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

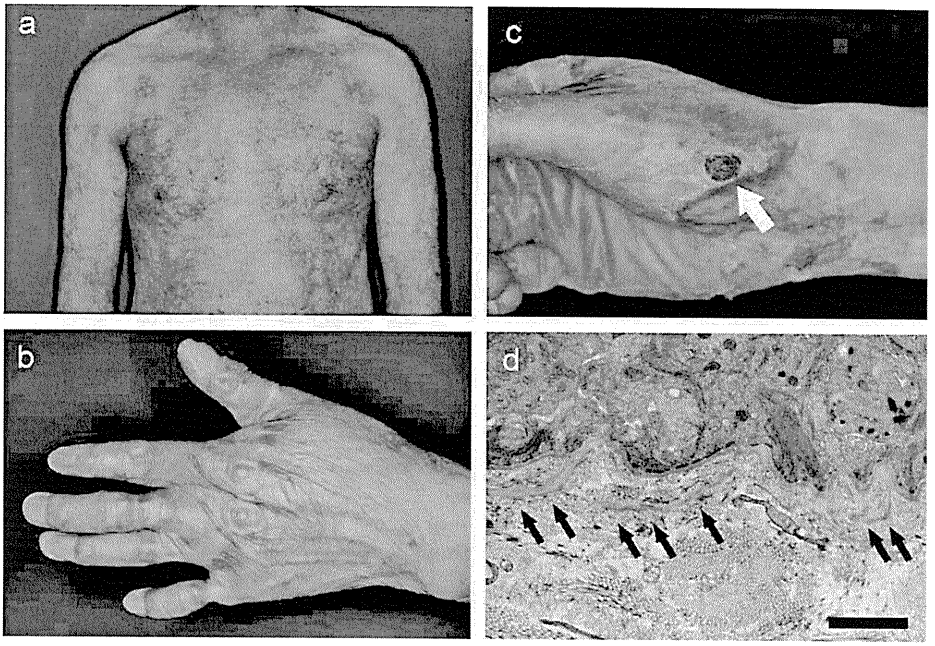

Fig. 1. Clinical and ultrastructural features of Patient 1. (a) Generalized poikiloderma is observed. (b) Pseudo-ainhum is seen from the left middle to little fingers. The middle and ring fingers are partly fused. (c) Recurrent squamous cell carcinoma (arrow) on the right palm at the age of 38. (d) Ultrastructural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

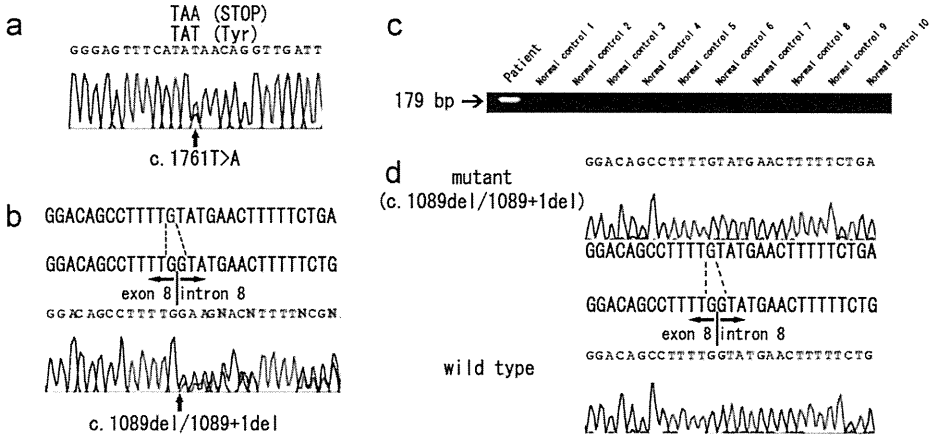

Fig. 2. *KIND1* mutation analysis of Patient 1. (a, b) Patient 1 is compound heterozygous for c.1761T>A (a) and c.1089del/1089+1del (b). An adenine that has replaced a thymine is indicated in red in the former mutation. A guanine deleted from two guanines at the exon 8–intron 8 boundary is indicated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

