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

Journal of Investigative Dermatology (2010) **130,** 2834–2836; doi:10.1038/jid.2010.218; published online 5 August 2010

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.

ACKNOWLEDGMENTS

We thank the patients and their families for their participation. We also thank Kaori Sakai for fine technical assistance and Dr James McMillan for proofreading and comments concerning this paper. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M Akiyama (Kiban B 20390304) and by the Health and Labour Sciences Research Grant (Research on Allergic Diseases and Immunology; H21-Meneki-Ippan-003) to H Shimizu. Filaggrin research in the McLean laboratory was supported by grants from The British Skin Foundation; The National Eczema Society; The Medical Research Council (Reference number G0700314); A*STAR, Singapore, and donations from anonymous families affected by eczema in the Tayside region of Scotland.

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See related commentary on pg 2703

RNase 7 Protects Healthy Skin from *Staphylococcus aureus* Colonization

Journal of Investigative Dermatology (2010) **130,** 2836–2838; doi:10.1038/jid.2010.217; published online 29 July 2010

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

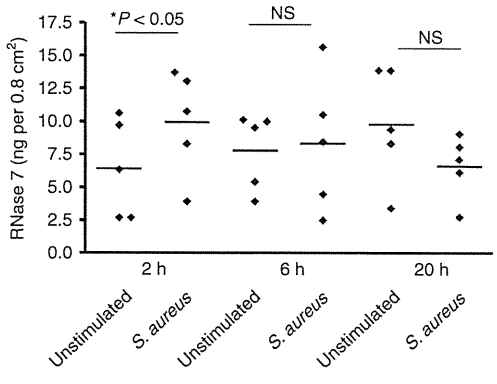

Figure 1. Induced secretion of RNase 7 on the skin surface on treatment with living *S. aureus*. Defined areas (0.8 cm^2) of skin explants derived from plastic surgery were incubated with or without approximately 1,000 colony-forming units of *S. aureus* (ATCC 6538) in 100 µl of sodium phosphate buffer. 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.

Journal of Dermatological Science 61 (2011) 38–44

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds

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

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ARTICLE INFO

Article history:
Received 22 July 2010
Received in revised form 8 November 2010
Accepted 13 November 2010

Keywords:
Epidermolysis bullosa
Exon-trapping system
Basement membrane zone
Skin atrophy
Pseudo-ainhum

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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0923-1811/$36.00
doi:10.1016/j.jdermsci.2010.11.008

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

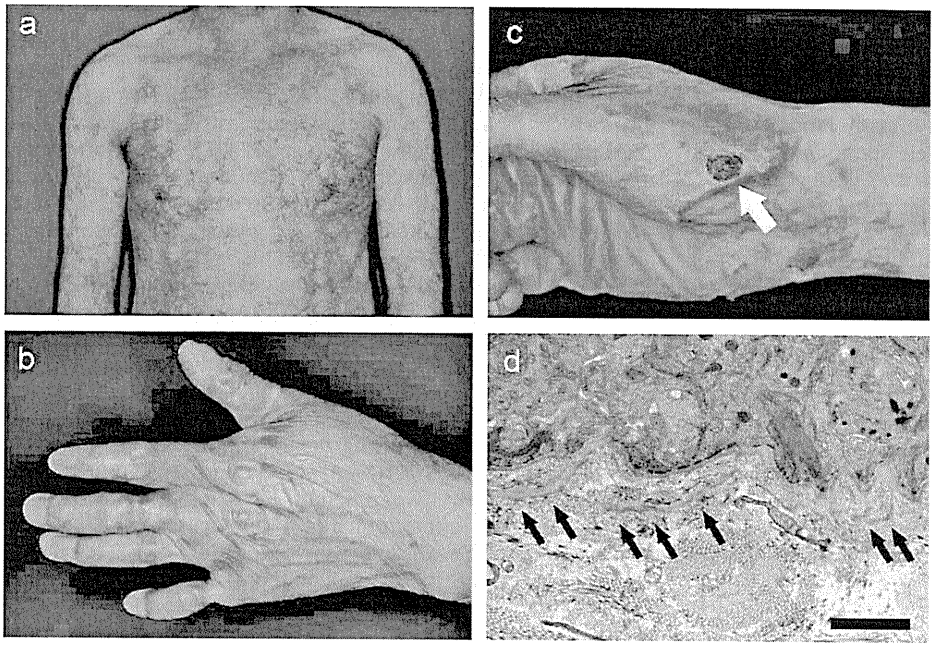

Fig. 1. Clinical and ultrastructural features of Patient 1. (a) Generalized poikiloderma is observed. (b) Pseudo-ainhum is seen from the left middle to little fingers. The middle and ring fingers are partly fused. (c) Recurrent squamous cell carcinoma (arrow) on the right palm at the age of 38. (d) Ultrastructural 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).

