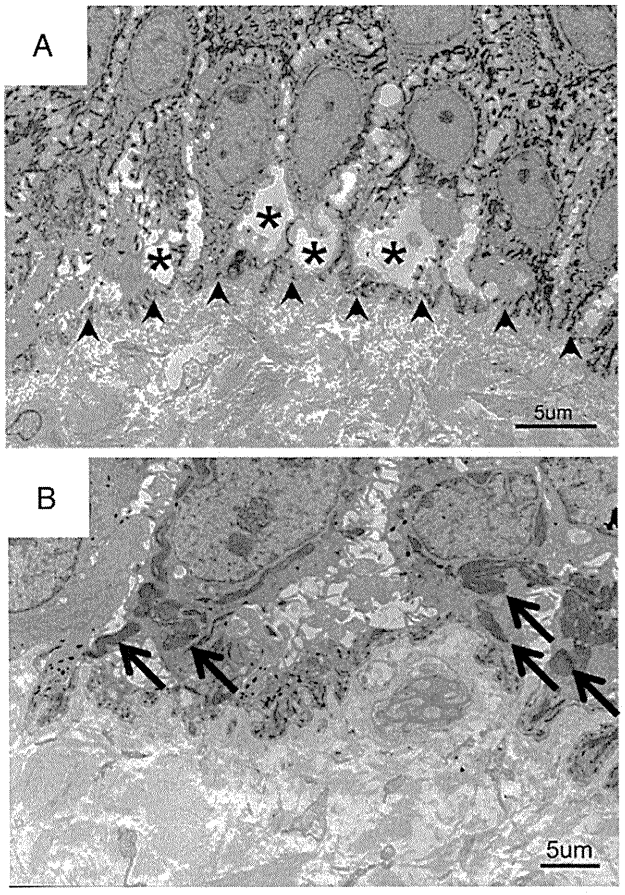

Fig. 3 Electron microscopic image of epidermolysis bullosa simplex shows (A) separation has occurred within the cytoplasm of the epidermal basal cells, which leads to intraepidermal blistering. The arrowheads indicate the lamina densa. The cytoplasm of the basal cells contains large vacuoles (asterisks) and show extensive damage. (B) Aggregation of keratin fibers is seen in epidermolysis bullosa simplex–Dowling-Meara (arrows).

to blister formation within the cytoplasm of the epidermal basal cells (Figure 3A).[27,28] In EBS-DM, in addition to the intraepidermal cleavage, clumping of degenerated keratin fibers can be observed within epidermal keratinocytes (Figure 3B).[29]

Rare types of EBS, including EBS with muscular dystrophy (EBS-MD) and EBS with pyloric atresia (EBS-PA), are caused by plectin gene mutations.[30-35] In EBS-MD and EBS-PA, the split occurs around the level of the HD inner plaque within the keratinocyte cytoplasm and is often associated with reduced numbers of poorly formed hypoplastic HDs, with reduced numbers of inner plaque and KIF association.[6,32]

Junctional EB

JEB can be further divided into three subtypes: Herlitz JEB, non-Herlitz JEB, and JEB with pyloric atresia.[1] All JEB subtypes are inherited in an autosomal-recessive manner and are characterized by blister formation in the lamina lucida.[36] Herlitz JEB, the most severe type, is caused by a complete absence of laminin 332.[37-39] Non-Herlitz JEB is caused by missense mutations leading to a reduction in functional laminin 332 or complete absence of collagen XVII.[37] JEB with pyloric atresia is caused by a genetic mutation in the integrin $\alpha 6$ or $\beta 4$ subunits that are the main receptor for ligand laminin 332 beneath HDs.[40,41]

Ultrastructurally, Herlitz JEB is characterized by widespread epidermal separation through the lamina lucida or by hypoplastic (small), or both, and a markedly reduced number of HDs (Figure 4).[6,22] In non-Herlitz JEB, HDs may appear normal or reduced in size or number.[6,42]

Dystrophic EB

DEB is caused by mutations in the gene that codes collagen VII, a major structural component of anchoring fibrils that is essential for connecting the dermis and the basal lamina and hence the epidermis.[3,9,43] Subepidermal blistering occurs in conjunction with reductions in anchoring fibril

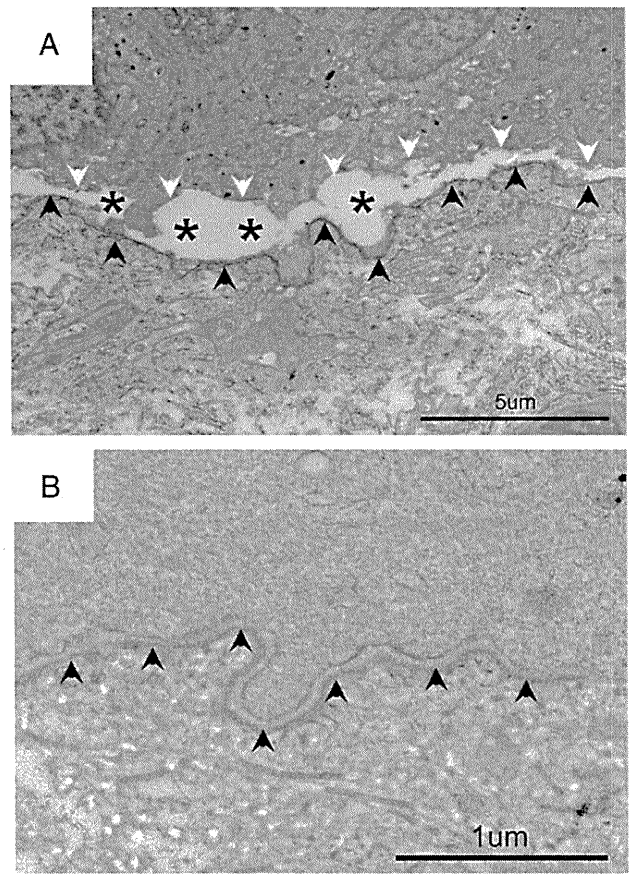

Fig. 4 Electron microscopic image of junctional epidermolysis bullosa (JEB) shows (A) a blister (asterisks) is present within the lamina lucida, between the plasma membrane of the basal keratinocytes (white arrowheads) and the lamina densa (black arrowheads). (B) The hemidesmosomes are rudimentary and reduced in number in Herlitz JEB.

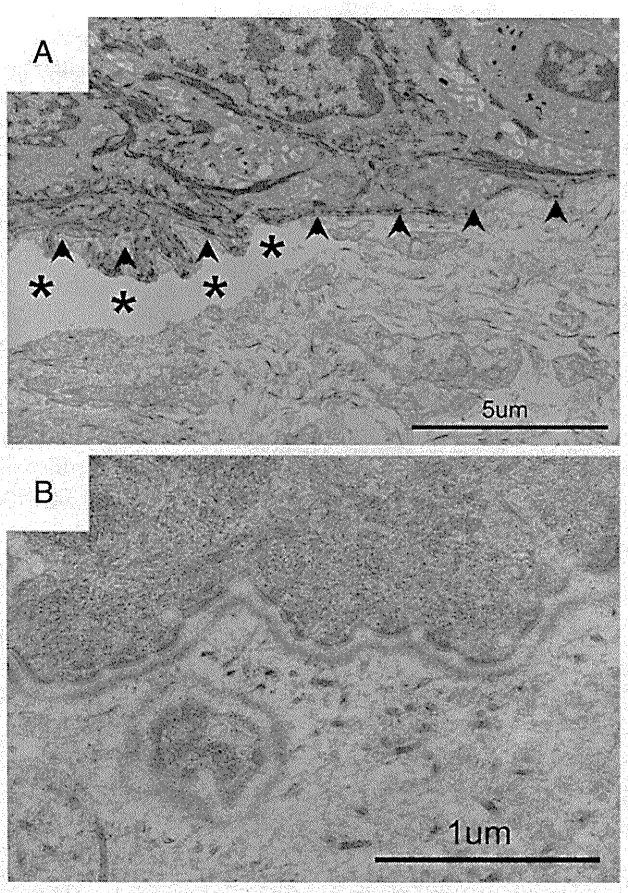

Fig. 5 Electron microscopic image of dystrophic epidermolysis bullosa shows (A) dissociation (asterisks) immediately below the lamina densa (arrowheads). (B) It is characterized by hypoplasia of anchoring fibrils.

numbers or with defects in normal anchoring fibril morphology, or both (Figure 5).[44] The phenotype of autosomal-dominant DEB (DDEB) is milder than that of recessive DEB (RDEB).[45] The most severe subtype of RDEB, severe generalized type, shows a severe reduction or lack of expression of collagen VII, which ultrastructurally results in rudimentary or absent anchoring fibrils (Figure 5).[44] By contrast, in the milder RDEB phenotype, termed "generalized other RDEB", shows reduced or rudimentary-appearing anchoring fibrils. In DDEB, anchoring fibrils are typically seen as normal in appearance or slightly decreased in number.

Kindler syndrome

Kindler syndrome has been added as a further, specific subtype of EB, in the latest classification of EB.[1,46] Kindler syndrome is inherited in an autosomal-recessive manner and is characterized by trauma-induced blistering, poikiloderma (skin atrophy and altered skin pigmentation), mucosal inflammation, and varying degrees of photosensitivity.[46] The pathogenesis of Kindler syndrome involves loss-of-function mutations in a newly recognized actin cytoskeleton-associated protein, now known as fermitin family homolog 1 and encoded by the gene *FERMT1*.[47] This protein has a role in controlling/activating β1 associated integrin cell adhesion and may play a role in the linkage of the actin cytoskeleton to β1 integrins and the extracellular matrix at sites of focal adhesion. Whereas EB is caused by abnormalities in HD-KIF cell attachment to the underlying basal lamina and dermis, Kindler syndrome is caused by defective activation of focal adhesion anchorage.[48] Ultrastructural examination of Kindler syndrome reveals a distinct disorganization below the