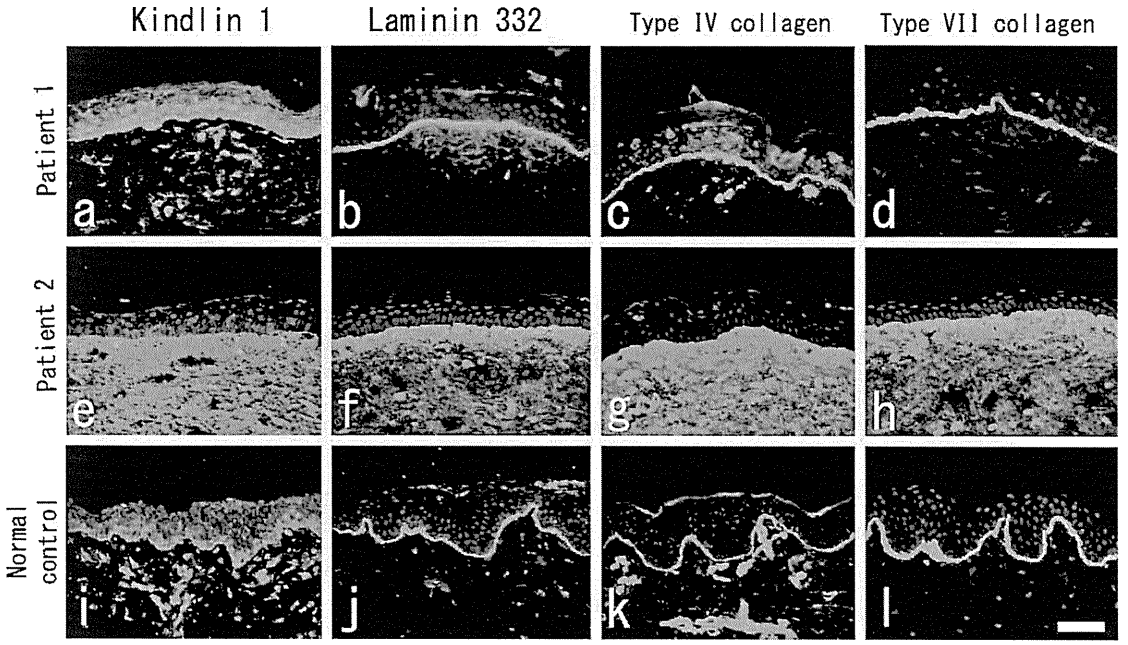

Fig. 3. Immunofluorescence analysis of skin specimens from KS with c.1089del/1089+1del. (a–d) Patient 1. (e–h) Patient 2 (a homozygote for c.1089del/1089+1del). (i–l) Normal control. Kindlin-1 labelling of skin specimens from Patients 1, 2 and the normal control show bright, linear staining at the DEJ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

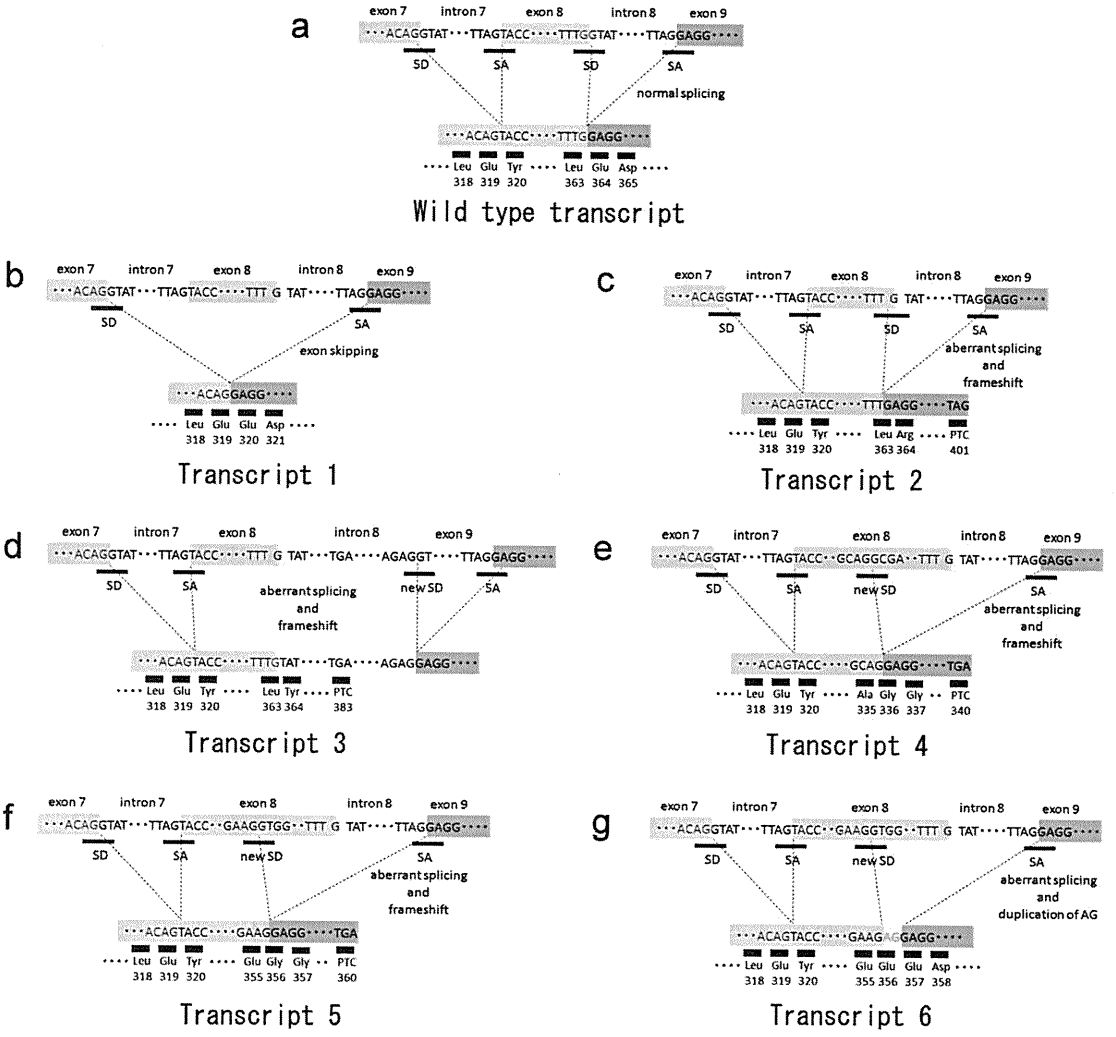

Fig. 4. Transcripts resulting from c.1089del/1089+1del. Wild-type (a) and mutant (b–g) sequences were subcloned in exon-trapping vector pSPL3 and transfected into HaCaT cells. Transcripts were derived from the pairing of vector and cloned splice signals. Boxes represent exons, blue underlines are splice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

