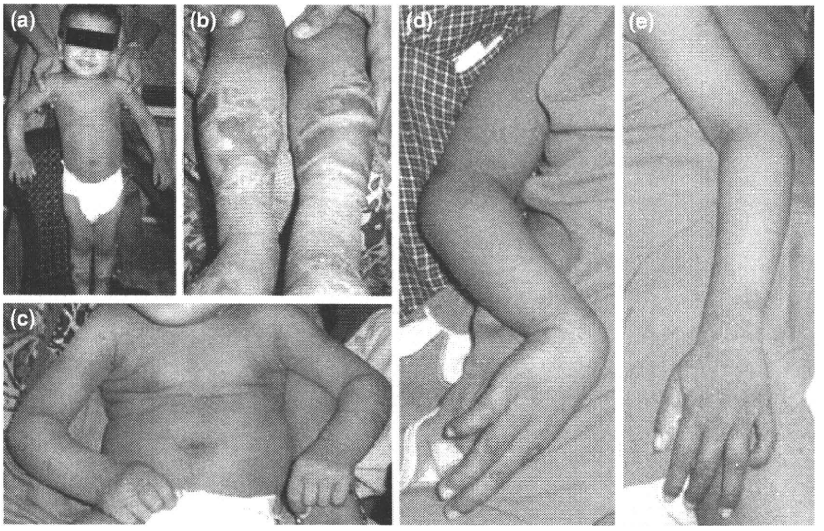

Figure 1 Clinical features of the Indian sisters with SLS. (a–c) The younger sister. Hyperkeratosis and scales cover whole body surface at 1.5 years of age (a). Dark brown scales are seen on the bilateral legs (b), the arms and the trunk (c). (d, e) The elder sister shows hyperkeratosis and brown scales on the bilateral arms at 4 years of age

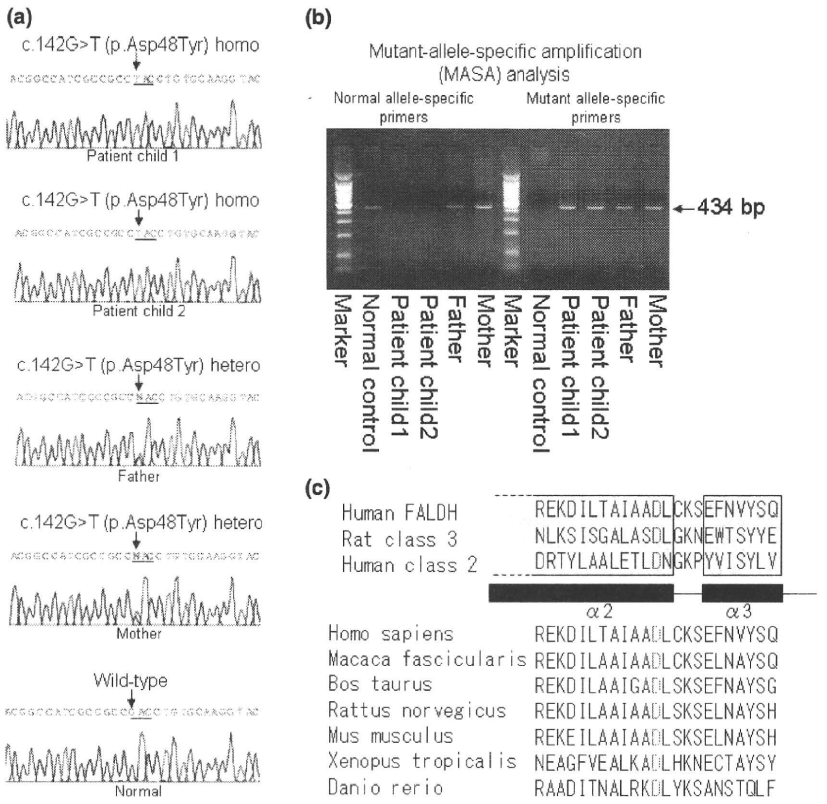

Figure 2 *ALDH3A2* mutation in the present SLS patients, and sequence alignments around the missense mutation. (a) Sequence analysis of *ALDH3A2*. In both patients, the younger sister (child 1) and the elder sister (child 2), a homozygous missense mutation c.142G>T (p.Asp48Tyr) in exon 1 derived from their parents was detected. The parents were heterozygous for the mutation. (b) Mutant allele-specific amplification analysis. With normal allele-specific primers, no amplification band is seen in the PCR products from the patients' DNA samples, suggesting that they have no normal allele. With mutant allele specific primers, the amplification band from the mutant alleles is detected as a 434-bp fragment in the PCR products from the DNA samples from the patients and their parents, and not in the PCR products from control DNA samples. This confirms the presence of the mutation c.142G>T in the patients. (c) Top: a sequence alignment between FALDH, rat class 3 and human class 2 ALDHs. Aspartic acid residue at codon 48 of FALDH is conserved. Secondary structure components found in the class 3 rat ALDH structure by Liu *et al.*[6] are presented with bars representing α-helices. Bottom: FALDH amino acid sequence alignment shows the level of conservation in diverse species of aspartic acid residue at codon 48 (D48) (red characters), which was altered by the missense mutation in the present family

tetraplegia.[3] In 1996, De Laurenzi *et al.*[4] reported that mutations in *ALDH3A2* underlie SLS. The present study reports a novel homozygous mutation in *ALDH3A2* in an Indian family with SLS.

The FALDH amino-acid sequence alignment shows that this aspartic acid residue at codon 48 is conserved among several diverse species. Compared with other aldehyde dehydrogenase (ALDH)-related sequences identified by Perozich *et al.*,[5] this aspartic acid is highly conserved among many members of the ALDH family (Fig. 2c). Analysis of the crystallized 3-D structure of the related class 3 rat cytosolic ALDH revealed that this aspartic acid is located in the C-terminal portion of the second α-helix strand, α2, of N-terminal four helices (Fig. 2c).[6] These findings strongly suggest that this aspartic acid residue is essential for the normal function of the FALDH. In the literature, missense mutation p.Ile45Phe in the α2 helix, three codons upstream of the present mutation site, was reported and the mutant enzyme was revealed to have only 9% residual enzyme activity compared with the wild-type enzyme.[7]

Until now, a number of mutations in *ALDH3A2* have been shown to be responsible for SLS in Europe, the Middle East, Africa, and North and South America.[1,7] However, in Asian populations, *ALDH3A2* mutations have been identified only in Japanese SLS patients.[1,2,8–10] Here, we report an *ALDH3A2* mutation for the first time in SLS patients in the Asian country other than Japan. The present results suggest that *ALDH3A2* is a gene responsible for SLS in Asian populations. Mutation analysis of the *ALDH3A2* gene is a highly sensitive method of confirming a diagnosis of SLS. It does not require a skin biopsy or FALDH enzymatic assays. We hope *ALDH3A2* mutation search will be globally available including many Asian countries in the future.

Acknowledgments

We thank Ms Akari Nagasaki, Ms Megumi Sato and Ms Yuki Miyamura for their technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M. Akiyama (Kiban B 20390304) and by a grant from Ministry of Health, Labour and Welfare of Japan (Health and Labour Sciences Research Grants; Research on Intrac\Diseases; H21-047) to M. Akiyama.