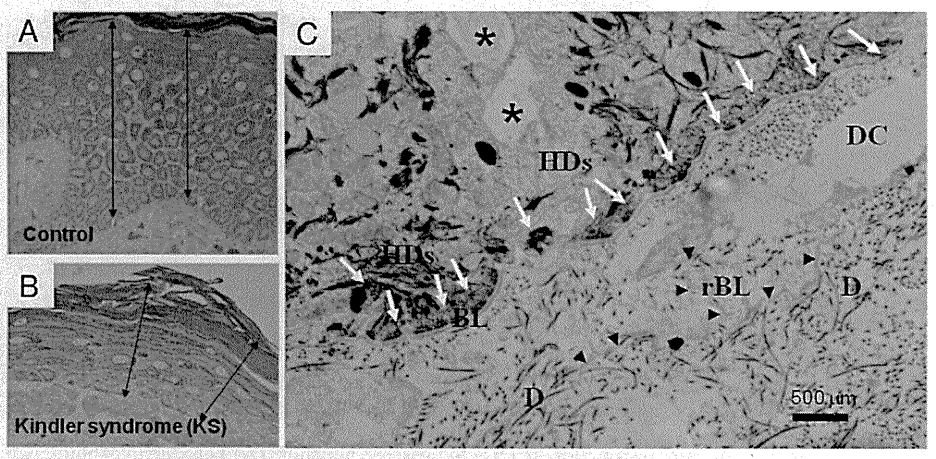

Fig. 6 Electron microscopic image of Kindler syndrome (KS) shows that (A) compared with the site-matched skin of healthy controls, (B) the skin of the KS patient has a thinner epidermis resulting from fewer cell layers. (C) Ultrastructurally, along the dermal-epidermal junction in the KS patient's skin, the hemidesmosomes (HDs) appear normal (white arrow); however, there may be signs of epidermal separation within the basal keratinocyte (asterisks) or immediately below the lamina densa as dermal clefts (DC). A common finding is the reduplication of the lamina densa (arrowheads) is seen in the upper dermis. Dermal cleft formation can occur together with reduplication of the lamina densa.

epidermal keratinocyte basement membrane exhibiting as lamina densa reduplication with branching, folding, and formation of loops and circles.[49] Cleft formation can occur at various sites along the dermal–epidermal junction, the largest and most common being below the lamina densa (Figure 6).[50] HDs and anchoring fibrils typically appear normal and with normal frequency, but there can be concomitant disturbances in the KIF network.

The role of electron microscopy in EB

Recent advances in genetic and IF techniques have enabled us to diagnose EB more rapidly and with greater accuracy regarding the particular underlying genetic defects.[1,2] We cannot, however, sufficiently predict precise clinical manifestations of each EB subtype using these techniques alone. Gene analysis cannot always precisely predict EB disease severity from novel mutations, although some successful genotype–phenotype correlations have been reported.[51,52] One reason is that most cases of JEB and RDEB are inherited in an autosomal-recessive manner and are thus caused by compound heterozygous gene mutations; therefore, it is usually difficult to assess the clinical phenotype and function of each mutant protein derived from different maternal or paternal mutations. Another reason is that there may exist, as yet undiscovered, modifier genes that influence EB disease severity, other than the causative gene.[53]

IF studies also have limited ability to assess disease severity by measuring the expression level of particular constitutive BMZ proteins, because the clinical severities of EBS, DDEB, and parts of autosomal-recessive EB with

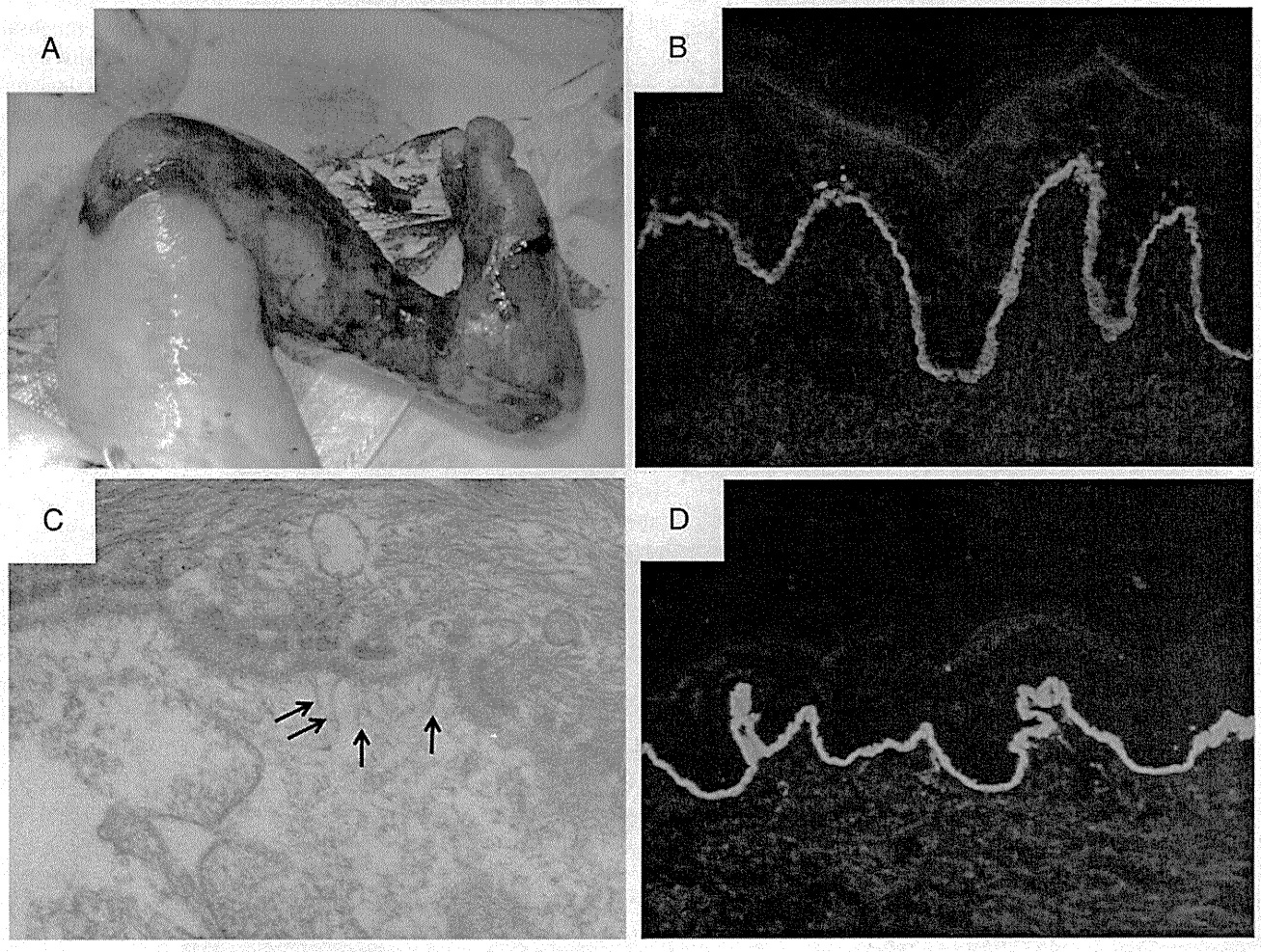

Fig. 7 A case of recessive dystrophic epidermolysis bullosa (EB) with missense mutation. (A) The patient exhibits a clinical severity similar to the most severe subtype of recessive dystrophic EB (DEB), the severe generalized type. (B) Immunofluorescence staining for collagen VII shows the expression level of collagen VII is just slightly reduced for the severe manifestation. (C) The electron micrograph of the DEB epidermal basement membrane zone. There are rudimentary-appearing anchoring fibrils, which are slightly reduced in number (arrow). (D) Normal control of immunofluorescence staining for collagen VII. In this case, the clinical severity correlates with the morphology of each mutant collagen VII anchoring fibril rather than the expression level.

missense mutations correlate with the combined function of the mutant proteins rather than the expression levels of both wild-type normal or abnormal protein expression examined by IF staining (Figure 7).[54] In these cases, determination of the precise molecular morphology of BMZ components provides important clues to predict their clinical severities, organ involvement, and overall patient prognosis. A careful ultrastructural examination can thus provide some estimate of EB clinical severity and disease progression, not only from a quantitative ultrastructural analysis but also from a morphologic examination. Taken together, we propose that electron microscopic evaluation remains an important technique acting as a bridge between genetic and immunohistologic tests and has the ability to provide extra diagnostic clues and subsequent beneficial practical and clinical information for EB patients and their health care providers.

Acknowledgments

Hideki Nakamura contributed to the transmission electron micrographs.

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Japanese-Specific Filaggrin Gene Mutations in Japanese Patients Suffering from Atopic Eczema and Asthma

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TO THE EDITOR

Mutations in *FLG*, the gene encoding profilaggrin/filaggrin, are the underlying cause of ichthyosis vulgaris (OMIM 146700) and an important predisposing factor for atopic eczema (AE) (Sandilands *et al.*, 2007). *FLG* mutations are also significantly associated with asthma with AE mainly in the European population (Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2010). The presence of population-specific *FLG* mutations has been reported in both the European and Asian races (Nomura *et al.*, 2007; Sandilands *et al.*, 2007). To clarify whether *FLG* mutations are a predisposing factor for asthma in the non-European population, we initially studied 172 Japanese AE patients (mean age, 24.8 ± 9.1 years) and 134 unrelated Japanese control individuals (healthy volunteers; mean age, 27.9 ± 6.0 years). All AE patients had been diagnosed based on widely recognized diagnostic criteria (Hanifin and Rajka, 1980). The majority of AE patients and control individuals were identical to those in a previous study (Nemoto-Hasebe *et al.*, 2010). In this AE cohort, 73 AE patients (mean age, 25.4 ± 8.9 years) experienced complications with asthma. Furthermore, we studied another Japanese asthma cohort (137 patients; mean age, 58.2 ± 16.9 years). Patients were considered asthmatic based on the presence of recurrent episodes of ≥2 of the three symptoms (coughing, wheezing, or dyspnea) associated with demonstrable reversible airflow limitation, either spontaneously or with an inhaled short-acting β2-agonist and/or increased airway responsiveness to methacholine (Isada *et al.*, 2010). Fully informed consent was obtained from the participants or their legal guardians for this study. This study had been approved by the Ethical Committee at Hokkaido University Graduate School of Medicine and was conducted according to the Declaration of Helsinki Principles.

FLG mutation screening revealed that 27.4% of patients in our Japanese AE complicated with asthma case series carried one or more of the eight *FLG* mutations (combined minor allele frequency of 0.151, $n=146$) (Table 1). Conversely, 26.3% of Japanese AE patients without asthma carried one or more of the eight *FLG* mutations (combined minor allele frequency of 0.147, $n=198$). The *FLG* variants are also carried by 3.7% of Japanese control individuals (combined minor allele frequency of 0.019, $n=268$). We found that all compound heterozygous mutations were present in *trans* by observing transmission or haplotype analysis (Nomura *et al.*, 2007, 2008). There is a statistically significant association between the eight *FLG* mutations and AE with asthma, and between the eight *FLG* mutations and AE without asthma (Table 1). Moreover, AE complicated with asthma manifested in heterozygous carriers of *FLG* mutations with an odds ratio for AE and asthma of 9.74 (95% confidence interval 3.47–27.32), suggesting a relationship between *FLG* mutations and AE with asthma.