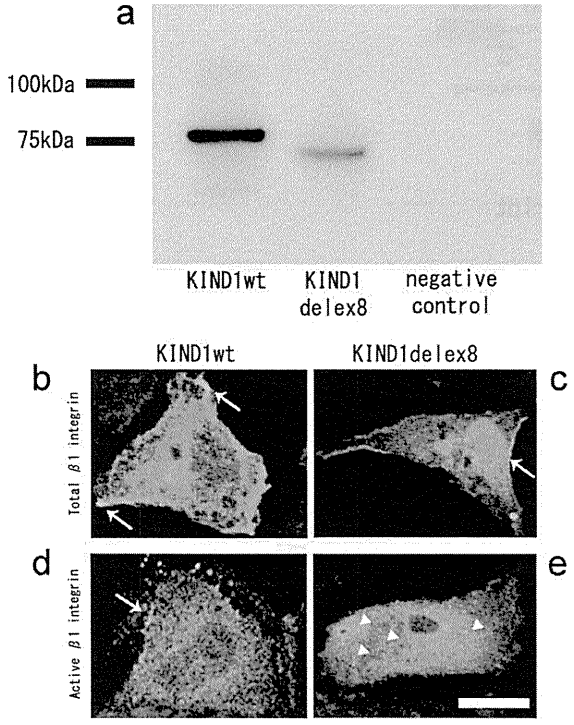

Fig. 5. Total and active β1 integrin expression in HeLa cells transfected with wild-type or mutated *KIND1* cDNA. (a) Immunoblot analysis of lysates from HeLa cells transfected with *KIND1* cDNA with (KIND1wt) or without exon 8 (KIND1delex8) confirms the expression of translated products (predicted sizes;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

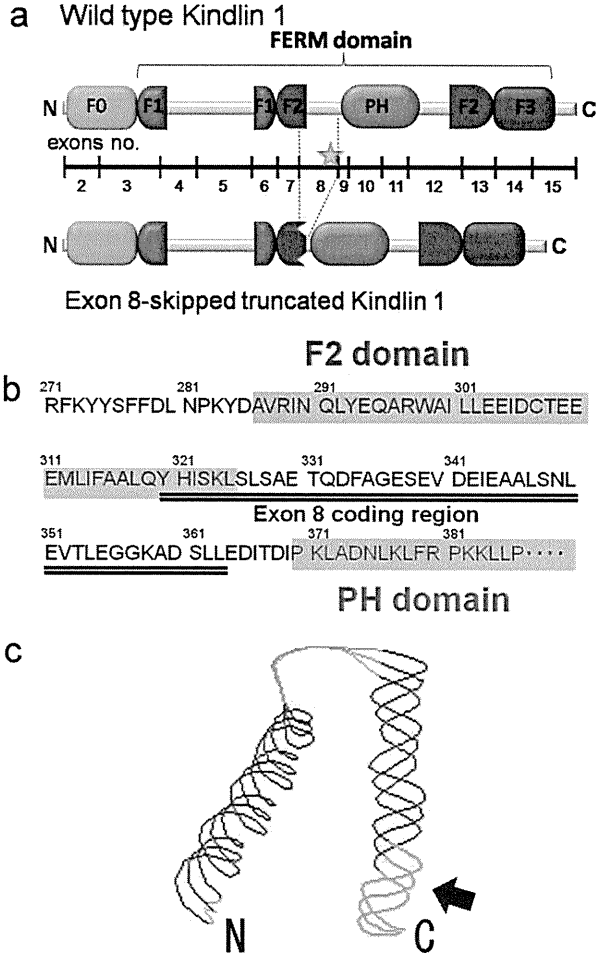

Fig. 6. Structural basis of exon-8-skipped truncated kindlin-1. (a) Schematic structure of wild-type and exon-8-skipped truncated kindlin-1. Kindlin-1 comprises the FERM domain, including F1, F2 and F3, flanked by the pleckstrin homology (PH) domain. A star indicates c.1089del/1089+1del at the exon 8/intron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

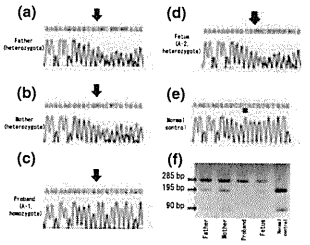

Figure 1. Prenatal diagnosis of junctional epidermolysis bullosa with pyloric atresia (family A). (a–e) Direct sequencing of *ITGB4*. The parents were heterozygous for c.1938delC in *ITGB4* (a, b). A-1, the proband, was homozygous for that mutation (c). A-2, the foetus, was found to be a heterozygous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

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

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).