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

Letter to the Editor

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

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

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

Accepted for publication 20 July 2010

Background

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

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

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

Experimental design

Patients

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

Mutation detection

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

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

Haplotype analysis

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

Prenatal diagnosis

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

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

Results

Recurrent c.1938delC in *ITGB4*

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

Founder effects of c.1938delC

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

Prenatal exclusion of JEB-PA

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

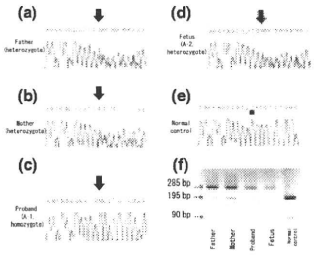

Figure 1. Prenatal diagnosis of junctional epidermolysis bullosa with pyloric atresia (family A). (a–e) Direct sequencing of *ITGB4*. The parents were heterozygous for c.1938delC in *ITGB4* (a, b). A-1, the proband, was homozygous for that mutation (c). A-2, the foetus, was found to be a heterozygous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

Conclusions

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

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

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

Acknowledgements

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

Conflicts of interest

The authors declare no conflicts of interest.

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Supporting Information

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

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

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DOI:10.1111/j.1600-0625.2010.01172.x
www.blackwellpublishing.com/EXD

Letter to the Editor

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

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

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

Accepted for publication 26 July 2010

Background

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

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

Question addressed

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

MUTATION IN BRIEF

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Plectin Deficiency Leads to Both Muscular Dystrophy and Pyloric Atresia in Epidermolysis Bullosa Simplex

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Communicated by Mireille Claustres

ABSTRACT: Plectin is a cytoskeletal linker protein which has a long central rod and N- and C-terminal globular domains. Mutations in the gene encoding plectin (*PLEC*) cause two distinct autosomal recessive subtypes of epidermolysis bullosa: EB simplex (EBS) with muscular dystrophy (EBS-MD), and EBS with pyloric atresia (EBS-PA). Previous studies have demonstrated that loss of full-length plectin with residual expression of the rodless isoform leads to EBS-MD, whereas complete loss or marked attenuation of expression of full-length and rodless plectin underlies the more severe EBS-PA phenotype. However, muscular dystrophy has never been identified in EBS-PA, not even in the severe form of the disease. Here, we report the first case of EBS associated with both pyloric atresia and muscular dystrophy. Both of the premature termination codon-causing mutations of the proband are located within exon 32, the last exon of *PLEC*. Immunofluorescence and immunoblot analysis of skin samples and cultured fibroblasts from the proband revealed truncated plectin protein expression in low amounts. This study demonstrates that plectin deficiency can indeed lead to both muscular dystrophy and pyloric atresia in an individual EBS patient. ©2010 Wiley-Liss, Inc.

KEY WORDS: basement membrane zone; skeletal muscle; mRNA decay; truncation

INTRODUCTION

Plectin is a 500-kDa protein of the plakin family, which interlinks different element of the cytoskeleton (Rezniczek, et al., 2010). Plectin is prominently expressed in muscle and in stratified and simple epithelia, including in the skin and gastrointestinal tract (Rezniczek, et al., 2010). In skin, plectin localizes to the inner plaque of the hemidesmosomes, at the site of interaction with intermediate filaments (Smith, et al., 1996). Plectin has a unique dumbbell-like structure with a central rod domain and N- and C-terminal globular domains (Wiche, et

Received 26 April 2010; accepted revised manuscript 7 July 2010.

DOI: 10.1002/humu.21330

al., 1991). Various types of plectin transcripts, including rodless ones, that do not encode for a central rod domain have been reported (Elliott, et al., 1997).

Epidermolysis bullosa (EB) comprises a group of heterogeneous congenital disorders characterized by dermal-epidermal junction separation. EB is subdivided into the three major groups of EB simplex (EBS), junctional EB and dystrophic EB, and the one minor group of Kindler syndrome, based on the level of blister formation (Fine, et al., 2008). So far, mutations in 14 different genes have been identified as underlying the EB subtypes (Fine, et al., 2008; Groves, et al., 2010). Among them, mutations in the gene encoding plectin, *PLEC* (MIM# 601282), have been known to be causal for two subtypes of autosomal recessive EBS (EBS with muscular dystrophy (EBS-MD) and EBS with pyloric atresia (EBS-PA)) and for one subtype of autosomal dominant EBS (EBS-Ogna) (Fine, et al., 2008).

Characteristic manifestations of EBS-MD are generalized skin blistering and late onset muscle weakness. Previous studies revealed defective expression of plectin in EBS-MD skin samples (Gache, et al., 1996; Shimizu, et al., 1999a; Shimizu, et al., 1999b) and mutations in *PLEC* in EBS-MD patients (McLean, et al., 1996; Pulkkinen, et al., 1996; Smith, et al., 1996; Takizawa, et al., 1999). The *PLEC* mutations detected in EBS-MD patients are mainly within exon 31, which encodes the large-rod domain of plectin (Natsuga, et al., 2010; Pfendner, et al., 2005; Sawamura, et al., 2007).

In contrast to patients with EBS-MD, those with EBS-PA typically develop a more severe phenotype that includes more generalized blistering and pyloric atresia (PA) (Nakamura, et al., 2005). The prognosis of EBS-PA is very poor, and affected patients usually die within months after birth (Nakamura, et al., 2005; Pfendner, et al., 2005; Pfendner and Uitto, 2005). *PLEC* mutations of EBS-PA were mostly located outside of exon 31 (Natsuga, et al., 2010).

Although both EBS-MD and EBS-PA are autosomal recessive EBS caused by *PLEC* mutations, the pathomechanisms distinguishing two subtypes were unclear. Recently, our group and others demonstrated that EBS-MD patients typically express a rodless plectin isoform, although the full-length plectin is absent (Koster, et al., 2004; Natsuga, et al., 2010). In contrast, both full-length and rodless plectin isoforms are deficient in EBS-PA patients, leading to the more severe disease phenotype (Natsuga, et al., 2010). In light of these findings, it has been postulated that EBS-PA patients could develop muscular dystrophy (MD) if they survived longer (Natsuga, et al., 2010). However, to our knowledge, there have been no EBS patients who suffered from both MD and PA.

Here, we report the first patient with EBS who developed both PA and MD. Both of the mutations identified in the patient were within the last exon (exon 32) of *PLEC*. Immunofluorescence and immunoblot analysis confirmed diminished and truncated plectin expression, using several antibodies against different domains of plectin. This study gives further insight toward improving our understanding of the genotype-phenotype correlation in EBS patients with *PLEC* mutations.

MATERIALS AND METHODS

Electron Microscopy

Skin biopsy samples were fixed in 2% glutaraldehyde solution, post-fixed in 1% OsO_4, dehydrated, and embedded in Epon 812. The samples were sectioned at 1 μm thickness for light microscopy and thin-sectioned for electron microscopy (70 nm thick). The thin sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

Mutation Detection

Genomic DNA (gDNA) was isolated from peripheral blood leukocytes of the proband and her parents. The mutation detection was performed after polymerase chain reaction (PCR) amplification of all *PLEC* exons and intron-exon borders, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The oligonucleotide primers and PCR conditions used in this study were derived from a previous report (Nakamura, et al., 2005). The gDNA nucleotides, the complementary DNA (cDNA)

nucleotides and the amino acids of the protein, were numbered based on the GenBank sequence information (accession no. NM_000445.3). PCR amplification of two parts of exon 32 was performed using the following primers. Primers 5'-GTGGAGACCACGCAGGTGTAC-3' and 5'-GGAGCCCGTGCCATAGAGG-3' for a single part of exon 32 synthesized a 420-bp fragment including c.10735 to c.11154. Primers 5'-AGCGGCTGACTGTGGATGAGG-3' and 5'-TGCGTGTCCTTGTTGAGGT-3' for another single part of exon 32 synthesized a 283-bp fragment including c.11230 to c.11512. Both of the mutations in the proband were confirmed by restriction digestion of PCR products. c.10984C>T and c.11453_11462del caused the generation of new restriction enzyme sites for *BsrI* and *BbvCI*, respectively.