In the Japanese general asthma cohort, 8.0% of the asthma patients carried one or more of the eight *FLG* mutations (combined minor allele frequency of 0.04, $n=274$) (Table 2). Whereas, of the Japanese patients with asthma complicated by AE, 22.2% carried one or more of the *FLG* mutations (combined minor allele frequency of 0.11, $n=36$). In contrast, 5.9% of asthma patients without AE carried one or more of the *FLG* mutations (combined minor allele frequency of 0.03, $n=238$). There was a statistically significant association between the eight *FLG* mutations and asthma with AE (Table 2). There was no statistically significant association between the *FLG* mutations and entire asthma patients, nor between *FLG* mutations and asthma without AE. We cannot exclude the possibility that this lack of significant association is due to the small number of the patients included in this study. We used the same control set for both case-controlled studies. Thus, strictly speaking, there is no independent replication for the control group.

Recent meta-analysis revealed that *FLG* mutations are significantly associated with asthma in the European population and there are especially, strong effects observed for *FLG* mutations for the compound phenotype, asthma in addition to eczema (Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2010). In contrast, there appeared to be no association of *FLG* mutations with asthma in the absence of eczema (Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2010).

This Japanese cohort has a completely different *FLG* mutation spectrum from those in the European and the North American populations. However, our results clearly confirm the strong association of *FLG* mutations with our Japanese cohort of AE patients with asthma complications, and the association of *FLG* mutations and asthma patients with AE complications, for the first time outside Europe or North America. Conversely, this study showed no significant correlation between general asthma patients and *FLG* mutations, suggesting that atopic asthma patients associated with *FLG* mutations are a minority among general asthma patients. The frequency of heterozygous, compound heterozygous, and homozygous *FLG* mutation carriers

Abbreviation: AE, atopic eczema

Table 1. Atopic eczema case–control association analysis for *FLG* null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		K4022X		Combined			
Genotype	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE	Con	AE (total)	AE (asthma+)	AE (asthma−)
AA	134	172	133	163	133	172	134	169	133	162	132	152	134	166	134	169	129	126	53	73
Aa	0	0	1	9	1	0	0	3	1	10	2	20	0	6	0	3	5	41	18	23
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5[1]	2	3
Total	134	172	134	172	134	172	134	172	134	172	134	172	134	172	134	172	134	172	73	99

Abbreviations: AE, atopic eczema; CI, confidence interval; Con, healthy control; OR, odds ratio.
For combined genotype: AE+asthma, exact *P*-value of Pearson χ^2-test=1.909×10^{-6}, OR and 95% CI for dominant models (AA vs aX)=9.737 (3.473–27.322); AE−asthma, exact *P*-value of Pearson χ^2-test=7.189×10^{-7}, OR and 95% CI for dominant models (AA vs aX)=9.191 (3.383–24.938); all AE, exact *P*-value of Pearson χ^2-test=1.189×10^{-7}, OR and 95% CI for dominant models (AA vs aX)=9.416 (3.625–24.450).
[1]All the five patients were compound heterozygotes for minor alleles.

Table 2. Asthma case–control association analysis for *FLG* null variants in Japan

	R501X		3321delA		S1695X		Q1701X		S2554X		S2889X		S3296X		K4022X		Combined			
Genotype	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma	Con	Asthma (total)	Asthma (AE+)	Asthma (AE−)
AA	134	137	133	137	133	137	134	137	133	133	132	132	134	136	134	136	129	126	14	112
Aa	0	0	1	0	1	0	0	0	1	4	2	5	0	1	0	1	5	11	4	7
aa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	134	137	18	119

Abbreviations: AE, atopic eczema; CI, confidence interval; Con, healthy control; OR, odds ratio.
For combined genotype: asthma+AE, exact *P*-value of Pearson χ^2-test=0.0122, OR and 95% CI for dominant models (AA vs aX)=7.3692 (1.7715–30.6748); asthma−AE, exact *P*-value of Pearson χ^2-test=0.5563, OR and 95% CI for dominant models (AA vs aX)=1.6124 (0.4979–5.2219); all asthma, exact *P*-value of Pearson χ^2-test=0.1968, OR and 95% CI for dominant models (AA vs aX)=2.2523 (0.7609–6.6667).

observed in our Japanese controls was only 3.7%, which was much lower than that seen in European general population, where it is approximately 7.5%. This suggested that there may be further mutations yet to be discovered in the Japanese. As we have sequenced more than 40 Japanese families with ichthyosis vulgaris, there is now little possibility that further highly prevalent mutations will be found in the Japanese population. However, it is still possible that there might be multiple, further low-frequency *FLG* mutations discovered in the Japanese population. In addition, because of the relatively small sample size of this genetic study, further replication in association studies will be required for *FLG* mutations and asthma in Japan.

In our cohorts, serum IgE levels were extremely high (median, 3141.9 IU ml^{-1}; 25th–75th percentiles, 1276.0–9753.0 IU ml^{-1}) in AE patients with asthma ($n=73$) in the AE cohort, compared with that in total asthma patients (median, 156.0 IU ml^{-1}; 25th–75th percentiles, 71.05–441.45 IU ml^{-1}, $n=137$) in the asthma cohort. These findings suggest that extrinsic allergic sensitization might have an important role in atopic asthma pathogenesis. Recent studies hypothesized skin barrier defects caused by *FLG* mutation(s) allow allergens to penetrate the skin, resulting in initiation of further immune response and leading to the development of systemic allergies, including atopic asthma (Fallon *et al.*, 2009). In patients with asthma that also harbor *FLG* mutations, we could not exclude the possibility that the systemic effects of early eczema might simply influence airway responsiveness (Henderson *et al.*, 2008).

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

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RNase 7 Protects Healthy Skin from *Staphylococcus aureus* Colonization

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TO THE EDITOR

The Gram-positive bacterium *Staphylococcus aureus* is an important pathogen that causes various skin infections (Miller and Kaplan, 2009). However, healthy skin is usually not infected by *S. aureus,* despite the high carrier rates in the normal population (Noble, 1998). This suggests that the cutaneous defense system has the capacity to effectively control the growth of *S. aureus*. There is increasing evidence that antimicrobial proteins are important effectors of the cutaneous defense system (Harder *et al.*, 2007). A recent study reported that keratinocytes contribute to cutaneous innate defense against *S. aureus* through the production of human β-defensin-3 (Kisich *et al.*, 2007). In addition to human β-defensin-3,, other antimicrobial proteins may also participate in cutaneous defense against *S. aureus*. One candidate is RNase 7, a potent antimicrobial ribonuclease that is highly expressed in healthy skin (Harder and Schröder, 2002; Köten *et al.*, 2009).

To investigate the hypothesis that RNase 7 may contribute to protect healthy skin from *S. aureus* colonization, we first incubated natural RNase 7 isolated from stratum corneum skin extracts (Harder and Schröder, 2002) with *S. aureus* (ATCC 6538). In concordance with our initial report about RNase 7 (Harder and Schröder, 2002), we verified that RNase 7 exhibited a high killing activity against *S. aureus* (lethal dose of 90% $=3$–$6\,\mu g\,ml^{-1}$).

Recently, we reported a moderate induction of RNase 7 mRNA expression in primary keratinocytes treated with heat-killed *S. aureus* (Harder and Schröder, 2002). To assess the induction of RNase 7 by *S. aureus* in the

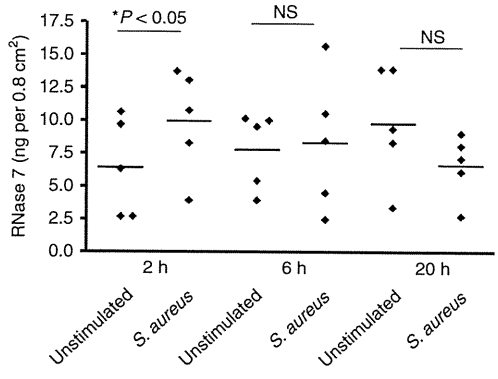

Figure 1. Induced secretion of RNase 7 on the skin surface on treatment with living *S. aureus*. Defined areas (0.8 cm^2) of skin explants derived from plastic surgery were incubated with or without approximately 1,000 colony-forming units of *S. aureus* (ATCC 6538) in 100 µl of sodium phosphate buffer. After 2, 6, and 20 hours, the concentration of secreted RNase 7 was determined by ELISA. Stimulation with *S. aureus* for 2 hours revealed a significant induction as compared with the unstimulated control after 2 hours (*$P<0.05$, Student's *t*-test; n.s. = not significant). Data shown are means of triplicates of five skin explants derived from five donors.

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Expression of exon-8-skipped kindlin-1 does not compensate for defects of Kindler syndrome

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ABSTRACT

Background: Kindler syndrome (KS) is a rare, inherited skin disease characterized by blister formation and generalized poikiloderma. Mutations in *KIND1*, which encodes kindlin-1, are responsible for KS. c.1089del/1089+1del is a recurrent splice-site deletion mutation in KS patients.
Objective: To elucidate the effects of c.1089del/1089+1del at the mRNA and protein level.
Methods: Two KS patients with c.1089del/1089+1del were included in this study. Immunofluorescence analysis of KS skin samples using antibodies against the dermo-epidermal junction proteins was performed. Exon-trapping experiments were performed to isolate the mRNA sequences transcribed from genomic DNA harbouring c.1089del/1089+1del. β1 integrin activation in HeLa cells transfected with truncated *KIND1* cDNA was analyzed.
Results: Immunofluorescence study showed positive expression of kindlin-1 in KS skin with c.1089del/1089+1del mutation. We identified the exon-8-skipped in-frame transcript as the main product among multiple splicing variants derived from that mutation. HeLa cells transfected with *KIND1* cDNA without exon 8 showed impaired β1 integrin activation. Exon-8-coding amino acids are located in the FERM F2 domain, which is conserved among species, and the unstructured region between F2 and the pleckstrin homology domain.
Conclusion: This study suggests that exon-8-skipped truncated kindlin-1 is functionally defective and does not compensate for the defects of KS, even though kindlin-1 expression in skin is positive.

1. Introduction

Kindler syndrome (KS) is classified as a novel subtype of epidermolysis bullosa (EB) according to a revised classification of EB [1] and characterized by photosensitivity, skin fragility, fused fingers, and generalized progressive poikiloderma [2]. A characteristic histological finding in KS skin is the variability of epidermal separation and clefting at the epidermal basement membrane [3,4]. Former studies have confirmed that mutations in *COL7A1* are not a factor in KS patients [3,5].

