mutation detection has become a powerful tool for the diagnosis of IBS. All the present four patients from three families had been misdiagnosed for years as having BCIE. However, from the present results of clinical and morphological observations and the mutation detection, a definite diagnosis of IBS could be made in all these patients. Indeed, during the period before K2e mutation analysis was available for the diagnosis of IBS, from 1937 when the first case of IBS was reported, until 1994, only five families were reported in the literature (K2e mutations were later detected in four of the five families).[9] On the other hand, since 1994 when the K2e mutation analysis became available to make a definite diagnosis of IBS, 19 new families were reported to harbour K2e mutations and were diagnosed as having IBS.[13–15] We have added three more families in the present report who were previously misdiagnosed as having BCIE. These data as well as the present patients suggest that direct sequencing of KRT2E is a powerful tool for the diagnosis of IBS and that IBS is not so extremely rare as was first thought when mutation analysis was not available for the diagnosis.

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Clinical Heterogeneity of 1649delG Mutation in the Tail Domain of Keratin 5: A Japanese Family with Epidermolysis Bullosa Simplex with Mottled Pigmentation

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Twenty-five- and 22-y-old Japanese women, who are cousins, presented with distal skin fragility, widespread small, pigmented macules, and toenail deformity. Blisters occurred between the epidermis and the dermis with degeneration of the basal cells, suggesting epidermolysis bullosa simplex with mottled pigmentation (EBS-MP). Electron microscopy of the pigmented spots demonstrated vacuolization of basal cells as well as disturbed junctional structures and incontinence of pigmentation. Gene analysis resulted in detection of a heterozygous deletion of a guanine nucleotide in exon 9 at position 1649. P25L mutation was not detected in either case. It is possible that EBS-MP occurs not only based on the P25L mutation of the keratin 5 molecule, but also because of other types of mutations of epidermal keratin genes.

Keywords: epidermolysis bullosa simplex with mottled pigmentation/1649 del G mutation of KS/vacuolization degeneration of basal cells
J Invest Dermatol 125:83–85, 2005

Epidermolysis bullosa simplex with mottled pigmentation (EBS-MP) is characterized by distal skin fragility, and widespread pigmented macules. All previously reported cases of EBS-MP examined demonstrated a heterozygous point mutation, P25L, in the non-helical V1 domain of keratin-5 (K5) molecule (Uttam *et al*, 1996; Irvine *et al*, 1997, 2001; Moog *et al*, 1999; Hamada *et al*, 2004). Recently, we encountered a Japanese female and her cousin affected with distal skin fragility, distribution of pigmented macules, toenail deformity, and histological findings of subepidermal blister with basal cell degeneration, compatible with EBS-MP. Gene analysis demonstrated, however, that both patients had no mutation in V1 domain of the KRT5, but 1649delG mutation in the V2 domain of the gene. We describe a possibility that EBS-MP is based on gene mutation other than P25L in V1 domain of K5.

Case Report

Case 1 A 25-y-old Japanese female presented with small blisters on the distal portion of the extremities and the abdomen. These blisters re-epithelized in a short period leaving no scarring (Fig 1*A*, *B*), but were recurrent. Blistering began in her infancy, and the degree had lessened as she aged, but showed exacerbation in the summer season. Small spots of striking polygonal hyper-pigmentation were also seen all over the trunk and extremities. It was the patient's statement that most of the pigmented spots were not the result of apparent blistering, although some of the pigmentation on the extremities seemed to be residual marks of blistering. On the abdomen and back, whitish spots were also seen, and these disturbed color tone giving an impression of dirty skin (Fig 1*C*). There were no other abnormalities detected on laboratory investigation, or examinations of bone structure and intelligence, but relatively small toenails, especially of fourth and fifth toes were noted. The histology of the blister revealed subepidermal separation with degeneration of the basal cells (Fig 1*D*), and that of the pigmented spot was vacuolization degeneration of the basal cells and incontinence of pigmentation in the upper dermis (Fig 1*E*). Ultrastructural examination of tissues adjacent to a blister did not detect any remarkable changes in the epidermis; normal arrangement of tonofibrils and absence of the clumping. On the other hand, a sample obtained from a pigmented spot demonstrated liquefaction degeneration-like vacuolization in the lower keratinocytes, deformed structure of epidermal–dermal junction, and protrusion of the cytoplasm of vacuolized keratinocytes through discontinued basal lamina. The accumulated melanosomes were detected in some lower epidermal cells. No remarkable abnormalities of individual tonofibril bundles were seen (Fig 1*F–H*).

Abbreviations: EBS-MP, epidermolysis bullosa simplex with mottled pigmentation; K5, keratin-5

Case 2 A 22-y-old female, a cousin of case 1 presented with a distribution of tiny pigmentation spots resembling

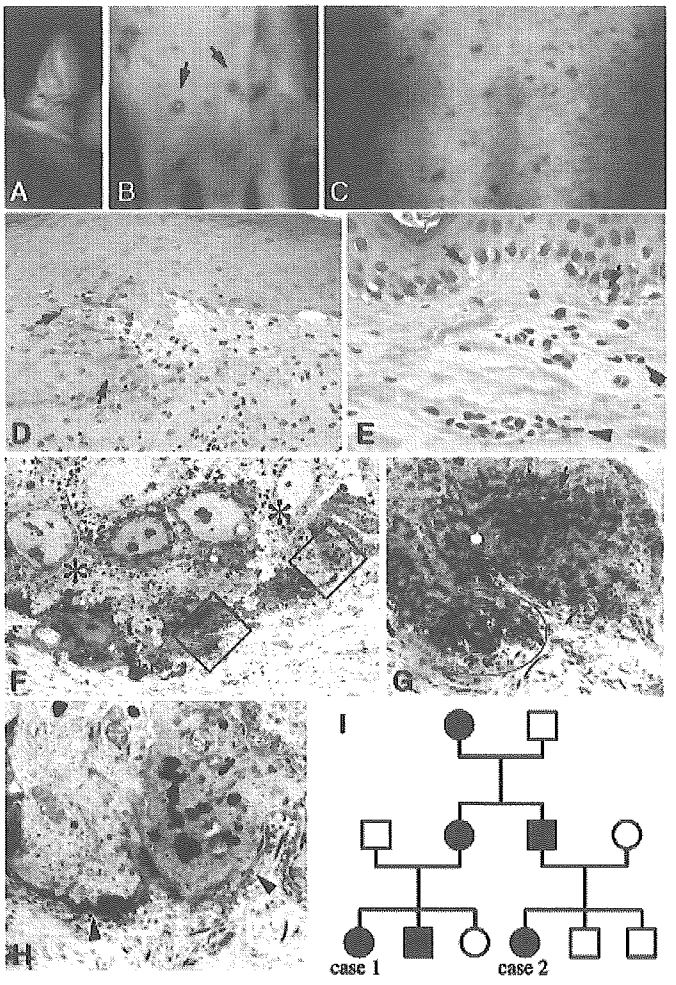

Figure 1
Clinical appearance, histology and family pedigree of the cases of epidermolysis bullosa simplex with mottled pigmentation. (*A*) A ruptured blister on the fourth toe. (*B*) Tiny blisters (*arrows*) on the left dorsal hand leaving faint pigmentations. (*C*) Dark brown-pigmented spots and whitish hypo-pigmented macules were distributed on the lower back. These spots were not residues of the blistering. (*D*) Histology of the blister showed subepidermal cleavage with degenerated basal cells (*arrows*). (*E*) Histology of the pigmented spot was vacuolization degeneration of the basal cells (*arrows*) and incontinence of pigmentation of the dermis (*arrowheads*). (*F*) Ultrastructural examination of the pigmented spot disclosed liquefaction degeneration-like vacuolization in the lower keratinocytes (*asterisks*). (*G*) Enlarged photograph of the left rectangular area of (*F*) presents intracytoplasmic hemidesmosomes (*arrows*), and indistinct hemidesmosomes and lamina demsa, indicating deformed structures of epidermal–dermal junction. (*H*) Enlarged photograph of the right rectangular area of F demonstrates protrusion of the cytoplasm (including clumped melanosomes) of vacuolized keratinocytes (*asterisk*) through the discontinuous basal lamina (between the arrowheads). (*I*) Pedigree of the family. The mother and younger brother of case 1, the father of case 2, and the grandmother were affected.

those of case 1. This patient had demonstrated blistering in her youth, but remarkable blisters had seldom recurred in recent years. Although blistering and pigmentation were less severe than those in case 1, the toenails were smaller than those of case 1. The mother of case 1 and the father of case 2, who were siblings, a younger brother of case 1, and their grandmother also demonstrated blistering and pigmentation (Fig 1*I*).

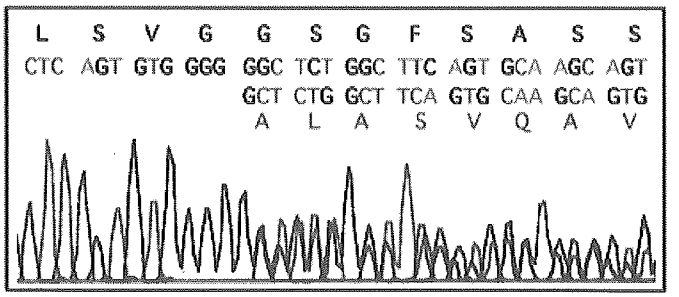

Figure 2
Mutation analysis of the epidermolysis bullosa simplex patients. The sequence analysis of the PCR fragments discloses a heterozygous deletion of 1649th guanine in exon 9 of KRT5 (*lower case*), leading to a frameshift and delayed termination codon (*upper case*).