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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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

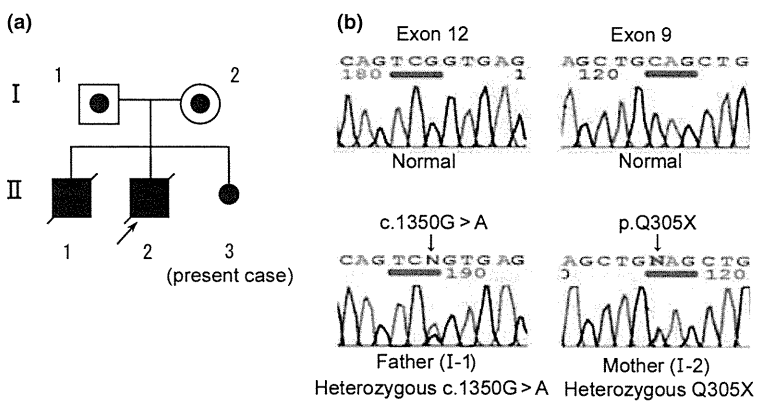

Figure 1 Family tree of the present case and the causative *PLEC* mutations. (a) The first and second newborns exhibited clinical features typical of EBS-PA and died shortly after birth. The proband (the second newborn) is indicated by an arrow. (b) The paternal splice-site mutation was a c.1350G>A transition 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)

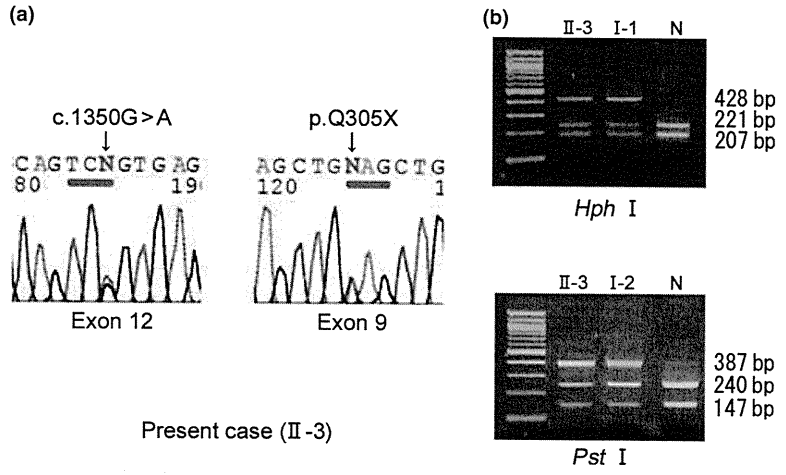

Figure 2 Analysis of the plectin gene mutations in genomic DNA from amniocytes of a fetus at risk. (a) Mutation analysis of genomic DNA from amniocytes shows both the c.1350G>A mutation in exon 12 and p.Q305X mutations in exon 9. (b) The presence of the mutations was verified by restriction enzyme digestion. 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)

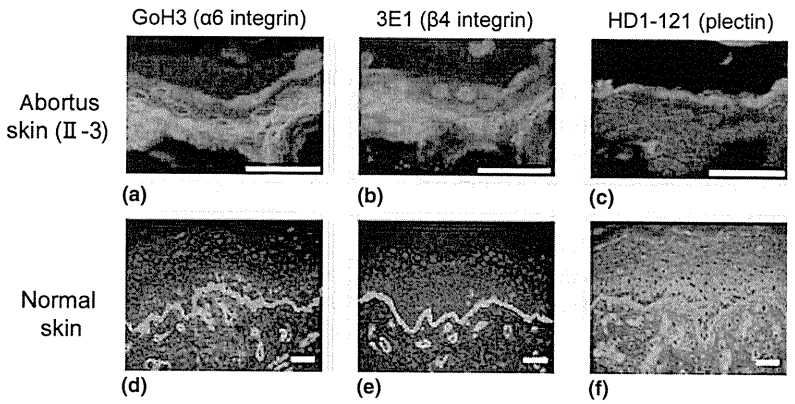

Figure 3 Absence of plectin expression in the abortus. α6 integrin (mAb GoH3) and β4 integrin (mAb 3E1) are expressed in the abortus skin (a, b) and the control skin (d, e). Staining with monoclonal antibody for plectin (mAb HD1–121) shows positive in the control skin (f) but negative in the skin of 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

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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