In 2003, mutations in the *KIND1* gene encoding kindlin-1 were detected in KS patients [6,7]. The *KIND1* gene was mapped to chromosome 20p12.3 [7]. The gene spans 48.5 kb of genomic DNA and contains 14 coding sequences (exons 2–15) and one non-coding exon (exon 1) [2,7]. The *KIND1* gene is the human homolog of the *Caenorhabditis elegans* gene, *unc-112*, which encodes a membrane-associated structural/signaling protein that has been implicated in linking the actin cytoskeleton to the extracellular matrix (ECM) [7,8]. Kindlin-1 deficiency is associated with cutaneous basement membrane zone abnormalities and reduced integrin activation [9]. Also, kindlin-1 is necessary for lamellipodia formation *in vitro*, which is mediated by RhoGTPase signaling [10]. To date, more than 30 different loss-of-function mutations in *KIND1* have been reported [2].

Splicing is a common mRNA modification after transcription, in which introns are removed and exons are joined. This is mandatory for typical eukaryotic mRNA before it can be used to produce an accurate protein through translation. Nucleotide alterations in positions close to the spliced sites affect correct splicing of the mRNA transcript and result in complete skipping of the exon, retention of the intron, or the introduction of a new splice site

Abbreviations: KS, Kindler syndrome; ECM, extracellular matrix; EB, epidermolysis bullosa; SCC, squamous cell carcinoma; MASA, mutant-allele-specific amplification; PTC, premature termination codon; NMD, nonsense-mediated mRNA decay; PCR, polymerase chain reaction; DEJ, dermo-epidermal junction.

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within an exon or intron. Several methods are available to predict the consequences resulting from splice site mutations, such as the use of neural network software [11] (http://www.fruitfly.org/seq_tools/splice.html) and GeneSplicer software [12] (http://cbcb.umd.edu/software/GeneSplicer/). However, these programs cannot distinguish between pseudo and real splice sites [13]; therefore, other functional testing is necessary to correctly predict the mRNA products. Use of an exon-trapping system (Invitrogen, Carlsbad, CA) is one such approach for directly isolating transcribed mRNA sequences from genomic DNA [14]. This system is a reliable and easy-to-use tool for assessing the effects of splice-site mutations on mRNA splicing in cell cultures [15].

This study highlights a recurrent c.1089del/1089+1del in *KIND1* in KS patients. To elucidate the pathogenic effects of this deletion mutation on mRNA splicing, exon-trapping experiments were performed. We found that in-frame exon-8-skipped transcripts were produced by c.1089del/1089+1del defects. Immunofluorescence analysis of the patient's skin showed positive kindlin-1 staining, which might have resulted from exon-8-skipped kindlin-1. *In vitro* analysis using living cells revealed the expression of truncated kindlin-1 lead to impaired activation of β1 integrin. This study clarifies the complex sequelae resulting from a splice-site deletion mutation and provides greater understanding of the pathomechanisms involved in KS disease.

2. Materials and methods

2.1. Mutation detection

gDNA was extracted from the patient's peripheral blood cells. The mutation detection strategy was implemented after polymerase chain reaction (PCR) amplification of all exons and the intron–exon border of *KIND1*, followed by direct automated sequencing using an ABI Prism 3100 genetic analyzer (Advanced Biotechnologies, Columbia, MD). Oligonucleotide primers and PCR conditions used in this study are described elsewhere [7]. The genomic DNA nucleotides, the complementary DNA nucleotides, and the amino acids of the protein were numbered based on the following sequence information: GenBank accession no. NM_017671 [7].

2.2. Mutant-allele-specific amplification analysis

To verify the c.1761T>A mutation, using PCR products as a template, mutant-allele-specific amplification (MASA) analysis was performed with mutant-allele-specific primers carrying the substitution of two bases at the 3′-end mutant-allele-specific primers [16,17]: forward, 5′-ACATTCTGGGAGTTTCATGA-3′; reverse, 5′-CAATTCTGAGGGACACACAT-3′. Only the 179-bp fragment derived from the mutant allele was amplified with these primers.

2.3. Electron microscopy

Electron microscopy was performed as previously described [18,19]. Briefly, skin biopsy samples were fixed in 2% glutaraldehyde solution, post-fixed in 1% OsO_4, dehydrated, and embedded in Epon 812. The samples were sectioned at 1 μm thickness for light microscopy and thin sectioned for electron microscopy (70 nm thick). The thin sections were stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope.

2.4. Antibodies

The following antibodies (Abs) were used: monoclonal antibody (mAb) HD1-121 against the rod domain of plectin; mAbs GoH3 and 3E1 against α6 and β4 integrins, respectively (Chemicon International, CA); mAb GB3 against laminin 332 (Sera-lab, Cambridge, UK); mAb LH7.2 against type VII collagen (Sigma, St. Louis, MO); mAb PHM-12+CIV22 against type IV collagen (NeoMarkers, Fremont, CA); S1193 against BP230; mAb HDD20 against type XVII collagen; anti-kindlin-1 Ab (ab68041) that recognizes the C-terminus of kindlin-1 (Abcam, Cambridge, UK); unconjugated and horseradish peroxidase conjugated anti-V5 Abs (Invitrogen); and mAbs 4B7R and 12G10 against β1 integrin (Abcam, Cambridge, UK). The following secondary antibodies were used: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ab (Jackson Immuno Research, West Grove, PA); FITC-conjugated goat anti-mouse Ab (Jackson Immuno Research); and TIRTC-conjugated goat anti-mouse Ab (SouthernBiotech, Birmingham, AL); horseradish peroxidase-conjugated goat anti-mouse Ab (Jackson Immuno Research). mAb GoH3 was a kind gift from Dr. A. Sonnenberg of the Netherlands Cancer Institute. mAbs HD1-121 and HDD20 were kind gifts from Dr. K. Owaribe of Nagoya University. The antibody S1193 was a kind gift from Dr. J.R. Stanley of the University of Pennsylvania.

2.5. Skin immunofluorescence studies

Indirect immunofluorescence analysis using a series of antibodies against antigens at the dermo-epidermal junction (DEJ) and cryostat skin sections was performed as previously described [3,20].

2.6. Exon-trapping experiments

Exon-trapping (Invitrogen, Carlsband, CA) is an approach used for the direct isolation of mRNA sequences transcribed from gDNA. To generate a *KIND1* genomic fragment extending from intron 6 to intron 9, we synthesized two primers (5′-GAATTCCTGAGCTGAAGTTTGCTGCA-3′ and 5′-GGATCCACCTTTGAACCATGAACCTG-3′) which contained the respective restriction enzyme sites: EcoRI and BamHI. PCR were performed using the patient's gDNA as a template. The DNA fragment was digested with EcoRI and BamHI and subcloned into the multi-cloning site of a pSPL3 expression vector, which contained a portion of the HIV-1 tat gene, an intron, splice donor and acceptor sites, and some flanking exon sequences. Sequence analysis selected constructs with or without the splice site mutation c.1089del/c.1089+1del. The constructs were transfected into HaCaT cells using lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted from the cultured cells and RT-PCR was performed using the trapping vector-specific oligonucleotide primers. The samples without transfection of the SPL3 were used as controls. The PCR products were subcloned into a TA cloning vector pCRII (Invitrogen).

2.7. In vitro analysis of truncated KIND1

cDNA containing the entire coding region of *KIND1* (FEMT1wt) was subcloned into the pcDNA3.1V5-His vector (Invitrogen). *KIND1* cDNA without exon 8 was subcloned to generate the same vector minus exon 8 (KIND1delex8) using PCR methods and the following flanking *KIND1* cDNA primers: sense, 5′-GAGGACATTACTGATATCCC-3′, anti-sense, 5′-CTGTAGAGCTGCAAAGATCA-3′. Two different KIND1wt and KIND1delex8 transfections were performed into HeLa cells, using Lipofectamine LTX (Invitrogen). For immunoblotting, HeLa cells 24 h after transfection were lysed in Laemmli buffer [21], cell debris was removed by centrifugation, and supernatant was collected. SDS-PAGE and immunoblotting were performed using standard techniques. For immunofluorescence, HeLa cells at 24 h after transfection were washed with phosphate-buffered saline and fixed with methanol. All cells were observed using a confocal laser scanning microscope (Olympus Fluoview FV300).

The medical ethics committee of Hokkaido University approved all described studies. The study was conducted according to Declaration of Helsinki Principles. The patients gave their written informed consent.

3. Results

3.1. Case description

Patient 1 was a 40-year-old Japanese male. He was the first child of non-consanguineous, healthy parents. Generalized skin fragility had been seen since infancy and early childhood. Physical examination revealed extensive poikiloderma with reticular pigmentation involving the entire skin surface (Fig. 1a). Pseudo-ainhum was noted on the left middle, ring and small fingers (Fig. 1b). He developed cutaneous squamous cell carcinoma (SCC) on his right palm at the age of 27. A wide local excision was performed on the SCC, but the carcinoma recurred four times (Fig. 1c) and was treated with additional local excision and radiation therapy.

Ultrastructural observation of the skin specimen from Patient 1 revealed scattered lamina densa reduplication (Fig. 1d). The ultrastructural appearance of the basal lamina, including the anchoring fibrils and hemidesmosomes, was normal (Fig. 1d).

3.2. Recurrent c.1089del/1089+1del KIND1 mutation in Japanese KS patients

KIND1 mutation analysis revealed that Patient 1 was compound heterozygous for c.1761T>A (p.Tyr587X) (Fig. 2a) and c.1089del/1089+1del (Fig. 2b). Mutant-allele-specific amplification (MASA) analysis demonstrated that a 179-bp fragment derived from the mutant allele containing c.1761T>A was amplified from the patient's gDNA, but not from the DNA of normal controls (Fig. 2c). c.1089del/1089+1del was confirmed by TA-cloning (Fig. 2d). c.1761T>A (p.Tyr587X) was novel, and c.1089del/1089+1del had been described in two unrelated Japanese KS patients, who were homozygotes for that mutation [7].

3.3. Kindlin-1 expression in KS skin with c.1089del/1089+1del

Kindlin-1 labelling of Patient 1's skin showed linear staining at the DEJ with weak labelling at the cell periphery in the basal keratinocytes (Fig. 3a), which was not distinctly different from normal human skin (Fig. 3i). Skin specimens from another Japanese KS patient (Patient 2), who is homozygous for c.1089del/1089+1del, revealed the same kindlin-1 labelling pattern (Fig. 3e). Patient 2 was described in previous reports [3,7]. Briefly, she was a 38-year-old Japanese female with a history of photosensitivity, blister formation, fusion of the fingers, and esophageal and vaginal stenosis. Her clinical manifestation was extensive poikiloderma involving the entire skin surface [3].