PCR Amplification and DNA Sequence

The following experiments were performed under the approval of medical ethical committee of the Osaka Red Cross Hospital, and were conducted according to Declaration of Helsinki Principles. Total genomic DNA was extracted from peripheral blood lymphocytes of both cases (obtained under written informed consent and permission), then subjected to mutation screening of the genes encoding K5 and K14, KRT5 (GeneBank Accession no. M21389) and KRT14 (GeneBank Accession no. J00124). The segments of the KRT5 and KRT14 genes including all exons and all exon–intron borders were amplified by PCR using pairs of oligonucleotide primers synthesized according to the previous report (Sorensen *et al*, 1999). For PCR amplification, approximately 200 ng of genomic DNA, 40 pmol of each primer, 0.5 mM $MgCl_2$, 20 μmol of each dNTP, and 1.25 U of Taq polymerase were used in a total volume of 50 μL. The amplification conditions were 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 56°C–62°C for 45 s, and 72°C for 45 s, and extension at 72°C for 10 min in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California). The PCR products were subjected to automated nucleotide sequencing in an ABI 3100 genetic analyzer (Perkin Elmer, Warrington, UK).

We could not find any potential pathogenic mutations in KRT14 gene. Although sequence analysis of exons 1–8 of KRT5 did not demonstrate any mutation, analysis of exon 9 of KRT5 demonstrated a heterozygous deletion of a G nucleotide at position 1649 (1649delG, Fig 2). As a result of the 1649delG mutation, the carboxyl-terminal domain is predicted to have been replaced by an aberrant sequence of 76 amino acids very rich in alanine and proline residues that is 35 amino acids longer than the wild type.

Discussion

Every keratin protein has a central α-helical rod domain, which is flanked on each end with non-helical amino- and carboxyl-terminal domains. The rod domains play a crucial role in intermediate filament assembly and organization, whereas non-helical domains, head, and tail domains, are thought to be less functional (Steinert *et al*, 1993). In review of previous reports, all the cases of Dowling–Meara and Koebner types of EBS showed mutations in 1A or 2B or L12 domain of K5 or K14. Mutations in the H1 domain, however,

can lead to the Weber–Cockayne type of EBS, the mildest form of EBS (Chan *et al*, 1993; Erhlich *et al*, 1995; Muller *et al*, 1998), whereas mutation in the V1 domain in EBS-MP cases, the second mildest form of EBS. Our cases demonstrated that not only P25L mutation of the V1 domain, but also 1649delG mutation of the V2 domain leads to EBS-MP.

It is of interest that the 1649delG mutation described here is identical to the mutation seen in EBS with migratory circinate erythema (Gu *et al*, 2003), which shows very different clinical features from ours; migrating circinate erythema with vesicles. Although adult members in the Korean family reported demonstrated hyper- and hypo-pigmentation patches after blistering, the striking clinical features of their cases are circinate, band-like erythema with blisters seen in infancy, which were never seen in our cases. Recent investigations disclosed that the relationship between types of genomic mutation and clinical phenotypes is not definitive but very complex, and similar genomic mutations can show different subtypes of EBS (Hut *et al*, 2000). For example, the identical L463P mutation in K5 was demonstrated in subtypes of Weber–Cockayne (Nomura *et al*, 1997) and Koebner (Dong *et al*, 1992) in EBS. The mutations in V2 domain of a keratin family are usually small insertions/deletions leading to frameshift, which results in translation of an aberrant tail peptide (Sprecher *et al*, 2001; Whittock *et al*, 2002; Gu *et al*, 2003; Sprecher *et al*, 2003). These mutations are strongly associated with unusual phenotypes and contribute phenotypic heterogeneity of keratin disorders (Sprecher *et al*, 2003). Of the previously reported V2 mutations only two, 1635delG (Sprecher *et al*, 2003) and 1649 delG (Gu *et al*, 2003) were in KRT5. Characteristic feature of the case with 1635delG was marked improvement of the skin fragility with age, while that of the case with 1649 delG was circinate erythema and the resulting brown pigmentations. Interestingly, the present case was harboring 1649-delG mutation. The differences in the clinical features between our cases of EBS-MP and cases of EBS with migratory circinate erythema cannot be explained by the disease severity, suggesting the presence of complicated mechanisms underlying the pathogenesis of EBS.

The liquefaction degeneration-like vacuolization of keratinocytes, specifically observed in pigmented spots in our cases, may elucidate the mechanism of formation of mottled pigmentation. Such findings of degeneration have already been reported in the literature. Prominent vacuolization of the basal cells with colloid bodies formation and discontinuous, as well as multiplied lamina densa (Coleman *et al*, 1993), and cytoplasmic projection of the basal cells through interrupted lamina densa (Bruckner-Tuderman *et al*, 1989) were observed in cases of EBS-MP, in which there were no DNA analyses performed. In the latter case, the pigmentation manner bears resemblance to our cases: distinct polygonal hyper-pigmentation. Recently, a Japanese family with EBS-MP were confirmed to have P25L mutation of K5 and showed basal vacuolization similar to our findings (Hamada *et al*, 2004). These findings, including those in our cases, suggest that the integrity of the dermal–epidermal junction is damaged. Dyschromatosis of EBS-MP, at least of some cases, may be because of subclinical disturbance of the junctional structures, the basal cells and the lamina densa.

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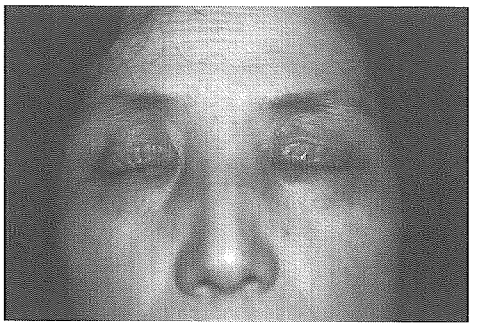

Fig 1. Facial lesions following the lactose oral challenge.

flatulence, and gas after drinking milk. Based on the clinical course, results of challenge tests, and family history, we diagnosed her fixed food eruption as being caused by lactose.

Lactose is a constituent of numerous foodstuff and orally administered medicines. Although there are many reports of intestinal and airway reaction to lactose, there are only two cases of cutaneous involvement: generalized eczema in a patient with lactose intolerance[1] and fixed drug eruption in a patient injected with botulinum toxin containing lactose.[2]

The patient reported here showed typical clinical appearance for fixed eruption. Fixed eruptions are defined as recurrent circumscribed pigmented lesions in the same site or sites after exposure to the causative agent.[3] Derbes[4] demonstrated that most of the causative factors were drugs, and termed "fixed drug eruption." Moreover, fixed eruption has also occurred after nondrugs. For example, ingestion of cheese crisp,[5] strawberry,[6] tartrazine-containing food,[7] lentil,[8] and tonic water[9] has also been reported. In such cases, "fixed food eruption," which Kelso[6] coined in 1996, should be an appropriate term. As a nondrug, lactose was the causative agent in our patient, and as the patient already noticed symptoms after digestion of dairy products before administrating drugs, we believed we should diagnose her as having fixed food eruption rather than fixed drug eruption.

The precise mechanism of fixed eruption remains elusive, but it is considered to be an allergic reaction or genetic disorder. In our case, because her family members had lactose intolerance, a genetic predisposition seems to be involved, similar to the case reported by Hatzis et al.[5]

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Focal palmoplantar callosities in non-Herlitz junctional epidermolysis bullosa

To the Editor: Hereditary palmoplantar keratodermas are usually linked to gene mutations of structual proteins in the epidermis, including many types of keratins, loricrin, and desmosomal cadherin.[1,2] Among 3 major categories of inherited epidermolysis bullosa (EB) classified on the basis of the ultrastructural level of skin cleavage (epidermolytic, junctional, and dermolytic), keratoderma of the palms and soles is characteristic of epidermolytic EB or EB simplex that is associated with keratin gene mutations.[3] We report a case of non-Herlitz junctional EB (JEB-nH) presented with focal palmoplantar callosities.

A 41-year-old man had a blistering disorder associated with hair and nail changes since shortly after birth. The blisters were precipitated by trivial trauma and healed with atrophy and scarring. On physical examination, generalized blisters and erosions were noted, associated with erythema and atrophy. A patch of atrophic alopecia was present at

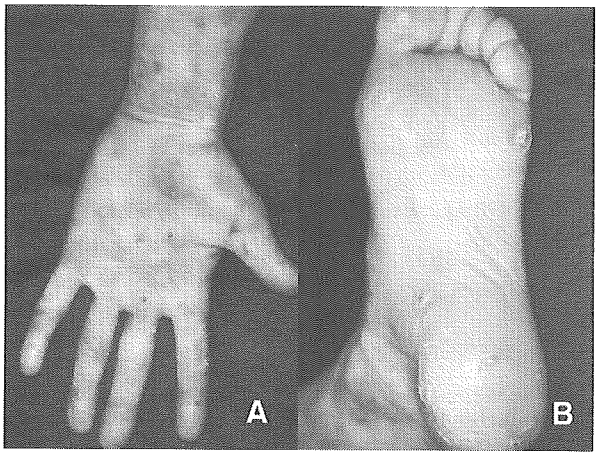

Fig 1. Focal callosities on palms (**A**) and soles (**B**).