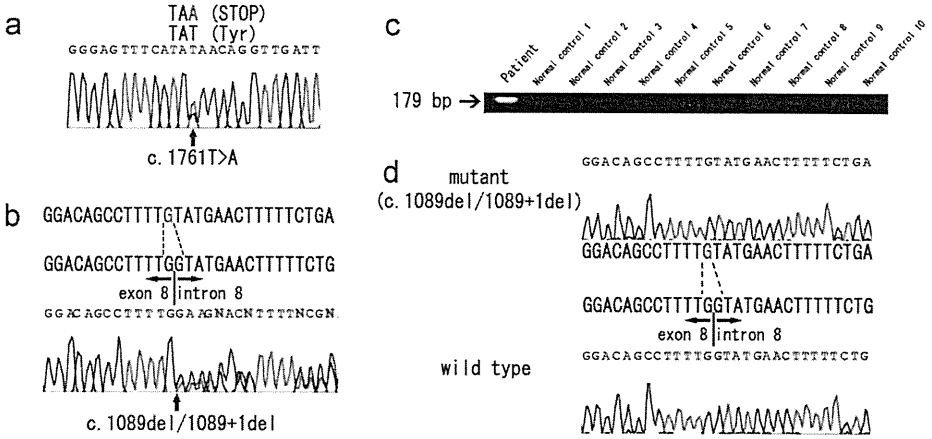

Fig. 2. *KIND1* mutation analysis of Patient 1. (a, b) Patient 1 is compound heterozygous for c.1761T>A (a) and c.1089del/1089+1del (b). An adenine that has replaced a thymine is indicated in red in the former mutation. A guanine deleted from two guanines at the exon 8–intron 8 boundary is indicated 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*

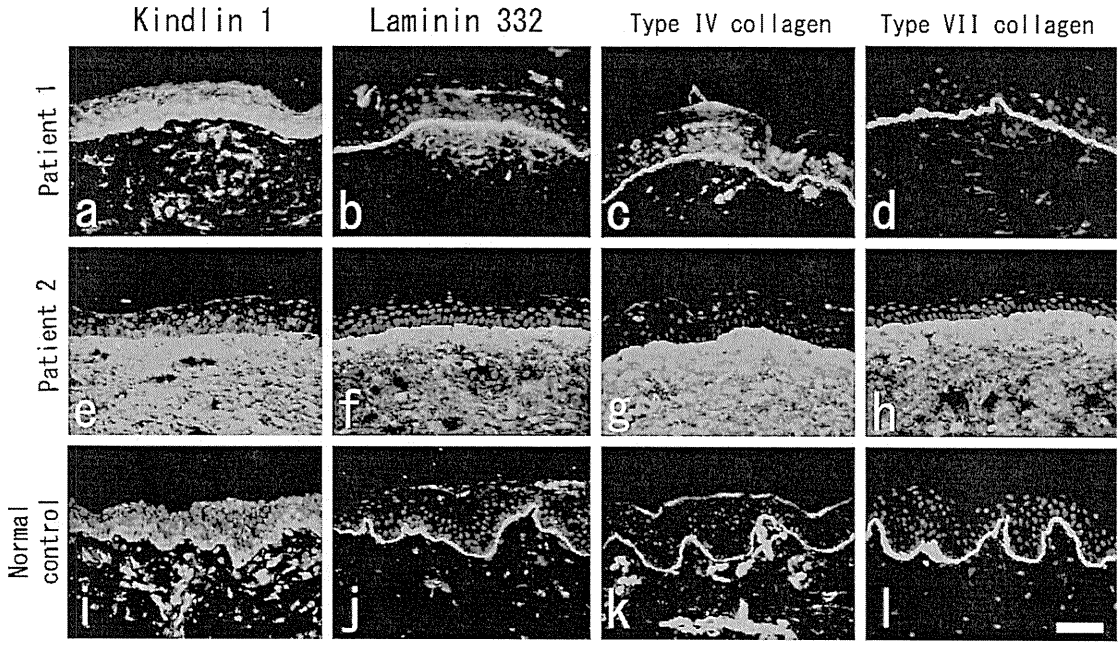

Fig. 3. Immunofluorescence analysis of skin specimens from KS with c.1089del/1089+1del. (a–d) Patient 1. (e–h) Patient 2 (a homozygote for c.1089del/1089+1del). (i–l) Normal control. Kindlin-1 labelling of skin specimens from Patients 1, 2 and the normal control show bright, linear staining at the DEJ 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).