Skin specimens from Patient 1 showed a thin linear expression pattern for all basement membrane proteins including α6 and β4 integrins, plectin, BP230, and type XVII collagen, using specific antibodies (data not shown). Laminin 332, type IV collagen and type VII collagen also revealed a thin linear labelling pattern (Fig. 3b–d). None of the immunohistochemical findings of interrupted or reduplicated dermo-epidermal junction that characterize typical KS were seen (Fig. 3b–d). On the other hand, laminin 332, type IV and VII collagen labelling of skin specimens from Patient 2 showed typical dermo-epidermal junction interruption, as previously described [3] (Fig. 3f–h).

3.4. In-frame skipping of exon 8 resulting from c.1089del/1089+1del

c.1089del/1089+1del was located at the splicing cryptic site. To analyse the transcripts resulting from c.1089del/1089+1del, a previously reported exon-trapping system was used [22,23]. We inserted the genomic fragments with or without c.1089del/1089+1del mutation into the pSPL3 vector, transfected these constructs into HaCaT cells and prepared total RNA from the cells. We then synthesized cDNA and amplified the extracted exons by PCR using vector-specific primers. PCR products were subcloned into TA-vector and sequenced. Sequence analysis revealed that all

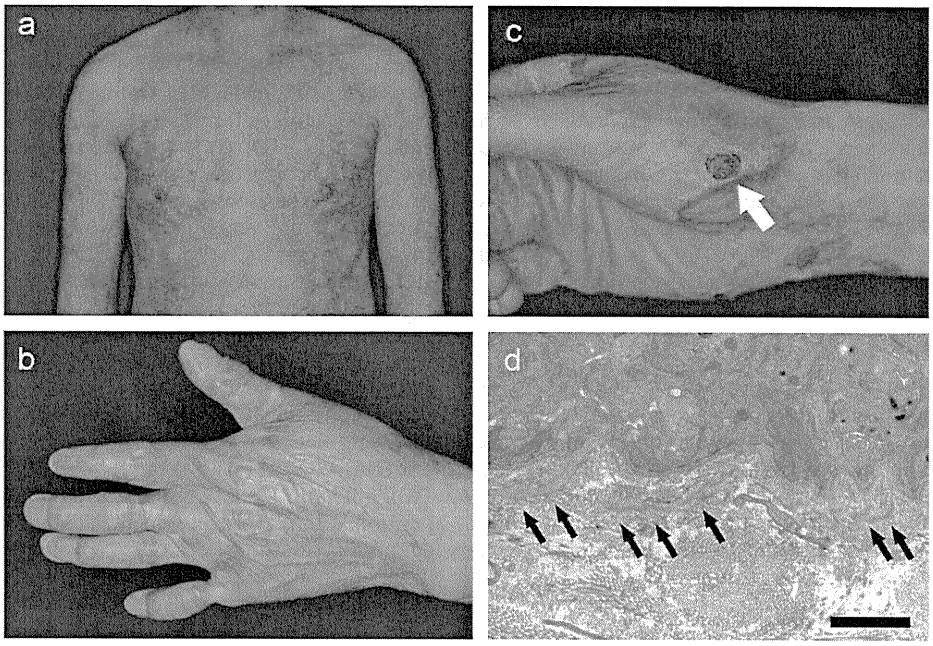

Fig. 1. Clinical and ultrastructural features of Patient 1. (a) Generalized poikiloderma is observed. (b) Pseudo-ainhum is seen from the left middle to little fingers. The middle and ring fingers are partly fused. (c) Recurrent squamous cell carcinoma (arrow) on the right palm at the age of 38. (d) Ultrastructural features of the skin specimens from Patient 1. Epidermal–dermal separation is not observed. Some reduplication of lamina densa is seen (arrows). No apparent abnormalities in hemidesmosomes and anchoring fibrils are detected (bar: 5 μm).

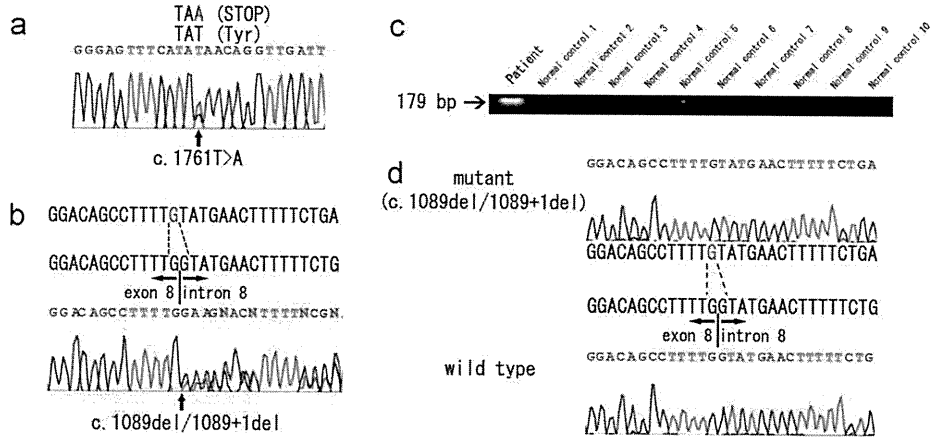

Fig. 2. *KIND1* mutation analysis of Patient 1. (a, b) Patient 1 is compound heterozygous for c.1761T>A (a) and c.1089del/1089+1del (b). An adenine that has replaced a thymine is indicated in red in the former mutation. A guanine deleted from two guanines at the exon 8–intron 8 boundary is indicated in red in the latter mutation. (c) Mutant-allele-specific amplification (MASA) analysis on c.1761T>A shows the amplification band from the mutant allele to be a 179-bp fragment from the patient's DNA sample, but no amplification was detected in normal controls. (d) TA-cloning of the PCR products derived from the patient's gDNA reveals wild-type (lower) and mutant (c.1089del/1089+1del, upper) subclones.

the clones without the mutation contained exons 7, 8 and 9 (wild-type transcript, Fig. 4a). The sequenced clones with the mutation showed six different transcripts (51 clones, transcripts 1–6, Fig. 4b–g). In most of the clones with the mutation (45 of 51 clones), skipping of exon 8 was observed (transcript 1, Fig. 4b). The size of exon 8 was 132 bp, so the deletion of exon 8 did not alter the coding frame and restored the translation of a polypeptide that was encoded by the downstream exons (Fig. 4b). In transcript 2 (1 of 51 clones), an exonic guanine deletion was seen at the 3′ end of exon 8, resulting in a frameshift followed by a premature termination codon (PTC) (Fig. 4c). A new splice donor site within intron 8, between nucleotides (nts) 1089 + 240 and 1089 + 241, was activated in transcript 3 (1 of 51 clones), also resulting in a frameshift and a subsequent PTC (Fig. 4d). In transcripts 4 and 5 (2 and 1 of 51 clones, respectively), two new splice donor sites within exon 8, between nts 1006 and 1007 and between nts 1066 and 1067, respectively, were activated, leading to a frameshift and a subsequent PTC (Fig. 4e and f). In transcript 6 (2 of 51 clones), the duplication of nts 1065–1066 (AG) compensated transcript 5, which did not alter the coding frame and restored the translation of a polypeptide that was encoded by the downstream exons (Fig. 4g).

3.5. Exon 8-skipped truncated kindlin-1 in vitro

To elucidate whether *KIND1* transcripts with in-frame deletion of exon 8 (transcript 1) express in living cells, we subcloned *KIND1* cDNA without exon 8 into pcDNA3.1V5-His vector (KIND1delex8).

Immunoblot analysis of lysates from HeLa cells transfected with KIND1delex8 showed a lower band than those of wild-type *KIND1*

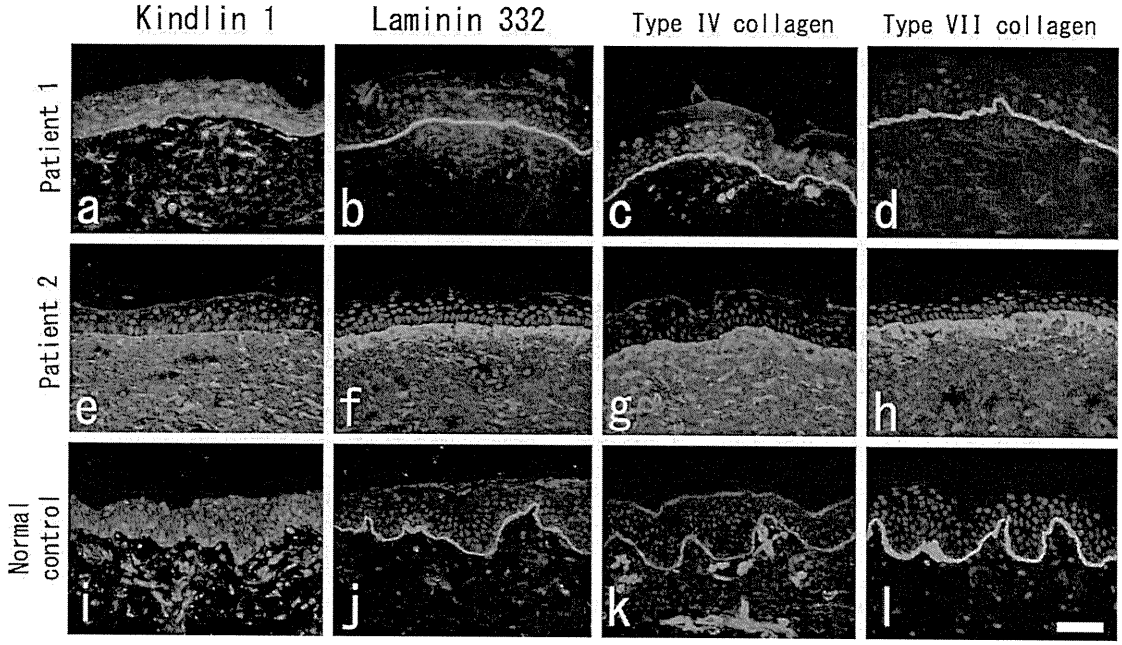

Fig. 3. Immunofluorescence analysis of skin specimens from KS with c.1089del/1089+1del. (a–d) Patient 1. (e–h) Patient 2 (a homozygote for c.1089del/1089+1del). (i–l) Normal control. Kindlin-1 labelling of skin specimens from Patients 1, 2 and the normal control show bright, linear staining at the DEJ as well as less intense labelling at the cell periphery in the basal keratinocytes (a, e, i). Thin, linear DEJ labelling with laminin 332, type IV collagen and type VII collagen is observed in skin samples from the present case (b–d) and normal control (j–l). Skin specimens from Patient 2 show interrupted or reduplicated DEJ (f–h) (bar: 50 μm).