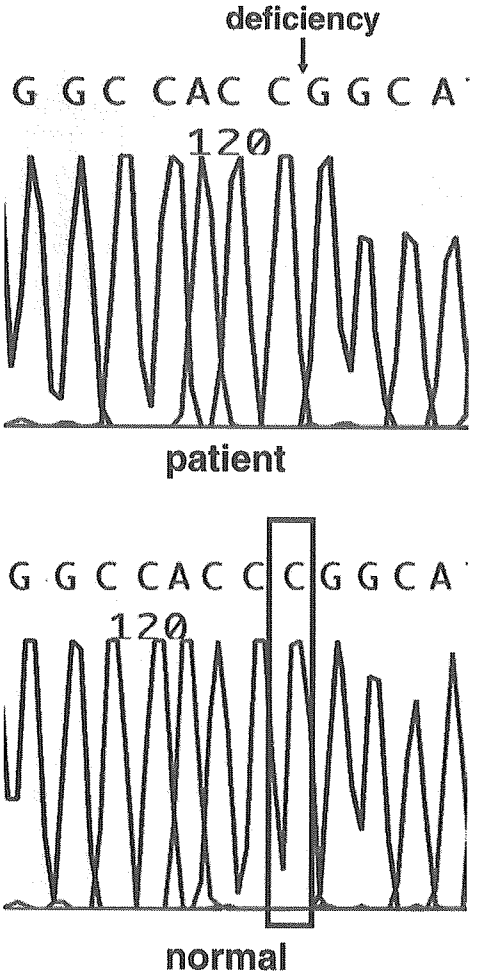

Fig 2. Direct sequencing of type XVII collagen gene (COL17A1). Proband was homozygous for mutation of 4335delC, in exon 53 of COL17A1, resulting in stop codon.

frontal area of the scalp. Most of the 20 nails were either dystrophic or absent. Pitting of teeth was noted. Of note, he had marked focal callosities on his palms and soles (Fig 1). Focal callosities first developed when he started to walk and slowly progressed throughout his life. Blistering on the palms and soles preceded and accompanied the formation of callosities. He worked as a clerk and had no history of occupationally traumatizing his hands or feet. A biopsy specimen from the edges of blisters showed subepidermal blistering without an inflammatory infiltrate. In immunofluorescence mapping studies type XVII collagen (bullous pemphigoid antigen-2) was absent at the basement membrane zone; laminin-5, bullous pemphigoid antigen-1, $\alpha 6$ integrin, $\beta 4$ integrin, plectin, type IV collagen, and type VII collagen were present. An electron microscopic study demonstrated dermoepidermal separation within the lamina lucida between the plasma membrane of the basal keratinocytes and the lamina densa. The majority of the hemidesmosomes were hypoplastic with thin attachment plaques and the absence of a subbasal dense plate. The shape and the number of the anchoring fibrils appeared normal. Analysis of the type XVII collagen gene (COL17A1) identified a homozygous mutation resulting in premature stop codon (Fig 2), indicating a definite diagnosis of JEB-nH.

As delineated above, some types of palmoplantar keratodermas are caused by mutations in keratins expressed in the ridged skin. Keratins K5 and K14 are expressed in basal keratinocytes all over the body, including palmoplantar areas, and keratins K1 and K10 are in suprabasal keratinocytes. Mutations in those keratins epidermolytic EB with focal or widespread callosities on palms and soles are common.[2,3] Furthermore, the epidermis of ridged skin expresses a more complex pattern of keratins than thin skin, including K6a, K6b, K16, K17, and K9, probably as an adaptation to greater stress experienced by this area of the skin.[2] Mutations in keratin K9 cause keratoderma strictly confined to the ridged skin. Because keratins K6a, K6b, K16, and K17 are predominantly expressed both in palmoplantar regions and nails, mutations in those keratins lead to pachyonychia congenita featured by hyperkeratosis of the ridged skin and nail dystrophy.[2] At present, it remains to be elucidated as to which precise biomechanisms cause palmoplanter keratoderma in junctional EB that is ascribed to mutations in type XVII collagen or laminin-5, namely proteins composing hemidesmosomes. However, at least 3 cases of JEB-nH presenting with palmoplantar callosities has been described in the literature (first case by Hashimoto in 1976,[4] case 2 by Paller et al,[5] and figure 40.17 in Rook-Wilkinson-Ebling Textbook of

Dermatology[6]), suggesting the coexistence of keratoderma in the ridged skin and JEB-nH is more than a coincidence. Focal palmoplantar callosities, if present, could be regarded as one of the clinical features of JEB-nH and might be useful for differential diagnosis in the early infantile period (as with this case). This is the first example of mutations in genes involving keratins that could produce, or at least be associated with, the formation of hyperkeratosis.

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NOTES & COMMENTS

Determine alpha-1 antitrypsin level and phenotype in patients with neutrophilic panniculitis

To the Editor: We read with interest the report in the February 2004 issue of the *Journal* by Sutra-Loubet et al entitled "Neutrophilic panniculitis."[1] We have seen several similar clinical presentations in our clinic. We often assess serum levels and phenotype determination of alpha-1 antitrypsin (AAT) in the evaluation of ulcers or plaques with histologic characteristics of lobular neutrophilic panniculitis. Phenotype determination is essential, as a normal level of a variant enzyme with altered activity can lead to clinical disease. More than 90 mutant alleles of the AAT gene are known; alleles are grouped into 4 categories based on acid starch gel mobility (F = fast, M = medium, S = slow, and Z = very slow).[2] While the ZZ and MZ variants are most often associated with cutaneous lesions, MS, SS, and SZ variants can result in panniculitis; serum levels are often normal in these instances. Our report of two patients with MS-variant panniculitis and review of 41 previously reported cases of AAT-associated panniculitis is forthcoming.[3] Similar to the cases reported by Sutra-Loubet et al, AAT-related panniculitis tends to be steroid responsive and tends to improve with dapsone or doxycycline. Determination of both the level and phenotype of the AAT status of the patient reported by Sutra-Loubet et al might be informative.

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Epidermolysis Bullosa Simplex Associated with Pyloric Atresia Is a Novel Clinical Subtype Caused by Mutations in the Plectin Gene (*PLEC1*)

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Epidermolysis bullosa (EB) is an inherited mechanobullous disorder of the skin, and is divided into three major categories: EB simplex (EBS), dystrophic EB, and junctional EB (JEB). Mutations in the plectin gene (*PLEC1*) cause EBS associated with muscular dystrophy, whereas JEB associated with pyloric atresia (PA) results from mutations in the α6 and β4 integrin genes. In this study, we examined three EB patients associated with PA from two distinct families. Electron microscopy detected blister formation within the basal keratinocytes leading to the diagnosis of EBS. Surprisingly, immunohistochemical studies using monoclonal antibodies to a range of basement membrane proteins showed that the expression of plectin was absent or markedly attenuated. Sequence analysis demonstrated four novel *PLEC1* mutations. One proband was a compound heterozygote for a nonsense mutation of Q305X and a splice-site mutation of 1344G→A. An exon-trapping experiment suggested that the splice-site mutation induced aberrant splicing of the gene. The second proband harbored a heterozygous maternal nonsense mutation, Q2538X and homozygous nonsense mutations R1189X. Analysis of the intragenic polymorphisms of *PLEC1* suggested that R1189X mutations were due to paternal segmental uniparental isodisomy. These results indicate that *PLEC1* is a possible causative gene in this clinical subtype, EBS associated with PA. Furthermore, two patients out of our three cases died in infancy. In terms of clinical prognosis, this novel subtype is the lethal variant in the EBS category. (*J Mol Diagn *2005, 7:28–35)

Epidermolysis bullosa (EB) comprises a group of genetically determined skin fragility disorders characterized by blistering of the skin and mucous membrane. EB has traditionally been divided into three main categories on the basis of the level of tissue separation within the cutaneous basement membrane zone (BMZ); tissue separations in EB simplex (EBS), dystrophic EB and junctional EB (JEB) occur in the basal keratinocytes, the dermis, and the lamina lucida of basement membrane, respectively (Table 1).[1] Recent advances in EB research, have allowed the identification of mutations in 10 different genes, which account for the clinical heterogeneity in EB (Table 1).[2] Dominantly inherited EBS results from mutations in the basal keratinocyte-specific keratin 5 and 14 genes, whereas mutations in the plectin gene (*PLEC1*) can cause recessive EBS complicated by muscular dystrophy (EBS-MD). Dystrophic EB is characterized by severe blistering and scarring, and is due to mutations in the type VII collagen gene. The defective genes in JEB include the three genes encoding for the laminin 5 chains, the type XVII collagen gene, or the genes encoding the hemidesmosome-associated integrin α6 and β4 subunits (*ITGA6* and *ITGB4*).

The subtype of JEB involving α6 and β4 integrins is associated with congenital pyloric atresia (PA).[3,4,5] Staining with specific antibodies to the α6 or β4 integrin subunits in the JEB-PA skin reveals reduced or absent staining.[6,7] JEB-PA is usually lethal, but non-lethal variants have also been reported. The affected individuals with the lethal forms usually die within the first weeks or months after birth, whereas in the non-lethal variants, the clinical severity tends to improve with age.[3,5,8,9,10]

However, there have been several recent reports demonstrating EB cases associated with PA showing an intraepidermal level of cleavage consistent with the diagnosis of EBS.[11,12,13,14] In this study, we have encountered three

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Table 1. Epidermolysis Bullosa (EB) Classification and the Causative Genes[1]

Major EB type	Major EB subtypes	Involved genes/protein
EB simplex (EBS)	Dowling-Meara EBS	K5, K14
	Koebner EBS	K5, K14
	Weber-Cockayne EBS	K5, K14
	EBS with muscular dystrophy	Plectin
Junctional EB (JEB)	Herlitz JEB	Laminin 5
	Non-Herlitz JEB	Laminin 5, BPAG2
	JEB with pyloric atresia	$\alpha 6\beta 4$ integrin
Dystrophic EB (DEB)	Dominant DEB	Type VII collagen
	Hallopeau-Siemens recessive DEB	Type VII collagen
	Non-Hallopeau-Siemens recessive DEB	Type VII collagen

similar cases of EBS associated with PA that demonstrated an abnormal epidermal expression of plectin. Furthermore, we have identified novel four mutations, Q305X, 1344G→A, R1189X, Q2538X in the *PLEC1* gene of those cases. Thus, this study furthers our understanding of the possible range of pathophysiology in EB and of the biology of the cutaneous basement membrane.