The mutation nomenclature follows the journal's guidelines (www.hgvs.org/mutnomen) according to the reference sequence NM_000445.3, with +1 as the A of the ATG initiation codon.

Haplotype analysis

Genotype analysis of this family to establish the *de novo* nature of c.11453_11463del in the proband was performed using three chromosome 8 markers (D8S272, D8S264, D8S270) and six non-chromosome 8 markers (D1S468, D1S252, D1S2842, D3S1297, D3S1566 and D3S1311). All microsatellite markers (ABI Prism Linkage Mapping Set Version 2.5; Applied Biosystems, Warrington, UK) were amplified with fluorescently labeled oligonucleotides and used under conditions recommended by the manufacturer. Electrophoretic analysis was performed on an ABI Prism 310 Genetic Analyzer with Performance Optimized Polymer 4 (POP4) using GeneScan software (Applied Biosystems). The allele sizes were analyzed using Genotyper software (Applied Biosystems).

Immunofluorescence Studies

Immunofluorescence analysis was performed using skin specimens from the proband as previously described (Natsuga, et al., 2010). Briefly, fresh-frozen skin specimens were embedded in optimal cutting temperature (OCT) compound and quickly frozen in isopentane cooled over liquid nitrogen. 5-μm cryostat sections were incubated with primary antibodies. After washing in phosphate-buffered saline, the sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate.

Antibodies

The following antibodies against basement membrane zone (BMZ) components were used: monoclonal antibody (mAb) PN643 against the N-terminal actin-binding domain of plectin; mAb HD1-121 against the rod domain of plectin; C20 and mAb PC-815 against the C-terminal globular domain of plectin (Fig. 1A); mAbs GoH3 and 3E1 (Chemicon International, CA) against α6 and ß4 integrins, respectively; mAb GB3 (Sera-lab, Cambridge, UK) against laminin 332; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen; mAb PHM-12^{+}CIV22 against type IV collagen (NeoMarkers, Fremont, CA); and S1193 and mAb HDD20 against BP230 and type XVII collagen, respectively. mAbs PN643, HD1-121 and PC815 were generously donated by Prof. K. Owaribe of Nagoya University, and antibody S1193 by Prof. J. R. Stanley of the University of Pennsylvania. C20, a goat polyclonal antibody against the C-terminus of plectin, was purchased from Santa Cruz. Anti-beta-actin mAb (AC15, Sigma, St. Louis, MO) was used to confirm equal protein loading.

Cell Culture and Immunoblot Analysis

Cell culture and immunoblot analysis was performed as previously described (Natsuga, et al., 2010). Cultured fibroblasts were obtained from skin biopsies of a normal human volunteer and the proband. Cultured fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. For sample preparation, cultured cells were lysed in Nonidet-40 (NP-40) containing buffer (1% NP-40, 25mM Tris-HCl (pH 7.6), 4mM EDTA, 100mM NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF), and proteinase inhibitor

cocktail (Sigma, St. Louis, MO)); cell debris was removed by centrifugation; and the supernatant was collected. Supernatants were boiled in Laemmli's sample buffer (Laemmli, 1970), applied to a 4–12% gradient Bis-Tris gel (Invitrogen, Carlsbad, CA), and transferred to a PVDF membrane. The membrane was incubated with PN643, HD1-121, C20 and AC15 followed by incubation with horseradish peroxidase (HRP) conjugated anti-mouse IgG (for PN643, HD1-121 and AC15) and HRP-conjugated anti-gout IgG (for C20). The blots were detected using ECL Plus Detection Kit (GE Healthcare, Fairfield, CT).

Semi-quantitative RT-PCR Analysis

Semi-quantitative reverse transcription PCR (RT-PCR) analysis was performed as previously described (Natsuga, et al., 2010). Total RNA was isolated from cultured fibroblasts (from normal human volunteers and the proband, using RNeasy kit (Qiagen, Valencia, CA)), and first-strand cDNA was made using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). First-strand cDNA was then amplified by PCR with primers specific for the exon boundaries flanking the rod domain of plectin as described previously (Koster, et al., 2004; Natsuga, et al., 2010). The following primers were used (Fig. 1B): 30F, 5'-CATCAGCGAGACTCTGCGGC-3'; 31R, 5'-TGCGCCTGTCGCTTTTGTGC-3'; 31F, 5'-AGCTGGAGATGAGCGCTGA-3'; 32R, 5'-TGCTGCAGCTCCTCCTGC-3'. To ensure equal loading, a housekeeping gene (GAPDH) was simultaneously amplified. The PCR products were assessed on a 2% agarose gel. The images were obtained with LAS-4000 mini (Fujifilm, Tokyo, Japan).

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

RESULTS

Case Description

The proband was a first child of non-consanguineous Japanese parents. There was no family history of bullous diseases. He was born by cesarean section after a 39-week gestation because of non-reassuring fetal status. Clinically the proband showed extensive blistering and aplasia cutis on the extremities (Fig. 2A, B). Routine abdominal X-ray revealed a single bubble sign, suggesting the presence of PA (Fig. 2C). Generalized muscle hypotonia, dysphagia and difficulty in breathing were also observed from birth. Laboratory examination at birth revealed markedly elevated levels of creatine kinase (CK) (11,852U/L, normal value; 60-400U/L). The skeletal muscle isoform of CK (CK-MM) was 84% of total CK (CK, 2058U/L at age 12 days). Elevated levels of muscle enzymes including CK and aldolase (normal value; 1.7-5.7U/L) persisted over the course of his life (CK, 1924U/L; aldolase, 40.0U/L at age 25 days). Based on the clinical features and laboratory data, the presence of MD was confirmed. Muscle biopsy and reconstructive surgery for PA was not performed because the parents did not consent. The proband died 3 months after birth. Permission for autopsy was refused.

Skin Separation in Basal Keratinocytes

Electron microscopy of the skin samples from the proband showed that the skin separation localized to the base of the basal keratinocytes (Fig. 2D). Hemidesmosomes were hypoplastic and found at the base of the intraepidermal split (Fig. 2D). Keratin clumps were not observed.