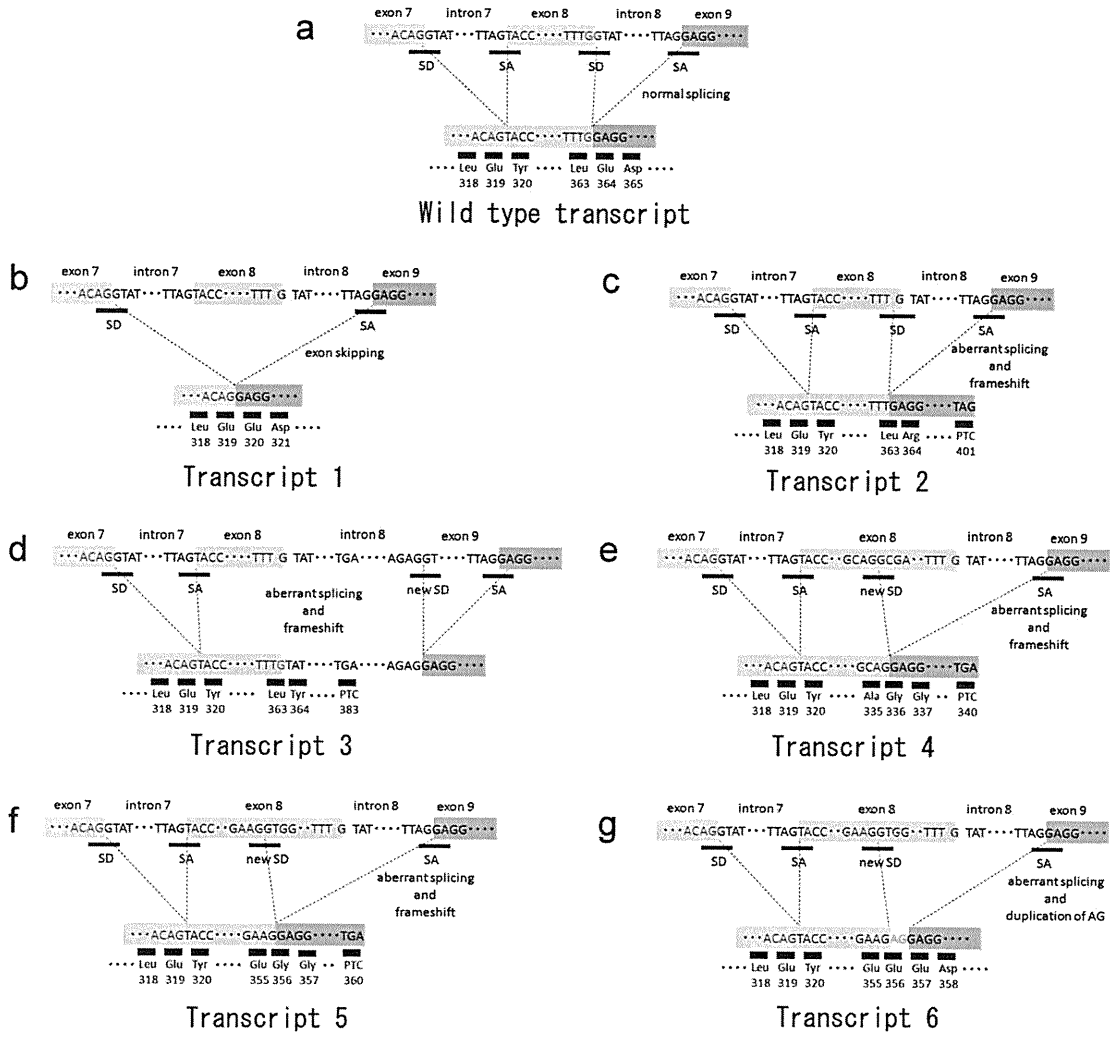

Fig. 4. Transcripts resulting from c.1089del/1089+1del. Wild-type (a) and mutant (b–g) sequences were subcloned in exon-trapping vector pSPL3 and transfected into HaCaT cells. Transcripts were derived from the pairing of vector and cloned splice signals. Boxes represent exons, blue underlines are splice 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

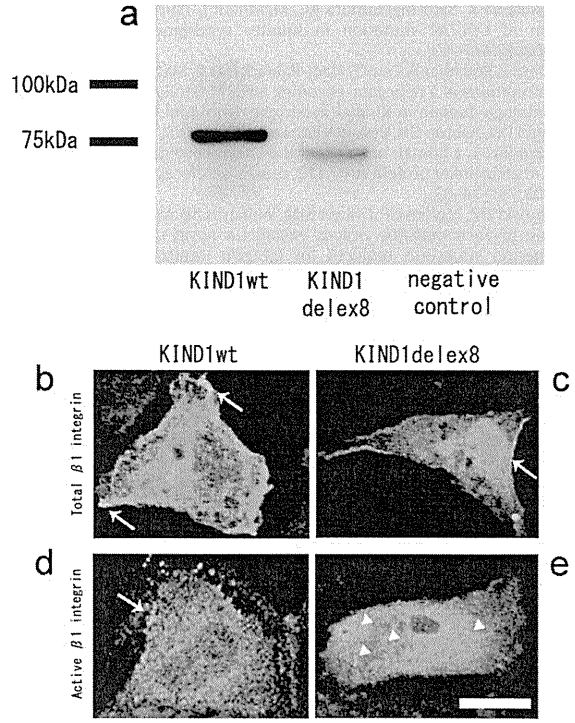

Fig. 5. Total and active β1 integrin expression in HeLa cells transfected with wild-type or mutated *KIND1* cDNA. (a) Immunoblot analysis of lysates from HeLa cells transfected with *KIND1* cDNA with (KIND1wt) or without exon 8 (KIND1delex8) confirms the expression of translated products (predicted sizes; 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).

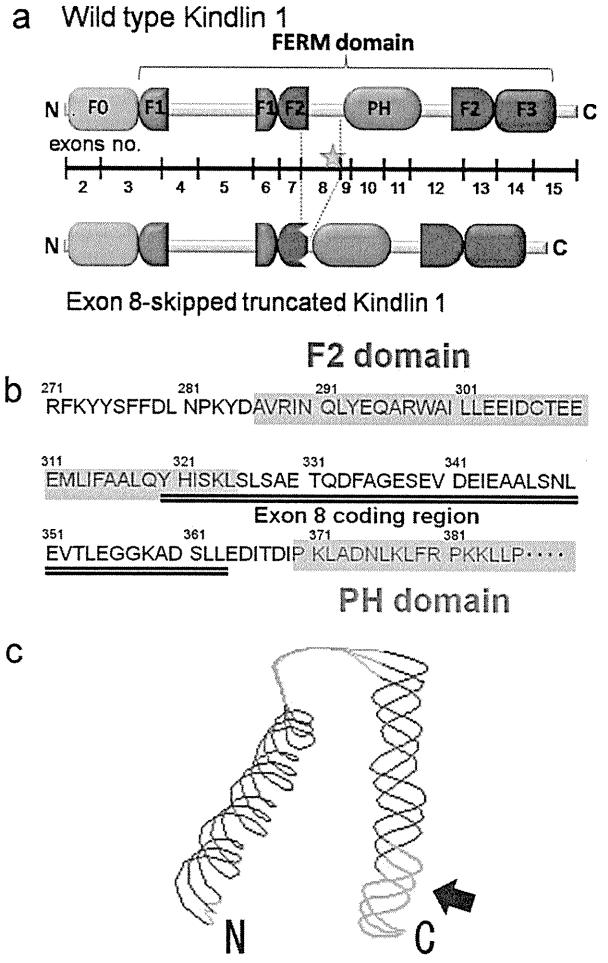

Fig. 6. Structural basis of exon-8-skipped truncated kindlin-1. (a) Schematic structure of wild-type and exon-8-skipped truncated kindlin-1. Kindlin-1 comprises the FERM domain, including F1, F2 and F3, flanked by the pleckstrin homology (PH) domain. A star indicates c.1089del/1089+1del at the exon 8/intron 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