Materials and Methods

Electron Microscopy

For electron microscopic examination, skin specimens were fixed in 5% glutaraldehyde and post-fixed in 1% osmium tetroxide, stained en-block in uranyl acetate. They were dehydrated in a graded series of ethanol solutions, then embedded in Araldite 6005 (NEM, Tokyo, Japan). Ultra-thin sections were cut, stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (H-7100, Hitachi; Tokyo, Japan) at 75kv.

Immunofluorescence Studies

Direct immunofluorescence analysis using a series of antibodies against BMZ antigens and cryostat skin sections was performed as described previously.[6,15,16] The following monoclonal antibodies (mAbs) against BMZ components were used: mAbs HD1-121, K15, 10F6 and 5B3 against the rod domain of plectin; mAbs GoH3 and 3E1 (Chemicon International, CA) against the $\alpha 6$ and $\beta 4$ integrins, respectively; mAb GB3 (Sera-lab, Cambridge, UK) against laminin 5 antibody; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen; mAb S1193 and HDD20 against BPAG1 and BPA2, respectively. The antibodies GoH3, S1193, and HDD 20 were kind gifts from Dr. A. Sonnenberg, the Netherlands Cancer Institute. The antibodies HD1-121 and K15 were kind gifts from Dr. K. Owaribe, Nogoya University.

Mutation Detection

Genomic DNA was obtained from both patients and the parents. The mutation detection strategy was performed after polymerase chain reaction (PCR) amplification of all exons and intron-exon borders, followed by direct automated nucleotide sequencing (Applied Biosystems, Foster City, CA). The genomic DNA nucleotides, the cDNA nucleotides and the amino acids of the protein were numbered based on the previous sequence information (GenBank Accession No. AH003623).[17] In particular, PCR amplification of exon 9, 12, 27, and one part of exon 32 was performed using following primers. Primers 5'-GTCGCTGTATGACGCCATGC-3'and 5'-TGGCTGGTAGCTCCATCTCC-3' for exon 9 produced a 387-bp fragment of the genomic DNA extending from g.2717 to g.3103, primers 5'-CCCACTCGCCTTAGGACAGT-3' and 5'-AAACCAACTCTGCCCAAAGC-3' for exon 12 synthesized a 428-bp fragment from g.3571 to g.3998, primers 5'-TTTCGAGGCTGGGGCTTCAT-3' and 5'-GCCTGGGTGATGGTGTGGTC-3' for exon 27 synthesized a 771-pb fragment from g.9681 to g.10451, and primers 5'-TCTGCTTTGGTGGGTGATGG-3'and 5'-AGCCTCTGGTTCTCCTCAGC-3' for a single part of exon 32 synthesized a 422-bp fragment from g.15326-g.15747. The PCR conditions of the amplification were 5 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 1 minute at 72°C. The informed consents both for studies and publication of the photographs were obtained from both families in this study.

Verification of Mutations

Each mutation was confirmed by restriction enzyme digestion of PCR products. The Q305X and 1395G->A mutations resulted in the loss of a restriction site for the *Pst*I and *Hph*I, respectively. The R1189X mutation caused the generation of a new restriction enzyme site for *Tsp45*I.

There was no proper restriction enzyme to verify the Q2538X mutation. PCR amplification was carried out using the following PCR primers, 5-TCTGCTTTGGTGGGTGATGG-3 and 5-CTCCAGCTTGGCCTTCTCCA-3 for generation of a 225-bp product. We changed the last base of the latter primer (underlined) from the original sequence, so that the combination of this change and the upstream sequence created a new *Alu*I site in the PCR product. Since the Q2538X mutation was also located just one base upstream from that primer, Q2538X abolished this *Alu*I site.

Exon-Trapping Experiments

Exon trapping system (Invitrogen, Carlsbad, CA) is an approach used for the direct isolation of transcribed sequences from genomic DNA.[18] To generate a *PLEC1* genomic fragment extending from intron 10 to intron 13, we synthesized two primers, 5′-AAACTCGAGGGCTGTCCCAGGTCTGGT-3′ and 5′-ATTGGATCCTGGGGCCGTGTGTACCTG-3′ which contained the following restriction enzyme sites, *Xho*I and *Bam*HI, respectively. PCR was performed using genomic DNA from the proband 1 as a template. The DNA fragment was digested with *Xho*I and *Bam*HI 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 1344G→A. The constructs were transfected into normal human epidermal keratinocytes using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (Roche Molecular Biochemicals). Total RNA was extracted from the culture cells and RT-PCR was performed using the trapping vector-specific oligonucleotide primers. The samples without transfection of the pSPL3 were used as controls. The PCR products were subcloned into a TA cloning vector pCR II (Invitrogen) and sequenced.

Results

Clinical Features of EB Associated with PA

The proband 1 was a male, born by spontaneous vaginal delivery after a 39-week gestation. His parents were unrelated and clinically normal. Pyloric atresia (PA) and polyhydramnios were suspected antenatally after ultrasound examination. Immediately after delivery, he presented with blisters and widespread ulcers on his trunk, genitalia, and legs. Findings from a routine abdominal radiograph led to a clinical diagnosis of EB associated with PA (Figure 1, A to C). Laparotomy revealed a severely distended stomach with a membrane at the pylorus. The membrane was excised and a pyloroplasty was performed. The histopathology of the septum showed re-epithelization of the epithelium, an atrophic lamina muscularis mucosae, and edematous submucosal tissue. He survived the operation, but still required intensive care at the age of 16 months. His elder brother, born 2 years before the proband 1, was also diagnosed as suffering from PA by routine abdominal X-ray. He manifested with multiple blisters and ulcers on his scalp, genitalia, and extremities, identical to those seen in the proband 1 (Figure 1, D to E). On the second day after birth, pyloroplasty and skin grafting were performed, but he later died of sepsis 4 months after birth.

The proband 2 was a female, born by induced vaginal delivery after a 36-week gestation, as the second child of non-consanguineous, healthy parents. She had a 1-year-old brother who was clinically unaffected. Antenatal ultrasonography had suggested a diagnosis of PA and

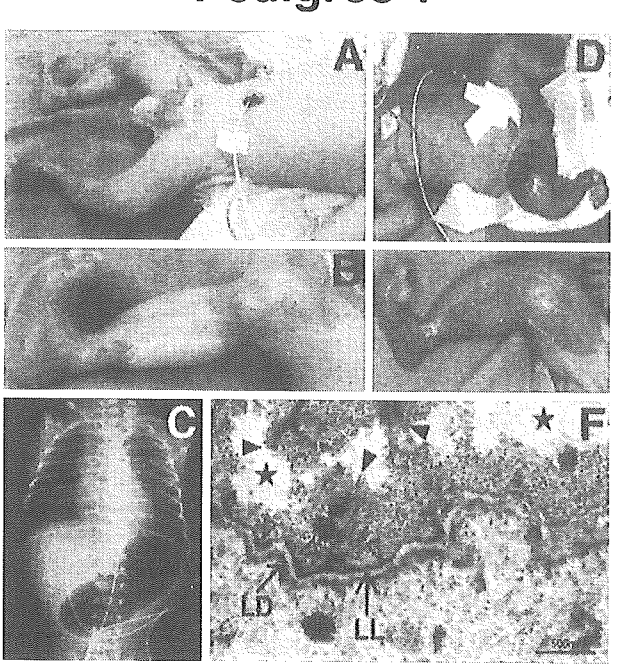


Figure 1. Clinical, X-ray, and ultrastructural findings of proband 1 in pedigree 1. **A:** Sharply demarcated erosions and ulcers on the trunk, genitalia, and lower extremity. **B:** Marked ulcers on the lower extremities. **C:** A single abdominal bubble of gas in an abdominal X-ray of proband 1. **D** and **E:** The same clinical manifestation of his elder brother. **F:** Electron microscopy of the skin from proband 1 shows tissue separation occurring at the base of the basal keratinocytes (**stars**). Keratin filaments are sparse and thin and not well associated with the hemidesmosome (HD) inner plaque (**full arrowheads**). Reduced numbers of hypoplastic HDs are recognized (**triangles**) and occasional HDs can be observed associated with thin bundles of keratin filaments within the basal keratinocyte (**open arrow**). The lamina densa (LD) and lamina lucida (LL) are present in the papillary dermis. **Bar**, 500 nm.

polyhydramnios. At birth, she presented with blisters and ulcers on the scalp, trunk, and extremities (Figure 2, A to D). Routine abdominal X-rays demonstrated a single abdominal bubble of gas, resulting in a clinical diagnosis of EB associated with PA. A laparotomy on the second day after birth showed a hugely distended stomach with a membrane at the pylorus. A gastroduodenostomy was subsequently performed. Histopathological analysis of the septum showed atrophic changes in general, normal appearance of the desquamative epithelium, and no increase in collagen fibers numbers. Due to her poor respiratory condition, she died of dyspnea at 31 days of age.