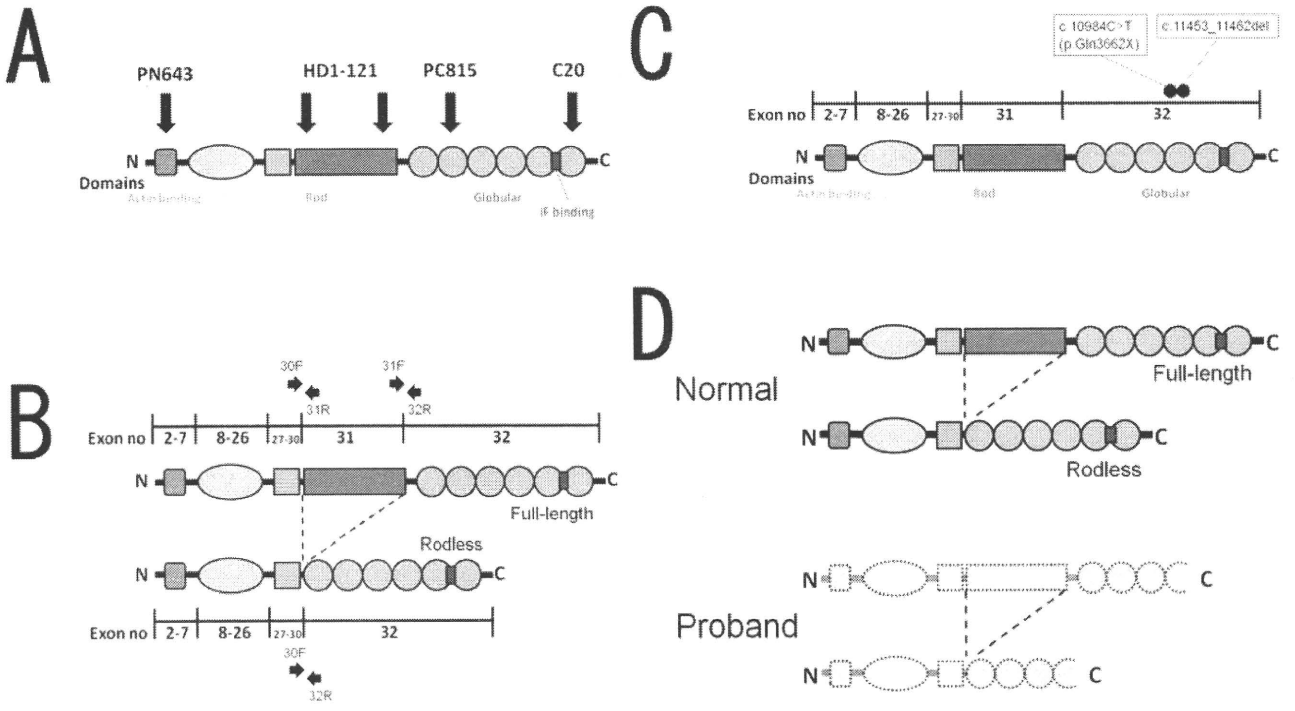

Figure 1. Plectin structure, antibodies against plectin, specific primers to amplify the full-length and the rodless plectin transcripts and *PLEC* mutations of the proband. (A) Plectin protein is composed of an actin-binding domain, N- and C-terminal globular domains, an intermediate filament (IF)-binding domain and a central rod domain. The C-terminal globular domain has 6 plectin repeat domains. The IF-binding domain is located between C-terminal repeats 5 and 6. PN643 is a monoclonal antibody (mAb) against the N-terminal actin-binding domain of plectin. HD1-121 is a mAb against the rod domain of plectin. PC815 is a mAb and C20 is a polyclonal antibody against the C-terminal globular domain of plectin. (B) The specific primers used to detect the presence of transcripts for full-length (30F/31R and 31F/32R) and rodless plectin (30F/32R) on cDNA synthesized from mRNA of normal humans and the proband's fibroblasts. (C) c.10984C>T and c.11453_11462del are located in the *PLEC* encoding C-terminal plectin repeat 4. (D) Normal humans express both full-length and rodless plectin. In our case, the *PLEC* mutations produced diminished and truncated plectin protein without the IF-binding domain.

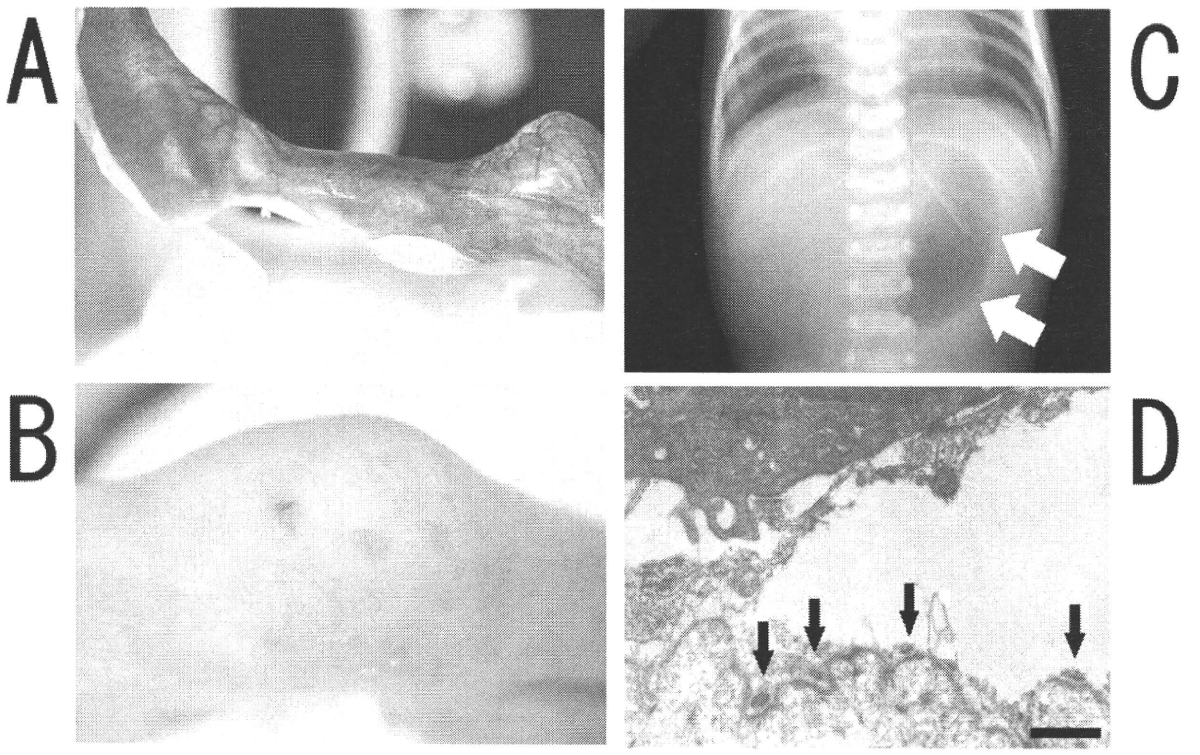

Figure 2. Clinical and ultrastructural features of the proband. (A) Aplasia cutis is observed on the left lower leg at birth. (B) Vesicles and erosions are scattered on the right knee. (C) Abdominal X-ray reveal single bubble sign (arrows), which indicated pyloric atresia. (D) Electron microscopy of the skin specimens from the proband reveals skin detachment within basal keratinocytes. Hemidesmosomes are hypoplastic and are observed at the base of the blisters (arrows) (Bar=1μm).

PLEC Mutations in Exon 32

PLEC mutational analysis demonstrated that the proband was compound heterozygous for maternal c.10984C>T (p.Glu3662X) and de novo c.11453_11462del in exon 32, the last exon of *PLEC* (Fig. 3A, 3B, 1C). The latter mutation is predicted to result in a frameshift that causes 88-amino-acid missense sequences followed by a premature termination codon (PTC). Both of the mutations were novel. c.10984C>T was confirmed by *BsrI* restriction enzyme digestion (Fig. 3C). c.11453_11462del was also confirmed by *BbvCI* restriction enzyme digestion (Fig. 3D) and TA-cloning (data not shown). Haplotype analysis of this family using microsatellite markers excluded false paternity as well as false maternity (data not shown) to establish the de novo nature of c.11453_11463del. The father's sperm has not been tested, although it might be beneficial to exclude the small possibility of paternal germ-line mosaicism through analyzing the father's sperm for any future prenatal diagnosis. In addition, c.7587G>A (p. =) transition in exon 32 was also detected in one allele of the proband and his father. This c.7587G>A transition was found in 3 of 100 normal unrelated alleles (50 healthy Japanese individuals), and was likely a polymorphism.