We thank Prof John McGrath for his critical comments on the present case (Patient 1), Dr James R. McMillan and Mr Michael O'Connell for their proofreading, and Ms Yuko Hayakawa for her technical assistance. This work was supported by Health and Labour Sciences Research grants for Research on Measures for Intractable Diseases, from the Ministry of Health, Labour, and Welfare of Japan (to H.S.).

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DOI:10.1111/j.1600-0625.2010.01177.x
www.blackwellpublishing.com/EXD

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

Accepted for publication 20 July 2010

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.

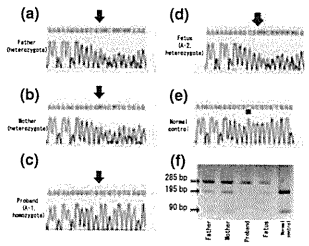

Figure 1. Prenatal diagnosis of junctional epidermolysis bullosa with pyloric atresia (family A). (a–e) Direct sequencing of *ITGB4*. The parents were heterozygous for c.1938delC in *ITGB4* (a, b). A-1, the proband, was homozygous for that mutation (c). A-2, the foetus, was found to be a heterozygous 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.

Acknowledgements

We thank Ms Yuko Hayakawa and Ms Yuki Miyamura for their technical assistance. This work was supported by Health and Labor Sciences Research grants for Research on Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare of Japan (to H.S.).

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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DOI:10.1111/j.1600-0625.2010.01172.x
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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*

Accepted for publication 26 July 2010

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

International Journal of
Dermatology

Medical genetics

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

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Conflicts of interest: The authors declare no conflicts of interest.

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.

Materials and Methods

The EBS-PA family

We previously reported this family with EBS-PA, in which the first and second newborns exhibited the clinical features of blistering and PA and died shortly after birth.[7] We then identified the precise genetic abnormality in the family through immunohistochemical analysis and genetic screening using the candidate gene approach. *PLEC* mutation analysis of genomic DNA from the parents and the proband demonstrated a paternal c.1350G>A splice-site mutation and a maternal p.Q305X nonsense mutation.[7] c.1350G>A was originally described as c.1344G>A and corrected according to the latest sequence information (GeneBank Accession No. NM_000445), plectin isoform 1c.[10] The parents were found to be heterozygous carriers, and the proband was compound-heterozygous (Fig. 1). The parents sought PND for a subsequent pregnancy.

PND

Amniocentesis was performed at 16 weeks gestation. Genomic DNA isolated from one-week-cultured amniocytes maintained in Amniomax medium (Invitrogen, Carlsbad, CA, USA) was subjected to polymerase chain reaction (PCR) amplification, followed by direct automated sequencing using an ABI Prism 3100 genetic analyser (Advanced Biotechnologies, Foster City, CA, USA). PCR amplification of the *PLEC* gene exons 9 and 12 was performed using the following primers. Primers 5′-GTCGCT GTATGACGCCATGC-3′ and 5′-TGGCTGGTAGCTCCATC TCC-3′ were used for amplification of exon 9, producing a 387-bp fragment. Primers 5′-CCCACTCGCCTTAGGACAGT-3′ and 5′-AAACCAACTCTGCCCAAAGC-3′ were used for amplification of exon 12, synthesizing a 428-bp fragment. PCR conditions were five minutes at 94 °C for one cycle, followed by 38 cycles of 45 seconds at 94 °C, 30 seconds at 57 °C or 60 °C, and one minute at 72 °C. The genomic DNA nucleotides, the cDNA nucleotides, and the amino acids of the protein were numbered based on the latest sequence information (GeneBank Accession No. NM_000445).

Written informed consent was obtained from the parents. PND was approved by the Institutional Ethical Committee of Hokkaido University Graduate School of Medicine. This study was conducted according to the Declaration of Helsinki Principles.

Immunofluorescence analysis

Immunofluorescence analysis using a series of antibodies against basement-membrane-associated molecules on cryostat skin sections was performed as previously described.[11] Skin biopsy was performed for the aborted fetus and a healthy volunteer as the normal control. The following monoclonal antibodies (mAbs) were used: mAb HD1–121 (a gift from Dr K. Owaribe of Nagoya University) against plectin; mAb GoH3 (a gift from Dr A. Sonnenberg of the Netherlands Cancer Institute) against α6 integrin; and mAb 3E1 (Chemicon, CA, USA) against β4 integrin.