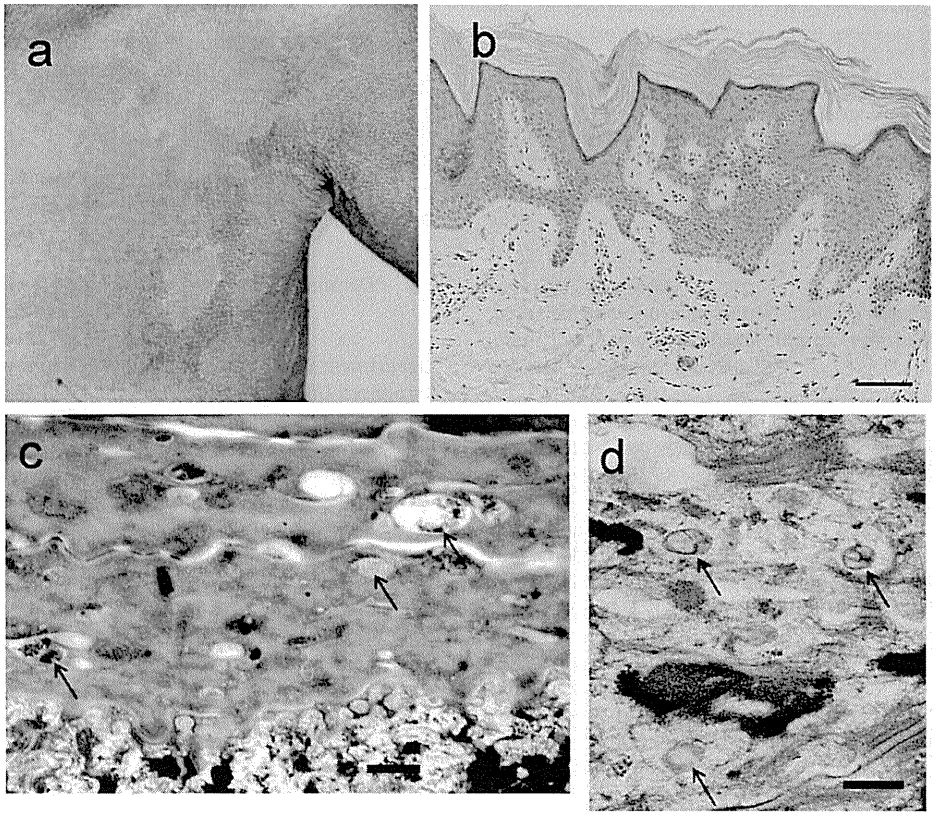

Fig. 1. Clinical appearance. (a) Scaly ichthyosiform erythema was apparent over the trunk. Morphological features of the patient's epidermis. (b) H&E staining of lesional skin from the patient's forearm. Orthohyperkeratosis, slightly thin granular layers and mild acanthosis with papillomatosis are noted, 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.

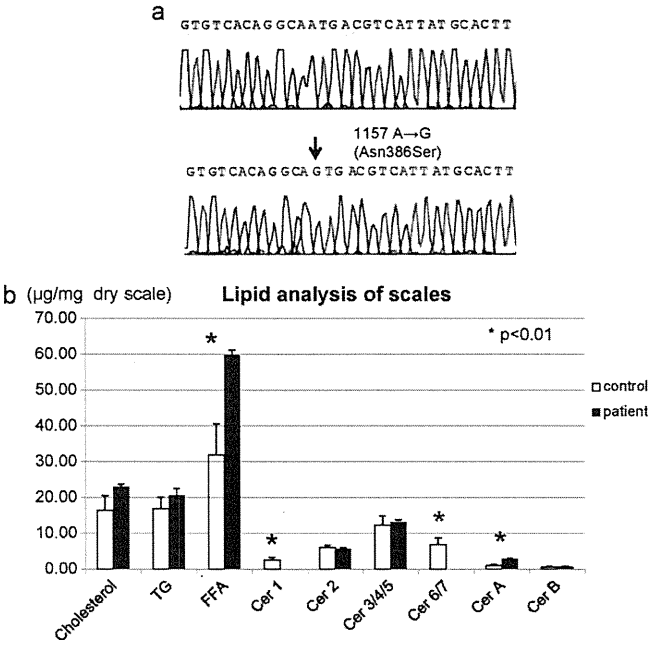

Fig. 2. (a) Sequencing analysis of FALDH gene. A homozygous point mutation (c.1157A>G) in the exon 8 that substitutes serine for asparagine at position 386 (p.Asn386Ser). (b) Lipid analysis of scales taken from sunburn lesions of a normal control individual (white bar) and from the patient's lesions (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.

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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

REVIEW

Definitions and outcome measures for bullous pemphigoid: Recommendations by an international panel of experts

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Sydney, Australia; Rouen, Bobigny, and Reims, France; Bern, Switzerland; Tokyo, Kurume, and Sapporo, Japan; Zagreb, Croatia; Buffalo and New York, New York; Marburg, Luebeck, and Dresden, Germany; Rome and Modena, Italy; Iowa City, Iowa; Groningen, The Netherlands; Dallas, Texas; Chapel Hill and Durham, North Carolina; Los Angeles, California; Philadelphia, Pennsylvania; Sao Paulo, Brazil; Barcelona, Spain; Salt Lake City, Utah; Manila, Philippines; Atlanta, Georgia; and Petah Tikva, Israel

Our scientific knowledge of bullous pemphigoid (BP) has dramatically progressed in recent years. However, despite the availability of various therapeutic options for the treatment of inflammatory diseases, only a few multicenter controlled trials have helped to define effective therapies in BP. A major obstacle in sharing multicenter-based evidences for therapeutic efforts is the lack of generally accepted definitions for the clinical evaluation of patients with BP. Common terms and end points of BP are needed so that experts in the field can accurately measure and assess disease extent, activity, severity, and therapeutic response, and thus facilitate and advance clinical trials. These recommendations from the International Pemphigoid

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Conflicts of interest: None declared.

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Committee represent 2 years of collaborative efforts to attain mutually acceptable common definitions for BP and proposes a disease extent score, the BP Disease Area Index. These items should assist in the development of consistent reporting of outcomes in future BP reports and studies. (J Am Acad Dermatol 10.1016/j.jaad.2011.06.032.)