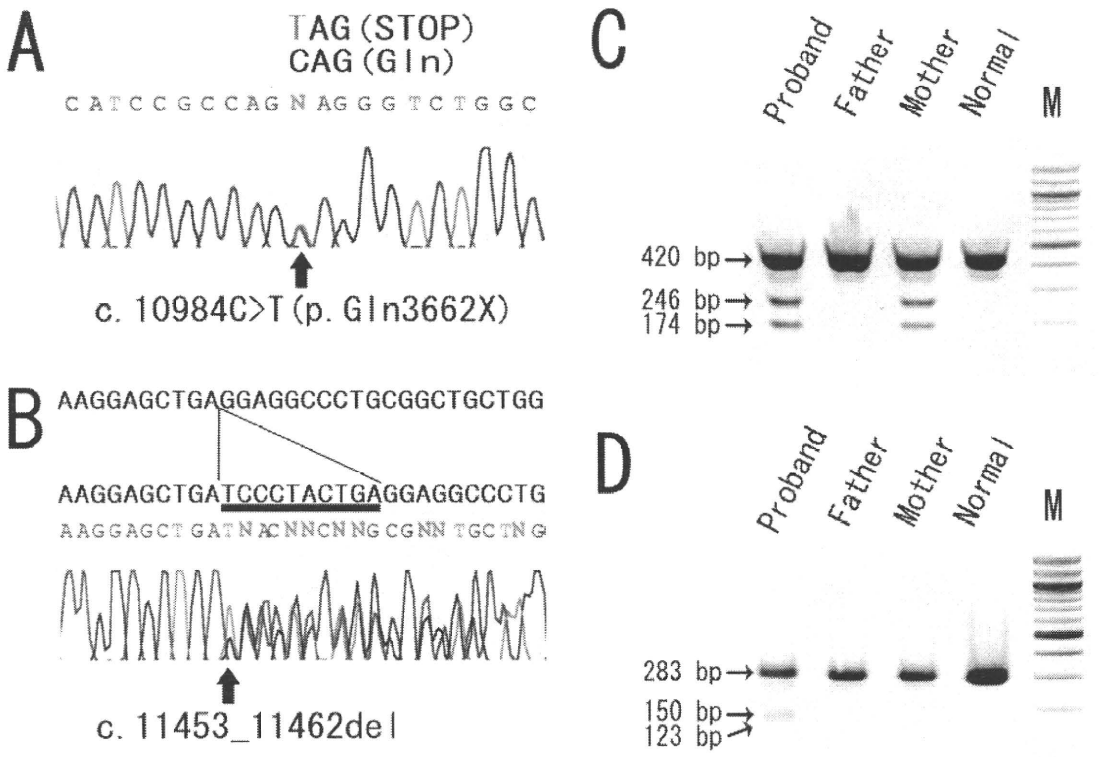

Figure 3. The novel *PLEC* mutations detected in the study. Maternal c.10984C>T (p.Gln3662X) (A) and de novo c.11453_11462del (B) in exon 32 were found in genomic DNA derived from the proband. A thymine substituted for a cytosine in the former mutation is indicated by the red character (A). Deleted nucleotides in the latter mutation are underlined (B). (C) c.10984C>T mutation caused the generation of a site for *BsrI* restriction enzyme. *BsrI* digestion of the 420-bp PCR product with and without the mutation resulted in a single band of 420-bp and in double bands of 246-bp and 174-bp, respectively. c.10984C>T was a maternal mutation. (D) c.11453_11462del caused the generation of a site for *BbvCI*. The 283-bp PCR product without the mutation was not digested by *BbvCI*. *BbvCI* digestion of the 273-bp PCR product with the deletion mutation showed two bands of 150 and 123-bp. c.11453_11462del was not detected in the parents' gDNA.

Diminished and Truncated Plectin Expression in Skin

We performed immunofluorescence analysis of the skin specimens from the proband using several antibodies that react with molecules of the dermo-epidermal junction (DEJ). To check plectin expression patterns in the skin specimens from the proband, we used four antibodies: PN643 (N-terminal globular domain), HD1-121 (rod domain), PC815 (C-terminal globular domain) and C20 (C-terminal globular domain) (Fig. 1A). Normal human control shows bright DEJ staining of all the antibodies tested (Fig. 4F-I). DEJ labeling of PN643, HD1-121 and PC815 was markedly diminished in the skin specimens from the proband (Fig. 4A-C). Staining of C20 was absent in the proband's skin (Fig. 4D). Immunostaining for type VII collagen (Fig. 4E), laminin 332, type IV collagen, type XVII collagen, α6 and β4 integrin, and BP230 revealed normal DEJ labeling patterns (data not shown).

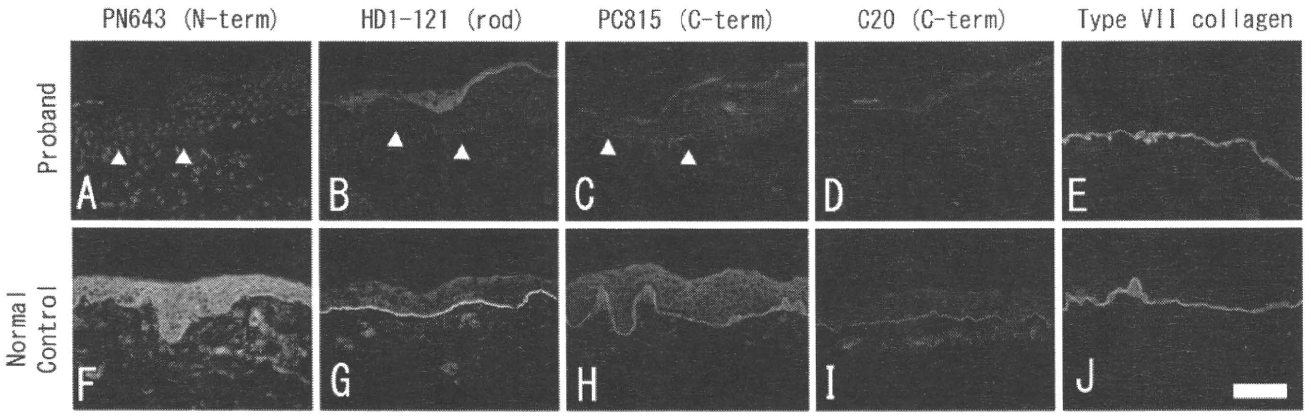

Figure 4. Immunofluorescence analysis of the proband's skin sample. In normal human skin, immunofluorescence shows that all of the antibodies against plectin (PN643, HD1-121, PC815 and C20) tested in this study bound to the dermal epidermal junction (DEJ) (F - I). DEJ labeling of PN643, HD1-121 and PC815 are weakly positive in the proband (A - C). In contrast, staining with C20 is negative in the proband's skin sample (D). Type VII collagen shows normal linear labeling in the proband and in the normal control (E, J). Weak labeling is indicated by arrowheads (Bar=100μm).

Diminished and Truncated Plectin in Cultured Fibroblasts

Immunoblot analysis of lysates from normal human cultured fibroblasts revealed that two closely spaced bands, corresponding to two forms of plectin (500kDa full-length and 390kDa rodless), reacted with PN643 and C20 antibodies recognizing the N- and C-termini of plectin (Fig. 5), as previously described (Natsuga, et al., 2010). HD1-121 against the rod domain reacted only with full-length plectin in normal human fibroblasts (Fig. 5). Lysates from cultured fibroblasts from the proband showed a faint band of PN643 and HD1-121 between 500kDa and 390kD, corresponding to truncated full-length plectin. C20 failed to react with lysates from the proband's cells (Fig. 5).

Full-length and rodless plectin transcripts are reduced in the proband's cultured fibroblasts

Using RT-PCR, the presence of mRNA that encodes full-length or rodless plectin was demonstrated in the normal human control as well as in the proband's cultured fibroblasts (Fig. 1B, 6). Judging from the PCR analysis results, the quantity of full-length and rodless plectin transcripts was markedly reduced in the proband's fibroblasts compared with those of the normal human control (Fig. 6).