Results

Mutation analysis of genomic DNA from amniocytes showed both paternal c.1350G>A splice-site mutation and maternal p.Q305X nonsense mutation (Fig. 2a). These mutation data were briefly mentioned in our recent paper on plectin expression patterns in patients with EBS.[12] Each mutation was confirmed by restriction enzyme digestion of PCR products. The c.1350G>A and p.Q305X mutations resulted in the loss of a restriction site for *Hph* I and *Pst* I, respectively (Fig. 2b). The prenatal molecular genetic diagnosis suggested that the fetus

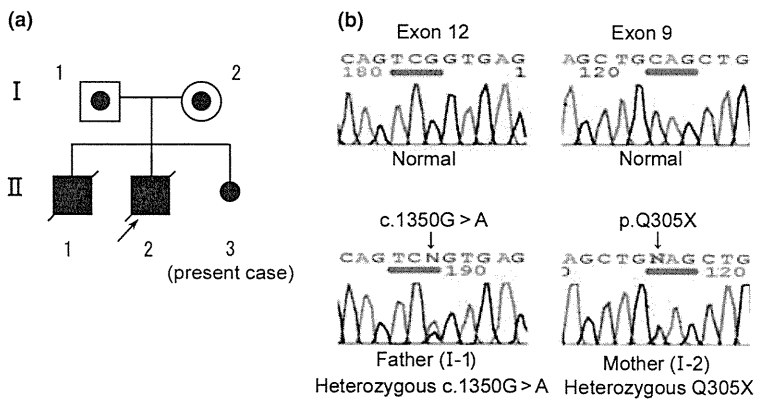

Figure 1 Family tree of the present case and the causative *PLEC* mutations. (a) The first and second newborns exhibited clinical features typical of EBS-PA and died shortly after birth. The proband (the second newborn) is indicated by an arrow. (b) The paternal splice-site mutation was a c.1350G>A transition at the end of exon 12. The maternal nonsense mutation was a c.913C>T transition in exon 9, leading to the substitution of glutamine 305 with a nonsense codon (p.Q305X)

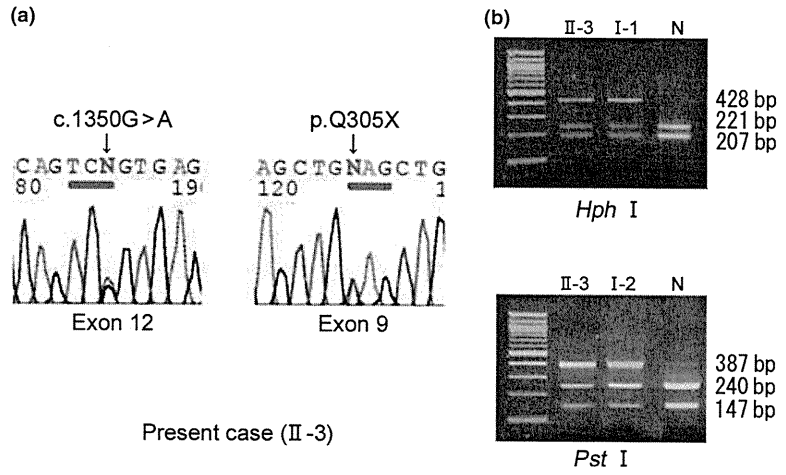

Figure 2 Analysis of the plectin gene mutations in genomic DNA from amniocytes of a fetus at risk. (a) Mutation analysis of genomic DNA from amniocytes shows both the c.1350G>A mutation in exon 12 and p.Q305X mutations in exon 9. (b) The presence of the mutations was verified by restriction enzyme digestion. The paternal mutation abolished a recognition site for the *Hph*I restriction enzyme. In the case of the normal allele, the 428-bp fragment was digested to 221 bp and 207 bp (lane N), whereas in the case of the mutant allele, a 428-bp fragment resisted digestion in the PCR product (father: lane I-1; present fetus: lane II-3). The maternal mutation also abolished a recognition site for the *Pst*I restriction enzyme. In the case of the normal allele, the 387-bp fragment was digested to 240 bp and 147 bp (lane N), whereas in the case of the mutant allele, a 387-bp fragment resisted digestion in the PCR product (mother: lane I-2; present fetus: lane II-3)

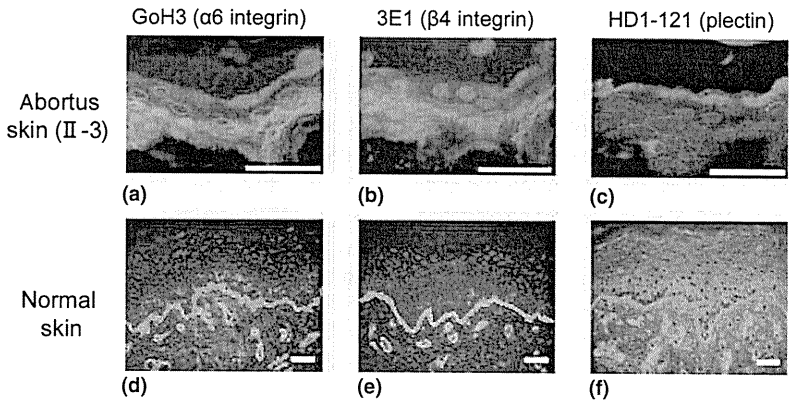

Figure 3 Absence of plectin expression in the abortus. α6 integrin (mAb GoH3) and β4 integrin (mAb 3E1) are expressed in the abortus skin (a, b) and the control skin (d, e). Staining with monoclonal antibody for plectin (mAb HD1–121) shows positive in the control skin (f) but negative in the skin of the abortus (c: blue frame). Note that the skin tissue from the abortus was subject to degeneration before skin sampling. Thus, protein localization cannot be evaluated in the degenerated tissue. Scale bar: 50 μm

was a compound-heterozygote and affected by JEB-PA. The parents elected for the fetus to be terminated at 20 weeks gestation.