Key words: bullous pemphigoid; consensus; definitions; outcome measures; severity score.

Bullous pemphigoid (BP) is a common autoimmune bullous disease typically affecting the elderly. There have been only a handful of well-designed randomized controlled trials assessing the effectiveness of therapies for BP.[1] In relatively rare diseases where it is difficult to include enough patients to have sufficient power to compare different treatments, meta-analysis is a powerful tool that is used to pool data across trials. However, it is impossible to compare the therapeutic outcomes from the majority of these BP studies using meta-analysis, as they have varying definitions and outcome measures.

CAPSULE SUMMARY

- It is impossible to compare the therapeutic outcomes from the majority of bullous pemphigoid studies using meta-analysis, as they have varying definitions and outcome measures.
- These recommendations, developed over the last 3 years by experts, provide appropriate definitions for the various stages of disease activity and therapeutic end points in bullous pemphigoid.
- These definitions can be used in case series and clinical trials to compare the efficacy of treatments for bullous pemphigoid.

PURPOSE

The purpose of this statement is to provide appropriate definitions for the various stages of disease activity, define therapeutic end points in BP, and to propose an objective disease extent measure that can be used in clinical trials. The use of the same definitions and outcome measures makes the results of trials more comparable. Since definitions and outcome measures for pemphigus[2-4] have been published, most trials in pemphigus and reports have begun adopting these systems or referring to them when their existing trials using other measures were unable to show a difference.[5]

METHODS

An international BP definitions committee was organized in 2008, at the point when the international pemphigus definitions committee completed its similar work on pemphigus.[2] The committee was an expansion of the first committee and convened 7 times over 2 years to discuss the appropriate definitions. These meetings were held at the American Academy of Dermatology (AAD) annual meeting in San Antonio, TX, in 2009 (D. F. M. and V. P. W.); European Society for Dermatologic Research in Budapest, Hungary, in 2009 (D. F. M. and P. J.); the European Academy of Dermatovenereology in Berlin, Germany, in 2009 (D. F. M. and L. B.); the AAD in Miami, FL, in 2010 (D.F.M. and V. P. W.); the Pemphigus 2010 Meeting in Bern, Switzerland (V. P. W. and D. F. M.); and the International Pemphigus and Pemphigoid Meeting at the National Institutes of Health in November 2010 (V. P. W. and D. F. M.), in Bethesda, MD. The final meeting was held at the AAD in 2011 in New Orleans, LA (D. F. M. and V. P. W.). Meetings were supported in part by local dermatology societies. The draft definitions and end points were electronically mailed to the larger group, allowing for comments between meetings.

THE RECOMMENDATIONS

Observation points

The end points are illustrated and summarized (Fig 1 and Table I).

Early end points

"Baseline" is the point at which a physician starts treatment for BP.

"Control of disease activity" (disease control; beginning of consolidation phase) is defined as the point at which new lesions or pruritic symptoms cease to form and established lesions begin to heal. The time to disease control is the time between baseline and this control point.

"End of the consolidation phase" is defined as the time at which no new lesions or pruritic symptoms have developed for a minimum of 2 weeks and the majority (approximately 80%) of established lesions has healed. At this point tapering of corticosteroids often occurs. The length of the consolidation phase is the time between disease control and the end of consolidation phase.

Abbreviations used:

AAD: American Academy of Dermatology
BP: bullous pemphigoid
BPDAI: Bullous Pemphigoid Disease Area Index
DAI: Disease Area Index
PDAI: Pemphigus Disease Area Index

"Transient lesions" are new lesions that heal within 1 week or pruritus lasting less than a week and clearing without treatment.

"Nontransient lesions" are new lesions that do not heal within 1 week or pruritus continuing more than a week with or without treatment.

Intermediate end points

During this period, the corticosteroids and other treatments are usually being tapered, but for some patients medication doses do not change because of flaring with attempts to taper treatment. "Complete remission during tapering" is the absence of nontransient lesions while the patient is receiving more than minimal therapy. There is no minimum time point here as the patient is under control but has not yet reached the desired outcome of disease remission on minimal or no therapy.

Late observation end points

Late observation end points of disease activity are identified as: (1) complete remission off therapy; and (2) complete remission on therapy, both of which only apply to patients who have had no new or established lesions for at least 2 months. "Complete remission off therapy" is defined as an absence of new or established lesions or pruritic symptoms while the patient is off all BP therapy for at least 2 months.

"Complete remission on therapy" is defined as the absence of new or established lesions or pruritus while the patient is receiving *minimal* therapy for at least 2 months. "Minimal therapy" is defined as less than or equal to 0.1 mg/kg/d of prednisone (or the equivalent) or 20 g/wk of clobetasol propionate and/or minimal adjuvant or maintenance therapy for at least 2 months, as shown in Fig 1 and discussed further below.