Skin Separation in Basal Keratinocytes

First, we examined the skin separation level within the epidermal BMZ. Electron microscopy of the skin samples from proband 1 revealed that the tissue separation was localized at the base of the basal keratinocytes and that hemidesmosomes (HDs) were reduced in frequency and hypoplastic (Figure 1F). In the skin sample from the proband 2 there was a reduction in HD numbers and many of the HDs appeared to be small or hypoplastic. The plane of separation was consistently within the basal keratinocyte, just above the HD inner plaque.[19] The ma-

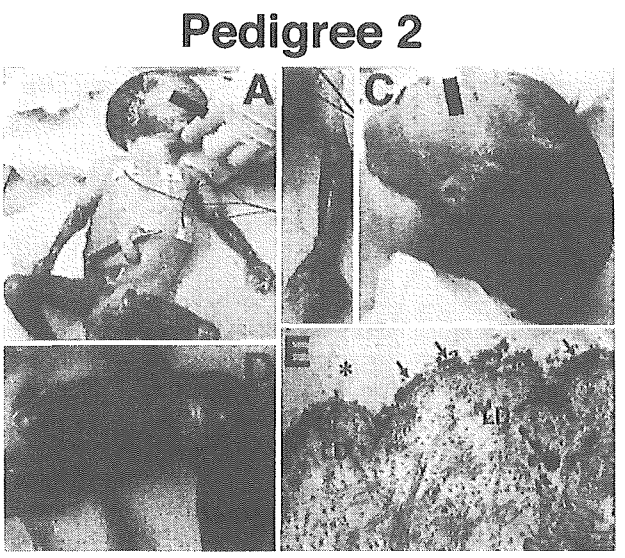


Figure 2. Clinical and ultrastructural findings of proband 2 in pedigree 2. **A:** Clearly demarcated blisters and ulcers on the scalp, abdomen, and extremities. **B:** Ulcers on the left arm. **C:** Localized erosions on the scalp. **D:** Ulcers on the left leg. **E:** Electron microscopy of the skin shows basal cell debris including flat electron densities (representing remnant hemidesmosome outer plaques (**arrows**) can be seen at the base of the intraepidermal split (**asterisk**). **Bar**, 1 μm.

jority of HD outer plaques remained attached to the underlying plasma membrane and extracellular electron-dense lamina densa. Keratin filaments within the basal cells appeared to form poor attachment to these rudimentary HDs, which remained firmly attached to the base of the separation (Figure 2E). The ultrastructural findings in both cases were consistent with these patients being classified as suffering from EBS.

Abnormal Expression of Plectin

An immunohistochemical study using monoclonal antibodies (mAbs) to a range of BMZ component proteins was performed (Figure 3). Immunoreactivity using the mAb HD1–121 against plectin was markedly attenuated in the proband 1 and completely lost in the proband 2. Analysis using other plectin mAbs K15, 10F6, and 5B5 revealed similar results, in which proband 1 showed markedly reduced expression while no immunoreactivity was detected in proband 2. Immunostaining for other BMZ proteins including the α6 and β4 integrins, laminin 5, type VII and IV collagens were normal (Figure 3). BPAG1 and BPAG2 also showed normal, bright, linear labeling of the BMZ in both probands (data not shown).

PLEC1 Mutations in Proband 1

Since abnormal expression of plectin was observed in both probands, we performed direct mutational analysis of the entire plectin gene. In this study, the cDNA nucleotides and the amino acids of the protein were numbered based on the previous sequence information (GenBank Accession No. AH003623).[17] Direct nucleotide sequencing of *PLEC1* from proband 1 demonstrated nonsense and splice-site mutations. The nonsense mutation was a C→T transition at nucleotide c.913 of cDNA in exon 9, resulting in the substitution of a glutamine (CAG) at position 305 with a stop codon (TAG) (Q305X). The splice-site mutation was a G→A substitution at nucleotide c.1344 in the cDNA that was located at the 3′ end of exon 12 (1344G→A) (Figure 4). The Q305X mutation was maternal and the 1344G→A mutation was paternal. These mutations were confirmed by restriction endonuclease analysis (Figure 4).

Since the splice site mutation of 1344G→A might just be a polymorphism, we examined 120 unrelated alleles as control and were unable to detected the same nucleotide change. Furthermore, we performed an exon-trapping experiment, as keratinocytes from the proband 1 or the parents were unavaliable. We inserted the genomic fragments with or without 1344G→A mutation into this vector, transfected these constructs into normal cultured human epidermal keratinocytes and prepared total RNA from the keratinocytes. We then synthesized cDNA, and amplified the extracted exons by PCR. Agarose gel electrophoresis showed strong 460-bp upper and weak lower 305-bp bands from the samples of the constructs containing the mutation, although a single 554-bp band was amplified from that construct without the mutation (data not shown).

Sequence analysis revealed that the upper 460-bp band contained exons 11 and 13 while the lower 305-bp band contained exon 11 alone. Conversely, exons 11, 12, and 13 were normally extracted from the DNA fragment without the mutation, resulting in a 554-pb band. Deletion of exon 12, which was 94 bp in size, caused a change in the mRNA coding frame and generation of a premature downstream stop codon. However, the combined size of exons 12 and 13 was 249 bp, so the deletion of these exons did not alter the coding frame and restored the translation of a polypeptide that was encoded by the downstream exons.

PLEC1 Mutations in Proband 2

Sequence analysis of the proband 2 revealed an unusual finding of both homozygous and heterozygous mutations. The homozygous mutation was a C→T transition at cDNA position c.3565 in exon 27, resulting in a substitution of an arginine (CGA) at residue 1189 with a stop codon (TGA) (R1189X). Conversely, the heterozygous mutation was a C→T transition at position c.7612 in exon 32, leading to an alteration of a glutamine (CAG) at 2538 to a stop codon (TAG) (Q2538X) (Figure 5). Nucleotide sequences of her parents disclosed that the father and mother were heterozygous for R1189X and Q2538X, respectively. These mutations were confirmed by restriction endonuclease analysis (Figure 5).

To eliminate a possibility that a sequence variation under one of the primers caused failure to amplify a maternal allele, we investigated by using a second set of primers for exon 27/intron 28. This also confirmed that the R1189X mutation was homozygous (data not shown).

Furthermore, comparison of 8 intragenic polymorphisms showed that the father and mother were heterozy-

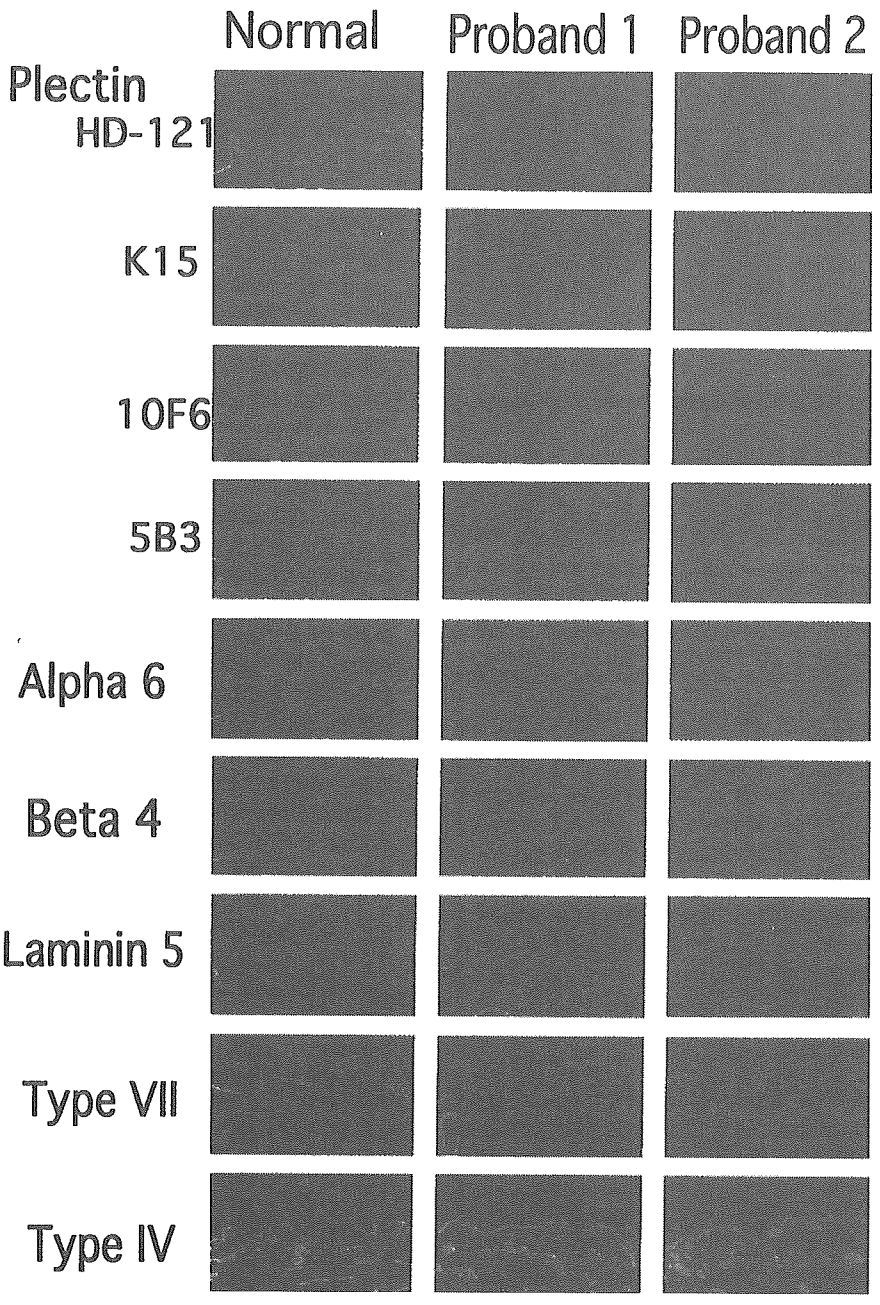


Figure 3. Immunofluorescence using antibodies against basement membrane zone components. Staining using HD1–121, K15, 10F6, and 5B3 monoclonal antibodies (mAbs) for plectin were markedly attenuated in the proband 1 and completely loss in the proband 2. Immunostaining for other proteins including $\alpha 6$ and $\beta 4$ integrins, laminin 5, type VII and IV collagens were normal in both probands and controls.

gous for 2 of 8 and 6 of 8 markers, respectively (Figure 6). The mutation R1189X and intron 28/10648 T→A were informative for the absence of a maternal allele in the proband 2. These results indicated that the mutations R1189X and their adjacent sequences were of paternal origin, suggesting possibility of segmental paternal uniparental isodisomy over this short area.