Immunofluorescence analysis showed that immunoreactivity using the mAbs HD1–121 (plectin), GoH3 (α6 integrin), and 3E1 (β4 integrin) was positive in the normal control skin (Fig. 3d–f). The skin sample obtained from the abortus tested positive for α6 integrin and β4 integrin (Fig. 3a,b) but negative for plectin (Fig. 3c).

Discussion

This is the first successful PND of plectin-deficient EBS-PA, and the correct diagnosis was reconfirmed in the skin of the abortus. Given the universal mortality of EBS-PA due to *PLEC* mutations, there might be unreported PND cases for this form of EB. The prognosis of plectin-deficient EBS-PA is poor, and most patients commonly die within the first year of life,[13] as happened in the first- and

second-born progeny in the present family. Fetuses at risk of this condition are frequently terminated during pregnancy, and DNA-based PND plays an important role in prohibiting unnecessary termination of healthy fetuses at risk. Due to the recent elucidation of the causative genetic defects for genetic skin disorders, it has become possible to make DNA-based PND for severe genodermatoses by sampling of the chorionic villus or amniotic fluid in the earlier stages of pregnancy with a lower risk to fetal health and with a reduced burden on the mothers.

Plectin, a component of the hemidesmosome inner plaque, is involved in the attachment and crosslinking of the cytoskeleton and intermediate filaments to specific membrane complexes.[10] It has been described that EBS associated with muscular dystrophy (EBS-MD) results from *PLEC* mutations.[14,15] Mutations in the rod domain of *PLEC* are known to cause EBS-MD.[9,14,15] In addition, recent reports have confirmed that some *PLEC* mutations also lead to EBS-PA.[7–9,13] One alternative splice *PLEC* mRNA transcript that lacks exon 31 encoding the central core rod domain was identified in rat tissues.[16] By plectin-domain-specific reverse transcriptase-PCR, expression of this rodless alternative spliced form was confirmed in human keratinocytes.[17] Recently, our group demonstrated that loss of the full-length plectin with maintenance of the rodless plectin leads to EBS-MD, whereas complete loss or marked attenuation of full-length and rodless plectin expression underlies the EBS-PA phenotype.[12] The present family further supports the hypothesis that homozygotes or compound-heterozygotes for mutations that cause plectin truncation outside the rod domain show the EBS-PA phenotype.

In summary, this is the first report of DNA-based PND of EBS-PA. EBS-PA has now been added to the list of severe genodermatosis for which DNA-based PND is feasible.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to H. Nakamura (Kiban C 1959129107) and H. Shimizu (Kiban A 21249063).

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26 November 2010
10 March 2011
29 March 2011

doi:10.1016/j.jdermsci.2011.03.011

Letter to the Editor

Altered lipid profiles in the stratum corneum of Sjögren-Larsson syndrome

Sjögren-Larsson syndrome (SLS) is a rare, autosomal recessive neurocutaneous disorder characterized by clinical triads, congenital ichthyoids, spasticity and mental retardation [1]. SLS is caused by mutations in fatty aldehyde dehydrogenase (*FALDH*) (or *ALDH3A2*) gene [1]. FALDH is a microtonal NAD-dependent enzyme, which oxidizes medium- to long-chain aliphatic aldehydes to fatty acids. Accumulation of fatty alcohol has been shown in cultured fibroblasts and in plasma from SLS patients [1]. Numbers of mutations of *FALDH* gene have been shown, although only three mutations have been identified in Japanese SLS patients [2–4]. We here report a SLS patient who is a homozygote for one of the known mutations. In addition to assessing skin phenotype, permeability barrier function and cutaneous morphology, biochemical analysis revealed novel alterations in lipid profiles in the stratum corneum associated with barrier function.

A 57-year-old Japanese woman complaining of slightly pruritic and dry skin with scaling visited our hospital. The patient has been suffering from scaly skin lesions over the entire body since her early childhood. She presented generalized dryness, widespread itchy hyperkeratosis scaly lesions with brown scaling plaques, and slight erythema on the trunk and extremities (Fig. 1a). The neurologic examination revealed severe spastic paraplegia in the lower limbs with an increased muscle tone, hyperreflexia in all limbs, and positive Babinski reflexes bilaterally. She also showed mental retardation (IQ 39). A skin biopsy specimen from the right arm revealed orthohyperkeratosis with thin granular layers and mild acanthosis with papillomatosis (Fig. 1b). Electron microscopic examination showed several lipid droplets without surrounding membrane in the cornified cells (Fig. 1c). Moreover, abnormal lamellar granules, which lacked lamellar contents, were present in the granular cells (Fig. 1d). From these clinical features and cutaneous morphology, this patient was diagnosed as SLS. Mutation analysis using a cDNA sample from the patient's peripheral white blood cells showed a homozygous point mutation c.1157A>G which results in alteration from asparagine to serine at cordon 386 (p.Asn386Ser) in the β-9 chains containing active domain of FALDH (Fig. 2a).

Transepidermal water loss (TEWL) of the ichthyosiform lesion on the extensor and flexor sides of the forearm and back (6.3, 12.2, 10.2 g h^{-1} m^{-2}, respectively) was within the normal range (0–10, very good; 10–15, good; 15–20, fair; 25–30, poor; more than 30, very poor). On the other hand, water retention capability was impaired in the lesion (25.5, normal > 60).