Minimal adjuvant therapy in BP corresponds to the following doses or less: methotrexate 5 mg/wk; azathioprine 0.7 mg/kg/d (with normal thiopurine s-methyltransferase level); mycophenolate mofetil 500 mg/d; mycophenolic acid 360 mg/d; or dapsone 50 mg/d. There has only been one small randomized controlled trial on tetracycline and niacinamide,[6] which was underpowered because of low numbers and was unable to demonstrate a difference. Nevertheless, the committee's expert opinion is that full therapeutic doses of the tetracyclines may work in localized forms of BP. As the tetracycline class of drugs is relatively nontoxic, the full therapeutic dose was listed among minimal therapies for BP.

"Partial remission off therapy" is defined as the presence of transient new lesions that heal within 1 week without treatment and while the patient is off all BP therapy for at least 2 months.

"Partial remission on minimal therapy" is defined as the presence of transient new lesions that heal within 1 week while the patient is receiving minimal therapy.

A newer term, "mild new activity," refers to fewer than 3 lesions a month (blisters, eczematous lesions, or urticarial plaques) that do not heal within 1 week, or the extension of established lesions or pruritus once per week but less than

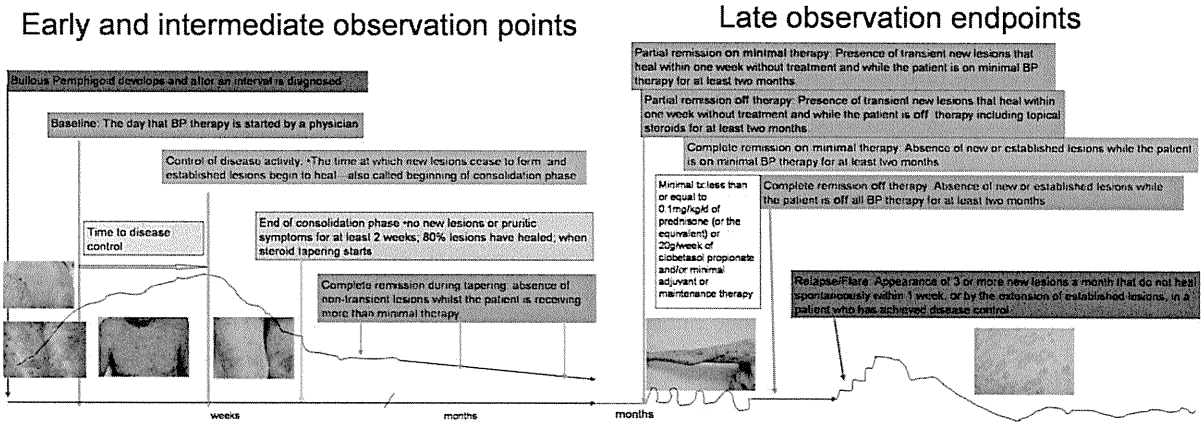

Fig 1. Pictorial depiction of end points in bullous pemphigoid.

Table I. Definitions for bullous pemphigoid

Early observation points	
Baseline	Day that BP therapy is started by physician
Control of disease activity	Time at which new lesions cease to form and established lesions begin to heal or pruritic symptoms start to abate
Time to control of disease activity (disease control; beginning of consolidation phase)	Time interval from baseline to control of disease activity
End of consolidation phase	Time at which no new lesions have developed for minimum of 2 wk and approximately 80% of lesions have healed and pruritic symptoms are minimal
Intermediate observation end points	
Transient lesions	New lesions that heal within 1 wk or pruritus lasting <1 wk and clearing without treatment
Nontransient lesions	New lesions that do not heal within 1 wk or pruritus continuing >1 wk with or without treatment
Complete remission during tapering	Absence of nontransient lesions while patient is receiving more than minimal therapy
Late observation end points	
Minimal therapy	≤0.1 mg/kg/d Of prednisone (or equivalent) or 20 g/wk of clobetasol propionate and/or minimal adjuvant or maintenance therapy
Minimal adjuvant therapy and/or maintenance therapy	Following doses or less: methotrexate 5 mg/wk; azathioprine 0.7 mg/kg/d (with normal thiopurine s-methyltransferase level); mycophenolate mofetil 500 mg/d; mycophenolic acid 360 mg/d; or dapsone 50 mg/d
Partial remission on minimal therapy	Presence of transient new lesions that heal within 1 wk while patient is receiving minimal therapy for at least 2 mo
Complete remission on minimal therapy	Absence of new or established lesions or pruritus while patient is receiving minimal therapy for at least 2 mo
Partial remission off therapy	Presence of transient new lesions that heal within 1 wk without treatment while patient is off all BP therapy for at least 2 mo
Complete remission off therapy	Absence of new or established lesions or pruritus while patient is off all BP therapy for at least 2 mo
Mild new activity	<3 Lesions/mo (blisters, eczematous lesions, or urticarial plaques) that do not heal within 1 wk, or extension of established lesions or pruritus once/wk but less than daily in patient who has achieved disease control; these lesions have to heal within 2 wk
Relapse/flare	Appearance of ≥3 new lesions/mo (blisters, eczematous lesions, or urticarial plaques) or at least one large (>10 cm diameter) eczematous lesion or urticarial plaques that do not heal within 1 wk, or extension of established lesions or daily pruritus in patient who has achieved disease control
Failure of therapy for initial control	Development of new nontransient lesions or continued extension of old lesions, or failure of established lesions to begin to heal or continued pruritus despite: Clobetasol propionate 40 g/d for 4 wk; or Prednisone 0.75 mg/kg/d equivalent for minimum of 3 wk with or without drugs used for maintenance therapy; or A tetracycline on full dosing for 4 wk; or Dapsone 1.5 mg/kg/d for 4 wk; or Methotrexate 15 mg/wk (if >60 kg and no major renal impairment) for 4 wk; or Azathioprine 2.5 mg/kg/d for 4 wk (if thiopurine s-methyltransferase level is normal); or Mycophenolate mofetil 40 mg/kg/d (if normal renal function, otherwise according to age/creatinine clearance) for 4 wk