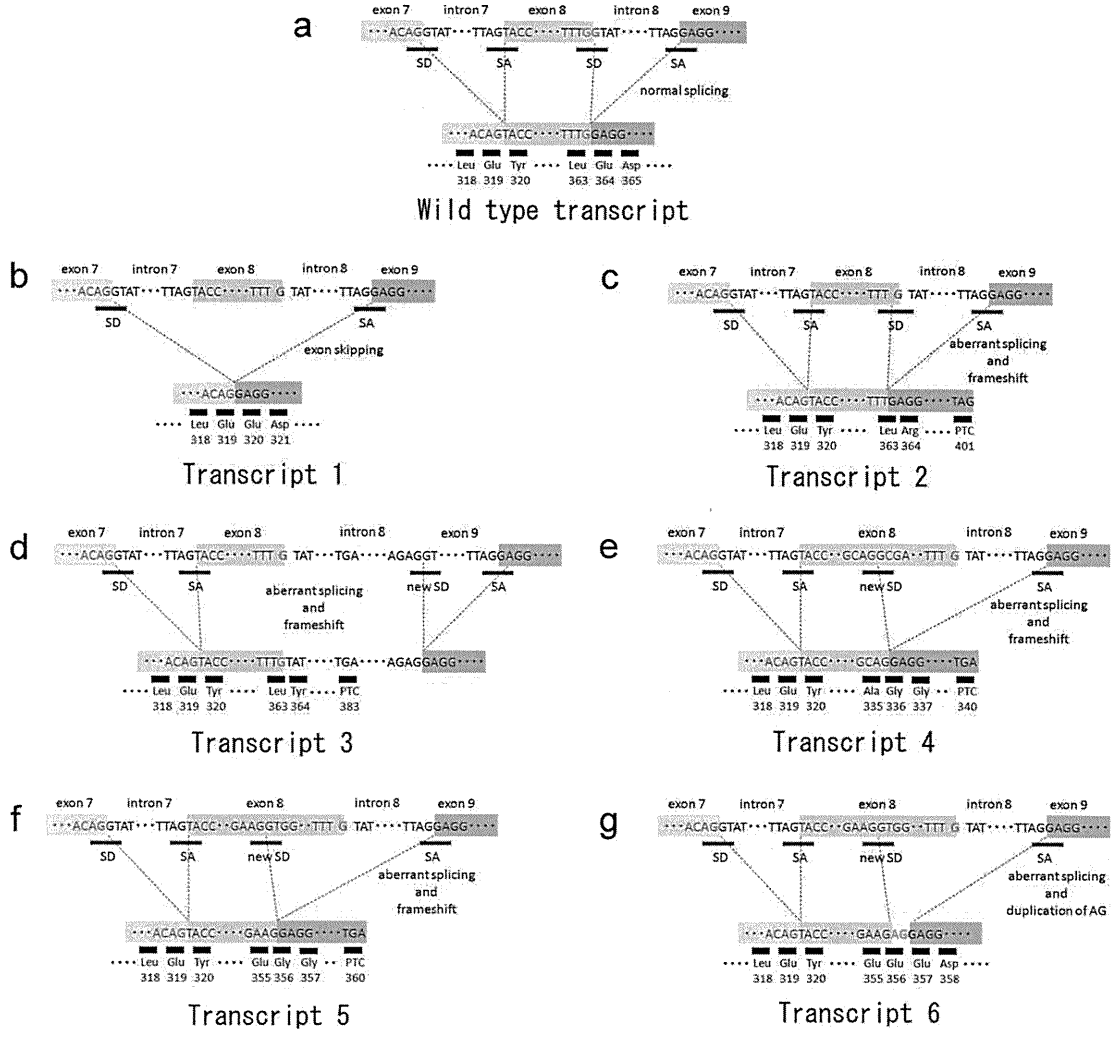

Fig. 4. Transcripts resulting from c.1089del/1089+1del. Wild-type (a) and mutant (b–g) sequences were subcloned in exon-trapping vector pSPL3 and transfected into HaCaT cells. Transcripts were derived from the pairing of vector and cloned splice signals. Boxes represent exons, blue underlines are splice sites (SD: splice donor site; SA: splice acceptor site) and black underlined regions are amino acids. Guanines at the exon 8/intron 8 boundary are in red. (a) The wild-type transcript contains exons 7, 8 and 9. (b) In transcript 1 (45 of 51 clones), exon 8 is skipped, and exon 7 is directly connected to exon 9. (c) In transcript 2 (1 of 51 clones), an exonic guanine is deleted at the 3′ end of exon 8, which results in frameshift and a premature termination codon (PTC). (d) In transcript 3 (1 of 51 clones), a new cryptic donor site within intron 8 is activated between nts 1089 + 240 and 1089 + 241. (e) Transcript 4 (2 of 51 clones) shows a new splice donor site within exon 8 between nts 1006 and 1007. (f) In transcript 5 (1 of 51 clones), another new splice donor site within exon 8 between 1066 and 1067 is activated. (g) Transcript 6 (2 of 51 clones) reveals a new splice donor site within exon 8 between 1066 and 1067 with duplication of nts 1065 and 1066 (AG).

cDNA (KIND1wt) (Fig. 5a). Total and activated β1 integrin was assessed as described previously [9]. Total surface β1 integrin detected by the mouse monoclonal anti-β1 integrin Ab (4B7R) was similar in the cells transfected with KIND1wt (Fig. 5b) and those transfected with KIND1delex8 (Fig. 5c). Localization of active β1 integrin labelling using the mouse monoclonal anti-β1 integrin Ab (clone 12G10) at focal adhesions was observed in the cells transfected with KIND1wt (Fig. 5d). However, active β1 integrin localized mostly at cytoplasms in the cells transfected with KIND1delex8 (Fig. 5e).

4. Discussion

Although the gene responsible for KS (*KIND1*) is now known, the complete KS pathomechanism has not been fully clarified. Our study revealed that the typical KS phenotype developed in KS patients with c.1089del/1089+1del possibly through defective β1 integrin activation even though kindlin-1 staining is positive as a result of the truncated protein.

KIND1 mutational analysis of Patient 1 identified one novel nonsense mutation c.1761T>A (p.Tyr587X). This nonsense mutation is thought to be targeted by nonsense-mediated mRNA decay (NMD). We also identified a recurrent mutation, c.1089del/1089+1del [7]. As two guanines exist at the exon/intron 8 boundary, it is impossible to determine whether the deleted guanine is from exon 8 or from intron 8 at the genomic DNA level. c.1089del, i.e. a guanine deletion from exon 8, predicts a frame-shifting change with leucine-97 as the first affected amino acid (p.Leu363fs) at the protein level [7]. Conversely, c.1089+1del, i.e. a guanine deletion from intron 8, should result in a splice donor site mutation [7]. The exon-trapping experiments in this study showed multiple transcripts produced by c.1089del/1089+1del. Exon 8 skipping produced a major transcript resulting from c.1089del/1089+1del (transcript 1, Fig. 4b).

Under immunofluorescence analysis, the skin specimens from Patient 1 and 2 tested positive for kindlin-1 (Fig. 3a and e). There are some reports describing KS patients with positive kindlin-1 staining [2,24]. From the data on transcripts from c.1089del/1089+1del, transcript 1 (in-frame deletion of exon 8, Fig. 4b) and

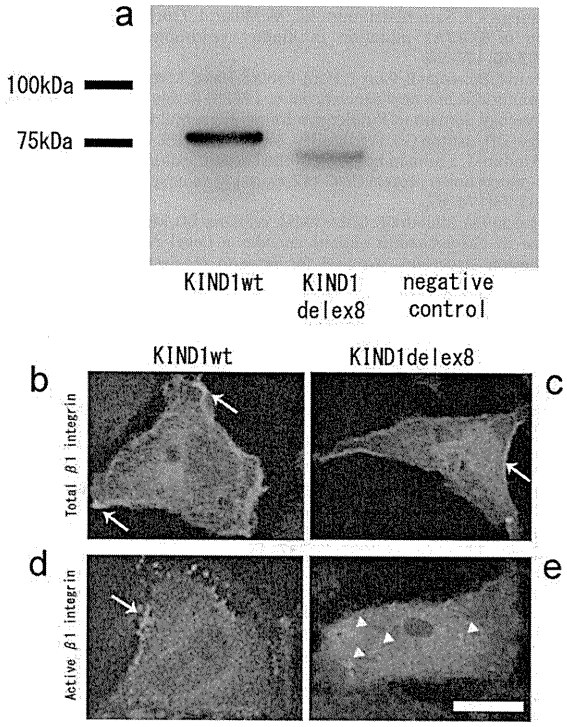

Fig. 5. Total and active β1 integrin expression in HeLa cells transfected with wild-type or mutated *KIND1* cDNA. (a) Immunoblot analysis of lysates from HeLa cells transfected with *KIND1* cDNA with (KIND1wt) or without exon 8 (KIND1delex8) confirms the expression of translated products (predicted sizes; 78 kDa and 74 kDa, respectively). pcDNA3.1V5-His vector without insert was used as negative control. (b, c) Total β1 integrin localization in HeLa cells transfected with KIND1wt (b) or KIND1delex8 (c). Only merged confocal microscopy images are shown. Total β1 integrin (red) localizes at focal adhesions (arrows) in both HeLa cells transfected with KIND1wt and those transfected with KIND1delex8. V5-tagged kindlin-1 is visualized with FITC-conjugated anti-V5 antibody. (d, e) Active β1 integrin labelling in HeLa cells transfected with KIND1wt (d) or KIND1delex8 (e). Active β1 integrin (red) localizes at focal adhesions (arrow) in the cells transfected with KIND1wt (d). In contrast, active β1 integrin is seen mostly in cytoplasms (arrowheads) in the cells transfected with KIND1delex8 (e) (bar: 5 μm).