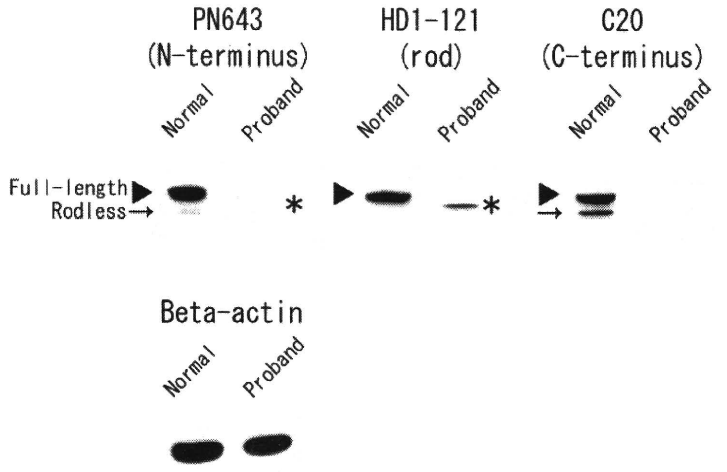

Figure 5. Immunoblot analysis of cultured fibroblasts from the normal human control and the proband. Immunoblot analysis of extracts from fibroblasts of the normal control and the proband by using PN643 against the N-terminal actin-binding domain, HD1-121 against the rod domain and C20 against the C-terminal plectin repeats. Rodless plectin (arrows), detected with PN643 and C20, migrates just below full-length plectin (arrowheads) in normal human fibroblasts. Using HD1-121, only full-length plectin is observed in the normal control. In contrast, fibroblasts of the proband contained smaller proteins than 500-kDa full-length plectin, the putatively truncated full-length plectin (asterisks), which was detected with PN643 and HD1-121. C20 did not react with lysates of the proband's fibroblasts. Equal protein loading was confirmed by reprobing with AC15 (anti-beta-actin antibody).

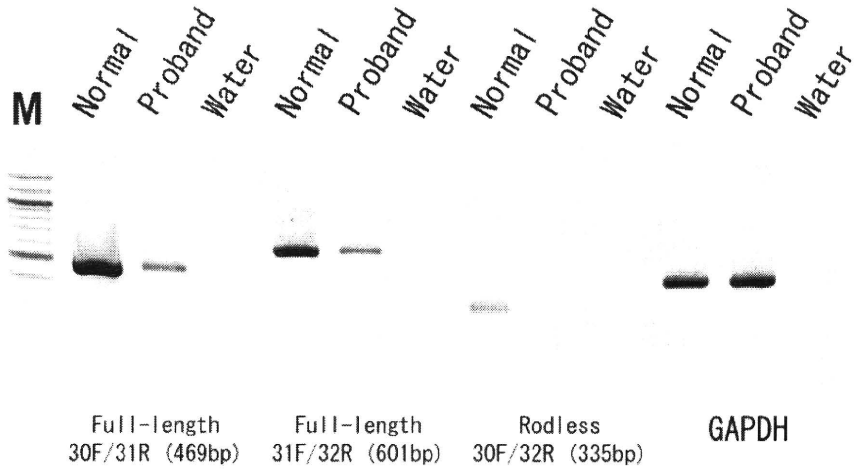

Figure 6. Semi-quantitative RT-PCR for full-length and rodless plectin transcripts. The quantity of full-length (30F/31R and 31F/32R) and rodless (30F/32R) plectin transcripts in the proband's cultured fibroblasts is reduced in comparison to those of the normal control. GAPDH mRNA expression was used as the loading control in these experiments. The negative control reaction (DNA-free water instead of cDNA) shows no PCR products. The molecular weight standard (lane M) is a 100-bp ladder.

DISCUSSION

This is the first report of EB complicated with both MD and PA. Skin detachment within basal keratinocytes was demonstrated by electron microscopy, which indicated the simplex subtype of EB. The proband's skin sample and cultured fibroblasts showed reduced and truncated plectin expression (Fig. 1D).

Both of the premature termination codon (PTC)-causing mutations detected in our case are within exon 32, the last exon of *PLEC*. Nonsense-mediated mRNA decay (NMD) is a quality-control mechanism that selectively degrades mRNAs with PTCs (Holbrook, et al., 2004). When the mRNA has a PTC more than a certain range upstream of one exon-exon junction, the transcript is down-regulated by NMD. In contrast, when PTCs are located in the last exon, NMD does not generally occur and abnormal mRNA is translated into truncated protein. However, some exceptions were described in a study in which transcripts underwent NMD despite having a PTC in the last exon (Chan, et al., 1998). Reduced amounts of full-length and rodless plectin transcripts in the proband's cells are explained by NMD, even though the PTCs of the proband are in the last exon.

One reported EBS-MD case was homozygous for a PTC-causing mutation (c.13458_13473dup) in exon 32 (Schroder, et al., 2002). c.13458_13473dup is at the downstream of the 6th plectin repeat and is predicted to cause a frameshift followed by a premature termination codon. The age of onset for MD in the patient with c.13458_13473dup was 4 years (Schroder, et al., 2002); in our case, severe muscle weakness was observed immediately after birth. This clinical difference might be explained by the length of truncated proteins identified in each patient. Compound heterozygous mutations of c.10984C>T and c.11453_11462del encode truncated plectin protein that does not include the intermediate filament (IF) binding site that was mapped to an approximately 50-amino-acid sequence between the 5th and 6th plectin repeat (Nikolic, et al., 1996; Rezniczek, et al., 2010). Therefore, the truncated plectin in our case might not have bound to IF including desmin in muscle tissues, which might account for the congenital muscle weakness. In contrast, the truncated plectin produced by c.13458_13473dup harbors the IF-binding site described above. Although the amount of plectin protein was slightly diminished, it may be that substantial amounts of truncated plectin with the residual IF-binding site delayed the development of muscular dystrophy and prevented pyloric atresia in the previous patient (Schroder, et al., 2002).

EBS-MD patients do not have muscular symptoms at birth, but muscle weakness appears later in their life. The type of *PLEC* mutations (PTC-causing mutations or in-frame insertions/deletions) influences the timing of MD onset (Chiaverini, et al., 2010). Also, it may be that, in most EBS-MD cases, the presence of residual rodless plectin resulting from PTC-causing mutations in exon 31 delays the onset of MD because of the remaining IF-binding site in rodless plectin.

It has been postulated that two pathologic elements are involved in the development of PA in EB patients: 1) the integrity of basement membrane and hemidesmosomes, and 2) the control of the normal process of fibrosis in the course of wound healing (Maman, et al., 1998). The sequence of events might be initiated by the separation of the intestinal mucosal layer as a result of disintegration of basement membrane and hemidesmosomes. Inflammatory responses cause massive fibrosis, which might lead to the obstruction of the intestinal lumina, especially in anatomically narrow passages, such as pylorus (Maman, et al., 1998). Previously described EBS-MD patients do not suffer from PA, which suggests that residual rodless plectin can prevent the development of PA. In our case, both full-length and rodless plectin proteins are quantitatively reduced and the shortened plectin might not have functioned normally, which might have lead to the PA phenotype.

It had been predicted that some cases of EBS-PA would develop MD, although no such case had been reported in the literature (Natsuga, et al., 2010). One possible explanation is that the poor systemic condition of EBS-PA and the limited observation period due to the patient's very short lifespan prevented the diagnosis of MD. Our data suggests that surgical correction of PA is insufficient to treat EBS-PA patients because they would most likely go on to develop MD even if they survive surgery. Therefore, we should look to develop more fundamental therapeutic options for those patients.

In summary, this study clearly shows that plectin mutations lead to both MD and PA phenotypes in an individual EBS patient.

ACKNOWLEDGMENTS

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

Contract grant sponsor: The Ministry of Health, Labour and Welfare; Contract grant number: H20-Nanchi-Ippan-028.