Discussion

Plectin, a component of the hemidesmosome (HD) inner plaque, is involved in the attachment and cross-linking of the cytoskeleton and intermediate filaments to specific membrane complexes.[20,21] Each monomer comprises a central rod domain of an α-helical coiled-coil structure and flanking amino-and carboxyl-terminal large globular domains.[22,23] It is widely distributed in almost all tissues and the exact function in each tissue is still unclear.[24,25] Although a family with EBS Ogna was reported to result from plectin mutation,[26] mutations in the plectin gene (*PLEC1*) generally cause EBS associated with muscular dystrophy (EBS-MD), which is characterized by generalized blistering, and a late-onset muscular dystrophy.[15,16,17,27,28,29]

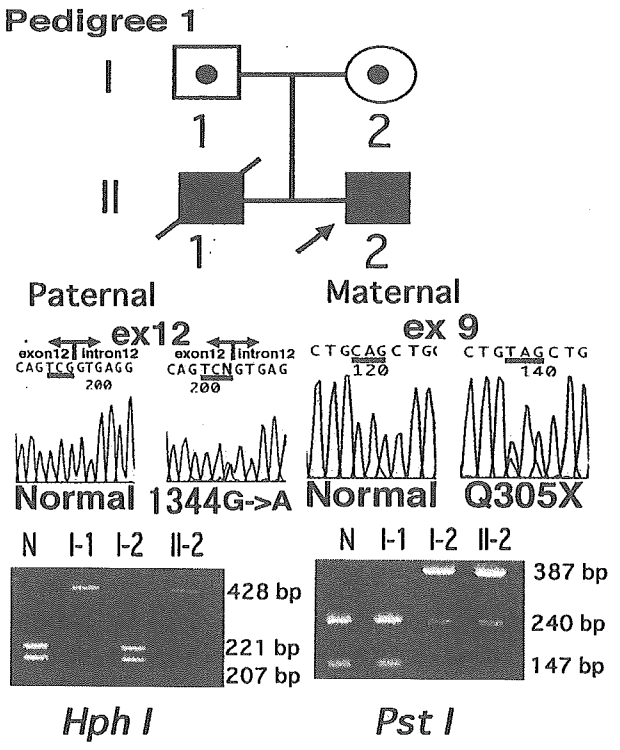


Figure 4. Mutation detection in pedigree 1. The proband harbored a G→A transition at position c.1344 in exon 12 within an intron-exon border (**middle-right panel**). He also possessed a heterozygous transition 913C→T (exon 9), leading to the substitution of glutamine 305 with a nonsense codon (Q305X). *HphI* digestion of the 428-bp fragment with and without the 1344G->A mutation product resulted in single band of 428 bp and double bands of 221 and 207 bp, respectively (**left panel**). The 387-bp PCR fragment containing the Q305X mutation was not digested by *PstI* whereas the digestion of the fragment without the mutation showed two bands of 240 and 147 bp (**right panel**). The 1344G→A mutation was paternal and the Q305X mutation was maternal.

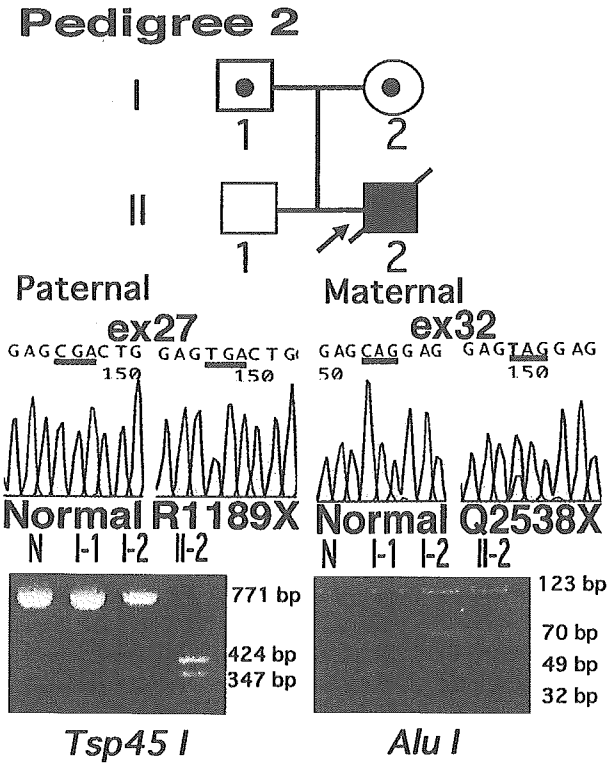
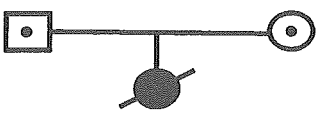


Figure 5. Mutation detection in pedigree 2. The proband 2 harbored a homozygous transition 3565C→T (exon 27) at codon 1189 producing a nonsense codon instead of arginine (R1189X) (**middle panel**). She also harbored a heterozygous C→T (c.7612) substitution (exon 32) in codon 2538, replacing glutamine with a nonsense codon (Q2538X). The R1189X mutation caused the generation of site for the *Tsp45I* restriction enzyme. The 771-bp PCR product with the mutation was digested by *Tsp45I* resulting in 424-bp and 347-bp bands (**left panel**). Since no proper restriction enzyme site was found around the Q2538X mutation, we changed one base of the PCR primer from the original sequence to create a site for *AluI* (see Materials and Methods). The digestion of 225-bp PCR product with Q2538X produced 70-bp band (**right panel**). The father and the mother are heterozygous for R1189X and Q2538X mutations, respectively.

The two probands in this study were both microscopically classified and clinically diagnosed as suffering from EBS associated with PA. We speculated at first, whether the causative mutations might lie in either *ITGA6* or *ITGB4* genes, known to cause PA-JEB. Surprisingly, however, the tissue separation was not within the lamina lucida as might be expected for JEB-PA, but was higher within basal keratinocyte, consistent with EBS. Immunohistochemical examination subsequently revealed abnormal plectin expression, leading us to speculate that mutations in *PLEC1* might be the underlying cause of this condition.

Indeed, mutational analysis detected novel nonsense and splice-site mutations on each *PLEC1* allele of the first proband (Figure 4). The splice-site mutation is thought to affect the splicing of the *PLEC1* gene, since the G nucleotide in the GT splice donor site in many human genes is relatively conserved within 78% of all splice junctions.[30] We first examined 120 unrelated alleles and could not detect the same nucleotide change. Furthermore, the exon-trapping experiments revealed that this mutation caused aberrant splicing of this region and suggested that this splice site mutation was likely to be pathogenic.

Immunofluorescence staining using the plectin antibodies, HD1–121, K15, 10F6, and 5B3, detected some plectin expression in proband 1 (Figure 3). HD1–121 recognizes multiple epitopes on the plectin rod domain.[31] In addition, the 10F6 and 5B3 epitopes were located in the central and carboxyl-terminal parts of the rod, respectively.[32] The 1344G→A mutation lies upstream of the region encoding the rod domain. As shown in the exon-trapping experiment, the presence of the low weaker band suggested some expression of the plectin



Exon 5/ 749 T→C	+	+	+	−	+	−
Intron 9/ 3023 C→G	+	+	+	−	+	−
Exon 15/ 4766 C→T	+	−	+	−	+	+
Intron 19/ 7407 T→C	+	+	+	−	+	−
Exon 27/ 10113(**R1189X**)	+	−	+	+	−	−
Intron 28/ 10648 T→A	+	+	+	+	−	−
Exon 31/ 12020 C →T	+	−	+	−	+	+
Exon 31/ 12271 T →C	+	+	+	−	+	−
Exon 32/ 15497(**Q2538X**)	−	−	−	+	+	−
Exon 32/ 15547 A →C	+	+	+	−	+	−

Figure 6. Detection of uniparental isodisomy. Comparison of 8 intragenic polymorphisms around the homozygous R1189 mutation showed that the father and the mother were heterozygous for 2 of 8 and 6 of 8 markers. The mutation R1189X and intron 28/10648 T→A were informative for the absence of a maternal allele in the proband.

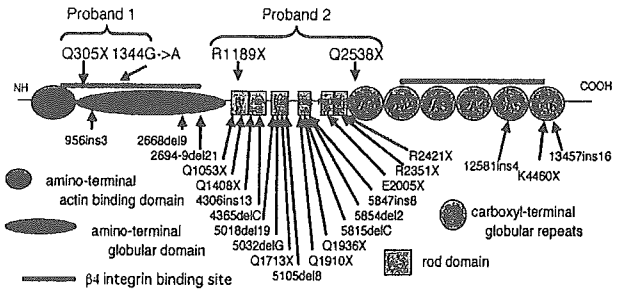


Figure 7. Database showing the position of mutations in *PLEC1*. Each functional domain is shown in the schematic model of plectin structure. The cDNA and the amino acids of the protein are numbered based on the previous sequence information (GenBank Accession No. AH003623).[17] Amino-terminal actin binding domain, amino-terminal globular domain, β4 integrin binding sites, rod domain, and carboxyl-terminal globular domain are shown.

rod domain, which is expected to be insufficient for the normal function of plectin.