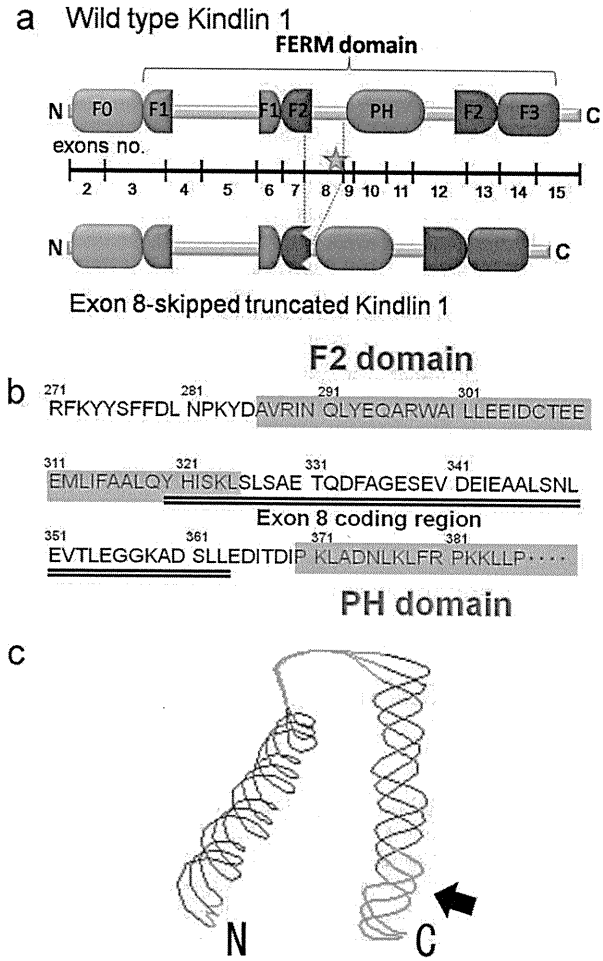

Fig. 6. Structural basis of exon-8-skipped truncated kindlin-1. (a) Schematic structure of wild-type and exon-8-skipped truncated kindlin-1. Kindlin-1 comprises the FERM domain, including F1, F2 and F3, flanked by the pleckstrin homology (PH) domain. A star indicates c.1089del/1089+1del at the exon 8/intron 8 boundary. (b) Exon 8 coding amino acids (black underline) are located in the F2 domain (red characters) and the unstructured area between the F2 and PH domains (blue characters). (c) Data on the 3D structure of the F2 domain of talin (PDB ID code: 2hrj) were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). 3D imaging of the F2 domain of talin (amino acids 209-247) corresponding to that of kindlin-1 (amino acids 286–325) was done using Swiss-PdbViewer (ver 4.01). Exon 8 coding amino acids (green) in the F2 domain are located in the α helix (arrow).

transcript 6 (a new splice donor site between nts 1066 and 1067 with duplication of nts 1065 and 1066, Fig. 4g) can be regarded as responsible for positive kindlin-1 staining in our cases, because, in Patient 1, another allele harbouring c.1761T>A is predicted to be targeted by NMD. There is a possibility that the anti-kindlin-1 Ab utilized in this study (ab68041) reacts with both kindlin-1 and kindlin-2 because of the similar amino acids sequences between two proteins. However, the staining pattern of kindlin-2 in epidermis is reported as pan-epidermal membranous labelling but with no staining along the lower pole of basal keratinocytes in contact with the basement membrane [24]. In contrast, the normal human skin samples stained with ab68041 showed a linear labelling pattern at DEJ, which might indicate that the antibody is mostly against kindlin-1. Also, the expression level of kindlin-2 in skin is described to be almost parallel with that of kindlin-1 [24]. From these facts, we believe that the staining pattern we observed in this study reflects the expression of kindlin-1.

Even though truncated kindlin-1 expression in patient's skin is predicted from *in vitro* assay using cultured cells transfected with mutated *KIND1* cDNA, most of the clinical features of the patients with c.1089del/1089+1del are common to those of typical KS patients: generalized poikiloderma, skin atrophy especially on the dorsal aspects of the hands, and development of SCC and pseudosyndactyly [2]. This might be explained by impaired activation of β1 integrin in the cells transfected with mutated *KIND1* cDNA.

The sequences of kindlin-1 are highly conserved and are very similar to those of the talin FERM domain, which is composed of F1, F2 and F3 [25] (Fig. 6a). F2 of kindlin-1 is interrupted by a large insert with a pleckstrin homology (PH) domain [26]. The exon 8 coding region is located in F2 and in an unstructured area between the F2 and PH domains (Fig. 6a). The 3D structure of the F2 domain indicates that the exon 8 coding amino acids in the F2 domain consist of the α helix (Fig. 6b and c). It is possible that exon-8-skipped truncated kindlin-1 leads to significant conformational change and loss of function and that truncated kindlin-1 expression resulting from c.1089del/1089+1del does not prevent KS development.

The immunohistochemical features of other DEJ molecules of Patient 1 were atypical. Previous studies revealed that antibodies against laminin-332, type IV collagen and type VII collagen shows a broad reticular staining pattern at the DEJ in typical KS patients (Fig. 3f–h) [3]. We were unable to observe such typical findings in the specimens of Patient 1, and only a thin linear labelling pattern was seen at the DEJ (Fig. 3b–d). A broad, reticular labelling pattern

at the DEJ is thought to correspond to marked reduplication of the lamina densa as a typical ultrastructural feature of KS patients [3]. Ultrastructural observation of Patient 1 also showed some lamina densa reduplication, but the extent was milder than that of typical KS (Fig. 1d). Limited reduplication of the lamina densa could explain the thin, linear labelling of laminin-332, type IV collagen and type VII collagen in Patient 1. However, the finding that skin specimens from Patient 2, who is homozygous for c.1089del/1089+1del, showed a broad reticular pattern at the DEJ (Fig. 3f–h) indicate that the presence of truncated kindlin-1 at the DEJ is not a cause of the linear thin immunofluorescence staining pattern of DEJ and the mild ultrastructural findings.

In summary, we have described a recurrent splice-site deletion mutation in *KIND1* in KS. The splice-site deletion produces multiple transcripts, most of which translatable into polypeptides that are encoded by the downstream exons. Although positive kindlin-1 staining in skin can therefore result from those transcripts, clinical manifestations of KS patients with c.1089del/1089+1del are as severe as those of KS with nonsense mutations because of defective integrin activation. This study, illumining the complicated pathomechanisms of KS, suggests that truncated kindlin-1 is functionally defective and cannot compensate for defects of KS.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Letter to the Editor

A founder effect of c.1938delC in *ITGB4* underlies junctional epidermolysis bullosa and its application for prenatal testing

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Abstract: Junctional epidermolysis bullosa associated with pyloric atresia (JEB-PA) is one of the most severe inherited skin diseases, characterized by generalized blister formation and occlusion of the pylorus at birth. Most JEB-PA patients have mutations in the gene encoding β4 integrin (*ITGB4*). No recurrent mutations in *ITGB4* have been described as having founder effects. We collected three JEB-PA families with c.1938delC in *ITGB4*. Haplotype analysis using single nucleotide polymorphism markers throughout *ITGB4* suggested one rare haplotype (2.8% of the Han Chinese and ethnic Japanese populations) in all alleles with c.1938delC. The parents of one of the three families sought prenatal diagnosis for a subsequent pregnancy. We succeeded in performing prenatal exclusion of JEB-PA using the foetal genomic DNA. Our study clearly demonstrated that recurrent c.1938delC in *ITGB4* is a founder mutation in JEB-PA patients, and that genotyping of the mutation can be utilized for prenatal diagnosis of JEB-PA.

Key words: basement membrane zone – haplotype analysis – single nucleotide polymorphism

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Background

Recurrent mutations in a population might be explained by founder effects, in which the mutant alleles of a few ancestors spread in the population because of limited gene pool, genetic drift and healthy carrier migration (1).

Epidermolysis bullosa (EB) comprises a group of disorders characterized by congenital skin fragility. EB has been classified into EB simplex, junctional epidermolysis bullosa (JEB), dystrophic EB and Kindler syndrome (2–4). JEB is subclassified into three clinical subtypes: Herlitz JEB, non-Herlitz JEB and JEB with pyloric atresia (JEB-PA). JEB-PA is characterized by generalized blistering and occlusion of the pylorus at birth, which usually leads to early demise (5). Mutations in the gene encoding α6 (*ITGA6*) or the β4 integrin subunit (*ITGB4*) are responsible for JEB-PA (6,7). Most patients with JEB-PA have mutations in *ITGB4* (8). No frequent prevalent mutations have been noted, except in the Hispanic population, where c.1802G>A (p.Cys601Tyr) is present on five of 10 alleles of JEB-PA patients (9).

Here, we have collected three JEB-PA families, in which c.1938delC in *ITGB4* is present. Haplotype analysis revealed c.1938delC as a founder mutation in JEB patients. Based on these data, we successfully performed prenatal exclusion of JEB-PA with this mutation.

Experimental design

Patients

Three unrelated non-consanguineous Japanese families (A, B and C) with JEB-PA in this study are summarized in Fig. S1a. Family A and B originate from Shikoku Island in Japan and family C is from other part of the country. A-1 and B-1 are newly identified JEB-PA patients. They died of disseminated intravascular coagulation 1 and 2 months after birth, respectively. Immunofluorescence study of skin specimens from both of the patients showed the absence of β4 integrin and weak expression of α6 integrin subunits (data not shown). Immunostaining for laminin 332, type IV collagen, type VII collagen, type XVII collagen, plectin and BP230 revealed normal linear labelling patterns (data not shown). C-2 is a patient with non-lethal variant of JEB-PA. The case description and mutational data of C-2 have been reported previously (10).

Mutation detection

Genomic DNA (gDNA) was extracted from blood cells of the probands and their parents. Mutation detection was performed after polymerase chain reaction (PCR) amplification of all exons and intron–exon borders of *ITGB4*, followed by direct sequencing using an ABI Prism 3100 genetic analyzer (Advanced Biotechnologies Inc., Columbia, MD, USA) (11–13). The genomic DNA nucleotides, the complementary DNA nucleotides and the amino

acids of the protein were numbered based on the following sequence information (GenBank accession No. NM_000213).

Haplotype analysis

To determine whether c.1938delC is a founder mutation, we performed haplotype analysis of three JEB-PA families. We constructed linkage disequilibrium (LD) blocks containing *ITGB4* using genotype data from the HapMap database (International HapMap Consortium, 2005). The haplotype structure with its tag-single nucleotide polymorphisms (SNPs) was determined using Haploview (14). We genotyped 15 tag-SNPs (Fig. S1b) using the ABI Prism 3100 genetic analyzer (Advanced Biotechnologies Inc.).