BP, Bullous pemphigoid.

daily, in a patient who has achieved disease control. This term was not included in the pemphigus definitions but the committee thought that it might be important to capture this phase during studies to determine if some patients with BP and certain characteristics or treatments experienced new mild activity not significant enough to constitute a flare. In this way, it could be determined in the future if these patients with BP might benefit from a change of treatment plan or not.

Relapse/flare

The terms "relapse" and "flare" are used interchangeably and are defined as the appearance of 3 or more new lesions a month (blisters, eczematous lesions, or urticarial plaques) or at least one large (>10 cm diameter) eczematous lesion or urticarial plaque that does not heal within 1 week, or the extension of established lesions or daily pruritus in a patient who has achieved disease control.

Treatment failure

"Failure of therapy for initial control" is defined as the development of new nontransient lesions or continued extension of old lesions, or failure of established lesions to begin to heal or daily pruritus despite certain strengths of corticosteroids with or without higher doses of adjuvants. The dose of prednisone defined as treatment failure is 0.75 mg/kg/d equivalent for minimum of 3 weeks. This dose was selected because the Cochrane review of interventions for BP[1,7] determined that in acute BP there was no purpose in using prednisone at a higher dose than this. Topical clobetasol propionate at 40 g/d for 4 weeks was selected on the basis of the randomized controlled trials conducted by the French group.[8,9] Other therapies include tetracycline at full doses for 4 weeks; dapsone 1.5 mg/kg/d for 4 weeks; methotrexate 15 mg/wk (if >60 kg and no major renal impairment) for 4 weeks; azathioprine 2.5 mg/kg/d for 4 weeks (if thiopurine s-methyltransferase level is normal); or mycophenolate mofetil 40 mg/kg/d (if normal renal function, otherwise according to age/creatinine clearance) for 4 weeks. The definition does not imply these drugs and their respective doses are equivalent in therapeutic efficacy. Rather it provides a standardized agreement as to what can be defined as a failure of therapy.

BP disease activity index

Like the Pemphigus Disease Area Index (PDAI),[3] the BP Disease Area Index (BPDAI) measure has separate scores for skin and mucous membrane activity. Damage scores are separate as well and are included to remind physicians that not all visible lesions in BP represent active disease. Areas of the skin predominantly affected in BP[10] were taken into account when selecting the skin sites so that trials would better differentiate clinical response in BP. Hence, additional weighting was given to the arms and legs and less emphasis to the face and scalp, slightly different from the PDAI. The mucous membrane areas were retained from the PDAI even though it is relatively rare to see mucous membrane involvement in BP, so that the activity could be compared with extent of mucous membrane involvement in different autoimmune bullous diseases. There are separate columns for the extent of blistering and for the urticarial/eczematous lesions that may be more extensive in BP.

BPDAI PRURITUS COMPONENT - VAS

DATE: .....

☐Baseline	☐ Beginning Consolidation
☐Consolidation phase	☐End of Consolidation
☐Tapering phase	☐Partial remission on minimal therapy
☐Complete remission on minimal therapy	☐Partial remission off therapy
☐Complete remission off therapy	☐Flare

A. How severe has your itching been over the last 24 hours?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

B. How severe has your itching been the past week?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

C. How severe has your itching been in the past month?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

Average INTENSITY SCORE FOR PAST MONTH = (A+B+C) = /30

OR

For BP patients with impaired mental functioning:

	No evidence of itch (no excoriations)	0
	Mild itch (isolated excoriations up to two body sites)	10
	Moderate itch (excoriations on ≥ 3 body sites, impairment of daily activity	20
	Severe itch (generalized excoriation, sleep impairment)	30
	TOTAL SCORE	/30

Fig 2. Subjective Bullous Pemphigoid (*BP*) Disease Area Index (*BPDAI*) pruritus score. *VAS*, Visual analog scale.

As a major symptom that may herald the onset and recurrence of BP is pruritus, a separate subjective component of the BPDAI is proposed to measure the severity of this (Fig 2). Naturally, other causes of pruritus in the elderly must be excluded, such as xerosis, dermatitis, renal impairment, liver impairment, and scabies. Providing that only pruritus related to BP is considered in the definitions and scored, this system can be used to subjectively grade the intensity of pruritus using a visual analog scale to answer the question, "How severe is your itching today?" and the patient marks an "x" on the 0- to 10-cm line where 0 is no itch and 10 is maximal itching. The degree of itching is measured as the distance in centimeters from 0, out of 10. This is repeated for the severity overall of itching in the past week and month. A total score is calculated from this out of 30. If the patient with BP is incapable of completing a reliable visual analog scale rating, for example, as a result of dementia, then the degree of pruritus is inferred, based on the extent of excoriations alone, also scored