Although we found nonsense mutations of both paternal and maternal origins in proband 2, it was unusual that R1189X mutation was homozygous. To further understand the mechanism causing this defect, we searched for several intragenic polymorphisms within the *PLEC1* gene (Figure 6). The results suggested that the R1189X mutations and their adjacent sequences had originated from one paternal allele. Uniparental disomy refers to the situation in which two copies of a chromosome come from the same parent.[33] The possibility of isodisomy can be hypothesized if two identical segments from one parental homologue are present in the offspring, whereas sequences of both homologues from the transmitting parent are present in the normal cause, heterodisomy. Angelman syndrome and Prader-Willi syndrome are examples of disorders caused by uniparental disomy.[34] This study indicates that the R1189X mutations and its adjacent sequences are likely to be derived from one paternal homologue and suggested possibility of segmental uniparental paternal isodisomy.

The next point we should discuss was how *PLEC1* mutation led to PA. We have reviewed the previously reported cases of plectin mutations in EBS-MD. In total, there were 22 mutations, of those, 16 mutations resided within the region encoding the rod domain (Figure 7). Of the 4 novel mutations from our cases, roughly, Q305X, 1344G->A, and Q2538X were not within the rod domain region and R1189X was in the very end of this domain. Plectin interacts with the cytoplasmic tail of β4 integrin via its carboxyl as well as amino-terminal domains (Figure 7),[23,35] and mutations in the β4 integrin gene result in PA. Therefore, we postulated that the development of PA in our cases is closely linked with the functions of plectin and the β4 integrin subunit. Interestingly, the mutations Q305X and 1344G→A in the first proband resided in the amino-terminal binding site for β4 integrin (Figure 7). However, the mutations of the second proband were not in the β4 integrin-binding site.

The level of tissue separation in EBS due to plectin gene mutations is different from that due to K5 or K14 gene mutations. In the latter cases there is a global cytolysis of the basal keratinocytes, while in case of the patients with *PLEC1* mutations the tissue separation is very low at the level of hemidesmosomes. Also, molecular consequences of deletion of the cytoplasmic domain of β4 integrin subunit or the type XVII collagen caused predominant features of EBS with intraepidermal separation.[36,37] Therefore, some investigators have proposed hemidesmosomal variants that include EBS with muscular dystrophy, JEB with pyloric atresia and generalized atrophic benign EB[2]. In this aspect, EBS with pyloric atresia in this study would belong to the variants.

Molecular mechanism of PA development even in JEB-PA has not yet been fully elucidated although phenotype and genotype information of this disease has been accumulated. Targeted ablation of both the *ITGA4* and *ITGB6* genes in mice clearly induced separation of the epidermis from the dermis as seen in the skin of JEB-PA.[38,39,40] In contrast, those model mice could not show straightforward evidence concerning mechanism of PA. Further studies are needed to clarify the pathogenesis of PA in all patients with EBS-PA as well as those with JEB-PA.

In this study, we have demonstrated for the first time that *PLEC1* mutations induce novel subtype of EB, EBS-PA. No definite conclusion has yet been reached regarding the occurrence of late onset muscular dystrophy phenotype of our three cases, since two patients have already died and one patient is still a neonate. However, this study furthers our understanding of the possible range of pathophysiology in EB and of the biology of the cutaneous basement membrane. In the previously reported four cases of EBS-PA,[11,12,13,14] two cases[11,12] also died shortly after birth although a search for plectin mutations were not carried out. In terms of clinical prognosis, this novel subtype EBS-PA is the lethal variant in the EBS category and will become a real target for gene therapy in the near future.

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Two cases of atypical melanocytic lesions in recessive dystrophic epidermolysis bullosa infants

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Summary

Atypical melanocytic lesions (AtML) are known to be associated with epidermolysis bullosa (EB), mainly with the junctional subtype. We report two cases of AtML in two female infants with recessive dystrophic epidermolysis bullosa (RDEB). Both lesions were dark brown- to black-coloured, asymmetric-shaped macules, 3–4 cm in size, with an irregular border and were located on the forearms of two unrelated, 1-year-old female infants. On a clinical and pathological basis, the pigmented macules were diagnosed as AtML in EB patients. There are only a few reports describing in detail the clinical and histopathological features of AtML in RDEB, especially in infant cases. AtML may easily be misdiagnosed as malignant melanoma and, even in infant patients with RDEB, this should be included as one of the differential diagnosis of pigmented lesions.

Report

Atypical melanocytic lesions (AtML) have previously been reported in epidermolysis bullosa (EB) patients, predominantly in the junctional subtype.[1–7] These lesions were described as clinically resembling malignant melanoma, although they were completely benign in nature. Only a few cases of AtML have been reported in recessive dystrophic type of EB (RDEB),[6,7] caused by mutations in the gene encoding type VII collagen (*COL7A1*).[8]

Case 1 was a 1-year-old Japanese female infant diagnosed as suffering from non-Hallopeau-Siemens RDEB from the ultrastructural and immunohistochemical features of her basement membrane zone which revealed markedly reduced staining with anti-type VII collagen antibody (LH 7.2). Mutation analysis revealed compound heterozygous type VII collagen mutations (*COL7A1* intron 81, 6576 + 1G → C; intron 109, 8109 + 2T → A). The former mutation was already known,[9] and the latter is to be reported elsewhere. She was a sporadic case and no other family members were affected.

She presented with a large pigmented lesion measuring 4 × 3 cm on her left forearm. The lesion was not present at birth but occurred at the end of the first year of life. It had irregular borders and a few small satellite lesions, and disappeared after two dye-laser treatments. However, the pigmentation recurred 3 months after the end of laser therapy (Fig. 1a). The dark brown- to black-coloured macule, 2.9 cm × 4.5 cm in size, was prominently asymmetric with irregular borders and had satellite lesions around it. We performed a punch biopsy from the skin lesion. Histopathological observations of haematoxylin & eosin and S-100 protein stained sections revealed an increased number of melanocytes in the basal layer of the lesional epidermis (Fig. 2). Neither dermal melanocytes nor nests of naevus cells were seen. We found no histopathological malignant features in the lesion. From these findings, we diagnosed the pigmented macule as AtML in an EB patient.

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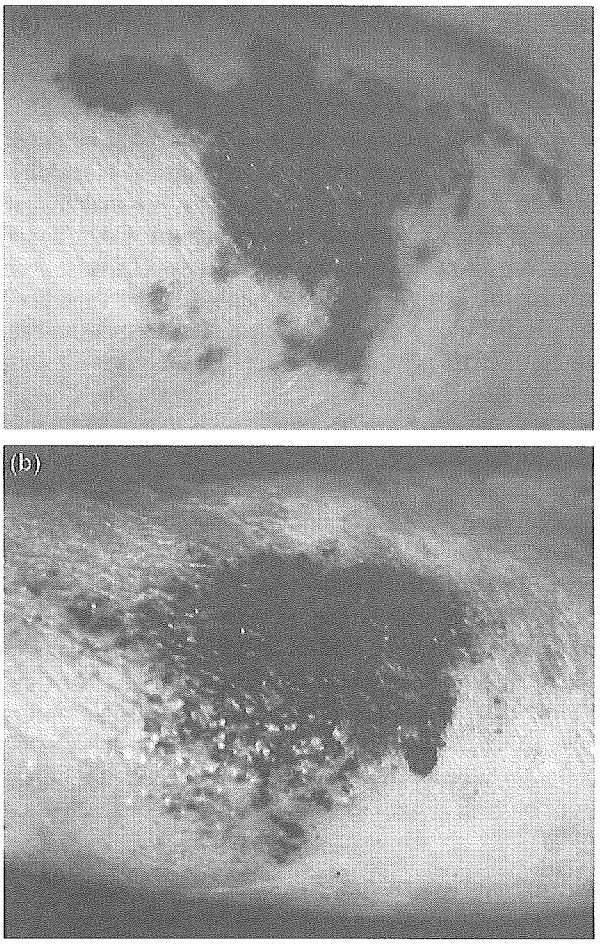

Figure 1 (a) Clinical features of case 1. A dark brown–black-coloured macule with irregular borders. Satellite lesions are also observed. (b) Clinical features of case 2. A dark brown–black-coloured macule with irregular borders.

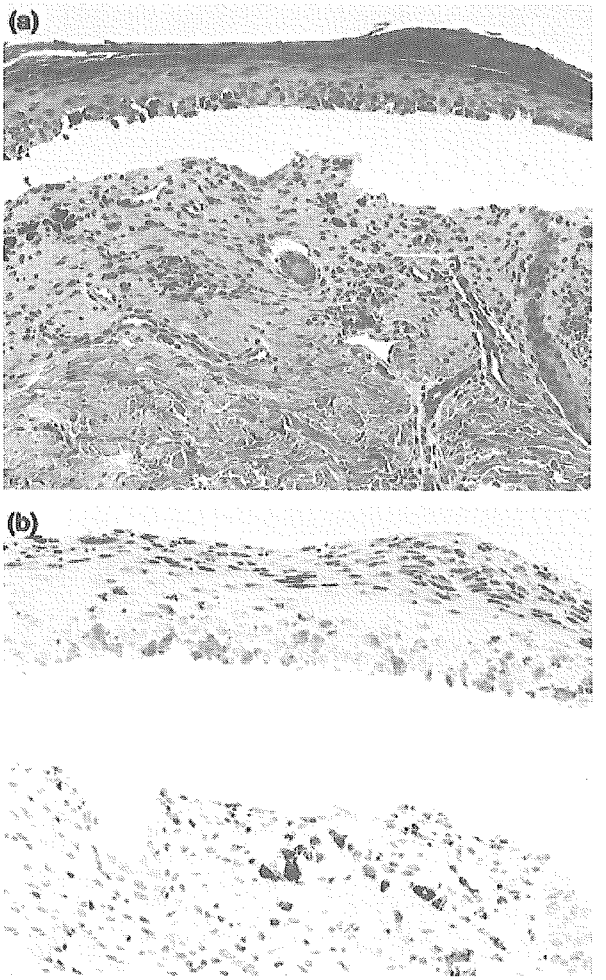

Figure 2 Histopathological features of case 1. (a) An increased number of melanocytes are scattered in the basal layer of the epidermis. Neither nests of naevus cells nor dermal melanocytes are seen (haematoxylin & eosin, original magnification ×200). (b) S-100 protein stain confirms the proliferation of melanocytes (original magnification ×250).