Major barrier lipid content of involved skin was assessed in comparison to non-ichthyotic scaly lesions from sunburn dermatitis as a control subject (note: we and others found that there is no significant difference in lipid content of sunburn scale and of non-sunburn scales from normal donors [5]). Although there was no difference in the quantity of cholesterol between the patient and control, free fatty acid (FFA) was increased by about two-fold over control (Fig. 2b). In contrast, ceramide (Cer) 1, 6, 7 were decreased in the patient's scales compared with those in control samples, while membrane-bound Cer species, Cer A, which are constituent of the corneocyte lipid envelope (CLE), were increased. We recently demonstrated that linoleate required for acylceramide synthesis is primarily derived from triglyceride (TG) [6]. However, TG content was not changed in SLS compared with that in control scales (Fig. 2b).

The identical mutation in our case was described in another Japanese patient with SLS [2]. The other mutations reported in the

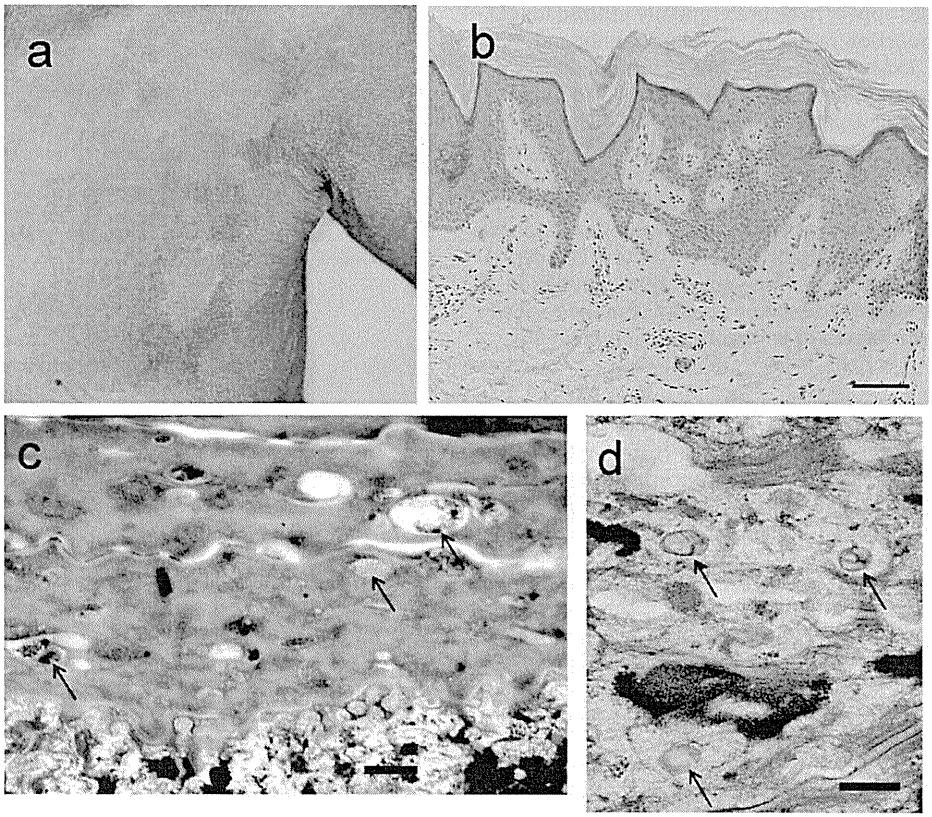

Fig. 1. Clinical appearance. (a) Scaly ichthyosiform erythema was apparent over the trunk. Morphological features of the patient's epidermis. (b) H&E staining of lesional skin from the patient's forearm. Orthohyperkeratosis, slightly thin granular layers and mild acanthosis with papillomatosis are noted, scale bar, 50 μm. (c) Ultrastructually, electron-lucent vacuoles are present within corneocytes (arrows) scale bar, 2 μm. (d) The presence of abnormal lamellar bodies lacking lamellar contents are evident in the cytoplasm of the granular cell (arrows) scale bar, 2 μm.

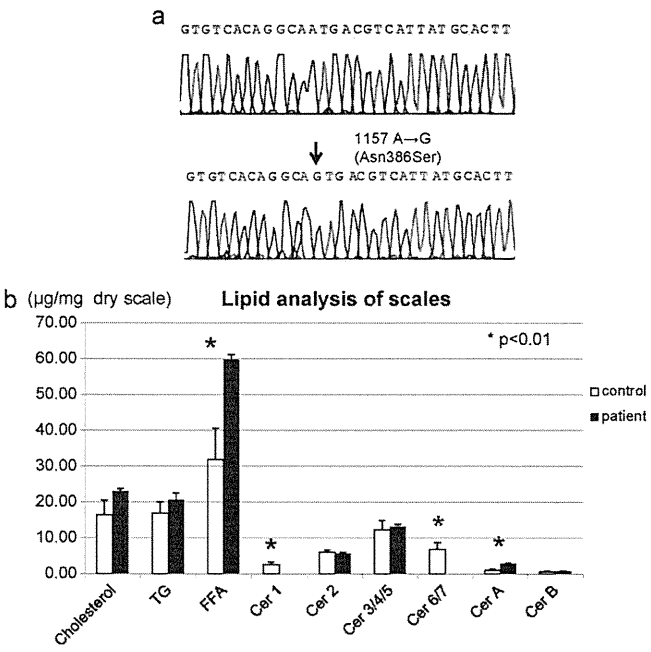

Fig. 2. (a) Sequencing analysis of FALDH gene. A homozygous point mutation (c.1157A>G) in the exon 8 that substitutes serine for asparagine at position 386 (p.Asn386Ser). (b) Lipid analysis of scales taken from sunburn lesions of a normal control individual (white bar) and from the patient's lesions (black bar) show increased FFA and Cer A level and decreased ceramide 1, 6, 7 levels in the patient's scale compared with control samples. Scales were taken from the upper back skin of the patient or control subjects. Gene and lipid analysis were performed as we described previously [4,6].