Prenatal diagnosis

We performed prenatal diagnosis of a foetus (A-2) at risk for JEB-PA from family A. A total of 30 ml of amniotic fluid was obtained under ultrasound guidance at 16 weeks' gestation. Foetal DNA was extracted from fresh cells from 10 ml of amniotic fluid. Genomic DNA isolated from amniotic fluid cells was subjected to polymerase chain reaction (PCR) amplification, followed by direct automated sequencing as described. The mutation site was sequenced using both forward and reverse strands and verified by *PmlI* (New England Biolabs Inc., Beverly, MA, USA) enzyme digestion of the PCR products.

The medical ethical committee of Hokkaido University and National Center for Child Health and Development approved all described studies. The study was conducted according to Declaration of Helsinki Principles. Participants gave their written informed consent.

Results

Recurrent c.1938delC in *ITGB4*

ITGB4 mutation analysis revealed that A-1 was homozygous for c.1938delC (Fig. 1c). The father and mother of A-1 were heterozygous for c.1938delC (Fig. 1a, b). B-1 was heterozygous for paternal c.1938delC and maternal c.4050_4057del (data not shown). c.1938delC was previously described in a patient with non-lethal variant of JEB-PA who is compound heterozygous for c.1938delC and c.2168C>G (p.Pro723Arg) (C-2) (10). c.4050_4057del was also reported in a JEB-PA patient who is compound heterozygous for c.4050_4057del and c.3434delT (12).

Founder effects of c.1938delC

The haplotype structure containing *ITGB4* was constructed using genotype data from the HapMap database (Fig. S1b, c). The haplotype block was represented by 16 haplotypes with >2% frequency (Fig. S1b, c). The chromosome containing c.1938delC in A-1 and B-1 had haplotype XI (GGGACGGGCGTCACC), which is seen in 2.8% of the Han Chinese and ethnic Japanese populations. The chromosome containing c.1938delC in C-2 might have had this haplotype although the phase was not determined.

Prenatal exclusion of JEB-PA

Direct sequencing of PCR products from the foetal gDNA (A-2) revealed the presence of c.1938delC in one allele and wild-type sequence in another allele (Fig. 1d). To confirm the results of direct sequencing, we performed restriction enzyme analysis. c.1938delC was found to result in the loss of a restriction enzyme for *PmlI*. The PCR product from the proband (A-1) after *PmlI* digestion revealed a 285-bp band, which indicated that she was homozygous for c.1938delC (Fig. 1f). In contrast, the PCR product from the parents and the foetus (A-2) after *PmlI* digestion showed 285-, 195- and 90-bp bands, which indicated that they were heterozygous for c.1938delC (Fig. 1f). Haplotype analysis of this family using microsatellite markers excluded maternal contamination of foetal cells (data not shown). These results predicted that the foetus would not be affected, and the pregnancy was continued. A neonate was born at full term in good health with completely normal skin.

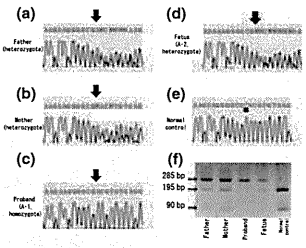

Figure 1. Prenatal diagnosis of junctional epidermolysis bullosa with pyloric atresia (family A). (a–e) Direct sequencing of *ITGB4*. The parents were heterozygous for c.1938delC in *ITGB4* (a, b). A-1, the proband, was homozygous for that mutation (c). A-2, the foetus, was found to be a heterozygous carrier (d). A cytosine at cDNA position 1938 in normal control is underlined (e). Arrows indicate a deleted cytosine in *ITGB4* sequence. (f) *PmlI* restriction enzyme digestion of the PCR products from the family members' genomic DNA. c.1938delC results in the loss of a site for *PmlI*. *PmlI* restriction enzyme digestion of the PCR products from normal control reveals 195- and 90-bp bands. Only a 285-bp band is observed in A-1 (the proband), who is homozygous for c.1938delC. In contrast, 285-, 195- and 90-bp bands are detected in the father, mother and A-2, suggesting that they are heterozygous for c.1938delC.

Conclusions

There are no recurrent *ITGB4* mutations that have been demonstrated to have founder effects in JEB-PA patients. Our study detected recurrent c.1938delC in *ITGB4* and revealed this to be a founder mutation in JEB-PA patients.

DNA-based prenatal testing of JEB-PA has been described (15–18). Our study has demonstrated the successful prenatal exclusion of JEB-PA with c.1938delC through mutation analysis of the foetal genomic DNA.

In summary, our study identified a founder c.1938delC in *ITGB4* and showed that this mutation can be applied for prenatal diagnosis of JEB-PA.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Haplotype analysis of the junctional epidermolysis bullosa families.

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Letter to the Editor

IL-1 signalling is dispensable for protective immunity in *Leishmania*-resistant mice

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Abstract: Leishmaniasis is a parasitic disease affecting ~12 million people. Control of infection (e.g. in C57BL/6 mice) results from IL-12-dependent production of IFNγ by Th1/Tc1 cells. In contrast, BALB/c mice succumb to infection because of preferential Th2-type cytokine induction. Infected dendritic cells (DC) represent important sources of IL-12. Genetically determined differences in DC IL-1α/β production contribute to disease outcome. Whereas the course of disease was not dramatically altered in IL-1RI$^{-/-}$ mice, local administration of IL-1α to infected C57BL/6 mice improved disease outcome. To definitively elucidate the involvement of IL-1 in immunity against leishmaniasis, we now utilized IL-1α/β-double-deficient C57BL/6 mice. C57BL/6 mice are believed to be a good surrogate model for human, self limited cutaneous leishmaniasis (CL). *Leishmania major*-infected IL-1α/β$^{-/-}$ mice were resistant to experimental CL comparable to controls. In addition, DC-based vaccination against leishmaniasis in C57BL/6 mice was independent of IL-1. Thus, in *Leishmania*-resistant C57BL/6 mice, IL-1 signalling is dispensable for protection.

Key words: IL-1 – dendritic cells – *L. major*

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Background

Leishmaniasis is a parasitic disease transmitted by the bite of a sand fly. The disease ranges from cutaneous leishmaniasis (CL) to visceral leishmaniasis and ~12 million people are affected worldwide (1). In murine experimental leishmaniasis, control of infection results from IL-12-dependent production of Th1/Tc1-derived IFNγ that activates infected macrophages (MΦ) to eliminate parasites (2–5). In disease-resistant C57BL/6 mice, skin DC infected with *Leishmania major* represent important sources of IL-12 (6). In contrast, BALB/c mice respond to infection with preferential Th2-type cytokine production, which is associated with disease progression. Genetically determined DC-derived factors that influence disease susceptibility of BALB/c mice include elevated levels of inhibitory IL-12p80 (7) and decreased release of IL-1α/β (8,9). Previously, we demonstrated that IL-1α/β facilitates Th1 induction in several inflammatory disease models (9–11). Treatment of BALB/c mice with IL-1 during T cell priming inhibited progressive disease by shifting the immune response towards Th1 (9). However, prolonged administration of IL-1α promoted Th2 expansion in already established infections and worsened disease outcome (11).

Abbreviations: CL, cutaneous leishmaniasis; DC, dendritic cells; MΦ, macrophages.

Question addressed

IL-1 is a key mediator of inflammation (12,13). IL-1α and IL-1β exert similar biological functions by binding to the IL-1 type I receptor (IL-1RI) (14). To definitively elucidate the involvement of IL-1 in immune responses in CL, we utilized IL-1α/β-double

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DNA-based prenatal diagnosis of plectin-deficient epidermolysis bullosa simplex associated with pyloric atresia

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Abstract

Background Mutations in the plectin gene (*PLEC*) generally lead to epidermolysis bullosa simplex (EBS) associated with muscular dystrophy. It has been recently demonstrated that *PLEC* mutations can also cause a different clinical subtype, EBS associated with pyloric atresia (EBS-PA), which shows early lethality. Prenatal diagnosis (PND) of EBS-PA using mutation screening of *PLEC* has not been described.

Objective This study aimed to perform DNA-based PND for an EBS-PA family.

Materials and methods The EBS-PA proband was compound-heterozygous for a paternal c.1350G>A splice-site mutation and a maternal p.Q305X nonsense mutation. Genomic DNA was obtained from amniocytes taken from an at-risk fetus of the proband's family. Direct sequencing and restriction enzyme digestion of polymerase chain reaction products from the genomic DNA were performed.

Results Mutational analysis showed that the fetus harbored both pathogenic mutations, suggesting that the fetus was a compound-heterozygote and therefore affected with EBS-PA. The skin sample obtained by autopsy from the abortus confirmed the absence of plectin expression at the dermal–epidermal junction.

Conclusions This is the first successful DNA-based PND for an EBA-PA family.

Introduction

Epidermolysis bullosa (EB) comprises a group of diseases that are classified into four categories – EB simplex (EBS), junctional EB (JEB), dystrophic EB, and Kindler syndrome – depending on the depth of the dermal-epidermal junction split.[1] The four categories are subcategorized into minor subtypes, some of which show severe prognosis and lead to early demise.

Prenatal diagnosis (PND) of lethal EB subtypes has been performed for more than two decades. Electron microscopy and immunofluorescence analysis of fetal skin samples were the mainstay for PND of EB fetuses.[2] However, morphologically based PND had technical difficulties and abortion risks from the fetal skin biopsies. As the genes responsible for EB have been indentified, DNA-based PND has been available for many lethal EB subtypes.[2,3] Recently, other techniques such as immunofluorescence analysis of villous trophoblasts,[4] preimplantation genetic analysis[5], and preimplantation genetic haplotyping[6] have been described as useful for PND of EB.

Among the lethal EB subtypes, EB associated with pyloric atresia (EB-PA) has been known to result from mutations in the genes encoding either plectin (*PLEC*), or α6 (*ITGA6*), or β4 integrin (*ITGB4*).[1] EB-PA can either manifest as JEB with PA (JEB-PA) or EBS with PA (EBS-PA) and is categorized as hemidesmosomal variant of EB. EB-PA due to *ITGA6* or *ITGB4* mutations is generally characterized by blister formation at the level of the lamina lucida as JEB-PA, although skin separation within basal keratinocytes has been described in a few cases.[1] In contrast, it has been recently reported that another subset of lethal EB-PA shows an intraepidermal level of cleavage consistent with EBS, caused by mutations in the gene encoding plectin (*PLEC*).[7–9] To date, PND of EBS-PA using mutation screening of *PLEC* has not been reported in the literature. This paper describes the first DNA-based PND for an EBS-PA family.