Case 2 was a 1-year-old Japanese female infant diagnosed as having Hallopeau-Siemens RDEB from her basement membrane zone ultrastructure and immunohistochemical features showing no labelling with LH 7.2. She was a sporadic case and no other family members were affected. She presented with a large pigmented lesion measuring 4 cm × 4 cm on her left forearm (Fig. 1b). The lesion was a black to brown macule with variegations in colour and with irregular borders. We rubbed the lesion, which led to the formation of a mechanical blister, and removed the roof of the blister corresponding to the epidermis in RDEB patients. The pigmented lesion disappeared completely after this simple mechanical procedure. At the light microscopic level, the pigmented lesion showed that abundant melanocytes were scattered throughout the basal layer of the lesional epidermis as seen in case 1 (data not shown).

Electron microscopic findings of the removed tissue showed a separation of the epidermis from the dermis immediately below the lamina densa of the epidermal basement membrane. The remnants of defective anchoring fibrils were seen with the epidermal layers (Fig. 3). Melanocytes showing no atypism, producing mature melanosomes were scattered in the lesional epidermis. Clusters of melanosomes were also observed in the cornified cell layer. From these observations, the pigmented lesion was diagnosed as AtML in an EB

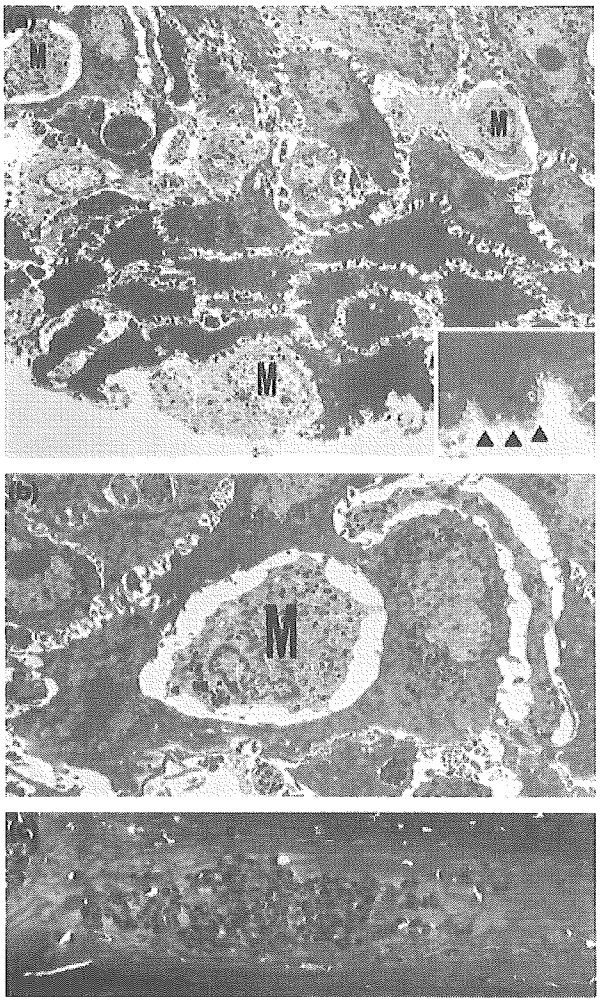

Figure 3 Electron micrograph of the pigmented plaque of case 2. (a) Subepidermal blister formation is seen. Melanocytes (M) are scattered in the epidermis. At the bottom of the epidermis, remnants of malformed anchoring fibrils are observed (inset, arrowheads) (original magnification, ×1300; inset ×6500). (b) A melanocyte (M) in the pigmented lesion contains a large number of mature melanosomes. Abundant melanosomes are also seen in the keratinocytes (original magnification, ×3200). (c) Clustered melanosomes (arrow) are found in the cornified cell layer (original magnification, ×6500).

patient. Neither recurrence nor metastases have been observed for 5 years after the operation.

To date, about 20 cases of AtML in EB patients have been reported,[1–7] while only a few cases of that lesion have been described in RDEB.[6,7]

Bauer *et al.*[7] proposed a classification of AtML in EB patients from the histopathological features. In the histological classification, AtML in EB were divided into two main types, the persisting naevus or pseudomelanoma, and the compound congenital naevus (in this report, we have used the term 'pseudomelanoma' not as a precursor of melanoma but as one histopathological subtype of benign pigmented lesions proposed by Bauer *et al.*).[7] In the former, an increased number of melanocytes in the basal layer were seen and the melanocyte proliferation was benign based upon histological criteria. In the latter type, nests of naevus cells were seen around the dermo-epidemal junction, in the superficial dermis and along the skin adnexa. The histopathologic findings of the present cases were both consistent with the former type.

While the exact age of onset of such lesions in EB was not clear, it has been thought that AtML in EB patients may develop during the later stages.[7] It has been hypothesized that recurrent blister formation leads to dissemination or the benign proliferation of melanocytes, resulting in AtML in EB.[6,7] In this context, the deep erosions and bullae formation in RDEB might contribute to the early onset of this type of AtML.

Our cases indicated that AtML can occur in infants of RDEB, and this should be included as one of the differential diagnoses of pigmented lesions seen in RDEB patients, in order to reduce the risk of clinical misdiagnosis of these lesions as malignant melanoma.

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Ultrastructural Features of Trafficking Defects Are Pronounced in Melanocytic Nevus in Hermansky–Pudlak Syndrome Type 1

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Hermansky–Pudlak syndrome (HPS) is an autosomal recessive disorder characterized by oculocutaneous albinism, a bleeding disorder, and ceroid lipofuscinosis in the lungs and gut. HPS is genetically heterogeneous and the most common variant, HPS type 1, is caused by mutations in *HPS1* gene. The protein encoded by *HPS1* is considered to facilitate the trafficking of melanocyte-specific gene products into the premelanosome. We report the ultrastructural findings in a melanocytic nevus seen in a 17-y-old Japanese female patient with HPS1 who is a compound heterozygote of *HPS1* mutations, including a novel mutation. Electron microscopy of a pinkish papule corresponding to the melanocytic nevus revealed markedly aberrant, immature melanosomes, large membranous structures, and giant melanosomes in the vicinity of trans-Golgi network, the characteristic abnormalities because of protein trafficking defects in *HPS1*. These ultrastructural features were far more clearly demonstrated in the nevus cells than in the epidermal melanocytes. Thus, ultrastructural analysis of nevus cells may be an additional diagnostic tool for HPS1 and could give us important clues to further understanding of the pathomechanisms of HPS.

Key words: Chediak–Higashi syndrome/melanocyte/vesicle formation
J Invest Dermatol 125:154–158, 2005

Hermansky–Pudlak syndrome (HPS; MIM 203300) is a rare, autosomal recessive disorder characterized by oculocutaneous albinism, bleeding diathesis, and lysosomal accumulation of ceroid lipofuscin. HPS was first described by Hermansky and Pudlak (1959). Mutations in seven genes, *HPS*1–7 have been identified to cause HPS in humans (Oh *et al*, 1996; Dell'Angelica *et al*, 1999; Anikster *et al*, 2001; Suzuki *et al*, 2002; Li *et al*, 2003; Zhang *et al*, 2003) and *HPS1* is the most common causative gene.

Proteins encoded by *HPS* genes are thought to be involved in trafficking of lysosome proteins and lysosome-related organelles, and abnormalities of lysosomes and other specific organelles, such as platelet dense granules, lung lamellar bodies, and melanosomes, have been observed during light and electron microscopic examination. Melanocytes in HPS contain predominantly premature melanosomes and abnormal, large membranous structures including tubulovesicular structures because of these trafficking defects (Witkop *et al*, 1973; Frenk and Lattion, 1982; Boissy *et al*, 1998; Husain *et al*, 1998).

In this study, we demonstrated ultrastructural features of the nevus cells from an HPS patient harboring one novel and one recurrent HPS1 mutations. The present findings seen in the nevus cells could provide us significant information for the diagnosis of HPS.

Abbreviations: CHS, Chediak–Higashi syndrome; HPS, Hermansky–Pudlak syndrome

Results

Clinical presentation The patient was a 17-y-old Japanese female. She was the second child of non-consanguineous, healthy parents. There was no family history of genodermatosis including albinism. She had one unaffected elder sister. She had presented with oculocutaneous albinism since birth and had a history of easy bruising. No infectious diathesis was noted. On physical examination, we noted that her skin was extraordinarily pale for a Japanese patient, and her hair was pale blonde (Fig 1*a*). Her eyes were pale red-brown. Ophthalmologic examination revealed mild horizontal nystagmus and slightly reduced visual acuity. A round, pinkish papule, measuring 5 mm in diameter, was seen on her neck (Fig 1*b*). The papule was resected and processed for morphological observation. Laboratory examination showed decreased platelet aggregation to collagen. Chest computed tomography demonstrated no evidence of interstitial pneumonitis. Gastrointestinal and colon endoscopies revealed no abnormality and granulomatous colitis was excluded.

HPS gene mutation analysis The mutation analysis for her *HPS1* gene revealed that the patient was a compound heterozygote for a novel mutation IVS6+1G>A (Fig 2*a*) (sequence according to GenBank accession No. AF450133) and a recurrent *HPS1* mutation IVS5+5G>A (Fig 2*b*), which has been reported in Japanese HPS patients (Oh *et al*, 1998; Horikawa *et al*, 2000). We previously reported