Japanese cases were c.481delA, c.1087_1089delGTA, c.332G>A (p.Trp111X) and c.636T>G (p.Ser212Arg) [3,4]. All the mutations found in Japanese families were distinct from one another and no founder effect was suggested in *ALDH3A2* mutations underlying Japanese SLS cases.

Recent studies by lanthanium perfusion assay, which is more sensitive for assessing permeability barrier function *in vitro* using skin sections than TEWL measurements employed in our study, reveals abnormal permeability barrier formation, structures, and function in SLS patients [7], while our present study is the first time for assessing both TEWL and hydration of SLS patient *in vivo*. Consistent with this prior study abnormal epidermal barrier structures [7] are evident in our patient, but alterations of TEWL were not observed. We assume that hyperkeratosis could attempt to compensate barrier dysfunction as previously suggested [8] and result in attempting to minimize barrier abnormality. Yet, decreased SC hydration in a SLS patient could alter normal SC environment, leading to abnormal epidermal homeostasis.

It remains to be resolved, however, why FFA level was high in spite of the deficient activity of FALDH, which was the enzyme catalyzing the sequential oxidation of fatty alcohol to fatty acid. It is likely that increased levels of wax esters and alkyl-diacylglycerol in scales and keratinocytes of SLS [9] derived from fatty alcohol may contribute to FFA production via hydrolysis with lipase, because the levels of these lipids were high.

Consistent with a prior study showing a deficiency of Cer 1, 6 in SLS patients' skin [10], Cer 1, 6, 7 were decreased in the epidermis of our case. We further demonstrated that the levels of CLE-bound ceramides, Cer A, which are produced from acylglucosylceramide, elevated in the scale from the patient, although Cer 1 (EOS) generated from the same precursors decreased. Therefore,

acylglucosylceramides appear to be preferentially utilized for CLE-bound ceramide production rather than free (CLE-unbound) lipid production in the SC. Exact mechanisms for CLE formation have not been elucidated yet and it remains to be resolved whether preferential utilization of acylglucosylceramide for CLE formation occurs only in the present case or also in other SLS patients. Moreover, it is unknown how decrease in Cer 1, 6, 7 occur and whether barrier lipid abnormality in the patient was a primary event or a secondary phenomenon in the pathogenesis of SLS skin lesions. Cer 1 is essential lipid species to form epidermal permeability barrier formation. Thus, not only accumulation of free fatty acids, but also deficiency of specific ceramide species might contribute to formation of ichthyotic phenotype in SLS.

Acknowledgements

We greatly thank Dr. Sumiko Hamanaka (Lab. for Molecular Membrane Neuroscience, RIKEN Brain Science Institute) and Dr. Ken Hashimoto (Department of Dermatology, Wayne State University School of Medicine) for helpful discussion.

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2 December 2010

doi:10.1016/j.jdermsci.2011.03.009

Letter to the Editor

BMP-4 down-regulates the expression of Ret in murine melanocyte precursors

Bone morphogenetic proteins (BMPs) have been implicated in a diverse array of biological processes including development and apoptosis [1]. Ret is involved in the physiological mechanisms of melanocyte activation and melanin production [2]. Ret expression in enteric neural precursors is initiated shortly after they emigrate from the neural plate.

We established three distinct cell populations of mouse neural crest (NC) cells, NCCmelb4, NCCmelb4M5 and NCCmelan5. NCCmelb4 cells have the potential to differentiate into mature melanocytes, but since they express melanocyte markers such as tyrosinase-related protein 1, DOPAchrome tautomerase and Kit, we consider them to be immature melanocytes, not multipotent precursors that can differentiate into neurons, as well as glia [3]. NCCmelb4M5 cells belong to the melanocyte lineage, but are less differentiated than NCCmelb4 cells [4]. NCCmelb4M5 cells do not express Kit and grow independently of the Kit ligand; these cells have the potential to differentiate into NCCmelb4 cells, which are Kit-positive melanocyte precursors. NCCmelan5 cells demonstrate the characteristics of differentiated melanocytes. We have also established an oncogene Ret-transgenic mouse line, line 304/B6, in which skin melanosis, benign melanocytic tumors and malignant melanomas develop in a stepwise fashion [2]. A malignant melanoma cell line, Mel-Ret, was established from the Ret-transgenic mouse. We found that all four cell lines express BMP receptors using Western blotting analysis (data not shown).

Western blotting revealed expression of the Ret protein in NCCmelb4M5 and in Mel-Ret cells, but in contrast, there was no expression of the Ret protein in NCCmelb4 or NCCmelan5 cells (Fig. 1A). Immunostaining also revealed that NCCmelb4M5 (Fig. 1B) and Mel-Ret cells are positive for Ret, but NCCmelb4 and NCCmelan5 cells are negative for Ret. Thus, Ret protein is expressed in most immature melanoblasts, while melanocytes are negative for Ret. We then analyzed Ret protein expression in BMP-4-treated NCCmelb4M5 cells by Western blotting (Fig. 1C–F). BMP-4 was added to the medium and incubated for 3 days at varying concentrations. After incubation with 10 ng/ml BMP-4 for 3 days, Ret protein expression was decreased, and disappeared completely