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BJD
British Journal of Dermatology

Partially disturbed lamellar granule secretion in mild congenital ichthyosiform erythroderma with *ALOX12B* mutations

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Accepted for publication
17 February 2010

Key words
ichthyosis, keratinization, lipid, lipoxygenase, LOX

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2010.09745.x

Congenital ichthyosiform erythroderma (CIE) (OMIM 242100) is a major type of autosomal recessive congenital ichthyosis (ARCI) showing generalized scaling and erythroderma without blister formation.[1] Mutations in *ALOX12B* (OMIM 603741), encoding 12R-lipoxygenase (LOX), were identified in patients with CIE in 2002.[2] To date, several *ALOX12B* mutations have been reported in CIE families.[3,4] LOXs are a family of nonhaem, iron-containing dioxygenases which catalyse dioxygenation of fatty acids with one or more (Z,Z)-1,4-pentadiene moieties.[5] Three members of the human LOX family, 15-LOX-2, 12R-LOX and eLOX-3, are preferentially expressed in the skin.[5,6] The 12R-LOX pathway leads to hepoxilin B3 and trioxilin B3[7] resulting in 20-carboxy-trioxilin A3,[5] which is thought to be a key biological regulator in the skin.[8] 12R-LOX deficiency results in a CIE phenotype in humans[2,9,10] and in mice.[11,12] We report that a Japanese patient with CIE, harbouring one previously unreported *ALOX12B* mutation p.Arg442Gln and another known mutation p.Arg432X, showed partially disturbed secretion of lamellar granule (LG) contents in the epidermis.

Case and methods

The patient was the first child of healthy, unrelated Japanese parents. There was no family history of any related disorders. The male child was born via uncomplicated, vaginal full-term delivery. The newborn was covered by a collodion membrane and showed thick scales on a background of erythroderma over his entire body, with skin fissures on the trunk (Fig. 1a–c). Moderate ectropion and eclabium were seen. Hands and feet were oedematous, and the palms and soles were involved. The patient was treated with a topical application of white petrolatum with an occlusive dressing technique in a humid incubator. The hyperkeratosis and oedema were remarkably reduced within 2 weeks. At age 3·5 months, the patient showed mild white to grey-coloured scales over the erythematous skin covering his entire body (Fig. 1d–f).

Mutation analysis of *ALOX12B* was performed using genomic DNA isolated from peripheral blood cells of the patient and his parents.

Results and discussion

Mutation analysis of *ALOX12B* revealed that the patient was a compound heterozygote for a known nonsense mutation p.Arg432X (c.1294C>T) in exon 10 and a previously unreported missense mutation p.Arg442Gln (c.1325C>T) in exon 10 (GenBank NM_001139.2) (Fig. 1g, h). The nonsense mutation p.Arg432X was present in a heterozygous fashion in his mother, although p.Arg442Gln was not found in the parents and was thought to be a *de novo* mutation. The mutations were verified by mutant allele-specific amplification analysis. No mutation was found in the sequence analysis of 200 alleles from 100 normal, unrelated Japanese individuals, and therefore it is unlikely to be a polymorphism (data not shown). No other pathogenic mutations were found in *TGM1* (OMIM 190195), *ABCA12* (OMIM 607800), *NIPAL4* (ichthyin; OMIM 609383), *CYP4F22* (previously known as *FLJ39501*; OMIM 611495) or *ALOXE3* (OMIM 607206) by direct sequencing analysis.

p.Arg432X results in a serious truncation of the 12R-LOX peptide, losing approximately half of the C-terminal catalytic LOX domain, and is thought to have a serious effect on the enzyme activity.

The arginine residue mutated by p.Arg442Gln is in the central part of the C-terminal catalytic LOX domain and is highly conserved among diverse species (Fig. 1i) and human LOX family members (Fig. 1i). These facts suggest that this arginine residue might be essential for enzyme activity and that the present missense mutation affects 12R-LOX activity.

Electron microscopy of a skin biopsy specimen from the trunk at age 6 days using ruthenium tetroxide postfixation revealed irregular-sized lipid droplets in the stratum corneum.

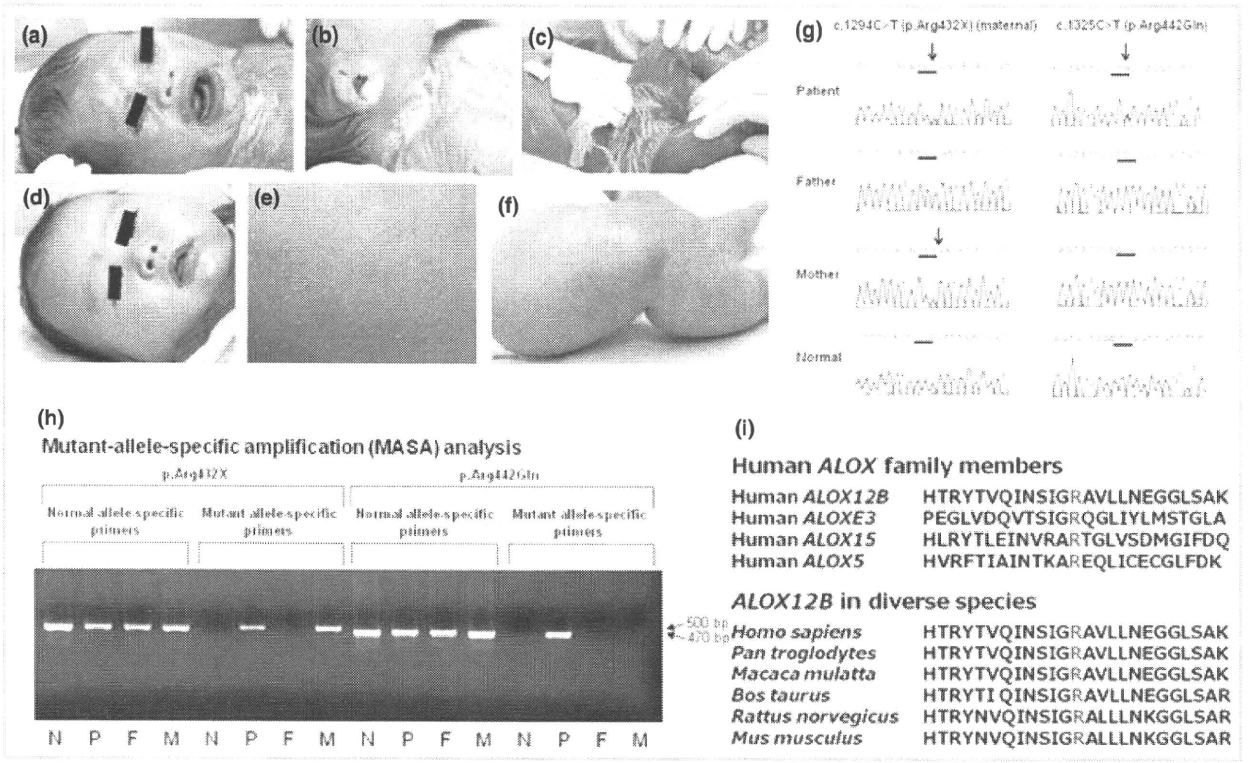

Fig 1. The patient's clinical features and compound heterozygous mutations in *ALOX12B*. (a–c) At 1 day of age: moderate ectropion and eclabium (a), a malformed auricle (b), and thick plate-like scales and fissures on the neck and the chest (b) and on the lower abdomen to the thigh (c). (d–f) At age 3·5 months: only slight, fine scales on the face (d), the back (e) and the thigh (f). (g) Compound heterozygous *ALOX12B* mutations, maternal p.Arg432X (c.1294C>T) and a novel missense mutation p.Arg442Gln (c.1325C>T) in exon 10 of the patient's genomic DNA. (h) Mutant allele-specific amplification (MASA) analysis showed a 500-bp band from p.Arg432X and a 470-bp band from p.Arg442Gln. N, normal; P, patient; F, father; M, mother. (i) Arginine 442 in 12R-lipoxygenase (LOX) altered by p.Arg442Gln; red character (R) is conserved among human *ALOX* family members (top) and diverse species (bottom).

Intact LGs were observed and intercellular lipid lamellae were seen in the intercellular space in the stratum corneum, although secretion of LG contents was disturbed in part in the granular layer cells (Fig. 2a–e).

Immunofluorescence staining revealed that one of the major basal keratins (keratin 5), differentiation-specific keratin (keratin 10) and ABCA12 were distributed normally in the basal layer, in the suprabasal layers, and in the granular layers, respectively, of the patient's epidermis (Fig. 2f, h, j). Immunoreactivity for cornified cell envelope-associated proteins involucrin, loricrin and transglutaminase 1 was seen normally distributed in the patient's upper epidermis (Fig. 2l, n, p).

Keratinocyte differentiation (keratinization)-specific molecules examined in the present study were normally distributed in the patient's epidermis. Also, keratinocyte differentiation defects were not morphologically or biochemically detected in *Alox12b*-disrupted mice.[11] These facts suggest that keratinocyte differentiation defects might not be involved in the pathogenesis of ichthyosis in CIE with *ALOX12B* mutations.

One report on ultrastructural features of the 12R-LOX-deficient human epidermis demonstrated lipid droplets in the cornified layers.[13] From the study of *Alox12b* mutant mouse skin transplants, it was suggested that 12R-LOX deficiency might affect the processing of LGs.[14] In *Alox12b*-disrupted mouse skin, irregular-sized vesicles were observed in the granular layers, and the stacks of lipid lamellae representing extruded content of LGs were seen in the transition zone between the uppermost granular and the first cornified cells.[11] In the present study, we clearly demonstrated lipid droplets in the cornified cell layers, and partially disturbed LG content secretion into the intercellular space in the patient's epidermis. Our results suggest that partially disturbed secretion of LG contents is involved in the pathogenesis of ichthyosis in patients with CIE with *ALOX12B* mutations. It has not been clarified completely yet how the partially disturbed secretion of LG contents contributes to the pathogenesis of CIE. Several ichthyosiform disorders have been shown to involve the abnormal function of LG.[1,15] Lipid contents secreted from LG are known to form the intercellular lipid layers in the stratum corneum.[16] The intercellular lipid layers are essential for the epidermal barrier function and the defective intercellular lipid layers caused by disturbed secretion of LG lipid contents are expected to result in skin barrier impairment.[17] Defective skin barrier function leads to compensatory mechanisms involving epidermal hyperproliferation and hyperkeratosis, which are

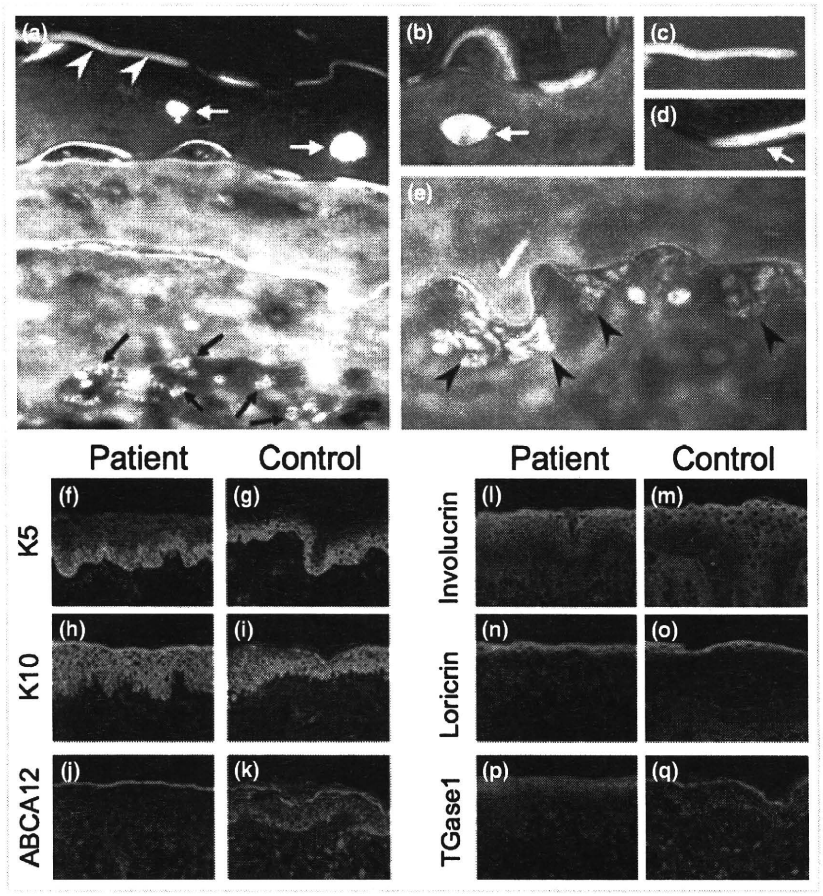

Fig 2. Disturbed secretion of lamellar granule (LG) contents from the granular layer cells and normal distribution of keratinization markers in the patient's epidermis. (a–e) Ultrastructural features of the skin biopsy specimen in the neonatal period. (a) Overall keratinization processes appeared normal. Black arrows, LGs; white arrowheads, intercellular lipid lamellae; white arrows, various-sized cytoplasmic lipid vacuoles. (b) An irregular-sized lipid vacuole (white arrow) containing the lamellar structure. (c) Intact intercellular lipid lamellae. (d) Normal cornified cell envelope (white arrow). (e) Partially congested LG secretion (arrowheads) in a granular layer cell. (f–q) Immunofluorescence staining (FITC) revealed that keratin 5, keratin 10 and ABCA12 were distributed normally in the basal layer, suprabasal layers and granular layers, respectively. For keratin 5 immunostaining, we used an antibody which cross-reacts with keratin 8. However, the labelling of this antibody is thought to reflect keratin 5 expression in this study, because keratin 8 expression is usually restricted to the simple epithelia. Cornified cell envelope-associated proteins, involucrin, loricrin and transglutaminase 1, were seen normally distributed in the upper epidermis. (f–q) Nuclear stain, red (propidium iodide). Original magnification: (a) × 6000, (b) × 12 000, (c, d) × 18 000, (e) × 10 000, (f–q) × 20.

observed frequently in ichthyosiform diseases.[16] In this context, we can hypothesize that the partially disturbed secretion of LG contents might cause defective intercellular lipid layers in the stratum corneum, resulting in skin barrier defects and subsequent compensatory hyperkeratosis in CIE.

Patients with CIE harbouring *ALOX12B* mutations have previously been reported in African and European populations.[3,4] Our previous studies failed to identify *ALOX12B* mutations in Japanese patients with ARCI[18] and demonstrated that the frequency of *ALOX12B* mutations is expected to be low in Japanese patients with ARCI. As far as we know, the present case is the first patient with CIE with detected *ALOX12B* mutations in the Asian area and our results also confirm *ALOX12B* as one of the CIE causative genes in the Asian population. Further accumulation of CIE cases is needed to clarify the frequency of *ALOX12B* mutations in patients with CIE in Asian countries.

Acknowledgments

We thank Dr James R. McMillan for proofreading this manuscript and Ms Yuki Miyamura for her fine technical assistance on this project. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to M.A. (Kiban B 20390304) and by a grant from the Ministry of Health, Labor and Welfare of Japan (Health and Labor Sciences Research Grants; Research on Intractable Diseases; H21-047) to M.A.

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